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BIOMECHANICAL REGULATION OF ARTICULAR CARTILAGE METABOLISM OF PROTEOGLYCAN 4 AND ARTICULAR SURFACE INTEGRITY

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in

Bioengineering

by

Gayle E. Nugent

Committee in charge:

Professor Robert L. Sah, Chair
Professor Jeff D. Esko
Professor Wayne R. Giles
Professor Richard L. Lieber
Professor L. Amy Sung

2006

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TABLE OF CONTENTS

Signature Page ..... **iii**

Table of Contents ..... **iv**

List of Figures and Tables ..... **viii**

Acknowledgments ..... **x**

Vita……..... **xiii**

Abstract… ..... **xv**

Chapter 1: Introduction **1**

1.1 Abstract 1

1.2 Structure, Composition, and Boundary Lubrication Function of Articular Cartilage 5

1.3 Proteoglycan 4 (PRG4) Lubricant Molecules 8

1.4 Mechanical Regulation of Chondrocyte Metabolism and the Use of Bioreactors 11

1.5 Chemo-Mechanical Regulation of PRG4 Metabolism 15

1.6 References 17

Chapter 2: Static and Dynamic Compression Regulate Cartilage Metabolism of Proteoglycan 4..... 27

2.1 Abstract..... 27

2.2 Introduction 29

2.3 Materials and Methods 32

2.4 Results 37

2.5 Discussion..... 42

2.6 Acknowledgments 47

2.7 References 48

Chapter 3: Dynamic Shear Stimulation of Bovine Cartilage Biosynthesis of Proteoglycan 4..... 52

3.1 Abstract..... 52

3.2 Introduction 54

3.3 Materials and Methods 57

3.4 Results 64

3.5 Discussion..... 71

3.6 Acknowledgments 77

3.7 References 78

Chapter 4: PRG4 Exchange Between Articular Cartilage Surface and Synovial Fluid 82

4.1 Abstract 82

4.2 Introduction 84

4.3 Materials and Methods 87

4.4 Results 92

4.5 Discussion 98

4.6 Acknowledgments 103

4.7 References 104

Chapter 5: Continuous Passive Motion Applied to Whole Joints Stimulates Chondrocyte Biosynthesis of PRG4 107

5.1 Abstract 107

5.2 Introduction 109

5.3 Materials and Methods 112

5.4 Results 120

5.5 Discussion 126

5.6 Acknowledgments 132

5.7 References 133

Chapter 6: Site- and Exercise Related Variation in Structure and Function of Cartilage from Equine Distal Metacarpal Condyle 139

6.1 Abstract..... 139

6.2 Introduction 141

6.3 Materials and Methods 144

6.4 Results 149

6.5 Discussion..... 156

6.6 Acknowledgments 160

6.7 References 161

Chapter 7: Conclusions..... 165

7.1 Summary of Findings 165

7.2 Discussion..... 168

7.3 Future Work..... 174

7.4 References 177

LIST OF FIGURES AND TABLES

Figure 1.1: Overall aims 4

Figure 1.2: Compartmental model of synovial joint 7

Figure 1.3: PRG4 structure 10

Figure 2.1: Experimental design 36

Figure 2.2: PRG4 secretion. 39

Figure 2.3: PRG4 immunolocalization. 40

Figure 2.4: Depth associated variation in PRG4 expression. 41

Figure 3.1: Mechanical stimulation protocol 63

Figure 3.2: Cartilage thickness 66

Figure 3.3: PRG4 secretion 67

Figure 3.4: Western blot 68

Figure 3.5: Chondrocyte PRG4 expression 69

Figure 3.6: Depth associated variation in PRG4 expression 70

Figure 4.1: Experimental design 91

Figure 4.2: Association experiments 95

Figure 4.3: Dissociation / repletion: quantification 96

Figure 4.4: Dissociaiton / repletion: immunolocalization 97

Figure 5.1: CPM bioreactor system 118

Figure 5.2: Experimental design 119

Figure 5.3: PRG4 secretion mappings 123

Figure 5.4: Chondrocyte viability, PRG4 expression, and PRG4 secretion 124

Figure 5.5: PRG4 immunolocalization 125

Figure 6.1: Sample sites 148

Table 6.1: Principal component analysis 152

Figure 6.2: Structural and biomechanical properties 153

Figure 6.3: Biochemical properties 154

Figure 6.4: Indentation stiffness vs structural and biochemical measurements 155

ACKNOWLEDGMENTS

I would like to thank several people without whom this work would not have been possible. Most importantly, I thank my thesis advisor, Dr. Robert Sah for giving me the opportunity to be part of his research group, and for all of the advice and support over the years. His hard work and dedication to high quality research and maintaining funding for the lab are important to the success of each of us, and he should probably be thanked more often for that!

Chapter 2 is reprinted from *Biorheology*, 43(3-4), G.E. Nugent, T.A. Schmidt, B.L. Schumacher, M.S. Voegtline, W.C. Bae, K.D. Jadin, and R.L. Sah, Static and dynamic compression regulate cartilage metabolism of PRoteoGlycan 4 (PRG4), p. 121-200, Copyright 2006, with permission from IOS Press. I thank the co-authors of the manuscript for their contributions: Tannin A. Schmidt, Barbara L. Schumacher, Dr. Michael S. Voegtline, Dr. Won C. Bae, and Kyle D Jadin. In addition, we thank the funding sources that supported this work: Arthritis Foundation, NASA, NIH, NSF, Whitaker Foundation (pre-doctoral fellowship to GEN).

Chapter 3 is reprinted in full from *Arthritis & Rheumatism*, 54(6), Nugent GE, Aneloski NM, Schmidt TA, Schumacher BL, Voegtline MS, Sah RL, Dynamic shear stimulation of bovine cartilage biosynthesis of proteoglycan 4, p. 1888-96, Copyright 2006, with permission from John Wiley & Sons, Inc. I thank the co-authors of the manuscript for their contributions: Aneloski NM, Schmidt TA, Schumacher BL, Voegtline MS. In addition, we thank the funding sources that supported this work: Arthritis Foundation, NIH, NSF, Whitaker Foundation (pre-doctoral fellowship to GEN).

For their contributions to chapter 4, I thank co-authors Amy H. Chan, and Barbara L. Schumacher. In addition, we thank the funding sources that supported this work: NIH, NSF, Whitaker Foundation (pre-doctoral fellowship to GEN), McNair Program (undergraduate scholarship to AHC).

For their contributions to chapter 5, I thank co-authors Tad Takara, James K. O'Neill, Sean B. Cahill, Simon Görtz, Terrence Pong, Hideru Inoue, Nicole M. Aneloski, Will W. Wang, Keeley I. Vega KI, Travis J. Klein, Nancy D. Hsieh-Bonassera, Dr. Won C. Bae, James D. Burke, and Dr. William D. Bugbee. This work was supported by NIH, NSF, Whitaker Foundation (pre-doctoral fellowship to GEN), Irwin and Joan Jacobs (undergraduate scholarship to JKO), Stein Institute for Research on Aging (scholarship to TT). We also thank Dr. Darryl D'Lima for advice on whole-joint preparations.

Chapter 6 is reprinted from *Osteoarthritis and Cartilage*, 12(10), Nugent GE, Law AW, Wong EG, Temple MM, Bae WC, Chen AC, Kawcak CE, Sah RL, Site- and exercise-related variation in structure and function of cartilage from equine distal metacarpal condyle, p. 826-33, Copyright (2004), with permission from Elsevier. The dissertation author (primary investigator) thanks the co-authors of the manuscript for their contributions: Amanda A. Law, Eric G. Wong, Dr. Michele M. Temple, Dr. Won C. Bae, Dr. Albert C. Chen, and Dr. Chris E. Kawcak. In addition, we thank the funding sources that supported this work: Arthritis Foundation, Global Equine Research Alliance, Marilyn M. Simpson Trust, NASA, NIH, NSF, and a pre-doctoral fellowship from the Whitaker Foundation (GEN).

I thank my dissertation committee members: Dr. Wayne Giles, Dr. Richard Lieber, Dr. Jeffery Esko, and Dr. Amy Sung, for their valuable input during my time at UCSD, and especially during my thesis proposal.

I am very lucky to have been part of a lab in which teamwork was always encouraged. I thank the many lab members who have provided much needed assistance along the way: Barb Schumacher and Tannin Schmidt, for all of their advice on experiments over the years, and stimulating conversations about lubrication and SZP; Van Wong for answers to countless computer-questions; Van Wong, Michele Temple, and Greg Williams, Nancy Hsieh, and Ken Gratz, for maintaining order in the lab during difficult times; Michele Temple, Albert Chen, and Won Bae, for getting me started learning lab techniques during my second year of school; Megan Blewis, for the runs and all the "new" stories; Travis Klein, Tannin Schmidt, Kyle Jadin, Megan Blewis, Ken Gratz for all the team cartilage running endeavors; Amy Chan, for countless hours of planning, tissue harvesting, pipetting, and discussion of results; Tad Takara and James O'Neill for all of their contributions to team CPM 2005-6 and their ability to keep it real; Simon Görtz for advice on aseptic and surgical techniques and for all the cleverly worded emails; Amanda Law and Eric Wong, for their help in indentation testing of equine cartilage; Nikki Aneloski, for help in designing the biaxial motion bioreactor; and all other lab members who have been around during my time at CTE!

Finally, I thank my family: Marilyn, Patrick, and Jeanne Nugent for providing real-world perspective when necessary, and my husband Austin Derfus for endless support and interest in my success.

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ABSTRACT OF THE DISSERTATION

BIOMECHANICAL REGULATION OF
ARTICULAR CARTILAGE METABOLISM
OF PROTEOGLYCAN 4 AND
ARTICULAR SURFACE INTEGRITY

by

Gayle E. Nugent

Doctor of Philosophy in Bioengineering
University of California, San Diego, 2006
Professor Robert L. Sah, Chair

Articular cartilage provides a low-friction, load bearing surface that allows the bones of diarthrodial joints to slide smoothly against each other, with the zonal variations in cartilage matrix structure imparting different functions to each zone. The ability of mechanical stimuli to regulate chondrocyte production of matrix molecules involved in load-bearing has been well documented. Recently it has been suggested that chondrocyte metabolism of specialized molecules secreted by the superficial zone, such as proteoglycan 4, that mediate the lubrication function of the articular surface, may also depend on biomechanical cues. The overall motivation of this

dissertation was to contribute to the understanding of the role of biomechanical stimulation in maintaining boundary lubrication of the articular surface by investigating the effects of various loading protocols *in vitro* (explanted cartilage disks), *ex vivo* (intact cartilage during whole-joint culture), and *in vivo*, on PRG4 metabolism and articular surface integrity.

In vitro studies demonstrated that static and dynamic compression and dynamic shear stimulation regulate PRG4 biosynthesis, both during loading and after unloading. Similarly, continuous rehabilitative motion applied to whole joints during culture stimulated PRG4 biosynthesis, in a manner dependent on region within the joint, as different regions experienced different loading environments. Marked site-associated variation in cartilage surface integrity was also shown to exist *in vivo*, likely due to variation in biomechanical environments with joint region. Finally, it was shown that PRG4 molecules can be removed from and re-attached to the articular cartilage surface, which suggests a mechanism by which secreted PRG4 molecules might maintain lubrication function.

Elucidating the role of mechanical stimuli, both *in vitro* and *in vivo,* in regulating cartilage metabolism of PRG4 and articular surface integrity could lead to an understanding of the processes involved in joint health and degeneration, and possible techniques for tissue-engineering of cartilage tissue with a functional surface layer.

CHAPTER 1

INTRODUCTION

1.1 General Introduction to the Dissertation

Articular cartilage provides a low-friction, load bearing surface that allows the bones of diarthrodial joints to slide smoothly against each other, with the zonal variations in cartilage matrix composition and structure imparting different functions to each zone. Effects of biomechanical stimuli on chondrocyte metabolism of cartilage extracellular matrix molecules such as collagen and glycosaminoglycans, which are essential to the load-bearing function of the middle and deep zones of cartilage, have been well documented both *in vitro* and *in vivo*. Only recently has it been suggested that chondrocyte metabolism of specialized molecules such as proteoglycan 4, that contribute to the lubrication function of the articular surface zone, may depend on biomechanical cues as well. The aim of this dissertation work was to contribute to the understanding of the role of biomechanical stimulation in maintaining the boundary lubrication function of the articular surface. Toward this goal, the effects of various mechanical loading protocols, both *in vitro*, (applied to a: explanted cartilage disks, or b: intact cartilage during whole-joint culture), and *in vivo*, on PRG4 metabolism and

articular surface integrity were investigated, as was the ability of PRG4 to bind to the articular cartilage surface (**Fig. 1.1**).

This chapter begins with a review of articular cartilage structure and function, focusing on the superficial zone and its structural properties that impart boundary lubrication function to the articular surface. A brief background on products of the proteoglycan 4 gene is then given, followed by an overview of methods used to investigate mechanical stimulation of chondrocyte metabolism and general conclusions regarding the effects of such stimuli on metabolism of cartilage matrix molecules (i.e. collagen, GAG: those involved in the weight bearing function of the tissue). Finally, a review of recent literature suggesting that chondrocyte metabolism of lubricant PRG4 molecules could be regulated by local microenvironment is presented.

Chapter 2, which is in press in *Biorheology [68]* describes the effects of static and dynamic compression of different magnitudes on PRG4 biosynthesis by chondrocytes in cylindrical cartilage disks during *in vitro* culture. This work demonstrated that both static and dynamic compressive stimulation inhibited PRG4 secretion during the loading period, but that following unloading, cartilage that had been dynamically stimulated secreted more PRG4 than unloaded controls.

Chapter 3, published in *Arthritis & Rheumatism* [66], describes the effects of dynamic tissue shear deformation on PRG4 biosynthesis by chondrocytes in cylindrical cartilage disks during *in vitro* culture. Dynamic shear stimulation led to marked up-regulation of PRG4 secretion relative to unloaded controls, both during stimulation and after unloading of the tissue.

Chapter 4 addresses the ability of PRG4 molecules to adhere to the articular surface of cartilage disks. Results suggest that native PRG4 at the articular surface does not readily exchange with PRG4 in the surrounding fluid, but can be removed by certain chemical treatments, and can be subsequently replaced with PRG4 from synovial fluid.

Chapter 5, submitted to the *Arthritis & Rheumatism*, addresses the effect of physiological joint motion, applied to a whole joint during culture *in vitro*, on chondrocyte PRG4 metabolism. Joint motion, applied using an adapted continuous passive motion device, up-regulated PRG4 synthesis for certain joint regions that were continuously or intermittently sliding relative to other tissues during the stimulation protocol.

Chapter 6, published in *Osteoarthritis and Cartilage* [67], describes the site-associated variation in articular cartilage surface integrity resulting from *in vivo* joint loading in an equine early exercise training model. Indentation stiffness and surface roughening varied markedly with region of the joint, likely due to distinct biomechanical environments experienced by different regions.

Finally, Chapter 7 summarizes the major conclusions from this work and discusses future directions for these projects.

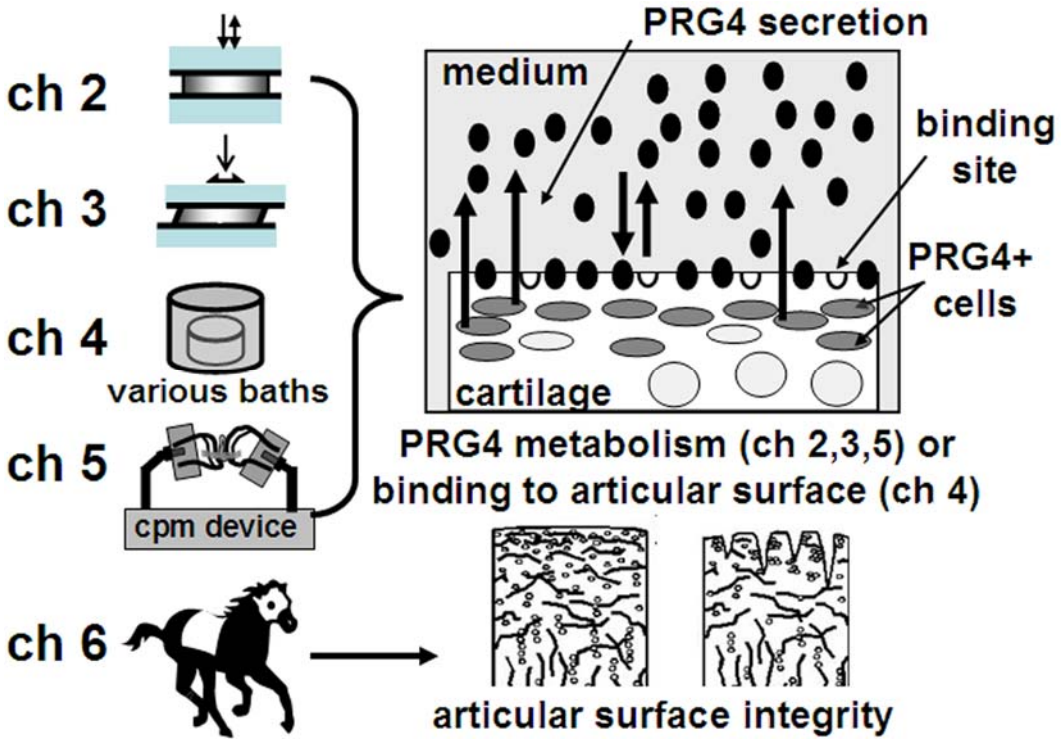


Figure 1.1. Overall aims of the dissertation.

1.2 Structure, Composition, and Boundary Lubrication Function of Articular Cartilage

Articular cartilage functions to provide a low-friction, load-bearing surface which allows the bones of diarthrodial joints to slide smoothly against each other while transmitting load. Cartilage tissue has classically been divided into three zones: superficial, middle, and deep, which are characterized by distinct compositions, structures, and functions [34]. The superficial zone has a high density of chondrocytes with flattened morphology [86], relatively low glycosaminoglycan (GAG) content, and collagen fibers running parallel to the articular surface. The middle and deep zones, on the other hand, have round, less densely packed chondrocytes, arranged in columns perpendicular to the articular surface [34, 35, 86], higher GAG content [58], and collagen fibers running perpendicular to the articular surface [5]. Thus the middle and deep zones of the tissue are thought to provide the load bearing function of cartilage, while the superficial zone mediates lubrication function.

While other modes of lubrication, e.g. fluid film lubrication, occur at some points during joint function, boundary mode lubrication dominates when a joint surface has been loaded in a stationary position, and then begins to articulate. In boundary mode lubrication, layers of molecules separate opposing surfaces and thus reduce frictional forces resulting from relative sliding of the two surfaces. In order for molecules to provide boundary lubrication, they must be adsorbed or bound to the surface they lubricate.

The superficial zone of articular cartilage is covered with an acellular, fibrous layer that varies in thickness from 1-10 μm [3, 25, 98]. This layer contains the lamina splendens, as well as other biomolecules such as hyaluronic acid [101] (HA), phospholipids [76, 102], proteoglycan 4 (PRG4), fibronectin [65, 102], albumin [65],

fibromodulin [65], decorin [65], biglycan [65], and collagen types I, III, but not type II [91]. Of these molecules, HA [36, 88, 89], phospholipids [30, 31, 84], and PRG4 [36-39] have been suggested to play roles in the boundary lubrication function at the articular cartilage-synovial fluid interface during joint motion, though the exact mechanism of lubrication and molecular mediators remain controversial. While previous studies had shown that PRG4 reduces friction in glass-on-latex [36, 37, 39] and cartilage-on-glass [87, 89] lubrication assays, recent work in our laboratory has shown that PRG4 also contributes to boundary lubrication function during cartilage-on-cartilage sliding [77, 80].

We have developed a compartmental model of the synovial joint (**Fig. 1.2**) and have used it as a framework within which to design experiments to better understand the roles of various joint tissues, and their production of putative lubricant molecules (especially PRG4), in maintaining joint lubrication function. This dissertation focuses on the contribution of PRG4 to the synovial joint environment by articular cartilage, and the role of mechanical stimuli in regulating that contribution.

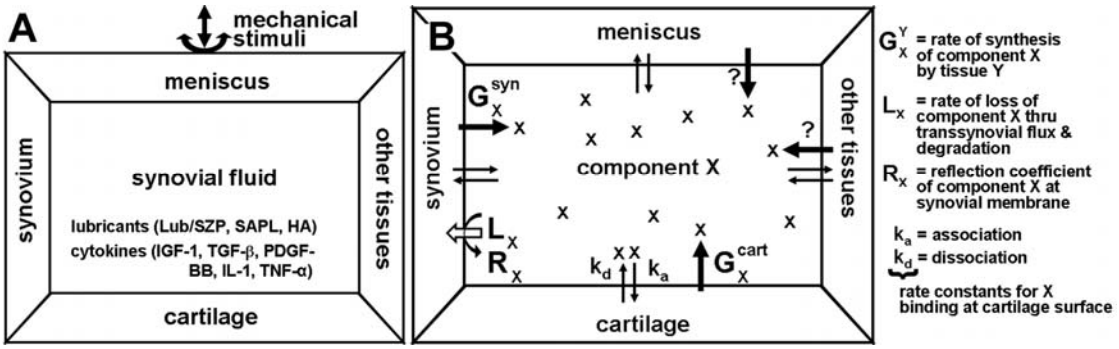


Figure 1.2. (**A**) Model of synovial joint in which each joint tissue comprises a compartment that contributes to the molecular contents of the synovial fluid environment. (**B**) Example of how synthesis of molecule X by tissue Y contributes to the concentration of component X in the synovial fluid compartment, and possibly to the attachment of component X to the surfaces of joint tissues.

1.3 Proteoglycan 4 (PRG4) Lubricant Molecules

The *Prg4* gene encodes multiple proteoglycan products, which have been identified in a number of tissues, and therefore are known by a variety of abbreviations, including megakaryocyte stimulating factor (MSF), camptodactyly-arthropathy-coxa vara-pericarditis (CACP), Lubricin, hemangiopoietin (HAPO), Superficial zone protein (SZP), and PRG4. MSF was originally purified from urine, and stimulates platelet-forming cells [62]. Mutations in the *prg4* gene cause CACP disease in humans [57], which results in non-inflammatory joint failure [2], demonstrating the importance of the gene *in vivo*. HAPO, an alternatively spliced isoform, simulates both hematopoietic progenitor and endothelial cells [55]. Lubricin, originally purified from synovial fluid [89, 90], is highly expressed by cells of the synovial lining, and functions to reduce friction in latex-on-glass friction assays [37, 38]. SZP was first isolated from conditioned medium of cultured superficial zone (but not deep zone) cartilage explants [82]. It is this 345 kDa glycoprotein product of the *Prg4* gene that will be the focus of this project, and it will be referred to as PRG4.

PRG4 Structure and Putative Molecular Interactions (Fig. 1.3). Since PRG4 has several regions that are highly homologous to certain domains of other well-characterized proteins, some interactions of PRG4 with other molecules have been hypothesized. Both the N-terminal and the C-terminal of PRG4 have regions with high sequence homology to certain sequences of vitronectin. In particular, PRG4 has a somatomedin-B (SMB) like domain near the N-terminal, and a hemopexin-like domain near the C-terminal. Purified hemopexin interacts with HA [32], suggesting that the hemopexin-like domain could also mediate PRG4 binding to HA present at or near the articular surface. In addition, lubrication studies have shown that HA and

PRG4 act in concert to reduce friction [36, 89], supporting the possibility of molecular interaction between the two at the articular surface.

The region of PRG4 encoded by exon 6 contains o-linked β(1-3)Gal-GalNAc oligosaccharides, which likely mediate the boundary lubrication function of PRG4 by organizing water molecules near the articular surface [38]. Just N-terminal to this mucin-like domain, exon 6 contains a covalently bound chondroitin sulfate (CS) chain [20, 82]. Such GAG chains could also mediate non-covalent binding of PRG4 to matrix molecules such as collagens [29] or fibronectins [4, 47] at the articular surface.

Another possibility is that PRG4-surface binding is lipid-mediated, as PRG4 has been shown to contain up to 11% lipid [84]. PRG4-lipid binding may involve hydrophobic-hydrophobic attraction [7, 92], covalent binding via palmitoylation [20, 84], or hydrophilic interaction [31], in which negatively charged phosphate ions render phosphatidylcholine (PC) molecules effectively cationic, allowing them to be strongly adsorbed to the negative portions of the proteoglycan.

Self-aggregation of PRG4 molecules, via disulfide bond formation between unmatched cysteine residues near the c-terminal [20], may also be important for its binding to the articular surface. Alternatively, the unmatched cysteine residue may form disulfide bonds with other molecules present at the articular surface and thus play a role in maintenance of PRG4 at the articular surface.

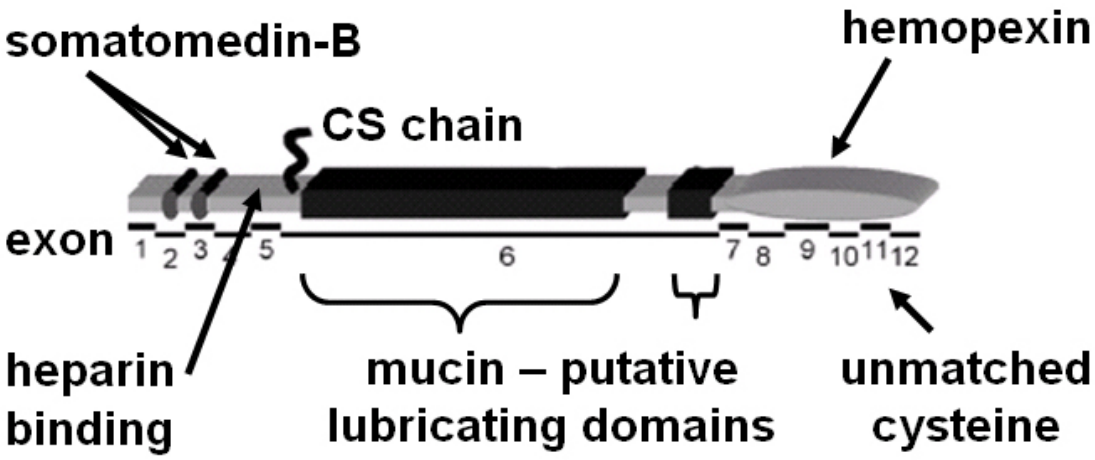


Figure 1.3. PRG4 structure (adapted from [74]) and binding motifs relevant to putative PRG4-articular surface attachment and boundary lubrication mechanisms.

1.4 Mechanical Regulation of Chondrocyte Metabolism of Matrix Molecules and the Use of Bioreactors

Experimental and clinical observations indicate that, *in vivo*, the mechanical environment of the joint provides physical cues that guide the development of normal, functional articular cartilage. Several studies have shown that elimination or reduction of load-bearing in young animals leads to abnormal development of articular cartilage. In embryonic chick limbs, paralysis during skeletal maturation leads to abnormal joint shapes and sizes [18]. Immobilization of a weight bearing limb in growing beagle dogs results in glycosaminoglycan (GAG) depletion [45] and reduced indentation stiffness [43] of the articular cartilage. In young beagle dogs subjected to a moderate running program, thickness and GAG content were augmented in regions bearing the highest loading surplus, indicative of a functional adaptation to use [46]. Similarly, mechanical [64] and biochemical [10, 63] properties vary with exercise level in a site-specific manner in skeletally immature (5-24 months) equine carpal cartilage. Furthermore, neonatal ovine [54] and equine [8, 9] articular cartilage shows no detectable site-associated variation in biochemical content, in contrast to the clear biochemical heterogeneity in the mature tissue.

Thus mechanical loading clearly affects chondrocyte metabolism *in vivo*, yet the *in vivo* mechanical environment of articular cartilage is complex, difficult to define theoretically, and is not isolated from other systemic stimuli such as biochemical signals. For this reason, cartilage explants of defined (usually cylindrical) geometry have been used to determine the effects of well-defined mechanical stimuli on chondrocyte metabolism. Such studies make use of custom-made bioreactors that

allow for application of some type of tissue deformation during *in vitro* culture. One common method for loading of cartilage explants in compression is to sandwich them between two platens, permeable or impermeable, during culture. In general, these studies have demonstrated that static unconfined axial compression tends to inhibit matrix synthesis, while dynamic compression in the range of 0.1-1 Hz tends to stimulate matrix synthesis [1, 11, 24, 26, 42, 44, 48, 51, 52, 69-71, 75, 81, 85, 93, 94]. Bioreactors have been used to apply other types of loading as well. Macroscopic tissue shear deformation (amplitude 0.5-6%, frequency 0.01-1 Hz) also stimulates proteoglycan and protein synthesis over control levels statically held at the same offset compression [21, 40, 41]. Hydrostatic pressures in the physiological range (5-15MPa) have also been shown to increase matrix synthesis in cultured bovine articular cartilage explants, in a manner dependent on the duration of loading and source site within the joint [27, 70], while lower pressures (0.5-2.0 MPa) inhibit biosynthetic activity in cultured bovine and human articular cartilage explants [53].

