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Feasibility of intra-articular adeno-associated virus-mediated proteoglycan-4 gene therapy to prevent osteoarthritis

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FEASIBILITY OF INTRA-ARTICULAR ADENO-ASSOCIATED VIRUS-MEDIATED PROTEOGLYCAN-4 GENE THERAPY TO PREVENT OSTEOARTHRITIS

by

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CERTIFICATE OF APPROVAL

PH.D. THESIS

This is to certify that the Ph.D. thesis of

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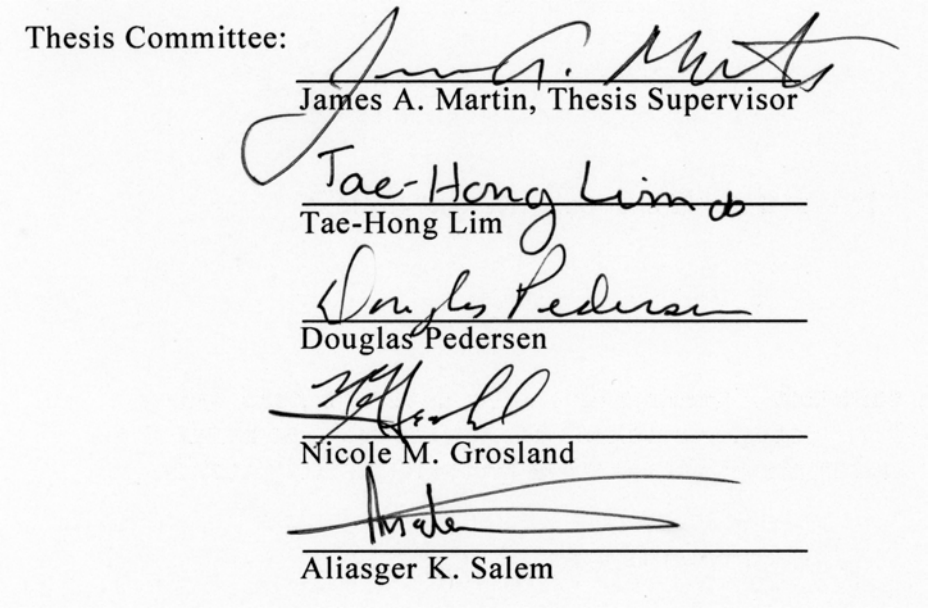
has been approved by the Examining Committee for the thesis requirement for the Doctor of Philosophy degree in Biomedical Engineering at the August 2015graduation.



To my family, wife

ACKNOWLEDGEMENTS

First of all, I thank God for allowing me to be here today after several years of study at the University of Iowa. I cannot express enough thanks to my committee for their continued support and encouragement: Dr. James A. Martin, the chair of the committee; Dr. Tae-Hong Lim, my academic adviser; Dr. Douglas Pedersen; Dr. Nicole M. Grosland; and Dr. Aliasger K. Salem. I offer my sincere appreciation for all the learning opportunities given to me provided by my committee.

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ABSTRACT

Lubricin, or proteoglycan 4 (PRG4), is a secreted, glycosylated protein that binds to cartilage surfaces, which functions as a boundary lubricant. The loss of lubricin from cartilage is identified as a major pathogenic factor in post-traumatic osteoarthritis (PTOA) that has now been the aim of therapeutic intervention. Intra-articular injection of PRG4 protein provides short-term benefits that might be extended using sustained delivery methods such as in gene therapy.

Here we describe the development and testing of such therapy using adeno-associated virus (AAV) as a vector for the transfer of PRG4-green fluorescent protein (GFP) fusion gene. Our recombinant PRG4 gene produces a PRG4-GFP fusion protein to facilitate tracking of its expression and distribution on joint surfaces. We hypothesized that PRG4-GFP is fully functional as a cartilage lubricant and that PRG4-GFP produced *in vivo* is expressed by synoviocytes and other joint cells, and cartilage surfaces remained coated for several weeks up to months after intra-articular injection of the virus.

PRG4-GFP showed lubricin-like cartilage binding *in vitro*, and lubrication immunoblot analysis confirmed that purified PRG4-GFP from cultured media conditioned by PRG4-GFP-transduced synoviocytes was heavily glycosylated, while confocal microscopy revealed binding of the fluorescent fusion protein to cartilage surfaces. Metal-on-cartilage friction tests showed that PRG4-GFP reduced friction coefficients to a degree comparable to that of synovial fluid and

had strong chondro-protective effects in explanted cartilage exposed to shear loading. The chondrocyte viability after shear loading showed that PRG4-GFP had a strong chondro-protective effect on par with that of the synovial fluid. Confocal microscopy and immunohistology confirmed that cartilage surfaces in the stifle joints of mice injected with viruses were coated with PRG4-GFP for up to 2 or 4 weeks after the treatment. The overexpression of PRG4-GFP and coating of cartilage surfaces in the stifle joints of mice injected with Adeno-Associated Virus for the transfer of PRG4-GFP fusion gene (AAV-PRG4-GFP) was confirmed by confocal microscopy and immunohistology for up to 2 or 4 weeks post-injection. The μCT imaging and immunohistology in AAV-PRG4-GFP injected rabbit knees showed stronger inhibition in degeneration of damaged tissues than in AAV-GFP injected control group. Collectively these findings indicate that AAV-PRG4-GFP transduction is a valuable new tool for evaluating the effects of long-term lubricant supplementation on PTOA in animal models.

PUBLIC ABSTRACT

There are several types of degenerative joint disease in human body joints; rheumatoid arthritis (RA), osteoarthritis (OA), and post-traumatic osteoarthritis (PTOA). This study focused on OA, or PTOA, which is caused by mechanical damage, aging joints, injury, and obesity. OA usually develops in joints that are injured by repeated overuse and exceeded mechanical force such as performing a particular task or playing a favorite sport or carrying around excess body weight.

Over the past few decades, many researchers and scientists introduced a plenty of gene therapy methods such as injection of anti-inflammatory drugs, nutrients, regeneration factors, and lubricants for OA cure and treatment. Nowadays, physical therapy and joint splinting and joint replacement surgery are also developed by a large number of researchers; however, there is neither an ideal treatment of OA, nor is there a complete understanding of OA progression mechanism. Over 27 million in the US population still suffer from OA/PTOA, and a tremendous amount of money and effort are poured into finding the cure for OA/PTOA.

In this study, we focused on development of enhanced gene therapy which uses the recombinant lubricin (PRG4-GFP). Lubricin, also known as proteoglycan 4 (PRG4), is the main lubricant acting as synovial fluid to reduce friction and wear. It is vital to maintain proper function within knee joints.

We developed and tested a gene therapy using adeno-associated virus (AAV) as a vector for the transfer of PRG4-green fluorescent protein (GFP)

fusion gene. A friction test with PRG4-GFP showed lubricin-like cartilage binding capacity and inhibiting progression of OA after damage and lubrication. An immunoblot analysis confirmed the production and function of PRG4-GFP. The expression of PRG4-GFP and the coating of cartilage surfaces in the stifle joints of mice/rabbits injected with AAV for the transfer of PRG4-GFP fusion gene (AAV-PRG4-GFP) were confirmed by a confocal microscopy and immunohistology.

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CHAPTER 1

INTRODUCTION

Up to 60% of patients who undergo anterior cruciate ligament (ACL) replacement surgery develop symptomatic knee osteoarthritis (OA) within 15 years.(1-4) The need for a new treatment option is urgent, as refinements in ACL repair techniques over the last few decades have not significantly reduced the risk for OA. Articular cartilage is vulnerable without joint lubrication, which is needed to minimize friction between joint surfaces. Lubricant failure and loss in the aftermath of ACL injuries are well documented, and it is clear that lubricant loss plays a major pathogenic role in OA, particularly in the ACL-deficient knee, where shear stresses are exacerbated by joint instability.(5)

Synovial fluid contains a number of substances that lubricate joint surfaces including hyaluronic acid, phospholipids, and lubricin, a heavily glycosylated protein secreted by synovial cells and chondrocytes.(6-8) Tissue and synovial fluid analyses from ACL transection models reveal that lubricin is released from cartilage surfaces following ligament injury, primarily due to proteolysis. Once lubricin is lost it is not easily replaced due to inhibition of its biosynthesis by pro-inflammatory cytokines.(9, 10) Lubricin supplementation *via* intra-articular injection mitigates cartilage degeneration in ACL transection models, indicating that its loss contributes to OA progression.(11) However, multiple injections are required for efficacy due to lubricin's relatively short half-life *in vivo* (Figure 2.4).(12) This limitation stands as a barrier to

implementation of lubricin therapy in humans at risk for OA who may require supplementation for months or years. Intra-articular gene therapy offers an appealing solution to this problem, as a single injection of viruses carrying proteoglycan, the gene encoding lubricin, which can provide a steady supply of the protein for many months. Although viral vectors for *in vivo* transduction have been available for years, the recently developed adeno-associated virus (AAV) vectors offer distinct advantages over other systems. Cells infected with AAV remain healthy and inflammatory responses to the virus are minimal. Moreover, in contrast to retroviral systems where oncogenic mutagenesis is a risk, AAV seldom inserts into the host's chromosomes and transgene expression is a function of multiplicity of infection.(13, 14) Green fluorescent protein (GFP) expression was maintained at a steady rate for more than a year after intra-articular injection of AAV-GFP in the rodent stifle joint (Figure 2.5).

Evaluating *in vivo* transgene expression can be greatly facilitated by combining sequences encoding fluorescence markers with the gene of interest in bicistronic clones. This technique allows the investigator to identify transduced cells, but also explains the fate of secreted products like PRG4, which may end up at some distance from the cell that produced it. Our recombinant PRG4-GFP (PRG4-GFP) clone, which produces a PRG4/GFP fusion protein, was designed to address this issue.(15) The enhanced GFP sequence was inserted into the PRG4 gene after removal of ~1/3 of the DNA encoding of the mucin-decorated domain, due to the limited carrying capacity of the AAV vector. Because this manipulation could have caused changes in protein performance, we sought first

to verify that PRG4-GFP was fully functional as a cartilage lubricant by conducting *in vitro* binding, friction, and shear loading tests in an explant model. Later, we studied the distribution of PRG4-GFP-expressing cells and the disposition of PRG4-GFP protein in the mouse/rabbit joint using confocal microscopy and GFP/PRG4 immunohistochemistry.

CHAPTER 2

BACKGROUND

2.1 Articular cartilage

Articular cartilage is an avascular and a lymphatic structure at diarthrodial joints which provide a smooth and lubricated surface for articulation with low friction coefficient. To understand the environment of joint lubrication, one needs to understand that cartilage has several important characteristics. Articular cartilage has its own regeneration capacity for intrinsic healing and repair, not through vascular system to the site of damage.(16)

2.1.1 Components of articular cartilage

Articular cartilage is composed of water, collagen, proteoglycans, and chondrocytes. Each component is related to support cartilage function and structure.

2.1.2 Water

Cartilage is mainly formed by water, with 80% being in the superficial zone and 65% in the deep zone. Water allows load-dependent deformation of the cartilage. It provides nutrition and medium for lubrication, creating a low-friction gliding surface. In osteoarthritis, water content becomes more than 90% due to increased permeability and disruption of the matrix. This leads to decreased modulus of elasticity and thus reduction in load bearing capability of the articular cartilage.