Similar types of bioreactors have been used to apply mechanical deformation to tissue engineered cartilage constructs during culture, to modulate the biochemical and biomechanical properties of the resultant cartilaginous tissue formed *in vitro* [14, 15, 28]. Static and dynamic tissue deformation in compression modulates matrix synthesis in chondrocytes seeded into agarose hydrogels [12, 50, 60, 61], polyglycolic acid (PGA) [16], and collagen type I [33] or type II [49] scaffolds. As with cartilage explants, static and low-frequency dynamic loading protocols generally inhibit GAG synthesis in constructs in a dose-and time dependent manner, while higher frequencies of dynamic loading tend to stimulate matrix synthesis [12, 16, 49, 50, 60, 61]. For

cartilaginous constructs, biosynthetic response to loading increases with time in culture [12, 49, 60, 61], suggesting that extracellular matrix, which builds up over time in culture, plays a role in chondrocyte detection of the loading signal. Intermittent application of cyclic shear forces to chondrocyte-seeded calcium polyphosphate substrates during 4 weeks of culture also led to more cartilaginous tissue production, which contained 40% more collagen and 35% more proteoglycan and was mechanically stronger than unstimulated controls [96, 97].

Other types of bioreactor culture systems have been designed to allow for the mechanical environment of cartilaginous constructs to be determined by the fluid flow pattern of the media through the system. For example, rotating wall bioreactors allow for continuous replenishment of nutrients and laminar flow shear stimulation of cells embedded in constructs. Cartilaginous constructs cultured in rotating wall bioreactors had higher cell numbers, wet weights, and extracellular matrix production than statically cultured controls [22, 73], as well as increased compressive properties [59, 95]. Another type of bioreactor allows direct perfusion of culture media through the forming cartilaginous tissue [19]. Chondrocyte-PGA constructs cultured in this type of bioreactor had higher cell proliferation [19] and increased sGAG synthesis [72] compared to statically cultured controls; effects that are both dose-dependent and time-dependent [17].

Thus many different approaches can be used to determine effects of mechanical stimulation on chondrocyte metabolism, and each experimental configuration allows for different types of hypotheses to be addressed. For example, the biaxial motion bioreactor developed in this thesis was developed to apply well-

defined mechanical stimuli, though chondrocytes in cartilage explants may not behave exactly the same way as chondrocytes in intact cartilage remaining in the joint. On the other hand, the whole-joint bioreactor was developed to apply more physiological, though more complex and less theoretically defined, mechanical stimulation to cartilage in its native configuration within the joint.

Finally, it should be noted that while certain mechanical stimulation protocols stimulate matrix synthesis in articular cartilage explants, loading amplitudes must also remain low enough to avoid causing cell death. Dynamic loading of 0.1 MPa does not cause cell death for up to 72 hours of loading, but loads of 1 MPa and above can lead to cell death and collagen network damage in the superficial zone within 1 hour of loading [13]. Static loads also cause cell death more quickly (3h) than dynamic loads (6h) of the same magnitude (1 MPa and above) [56].

1.5 Chemo-Mechanical Regulation of PRG4 Metabolism

PRG4 synthesis and expression by chondrocytes in culture are markedly regulated by chemical factors. Inclusion of fetal bovine serum (FBS) and ascorbate (Asc) in culture media upregulates PRG4 secretion levels [79]. Certain growth factors and cytokines that are present at high levels in injury and arthritis also affect PRG4 metabolism. For example, transforming growth factor-β1 (TGF-β1) increases PRG4 mRNA synthesis by chondrocytes cultured in monolayer [6], and protein secretion by chondrocytes in explants [78] and 3D constructs [20]. Conversely, interleukin-1 (IL-1α) has inhibitory effects on PRG4 mRNA [20] and protein synthesis [78]. Thus the chondrocyte microenvironment is a key determinant of PRG4 metabolism.

Since mechanical loading also affects chondrocyte microenvironment, it has been hypothesized that mechanical stimuli can also regulate PRG4 metabolism, and recent *in vivo* experimental observations support this hypothesis. During embryonic development of the mouse elbow joint, PRG4 mRNA expression begins at the onset of joint cavitation [74], suggesting that PRG4 expression might be induced by the initiation of relative motion between the articular surfaces. A similar pattern is seen during post-natal growth, where fetal bovine cartilage exhibits inconsistent PRG4 expression by chondrocytes near the articular surface, in contrast with adult tissue, which has abundant PRG4-expressing cells near the surface [83]. In both cases (*in utero* and *in vivo*), increased chondrocyte expression of PRG4 coincides with increased joint motion. Furthermore, in a meniscectomy-induced (i.e., mechanically induced) osteoarthritis model in sheep, abnormal joint motion resulted in degeneration

of articular cartilage in certain regions of the tibial plateau, with decreased PRG4 proteoglycan staining in these regions [100]. Staining was also higher in covered than uncovered regions for both lateral and medial plateaus, suggesting that intrinsic site-associated variations in PRG4 expression could be due to mechanical factors as well.

In vitro studies, published during the time of this dissertation work, have also demonstrated that certain mechanical stimuli can regulate PRG4 mRNA expression by chondrocytes cultured in cartilaginous constructs. Dynamic surface motion applied to chondrocytes embedded in polyurethane constructs [23], and cyclic tensile strain [99] applied to chondrocytes in alginate constructs resulted in increased PRG4 mRNA expression compared to that of unloaded controls, further supporting the hypothesis that chondrocyte PRG4 metabolism depends on mechanical loading. However, the effects of mechanical stimulation on PRG4 protein production by chondrocytes in their native extracellular matrix remain to be elucidated, and are therefore investigated in this dissertation.

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CHAPTER 2

STATIC AND DYNAMIC COMPRESSION REGULATE CARTILAGE METABOLISM OF PROTEOGLYCAN 4

2.1 Abstract

The boundary lubrication function of articular cartilage is mediated in part by molecules at the articular surface and in synovial fluid, encoded by *Prg4*. The objective of this study was to determine whether static and dynamic compression regulate PRG4 biosynthesis by cartilage explants. Articular cartilage disks were harvested to include the articular surface from immature bovines. Some disks were subjected to 24 h (day 1) of loading, followed by 72 h (days 2-4) of free-swelling culture to assess chondrocyte responses following unloading. Loading consisted of 6 or 100 kPa of static compression, with or without superimposed dynamic compression (10 or 300 kPa peak amplitude, 0.01 Hz). Other disks were cultured free-swelling as controls. PRG4 secretion into culture medium was inhibited by all compression protocols during day 1. Following unloading, cartilage previously subjected to dynamic compression to 300 kPa exhibited a rebound effect, secreting more PRG4 than did controls, while cartilage previously subjected to 100 kPa static loading secreted less PRG4. Immunohistochemistry revealed that all compression protocols also affected the number of cells expressing PRG4. The paradigm that mechanical

stimuli regulate biosynthesis in cartilage appears operative not only for load bearing matrix constituents, but also for PRG4 molecules mediating lubrication.

2.2 Introduction

Articular cartilage functions to provide a low-friction, load bearing surface which allows the bones of diarthrodial joints to slide smoothly against each other while transmitting load [32]. Cartilage tissue has classically been divided into three zones: superficial, middle, and deep, with distinct biochemical content and organization that impart specific functions to each zone. The middle and deep zones provide load bearing, while the superficial zone mediates low-friction sliding at the cartilage surface [3]. This lubrication function is mediated in part by proteoglycan molecules synthesized from the proteoglycan 4 (*Prg4*) gene by tissues surrounding the joint cavity [11-13, 27, 33, 34].

The *Prg4* gene encodes multiple similar proteins, which have been identified in a number of tissues and are therefore known by several names: MSF, CACP, HAPO, Lubricin, SZP, and PRG4. Megakaryocyte stimulating factor (MSF) was originally purified from urine, and stimulates platelet-forming cells [21, 37]. Mutations in the PRG4 gene cause camptodactyly-arthropathy-coxa vara-pericarditis (CACP) syndrome in humans [20], which results in early onset non-inflammatory joint failure [1], demonstrating the functional importance of expression of this gene *in vivo*. Hemangiopoietin (HAPO), an alternatively spliced isoform, simulates both hematopoietic progenitor and endothelial cells [19]. Lubricin, originally purified from synovial fluid [34-36], is highly expressed by cells of the synovial lining, and functions to reduce friction in latex-on-glass [11-13] and cartilage-on-glass [33, 34] friction assays. Superficial zone protein (SZP) was first isolated from conditioned

medium of cultured superficial zone (but not deep zone) cartilage explants [28], and also reduces friction in cartilage-on-cartilage sliding [27]. In this paper these molecules will be collectively referred to as PRG4 [10].

Biomechanical regulation of metabolism of matrix molecules such as aggrecan and collagen, by chondrocytes is well documented for both *in vivo* and *in vitro* stimulation protocols (reviewed in [7, 9]). In general, static compression inhibits matrix biosynthesis, while dynamic compression and dynamic shear at certain frequencies and amplitudes can stimulate matrix metabolism in cartilage explants [14, 23]. While effects of mechanical stimuli on lubricant molecules are not as widely studied, recent evidence suggests that mechanical stimuli might also regulate PRG4 metabolism. During embryonic development of the mouse elbow joint, PRG4 mRNA expression begins at the onset of joint cavitation [22], suggesting that PRG4 expression might be turned on by the initiation of loading of the articular surfaces. A similar pattern is seen during post-natal growth, as fetal bovine cartilage exhibits inconsistent PRG4 staining at the articular surface and in cells near the surface, in contrast with adult tissue, which stains intensely for PRG4 in these locations [29]. In both cases (in utero and *in vivo*), increased expression of PRG4 coincides with increased joint loading. Regulatory effects of mechanical loading *in vitro* have also been reported, as dynamic tension [38] and dynamic surface motion [6] can upregulate PRG4 mRNA levels in chondrocyte-seeded constructs.

While these studies implicate a role for mechanical stimuli in regulating chondrocyte expression of PRG4, the effects of such stimulation on PRG4 expression by chondrocytes within their native extracellular matrix remain unknown. Thus the

objective of this study was to determine whether graded levels of static and dynamic compression regulate PRG4 metabolism in cartilage explants, assessed by (1) PRG4 secretion into culture media, and (2) depth-associated variation in localization of PRG4 expressing cells.

2.3 Materials and Methods

Cartilage explant. Cartilage disks were obtained as described previously [18]. Briefly, knees from 4 immature (1-3 week old) bovines were obtained from an abattoir. Under sterile conditions and with irrigation using phosphate buffered saline (PBS) supplemented with antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B; all from Gibco BRL, Grand Island, NY), 9 mm-diameter osteochondral cores were harvested from the patellofemoral groove using Osteochondral Autograft Transfer System (Arthrex, Naples, FL). These cores were cut parallel to the articular surface with a sledge microtome (Microm, Waldorf, Germany) to obtain slices (1 mm thick) including the intact articular surface. From these slices, smaller disks (3 mm diameter) were obtained using a stainless steel dermal punch (Miltex GmbH, Tuttlingen, Germany).

Culture and mechanical stimulation of cartilage explants. Cartilage disks were incubated in a humidified atmosphere of 5% CO_2, 95% air at 37ºC with medium (low-glucose Dulbecco's modified Eagle's medium, 10 mM HEPES buffer, 0.1 mM non-essential amino acids, 0.4 mM L-proline, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B; 1 ml per disk per day), supplemented with 10% FBS and 50 μg/ml ascorbate. Some disks were subjected to 24 h (day 1) of graded levels of continuous static (6 kPa (**S_6**) or 100 kPa (**S_{100}**)) or dynamic (3-10 kPa (**D_{10}**) or 3-300 kPa (**D_{300}**), 0.01 Hz) axial loading (**Fig. 2.1**) in load-controlled unconfined compression with fluid-impermeable polysulphone platens using custom-compression chambers described previously [18]. Static

compression was applied as a step-load, and amplitudes were equal to the static offset of the dynamic waveforms. Other disks were cultured free-swelling (unloaded) as controls. All disks were then placed into free-swelling culture for a subsequent, 72 h recovery period (days 2, 3, 4) to assess chondrocyte responses following unloading (**Fig. 2.1**). Control studies, with staining for live and dead cells using calcein AM and ethidium homodimer (Live-Dead®, Molecular Probes, Eugene, OR) and threshold-based image analysis with Matlab 7.0 software (The Mathworks, Inc., Natick, MD), confirmed that the loading protocols did not affect chondrocyte viability (84-95%, p=0.62). The automated image analysis software was validated by comparison with manual counts of live and dead cells.

PRG4 Secretion. Conditioned medium samples, collected and replaced after each 24 h period (**Fig. 2.1**), were quantitatively analyzed for PRG4 by indirect ELISA, as previously described [16] using mAb 3-A-4 (a generous gift from Dr. Bruce Caterson, University of Wales, Cardiff, UK) [29]. Briefly, samples were diluted serially, adsorbed, and then reacted with mAb 3-A-4, horseradish peroxidase-conjugated secondary antibody, and ABTS substrate, with 3 washes with PBS-0.1% Tween (Bio-Rad, Hercules, CA) between each step. A standard curve was generated from samples containing known amounts of PRG4, obtained from conditioned medium from explants from the superficial zone of bovine calf cartilage as previously described [28]. The protein-equivalent amount of PRG4 in each sample was calculated from the linear region of the standard curve (between 0.078 and 5 μg/ml of PRG4), as described elsewhere [16, 30]. Sample PRG4 protein content is expressed normalized to cartilage surface area. Control studies indicated that cartilage disks contained PRG4

in amounts, ~1 μg/cm^2, that were small relative to that secreted into the medium, so that the secreted quantities were representative of biosynthesis levels. Cartilage from the middle zone (1 mm thick, from 1 to 2 mm below the articular surface) subjected to the same loading protocols did not secrete PRG4 at levels above the detection of our assay for any of the loading conditions, and therefore were not analyzed further.

PRG4 Immunolocalization. The presence of PRG4 within chondrocytes was determined qualitatively in disks from cultures terminated at 24, 48, and 72 h (i.e., at the end of loading, and 24 or 36 h after unloading, **Fig. 2.1**). For the 4 h just prior to termination, these disks were incubated with medium supplemented with 1 μM monensin. Upon termination, the disks were frozen in Tissue Tek OCT (Sakura USA, Torrance, CA) and sectioned (5 μm slices) normal to the articular surface. Immunohistochemistry (IHC) was performed as described previously [16]. The sections were reacted with mAb 3-A-4, and detected with a peroxidase-based system. The stained samples were viewed to identify immunoreactive cells, indicating synthesis of PRG4. Sections probed with a non-specific mouse IgG antibody served as negative controls. Qualitative results were documented by photomicroscopy. The number of cells that were PRG4+ was determined by manual identification, and counted as a function of depth from the articular surface using custom Matlab code. Briefly, PRG4+ cells were counted in a representative 300 μm wide x 400 μm deep region of each section. Results are expressed as number of PRG4+ cells per area in each successive 50 μm bin below the articular surface, and as cumulative number of PRG4+ per area with depth from the articular surface. Since the thickness of free-swelling cartilage increased (approximately 20%, data not shown) during culture, and

that of compressed tissues did not (neither during loading nor after unloading), bin sizes were adjusted for compressed tissues. For these tissues, bins near the articular surface were 35 μm thick, and increased with depth from the articular surface to 49 μm thick at 320 μm total depth (equivalent to the 400 μm depth on unloaded tissues that swelled 20%). This modified strain distribution was approximated for unconfined compression based on axial strain profiles determined previously [15] for axially compressed immature bovine cartilage explants including the articular surface.

Statistical Analysis. Data are expressed as mean ± SEM. N=5-12, from 2-4 animals. PRG4 secretion data were log-transformed for statistical analysis to improve the uniformity of variance, but results are presented untransformed (**Fig. 2.2A**) and normalized to unloaded secretion levels (**Fig. 2.2B**) for ease of interpretation. Effects of loading condition on PRG4 secretion and number of PRG4+ cells were assessed using ANOVA with repeated measures for day of culture (PRG4 secretion) and depth from articular surface (number of PRG4+ cells). Dunnett's post hoc tests were used to compare secretion and expression by control cartilage to that of each loading condition. Statistical analysis was implemented with Systat 10.2 (Systat Software, Inc., Point Richmond, CA).

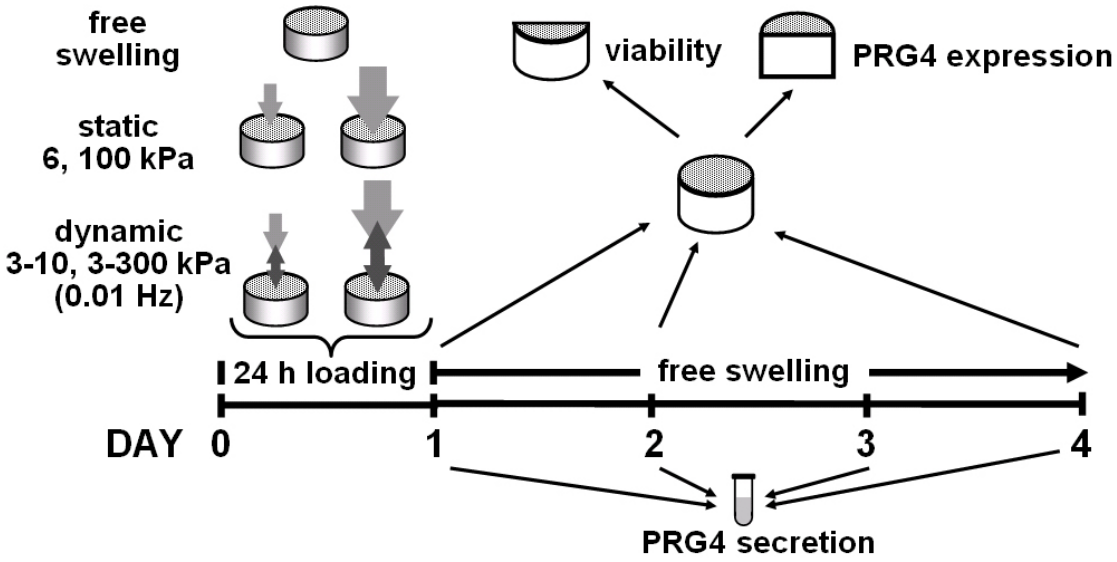


Figure 2.1. Experimental design. Cartilage was loaded for 24 h (day 1), followed by 72 h (days 2, 3, 4) unloaded culture. PRG4 secretion (by indirect ELISA) into culture media was determined at the end of each 24 h period, while chondrocyte viability (by Live-Dead stain) and PRG4 expression (by immunohistochemistry) were assessed at the end of days 1, 2, and 4. Loading protocols included static compression to 6 kPa (S_6) or 100 kPa (S_{100}), alone or with superimposed dynamic (0.01 Hz) loading with peak amplitudes of 10 kPa (D_{10}) or 300 kPa (D_{300}).

2.4 Results

PRG4 secretion into culture media by cartilage disks was affected by both static and dynamic compression at both amplitudes tested (**Fig. 2.2**). Secretion was inhibited by all compression protocols (from 21.0±1.7 μg/cm^2 by free-swelling disks to an average 10.5±1.3 μg/cm^2 by loaded disks, $p<0.01$ each) during the 24 hours of continuous static and dynamic loading (day 1). Following unloading (day 2), secretion rates returned to control levels (28.7±3.7 μg/cm^2) for all cartilage except that in group **D_{300}**, which on average secreted 46% more than controls ($p<0.001$). During subsequent days following unloading (days 3 & 4) all cartilage continued to secrete similar levels of PRG4 regardless of day 1 loading condition, except **S_{100}**, which on average secreted only 45% of the control level on day 3 ($p=0.057$), and 40% on day 4 ($p<0.05$).

Depth-associated variation in chondrocyte expression of PRG4 was also modulated by static and dynamic compression. In free-swelling tissue, many cells were PRG4+ in the top 0 to 100-200 μm (**Fig. 2.3B,H,N, and 2.4**), with very few cells deeper than 200 μm expressing PRG4. This pattern was consistent throughout the duration of culture (**Fig. 2.4**). In contrast, during the 24 h loading period (Day 1, **Fig. 2.3B-F, 2.4A,B**), all compressed tissues had more PRG4+ cells deeper than 200 μm from the articular surface than did controls, a trend appearing opposite to the decreased secretion of PRG4 by compressed tissues during loading. During the 24 h following unloading (**Fig. 2.3H-L, 2.3C,D**), the number of PRG4+ cells below 200 μm remained elevated over control levels for all previously loaded cartilage, except

that previously statically loaded to 100 kPa, which had very few PRG4+ cells, even in the top 100 μm. By day 4, the profile of PRG4 expressing cells was again similar to that of controls (PRG4+ cells only in the top ~100 μm) for all loaded groups (**Figs. 2.3N-R, 2.4E,F**), except in $\mathbf{S_{100}}$, where very few cells were PRG4+ anywhere in the tissue (**Figs. 2.3P, 2.4E,F**), consistent with the return of PRG4 secretion to control levels all by groups except $\mathbf{S_{100}}$, which secreted less PRG4 than controls. Chondrocytes below 400 μm of depth did not express PRG4 under any of the imposed conditions (data not shown). IgG controls were appropriately PRG4 negative (**Fig. 2.2A,G,M**) for all conditions.

The total number of PRG4+ chondrocytes (deepest bins, **Fig. 2.4B**) in the area counted was higher ($p<0.01$) in cartilage subjected to $\mathbf{S_{100}}$ and $\mathbf{D_{300}}$ than in unloaded cartilage during day 1. Cartilage subjected to $\mathbf{S_6}$ and $\mathbf{D_{10}}$ showed similar, though not statistically significant trends. All loading groups exhibited total numbers of PRG4+ cells statistically similar to those of controls on day 2 (**Fig. 2.4D**) and day 4 (**Fig. 2.4F**), except cartilage subjected to $\mathbf{S_{100}}$, which had fewer ($p<0.01$) PRG4+ cells than controls.

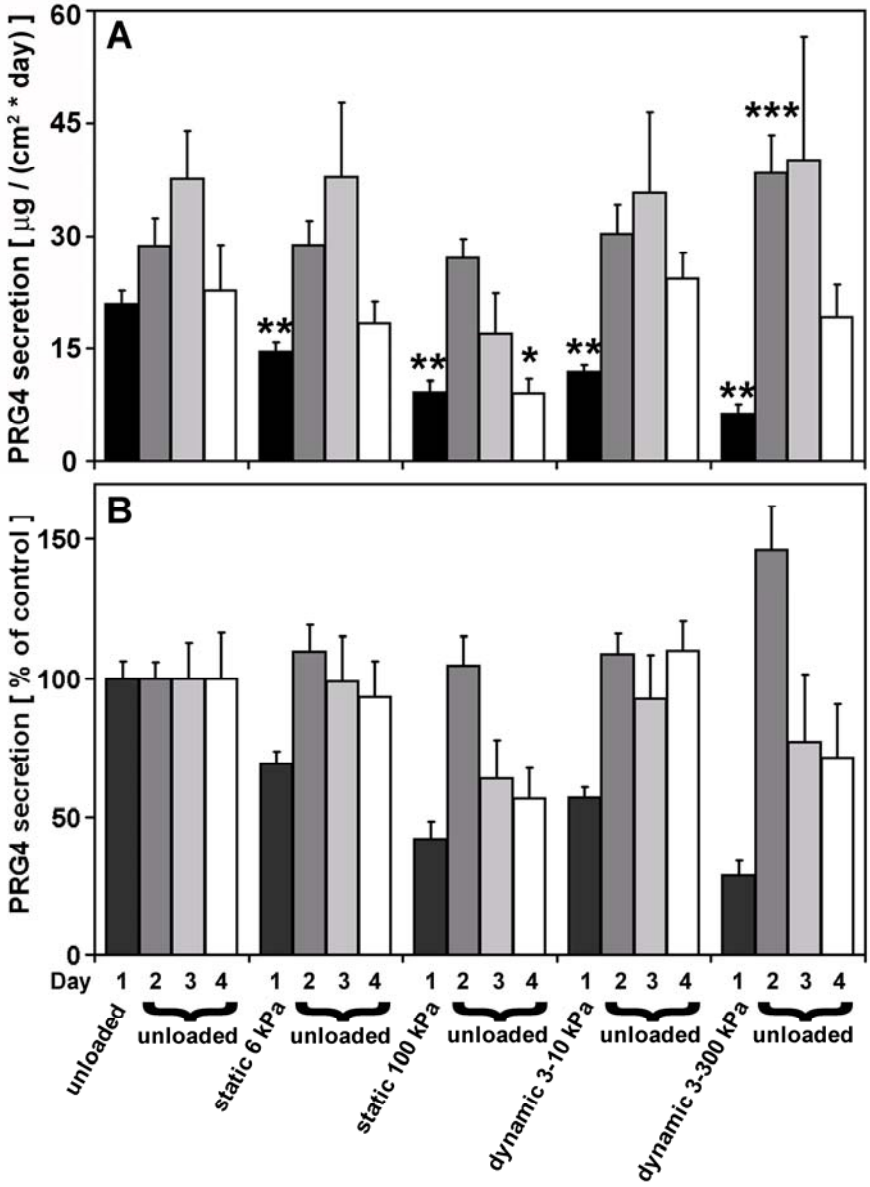


Figure 2.2. Absolute (**A**) and normalized (**B**) PRG4 secretion by cartilage during 24 hours of continuous static or dynamic compression of various magnitudes (Day 1), followed by 72 hours (Days 2-4) of unloaded culture. Mean±SEM, n=5-12. Differences from unloaded values for a given day are represented by *($p<0.05$), **($p<0.01$), ***($p<0.001$).

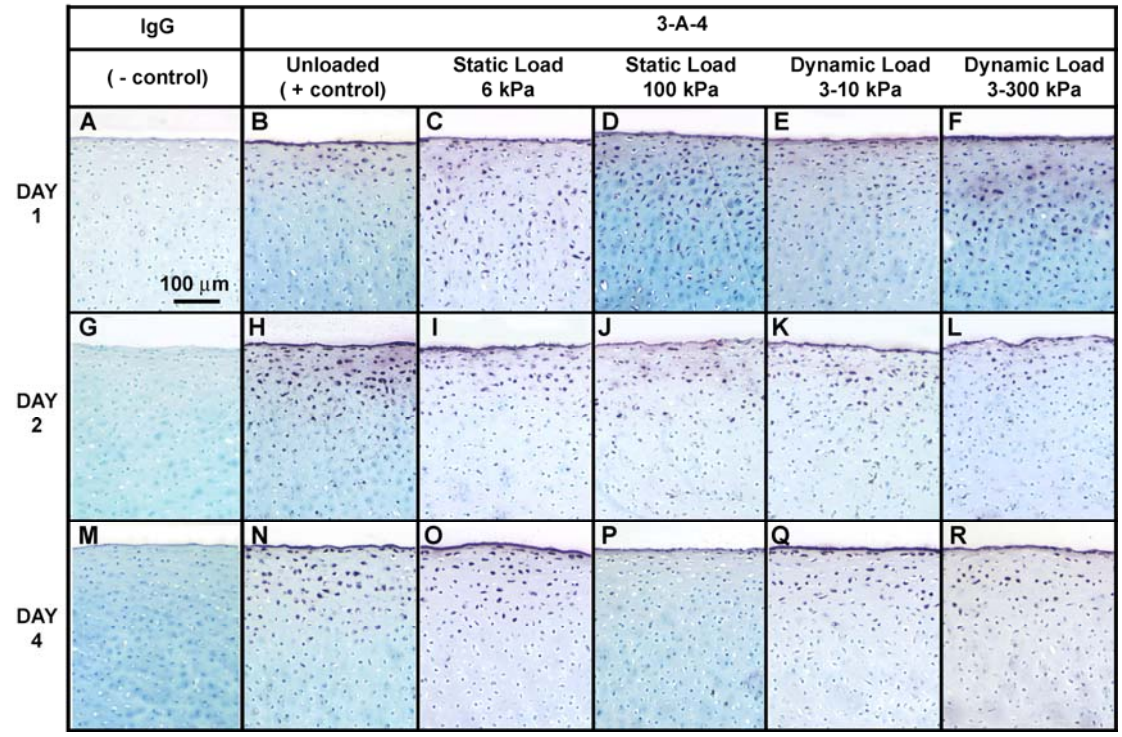


Figure 2.3. PRG4 immunolocalization in tissue subjected to 24 hours of continuous static or dynamic loading of various magnitudes, immediately following loading (**A-F**), 1 day after removal of load (**G-L**), and 3 days after removal of load (**M-R**). IgG labeled controls (**A,G,M**) show PRG4 negative surfaces and cells, while unloaded controls labeled with 3-A-4 antibody (**B,H,N**) show PRG4 positive surfaces and PRG4 positive cells in the top 100 μm.

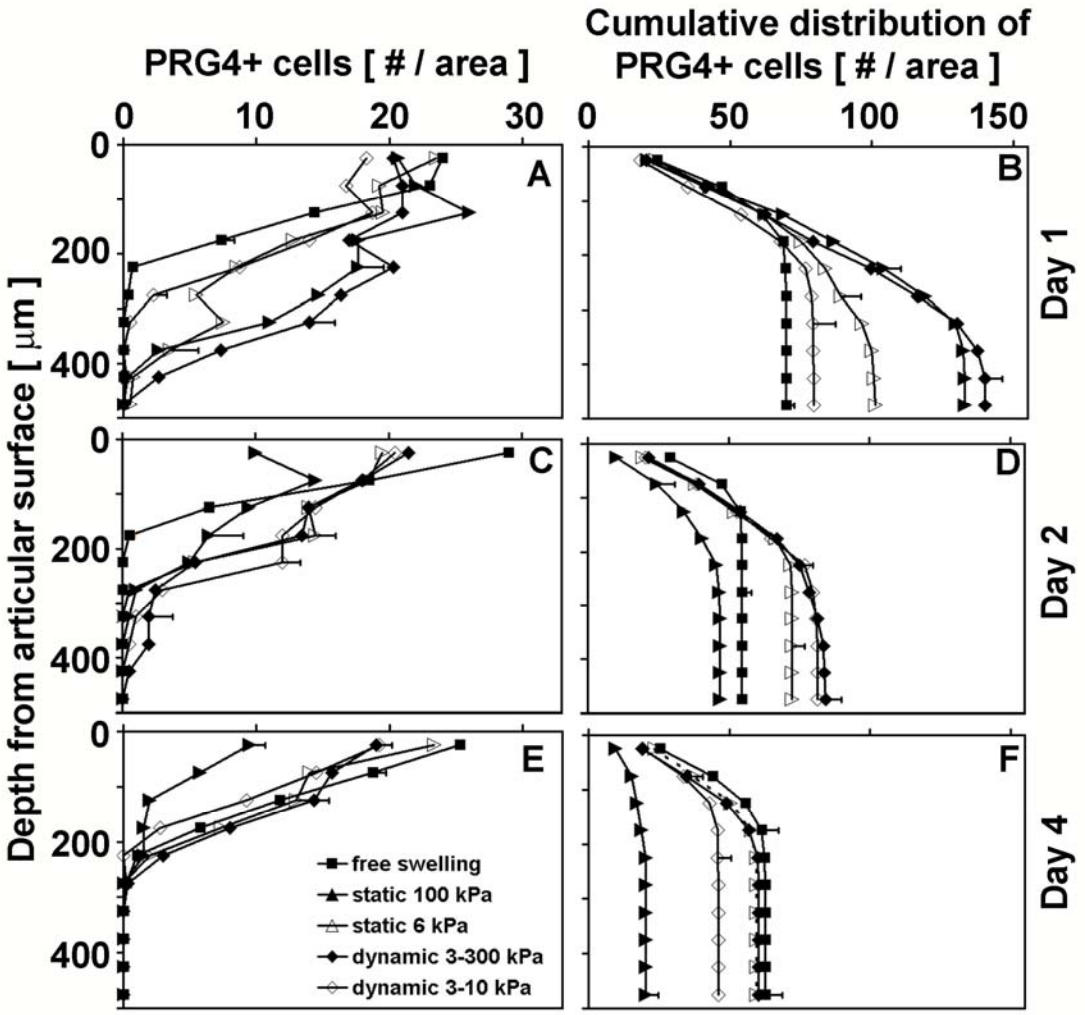


Figure 2.4. Depth-associated variation in chondrocyte expression of PRG4 in unloaded and loaded cartilage, represented as number of cells per area (**A,C,E**), and as cumulative number of PRG4+ cells with depth from surface (**B,D,F**), on day 1 (**A,B**), 2 (**C,D**), and 4 (**E,F**). Mean±SEM, with representative SEM shown for each loading condition, n=5-12.

2.5 Discussion

These results demonstrate marked magnitude- and time-dependent regulatory effects of mechanical stimuli on cartilage biosynthesis of PRG4 lubricant molecules. PRG4 secretion was inhibited by all compression protocols during the 24 h loading period (**Fig. 2.2**), but chondrocyte response following unloading depended on previous loading condition, with secretion returning to (**S_6** and **D_{10}**), exceeding (**D_{300}**), or remaining below (**S_{100}**) control levels. All compression protocols increased the number of PRG4+ cells below 200 μm during loading, with expression patterns more similar to those of controls following unloading, except for cartilage previously subjected to **S_{100}**, in which expression remained low throughout the duration of culture (**Figs. 2.3, 2.4**). Thus the paradigm that mechanical stimuli regulate the synthesis, assembly, and release of molecules in cartilage tissue appears operative not only for aggrecan and collagen constituents involved in load bearing, but also for PRG4 molecules that mediate lubrication.

These results support the hypothesis that chondrocyte metabolism depends on its local microenvironment, which is determined by a complex combination of imposed chemical and mechanical stimuli. Previous work has shown that PRG4 synthesis and expression by chondrocytes in culture are markedly regulated by chemical factors. Inclusion of fetal bovine serum (FBS) and ascorbate in culture media upregulates PRG4 secretion levels [26]. Certain growth factors and cytokines that are present at high levels in injury and arthritis also affect PRG4 metabolism. For example, transforming growth factor-β1 (TGF-β1) increases PRG4 mRNA synthesis

by chondrocytes cultured in monolayer [2], as well as protein secretion by chondrocytes in explants [25] and 3D constructs [5]. Conversely, interleukin-1 (IL-1α) has inhibitory effects on PRG4 mRNA [5] and protein [25] synthesis. These results, combined with the present work, demonstrate that both chemical and mechanical stimuli can independently up- or down-regulate PRG4 metabolism by chondrocytes.

Compression protocols were selected based on previous work [18], in which $\mathbf{D_{300}}$ and $\mathbf{S_{100}}$ regulated biosynthesis of matrix molecules for immature bovine cartilage explants of this geometry. While 0.01 Hz is not a loading frequency experienced in common daily activities such as running or walking, it is within the range of frequencies that consistently upregulate matrix biosynthesis over a range of loading amplitudes [23] throughout 3 mm diameter cartilage disks, whereas compression at 0.1 Hz increases biosynthesis only in the outer radial periphery [14]. In addition, continuous passive motion treatments *in vivo* use frequencies (0.025 Hz) of this order of magnitude [24]. The load magnitudes used here are low to intermediate within the range used typically for stimulating cartilage explants [4, 8, 17, 31], but since $\mathbf{D_{300}}$ and $\mathbf{S_{100}}$ had significant effects on matrix biosynthesis in previous studies [18], their effects on PRG4 metabolism in this explant loading configuration were deemed relevant. Lower level static and dynamic loading protocols ($\mathbf{S_6}$, $\mathbf{D_{10}}$) were also employed to determine whether the effects of compression were dose- (magnitude-) dependent. Compression with impermeable platens may have decreased the ability of PRG4 to exit the tissue into the culture medium. However, if this was the only effect resulting in decreased secretion during loading, presumably all of the PRG4 synthesized during this time would be free to exit the tissue upon unloading, resulting

in day 2 secretion levels high enough to make up for the decrease seen in day 1, which did not occur (**Fig. 2.2**).

These results are consistent with the paradigm that in general, static compression to a high enough level inhibits cartilage biosynthesis, while dynamic compression can have stimulatory effects, and expand this paradigm to include regulation of PRG4 lubricant biosynthesis. The findings also expand upon the results from a recent study [6] in which chondrocyte-seeded polyeurethane constructs were subjected to compressive stimulation to 10, 20, or 40% peak strain. PRG4 mRNA expression was decreased somewhat by loading to 10% peak strain whereas higher level loading did not affect expression. Together, this result and the current data suggest that compressive regulation of PRG4 secretion may occur at the translation level, while transcription remains relatively unchanged. In contrast, in that same study Grad et al did not detect PRG4 in conditioned medium from compressed or unloaded constructs, though this discrepancy may be due to differences in the sensitivity of the ELISA method used here, and the Western blot assay following purification steps used in that study.

As IHC results provide snapshots in time of the pattern of chondrocyte PRG4 expression, while PRG4 detected in medium samples represents an average of secretion over 1 day, it is not surprising that increased secretion concurred with increased expression by deeper chondrocytes (**Fig. 2.4A,C,E**), and total number of PRG4+ cells in the areas counted (**Fig. 2.4B,D,F**) in some cases, but not in others. A possible explanation results from the qualitative nature of IHC methods: even though compression clearly stimulates cells deeper in the tissue to express PRG4, the quantity

of PRG4 produced may in fact be very small relative to that produced by more superficial chondrocytes, and thus would not affect the quantity measured in the media. Alternatively, PRG4 released from cells deeper in the tissue may get trapped in the tissue as matrix molecule concentrations increase with depth from the surface, and may prevent large molecules such as PRG4 from being easily transported out of the tissue.

Nevertheless, the marked increase in expression by deeper cells due to compression suggests that these cells are receiving some signal that unloaded cells at this depth do not receive. Since the deformation experienced by cartilage explants under compression is not homogeneously distributed throughout the depth of the tissue [15], with highest strain at the articular surface that decreases rapidly with depth to approximately 400 μm, perhaps the chondrocytes alter PRG4 synthesis in response to strain above a certain threshold. This would be consistent with the fact chondrocytes deeper than 400 μm did not express PRG4 under any conditions tested.

Understanding the role of mechanical stimuli in regulating PRG4 metabolism could have implications for tissue engineering strategies that aim to replicate lubrication function at the surface of cartilaginous constructs formed *in vitro*, and for understanding the role of mechanical stimuli in regulating the processes of wear and degeneration. If PRG4 accumulation in the synovial fluid determines its function as a boundary lubricant at the articular surface, it is important to understand the contribution by cartilage, and how this contribution can be regulated by mechanical factors. Loss of PRG4 molecules in the synovial fluid, due to decreased secretion by the surrounding tissues, could lead to tissue damage or even degeneration, so

mechanical stimuli that increase PRG4 secretion could help maintain a healthy joint, while those that decrease secretion could result in damage to the articular surface.

2.6 Acknowledgments

This chapter is a reprinted from *Biorheology*, 43(3-4), G.E. Nugent, T.A. Schmidt, B.L. Schumacher, M.S. Voegtline, W.C. Bae, K.D. Jadin, and R.L. Sah, Static and dynamic compression regulate cartilage metabolism of PRoteoGlycan 4 (PRG4), p. 121-200, Copyright 2006, with permission from IOS Press. The dissertation (primary investigator) thanks the co-authors of the manuscript for their contributions: Tannin A. Schmidt, Barbara L. Schumacher, Dr. Michael S. Voegtline, Dr. Won C. Bae, and Kyle D Jadin. In addition, we thank the funding sources that supported this work: Arthritis Foundation, NASA, NIH, NSF, Whitaker Foundation (pre-doctoral fellowship to GEN).

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CHAPTER 3

DYNAMIC SHEAR STIMULATION OF BOVINE CARTILAGE BIOSYNTHESIS OF PROTEOGLYCAN 4

3.1 Abstract

Objective. The boundary lubrication function of articular cartilage is mediated in part by proteoglycan 4 (PRG4) molecules at the articular surface and in synovial fluid. The objective of this study was to determine the effects of dynamic shear stimulation on PRG4 biosynthesis by bovine cartilage explants. Methods. Cartilage disks with intact articular surfaces were harvested from immature bovines. Some disks were subjected to 24 hours (day 1) of loading, consisting of a step load of 20% static compression either alone or with superimposed dynamic shear (3% amplitude and 0.1 Hz), while other disks were cultured free-swelling as controls. After the 24-hour loading period, disks were terminated or were further incubated for up to 72 hours (days 2–4) in free-swelling culture to assess chondrocyte responses to, and following, unloading. PRG4 products secreted into culture medium were quantified by enzyme-linked immunosorbent assay and characterized by Western blotting. Chondrocytes

expressing PRG4 were localized by immunohistochemistry, and depth-associated variations in chondrocyte PRG4 expression were quantified by image analysis. Results. Dynamic shear stimulation increased PRG4 secretion to 3–4 times that of unloaded controls and statically compressed samples. Sheared cartilage secreted more PRG4 of 345 kd relative to smaller molecular weight species, as compared with unloaded controls. Immunohistochemistry revealed that shear stimulation also increased the total number of cells expressing PRG4 by inducing expression by cells at a depth of 200–400 μm. Conclusion. The paradigm that certain mechanical stimuli up-regulate biosynthesis in cartilage appears operative not only for load-bearing matrix constituents, but also for PRG4 molecules that mediate lubrication.

3.2 Introduction

Articular cartilage functions to provide a low-friction, load-bearing surface that allows the bones of diarthrodial joints to slide smoothly against each other while transmitting load. Cartilage tissue has classically been divided into 3 zones (superficial, middle, and deep) with distinct biochemical content and organization that impart specific functions to each zone. Chondrocytes in the middle and deep zones produce large amounts of aggrecan and collagen, which provide the load-bearing function of these zones. In addition to producing aggrecan and collagen, chondrocytes of the superficial zone also secrete specialized molecules, one of which is encoded by the proteoglycan 4 (PRG4) gene (GenBank accession no. AF056218 for bovine partial sequence and U70136 for complete human sequence) [5, 14, 26, 35].

PRG4 molecules mediate, at least in part, the lubrication function of the articular cartilage surface layer [15-17, 34, 39, 40]. Mutations in the PRG4 gene can cause camptodactyly-arthropathy–coxa vara–pericarditis syndrome in humans [24], which results in early-onset noninflammatory joint failure [1] that is due in part to articular surface alterations and subsequent cartilage deterioration [29], demonstrating the functional importance of the gene *in vivo*. Lubricin, a 227-kd product of the PRG4 gene originally purified from synovial fluid [40, 41], is highly expressed by cells of the synovial lining and functions to reduce friction in latex-on-glass [15-17] and cartilage-on-glass [39, 40] friction assays. Superficial zone protein (SZP), a 345-kd product of the PRG4 gene, was first isolated from conditioned medium derived from cultured explants of cartilage from the superficial zone, but not the deep zone [35].

SZP reduces friction in cartilage-on-cartilage sliding [34]. These proteoglycan products of the PRG4 gene [14] will also be referred to below as PRG4.

Biomechanical regulation of chondrocyte metabolism of cartilage matrix molecules, such as aggrecan and collagen, is well documented both *in vivo* and *in vitro* (for review, see refs. [10, 11]). In general, static compression inhibits matrix biosynthesis, whereas dynamic compression and dynamic shear at certain frequencies and amplitudes can stimulate matrix metabolism, as demonstrated for cultured cartilage explants [6, 18, 19, 30]. While mechanical regulation of the metabolism of lubricant molecules is not as widely studied, recent evidence suggests that mechanical stimuli might also regulate PRG4 expression in the synovial joint. During embryonic development of the mouse elbow joint, PRG4 messenger RNA (mRNA) expression begins at the onset of joint cavitation [29], suggesting that PRG4 expression might be induced by the initiation of relative motion between the articular surfaces. A similar pattern is seen during postnatal growth, where fetal bovine cartilage exhibits inconsistent PRG4 expression by chondrocytes near the articular surface; in contrast, adult tissue has abundant PRG4-expressing cells near the surface [36]. In both cases (in utero and *in vivo*), increased chondrocyte expression of PRG4 coincides with increased joint motion.

While these studies implicate a role of mechanical stimuli in regulating chondrocyte expression of PRG4, a variety of other factors present *in vivo* may also affect PRG4 expression and metabolism. For example, certain chemical factors, including those present at elevated concentrations in the synovial fluid in osteoarthritis, markedly regulate chondrocyte PRG4 metabolism in cartilage explants

that include the superficial zone [32, 33]. In explant culture, the exogenous chemical stimuli present *in vivo* can be eliminated or controlled to more precisely determine the effects of mechanical stimuli. Cartilage explant cultures also allow for application of well-defined mechanical stimuli to phenotypically stable chondrocytes, since they are still embedded within their native extracellular matrix.

The effects of mechanical stimulation on PRG4 protein expression by chondrocytes within their native extracellular matrix, however, remain unknown. Therefore, the objectives of this study were to determine the effects of dynamic tissue shear stimulation on cartilage metabolism of PRG4, as assessed by the quantity and structure of PRG4 products secreted into medium by chondrocytes in extracellular matrix of their native superficial and middle zones, and to determine chondrocyte expression of PRG4 and cartilage depth–associated variations in cells expressing PRG4.

3.3 Materials and Methods

Cartilage explants. Cartilage disks were obtained as described previously [23]. Briefly, stifle joints from 3 immature bovines (1–3 weeks old) were obtained from an abattoir. Under sterile conditions and with irrigation using phosphate buffered saline (PBS) supplemented with antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B), osteochondral cores (9 mm in diameter) were harvested from the patellofemoral groove using an Osteochondral Autograft Transfer System (Arthrex, Naples, FL). These cores were cut in a sledge microtome (Microm, Waldorf, Germany) to obtain slices (1 mm in thickness) that consisted of the intact articular surface (superficial zone; 0–1 mm from the articular surface) or the middle and deep zones (middle zone; 1–2 mm from the articular surface). From these slices, smaller disks (3 mm in diameter) were punched using a stainless steel dermal punch (Miltex, Tuttlingen, Germany). Finally, day 0 thickness was determined prior to the beginning of culture using a contact-sensing micrometer.

Culture and mechanical stimulation. For the first day, all disks were incubated in medium (low-glucose Dulbecco's modified Eagle's medium, 10 m*M* HEPES buffer, 0.1 m*M* nonessential amino acids, 0.4 m*M* L-proline, 2 m*M* L-glutamine, 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 0.25 μg/ml of amphotericin B) supplemented with 10% fetal bovine serum and 50 μg/ml of ascorbate. Incubations were performed in a custom biaxial motion bioreactor designed by the authors. The bioreactor (similar to that described by Frank et al [6]) consisted of 2 polysulphone parts attached to a stand-alone mechanical tester (model V500cs;

BioSyntech, Laval, Quebec, Canada). The bottom part had 12 independent culture wells (1 disk per well), and the top part had loading rods aligned with 8 wells, such that the other 4 wells allowed simultaneous free-swelling culture. The bioreactor also provided an enclosed, sterile atmosphere containing 5% CO_2 at a temperature of 37°C.

During the first day of culture, disks were subjected to 24 hours (day 1) of 1 of 3 mechanical stimulation conditions: free-swelling (**Fig. 3.1A**), 20% static compression relative to swollen thickness (**Fig. 3.1B**), or 20% static compression with superimposed dynamic sinusoidal simple shear of 0.1 Hz and 3% amplitude, centered about the axis of the disk (**Fig. 3.1C**). In each experiment, 2 experimental groups were subjected to stimulation conditions: groups 1 and 2 or groups 1 and 3. Some disks were terminated immediately upon unloading (lifting the top part of the bioreactor off the samples), at which time day 1 thickness was measured, and metabolic analyses were performed (see below). Other disks were then placed into free-swelling culture in a tissue culture plate and put in a standard incubator for a subsequent 72-hour recovery period (days 2, 3, and 4) to assess chondrocyte metabolic responses to, and following, unloading and were terminated on day 2 or day 4. Disk thickness was also measured at the end of culture (day 4). Compression and shear were applied via movement of the vertical and horizontal actuators, respectively, of the mechanical tester operating in the displacement mode of control. The amplitude of the horizontal load waveform recorded during mechanical stimulation was stable (within 4%) between 1 and 24 hours of loading. For this reason, load and displacement data sets of 1 minute duration (partial set shown in **Fig. 3.1D**) were recorded (using Mach-1 software; BioSyntech) after 1 hour of stimulation as well as 1 hour prior to the end of

stimulation (i.e., n = 4 total for 2 runs of the bioreactor). Cartilage shear modulus (G) was computed from the load and displacement waveforms as $G=\tau/\gamma$ where τ is the amplitude of the horizontal force recorded during stimulation and normalized to the cartilage surface area and γ is the amplitude of the applied horizontal motion divided by the thickness of the tissue [7]. The total harmonic distortion (calculated as 100% times the ratio of a, the square root of the sum of the squares of the powers of all higher harmonic frequencies, to b, the power of the fundamental frequency) of the load waveforms was determined as a measure of the quality of the sinusoidal shear stress imparted to the cartilage.

Metabolic analyses. Enzyme-linked immunosorbent assay (ELISA) for quantification of secreted PRG4. Medium samples that had been collected from cartilage cultures and replaced after each 24-hour period were quantitatively analyzed for PRG4 by indirect ELISA, as previously described [21], using monoclonal antibody (mAb) 3-A-4 (a generous gift from Dr. Bruce Caterson, University of Wales, Cardiff, UK) [36]. Briefly, samples were diluted serially, adsorbed, and then reacted with mAb 3-A-4, horseradish peroxidase–conjugated secondary antibody, and ABTS substrate, with 3 washes of PBS plus 0.1% Tween between each step. A standard curve was generated from samples containing known amounts of PRG4 purified from conditioned medium derived from explants of the superficial zone of bovine calf cartilage, as previously described [35]. The protein-equivalent amount of PRG4 in each sample was calculated from the linear region of the standard curve (between 0.078 μg/ml and 5 μg/ml of PRG4), as described elsewhere [21, 37]. The amount of PRG4 was normalized to the cartilage articular surface area.

Control studies indicated that all cartilage disks contained PRG4 at low levels (~1 μg/cm^2; quantified as previously described for cartilage meniscus [37]) relative to the amount secreted into the medium. Thus, the secreted quantities were representative of biosynthetic levels. Unlike superficial zone disks, cartilage middle zone disks subjected to the same loading protocols did not secrete PRG4 at levels above the detection limits of the assay (~0.1 μg/[cm^2*day]) for any of the loading conditions and were therefore not analyzed further.

Western blotting for characterization of secreted PRG4. Equal portions of medium samples collected from all 4 days of all free-swelling cultures were pooled, as were samples from all dynamically sheared cultures. PRG4 was purified from pooled samples by anion-exchange chromatography with DEAE Sepharose gel, collecting the 0.3–0.6*M* NaCl eluate, and then concentrated with a Centricon Plus filter with a 100-kd molecular size cutoff. These samples were then reduced with 10 m*M* dithiothreitol for 30 minutes at 37°C and alkylated with 50 m*M* iodoacetamide for 30 minutes at 37°C. Samples (0.5 μg of total PRG4 per lane, as determined above by ELISA with mAb 3-A-4) were separated by sodium dodecyl sulfate– polyacrylamide gel electrophoresis on a 4–20%-gradient polyacrylamide gel, transferred to a polyvinylidene difluoride membrane, and probed with mAb 6-A-1 (also a gift from Dr. Caterson), with the ECL Plus detection system (Amersham, Arlington Heights, IL). For visualization, the membrane was exposed to film for 30 seconds.

Immunohistochemistry for chondrocytes expressing PRG4. For free-swelling and dynamically sheared cartilage, the presence of PRG4 within chondrocytes was determined qualitatively in disks from cultures terminated at 24, 48,

and 96 hours (i.e., at the end of loading and at 24 and 72 hours after unloading). For the 4 hours just prior to termination, these disks were incubated with medium supplemented with 1 μ*M* monensin. Upon termination, the disks were frozen in TissueTek OCT and sectioned (5-μm slices) normal to the articular surface. Immunohistochemistry was performed as described previously [21]. The sections were reacted with mAb 3-A-4, which was detected with a peroxidase-based system. The stained samples were examined to identify immunoreactive cells, indicating synthesis of PRG4. Sections probed with a nonspecific mouse IgG antibody served as negative controls. Qualitative results were documented by photomicroscopy.

From these photomicrographs, the depth-associated variation in chondrocyte PRG4 expression was determined. Using a custom program written in Matlab 6.5 software (MathWorks, Natick, MA), we analyzed a representative region of each section measuring 300 μm wide by 400 μm deep. Chondrocytes expressing PRG4 were identified manually. Then, the total number of PRG4+ cells and the number of PRG4+ cells as a function of depth from the articular surface were counted. Results are expressed as the number of PRG4+ cells per area in each successive 50-μm bin below the articular surface. Since the thickness of free-swelling cartilage increased during culture and the thicknesses of all loaded tissues did not (neither during loading nor after unloading), bin sizes were adjusted for loaded tissues based on thickness data collected here as well as axial strain profiles determined previously [20] for axially compressed immature bovine cartilage explants including the articular surface. Thus, for the compressed tissues, bins near the articular surface were 35 μm thick and

increased with depth from the articular surface to 49 μm thick at a total depth of 320 μm (equivalent to the 400-μm depth on unloaded tissues that swelled 20%).

Statistical analysis. Data are expressed as the mean ± SEM. The effects of loading condition on cartilage thickness and PRG4 secretion were assessed using analysis of variance with repeated measures for day of culture. The effects of loading on the total number of PRG4+ cells in the area analyzed, as well as the number of PRG4+ cells in the area at particular depths, were analyzed by unpaired *t*-test. Tukey's post hoc test was performed to determine the effects of load within a given day. Statistical analysis was implemented with SyStat 10.2 (SyStat, Evanston, IL).

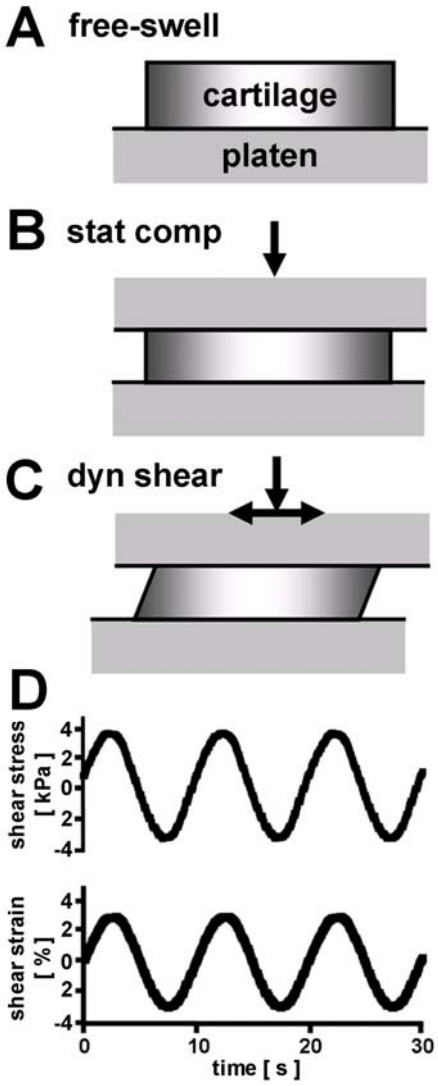


Figure 3.1. Mechanical stimulation of bovine articular cartilage. Cartilage was cultured under one of the following conditions: (**A**) free swelling (free-swell), (**B**) with static compression (stat comp), and (**C**), with dynamic shear stimulation (dyn shear) superimposed on static compression. Arrows indicate the direction of applied force. (**D**), Shear strain and shear stress waveforms were computed from data recorded during shear stimulation.

3.4 Results

Biomechanical characterization of the load and displacement data showed that tissue shear deformation was applied in a reproducible manner. The total harmonic distortion of the load waveforms was 8.1 ± 0.2% (mean ± SEM), and both displacement and load waveforms were smooth (no visible distortion) (**Fig. 3.1D**). Cartilage shear modulus was 0.11 ± 0.01 MPa (mean ± SEM).

Dynamic shear and static compression inhibited the increase in cartilage thickness that occurred during free-swelling culture (load/day interaction $P < 0.05$) (**Fig. 3.2**). Immediately following harvest (day 0), cartilage thickness was similar ($P = 0.55$) for all explants (mean ± SEM 1.02 ± 0.02 mm for each loading group). However, while the thickness of compressed and sheared cartilage remained essentially unchanged (within ±3%) during the 24 hours of stimulation, free-swelling cartilage thickness increased 14% during that same 24 hours. By the end of culture period (day 4), the thickness of free-swelling tissue had increased by a total of 22% over the initial thickness, while compressed and sheared cartilage remained within 3% of the initial thickness.

PRG4 secretion into culture media was markedly up-regulated by dynamic shear stimulation. Sheared cartilage superficial zone disks secreted 3–4 times the amount of PRG4 secreted by controls (**Fig. 3.3**). Not only did shear stimulation up-regulate PRG4 secretion during the 24 hours of continuous loading, but this effect was maintained for the next 3 days following unloading ($P < 0.05$ for each day). In contrast, statically compressed controls secreted PRG4 at the same level as free-

swelling controls (P =0.62 for each day) and were therefore not analyzed further. Cartilage middle zone disks subjected to the same loading protocols did not secrete PRG4 at levels above the detection limit of the assay (~0.1 μg/[cm^2*day]) for any of the loading conditions (data not shown) and, thus, were also not analyzed further.

Western blot analysis (**Fig. 3.4**) showed that medium from both free-swelling cartilage and dynamically sheared cartilage contained PRG4, predominantly of ~345-kd. Some smaller molecular weight immunoreactive species (~220, ~97, and ~20 kd) were detected in medium from both free-swelling and dynamically sheared cultures, although medium from dynamically sheared tissue appeared to contain relatively more PRG4 of ~345 kd than the smaller species, as compared with medium from free-swelling cartilage.