2.1.3 Collagen

Collagen occupies 10–20% of the wet weight of the articular cartilage. Among total collagen, Type II collagen forms the principal component (90–95%) of the microfibrillar framework and provides a tensile strength to the articular cartilage.

2.1.4 Proteoglycans

Proteoglycans are protein polysaccharide molecules which form 10–20% of the wet weight and provide a compressive strength to the articular cartilage. There are two major classes of proteoglycans found in articular cartilage: large aggregating proteoglycan monomers or aggrecans, and small proteoglycans including decorin, biglycan and fibromodulin. They are produced inside the chondrocytes and secreted in the matrix. The subunits of proteoglycans are called as glycosaminoglycans (GAGs). These are disaccharide molecules, with mainly two types, chondroitin sulphate and keratin sulphate. GAGs are bound to the protein core by means of sugar bonds, to form aggrecan molecule. Link protein stabilizes this chain with a central hyaluronic acid chain to form an intricate structure of the GAG molecule.(17-19)

2.1.5 Chondrocytes

Chondrocytes are highly specialized cells, forming only 1–5% of cartilage volume, which are sparsely spread within the matrix. Chondrocytes receive their nutrition by diffusion through the matrix. Chondrocytes are specialized cells for synthesizing and maintaining the matrix infrastructure. Chondrocytes synthesize all the matrix components and regulate matrix metabolism.(20, 21)

2.1.6 Structures of articular cartilage

Cartilage can be categorized into several zones by their characteristic features, roles, and components. These are superficial (tangential) zone, transitional zone, middle (radial) zone, and calcified cartilage zone.(22) There are several factors related to lubricating condition in the superficial zone (Figure 2.1, 2.2).(23) Being the thinnest of all layers, the superficial zone is predominantly related with joint lubrication and composed of latten ellipsoid cells. They lie parallel to the joint surface, and are covered by a thin film of synovial fluid, called ‘lamina splendens’ or ‘lubricin’. This protein is ultimately responsible for providing a gliding surface of the articular cartilage. Chondrocytes in this zone synthesize high concentration of collagen and low concentration of proteoglycans, thus making it as the highest water content zone. Parallel arrangement of the fibrils are responsible for providing the greatest tensile and shear strength. Disruption of this zone alters the mechanical properties of the articular cartilage and thus contributes to the development of osteoarthritis. This layer is also related to filtering for the large macromolecules and protecting the cartilage from synovial tissue immune system.

2.2 Osteoarthritis and post-traumatic osteoarthritis

Osteoarthritis (OA) is a painful, degenerative joint disease in body joints caused by aging joints, injury, and obesity.(17, 24, 25) OA usually develops in joints that are injured by repeated overuse and exceeded mechanical force such as performing a particular task or playing a favorite sport or carrying around excess body weight. This injury or repeated impact results in thins or wears away

the cartilage that cushions the ends of the bones in the joint. As a result, the bones rub together, causing a grating sensation, reducing joint flexibility, developing of bone spur, and swelling of the joint. The first symptom of OA is commonly pain that worsens following exercise or immobility. In recent studies, many researchers were introduced drugs such as analgesics, nonsteroidal anti-inflammatory drugs or appropriate exercises and physical therapy or joint splinting or joint replacement surgery for seriously damaged larger joints for OA treatment (Figure 2.3).

Nowadays, 10% of people in the world and 27 million people in the US are suffering with OA and the medical treatment costs is over 120 billion dollars per year.(26-30) Furthermore, post-traumatic osteoarthritis (PTOA) population is also continually increasing. PTOA is a common form of OA caused by a previous injury or other form of trauma such as athletic injury and past joint surgery. PTOA may occur in any joint, including the shoulder, wrist, and knees.

2.3 Joint lubrication phenomenon

Joint lubrication is largely classified as boundary lubrication, fluid-film (mixed) lubrication, and hydrodynamic lubrication. The boundary lubrication refers to the situation where a lubricant film is present between two rubbing surfaces but its thickness is insufficient to prevent asperity contact through the film. The boundary effect of synovial fluid in a cartilage-on-cartilage system is that the synovial fluid readily adheres to the cartilage surfaces, helping to keep them apart and decreasing frictional forces.(31) The contact lubrication mechanism is affected by lubricants of synovial fluid, which sticks firmly to the

articular cartilage surfaces. The frictional characteristics are determined by the properties of the solids and the lubricant film at the common interfaces.(32) It was shown that the fat in the surface layer of articular cartilage was important, since removing it chemically without apparent damage to other structures increases the coefficient of friction. Boundary lubrication of articular cartilage is extremely effective in preventing wear due to motion but loses its protective abilities under high loads. Therefore, for resisting under high loads, other lubricating mechanisms must be at work.

The fluid-film (mixed) lubrication is an important geometrical feature of fluid-film contacts that the films are many times thicker than the height of the irregularities on the surfaces of the solids. Load on the bearing surfaces in fluid-film lubrication is supported by the pressure in the film. The excellence of the lubrication system in this tissue significantly decreased the value of the coefficient of friction.(33)

Hydrodynamic lubrication is lubrication mechanism which is generally characterized by conformal surfaces. If the bearing is of convergent shape in the direction of motion, the fluid adhering to the moving surface will be dragged into the narrowing clearance space, thus building-up a pressure sufficient to carry the load. The viscosity of the fluid, the geometry and relative motion of the surfaces may be used to generate sufficient pressure to prevent solid contact. The magnitude of the pressure developed is not generally large enough to cause significant elastic deformation of the surfaces. The minimum film thickness in a

hydrodynamically lubricated bearing is a function of normal applied load, surface velocity, lubricant viscosity and geometry.(34)

The interaction of asperities or surface roughness can cause friction and wear of two unlubricated surfaces. The highest asperities come into contact with each other forming junctions which are sheared off as the surfaces slide on one another. The coefficient of friction of sliding dry surfaces depends on the shear strength of the contact junctions and the true area of contact; this area increases as the applied load is increased (Figure 2.7). Typical dry coefficients of friction are about 0.1 to 0.3 for plastic on plastic and about 0.3 to 0.8 for metal on metal.(35)

2.3.1 Synovial joints

Normal synovial joints operate with a relatively low coefficient of friction, about 0.001 (Table 2.1, 2.2). In comparison, polytetrafluoroethylene (Teflon) sliding on Teflon has a coefficient of friction of about 0.04, an order of magnitude higher than that for synovial joints. Identifying the mechanisms responsible for the low friction in synovial joints has been an area of ongoing research for decades. Both fluid film and boundary lubrication mechanisms have been investigated (Table 2.3).(31)

For a fluid film to lubricate moving surfaces effectively, it must be thicker than the roughness of the opposing surfaces. The thickness of the film depends on the viscosity of the fluid, the shape of the gap between the parts, and their relative velocity, as well as the stiffness of the surfaces. A low coefficient of friction can also be achieved without a fluid film through a mechanism known as

boundary lubrication. In this case, molecules adhered to the surfaces are sheared rather than the film. It now appears that a combination of boundary lubrication at low loads and fluid film lubrication at high loads is responsible for the low friction in synovial joints.(36, 37)

2.4 Lubricants in joint surface

The articular cartilage is a boundary gel film adhered to the articulating joint surface, with the presence of absorbed glycoproteins, such as lubricin, as well as macromolecular constituents, mainly made up of hyaluronic acid (HA), in natural synovial fluids, which effectively increase fluid viscosity and result in a thick lubricating fluid film between the joint surfaces.(38, 39) Articular cartilage forms a smooth, tough, elastic, and flexible surface that facilitates bone movement. The synovial space is filled with the viscous synovial fluid containing HA and the glycoprotein lubricin. Many researchers reported that HA and Lubricin and, SAPL are most important lubricant in boundary lubrication in joint surface.(40-45)

2.4.1 Hyaluronic acid (HA)

HA is a polymer of D-glucuronic acid and D-N-acetylglucosamine, which degrades under inflammatory conditions such as in OA. HA plays a major role in lubrication, shock absorption, and visco-elastic behavior of SF as a result of HA molecules and proteins/HA electrostatic interactions.(46-48) HA also exerts biological activities such as promotion of endogenous high MW HA production, interaction with pain receptors, and inhibition of pro-inflammatory mediators synthesis by joint cells.(49-54)

2.4.2 Surface active phospholipids (SAPL)

Also, Schwarz and Hills demonstrated that SAPL can facilitate joint lubrication with lubricin in articular cartilage. Hills and co-workers demonstrated that OA joints have a SAPL deficiency and that injection of the SAPL 1,2-dipalmitoylphosphtidylcholine (DPPC) in propylene glycol into human OA joints resulted in mobility improvement lasting up to 14 weeks without major side effects.(55)

2.4.3 Lubricin

Lubricin has been studied by investigators who were interested in the superficial layer of articular surfaces. Biochemical extraction of superficial zone cartilage led to the purification of a 345-kDa protein with minimal glycosaminoglycan substitutions, named superficial zone protein (SZP). The secreted protein lubricin is a major component of synovial fluid that reduces friction in both cartilage bearings and can also reduce friction between synthetic surfaces,(56-61) however, it can easily be degraded and fragmented by de-glycosylation.(15) The gene responsible for lubricin expression was initially named megakaryocyte-stimulating factor (MSF), owing to the discovery that the 32-kDa amino terminal fragment of the protein is able to stimulate megakaryocyte growth *in vitro* (Figure 2.6).(62, 63)

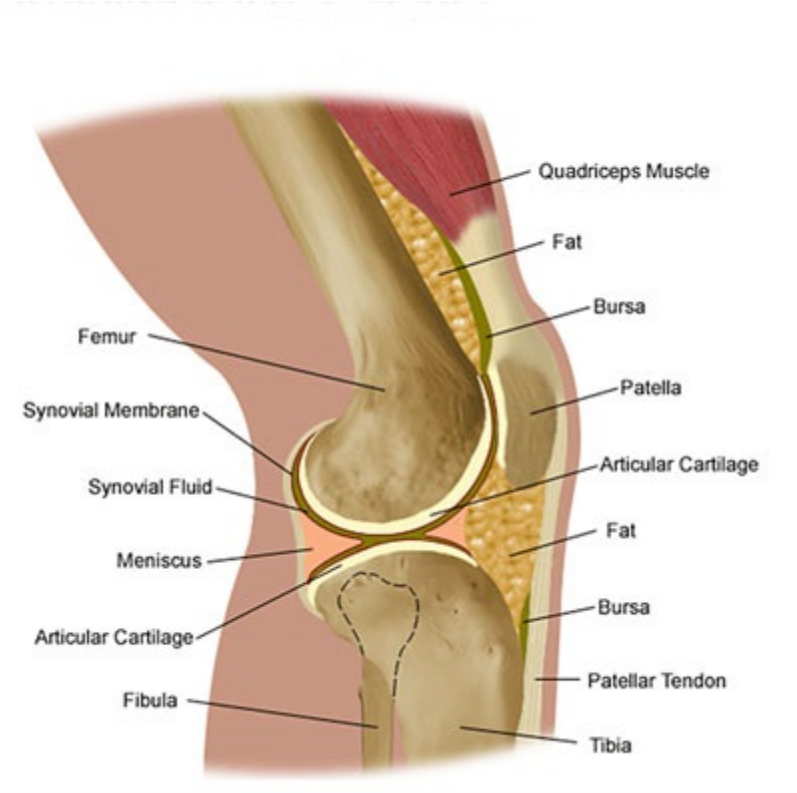

Figure 2.1 Structure of knee joints. The knee joints are composed of femur, tibia, articular cartilage, meniscus, ligaments, synovial membranes, patella and tendons.(64)