The total number of PRG4+ cells and the depth-associated variation in chondrocyte expression of PRG4 were also modulated by dynamic shear stimulation ($P < 0.001$ and $P < 0.05$, respectively). During loading, cartilage subjected to dynamic shear had a mean ± SEM of 90 ± 10 total PRG4+ cells across the 0.3-mm width of the immunohistochemistry sample counted, while free-swelling tissue had 70 ± 3 PRG4+ cells. On days 2 and 4, sheared tissues had 120 ± 9 and 88 ± 9 PRG4+ cells, respectively, while free-swelling tissues had only 55 ± 4 PRG4+ cells on day 2 and 62 ± 6 PRG4+ cells on day 4. In free-swelling tissue, many cells in the top 0 to 100–200 μm were PRG4+ (**Figs. 3.5B, 3.6A–C**), with very few cells deeper than 200 μm expressing PRG4. This pattern was consistent throughout the duration of culture (**Fig. 3.6**). In contrast, sheared tissues exhibited more ($P < 0.05$) PRG4+ cells in certain bins deeper than 200 μm, both during the 24-hour loading period and after 24 or 72 hours

following unloading (**Figs. 3.5C, 3.6A–C**). Chondrocytes deeper than 400 μm from the articular surface did not express PRG4 under any conditions tested.

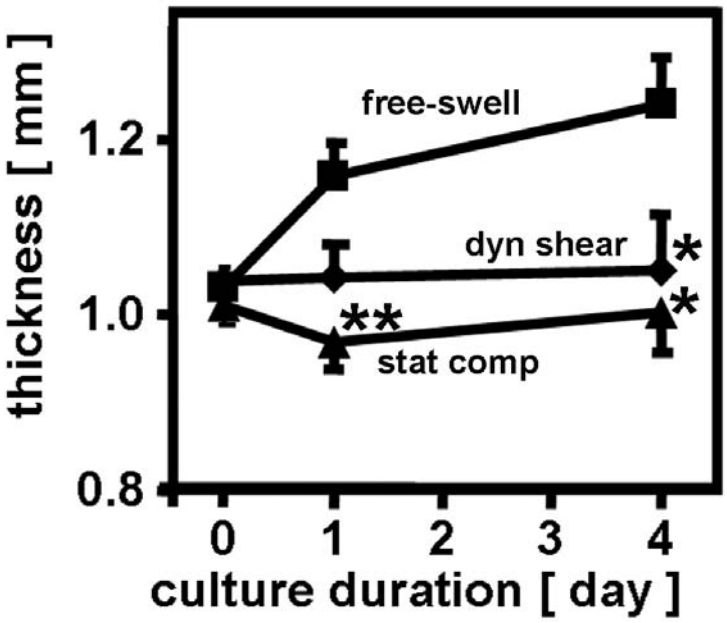


Figure 3.2. Thickness of free-swelling (free-swell), statically compressed (stat comp), and dynamically sheared (dyn shear) cartilage before culture (day 0), upon termination of loading (day 1), and upon termination of 72 hours of unloaded culture (day 4). Values are the mean and SEM of 4–8 samples. * = $P < 0.05$; ** = $P < 0.01$ versus free-swelling cartilage, by unpaired *t*-test.

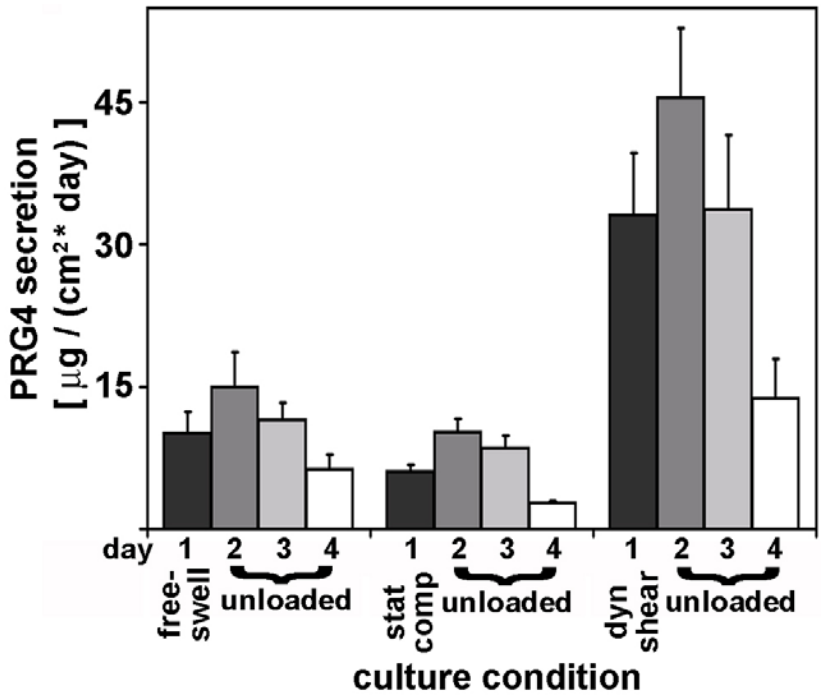


Figure 3.3. Secretion of proteoglycan 4 (PRG4) by free-swelling (free-swell), statically compressed (stat comp), or dynamically sheared (dyn shear) cartilage during 24 hours of continuous loading (day 1), followed by 72 hours (days 2–4) of unloaded culture. Values are the mean and SEM of 8–12 samples.

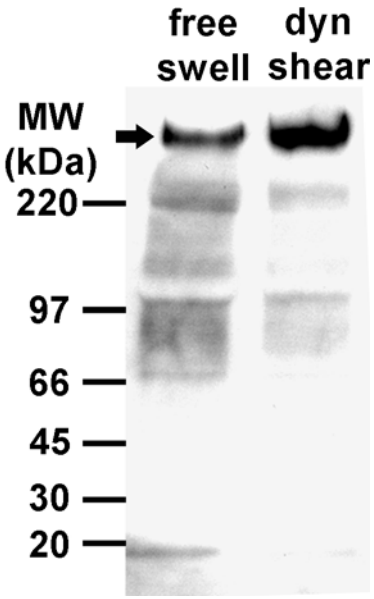


Figure 3.4. Characterization of proteoglycan 4 (PRG4) secreted into cultures of free-swelling and dynamically sheared cartilage. Western blot, showing that PRG4 secreted under free-swelling (free swell) and dynamically sheared (dyn shear) conditions (0.5 μg of total PRG4 per lane, as determined by enzyme-linked immunosorbent assay with monoclonal antibody 3-A-4) was predominantly of ~345-kd molecular weight (arrow).

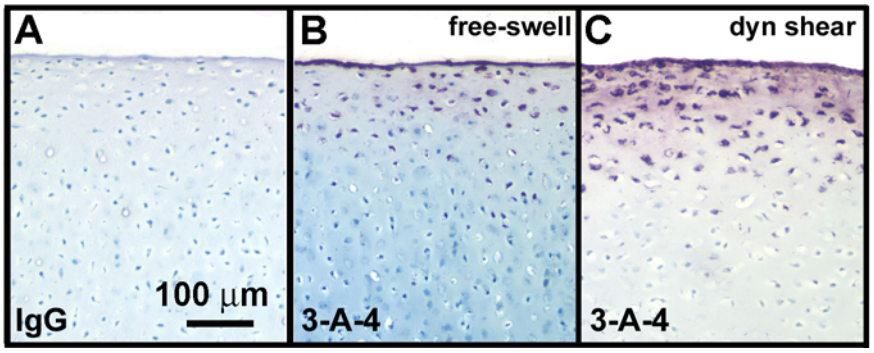


Figure 3.5. Immunohistochemistry for the presence of proteoglycan 4 (PRG4) within chondrocytes at different depths of cartilage. Shown are representative images of (**A**), cartilage probed with a nonspecific mouse IgG antibody (control), (**B**), free-swelling (free-swell) cartilage probed with monoclonal antibody 3-A-4, and (**C**), dynamically sheared cartilage probed with monoclonal antibody 3-A-4.

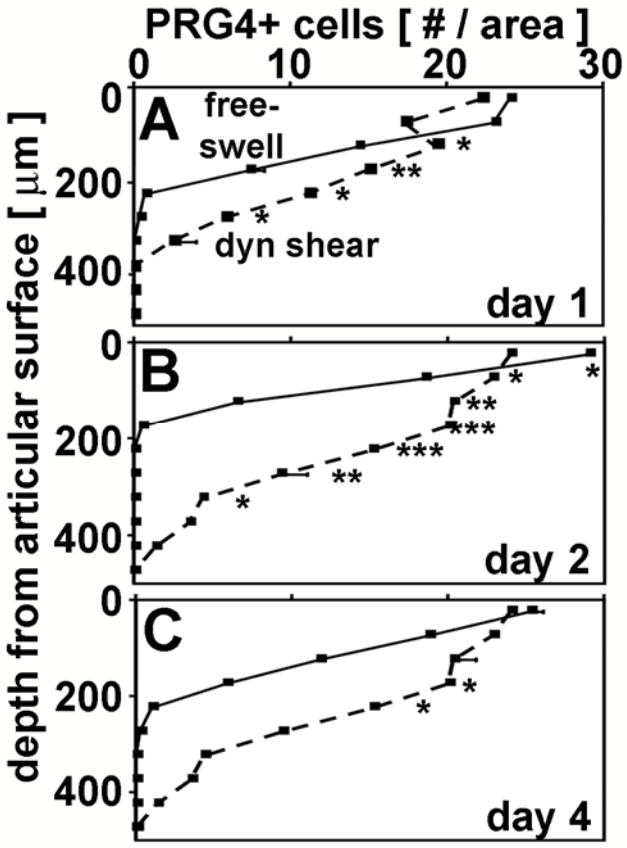


Figure 3.6. Depth-associated variation in chondrocyte expression of proteoglycan 4 (PRG4) in free-swelling (free-swell) and dynamically sheared (dyn shear) cartilage. Data are presented as the number of cells per area as determined on (**A**) day 1, (**B**) day 2, and (**C**) day 4. Values are the mean of 8–12 samples; bars show the representative SEM. * = $P < 0.05$; ** = $P < 0.01$;*** = $P < 0.001$ for the effect of loading, by unpaired *t*-test.

3.5 Discussion

The results of our study demonstrate that dynamic shear stimulation markedly up-regulates cartilage biosynthesis of PRG4. The quantity of total PRG4 secreted was higher for cartilage subjected to dynamic shear stimulation than for unloaded and statically compressed cartilage (**Fig. 3.3**). The number of cells expressing PRG4 was also higher in dynamically sheared cartilage than in unloaded cartilage (**Figs. 3.5, 3.6**). In addition, dynamic shear induced PRG4 expression by cells at depths of 200–400 μm from the articular surface, a region which did not express PRG4 under control conditions. Furthermore, shear stimulation may have affected the forms (different molecular weights) of PRG4 secreted and metabolized during culture (**Fig. 3.4**). Static compression and dynamic shear stimulation protocols both prevented the increase in thickness typical of cartilage explants cultured under free-swelling conditions (**Fig. 3.2**). These results suggest a possible feedback mechanism for mechanical regulation of the production of lubricant PRG4 molecules *in vivo*, that is, the tissue strains imparted to cartilage during motions utilizing, and thereby depleting, boundary lubricant molecules at the articular surface simultaneously signal the nearby chondrocytes to secrete more PRG4.

The bioreactor we used was developed based on a previous design used to study the effects of shear stimulation on cartilage protein and proteoglycan synthesis [6], with additional adaptations to provide tissue culture conditions without the use of an incubator. It was designed to impart dynamic shear frequencies and amplitudes that up-regulate chondrocyte metabolism of extracellular matrix molecules such as

aggrecan and collagen [6, 18] in cartilage explants. In addition, the compression we applied was in the range of cartilage deformation that has been shown experimentally to occur during motion *in vivo* [12]. While the exact nature of the cartilage deformation during shearing was not quantified, the low total harmonic distortion, along with the smooth (no visible distortion) shape of the load waveforms (**Fig. 3.1D**), indicated that the platens did not slip relative to the articular surface. In addition, the computed cartilage shear modulus was consistent with previously reported values [6, 38, 46], suggesting that the cartilage was indeed subjected to tissue-shear deformation (as opposed to surface motion, where the platen slides across the cartilage surface). The shear modulus reported herein is at the low end of the range (0.18–2.5 MPa) previously reported for middle zone immature bovine cartilage tested under simple shear [6] or pure shear [46], as well as for full-thickness adult bovine cartilage under simple shear [38], analogous to the lower compressive modulus in the superficial zone of cartilage compared with that of deeper zone and full-thickness cartilage [31].

PRG4 secretion and expression were analyzed to give a quantitative measure of PRG4 production as well as qualitative information to identify chondrocyte populations that were expressing PRG4 under the various loading conditions. Since the initial PRG4 content of cartilage disks is an order of magnitude lower than that detected in culture medium, the PRG4 detected in medium samples provided an index of that synthesized during culture. Such secretion levels could result in an underestimation of the total synthesis, depending on the partition coefficients of PRG4 in cartilage under various conditions [25], which remain unknown. It would be of

interest to determine how the level of PRG4 secretion relates to the amount of PRG4 that is bound and functional as a lubricant at the articular surface.

These results support the hypothesis that chondrocyte metabolism depends on the local microenvironment, which is determined by a complex combination of chemical and mechanical stimuli. Previous studies have shown that PRG4 synthesis and expression by chondrocytes in culture are markedly regulated by chemical factors. Inclusion of fetal bovine serum and ascorbate in culture media up-regulates PRG4 secretion levels [33]. Certain growth factors and cytokines that are present at high levels in injury and arthritis also affect PRG4 metabolism. For example, transforming growth factor β1 increases PRG4 mRNA synthesis by chondrocytes cultured in monolayer [2, 4], as well as protein secretion by chondrocytes in explants [32] and 3-dimensional constructs [5]. Conversely, interleukin-1α has inhibitory effects on PRG4 mRNA expression [5] and protein synthesis [32].

The microenvironmental mechanisms by which mechanical stimuli regulate chondrocyte biosynthesis, however, remain to be established. In contrast to pure shear, where no fluid flow is expected, the tissue shear configuration used in our studies results in a small amount of fluid flow [18], which appears to be partly responsible for the stimulation of matrix biosynthesis by dynamic compression [3, 19]. Dynamic compression applied for the same duration did not result in the marked up-regulation of PRG4 secretion seen with dynamic shear [27]. Alternatively, cell matrix deformation itself may cause changes in metabolism. Using a cell-tracking methodology [31], a preliminary study (Bae W: personal communication) demonstrated that application of ~4% shear strain to full-thickness human cartilage

resulted in a level of shear strain that is high near the articular surface and decreases with depth, with ~8–13% shear strain in the superficial zone (top 400 μm) and ~3% shear strain in the middle zone (30–60% of the cartilage depth). The region of high shear strain, the superficial zone of the tissue, is the same region in which the percentage of chondrocytes expressing PRG4 is high, suggesting that chondrocyte responsiveness may be due, at least in part, to the local shear strain to which the chondrocytes are exposed.

The regulatory effect of dynamic shear stimulation reported herein is also consistent with recent evidence that certain mechanical stimuli, including cyclical tension [45] and articular surface motion [8], can up-regulate chondrocyte PRG4 mRNA expression by chondrocyte-seeded cartilaginous constructs, and our findings extend the current knowledge to include effects on secretion of PRG4 protein products by chondrocytes within their native extracellular matrix. The extent to which PRG4 synthesis was up-regulated by dynamic shear (300–400% over unloaded controls), was similar in magnitude to the 300–700% up-regulation of PRG4 mRNA expression due to surface motion reported by Grad et al [8]. In contrast, shear stimulation of cartilage explants and engineered tissue up-regulates collagen and matrix proteoglycan synthesis by only 18–50% and 19–35%, respectively, over unloaded controls [6, 18, 43, 44]. In general, mechanical loading of cartilage regulates the expression of a large number of genes, including those sometimes considered to be constitutively expressed, such as GAPDH [22]. The overall regulatory effects of shear loading on cartilage metabolism may be further clarified by simultaneous analysis of the expression of a broad range of genes.

The possibility that dynamic shear influences the structure of PRG4 products synthesized would supplement recent findings that joint motion applied to chondrocytes in polyurethane constructs differentially regulated mRNA expression of different PRG4 isoforms [9]. Alternatively, the lower molecular weight bands could represent posttranslational cleavage of PRG4 products that were secreted as full-length molecules [28]. Disruption of disulfide bonds could also have allowed the separation of several species of various molecular weights under reducing conditions that are normally held together under nonreducing conditions. Taken together, these results suggest that mechanical stimuli may affect the transcription, translation, or secretion of PRG4, and additional studies are needed to elucidate the molecular details of these regulatory effects.

The molecular mechanism of joint lubrication remains a somewhat controversial subject (see, for example, refs. [13, 16, 42]). However, if PRG4 molecules accumulate in synovial fluid as a lubricant for the articular cartilage surface in a manner that is dependent upon secretion rates by the surrounding tissues, the contribution by cartilage itself could significantly impact the low-friction properties of the surface during joint motion. Understanding the role of mechanical stimulation in maintaining PRG4 levels in the synovial fluid could therefore lead to possible treatments to prevent the loss of the low-friction function of articular cartilage. In addition, the ability of dynamic shear stimulation to induce PRG4 expression in chondrocytes at a depth of 200–400 μm from the articular surface, but not those below 400 μm, may also prove useful for creating stratified tissue-engineered cartilaginous

constructs from isolated chondrocyte subpopulations that have functional, lubricating articular surfaces.

3.6 Acknowledgments

This chapter is reprinted in full from *Arthritis & Rheumatism*, 54(6), Nugent GE, Aneloski NM, Schmidt TA, Schumacher BL, Voegtline MS, Sah RL, Dynamic shear stimulation of bovine cartilage biosynthesis of proteoglycan 4, p. 1888-96, Copyright (2006), with permission from John Wiley & Sons, Inc. The dissertation author (primary investigator) thanks the co-authors of the manuscript for their contributions: Aneloski NM, Schmidt TA, Schumacher BL, Voegtline MS. In addition, we thank the funding sources that supported this work: Arthritis Foundation, NIH, NSF, Whitaker Foundation (pre-doctoral fellowship to GEN).

3.7 References

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CHAPTER 4

PRG4 EXCHANGE BETWEEN THE ARTICULAR CARTILAGE SURFACE AND SYNOVIAL FLUID

4.1 Abstract

The boundary lubrication function of articular cartilage is mediated in part by proteoglycan 4 (PRG4) molecules, found both in synovial fluid (SF) and bound to the articular cartilage surface. Currently the mechanism by which PRG4 binds to the articular surface is not well understood. The objectives of this study were to determine 1) the effect of bathing fluid contents on PRG4 concentration at the articular surface ($[PRG4]^{cart}$), and 2) whether native PRG4 can be removed from the surface and subsequently repleted with PRG4 from synovial fluid. In one experiment, cylindrical cartilage disks were stored in solutions of various PRG4 concentrations, either in phosphate buffered saline (PBS) or SF as the carrier fluid. In a separate experiment, cartilage disks were stored in solutions expected to remove native PRG4 from the articular surface and allow subsequent repletion with PRG4 from SF. $[PRG4]^{cart}$ was independent of PRG4 concentration of the bathing fluid, and was similar for both carrier fluids. PRG4 was removed from cartilage by treatment with hyaluronidase, reduction/alkylation, and sodium dodecyl sulphate, and was repleted fully by

subsequent bathing in SF. These results suggest that the articular surface is normally saturated with tightly bound PRG4, but this PRG4 can exchange with the PRG4 in SF under certain conditions. This finding suggests that all tissues surrounding the joint cavity that secrete PRG4 into the SF may help to maintain lubrication function at the articular surface.

4.2 Introduction

Articular cartilage normally functions to provide a low-friction, load-bearing surface that allows the bones of synovial joints to slide smoothly against each other during joint motion. Boundary lubrication is one mechanism by which the articular surface of cartilage provides low-friction properties, and is mediated in part by proteoglycan 4 (PRG4) molecules [10, 12, 29, 30], found both in synovial fluid (SF) and in a layer bound to the articular cartilage surface [26]. In classical descriptions of lubrication modes, boundary lubrication during surface-to-surface contact is mediated by a molecular surface layer or film attached to one or both surfaces [32], suggesting that the PRG4 bound to the articular cartilage surface mediates boundary lubrication function in the joint. PRG4 molecules are secreted into SF by several tissues surrounding the joint cavity, including chondrocytes of superficial zone [25], synoviocytes [11, 15], meniscal cells [27], though these tissues vary in their rates of secretion of PRG4 products. All PRG4 molecules in the fluid bathing articular cartilage could, in theory, contribute to the boundary lubrication function of the articular surface, provided they can attach to the articular surface.

A recent observation during mechanical characterization of cartilage lubrication suggested qualitatively that PRG4 attachment to the articular surface could indeed be dependent upon the quantity of PRG4 in the bathing fluid. In these experiments, cartilage-on-cartilage sliding in phosphate buffered saline (PBS) resulted in removal of PRG4 from the surface while sliding in SF resulted in the deposition of additional PRG4 to the surface in the region of cartilage-cartilage contact [24].

However, a definitive and quantitative relationship between PRG4 in SF and that bound to the articular surface remains to be established.

The molecular mechanism by which PRG4 molecules are held at the articular cartilage surface also remains unknown, but may involve one or more of the putative binding motifs previously reported for PRG4. Both the N-terminus and the C-terminus of PRG4 have regions with high sequence homology to certain sequences of vitronectin. In particular, PRG4 has a hemopexin-like domain near the C-terminus. Purified hemopexin interacts with hyaluronan (HA) [9], suggesting that the hemopexin-like domain could also mediate PRG4 binding to HA present at or near the articular surface, and recombinant fragments of the C-terminal domain of PRG4 have been shown to bind to HA [16]. In addition, lubrication studies have shown that HA and PRG4 act in concert to reduce friction [14], supporting the possibility of molecular interaction between the two at the articular surface. Just N-terminal to the mucin-like domain that mediates lubrication function [13], exon 6 contains a covalently bound chondroitin sulfate chain [6, 25]. Such chains could also mediate non-covalent binding of PRG4 to cartilage matrix molecules including collagens [7] and fibronectins [1, 19] at or near the articular surface. Another possibility is that PRG4-surface binding is lipid-mediated, as PRG4 has been shown to contain up to 11% lipid [28], and this interaction may involve hydrophobic [2, 31] or hydrophilic attraction [8]. Finally, PRG4 attachment to the articular surface may be maintained by disulfide bond formation, via the unmatched cysteine residue near the C-terminus [6]. This could result in self-aggregation of PRG4 molecules, or PRG4 binding to other molecules present at the articular surface.

We hypothesized that PRG4 binding to the articular surface depends on both PRG4 concentration of the surrounding fluid and PRG4 interactions with other molecules at the articular surface. The first aim ("association experiment") was to determine the effect of i) total PRG4 concentration in the bathing fluid ($[PRG4]^{bath}$) and ii) carrier fluid (PBS or SF) on concentration of PRG4 at the articular surface ($[PRG4]^{cart}$) for cartilage explants starting with intact native $PRG4^{cart}$. The second aim ("dissociation/repletion experiment") was to determine whether native $PRG4^{cart}$ can be removed (dissociated) by various methods expected to disrupt putative molecular interactions of PRG4, and subsequently replaced (repleted) with $PRG4^{bath}$ from SF.

4.3 Materials and Methods

Cartilage harvest. Cartilage disks (6 mm diameter, 0.4 mm thick) were explanted from the patellofemoral groove and lateral and medial femoral condyles of adult (1 year old) bovine stifle joints, under irrigation with PBS. The disks were cut either to include the superficial zone and the intact articular surface (superficial, 0-0.4 mm from articular surface) or from the middle zone (middle, 0.4-0.8 mm from articular surface) [18].

Synovial fluid/purified PRG4. Synovial fluid was obtained from bovine joints, and its PRG4 content was determined to be 450 μg/ml by indirect ELISA and semi-quantitative Western Blot analysis. For purified PRG4, cartilage disks were harvested as described above from the superficial zone of immature (1-3 week) bovines and cultured in medium supplemented with 0.1% bovine serum albumin and 10 ng/ml transforming growth factor-β1. PRG4 was purified from culture medium by anion exchange chromatography (DEAE-Sepharose^{TM}, 0.3M NaCl to 0.6M NaCl eluate collected) and concentrated with a Centricon® Plus 100 kDa MW cutoff filter [25].

PRG4 association experiment (Fig. 4.1). Superficial disks (n=160 total, 20 per group) and middle disks (n=160 total, 20 per group) were harvested as above, and stored for 72 h at 4ºC in baths of varying PRG4 concentration (5, 50, 150, 450 μg/ml total PRG4), in one of 2 carrier fluids (PBS or SF). Upon termination of storage, cartilage was rinsed 6x10 min in PBS with gentle nutation to remove bathing fluids and any loosely bound PRG4. $[PRG4]^{cart}$ was then quantified, and for some bathing

fluids (those with $[PRG4]^{bath}$ = 150 μg/ml, both in PBS and SF), $[PRG4]^{cart}$ was visualized by *en bloc* immunohistochemistry as described below.

PRG4 dissociation/repletion experiment (Fig. 4.1). Superficial disks (n=195 total) with the surface intact and middle disks (n=195 total) were harvested as above. For some disks (n=75), $[PRG4]^{cart}$ was quantified following harvest to assess content at day 0. Other disks (n=24 per group) were stored for 24 h at 25ºC in solutions expected to disrupt (or not to disrupt, in certain cases) putative molecular mechanisms of PRG4 binding to articular surface. These methods included (1) PBS, (2) 2M NaCl in PBS (2M NaCl), (3) 1% sodium dodecyl sulfate (SDS) after wipe with cotton swab soaked in 10% SDS [5], (4) reduction in 10mM dithiothreitol, followed by alkylation for 1h in 55mM iodoacetamide (Red/Alk), and (5) 10 U/ml streptomyces hyaluronidase (HAase). After all dissociation treatments, cartilage disks were rinsed 6x10 min in PBS with gentle nutation, and some disks (n=12 per group) were stored for an additional 24 h at 25ºC in PBS ($[PRG4]^{bath}$ = 0 μg/ml) to maintain $[PRG4]^{cart}$ at the level remaining after dissociation. Other disks (n=12 per group) were instead stored for an additional 24 h at 25ºC in SF ($[PRG4]^{bath}$ = 450 μg/ml), which was expected to result in repletion, or re-binding of PRG4 to the articular surface. All disks were again rinsed 6x10 min in PBS with gentle nutation, and $[PRG4]^{cart}$ was then quantified or visualized as described below. Control studies, in which bathing fluids were tested for GAG content using the dimethylmethylene blue binding assay [4], showed that HAase treatment removed nearly all GAG from the tissue (25-40 mg/g wet weight), whereas PBS, 2M NaCl, and Red/Alk removed smaller amounts of GAG

(15-20 mg/g wet weight). SDS bathing fluids were not tested for GAG content, as they interfere with the assay.

[PRG4]^{cart} quantification in cartilage disks. Upon termination of association, dissociation, and repletion treatments described above, cartilage disks were treated in 4M GuHCl, 0.02M Tris, with protease inhibitors (0.005M Benzamidine-HCl, 0.01M N-ethylmaleimide, 0.005M disodium EDTA, and 0.001M phenylmethylsulfonylfluoride) and nutation overnight at 4ºC to remove adherent PRG4. PRG4 content of the extract solution was determined by ELISA as described previously [27] using anti-PRG4 mAb 3-A-4 (gift from Dr. Bruce Caterson). [PRG4]^{cart} was presented normalized to articular surface area.

[PRG4]^{cart} visualization. Other disks from the association experiment were placed into 4% paraformaldehyde upon termination of culture, and PRG4 bound to the cartilage was visualized by *en bloc* immunohistochemistry (IHC), as described previously [3]. After both dissociation and repletion steps, cartilage disks were frozen in Tissue Tek OCT (Sakura USA, Torrance, CA) and sectioned (5 μm slices) perpendicular to the articular surface, and PRG4 was visualized by IHC as described previously [18]. For both types of IHC, samples were reacted with mAb 3-A-4 and detected with a peroxidase-based system (Vector Labs). Stained cartilage surfaces were viewed to identify the location of bound PRG4 (dark purple color). Sections probed with a non-specific mouse IgG served as negative controls. Results were documented by digital imaging and photomicroscopy.

Statistical analysis. All data are expressed as mean ± standard error of the mean (SEM), and statistical analyses were performed using Systat 10.2 software. For

the PRG4 association experiment, effects of $[PRG4]^{bath}$ and carrier fluid on $[PRG4]^{cart}$ were assessed by 2-way ANOVA. Differences between $[PRG4]^{cart}$ after dissociation and repletion treatments and $[PRG4]^{cart}$ at day 0 were assessed by Dunnett's tests.

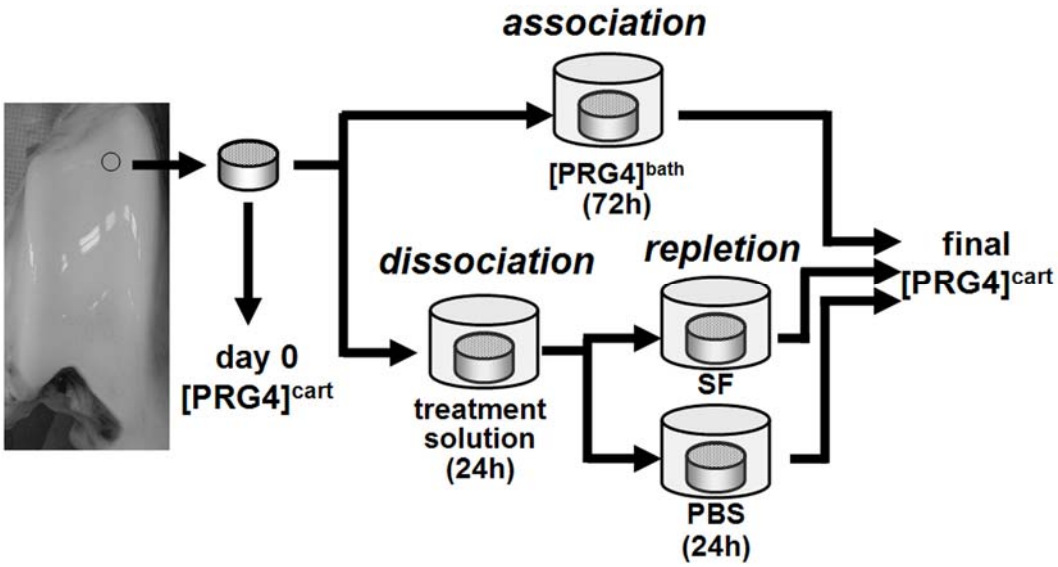


Figure 4.1. Experimental design to investigate PRG4 attachment to the articular surface.

4.4 Results

PRG4 association experiment. $[PRG4]^{cart}$, measured after cartilage had been stored for 72 h in various bathing solutions, was independent of the contents of the bathing fluid for both superficial and middle cartilage disks (data for superficial disks shown in **Fig. 4.2**). Averaged over all bath conditions, $[PRG4]^{cart}$ was higher ($p<0.001$) in superficial disks (0.72 ± 0.05 $\mu g/cm^2$) than middle disks (0.07 ± 0.01 $\mu g/cm^2$). Since the quantity of PRG4 in middle disks was near the lower limit of detection of the assay for all conditions, $[PRG4]^{cart}$ of these disks was not further analyzed. For superficial disks, $[PRG4]^{cart}$ was independent of $[PRG4]^{bath}$ ($p=0.80$) and carrier fluid (PBS vs SF) ($p=0.10$), and no significant interaction between $[PRG4]^{bath}$ and carrier fluid ($p=0.79$) was detected.

Immunolocalization of $[PRG4]^{cart}$ on cartilage disk surfaces agreed qualitatively with the quantitative data above. The articular surface of superficial disks (**Fig. 4.2Bi-iv**) stained more intensely for PRG4 than the cut surfaces of the same disks (not shown), and the (cut) surfaces of middle disks (**Fig. 4.2Bv-viii**). As expected, cartilage surfaces probed with non-specific IgG (**Fig. 4.2Biii,iv,vii,viii**) stained less intensely than their counterparts probed with anti PRG4 mAb 3-A-4 (**Fig. 4.2Bi,ii,v,vi**). The articular surface of the superficial disk from the SF bath (**Fig. 4.2Bii**) stained more intensely for PRG4 (with mAb 3-A-4) than the articular surface of the superficial disk from the PBS bath (**Fig. 4.2Bi**). However, the additional staining may be non-specific, since cartilage surfaces from the SF bath also stained

somewhat more intensely than those from the PBS bath when these same surfaces were probed with non-specific IgG antibodies (**Fig. 4.2Biii,iv**).

PRG4 dissociation/repletion experiment. Certain dissociation treatments resulted in reduced $[PRG4]^{cart}$ compared to that at day 0 (**Fig. 4.3**, filled boxes). $[PRG4]^{cart}$ at day 0 (quantified at the time of harvest) was higher ($p<0.001$) in superficial cartilage disks (0.50 ± 0.09 $\mu g/cm^2$), than in middle disks (0.09 ± 0.06 $\mu g/cm^2$). After both dissociation and repletion treatments, $[PRG4]^{cart}$ remained low for middle disks from all groups (average 0.09 ± 0.03 $\mu g/cm^2$), and was therefore not analyzed further. For superficial disks, treatment with PBS and 2M NaCl resulted in $[PRG4]^{cart}$ of 0.43 ± 0.05 and 0.33 ± 0.08 $\mu g/cm^2$, respectively, which were not statistically different from $[PRG4]^{cart}$ at day 0 ($p=0.49$, 0.46, respectively). Treatment with SDS, Red/Alk, and HAase, on the other hand, resulted in $[PRG4]^{cart}$ of 0.11 ± 0.03, 0.19 ± 0.05 and 0.04 ± 0.02 $\mu g/cm^2$, respectively, which were significantly lower than $[PRG4]^{cart}$ at day 0 ($p<0.001$ each for SDS and HAase, $p<0.01$ for Red/Alk).

After dissociation treatments, subsequent storage of cartilage disks in SF resulted in repletion of PRG4 at the articular surface, with $[PRG4]^{cart}$ returning to at least that of day 0 for all groups (**Fig 4.3**, open boxes). For cartilage previously treated with PBS, 2M NaCl, SDS, and Red/Alk, $[PRG4]^{cart}$ after treatment with SF was not different from the initial $[PRG4]^{cart}$ ($p=0.50$, 0.14, 0.06, and 0.99, respectively). In contrast, for cartilage previously treated with HAase, $[PRG4]^{cart}$ was higher (1.3 ± 0.3 $\mu g/cm^2$, $p<0.001$) than that at day 0.

Immunolocalization of $[PRG4]^{cart}$ in cartilage sections (**Fig. 4.4**) agreed qualitatively with the quantitative data above, and suggested that PRG4 added to cartilage from SF baths (during repletion treatment) was added to the articular surface, and not to the other cut surfaces in most cases. After dissociation treatments, cartilage treated with PBS and 2M NaCl (**Fig. 4.4H,I**) exhibited intense staining for PRG4 at the articular surface, similar to that seen for day 0 cartilage (**Fig. 4.4G**), whereas cartilage treated with SDS (**Fig. 4.4J**), Red/Alk (**Fig. 4.4K**), and HAase (**Fig. 4.4L**) did not. For all groups, cartilage probed with a non-specific IgG antibody (**Fig. 4.4A-F**) did not exhibit staining at the articular surface. After repletion of PRG4 by treatment with SF, cartilage previously treated with PBS and 2M NaCl (**Fig. 4.4M,N**) continued to exhibit PRG4 staining at the articular surface, and cartilage that had previously had its surface PRG4 removed by SDS, Red/Alk, and HAase (**Fig. 4.4O-Q**) again exhibited PRG4 at the articular surface. In contrast to the intense staining for PRG4 at the articular surface of the cartilage from all groups after repletion treatment (**Fig. 4.4M-Q**), there was very little staining within the tissue or at the cut surfaces of these disks (**Fig. 4.4R-V**), with the exception of cartilage disks previously treated with HAase which, upon treatment with SF, stained faintly for PRG4 throughout the depth of the tissue (not shown).

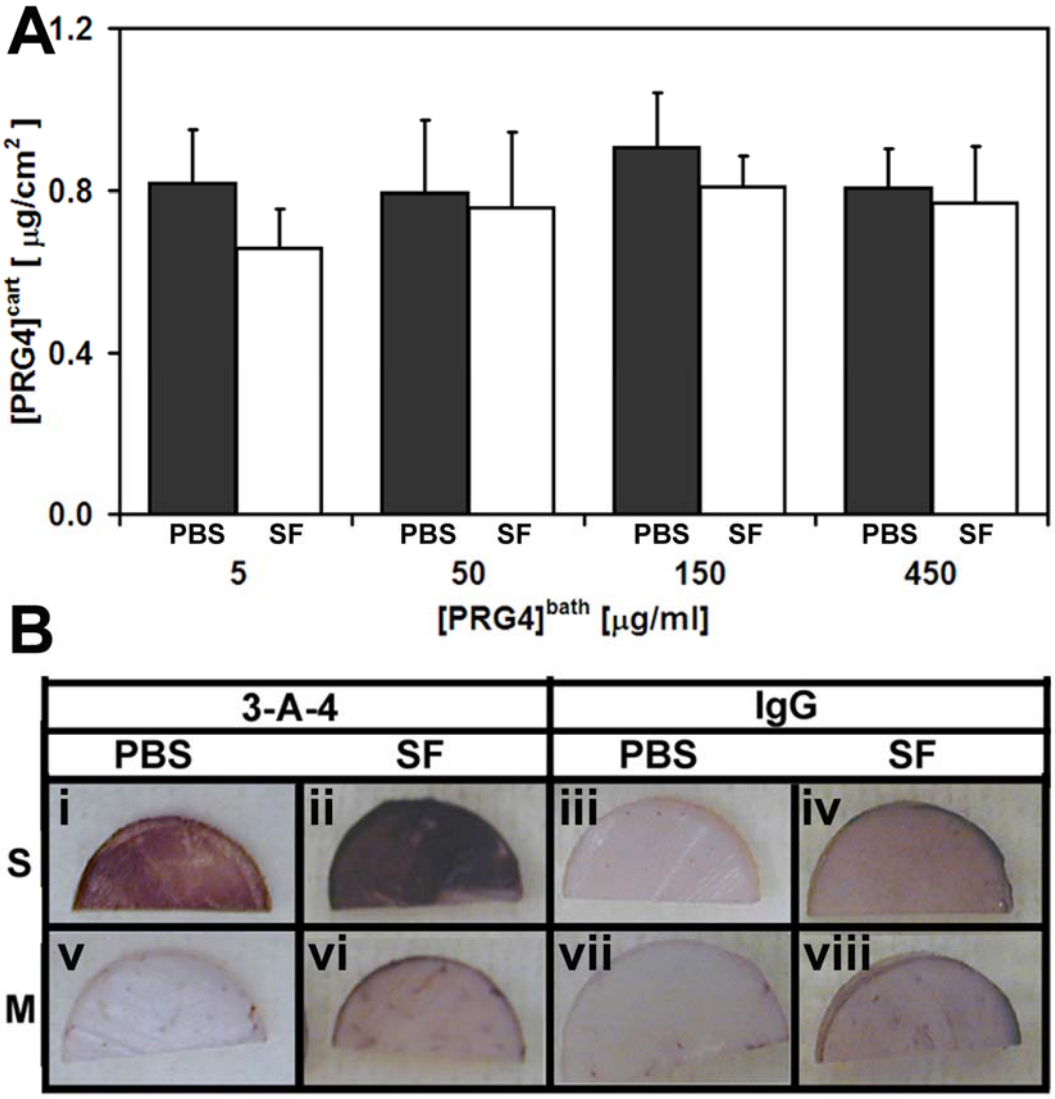


Figure 4.2. (**A**) $[PRG4]^{cart}$ after 72h in PRG4 baths of various concentrations ($[PRG4]^{bath}$), in either PBS (■) or SF (□) as the carrier fluid. Mean±SEM, n=20, from 2 animals. (**B**) $PRG4^{cart}$ visualized by mAb 3-A-4 (**i,ii,v,vi**) for the articular surface of superficial (S) disks (**i-iv**) and the surface of middle (M) disks (**v-vii**) after $[PRG4]^{bath}$ = ~150 μg/ml, in PBS (**i,iii,v,vii**) or SF (**ii,iv,vi,viii**). Controls were probed with non-specific IgG (**iii,iv,vii,viii**).

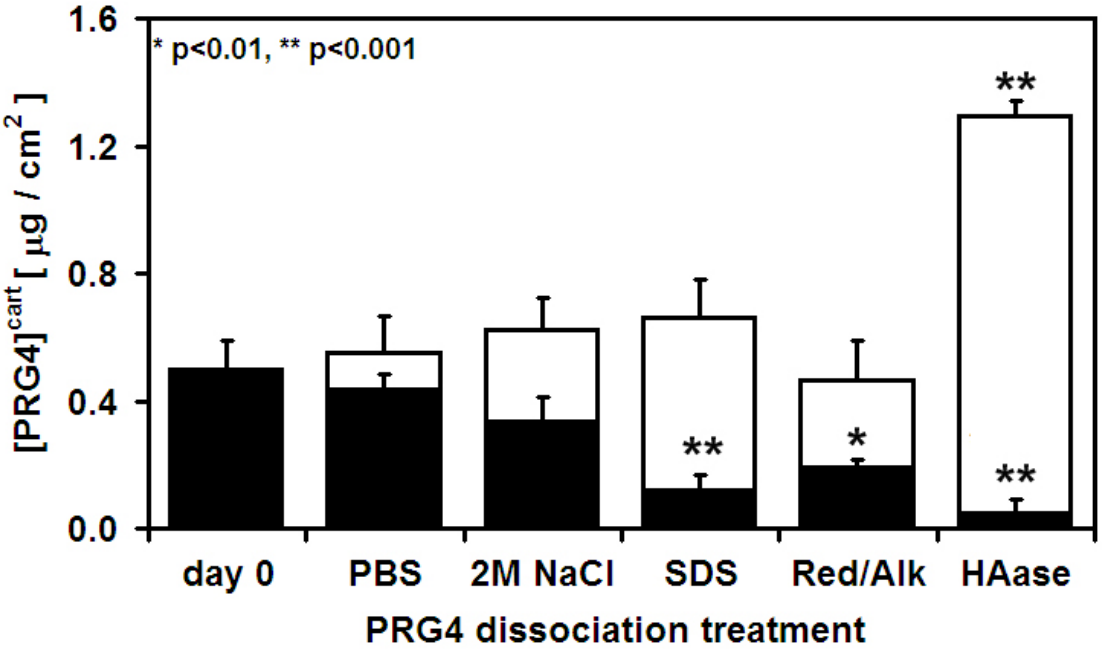


Figure 4.3. $[PRG4]^{cart}$ of superficial cartilage disks after dissociation (■) and $[PRG4]^{cart}$ repleted from SF (□). Mean±SEM, n=12 (67 for day 0 samples), from 2 animals.*$p<0.05$, **$p<0.001$ for differences from day 0 $[PRG4]^{cart}$.

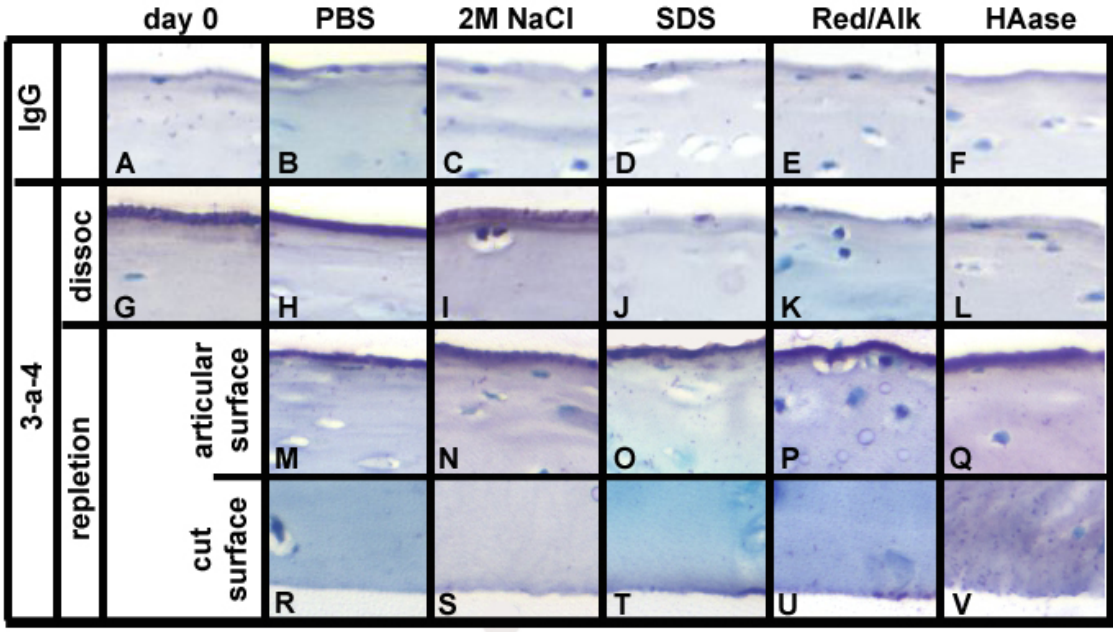


Figure 4.4. Immunohistochemistry for the presence of PRG4 (purple staining) at the articular surface (**A-Q**) or cut surface (**R-V**) of cartilage disks probed with mAb 3-A-4 (**G-V**) or a non-specific IgG (**A-F**), either after dissociation treatment (**A-L**) or after repletion treatment (**M-V**).

4.5 Discussion

These results provide insight into the nature of the interactions between PRG4 molecules bound to the articular cartilage surface and those present in the fluid bathing the tissue. $[PRG4]^{cart}$ did not depend on $[PRG4]^{bath}$ or carrier fluid in the association experiments, where native PRG4 at the articular surface was left intact before treatment (**Fig. 4.2**). However, native PRG4 could be removed by various dissociation treatments, including SDS, Red/Alk, and HAase (**Figs. 4.3, 4.4G-L**) and then repleted with PRG4 from SF (**Figs. 4.3, 4.4M-Q**). Immunolocalization of PRG4 in vertical sections of cartilage also suggested that PRG4 repletion occurred primarily at articular surface (**Fig. 4M-Q**), as opposed to the cut surfaces (**Fig. 4.4R-V**). Taken together, these results suggest that PRG4 at the articular surface is held quite tightly and does not readily exchange with PRG4 in the fluid bathing cartilage, but can be removed under certain conditions, and replaced with PRG4 from the fluid.

Reaction conditions were chosen to focus on binding rather than metabolic processing, and to simplify the system to ensure that the contents of the bath solutions were well-defined. While the kinetics of molecular interactions are generally temperature dependent [20], and may be different at 4°C and 25°C than at the physiological temperature of 37°C, the association experiments here were carried out at 4°C to slow cartilage metabolism, thus minimizing changes in $[PRG4]^{cart}$ due to biosynthesis, which would have resulted in time-varying $[PRG4]^{bath}$. Dissociation/repletion experiments were carried out at room temperature, also to slow cartilage metabolism but still allowing certain chemical treatments to proceed. In

addition, these experiments were carried out on free-swelling cartilage, without adjacent tissue surfaces in contact with the articular surface, eliminating the possible effects of mechanical stimuli on PRG4 binding to the articular surface.

Quantification of $[PRG4]^{cart}$ by extraction and ELISA allowed for detection in the range of 0.01-1 $\mu g/cm^2$, making this method sufficiently sensitive to detect the small amount of PRG4 at the articular surface (relative to the quantity of PRG4 typically secreted into medium during culture [23]). As PRG4 was extracted by submerging the entire cartilage disk in extraction solution, the quantity of PRG4 detected was representative of that in the whole disk. However, since PRG4 immunolocalization was much more intense at the articular surface than at the cut surfaces or within the tissue for most treatment groups, the majority of the PRG4 detected was likely that bound to the articular surface.

In the association experiments, the finding that $[PRG4]^{cart}$ was independent of the concentration of purified PRG4 (in PBS) at various concentrations in the physiological range [21] suggests that binding sites for PRG4 at the articular surface were saturated. Alternatively, this result might be attributed to possible alterations of the molecular structure of PRG4 resulting from the purification process, which could disrupt the binding mechanism. However, PRG4 purified in this manner remains functional in terms of its contribution to boundary lubrication [22, 24], suggesting that it has also retained its ability to bind to the articular surface. In addition, the PRG4 in SF bathing fluid had not been subjected to any purification, and should therefore be expected to behave as it does *in vivo*. Yet $[PRG4]^{cart}$ remained independent of

$[PRG4]^{bath}$ even when the carrier fluid was SF. Thus it is not likely that the loss of binding capability of PRG4 due to purification could explain these results.

Each dissociation treatment was chosen to address a specific hypothesis for the mechanism by which PRG4 binds to the articular surface. If the interactions between $[PRG4]^{cart}$ and $[PRG4]^{bath}$ were based on an equilibrium between the two concentrations, as in receptor-ligand kinetics [20], PBS would have been expected to remove some PRG4 from the articular surface. In this case the initial $[PRG4]^{bath}$ was 0 μg/ml, so the concentration gradient would have driven some PRG4 into the PBS solution. However, consistent with results from the association experiments, treatment with PBS alone did not alter $[PRG4]^{cart}$. Treatment with 2M NaCl was expected to disrupt possible electrostatic interactions between PRG4 and other molecules at the articular surface. This treatment also did not remove PRG4, which may suggest that PRG4 is held at the articular surface by another mechanism, or by higher ionic strength interactions. This is, however, in contrast to recent findings where 1.5M NaCl was sufficient to remove PRG4 from the articular surface of immature bovine cartilage disks [17]. This discrepancy could be due to differences in the strength with which PRG4 is held at the surfaces of immature and adult tissue.

Other dissociation treatments were sufficient to remove PRG4 from the articular surface. Treatment with SDS, expected to disrupt non-covalent bonds and denature proteins such that a binding mechanism based on 3D protein structure of PRG4 would also be disrupted, resulted in the loss of 77% of $[PRG4]^{cart}$. Reduction and alkylation, which breaks disulfide bonds and prevents their reformation, also removed 63% of $[PRG4]^{cart}$, suggesting that PRG4 could be held at the articular

surface by disulfide bonding to other molecules or by self-aggregation via the odd number of cysteines in the PRG4 sequence [6]. Finally, treatment with HAase removed nearly all (92%) of the $[PRG4]^{cart}$ present at day 0, which would suggest that HA may be necessary for PRG4 binding to the articular surface. Alternatively, since HAase also removed nearly all of the glycosaminoglycans from the tissue, this treatment may have removed other cartilage matrix molecules that mediate PRG4 binding to the articular surface as well.

The repletion of PRG4 at the articular surface with PRG4 from the bathing fluid (in this case SF) suggests that all of the tissues surrounding the joint that produce PRG4 could contribute to the boundary lubrication function of synovial fluid and the articular surface. If certain types of mechanical loading or even chemical signals resulted in the removal of PRG4 from the articular surface, then the PRG4 supply contained in the SF could be used to replace what was lost. SF also contains many other molecular components, such as HA, that may facilitate the binding of PRG4 to the articular surface. It would be interesting to determine whether repletion would occur to the same extent if the bathing fluid contained purified PRG4 in PBS, therefore eliminating the contributions from putative "binding cofactors" such as HA in the repletion process. The large amount of repletion that occurred for disks previously treated with HAase could be due to non-specific binding of PRG4 throughout the depth of the tissue for this group, which was suggested by the faint staining for PRG4 in all parts of the tissue seen in the vertical sections.

These results provide insight into the interactions between PRG4 bound to the articular surface and that in the surrounding fluid. The results of the dissociation and

repletion experiment are consistent with the hypothesis that PRG4 binding to the articular surface is mediated by the 3D structure of PRG4, disulfide bonding of PRG4 with itself or other molecules, or interactions with hyaluronan, though further work is needed to identify which mechanism(s) is (are) responsible for the attachment of PRG4 at the surface. Understanding how lubricant molecules bind to the articular surface could lead to the ability to manipulate the amount of PRG4 at the articular surface, and therefore, to modulate lubrication function.

4.6 Acknowledgments

This chapter has been submitted to *Journal of Orthopedic Research*. The dissertation author (primary investigator) thanks the co-authors of the manuscript for their contributions: Chan AH, and Schumacher BL. In addition, we thank the funding sources that supported this work: NIH, NSF, Whitaker Foundation (pre-doctoral fellowship to GEN), McNair Program (undergraduate scholarship to AHC).

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CHAPTER 5

CONTINUOUS PASSIVE MOTION APPLIED TO WHOLE JOINTS STIMULATES CHONDROCYTE BIOSYNTHESIS OF PRG4

5.1 Abstract

Background: Continuous passive motion (CPM) is currently a part of patient rehabilitation regimens after a variety of orthopedic surgical procedures. While CPM can enhance the joint healing process, the direct effects of CPM on cartilage metabolism remain unknown. Recent *in vivo* and *in vitro* observations suggest that mechanical stimuli can regulate articular cartilage metabolism of proteoglycan 4 (PRG4), a putative lubricating and chondroprotective molecule found in synovial fluid and at the articular cartilage surface. **Hypothesis:** A CPM device can be applied to whole joints in a bioreactor to maintain chondrocyte viability and stimulate PRG4 synthesis. **Study Design:** Controlled Laboratory Study. **Methods:** A bioreactor was

developed to apply CPM to bovine stifle joints *in vitro*. Effects of 24 hours of CPM on chondrocyte viability and PRG4 biosynthesis were determined. **Results:** Chondrocyte viability was maintained in the CPM bioreactors and rehabilitative joint motion applied in the form of CPM regulated PRG4 biosynthesis, in a manner dependent on the duty cycle of cartilage sliding against opposing tissues. Specifically, in certain regions that were continuously or intermittently sliding against meniscus and tibial cartilage during CPM, chondrocyte PRG4 synthesis was higher with CPM than without. **Conclusions:** Rehabilitative joint motion, applied in the form of CPM, stimulates chondrocyte PRG4 metabolism while maintaining cartilage viability. **Clinical Relevance:** The stimulation of PRG4 synthesis suggests one mechanism by which CPM benefits cartilage and joint health in post-operative rehabilitation.

5.2 Introduction

Articular cartilage functions to provide a low-friction, load-bearing surface which allows the bones of diarthrodial joints to slide smoothly against each other while transmitting load. Cartilage tissue has classically been divided into three zones: superficial, middle, and deep, with distinct biochemical content and organization that impart specific functions to each zone. For example, chondrocytes of the superficial zone of cartilage secrete specialized molecules, encoded by the proteoglycan 4 gene (PRG4, GenBank Accession Numbers: AF056218 for bovine partial sequence, U70136 for complete human sequence, also termed lubricin, SZP, CACP) [8, 16, 46], that are not expressed by chondrocytes in the deeper zone [46]. These PRG4 molecules mediate, at least in part, the boundary lubrication function of the articular cartilage superficial zone [19-21, 45, 51, 52], and have been suggested to protect the cartilage surface by preventing cellular adhesion to the surface [8]. Mutations in the PRG4 gene cause camptodactyly-arthropathy-coxa vara-pericarditis (CACP) disease, which results in early onset non-inflammatory joint failure [2, 27, 35], demonstrating the functional importance of the gene *in vivo*.

Recent experimental observations support the hypothesis that mechanical stimuli play a role in regulating PRG4 expression in articular cartilage *in vivo*. During embryonic development of the mouse elbow joint, PRG4 mRNA expression begins at the onset of joint cavitation [35], suggesting that PRG4 expression might be induced by the initiation of relative motion between the articular surfaces. A similar pattern is seen during post-natal growth, where fetal bovine cartilage exhibits inconsistent PRG4

expression by chondrocytes near the articular surface, in contrast with adult tissue, which has abundant PRG4-expressing cells near the surface [47]. In both cases (*in utero* and *in vivo*), increased chondrocyte expression of PRG4 coincides with increased joint motion. Furthermore, abnormal mechanical stimuli may result in decreased PRG4 expression. In a meniscectomy-induced (i.e., mechanically induced) osteoarthritis model in sheep, abnormal joint motion resulted in degeneration of articular cartilage in certain regions of the tibial plateau, with decreased PRG4 expression in these regions [56]. PRG4 expression was also higher in covered (by meniscus) than uncovered regions for tibial plateaus of normal ovine joints, suggesting that site-associated variations in intrinsic PRG4 expression could be due to mechanical factors as well [56].

Other studies have demonstrated that certain mechanical stimuli can regulate PRG4 metabolism by chondrocytes cultured in various configurations *in vitro*. Dynamic surface motion applied to chondrocytes embedded in polyurethane constructs [11], and cyclic tensile strain applied to chondrocytes in alginate constructs [55] resulted in increased PRG4 mRNA expression. Similarly, certain magnitudes of static and dynamic compression [32] and dynamic tissue shear [31] applied to cartilage explants altered PRG4 proteoglycan secretion relative to that of unloaded controls, both during loading and following unloading. Such *in vitro* studies allow the application of well defined mechanical stimuli under controlled biochemical environments by eliminating a variety of systemic factors present *in vivo* that affect chondrocyte metabolism. However, even in cylindrical cartilage explants, where chondrocytes are still embedded in their native matrix, the micro-environmental

phenomena (fluid flow, cell and matrix deformation) resulting from *in vitro* mechanical loading may be quite different from those experienced by intact articular cartilage in its native configuration covering bones, under physiological joint motion.