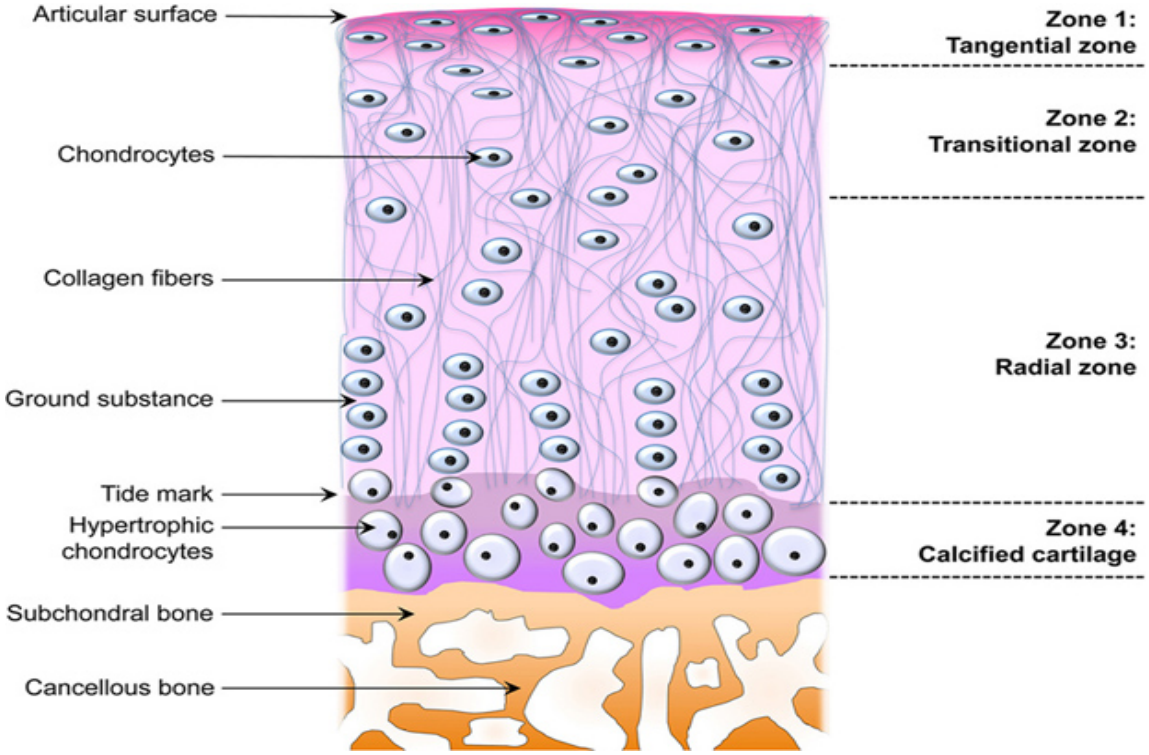

Figure 2.2 Articular cartilage simple illustration. Articular cartilage is classified into four different zones; tangential zone (zone 1), transitional zone (zone 2), radial zone (zone 3), and calcified zone (zone 4). Depending on depth, the morphology and cellularity of chondrocytes and the density of collagen and proteoglycans have differences.(65)

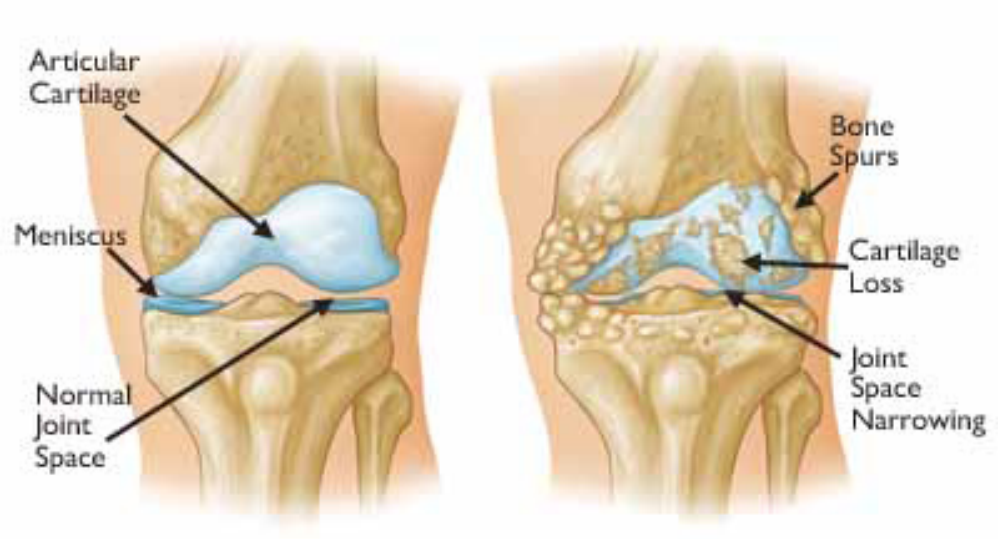

Figure 2.3 Normal cartilage and Osteoarthritis (OA). OA can be characterized as a degenerative disease of synovial joints with progressive loss of articular cartilage, bone spurs, joint space narrowing, and restricted motion.(66)

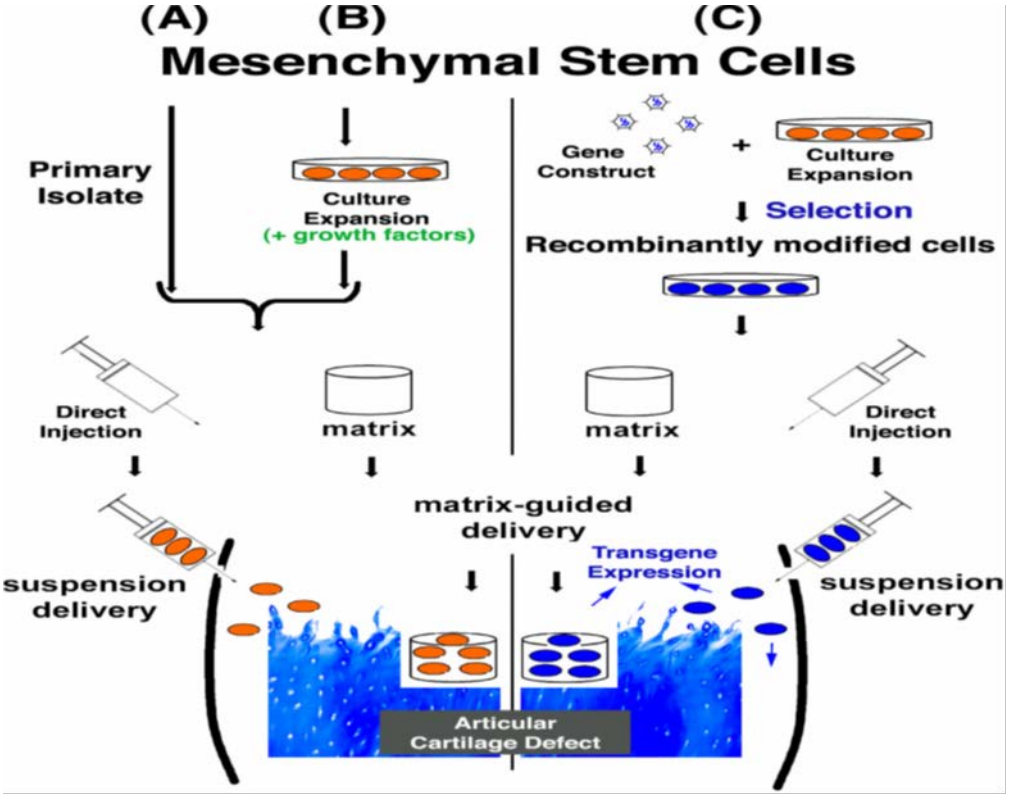

Figure 2.4 The general gene therapy for cartilage repair. Tissue engineering application of mesenchymal stem cells, illustrated using different approaches to articular cartilage repair. (A) Injection of a suspension of native mesenchymal stem cells, either directly without expansion or after culture expansion, into the joint space, where they encounter all intra-articular tissues. (B) Matrix-guided approach to repair deep cartilage defects in osteoarthritis, involving cell-seeding into biodegradable scaffold for controlled local application. (C) Cells recombinantly modified *via* gene transduction ex vivo, selected/expanded ex vivo, and injected directly into the joint space or seeded into matrix and implanted into cartilage defects, for global or local release of therapeutic transgene products.(67)

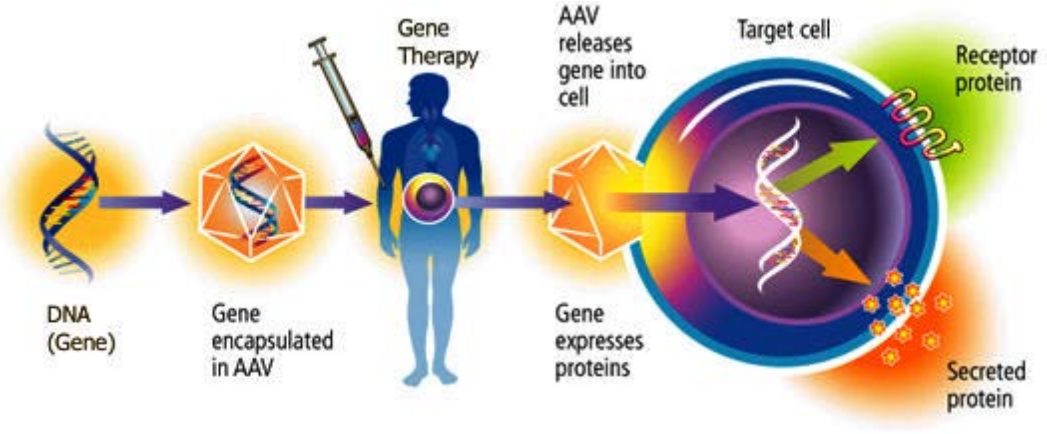

Figure 2.5 AAV gene therapy for cartilage repair. AAVs are non-pathogenic in humans, do not cause mutations and once integrated are stable leading to long term genetic expression. AAVs are that proliferating cells are not a requirement for transfection, the genome is small, and easy to manipulate. A disadvantage of the small AAV genome is that the transferred genetic material is limited in size to a maximum of 4.9kb.(68)