Continuous passive motion (CPM) was originally proposed as a therapeutic alternative to the traditional rehabilitative practice of joint immobilization for many orthopedic disorders and injuries [40]. CPM stimulation utilizes an external motorized device to move joints passively through a specified range of motion [4]. CPM is currently applied post-operatively to enhance patient recovery after anterior cruciate ligament reconstruction [30, 36]. CPM was also found in experimental studies to promote healing of articular cartilage defects [40, 41], and a variety of other joint afflictions [38, 39], supporting its use in post-operative rehabilitation of cartilage defect repair by autologous chondrocyte implantation [13, 28, 34] and microfracture [50]. However, the direct effects of CPM on chondrocyte metabolism remain unknown.

The hypothesis tested here was that a CPM device could be used to stimulate a whole joint during culture, and that this rehabilitative motion, applied to cartilage in its native configuration, regulates chondrocyte metabolism. The objectives of this study were to a) determine the spatial variation in intrinsic cartilage PRG4 secretion before bioreactor joint culture, and b) determine effects of CPM on chondrocyte viability and PRG4 biosynthesis using a bioreactor for culture of whole joints.

5.3 Materials and Methods

Joint Isolation. Immature bovine stifle joints (1-3 wk) were obtained from an abattoir. Soft tissues were cleared away with a scalpel, and the femur and tibia bones were cut with a bone saw such that the total length of the extended joint was ~25 cm. The contents of the bone marrow cavity were removed with a curette and the cavity was washed 3 times with 3% hydrogen peroxide. The outside of the joint was then scrubbed with 3% hydrogen peroxide and patted dry with gauze pads. The following steps were performed using aseptic technique, and all materials used including dissection surface, were initially sterile. The joint was scrubbed with sponges soaked with 7.5% povidone-iodine solution, and patted dry again. Under continuous irrigation with phosphate buffered saline with penicillin-streptomycin-fungizone, some joint tissues (muscle, periosteum, patella, patellar tendon, synovium, and fat) were cleared away using surgical scissors, a scalpel, and a periosteal elevator. The joint tissues remaining for bioreactor culture were the femoral and tibial bones and associated articular cartilage, the ligaments necessary to provide joint stability (anterior and posterior cruciate, lateral and medial collateral), and lateral and medial menisci.

Bioreactor Culture of Whole Joints & CPM Stimulation (Fig. 5.1). The cut ends of the bones were secured into custom designed polysulphone bone clamps, and a flexible, autoclavable polypropylene enclosure was placed around the joint and sealed tightly around the bone clamps to maintain a closed, sterile environment for the joint. Tygon tubing (Cole Parmer®) connected the bioreactor environment to a reservoir containing 2 L of culture medium (low-glucose Dulbecco's modified Eagle's medium

(DMEM), 10 mM HEPES buffer, 0.1 mM non-essential amino acids, 0.4 mM L-proline, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B) supplemented with 5% fetal bovine serum (FBS) and 25 μg/ml ascorbate. The bioreactor was filled with medium from the reservoir using a 3 channel high-flow rate peristaltic pump (Wiz Model HF, Teledyne Isco Inc.), operating at 83 ml/min, which also maintained medium circulation between the reservoir and the bioreactor throughout the duration of joint culture. The medium reservoir was submerged in a water bath to maintain the bioreactor temperature at 37°C and humidified 95% air/5% CO_2 was pumped through a 0.22 μm sterile filter into the reservoir to maintain the medium at pH 7.4. The bioreactor was adapted for attachment to a CPM device (FlexMate K500, BREG, Inc.), which can apply joint motion ranging between -10° and 120° of flexion (**Fig. 5.1C**), at rates between 30°/min and 150°/min.

For the experiments described here, CPM stimulation consisted of 24 h of continuous motion, with the joint oscillating between 10° and 46° of flexion at 110 º/min (43 s per cycle, or ~0.025 Hz). Intrinsic tension in the 4 intact ligaments maintained the articulating surfaces of the joint in contact, and no additional axial load was applied. Since the patella was discarded during dissection, cartilage from the patellofemoral groove (PFG) was not in direct contact with other tissues during bioreactor culture with or without continuous passive motion stimulation. In contrast, certain regions of the lateral and medial femoral condyles (LFC and MFC) slide against the adjacent menisci and tibial plateau cartilage during CPM stimulation. Thus cartilage from the PFG was in a similar mechanical environment for joints in both

bioreactor cultures, with or without CPM, and viability and metabolism of chondrocytes in this region were expected to be independent of CPM stimulation. Cartilage from the LFC and MFC of the joints cultured with CPM was compared to cartilage from those regions of joints cultured without CPM to determine direct effects of rehabilitative motion on chondrocyte health. The LFC and MFC were further divided into three sub-regions based on the type of contact the sub-region had with adjacent tibial cartilage and meniscus during the motion protocol. These sub-regions were designated as continuously, intermittently, or never sliding against the adjacent tissues, and this effect is referred to herein as sliding duty cycle (**Fig. 5.1D**).

Experimental Design (Fig. 5.2). ***Experiment I.*** Some joints were used to determine the intrinsic level of PRG4 secretion without bioreactor culture. Following isolation of these joints (n=4, from different animals), cylindrical cartilage disks (3 mm diameter, ~0.5 mm thick) were explanted from 12, 29, and 36 sites on the PFG, LFC, and MFC, respectively (sites shown in **Fig. 5.3B**), and cultured for 2 days in 48-well tissue culture plates and incubators. Medium (as described above, but with 10% FBS, 0.5 ml per disk) was collected and replaced daily for analysis of PRG4 secretion. Control studies in which cartilage disks from these locations were cultured for 4 days showed that site-associated variation in PRG4 secretion (relative secretion among sites) was stabilized by the 2nd day in culture, such that culturing the disks for 2 days was sufficient to determine differences in secretion values among explant sites. For this reason PRG4 secretion over the first 2 days of culture is presented here for comparison of results of experiment I with those of experiment II. ***Experiment II.*** Paired stifle joints from each of 4 animals were isolated as described above, and

transferred to bioreactors for whole joints. One bioreactor was attached to the CPM device, while the other was placed on a bench-top for culture without mechanical stimulation. Following 24 h of joint culture in bioreactors, cartilage disks were explanted under sterile conditions from 24 sites on the PFG, and 30 sites each on the LFC and MFC (sites shown in **Fig. 5.3C**). Sites on the femoral condyles were chosen to represent the 3 sliding duty cycle conditions (continuously, intermittently, and never sliding against another tissue). Each cartilage disk was analyzed for either a) chondrocyte viability (8 disks per region), PRG4 immunolocalization within chondrocytes (4 per region), or PRG4 secretion (12-18 per region).

Cartilage Analysis. *Chondrocyte Viability.* Cartilage disks were stained with Calcein-AM and ethidium homodimer-1 to localize live and dead cells, respectively, and *en face* images of the articular surface were obtained by photomicroscopy. *PRG4 Immunolocalization*. The presence of PRG4 within chondrocytes was determined qualitatively from cartilage disks that were incubated for 4 h following bioreactor culture in medium supplemented with 1 μM monensin, essentially as described previously [24]. Upon termination of culture, the disks were frozen in Tissue Tek OCT (Sakura USA) and sectioned (5 μm slices) perpendicular to the articular surface. The sections were reacted with mAb 3-A-4 (a generous gift from Dr. Bruce Caterson, University of Wales, Cardiff, UK [47]), and detected with a peroxidase-based system (Vector Labs). Stained samples were viewed to identify immunoreactive cells, indicating synthesis of PRG4. Sections probed with a non-specific mouse IgG antibody served as negative controls. Qualitative results were documented by photomicroscopy using brightfield illumination. *PRG4 Secretion.* Culture medium was

collected and replaced every 24 h for cartilage disks incubated for 2 days. As described previously [24], conditioned medium samples were quantitatively analyzed for PRG4 content by indirect ELISA using mAb 3-A-4. Briefly, samples were diluted serially, adsorbed, and then reacted with mAb 3-A-4, horseradish peroxidase-conjugated secondary antibody, and ABTS substrate, with 3 washes with PBS-0.1% Tween (Bio-Rad) between each step. A standard curve was generated from samples containing known amounts of PRG4, obtained from conditioned medium from explants from the superficial zone of bovine calf cartilage as previously described [46]. The protein-equivalent amount of PRG4 in each sample was calculated from the linear region of the standard curve (between 0.078 and 5 μg/ml of PRG4), as described previously [24, 48]. Control studies indicated that cartilage disks contained PRG4 in amounts, ~1 $\mu g/cm^2$, that were small relative to that secreted into the medium, so that the secreted quantities were representative of biosynthesis levels.

Data Reduction. *Chondrocyte Viability.* Live and dead chondrocytes were identified by threshold-based image analysis with Matlab 7.0 software (The Mathworks, Inc., Natick, MD). Chondrocyte viability is presented as % live cells = (100% * # live cells) / (# live cells + # dead cells), for the articular surface area analyzed. *PRG4 immunolocalization.* Chondrocytes staining for PRG4 (PRG4+) as well as those not expressing PRG4 (PRG4-) were identified manually in a 300μm x 400μm area of each vertical section, and counted by image analysis with Matlab 7.0. Since total number of cells varied with joint region, PRG4 expression was reported as a percentage of the total cell number in the area counted: % PRG4+ = (100% * # PRG4+ chondrocytes) / (#PRG4+ chondrocytes + #PRG4- chondrocytes). *PRG4*

secretion. PRG4 protein secretion was reported normalized to cartilage disk surface area, and was also represented as a contour plot. The contour plot was created using Matlab 7.0 to interpolate between input mean secretion values at the locations of each cartilage disk, and mapped onto the joint surface, with color indicating the level of secretion by explants taken from that location, on a logarithmic scale.

Statistical Analysis. All data are expressed as the mean ± standard error of the mean (SEM). Statistical analyses were performed using Systat 10.2 software. ***Experiment I.*** PRG4 secretion values were log transformed for normality, and effects of joint region (PFG, LFC, MFC) and site (indicated by dots, **Fig. 5.3B**) on PRG4 secretion by cartilage explants from freshly harvested joints were determined by repeated measures ANOVA (region and site as repeated factors) and post-hoc comparisons of the interaction between region and site. ***Experiment II.*** *Chondrocyte viability:* Percentage data were arcsine transformed and effects of CPM (- or + CPM) and sliding duty cycle (continuous, intermittent, or never) were assessed by repeated measures ANOVA, where CPM and sliding duty cycle were repeated factors. *PRG4 expression:* Percentage data were arcsine transformed, and effects of CPM and duty cycle were determined by ANOVA with Tukey post-hoc tests. *PRG4 secretion:* Secretion values were log transformed and effects of CPM and joint region were determined by repeated measures ANOVA.

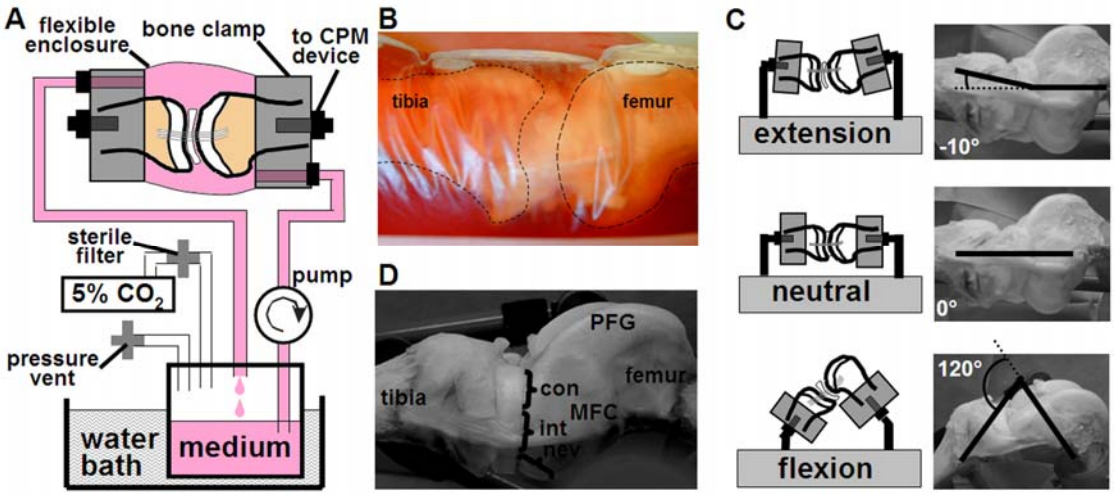


Figure 5.1 Continuous passive motion bioreactor system for whole joints. (A) Bioreactor provides tissue-culture conditions during continuous passive motion stimulation of the joint. (B) Actual joint in bioreactor. (C) During motion stimulation, the joint oscillates between two positions within the range of -10° to 120° flexion, such that certain regions of the femoral condyles (D) are continuously (con), intermittently (int), or never (nev) sliding against opposing tissues during stimulation.

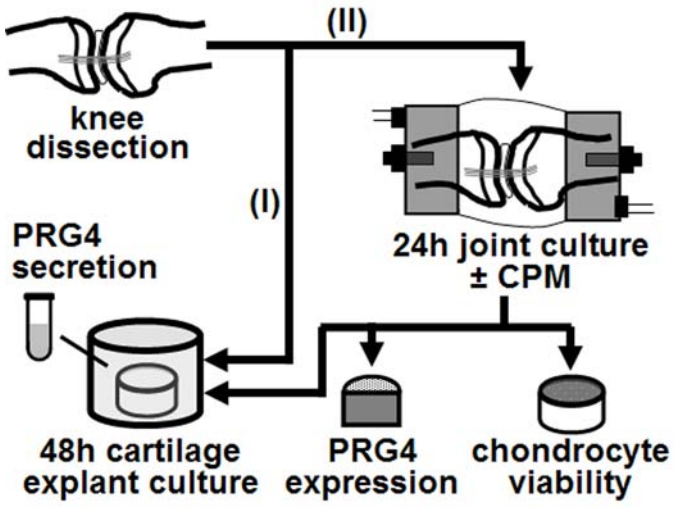


Figure 5.2. Bovine stifle joints were isolated, dissected, and used in one of two separate experiments. (I) Cylindrical cartilage disks were explanted and cultured for 2 days. (II) Joints were cultured for 24 hours in the bioreactor with or without CPM stimulation, and cartilage disks were then explanted and analyzed for chondrocyte viability or chondrocyte PRG4 expression, or cultured for 2 days. PRG4 secreted by cartilage explants during culture was quantified and characterized from the conditioned medium samples.

5.4 Results

Experiment I. Intrinsic chondrocyte PRG4 secretion, determined from freshly isolated joints, was highly dependent on cartilage location (**Fig. 5.3B**). PRG4 secretion varied markedly with cartilage explant site (indicated by dots, **Fig. 5.3**) within the LFC ($p<0.01$) and MFC ($p<0.001$) regions, but not within the PFG region ($p=0.78$). Averaging secretion values over all sites within a given region suggested ($p=0.1$) that in general, cartilage from the PFG and MFC secreted similar amounts of PRG4 on average (55±18, 42±7 μg/(cm^2*day), respectively), while cartilage from the LFC secreted only 27±5 μg/(cm^2*day). Finally, PRG4 secretion rates varied strikingly among the 4 animals, with the coefficient of variation (CV) of PRG4 secretion values at a given cartilage explant site ranging from 13 to 158%.

Experiment II. Cartilage viability was maintained during bioreactor culture, with or without CPM stimulation. Chondrocyte viability (**Fig. 5.4A**) was high (80-93%) for all regions and loading conditions, and varied somewhat with joint region ($p=0.07$), but not in a CPM-dependent manner ($p=0.42$ for interaction between CPM stimulation and joint region).

CPM stimulation up-regulated PRG4 biosynthesis in cartilage from certain locations of the joint. PRG4 immunolocalization (**Figs. 5.4B, 5.5**) revealed that the percent of the chondrocyte population of expressing PRG4 was regulated by CPM in a sliding duty cycle dependent manner ($p<0.001$ for interaction between CPM and sliding duty cycle). The regions of the femoral condyles that were continuously sliding against cartilage/meniscus in the joint cultured with CPM (**Fig. 5.5I,L**) contained

more PRG4+ chondrocytes (LFC: 37±4%, $p<0.001$; MFC: 40±5%, $p<0.01$), compared to the same regions of the joint cultured without CPM (**Fig. 5.5B,E**), where 13±3% and 22±2% of chondrocytes expressed PRG4, respectively, in the LFC and MFC. In addition, cartilage from the region of the LFC that was intermittently sliding against cartilage/meniscus (**Fig. 5.5C,J**) had higher PRG4 expression ($p<0.01$) with CPM (36±8%) than without (13±4%), though an effect of CPM was not detected for the intermittently sliding region of the MFC ($p=0.99$, **Fig. 5.5F,M**), where 37±5% of chondrocytes were PRG4+. As expected, PRG4 expression was independent of CPM for the PFG region (52±2%, $p=0.99$, **Fig. 5.5A,H**) and for regions of the LFC and MFC that were never sliding against cartilage/meniscus during CPM (43±1% and 40±5%, respectively, $p=1.0$ each, **Fig. 5.5D,K,G,N**).

Bioreactor culture with CPM appeared to also maintain chondrocyte PRG4 secretion in a sliding duty-cycle dependent manner, compared to bioreactor culture without joint motion (**Figs. 5.3C, 5.4C**). Consistent with effects on PRG4 expression, PRG4 secretion in continuously sliding regions of the LFC tended to be higher ($p=0.2$) in cartilage from the CPM stimulated joint (11±5 μg/(cm^2*day)) than in cartilage from the same region of the joint cultured without CPM (4±2 μg/(cm^2*day)). Averaged over all sites in a given region, PRG4 secretion also varied with joint region ($p<0.05$), consistent with the trend for cartilage from freshly isolated joints (Experiment I). The PFG and MFC secreted more PRG4 (40±7 and 27±8 μg/(cm^2*day), respectively, $p<0.05$ each) than cartilage from the LFC (10±2 μg/(cm^2*day)). Cartilage from the PFG regions of joints cultured both with CPM and without CPM secreted similar amounts of PRG4 ($p>0.9$). As expected, CPM stimulation did not affect PRG4

secretion by cartilage from sub-regions of the LFC (12±5 μg/(cm^2*day), p=1.0) and MFC (12±4 μg/(cm^2*day), p=0.56) that were never sliding against cartilage/meniscus during CPM stimulation. Secretion values were also unaffected by intermittent sliding (LFC: 14±5 μg/(cm^2*day); MFC: 27±10 μg/(cm^2*day), p>0.9 each).

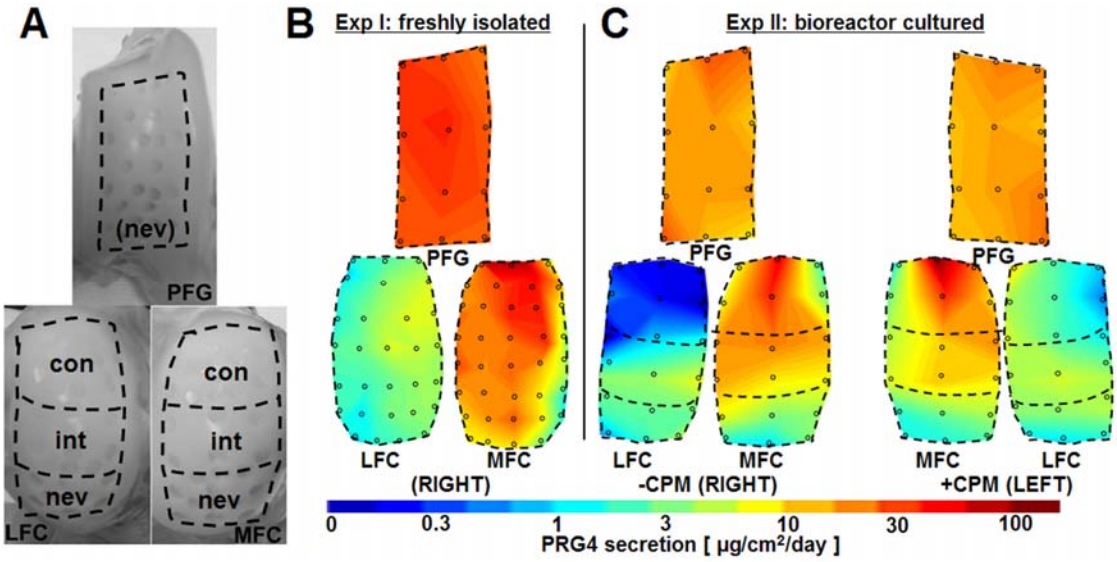


Figure 5.3. (A) Cartilage disks were explanted from various sites on the patellofemoral groove (PFG) and lateral and medial femoral condyles (LFC, MFC), and grouped into sub-regions that continuously (con), intermittently (int), or never (nev) slide against opposing tissues during CPM stimulation. (B) PRG4 secretion by cartilage disks from freshly harvested (Experiment I) or bioreactor cultured (Experiment II, with or without CPM stimulation) joints, represented as a logarithmic color scale contour mapping of onto the joint regions. (Explant sites are shown as open dots.)

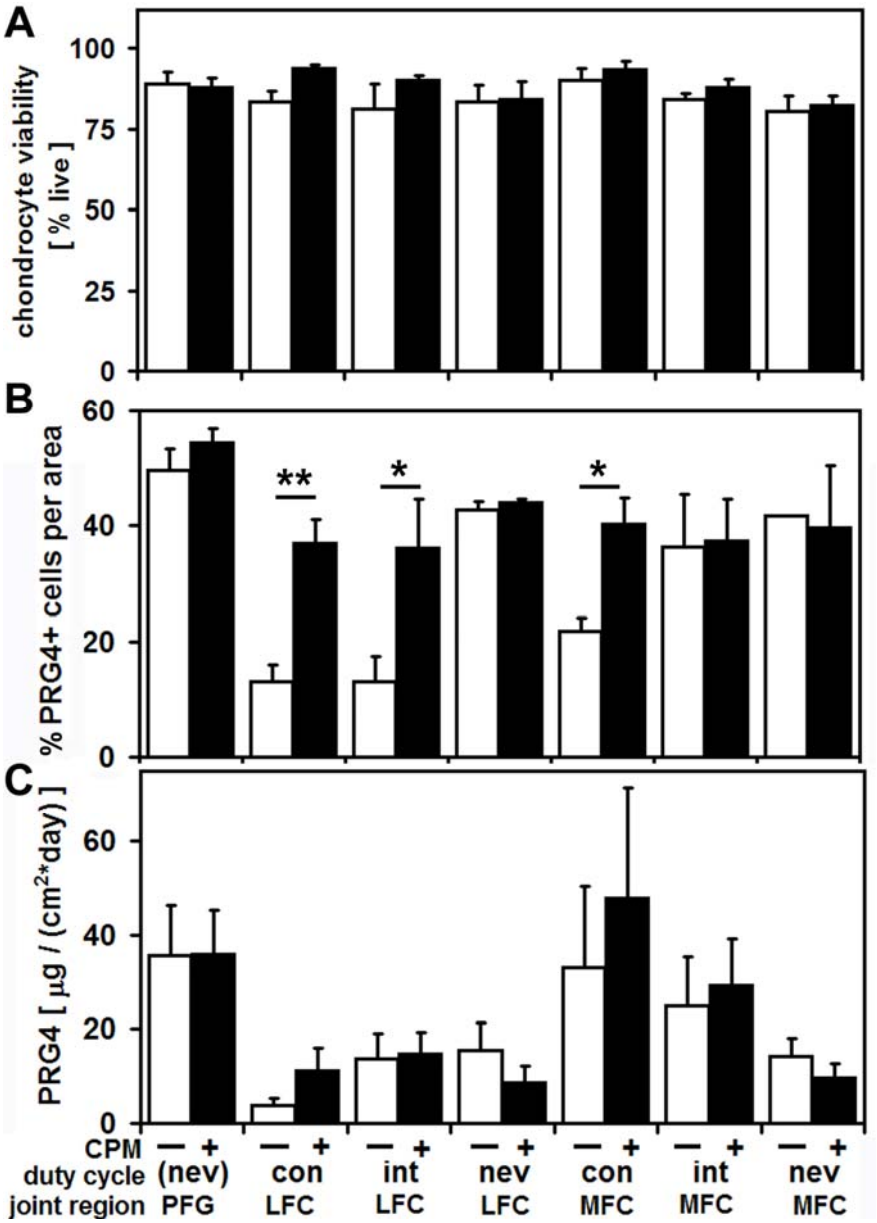


Figure 5.4. (A) Chondrocyte viability determined from *en face* images of the articular surface, (B) chondrocyte PRG4 expression in cartilage sections, and (C) PRG4 secretion of cartilage disks from the patellofemoral groove (PFG) and various sub-regions (based on sliding duty cycle) of the lateral and medial femoral condyles (LFC, MFC) of bovine stifle joints after culture in a whole joint bioreactor with (■) or without (□) CPM stimulation. Mean±SEM. n=3-4 animals. *$p<0.01$, **$p<0.001$.

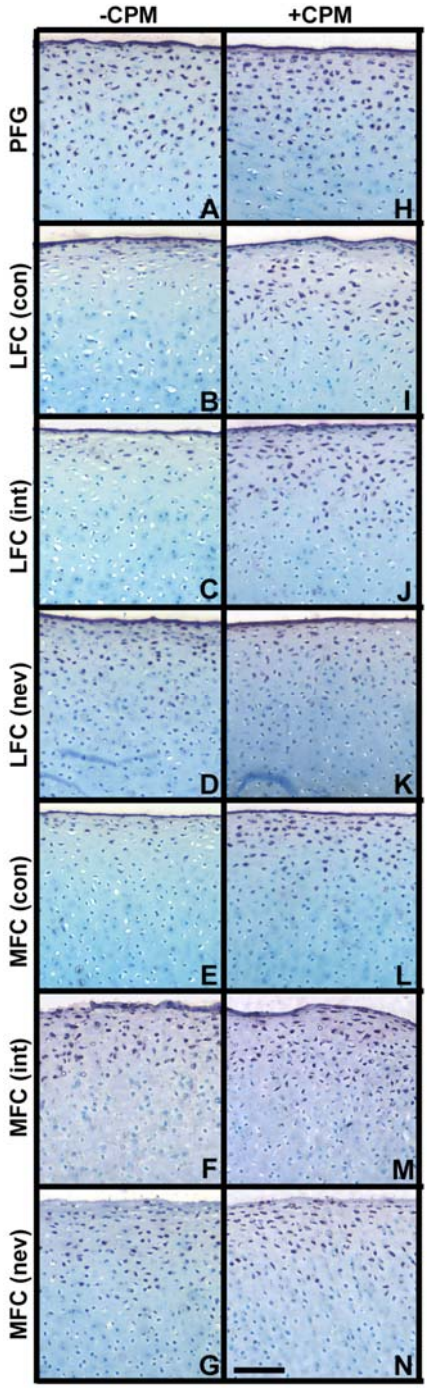


Figure 5.5. Immunolocalization of PRG4 (dark purple stain) within chondrocytes. Shown are representative images of cartilage samples from various joint regions, after bioreactor culture without (**-CPM, A-G)**, or with CPM stimulation (**+CPM, H-N**). Bar = 100 μm.

5.5 Discussion

These results identify a mechanism by which joint motion and CPM may contribute to maintenance of joint health, and demonstrate the ability to culture whole joints in a bioreactor. CPM increased the percentage of chondrocytes expressing PRG4 (**Figs. 5.4B, 5.5B,E,I,L**) and tended to increase PRG4 secretion (**Figs. 5.3C, 5.4B**) for cartilage in the regions of the femoral condyles that were continuously sliding against adjacent meniscus/cartilage, compared to those values for the corresponding regions of joints cultured without CPM. Intermittent sliding against adjacent meniscus/cartilage was also sufficient to up-regulate chondrocyte PRG4 expression in cartilage from the LFC (**Figs. 5.4B, 5.5C,J**). Chondrocyte viability remained quite high during bioreactor culture of joints and was independent of applied continuous passive motion (**Fig. 5.4A**), suggesting that differences in PRG4 metabolism were due to mechanical regulation in certain regions, rather than cell death. In addition, the marked site-associated variation in intrinsic chondrocyte PRG4 secretion over the joint surface was revealed (**Fig. 5.3B**).

The parameters of rehabilitative joint motion, applied through CPM, were chosen based on previous protocols found to be stimulatory, and also consideration of knee joint kinematics. For example, the cycling frequency (43 seconds per cycle) is similar to that found most effective in healing cartilage defects in rabbits [37], and is in the range of frequencies typically used for *in vitro* experimentation with CPM (40-45 seconds per cycle [22, 29, 41, 54]). The range of motion used here was within the range of flexion angles typically experienced during walking (0-41° minimum to 50-

77° maximum flexion angle) for both human knee joints [1, 6, 12] and ovine stifle joints [53]. This study did not attempt to reproduce joint muscle forces, which also contribute to normal active loading of the joint (total joint compressive forces up to 3 times body weight [42]). Axial loading in the present experiments was due to forces in the intact cruciate and collateral ligaments [9]. Thus the loading applied here is similar in nature to that applied during CPM rehabilitative therapies. The study of physiological levels of axial compression in addition to the CPM stimulation protocol could provide additional insight into the biomechanical regulation of chondrocyte metabolism *in vivo*.

Also, since the goal of this study was to determine, in a controlled manner, the effects of continuous passive motion on chondrocyte metabolism, several joint tissues were removed. While patellofemoral joint forces contribute to the kinematics of knee motion *in vivo* [25, 49, 57], removal of the patella allowed for an internal control region (i.e. PFG) of cartilage that experienced the same mechanical environment in joints cultured both with and without CPM. Synovial fluid was also removed, and the medium used to bathe the joint was a baseline formulation typically used for cartilage and chondrocyte cultures, different in composition from the synovial fluid that normally bathes cartilage *in vivo*. This created a well-defined biochemical environment that allowed effects of CPM itself to be determined. Fetal bovine serum was added during bioreactor culture, as it has been shown to maintain cartilage proteoglycan synthesis *in vitro* [14]. Specific chemokines and growth factors could also be added during bioreactor culture, without CPM to assess the effects of

biochemical stimuli on chondrocytes in intact cartilage, or with CPM to investigate possible interactive effects of biomechanical and biochemical stimuli.

PRG4 metabolism was chosen for study because it is a functional lubricant whose expression is mechanosensitive. First, since PRG4 molecules function to provide boundary lubrication during cartilage-on-cartilage sliding [45], it was hypothesized that mechanical conditions that require lubrication (cartilage sliding against cartilage and meniscus in a physiological configuration) might result in increased PRG4 production. Furthermore, chondrocyte PRG4 metabolism is markedly regulated by micro-environmental cues during short term culture, including both biochemical [43, 44] and biomechanical [31, 32] stimuli, such that effects of rehabilitative joint motion were likely to be detected. PRG4 expression (by immunolocalization) provided a snapshot of chondrocyte populations expressing PRG4 immediately upon termination of CPM, while PRG4 secretion provided a functional measure of lubricant production over 2 days following bioreactor culture. The marked intrinsic topographical variation in these measures may be due to the high mechano-sensitivity of chondrocyte expression of PRG4, combined with wide variations in biomechanical environments experienced by different joint regions. The high inter-animal variation could account for the slightly lower overall PRG4 secretion levels in the bioreactor cultured joints compared to the levels secreted by freshly harvested joints, which were from different animals. However, the use of animal-matched joints (right and left from the same animal) for each run of experiment II eliminated this issue for comparison of joints cultured in bioreactors with and without CPM.

The novel bioreactor for CPM stimulation of whole joints developed in this study provided the means to investigate mechano-regulation of PRG4 metabolism in a loading environment that is more physiological than that of traditional bioreactor culture systems, while at the same time eliminating some of the confounding factors present with *in vivo* studies. The marked site-associated variation in chondrocyte PRG4 secretion and expression and the apparent dependence on local mechanical environment, taken together with the altered PRG4 expression patterns seen in tibial cartilage subjected to abnormal mechanical stimulation [56], further support the hypothesis that mechanical factors are key determinants of PRG4 metabolism *in vivo*. CPM stimulation resulted in up-regulation of PRG4 secretion and expression levels by nearly 3-fold in the continuously stimulated region of the LFC, consistent with the 3-fold increase in proteoglycan production resulting from tissue shear of cylindrical cartilage explants [31], and the 3 to 7-fold up-regulation of PRG4 mRNA due to surface motion applied to chondrocytes in cartilaginous constructs [11]. These results also raise the possibility that regulation of PRG4 metabolism may depend on loading duty cycle, as intermittent cartilage sliding was sufficient to alter PRG4 expression in the LFC, but not in the MFC. Since intermittent sliding did not appear sufficient to alter chondrocyte secretion of PRG4 in either femoral condyle, it may be that intermittent sliding led to transient up-regulation, whereas continuous sliding resulted in longer-lasting effects.

The finding that CPM during culture maintains chondrocyte viability and metabolic function supports the use of CPM as a post-operative treatment, and may indicate a mechanism by which CPM is beneficial to the health of cartilage and joints

in vivo. In addition, PRG4 synthesis was very low in cartilage from certain regions of the joint cultured without CPM, consistent with previous reports that joint immobilization causes decreased chondrocyte metabolism of matrix molecules, which results in decreased cartilage thickness and mechanical integrity [3, 5, 17, 23]. Thus, these results indicate that the negative effects of immobilization may also be due to down-regulation of chondrocyte synthesis of lubricant and chondroprotective PRG4 molecules.

The higher up-regulation of PRG4 secretion and expression in the LFC (3-fold for both measures) compared to the MFC (1.5-fold for secretion, ~1.8-fold for expression) in the continuously sliding region, as well as the ability of intermittent sliding to up-regulate PRG4 expression in the LFC but not the MFC, could be due to differences in biomechanical environment experienced by the two regions during CPM. In humans, the LFC and MFC experience distinct motion patterns relative to the adjacent tissues during knee flexion, with the MFC tending to move by sliding against the tibial plateau, while the LFC tends to "roll" across the tibial plateau [15, 18, 26, 33]. In digital video images obtained during pilot experiments in which CPM was applied to joints without medium or bioreactor enclosures, it appeared that this may also be the case for the bovine stifle joint, although with anatomically opposite tendencies (LFC sliding, MFC rolling), as the relative size and shape of the bovine femoral condyles and patellofemoral groove is opposite to that of humans. These observations suggest that sliding (which would impart shearing deformation to the tissue) leads to more up-regulation of PRG4 secretion than rolling (which would sequentially impart compressive deformation to different sites). Alternatively, the up-

regulation of PRG4 expression and secretion in certain regions could be due to increased nutrient transport to those regions resulting from motion of the joint, compared to the transport level during bioreactor culture without motion.

The CPM bioreactor for whole joints developed in this study provides a platform for possible future *in vitro* studies and applications, including the assessment of the effects of CPM on metabolism of various joint tissues and possible interactions of biomechanical and biochemical signals. This system could also be used to investigate the effects of rehabilitative joint motion on the efficacy of various cartilage defect repair strategies in live tissue *in vitro*, as described for post mortem tissue [7], before moving on to more costly and complicated *in vivo* studies [10]. The current system allows for the simultaneous culture of two joints, but could be scaled up for culture of many joints in parallel. In addition, his bioreactor may be useful for *in vitro* culture of biological joints which could ultimately be used as restorative implants for large cartilage defects, and possibly whole joints.

5.6 Acknowledgments

This chapter, in full, has been submitted to *Osteoarthritis & Cartilage*. The dissertation author was the primary investigator and thanks co-authors Tad Takara, James K. O'Neill, Sean B. Cahill, Simon Görtz, Terrence Pong, Hideru Inoue, Nicole M. Aneloski, Will W. Wang, Keeley I. Vega KI, Travis J. Klein, Nancy D. Hsieh-Bonassera, Dr. Won C. Bae, James D. Burke, and Dr. William D. Bugbee. This work was supported by NIH, NSF, Whitaker Foundation (pre-doctoral fellowship to GEN), Irwin and Joan Jacobs (undergraduate scholarship to JKO), Stein Institute for Research on Aging (scholarship to TT). The authors also thank Dr. Darryl D'Lima for advice on whole-joint preparations.

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CHAPTER 6

SITE- AND EXERCISE-REATED VARIATION IN STRUCTURE AND FUNCTION OF CARTILAGE FROM EQUINE DISTAL METACARPAL CONDYLE

6.1 Abstract

OBJECTIVE: Determine (1) the site-associated response of articular cartilage of the equine distal metacarpal condyle to training at a young age as assessed by changes in indentation stiffness and alterations in cartilage structure and composition, and (2) relationships between indentation stiffness and indices of cartilage structure and composition. METHOD: Experimental animals (n=6) were trained on a track (increasing exercise to 1 km/day by 5 months); controls (n=6) were pasture-reared. Animals were euthanized at 18 months and four osteochondral samples were harvested per metacarpal condyle from dorsal-medial, dorsal-lateral, palmar-medial, and palmar-lateral aspects. Cartilage was analyzed for India ink staining (quantified as reflectance score (RS)), short-term indentation stiffness (sphere-ended, 0.4 mm diameter), thickness, and biochemical composition. RESULTS: Cartilage structural, biochemical and biomechanical properties varied markedly with site in the joint. Sites

just medial and just lateral to the sagittal ridge showed signs of early degeneration, with relatively low RS, indentation stiffness, and collagen content, and relatively high water content. Effects of exercise and side (left vs right) were not detected for any measure. Overall, indentation stiffness correlated positively with RS and collagen content, and inversely with thickness and water content. CONCLUSION: Gentle exercise-imposed mechanical stimulation did not markedly affect articular cartilage function or structure. However, the marked site-associated variation suggests that biomechanical environment can initiate degenerative changes in immature cartilage during joint growth and maturation.

6.2 Introduction

The processes responsible for mechanical regulation of normal growth and maturation of articular cartilage remain to be elucidated. Experimental and clinical evidence indicates that mechanical loading is a crucial factor in the functional adaptation of the articular surface to meet the demands it faces [18]. Several studies have shown that elimination or reduction of load bearing in young animals leads to abnormal development of articular cartilage. In embryonic chick limbs, paralysis during skeletal maturation leads to abnormal joint shapes and sizes [12]. Immobilization of a weight bearing limb in growing beagle dogs results in glycosaminoglycan (GAG) depletion [27] and reduced indentation stiffness [22] of the articular cartilage. In young beagle dogs subjected to a moderate running program, thickness and GAG content were augmented in regions bearing the highest loading surplus, indicative of a functional adaptation to use [29]. Furthermore, neonatal ovine articular cartilage shows no detectable site-associated variation in proteoglycan content, in contrast to the clear biochemical heterogeneity in the mature tissue [31]. Similarly, neonatal equine carpal articular cartilage exhibits no site-associated variation in water content, DNA, GAG content, or collagen characteristics, while significant site-associated variation in these properties is apparent after the first 5 months post partum [6-8]. Lack of exercise during the first few months of life also appears to delay this development of biochemical heterogeneity in equine carpal cartilage [8]. Taken together, these studies reveal the importance of mechanical loading of joints for normal growth and maturation of articular cartilage.

While mechanical stimulation appears necessary for proper joint formation, excessive or abnormal mechanical stimuli may predispose cartilage to degenerate at specific sites. Mice undergoing a lifelong training regimen showed increased incidence of osteoarthritis in the knee joint compared to non-trained controls [30]. Young beagle dogs subjected to long-term strenuous exercise regimens demonstrated a site-dependent loss of GAG content [2, 28] and reduced stiffness [1, 21] in the femoral cartilage, alterations that are most likely degenerative in nature. In racing horses, the metacarpophalangeal, or fetlock, joint (**Fig. 6.1A,B**) appears particularly vulnerable to mechanically induced site-specific degeneration, as it experiences more degenerative lesions than any other limb joint [39]. Since these lesions have not been observed in non-racing horses, the equine fetlock joint is a location in which the articular cartilage may respond to alterations in mechanical loading during growth.

Before deterioration becomes symptomatic clinically, degenerative changes in articular cartilage can be manifest as more subtle alteration of the function, structure, and composition of the articular surface. Surface roughening can be quantified by staining the articular surface with India ink and subsequently measuring the light reflectance from the articular surface [11]. Mechanical softening can be detected as a reduction in short-term indentation stiffness [15]. Short-term indentation stiffness is sensitive to early-stage degeneration [5, 26, 40] and insensitive to thickness when the indenter tip radius is less than the thickness of the cartilage [17]. The biochemical composition of articular cartilage has also been used as an indication of integrity, and correlations between mechanical properties, structure and composition may help elucidate the structure-function relationships that govern cartilage behavior.

The structure, function, and composition of equine articular cartilage vary with site, age, and exercise. Aggregate modulus, determined by indentation testing [35], and biochemical properties [10, 34] vary with exercise level in a site-specific manner in skeletally immature (5-24 months) equine carpal cartilage. Conversely, indentation aggregate modulus of mature (~8 year old) carpal cartilage does not vary with exercise, although strong site-associated variations were evident [37]. For the equine distal metacarpal condyle, the site variation in biochemical composition has been mapped for normal adult articular cartilage [9], providing a baseline for comparison. However, the biomechanical properties and the effects of exercise at a young age on structure and composition have not yet been reported for the metacarpal condyle. The common practice of initiating racehorse exercise at a very young age provides a convenient model in which to study these properties and relationships among the properties, which may lead to better understanding of the processes involved in the development of both normal and degenerate tissue.

Thus, the objectives of this study were to determine (1) the site-associated response of articular cartilage of the equine distal metacarpal condyle to exercise training at a young age as assessed by changes in indentation stiffness and concomitant alterations in cartilage structure and composition, and (2) the relationships between indentation stiffness and indices of cartilage structure and composition. This study was part of a collaborative effort among Massey University, NZ; Colorado State University, USA; Royal Veterinary Collage, UK; and Utrecht University, NL, with the overall goal of reducing the incidence of injury in the racehorse by application of appropriate training in the young animal.

6.3 Materials and Methods

The study performed here was part of a larger project organized by the Global Equine Research Alliance, to reduce the incidence of musculoskeletal injury in the racehorse by determining the effect of training conditions on the young animal. Twelve newborn New Zealand Thoroughbred equine animals were used with IACUC approval. The animals were randomly divided into exercise (n=6) and control (n=6) groups, each consisting of three geldings and three fillies. All animals were raised on a pasture throughout the duration of the experiment. The exercise group of horses was subjected to an additional training regimen starting at 10 days of age. They were exercised 5 days/week at the minimum velocity necessary to maintain a canter on a 500 m long oval track in a paddock. The direction of movement around the track was alternated daily. Distance and velocity were gradually increased until 1 month of age, and thereafter increased to at least 1020 m at 6.7 m/s per day by 5 months. According to stride length data from kinematic studies of equine running [20, 24, 41], this corresponds to approximately 300-400 loading cycles per bout of exercise, or 1500-2000 loading cycles per week. The animals were euthanized at the age of 18 months and the left and right distal metacarpal condyles were harvested for analysis. From these sample materials received from the Global Equine Research Alliance, four osteochondral samples were harvested from each condyle at the following locations (**Fig. 6.1C**): dorsal-lateral (DL), dorsal-medial (DM), palmar-lateral (PL), and palmar-medial (PM). Samples were wrapped in gauze soaked in phosphate buffered saline solution (PBS; 2.667 mM KCl, 1.471 mM KH_2PO_4, 138 mM NaCl, 8.1 mM Na_2HPO_4-$7H_2O$, pH 7.2) with protease inhibitors (PI; 1 mM phenylmethanesulfonyl

fluoride, 2 mM disodium ethylenediamine tetraacetate, 5 mM benzamidine-HCl, and 10 mM N-ethylmaleimide) and stored at -70°C. Sites 1-5 were chosen to represent a range of susceptibility to degenerative clinical lesions [39], and identified using templates so that they were in the same relative anatomical location, regardless of joint size, for each sample (**Fig. 6.1C**). Sites 1 and 2 were located on the sagittal ridge, site 3 was just lateral or just medial to the sagittal ridge, and sites 4 and 5 were in more peripheral locations.

Structural Analysis. Cartilage thickness was determined by imaging. Digital photographs were taken from two sides of each sample with the camera perpendicular to the cut osteochondral surface. Cartilage thickness was determined from the digital images at each of the five testing sites using Scion Image software (Scion Corporation, Frederick, Maryland). The average of the two thickness measurements is reported for each site. Samples were analyzed by India ink staining and video image analysis to characterize surface roughness properties, as described previously [11]. Briefly, the articular surface was painted with a solution of India ink in PBS+PI (1:5), wiped to remove excess ink, and imaged at a resolution of 0.1 mm x 0.1 mm per pixel (8-bit, gray scale, 640x480 pixels). For each of sites 1-5, normalized light reflectance scores (RSs) were determined using gray scale calibration values (Q13, Eastman Kodak Co., Rochester, NY) that were chosen to approximate the reflectance of light from normal, non-staining cartilage (Gray #3, normalized value of 1), and fibrillated, maximally ink-stained cartilage (Gray #19, normalized value of 0). A relatively low RS indicates relatively high ink staining and cartilage degeneration.

Biomechanical Analysis. The indentation stiffness of the articular cartilage at each site was determined by mechanical testing. Prior to testing, samples were thawed at room temperature in PBS+PI, and the bone portion of each sample was mounted in dental plaster (Labstone, Modern Materials®), which fit into a customized holder with enough degrees of freedom to allow for positioning with the indenter tip perpendicular to the articular surface at each test site. The sample was mounted on a mechanical testing apparatus (V500cs, BioSyntech Canada Inc, Quebec, Canada) and hydrated with PBS+PI with a peristaltic pump (Fisher) for the duration of testing. Indentation testing consisted of application of a 0.16 MPa tare load (averaged over the largest cross-sectional area of 0.4 mm diameter, sphere-ended Al_2O_3 indenter tip), followed by three rapid 50 μm test displacements (applied at 0.5 mm/s, and held for 0.5 s each). The instantaneous force response was determined as the average of the three jumps in force measured during the three displacements. Indentation stiffness was defined as instantaneous force response normalized to displacement.

Biochemical Analysis. For selected sites showing marked variation in biomechanical and structural properties, full thickness cartilage samples were removed for additional biochemical analysis. From sites 1, 3, and 5, cartilage disks 3 mm in diameter were harvested, weighed wet, lyophilized, and weighed dry. Water content was computed as (wet weight - dry weight)/ wet weight. The cartilage disks were then solubilized with proteinase K (Roche) at 60°C for 16 h. Portions of the digest were analyzed to quantify the content of sulfated GAG by the dimethylmethylene blue dye binding assay [13], collagen (COL) by the p-dimethylaminobenzaldehyde binding assay for hydroxyproline [42], and DNA by

PicoGreen® [32] fluorescence assay. The hydroxyproline content was converted to collagen content assuming a mass ratio of collagen to hydroxyproline of 7.25 [19, 36]. All biochemical measures were normalized to wet weight.

Statistical Analysis. One way ANOVA was implemented using Systat 10.2 software (Systat, Inc, Evanston, IL) to analyze the effect of exercise group, with repeated measures for left-right leg (side), dorsal-palmar aspect (D-P), lateral-medial aspect (L-M), and site (1-5) comparisons. When significant effects ($P<0.05$) of site were detected, post hoc tests for repeated measures, with a Bonferroni correction, were used to compare the sites exhibiting most degenerative types of changes to other sites. All data are expressed as mean ± standard error of the mean (S.E.M.). The dependencies of biomechanical properties (indentation stiffness) on structural measures (thickness, RS) and biochemical constituents (water content, GAG, COL, DNA) were assessed by linear regression. In addition, to determine which of the many parameters were closely related to each other, a factor analysis by the method of principal components was performed. Indentation stiffness, RS, thickness, water content, GAG, collagen and DNA were the parameters included in this analysis. The principal components matrix was computed, and the loading coefficients (describing how each parameter contributed to each factor) were obtained. For each factor, the dominant contributors were identified as those whose coefficients (ranging from -1 to +1) had an absolute value greater than 0.5.

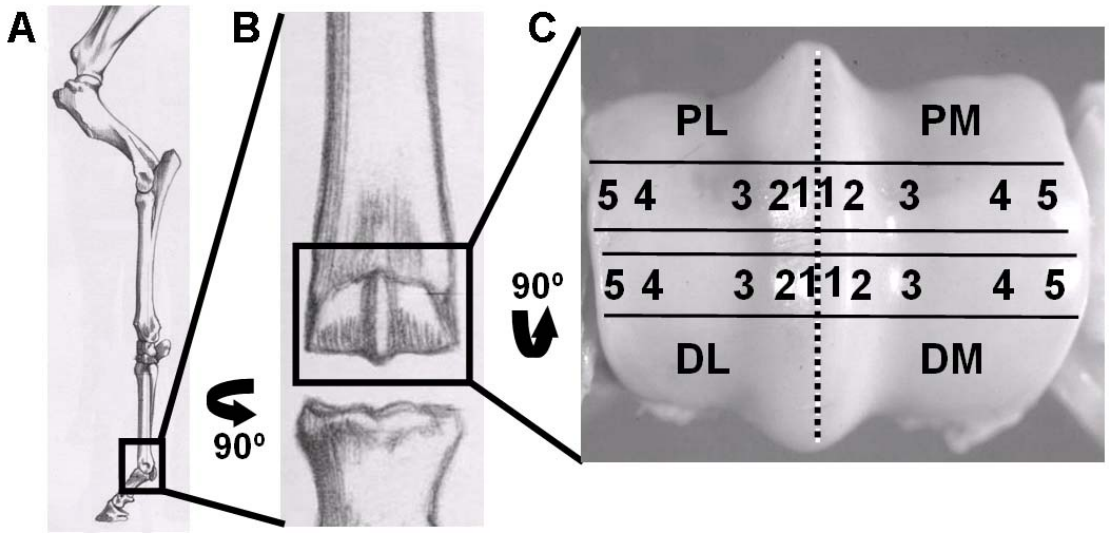


Figure 6.1. (**A**) Equine forelimb and (**B**) fetlock joint (from Ref. [14]). (**C**) Samples were harvested from palmar-lateral (PL), palmar-medial (PM), dorsal-lateral (DL), and dorsal-medial (DM) aspects of the distal metacarpal condyle and tested at sites 1-5. Dotted line indicates sagittal ridge.

6.4 Results

Cartilage thickness (**Fig. 6.2A,B**) varied with site ($P<0.001$), with site 3 being 22% thicker than sites 1, 2, 4 and 5 ($P<0.05$, each). Cartilage of the palmar aspect of the metacarpal condyle (0.81±0.01 mm) was thicker ($P<0.001$) than cartilage of the dorsal aspect (0.70±0.01 mm) as well. Cartilage thickness did not vary significantly with exercise ($P=0.72$) or side ($P=0.37$).

Cartilage surface structure, quantified as RS (**Fig. 6.2C,D**), also exhibited a significant site variation ($P<0.001$), with an interaction effect ($P<0.001$) between site and aspect (D-P). This interaction was evident as a low RS at site 2 in the palmar samples but not the dorsal samples. RS at site 3 (0.787±0.009) was slightly (5%) lower than that of sites 1, 4 and 5 on average ($P<0.05$ each). RS was lower ($P<0.05$) in palmar sites (0.807±0.006) than in dorsal sites (0.840±0.004). RS did not vary significantly with exercise ($P=0.65$) or side ($P=0.80$). Typical staining patterns for each aspect are shown in (**Fig. 6.2G**).

Indentation stiffness (**Fig. 6.2E,F**) varied significantly with site within the joint ($P<0.001$). Relative to sites 1, 2, 4 and 5, site 3 was, on average, 48% softer (each, $P<0.05$). In addition, cartilage of the palmar aspect (2.8±0.07 N/mm) was softer ($P<0.05$) than that of the dorsal aspect (3.54±0.07 N/mm), and cartilage of the medial aspect (3.12±0.07 N/mm) was softer ($P<0.05$) than that of the lateral aspect (3.21±0.4 N/mm). Stiffness in the exercised group (3.09±0.07 N/mm) was similar ($P=0.24$) to that of the control group (3.25±0.08 N/mm). Likewise, a difference between left and right sides was not detected ($P=0.98$).

Cartilage composition also varied with site and aspect, and did not vary with exercise or side. Water content (**Fig. 6.3A,B**) was higher ($P<0.05$) in palmar cartilage (72.3±0.4%) than in dorsal cartilage (70.3±0.4%), and also varied with site ($P<0.01$), with an interaction effect between aspect (L-M) and site ($P<0.05$). GAG content (**Fig. 6.3C,D**) was lower ($P<0.01$) in palmar (26.4±0.9 mg/g wet weight) than in dorsal (30.8±1.3 mg/g wet weight) cartilage, with a significant interaction between aspect (D-P) and site ($P<0.05$). Collagen content (**Fig. 6.3E,F**) showed site variation ($P<0.05$), as well as interaction effects (each, $P<0.05$) between aspect (L-M) and site, and between aspect (L-M) and exercise group. DNA content (**Fig. 6.3G,H**) varied significantly with site ($P<0.001$) and aspect (D-P) ($P<0.01$), with interaction between the two effects ($P<0.01$), and among sites, aspect (D-P), and training group ($P<0.05$). In terms of composition, site 3 exhibited relatively high water content, low collagen content, and low DNA content.

Biomechanical properties correlated significantly with both structural and biochemical properties. Indentation stiffness exhibited a strong inverse relationship with thickness [$R^2=0.71$, $P<0.001$, **Fig. 6.4A**), and significant positive relationships with RS [$R^2=0.18$, $P<0.01$, **Fig. 6.4B**] and COL content [$R^2=0.21$, $P<0.05$, **Fig. 6.4E**]. Stiffness also showed a significant inverse relationship with water content [$R^2=0.25$, $P<0.05$, **Fig. 6.4C**]. There was no detected relationship between indentation stiffness and GAG content [$R^2=0.07$, $P=0.20$, **Fig. 6.4D**]. Principal component analysis (Table I) agreed with the linear regression analysis, demonstrating that indentation stiffness, thickness, collagen content, and water content were strongly related, while DNA

content and GAG content were related to each other, but not as strongly to mechanical and structural properties.

Table 6.1. Principal Component Analysis.

Parameters	Factor 1	Factor 2
Stiffness	**0.885**	0.140
Thickness	**-0.923**	0.025
Reflectance Score	**0.637**	0.131
Collagen content	**0.654**	0.056
DNA content	0.276	**0.916**
GAG content	0.419	**-0.816**
Water content	**-0.750**	0.178
% of total variance explained by factor:	46.9	22.5

Dominant parameters are indicated in **bold-face**.

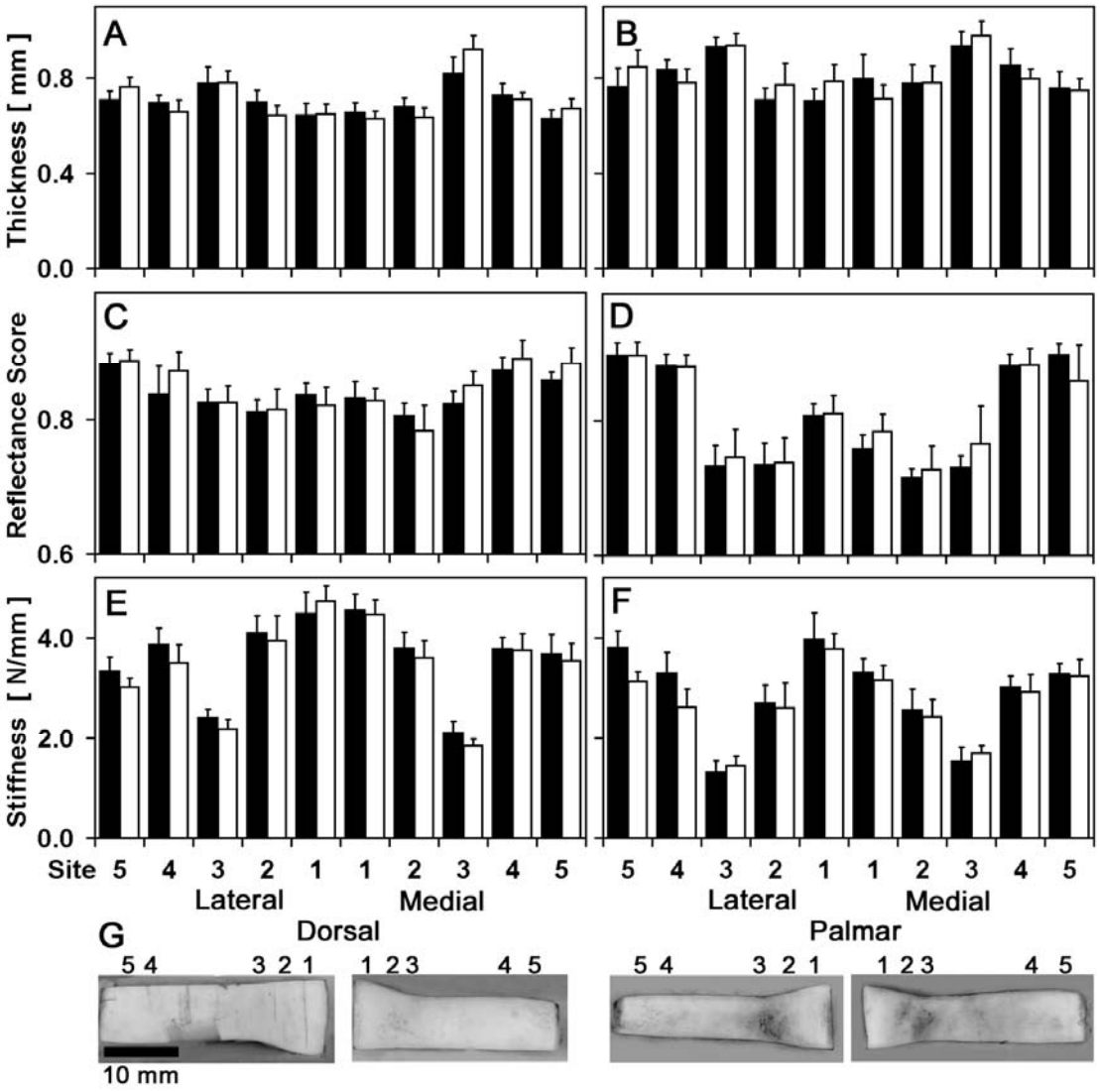


Figure 6.2. Variation in cartilage structural (**A-D**) and mechanical (**E,F**) properties associated with sites (see Fig. 1) from lateral to medial on dorsal and palmar aspects of control (□) and exercise (■) groups. Mean±S.E.M., n=6. (**G**) India ink-stained images typical for each aspect are shown below the respective plots, with testing sites labeled.