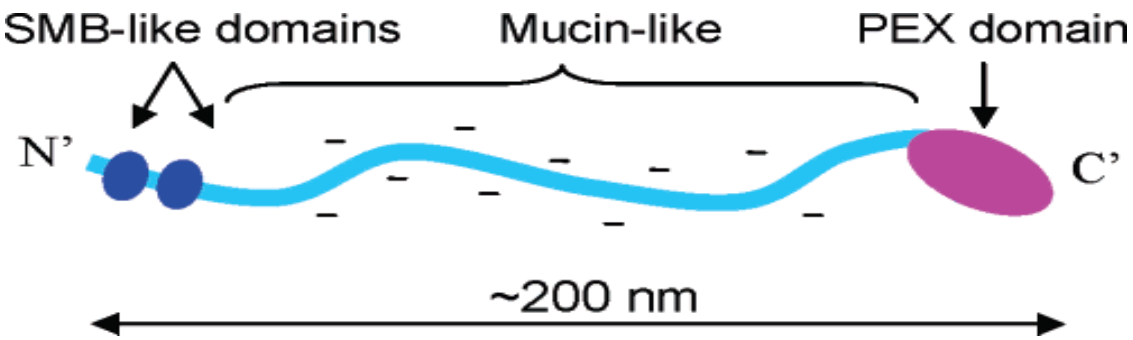

Figure 2.6 Schemic lubricin structure image. Schematic depicting the domain structure of lubricin and the portions encoded by each of the PRG4 gene's 12 coding exons. The amino-terminus is at the left and the carboxyl-terminus at the right. The amino-terminal portion contains 2 domains of 60% similarity to the somatomedin B (SMB) domains of vitronectin. The mucin-like domain, encoded by exon 6, comprises 25 and 6 repeats of the sequences KEPAPTTT/P and XXTTTX, respectively. Carboxyl-terminal to the mucin-like domains is a hemopexin (PEX) domain, whose similarity with vitronectin is 50% and with several members of the matrix metalloproteinase family is 40%. (12, 69)

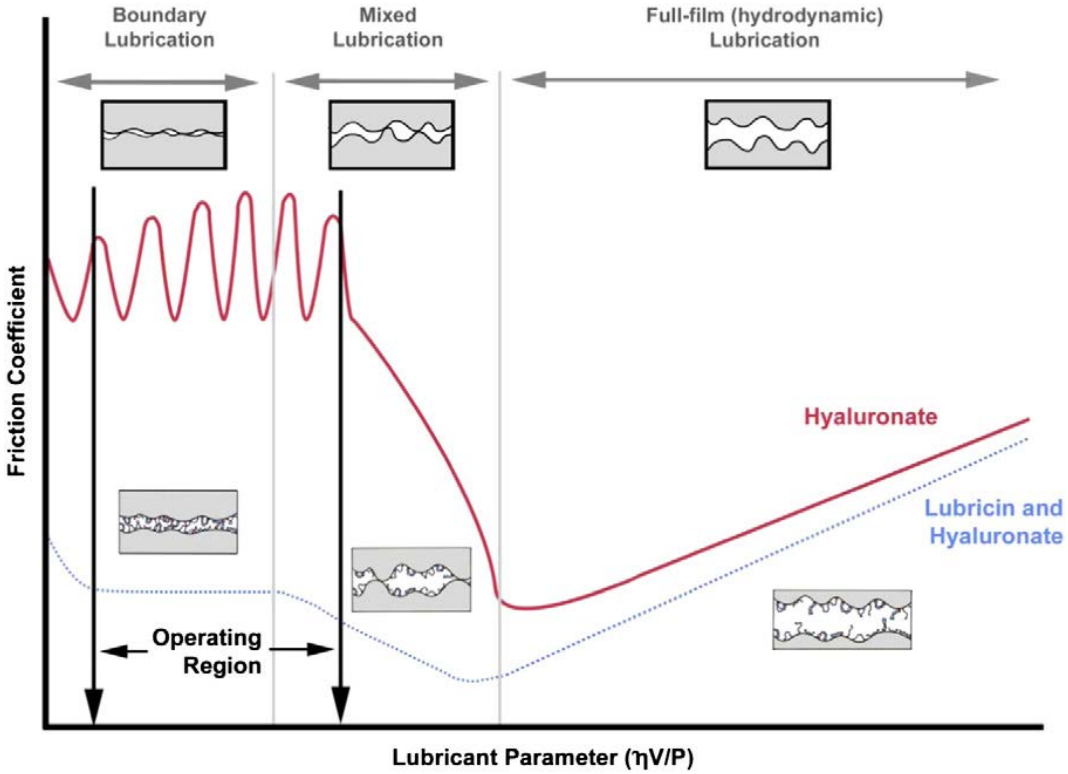

Figure 2.7 General lubrication phenomenon in joint knee. Model Stribeck curve where the friction coefficient at the fluid film thickness are plotted as a function velocity, fluid viscosity and load for the boundary lubrication, mixed, and hydrodynamic lubrication.(70)

Intact joints		
Friction coefficient	**Joint**	**Reference**
0.014-0.024	Human ankle	(71)
0.005-0.01	Canine ankle	(72)
0.02-0.042	Human hip	(72)
0.007	Canine hip	(73, 74)
0.005-0.02	Human knee	(71)
0.075-0.13	Guinea pig knee	(72)
0.0009-0.0029	Murine knee	(72)
0.01-0.07	Bovine knee	(75, 76)

Table 2.1 Experimental friction coefficient friction values of intact joints. The friction coefficient was obtained with pendulum systems or custom-built apparati at different intact joints.

Articular cartilage			
Friction coefficient	**Contact**	**Lubricants**	**Reference**
0.003-0.1	Glass	Synovial Fluid	(77)
0.0014-0.07	Glass	Synovial Fluid	(78)
0.1-0.9	Glass	Synovial Fluid	(79, 80)
0.01-0.1	Glass	Synovial Fluid	(81)
0.02-0.2	Metal	Saline	(82)
0.003-0.35	Metal	Synovial Fluid	(83, 84)
0.075-0.3	Cartilage	Saline, HA	(85)
0.024-0.055	Cartilage	Saline	(86, 87)

Table 2.2 Experimental friction coefficient friction values of articular cartilage. The friction coefficient values collected from pin-on-disc cartilage plug systems with a variety of opposing surfaces and lubricants.

Constituent	Concentration (mg/ml)		
	Normal	OA	Acute Injury
Hyaluronic acid (HA)	1-4	0.7-1.1	0.4-1.0
Lubricin	0.05-0.35	0.25-0.75	0.02-0.1
Phospholipids	0.1	0.2-0.3	0.02-0.08
Albumin/Globulins	15-25	29-39	>25
Cells (10^6 WBC/ml)	<0.2	<2	2-100

Table 2.3 Constituent of synovial fluid lubricants. The constituent of synovial fluid shows different pattern in OA conditions or acute injury in joints compare with normal condition.(88, 89)

CHAPTER 3

MOLECULAR CLONING AND CHARACTERIZATION OF A LUBRICIN-GREEN FLUORESCENT PROTEIN (GFP) FUSION PROTEIN

3.1 Background and significance

Lubricin is a secreted, glycosylated protein that binds to cartilage surfaces, where it functions as a boundary lubricant.(12) Lubricin is originally discovered by Swann(90, 91) and its lubricating properties were further characterized by Jay through a series of bench top investigations.(92-95) Lubricin is a mucinous glycoprotein encoded by the gene PRG4 containing twelve coding exons which include: two somatomedin B-like domains, a heparin binding domain, chondroitin sulfate attachment sites, two O-linked glycosylated mucin-like domains, and a hemopexin-like domain.(12, 96) In addition to being a major component of synovial fluid, it is also produced by chondrocytes in superficial zone and found on the articulating surface of the cartilage. Thus, we designed the experiment using one step lubricin gene therapy, relatively an unknown lubricant compared to HA and SAPL.(97)

Molecular cloning with adeno-associated virus (AAV) is widely used for its predictable expression in the integrated gene. AAV is a small virus which infects humans and some other primate species. AAV is not currently known to be pathogenic. The virus causes a very weak immune response, lending further support to its apparent lack of pathogenicity. Gene therapy vectors using AAV

can infect both dividing and quiescent cells and persist in an extrachromosomal state without integrating into the genome of the host cell, although in native virus, some integration of virally carried genes into the host genome do occur.(98) AAVs are non-pathogenic in humans and once integrated are stable rendering a long term genetic expression without mutations. AAV is an attractive tool for the management of chronic diseases from single gene mutation as well as acquired disorders. AAV is useful in that proliferating cells are not a requirement for transfection. It is relatively non-immunogenic, and the genome is small and easy to manipulate. However, the transferred genetic material is limited in size to a maximum of 4.9kb, due to the limited carrying capacity of the vector. It is also challenging to produce this vector in large amounts without delivering an equally large amount of the contaminating helper virus. Despite its limitation, AAV is a very attractive candidate for creating viral vectors for gene therapy, and for the creation of isogenic human disease models.(99) Recent human clinical trials using AAV for gene therapy in the retina have shown some promise.(100) Using AAV, establishing of AAV-PRG4-GFP will be a good source to help in company with existing gene therapy for OA.

3.2 Specific aims and hypotheses

PRG4-GFP has been cloned in a retroviral vector (pBNN-1-GFP, Addgen) for permanent transduction of cultured cells and in adeno-associated viral vectors (AAV2/5) for *in vivo* studies. A high level of transgene expression is driven by the cytomegalovirus (CMV) promoter. Recombinant proteins secreted by transduced synoviocytes and chondrocytes in culture have been extensively

characterized. We hypothesized that we could develop reliable and reproducible *ex vivo* methods through infection of molecular cloned AAV (AAV-PRG4-GFP).

Although, both of these cell types express lubricin, an increase in its expression was clearly associated with viral transduction. Western blots of conditioned media indicated that the added lubricin C (LubC), contributed by expression of the transgene, was of the same molecular weight as that of the endogenous form expressed in control cells transduced with an empty vector. Moreover, western blots probed with peanut agglutinin (PNA), a lectin that binds to lubricin and other heavily glycosylated proteins, revealed substantially heavier staining of high molecular weight proteins in media from LubC-transduced cells than controls. O-glycanase, which strips mucins from the lubricin core protein, dramatically depleted PNA staining in LubC transductants and controls. This occurred only in the presence of sialidase, which removes residues that block PNA binding to lubricin. Taken together, these data indicated that our recombinant LubC is appropriately glycosylated by synoviocytes.

The binding affinity of PRG4-GFP to cartilage surfaces were tested on cartilage surface with produced recombinant PRG4-GFP. A fluorescence assay of this conditioned medium indicated that the GFP concentration was 1.5 μg/ml, or ~0.4% of the total protein present in the sample. Fluorescence microscopy of the explant showed strong surface signal even after extensive washing with fresh medium, indicating that the GFP label was strongly bound to the cartilage surface. In ongoing work we are testing the effects of mechanical shear stress on surface binding, and we expect these studies will confirm that the cartilage

matrix-binding hemopexin domain is functional in the PRG4-GFP recombinant protein.

Specific Aim 1: Molecular cloning and characterization of a lubricin-green fluorescent protein (GFP) fusion protein.

Hypothesis 1: Human lubricin cDNA is cloned into adeno-associated virus (AAV) vector and PRG4-GFP proteins are expressed from AAV-PRG4-GFP.

a. Identify the construction of AAV-PRG4-GFP

b. Identify the expression of PRG4-GFP from infected cells

Specific Aim 2: Production and purification of a recombinant green fluorescent protein-lubricin (PRG4-GFP).