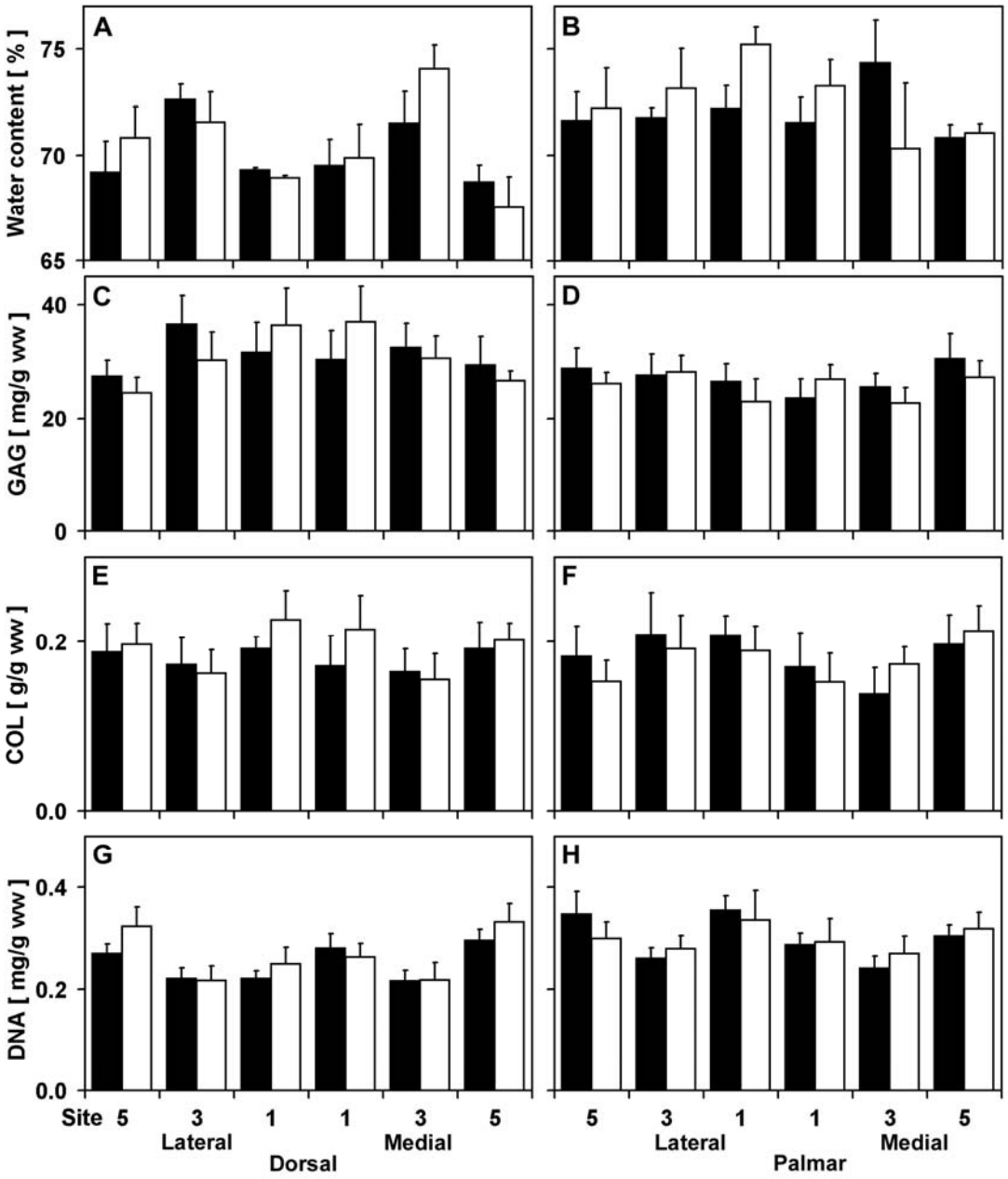


Figure 6.3. Variation in cartilage composition associated with sites (see **Fig. 1**) on dorsal (**A,C,E,G**) and palmar (**B,D,F,H**) aspects of control (□) and exercise (■) groups. Mean±S.E.M., n=3 for water content, n=6 for all other measures.

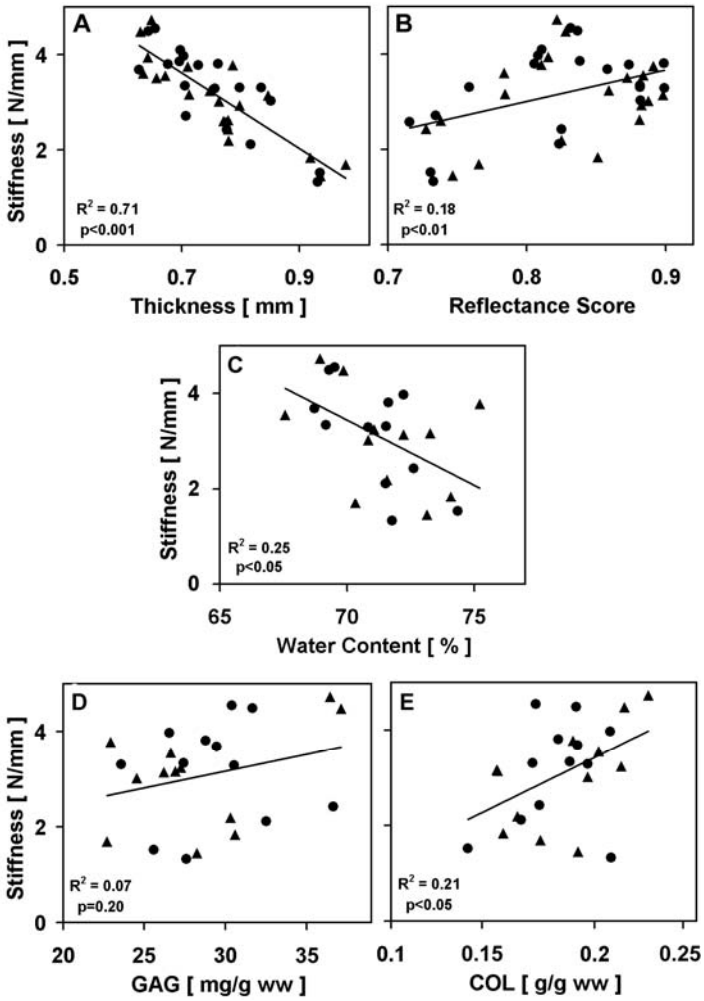


Figure 6.4. Linear regression of indentation stiffness vs structural and biochemical measurements for control (□) and exercise (■) groups.

6.5 Discussion

These results demonstrate marked site-associated variations in cartilage biomechanical, structural, and biochemical properties within the equine distal metacarpal condyle, without detectable effects of exercise training. Site 3 (adjacent to the sagittal ridge), especially on palmar aspect, exhibited hallmarks of early cartilage degeneration as indicated by softening (**Fig. 6.2C**), increased India ink staining [RS, **Fig. 6.2B**] and higher water content (**Fig. 6.3A**). Considering all sites tested, indentation stiffness appeared to be affected by structural and compositional features, being diminished with decreased RS (increased staining) and collagen content, and increased thickness and water content (**Fig. 6.4**). The strong site-dependence of these properties supports the idea that variations in loading in different regions of an articular surface influence the structure, composition, and biomechanical properties of the tissue. The subtle biomechanical, structural, and biochemical differences detected at sites prone to cartilage lesions suggest that predilection for further aging-associated degeneration in articular cartilage may begin early, during maturation of the joint.

Biochemical properties determined here are consistent with previously reported values for equine carpal cartilage in the young animal, and extend the available information for equine metacarpal cartilage. Water and collagen contents were similar to those for cartilage from the metacarpal condyle in adult (5-9 years old) horses of unknown exercise history [9] and for immature (5-11 months old) carpal cartilage in horses of various exercise levels [10]. GAG and DNA contents, however, were higher than those in adult metacarpal cartilage, and agreed more closely with the

levels in immature carpal cartilage. Since both GAG and DNA contents decrease markedly with age during growth and maturation of the equine animal, while changes in water and collagen content are less dramatic [10], these results seem reasonable for the cartilage from 18-month-old animals used in this study. The distribution of biochemical components throughout the condyle also corresponded to that of adult equine metacarpal cartilage [9]. Specifically, water and DNA content were higher, and GAG content was lower in the palmar aspect than in the dorsal aspect, suggesting that the articular cartilage in both control and exercised horses was developing the biochemical heterogeneity typical of this joint.

The dependence of indentation stiffness on degeneration at the articular surface agrees with a previous study [5] that also found a positive correlation of indentation stiffness with RS. Reflectance of ink-stained surfaces reveals the ability of ink particles to penetrate the tissue surface, which becomes more roughened and more permeable during degeneration. It is somewhat surprising that indentation stiffness correlated inversely with thickness since indentation stiffness has been calculated to be relatively independent of thickness until the indenter tip radius approaches the thickness of the cartilage [5]. Since the tip radius was 0.2 mm, and the thinnest cartilage was ~0.6 mm, the correlation between indentation stiffness and sample thickness may indicate that both properties depend on biomechanical environment in a similar manner. In previous studies indentation aggregate modulus was lower in sites with lower proteoglycan content [2, 21, 25, 28, 35] while in this study indentation stiffness correlated more strongly with water content and collagen content. In the present study, the lack of detected correlation between indentation stiffness and GAG

content may reflect the relatively large effect of other factors, varying among the different sites, and relatively modest variation in GAG content. This finding is also consistent with previous observations that short-term compressive stiffness is not directly dependent on proteoglycan content [3, 4, 15, 23, 33, 40].

This work extends the current understanding of the interrelationships among exercise, age, and site as determinants of the biomechanical, biochemical, and structural properties of articular cartilage during growth and maturation. The lower biomechanical integrity at sites of frequent clinical lesions is consistent with previous results in equine carpal cartilage [35, 37]. In canine animals, moderate loading levels tended to increase GAG production [29], while strenuous exercise led to GAG depletion in high-loading areas [2, 28]. Such exercise-associated variations in biochemical properties, however, were not detected in this study, possibly because of differences in training regimens and loading stimuli. In addition, direct comparison of these studies is difficult, as each species may also respond differently to various loading regimes, and the response may also depend on joint geometry, which determines spatial loading patterns and magnitudes resulting from imposed exercise. The lack of exercise-associated variation in structural, biomechanical and biochemical properties in the young equine animal suggests that moderate levels of early training do not predispose the articular cartilage to degeneration. This training regimen was used to introduce the foals to exercise, and was therefore gentle compared to typical training for adult racehorses [16]. Exercise protocols with increased training could lead to more discernible effects on cartilage properties. Furthermore, this result is consistent with the absence of discernible effects of exercise at the age of 11 months

on the articular cartilage of horses trained from the age of 1 week to the age of 5 months [10]. However, the present findings do not rule out the possibility of exercise-induced effects on other tissues that are more responsive to loading. It should also be noted that the present study examined exercise sessions of specific intensities, durations, and duty cycles per session. Variations in these parameters may also be important determinants of the effects on cartilage [18, 38].

The low stiffness and RS at site 3 compared to sagittal ridge (sites 1, 2) and peripheral locations (sites 4, 5) indicate relatively low tissue integrity [5, 11] of the articular cartilage at that site, which is in fact a location of frequent clinical lesions observed in racehorses [39]. Such clinical lesions are especially prevalent in the palmar aspect, which also exhibited lower indentation stiffness and RS than the dorsal aspect in this study. These subtle differences in biomechanical properties, structure, and composition detectable in the immature animal, which are especially interesting in the control animals, at sites prone to cartilage lesions suggest that degenerative changes in articular cartilage may begin before skeletal maturation is complete. The athletic equine animal may be a useful model for further investigation of the process of age-associated degeneration of articular cartilage, especially to follow the progression of site 3 properties throughout the life of the animal.

6.6 Acknowledgments

This chapter is reprinted from *Osteoarthritis and Cartilage*, 12(10), Nugent GE, Law AW, Wong EG, Temple MM, Bae WC, Chen AC, Kawcak CE, Sah RL, Site- and exercise-related variation in structure and function of cartilage from equine distal metacarpal condyle, p. 826-33, Copyright 2004, with permission from Elsevier. The dissertation author (primary investigator) thanks the co-authors of the manuscript for their contributions: Amanda A. Law, Eric G. Wong, Dr. Michele M. Temple, Dr. Won C. Bae, Dr. Albert C. Chen, and Dr. Chris E. Kawcak. In addition, we thank the funding sources that supported this work: Arthritis Foundation, Global Equine Research Alliance, Marilyn M. Simpson Trust, NASA, NIH, NSF, and a pre-doctoral fellowship from the Whitaker Foundation (GEN).

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CHAPTER 7

CONCLUSIONS

7.1 Summary of Findings

This dissertation has investigated the influence of various mechanical stimuli on cartilage metabolism of the putative lubricant PRG4 and on cartilage surface integrity, as well as the mechanism by which PRG4 binds to the articular surface, where it provides lubrication function. Effects of static and dynamic compression of various magnitudes on chondrocyte PRG4 metabolism were determined using bioreactors developed previously in the laboratory for culture of cartilage tissue fragments under uniaxial loading. A new bioreactor system was developed as part of this work to apply biaxial deformation to cartilage fragments during culture, and was used to assess the effects of dynamic tissue shear stimulation on PRG4 metabolism. A third type of bioreactor was developed as part of this work, through collaboration with BREG, Inc., to apply physiological joint motion during culture of whole joints. To address the effects of *in vivo* loading on articular surface integrity, an equine exercise model was employed as part of collaboration with Colorado State University. Finally, to investigate the binding of PRG4 to the articular cartilage surface, cartilage tissue fragments were stored in various treatment solutions under unloaded conditions.

In vitro loading of cylindrical disks of cartilage tissue (Chapters 2, 3) demonstrated that mechanical stimuli can markedly affect PRG4 metabolism. Static compression of both high and low magnitudes resulted in down-regulation of PRG4 secretion compared to that of unloaded cartilage. For lower level static compression, this effect was transient, such that upon unloading PRG4 secretion returned to control levels. For cartilage subjected to static compression to a higher level, however, PRG4 secretion remained low even after unloading. Dynamic compression of both low and high magnitude also down-regulated PRG4 secretion during the application of loading. As with static compression to a low level, this effect was transient for low level dynamic loading. In contrast, upon unloading, cartilage that was previously dynamically compressed at a higher magnitude secreted 1.5 times more PRG4 than controls. Dynamic tissue shear deformation, in contrast to the compression protocols, led to marked up-regulation of PRG4 secretion during the application of load, and this effect was maintained during 3 days of culture following unloading. For all of these studies, chondrocyte viability remained high and was unaffected by the loading protocols, suggesting that any changes in PRG4 metabolism were due to mechanical stimuli, rather than to cell death.

Rehabilitative loading, applied in the form of CPM during bioreactor culture of whole joints (Chapter 5), resulted in increased PRG4 synthesis in cartilage from certain regions of the joint that were continuously or intermittently sliding against other cartilage and meniscus surfaces during culture. As expected, for regions of cartilage that were not sliding against other tissues, PRG4 synthesis was independent of whether or not the joint was cultured with CPM. As with bioreactor culture of

cartilage tissue fragments, bioreactor culture of whole joints maintained high chondrocyte viability, and viability was also unaffected by CPM stimulation. This study also demonstrated the marked topographical variation in PRG4 secretion levels throughout the joint, and that the general pattern of variation was maintained during bioreactor culture of whole joints.

The articular cartilage of the equine fetlock joint exhibited marked site-associated variation in surface integrity, assessed by roughness and indentation stiffness (Chapter 6), likely due to varied biomechanical environments experienced by different regions of the joint during *in vivo* loading. In this model, where immature animals were introduced to gentle racehorse training, exercise did not appear to affect cartilage surface integrity, compared to that of pasture-reared control animals.

Treatment of the articular cartilage surface with various solutions (Chapter 4) provided insight into the mechanism by which PRG4 adheres to the cartilage surface. Treatment with bathing fluids of various physiological PRG4 concentrations revealed that native PRG4 at the articular surface is held quite tightly under free-swelling (not contacting adjacent tissues) conditions, and does not readily exchange with PRG4 in the bathing fluid. However, native PRG4 can be removed by certain chemical treatments, and subsequently replaced at the articular surface with PRG4 from a synovial fluid bath.

7.2 Discussion

Major contributions of the current work include: development of two new bioreactor systems for the study of mechanical regulation of chondrocyte metabolism; extension of the current paradigm that mechanical stimuli regulate cartilage metabolism to include regulation of lubricant molecule synthesis; observation of the correlation between depth-varying pattern of chondrocyte PRG4 expression and previously determined tissue strain profiles for cartilage under the same types of mechanical stimuli; demonstration of marked topographical variation in both surface integrity and PRG4 expression; and insight into the dynamics of PRG4 exchange between the articular cartilage surface and the synovial fluid that bathes it. Clinical implications of this work include experimental support for the use of CPM as a post-operative treatment, and also the possibility of creating tissue engineered cartilaginous constructs with lubrication function.

During this work, two novel bioreactors were developed: one for culture of articular cartilage fragments, and the other for culture of whole joints. The bioreactor for tissue fragments allowed for application of biaxial deformation to cylindrical cartilage disks during culture. This bioreactor was based on one developed previously for use in a standard incubator [2], but had additional adaptations to create tissue culture conditions outside of an incubator. This type of bioreactor allows for the application of mechanical stimuli that are well-defined in terms of biomechanical theory, though the resultant micro-environmental phenomena (fluid flow, electrochemical gradients, etc.) may be different from those experienced *in vivo*. The

second bioreactor, to the authors' knowledge, represents the first bioreactor for culture of whole joints with applied continuous passive motion. This type of bioreactor provides a novel platform for *in vitro* experiments of many types. One advantage of studying CPM as a mechanical stimulus is that cartilage loading in this configuration is more physiological than that of bioreactors for smaller tissue fragments. Advantages of whole joint culture, compared to *in vivo* studies, include the lower cost of maintaining a joint compared to that of maintaining an animal, and the well defined biochemical environment in which to test specific hypotheses about physiological loading.

These results obtained using these bioreactors have contributed to the understanding of the role of mechanical stimuli in regulating PRG4 metabolism. The general trends (down-regulation by static compression of sufficient magnitude, up-regulation by certain magnitudes and frequencies of dynamic shear simulation) are consistent with those seen for chondrocyte metabolism of matrix molecules involved in weight-bearing, such as GAG and collagen. The extent of up-regulation of lubricant PRG4 metabolism by dynamic shear (~3-fold) is particularly interesting, as the up-regulation of weight-bearing GAG and collagen production by mechanical stimuli is usually around 1.3-1.5 fold [2, 5, 16, 17]. While other studies have demonstrated mechanical regulation of PRG4 mRNA expression [3, 19], this study directly addressed the effects of mechanical stimuli on PRG4 protein synthesis. The marked up-regulation of PRG4 secretion due to both shear stimulation and CPM was consistent with the 3-7 fold increase in mRNA expression due to surface motion [3]. Compressive stimulation, in contrast, did not affect PRG4 mRNA expression [3] but

did affect PRG4 secretion here. Taken together these results suggest that mechanical regulation of PRG4 expression can occur at the levels of both transcription and translation, and that the strains experienced by chondrocytes during shear deformation may be different from those experienced during compression. Further studies are needed to fully understand the mechanisms by which these distinct mechanical stimuli differentially affect PRG4 metabolism.

PRG4 immunolocalization provided some insight into possible mechanisms by which mechanical stimuli may affect chondrocyte metabolism. For all loading protocols examined in this work, expression of PRG4 was limited to the chondrocytes in the upper 200-400 μm of depth from the articular surface. For unloaded cartilage, many chondrocytes in the upper 0-200 μm, but very few chondrocytes below 200 μm of depth, expressed PRG4. Certain mechanical stimulation protocols, however, led to markedly increased PRG4 expression by cells between 0 and 200 μm of depth, and induction of PRG4 expression by cells between 200 and 400 μm of depth. However, under no conditions did chondrocytes deeper than 400 μm express PRG4. A chondrocyte-tracking methodology has been used to show that tissue strains were highest near the articular surface, decrease with tissue depth, and are very low at depths beyond 400 μm during compression of immature bovine cartilage [6]. Similar results were obtained for tissue shear of human cartilage (Dr. W. Bae: personal communication) using a similar cell-tracking methodology [13]. Taken together these results support the hypothesis that induction of PRG4 secretion in chondrocytes between 200-400 μm of depth and increased overall secretion levels resulted from tissue strains above a certain level, and only chondrocytes in the top 400 μm of depth

experienced sufficient strains to influence PRG4 synthesis. Alternatively, it is possible that only chondrocytes in the upper ~400 μm are capable of making PRG4, regardless of the strains they experience. Further studies are needed to elucidate the mechanism by which distinct mechanical stimuli differentially regulate PRG4 metabolism.

The marked site-associated variation seen here for both articular surface integrity in the equine model and intrinsic PRG4 synthesis in the bovine model are likely due to varied biomechanical environments experienced by different joint regions during in vivo loading. It would be interesting to determine functional relationships between PRG4 metabolism and cartilage surface integrity, which could lead to eventual use of PRG4 as a strategy to prevent wear of the articular surface. The topographical variation in PRG4 secretion among regions of the bovine stifle joint also implies that for future studies of PRG4 metabolism, care should be taken to use site-matched cartilage samples to ensure that results are representative of experimental treatments, and not just site-associated variation in intrinsic PRG4 metabolism. Specifically, use of cartilage from the patellofemoral groove is recommended, as PRG4 secretion by cartilage in the bovine patellofemoral groove is quite uniform compared to that of the femoral condyles.

Results of cartilage storage studies have suggested that the PRG4 content of synovial fluid provides a supply for replenishing PRG4 that is lost from the articular surface. The finding that PRG4 in the synovial fluid and that at the articular surface did not exchange readily or even in low ionic strength solutions, suggests that PRG4 is bound quite tightly to the surface under unloaded conditions. Since PRG4 could be removed from the surface by treatment with SDS, Hyaluronidase, and

reduction/alkylation, the mechanism of binding could involve 3D proteins structure, interactions with hyaluronan, or aggregation via disulfide bonding, though further studies are needed to fully characterize the binding mechanism. In addition, the ability to replace PRG4 at the articular surface after it has been removed suggests that all of the tissues surrounding the joint compartment that contribute PRG4 to the synovial fluid could influence boundary lubrication function in the joint.

Stimulation of PRG4 metabolism in certain regions subjected to CPM stimulation suggests one mechanism by which CPM may be beneficial to cartilage health. CPM has been shown to aid in healing of cartilage defects in rabbits [11, 12], and is frequently used clinically as a post-operative treatment after cartilage defect repair [8, 10, 15] and ACL reconstruction [9], but the direct effects of CPM on chondrocyte metabolism were unknown. Thus this study provided experimental evidence supporting the use of CPM rather than joint immobilization as a post-operative treatment. While these studies focused on chondrocyte metabolism of lubricant molecules, further studies could be employed to determine if there are beneficial effects of CPM on chondrocyte metabolism of the matrix molecules involved in weight-bearing as well, to further support the use of CPM as a rehabilitative treatment for patients with various knee injuries and diseases.

The findings of this work have implications for tissue engineering of cartilaginous constructs with functional lubricating articular surfaces for repair of cartilage defects as well. For example, tissue constructs that mimic the zonal architecture of native articular cartilage, developed from chondrocyte sub- populations [7], could be subjected to dynamic shear stimulation prior to implantation in order to

increase PRG4 production by chondrocytes in the superficial zone of the constructs. In addition, since the attachment of PRG4 to the articular surface is thought to mediate its boundary lubrication function, understanding the mechanism of binding of lubricant molecules to the articular surface could lead to the ability to manipulate the amount of PRG4 at the articular surface.

Taken together, the results of this dissertation work have contributed to the development of a compartmental model of the synovial joint in which the metabolism and function of all of the joint components are interrelated. The findings reported here are consistent with the overall hypothesis that the health of the articular cartilage surface depends on its ability to provide boundary lubrication, and that this is mediated by PRG4 binding to the articular surface, which may be influenced by the amount of PRG4 produced by the tissues surrounding the synovial joint. More studies are needed, however, to establish mechanistic relationships among all of these phenomena, and to fully understand the role of each joint compartment in maintaining the lubrication function, and ultimately, the health of the joint.

7.3 Future Work

The current work can be expanded in the future in a number of ways, from further characterization of loading applied in the bioreactors, to investigation of the mechanism by which these stimuli take effect, to continuation of the analysis of the interactions between PRG4 at the articular surface and that in synovial fluid.

Given the dramatic effect that tissue shear stimulation had on PRG4 expression by chondrocytes in cartilage explants, it would be interesting to further investigate the cellular mechanisms underlying this phenomenon. Experiments using fluorescently stained cell nuclei as fiducial markers (for tracking with both photomicroscopy and videomicroscopy) are being undertaken to characterize tissue strains occurring during shearing of cartilage tissue [18]. It would be interesting also to compare the tissue strains imparted to cartilage with the polysulphone platens of the biaxial motion bioreactor, to those imparted to the tissue during cartilage-on-cartilage sliding in the whole joint bioreactor. Furthermore, tissue strain maps could be compared to the patterns of chondrocyte populations expressing PRG4, to possibly correlate a strain threshold with induction of PRG4 expression. In addition, synergistic effects of biochemical and biomechanical stimuli on PRG4 metabolism might be investigated, as these two types of stimuli have been shown to interactively regulate cartilage metabolism of matrix molecules such as glycosaminoglycan and collagen [4].

The bioreactor developed here for culture of whole joints represents a step toward the ultimate goal of culturing a biological replacement joint for implantation in place of traditional metal and plastic artificial joints. Biological joints could, in theory, offer a longer lasting remedy compared to non-biological implants, as they could integrate better with the host tissue and remodel themselves as necessary. For this to be practical in a clinical setting, the bioreactor system would have to be modified to

accommodate several joints simultaneously. In addition, harvest protocols and controls of tissue culture conditions should be optimized for cost-effective, long-term maintenance of joints in culture without infection.

The current CPM bioreactor system also has practical utility in the short term, as it provides a platform for many types of *in vitro* studies. The current system allows for the simultaneous culture of two joints, but could be scaled up in order to conduct *in vitro* experiments in a high throughput manner. To do this, the culture of smaller joints might be considered in order to limit the amount of supplies and space needed for the system. Possibilities for future *in vitro* studies include the use of the bioreactor to assess defect repair under physiological joint motion [1], but without the costs of keeping animals alive, and without systemic biochemical effects associated with *in vivo* studies. In addition, specific chemical stimuli could be added to the culture medium during joint culture with CPM to investigate interactive effects on PRG4 metabolism. Effects of CPM on metabolism of other extracellular matrix molecules produced by cartilage could be assessed, although the culture duration may need to be increased in order to detect effects of loading.

While the current studies have implicated certain mechanisms by which PRG4 may bind to the articular surface, the exact mechanism remains to be determined. Studies are underway to use Western Blot to determine the structure of PRG4 that is removed, PRG4 that remains at the articular surface under various conditions, as well as the PRG4 that is replaced at the articular surface upon bathing in synovial fluid. In addition, it would be interesting to determine whether repletion of PRG4 at the surface, after native PRG4 has been dissociated, can occur with purified PRG4 in PBS, and whether this effect is dependent on the concentration of PRG4 in the bathing fluid. Finally, since these studies were motivated by experimental observations that

the layer of PRG4 at the articular surface appeared disrupted by certain mechanical stimuli, it would be interesting to quantify the effects of mechanical stimuli on the amount of PRG4 at the articular surface. Such studies could be performed using both types of bioreactors developed in this work. It would then be important to relate the amount of PRG4 at the articular surface to the boundary lubrication function, using a cartilage-on-cartilage friction test that has been developed in our laboratory [14].

Finally, *in vivo* studies similar to that reported here for the equine exercise model, but with PRG4 metabolism and surface binding as endpoint measurements, could help to elucidate how mechanical stimuli regulate these processes. This could lead to further understanding of the interrelationships among PRG4 secretion by synovial joint tissues, PRG4 binding to the articular surface, and the integrity of the articular surface *in vivo*.

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