Hypothesis 1: The expressed and secreted PRG4-GFP can be purified by affinity chromatography.

a. Identify the expression level of PRG4-GFP

b. Identify the purification and yield of affinity chromatography (CNBr-activated sepharose 4B)

3.3 Materials and methods

3.3.1 Molecular cloning and characterization of PRG4-GFP fusion protein in adeno-associated virus

The domain structure of lubricin is described in Figure (Figure 3.1A). LubC was chosen for the experiment because of its modified, shortened size for GFP stable cloning. LubC has the water-binding, mucin-rich domain and the cartilage-binding hemopexin (HP) domain, both of which are essential for

boundary lubrication in cartilage. The heparin sulfate (HS) binding domain was deleted in order to decrease its domain size. The threonine-rich amino acid sequence found in the domain (KEPAPTT) is repeated 76 times. Enhanced green fluorescent protein (E-GFP) replaces a part of the original mucin domain.(101) E-GFP size is ~32.7 kDa with 293 amino acids.

The human lubricin cDNA was cloned through adeno-associated virus (AAV 2/5) vector by multiple fragment cloning methods. LubC gene was inserted into the AAV vector (pFBAAVCMVmscBGHPA; Vector Core, University of Iowa, Iowa City, IA) between the enzyme sites of Xho I and Not I. E-GFP DNA was amplified by PCR method and was inserted into the construct AAV-LubC, specifically between BamH I and Apa I enzyme sites. The final construct (AAV-PRG4-GFP) was confirmed by sequencing and was named PRG4-GFP. The sizes of PRG4-GFP and LubC are 3,036 and 3,951 bp, respectively.(102)

3.3.2 Osteochondral explants harvest and impact

Fresh bovine stifle joints from young adult cattle (15-24 months-old) were received from a local abattoir (Bud's Custom Meats, Riverside, IA). Osteochondral plugs (12-mm-diameter and 10-mm-height) were prepared using a biopsy punch (Miltex Inc., York, PA) from the femoral condyles. The plugs were washed twice in Hanks Balances Salt Solution (HBSS) and cultured in 1:1 mixture of Dulbecco's modified Eagle medium (DMEM) and Ham's F-12 medium (F-12) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA), 100 U/ml penicillin, 100 µg/ml streptomycin, and 2.5 µg/ml

Fungizone. All plugs were cultured in a 37 °C incubator with 5% O_2/CO_2. After two days in culture the osteochondral plugs were injured by 7 J/cm^2 blunt impact *via* a 4 mm-diameter flat-ended platen using a drop tower device.(103, 104)

3.3.3 Cell isolation

Chondrocytes, synoviocytes and meniscus cells were isolated using 2.5 mg/ml collagenase (Sigma-Aldrich, St. Louis, MO) and 2.5 mg/ml pronase (Sigma-Aldrich) digestion. The isolated cells were sub-cultured and used for infection efficiency of AAV-PRG4-GFP.

3.3.4 Infection efficiency test

Isolated cells (1.5 x 10^5) were seeded in 6-well plates and were infected with or without 2 x 10^5 vector genomes (v.g) of AAV-PRG4-GFP. For increasing infection efficiency, 1 µl/ml Hoechst 33342 (Sigma-Aldrich) and 2% FBS were added in DMEM for the first 6 hours. The medium was then replaced by serum-free DMEM with 1 µg/ml ITS premix (BD Biosciences, San Jose, CA) and cultured for 2 days. Positive cells (green) and negative cells (no color) were counted through the confocal examination which allowed the calculation of the percentage of infection.

3.3.5 Molecular cloning of PRG4-GFP fusion protein in retrovirus

For retroviral-transduced synoviocytes, we used RetroPack™ PT67 Cell Line (PT67) for producing retroviral-PRG4-GFP. PT67 plated on 6 wells (1.5 x 10^5 cells) in 2 ml DMEM/F12/10% FBS and cultured for one day. After washing the wells, we gently mixed Lipofectamine™ 2000 (infection reagent) 100 µl and diluted DNA (PRG4-GFP inserted Retrovirus) 100 µl in Opti-MEM® I medium.

After 6 hours culture, we replaced medium to DMEM/F12/2% FBS. To acquire pure PT67-PRG4-GFP (PT67 cells which can provide retroviral-PRG4-GFP), we performed fluorescence-activated cell sorting (FACS). The sorted PT67-PRG4-GFP again plated on T225 plate and cultured in 15 ml DMEM/F12 during 2 to 3 days. The retroviral-PRG4-GFP were secreted during culturing period, which was harvested and then treated by conditioned medium on prepared synoviocytes (1.0×10^6). After 2 days of culturing, green-fluorescent cells were selected by FACS.

3.3.6 Confocal microscopic imaging

The bovine cartilage plugs and murine knee joints were stained with 1 μg/ml calcein AM (Invitrogen; green) and 1 μg/ml ethidium homodimer (Invitrogen; red) and viewed through Olympus Fluoview 1000 Confocal Laser Scanning Microscope (Olympus America Inc., Center Valley, PA). The sites were scanned at about 500 μm depth and at 50 μm intervals.

3.3.7 Western blot analysis of PRG4-GFP in conditioned medium

The conditioned medium which contained the secreted PRG4-GFP from infected synoviocytes was collected, and increased its concentration tenfold using Amicon® Ultra centrifugal Filters 50K (Millipore, Billerica, MA) under 1,600 g at 4 °C for 10 minutes. The concentrated medium was mixed with 1% protease inhibitor cocktail (EMD Millipore, Billerica, MA) and stored at -20 °C.

Protein concentrations were determined by BCA method (5) using bovine serum albumin (BSA), and 2.5 mg of the concentrations were loaded into a 5%

SDS-PAGE gel. The blot was probed with a rabbit polyclonal antibody (Abcam Inc., Cambridge, MA) raised against GFP at ~ 250 kDa.

3.3.8 Statistical analysis

General statistical analysis was performed using software package SPSS (IBM, Armonk, NY, USA). One-way analysis of variance (ANOVA) with the Tukey *post hoc* test was performed to test all possible pairwise comparisons. The level of significance was set at least at $p<0.05$.

3.4 Results

3.4.1 Efficiency of AAV-PRG4-GFP infection

PRG4-GFP infection test was necessary to determine the efficiency and to check the presence of GFP-labeled cells. The infected cells were detected with green fluorescence and showed better infection rate in both synoviocytes and meniscus cells (Figure 3.4B and 3.4C) compared to chondrocytes (Figure 3.4A). Similarly, the number of quantified positive cells was significantly higher in synoviocytes (49.4% ± 9.4) and meniscus cells (43.7% ± 9.6) than chondrocytes (4.9% ± 2.0, $p<0.001$) (Figure 3.4D).

3.4.2 Expression of retroviral-transduced cells

Several types of retroviral-transduced cell lines were developed for permanent and stable transduction of cultured cells. Infection efficiency of retroviral vector inserted with PRG4-GFP in monolayer was almost ~90% (Figure 3.5). These cells are acquired and sorted by FACS and used for producing PRG4-GFP for the friction test.

3.4.3 PRG4-GFP production and analysis

Synoviocytes transduced with PRG4-GFP were layered on the surface of an osteochondral explant to confirm the binding of PRG4-GFP protein. The secreted protein was found successfully bound on the cartilage surface with a strong GFP signal.

3.4.4 Western blot probed by PNA and GFP antibody

The conditioned medium secreted from infected synoviocytes was processed for western blot. Infected cells with PRG4-GFP were strongly detected with the lubricin protein at ~250 kDa with PNA compared to the control group (Figure 3.7). In the enzyme treatment group (Sialidase-A + O-Glycanase), both showed the band of de-glycosylation. We are convinced that AAV-PRG4-GFP infection can produce functional lubricin.

Furthermore, we tried to purify PRG4-GFP. Infected cells with PRG4-GFP were strongly detected with the lubricin protein probed by GFP antibody at ~250 kDa, while there was no detection in either GFP or control groups (Figure 3.8).

3.5 Discussion and conclusions

We observed that PRG4-GFP inserted well in AAV *via* AAV-PRG4-GFP monolayer infection. The Retroviral-PRG4-GFP is also stably established in chondrocytes, synoviocytes, and human chondrocytes for permanent transduction. These cells express lubricin, and an increase in its expression was clearly associated with viral transduction. The AAV-PRG4-GFP infected chondrocytes secreted PRG4-GFP, which was layered on the surface of an osteochondral explant. It shows that surface binding capacity of PRG-GFP is functionally

strong. The binding capacity image was taken after incubating and washing with culture medium. The cartilage was cryo-sectioned first and they imaged (Figure 3.6). Fluorescence microscopy of the explant showed strong surface signal even after extensive washing with fresh medium, indicating that the GFP label was strongly bound to the cartilage surface.

We found that western blots of conditioned media indicated that the added lubricin C (LubC) contributed by expression of the transgene was of the same molecular weight as that of the endogenous form expressed in control cells transduced with an empty vector. Moreover, western blots probed with peanut agglutinin (PNA), a lectin that binds to lubricin and other heavily glycosylated proteins, revealed substantially heavier staining of high molecular weight proteins in media from LubC-transduced cells than in controls. O-glycanase, which strips mucins from the lubricin core protein, dramatically depleted PNA staining in LubC transductants and controls. This occurred only in the presence of sialidase, which removes residues that block PNA binding to lubricin. Taken together, these data indicated that our recombinant LubC is appropriately glycosylated by synoviocytes.

The recombinant PRG4-GFP fusion protein produced by transduced synoviocytes in monolayer culture was of the expected molecular weight, appropriately glycosylated, and was found to bind to cartilage surfaces (Figure 3.7, 3.8). The produced PRG-GFP tests on friction test in further studies.

The success of molecular cloning of human lubricin cDNA into AAV vector and construction of AAV-PRG4-GFP is verified in the expression of PRG4-GFP from infected cells in cartilage surface area.

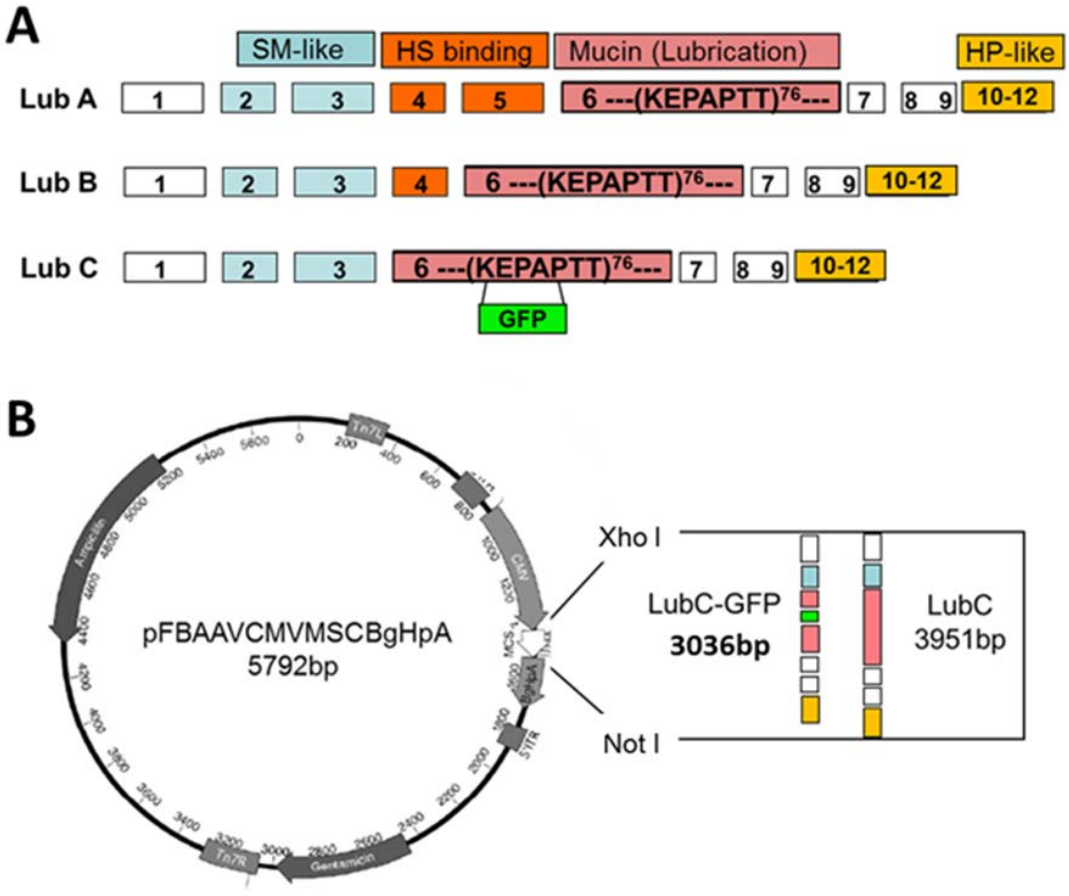

Figure 3.1 Molecular cloning of PRG4-GFP fusion protein. (A) Domain structures of Lub A, B, and C. LubC lacks the heparin sulfate (HS) binding domains present in A and B, but retains the water-binding mucin-rich domain and the cartilage-binding hemopexin (HP) domain, both of which are essential for boundary lubrication in cartilage. The threonine-rich amino acid sequence indicated in the mucin domain (KEPAPTT) is repeated 76 times, allowing for dense o-linked glycosylation (O-GlcNAc). Enhanced green fluorescent protein (E-GFP) replaces a part of the original domain 6 sequences in the mucin-rich domain. SM; somatomedin B, HS; heparin sulfate, HP; hemopexin (B) Cloning in AAV. Human lubricin cDNA was cloned into adeno-associated virus (AAV) vector by multiple fragment cloning methods. The LubC gene was inserted into the AAV vector between the enzyme site of Xho I and Not I. E-GFP DNA was amplified by PCR method. The E-GFP gene was inserted into AAV-LubC between BamH I and Apa I enzyme site within the lubricin gene.

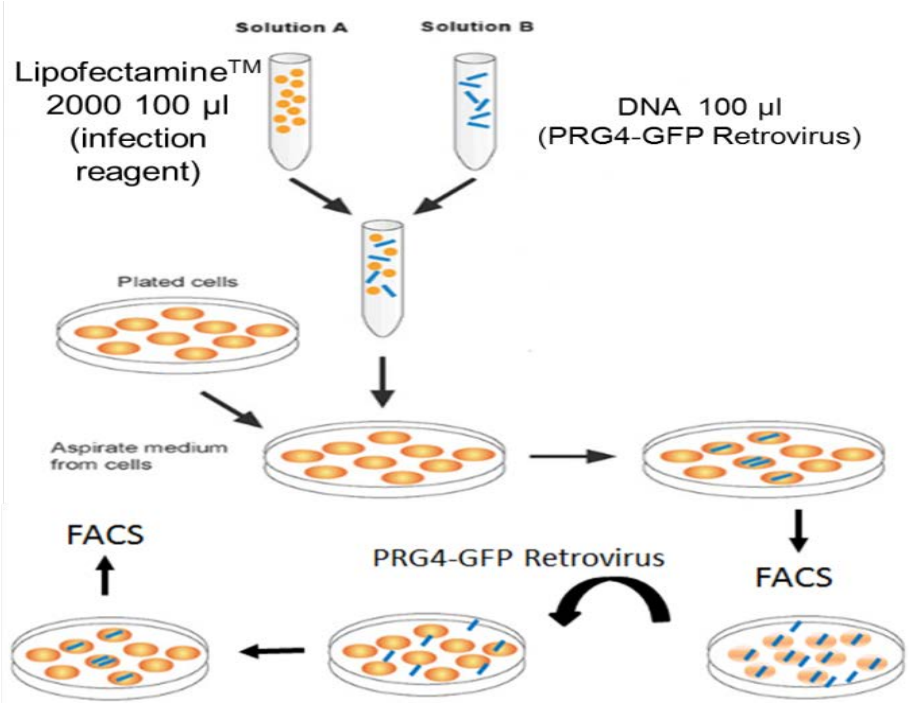

Figure 3.2 Molecular cloning of retrovival-PRG4-GFP fusion protein. Several types of retroviral-transduced cell lines were developed. Infection efficiency of retroviral vector inserted with PRG4-GFP in monolayer was almost ~90%

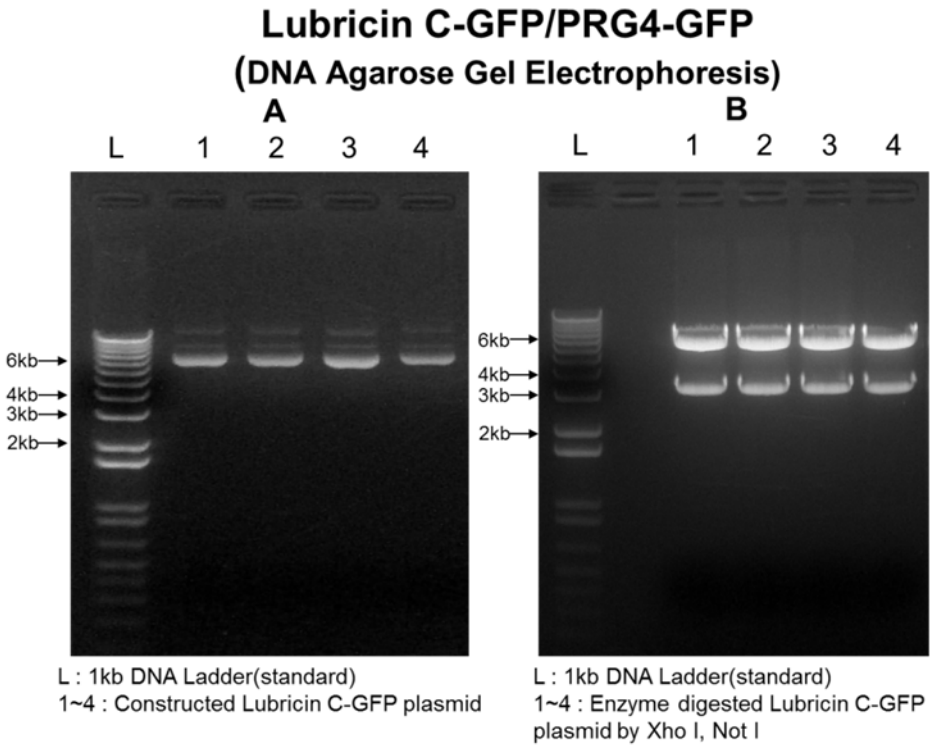

Figure 3.3 Molecular cloning confirmation of AAV-PRG4-GFP by PCR. The inserted PRG4-GFP gene into the AAV vector was verified by restriction enzyme Xho I and Not I.

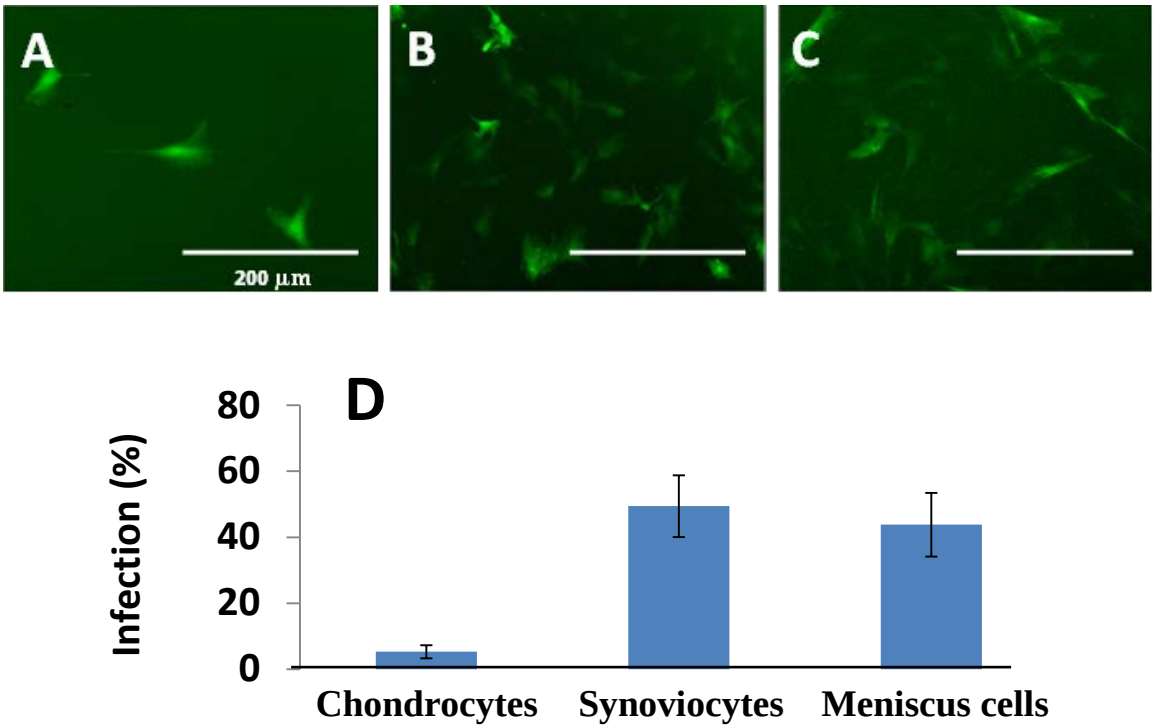

Figure 3.4 Infection efficiency of adeno-associated virus (AAV) inserted with PRG4-GFP in monolayer culture of chondrocytes. (A) Chondrocytes (B) Synoviocytes (C) Meniscus cells (D) Infection percentage of AAV. (n = 4-10, ***p<0.001)

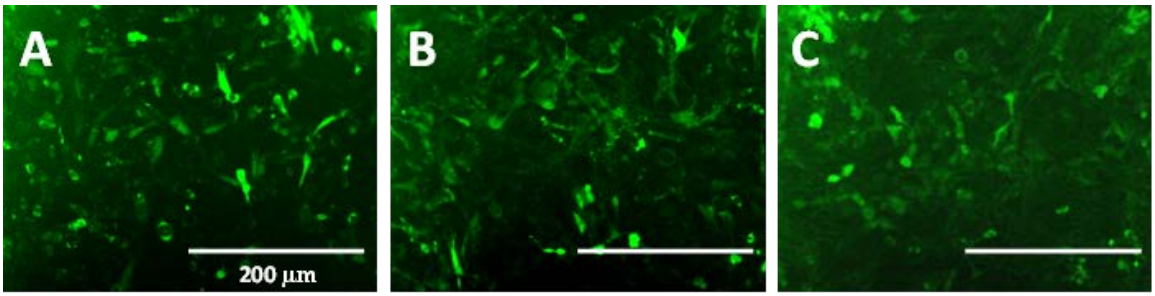

Figure 3.5 Infection efficiency of retroviral vector inserted with PRG4-GFP in monolayer culture of chondrocytes. (A) Chondrocytes (B) Synoviocytes (C) h/N double transduced cell

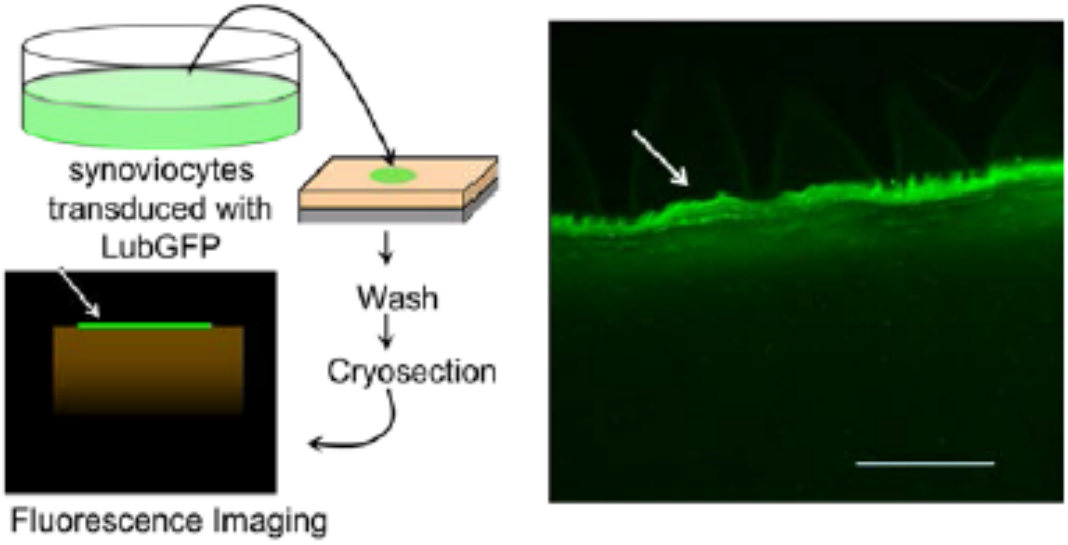

Figure 3.6 PRG4-GFP secreted by synoviocytes was layered on the surface of an osteochondral explant. After incubating and washing with culture medium, the cartilage was cryo-sectioned and imaged. A representative image shows strong GFP signal on the cartilage surface. Scale bars = 200 μm.

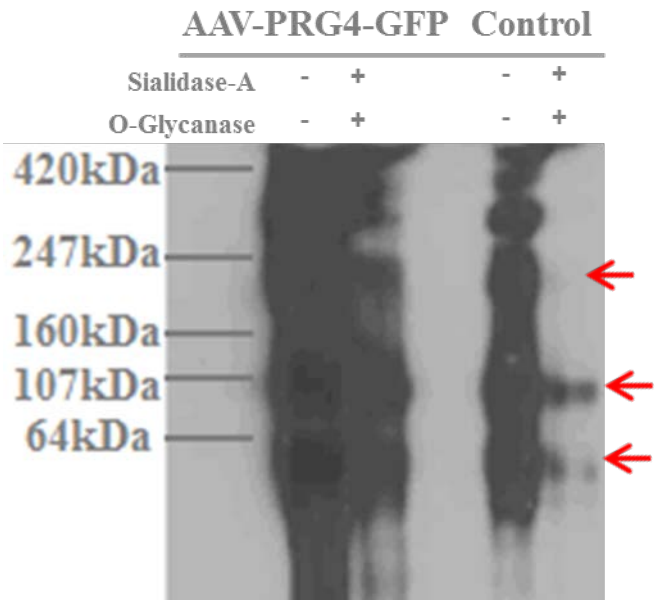

Figure 3.7 Immunoblot analysis of lubricin in conditioned medium. The conditioned medium from AAV-PRG4-GFP infected synoviocytes and normal synoviocytes were loaded in each lane of a 5% SDS-PAGE gel. The blot was probed with peanut agglutinin (PNA).

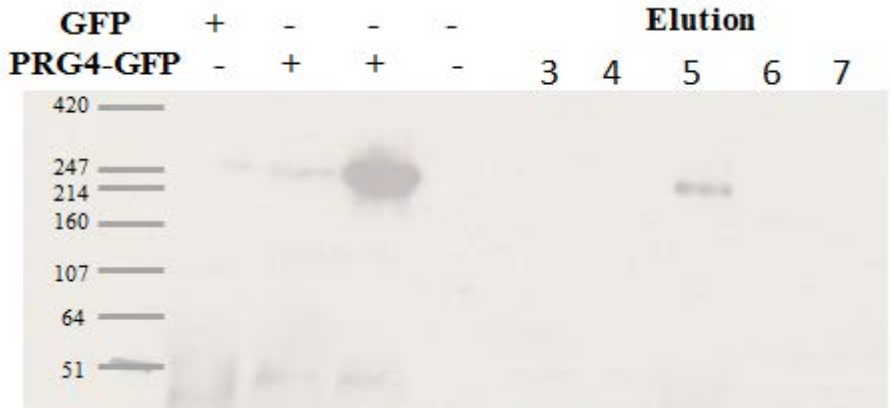

Figure 3.8 PRG4-GFP production and purification by affinity chromatography (CNBr-Activated Sepharose 4B). Western blot of conditioned medium from transduced synoviocytes indicated that PRG4-GFP strongly expressed the lubricin protein at ~250 kDa. The blot was probed with GFP antibody.

CHAPTER 4

PRG4-GFP PROTECTS CHONDROCYTES FROM DEATH AND DAMAGE INDUCED BY SHEAR LOADING

4.1 Background and significance

Friction coefficient is the maximum value of the frictional force divided by the normal force. Friction coefficient testing is used for a variety of materials from lubricants to films and household items, to determine the frictional characteristics of a material. The dimensionless value of the friction coefficient is the ratio of the force required to slide the surface to the force perpendicular to the surface. A low friction coefficient indicates that the surfaces are smoother, less resistant.

Over the past several decades, many researchers and laboratories have measured the friction coefficient in body joints. These investigations have examined a variety of species and joints across a range of methods. While most early studies utilized methods to study the frictional properties of intact synovial joints, more recent work has employed techniques and equipment to look more closely at cartilage alone. Methods include whole joint friction pendulum systems and custom-made friction simulators,(71, 105, 106) and the sliding of excised cartilage plugs against various surfaces.(81-84, 107) There are advantages and disadvantages related to both the use of intact joints and the use of cartilage plug samples.

Tribological studies that investigate the frictional properties of intact joints allow for the preservation of physiological conditions. These studies require custom-built systems, such as pendulums or arthrotripsometers, in which the joint serves as a fulcrum that the apparatus pivots around. Reported frictional coefficients of articular cartilage measured from intact joints range from as low as 0.001 to as high as 0.35. Further studies have demonstrated that the use of cartilage samples against artificial flat surfaces allows for the direct measurement of the friction coefficient. Frictional coefficients measured with cartilage plugs utilizing lubricants such as synovial fluid and saline with cartilage against glass, metal, and cartilage range from 0.0015 to 0.9.

While there has been decades of research investigating the tribological properties of articular cartilage and synovial joints, additional work is needed to examine the response of friction, lubrication and wear to biomechanical testing, such as cyclic loading.(108) Exposing test specimens to biomechanical testing leads to greater insight and allows for a simulation of *in vivo* joint conditions. McCann groups demonstrated that cyclic loading with a pendulum system resulted in friction coefficient increases *via* the medial compartment of bovine knee joints.(75) Forster and Fisher also showed that cyclic loading of bovine cartilage plugs with a custom-made apparatus yields an increase in friction coefficient.(84) Nugent Derfus groups determined that cyclic loading of bovine stifle joints has beneficial effects upon lubrication; they were able to demonstrate that continuous passive motion upregulated the production of lubricin.(109)

In this study, we developed the cyclic shear loading device for *in vitro* friction experiment based on tribological studies. Then, we prepared injured bovine osteochondral explants model and treated with several lubricants on that model. Friction coefficient was measured by LabVIEW software connected to friction device. This study will determine PRG4-GFP's cytoprotective effect on shear stress and the relationship between lubricants and friction coefficient.

4.2 Specific aims and hypotheses

We tested possible lubricants in knee joint *in vitro* model. The explants were then incubated for 4 hours in pure Hanks balanced salt solution (HBSS) or in HBSS containing 20% bovine synovial fluid, or 0.3 μM PRG4-GFP (0.1 mg/ml), or 0.3 μM BSA (0.03 mg/ml). Then, explants were moved into a custom built shear loading device and a 1.0 kg vertical load was applied to a smooth acrylic ball resting on the impact site. After preloading in place for 30 minutes the ball was driven back and forth across the impact site over a distance of 5 mm at sliding velocity of 1.0 mm/sec for 60 minutes.

PRG4-GFP showed lubricin-like cartilage binding capacity to cartilage surfaces *in vitro*. We hypothesized that metal-on-cartilage friction tests would show that PRG4-GFP reduced friction coefficients to a degree comparable to that of synovial fluid and had strong chondroprotective effects in explanted cartilage exposed to shear loading.(84) Assessments of chondrocyte viability after shear loading showed that PRG4-GFP had a strong chondroprotective effect on par with that of synovial fluid.

Specific Aim 1: PRG4-GFP protects chondrocytes from death and damage induced by shear loading.

Hypothesis 1: PRG4-GFP prevents shear loading damage on articular cartilage and decreases chondrocyte death.

a. Construct the shear loading device.

b. Verify the lubricant's preventing effect on cell death on damaged articular cartilage compared to control group

4.3 Materials and methods

4.3.1 Characterization of friction device

A custom-designed friction device was used in this study (Figure 4.1A). A stepper motor actuator (NEMA 17 Stepper, Ultra Motion, Cutchogue, NY) connected with a 10 lb load cell (Honeywell, Columbus, OH) created shear loading on flat surface of the specimens. A constant normal load was also applied on the specimen *via* a 1 kg stainless steel bar. The specimen was securely fixed in a custom-designed molder and contacted with a 1 x 1 cm flat steel bar (Figure 4.1B). The entire device was located in a 37 °C incubator and operated using LabVIEW software (National Instruments Corp., Austin, TX). For the validation of the device, articular cartilage, rubber and wood were tested to measure the friction coefficient (μ).(75, 110, 111)

4.3.2 Friction test on explants

Fresh bovine osteochondral explants were anchored along the bone surface to the base plate with poly caprolactone (PCL). The centers of the explants were subject to a high energy impact load *via* a 4 mm platen, which

caused superficial chondrocyte death and mild fissuring of the superficial zone. Osteochondral explant plugs with 7 J/cm^2 impact injury were incubated in various lubricants, HBSS, 20% bovine synovium fluid (SF), or PRG4-GFP with 50, 100, and 200 μg/ml for 30 minutes. Next, the plugs were moved into a custom built shear loading device and were then equilibrated with 1 kg pre-loading on the impact site. After pre-loading in place for 30 minutes the ball was driven back and forth across the impact site over a distance of 5 mm at a sliding velocity of 1.0 mm/sec for 60 minutes. After staining with calcein AM and ethidium homodimer the explants were imaged on a confocal microscope to observe the percentage of viable cells at the center of the impact site. Imaging was repeated at 24 hours and 72 hours, and 168 hours post-op.

For measuring friction coefficient, fresh bovine osteochondral explant plugs (12 mm) were anchored along the bone surface to the base plate with new designed plate. The centers of the explants were subject to a high energy impact load *via* a 4 mm platen, and then the explant plugs were incubated for 30 minutes in pure HBSS or 20% bovine SF or PRG4-GFP with 50, 100, and 200 μg/ml. Then, explant plugs were moved into a custom built shear loading device and a 1.0 kg vertical load was applied to a smooth metal surface resting on the impact site. After pre-loading in place for 10 minutes the metal surface was driven back and forth across the impact site over a distance of 3 mm at a sliding velocity of 1.0 mm/sec for 3 cycles. Friction coefficient was calculated by the kinetic equation ($F = \mu N$; F; friction force, μ; friction coefficient, and N; normal force).

4.3.3 Confocal examination

The bovine cartilage plugs and murine knee joints were stained with 1 μg/ml calcein AM (Invitrogen; green) and 1 μg/ml ethidium homodimer (Invitrogen; red) and viewed through Olympus Fluoview 1000 Confocal Laser Scanning Microscope (Olympus America Inc., Center Valley, PA). The sites were scanned at about 500 μm in depth and at 50 μm intervals.

All confocal images were stacked in Z-project by Image J software (rsb.info.nih.gov/ij) and cell viability was calculated using quantitative cell image processing (QCIP^{TM}).(112) The values at each time period (days 1, 3 and 7) were normalized to those at day 0.

4.3.4 Statistical analysis

Cell viability among tested groups was compared with the statistical analysis software package SPSS (IBM, Armonk, NY, USA). One-way analysis of variance (ANOVA) with the Tukey *post hoc* test was conducted to test all possible pairwise comparisons. The level of significance was set at $p<0.05$.

4.4 Results

4.4.1 Effect of PRG4-GFP on cartilage friction

The lubricating function of attached PRG4-GFP was evaluated using the friction test. A custom-designed friction device (Figure 4.1A, 4.1B) was validated using articular cartilage, rubber and wood (Figure 4.2A). Friction coefficient of articular cartilage was extremely low (0.015 ± 0.004) which was consistent with literatures showing 0.0005-0.04 for articular joint (113), while

the coefficient of rubber and wood were 0.25 ± 0.014 and 0.256 ± 0.014, respectively.

Injured osteochondral plugs were treated with PRG4-GFP with concentrations of 50, 100 and 200 μg/ml and bovine SF as a positive control (Figure 4.2B). The friction coefficient of injured cartilage with 7 J/cm^2 showed 5-times higher (0.073 ± 0.013) than that of an intact cartilage. This high friction value was reduced in PRG4-GFP treatment groups as well as the bovine SF group. In particular, the coefficients of PRG4-GFP ranged from 100 (0.011 ± 0.011, $p<0.001$) to 200 μg/ml (0.014 ± 0.006, $p<0.001$) were significantly low without any difference in intact cartilage.

4.4.2 Cytoprotective effect of PRG4-GFP on chondrocyte

Figure 4.3 shows representative images on the surface of cartilage at day 0 (post-impact and -friction) and at day 7. The cartilage plug cultured in HBSS was severely damaged with 20-30% initial death of chondrocyte after an impact-injury. This cell death dramatically increased at day 7 (over 60%). There was cytoprotective effect in groups of BSA, SF and PRG4-GFP at days 0 and 7.

The chondrocytes are continuously die from impact damage and friction stress. The viability was quantified using QCIP^{TM} software and resulted in significant improvement of protecting cell death in groups of 20% SF and 100 μg/ml PRG4-GFP (Figure 4.4). The percentage of viable cells in HBSS was approximately 40%, in negative control group (BSA and CM) was 60%, while PRG4-GFP treatment maintained the viability with over 80% at day 7 ($p<0.01$).

4.5 Discussion and conclusions

A custom-designed friction device was well developed with stepper motor, cyclic actuator, 10 lb load cell, LabVIEW software, and 1 kg shear loader. A constant normal loader was also applied on the specimen *via* a 1 kg stainless steel bar. For the validation of the device, articular cartilage, rubber and wood were tested to measure the friction coefficient. The friction device (Figure 4.1) was validated using articular cartilage, rubber and wood (Figure 4.2A). Friction coefficient of normal articular cartilage was extremely low (0.015 ± 0.004), while the coefficient of rubber and wood were 0.25 ± 0.014 and 0.256 ± 0.014, respectively. We are convinced that our friction test device functions within normal units.

PRG4-GFP was treated on injured model to check the cytoprotective effect. Injured osteochondral plugs were treated with PRG4-GFP with concentrations of 50, 100 and 200 μg/ml, and the bovine SF was set as a positive control (Figure 4.2B). The friction coefficient of injured cartilage showed 5-times higher (0.073 ± 0.013) than that of intact cartilage (0.015 ± 0.004). This high friction value was reduced in PRG4-GFP treatment groups as well as the SF group. In particular, the coefficients of PRG4-GFP ranged from 100 (0.011 ± 0.011, $p<0.001$) to 200 μg/ml (0.014 ± 0.006, $p<0.001$) were significantly low without any difference in intact cartilage. We recognized that recombinant PRG4-GFP has cytoprotective and the capacity of decreasing friction as synovial fluid, and that PRG4-GFP treatment has a favorable potential for gene therapy. Friction test results showed that the cartilage plug cultured in HBSS was

severely damaged with 20-30% initial death of chondrocyte after an impact-injury. While this cell death dramatically increased at day 7 in groups HBBS, BSA (over 60%), there was cytoprotective effect in groups of SF and PRG4-GFP at days 0 and 7 (Figure 4.3). The cell viability was calculated by confocal imaging with QCIP^{TM}.

The viability resulted in a significant improvement of protecting cell death in groups of 20% SF and 100 μg/ml PRG4-GFP like being seen as confocal imaging (Figure 4.4). The percentage of viable cells in HBSS was approximately 40%, while PRG4-GFP treatment maintained the viability with over 80% at day 7 ($p<0.01$). Depending on high cell viability and decreasing friction coefficient after PRG4-GFP treatment, we predicted *in vivo* gene therapy using PRG-GFP would be a good source for curing OA.

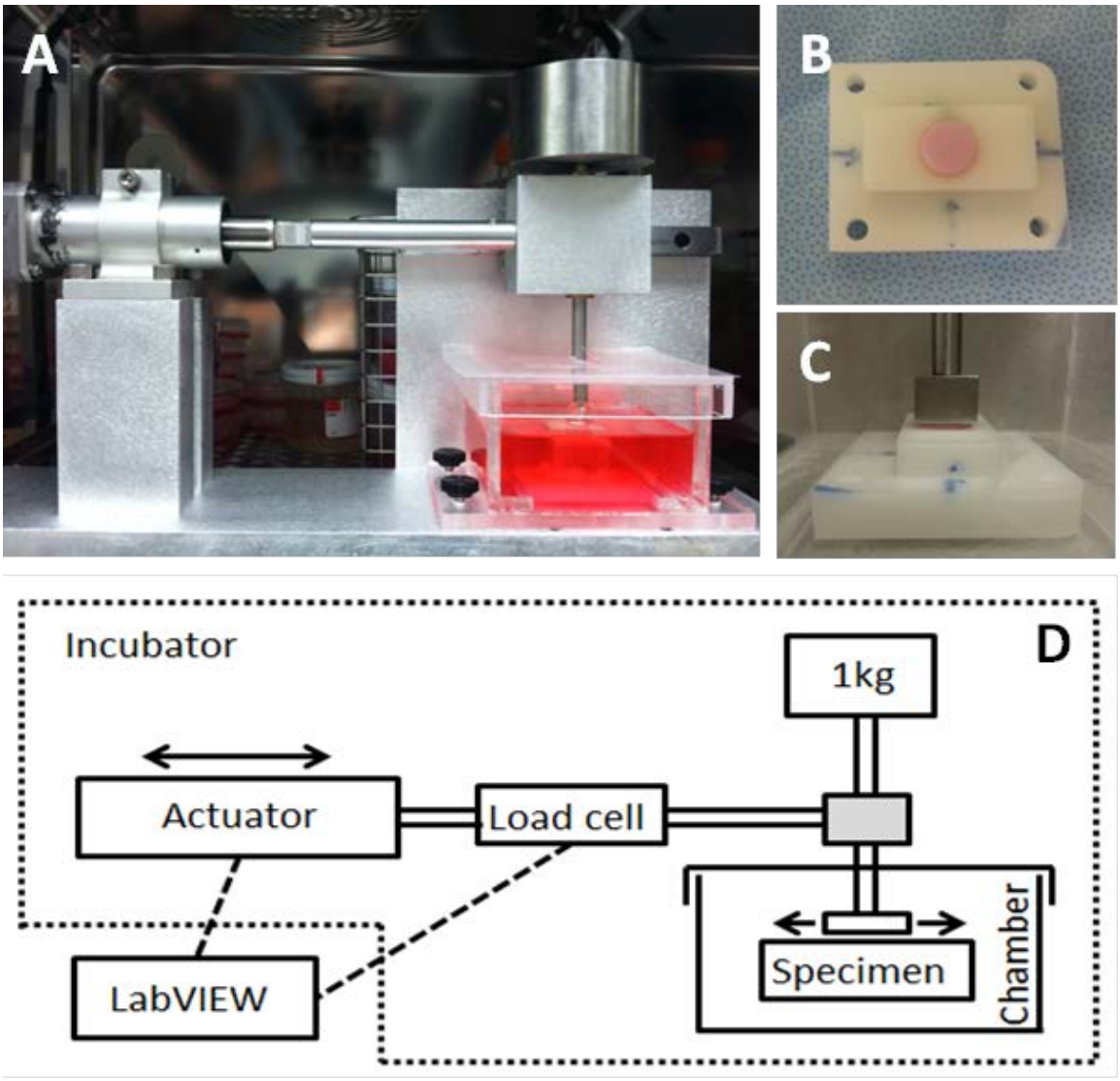

Figure 4.1 Friction device for friction test (A) Friction device model. Specimens were applied to both shear load and normal load *via* a stepper motor actuator and a 1 kg mass. (B) Osteochondral explants (12 mm diameter and 10 mm height) impacted with 7 J/cm^2 using a drop tower was securely fixed with screws in a custom-designed molder. (C) 1 kg mass loading image on explant (D) The scheme of friction device

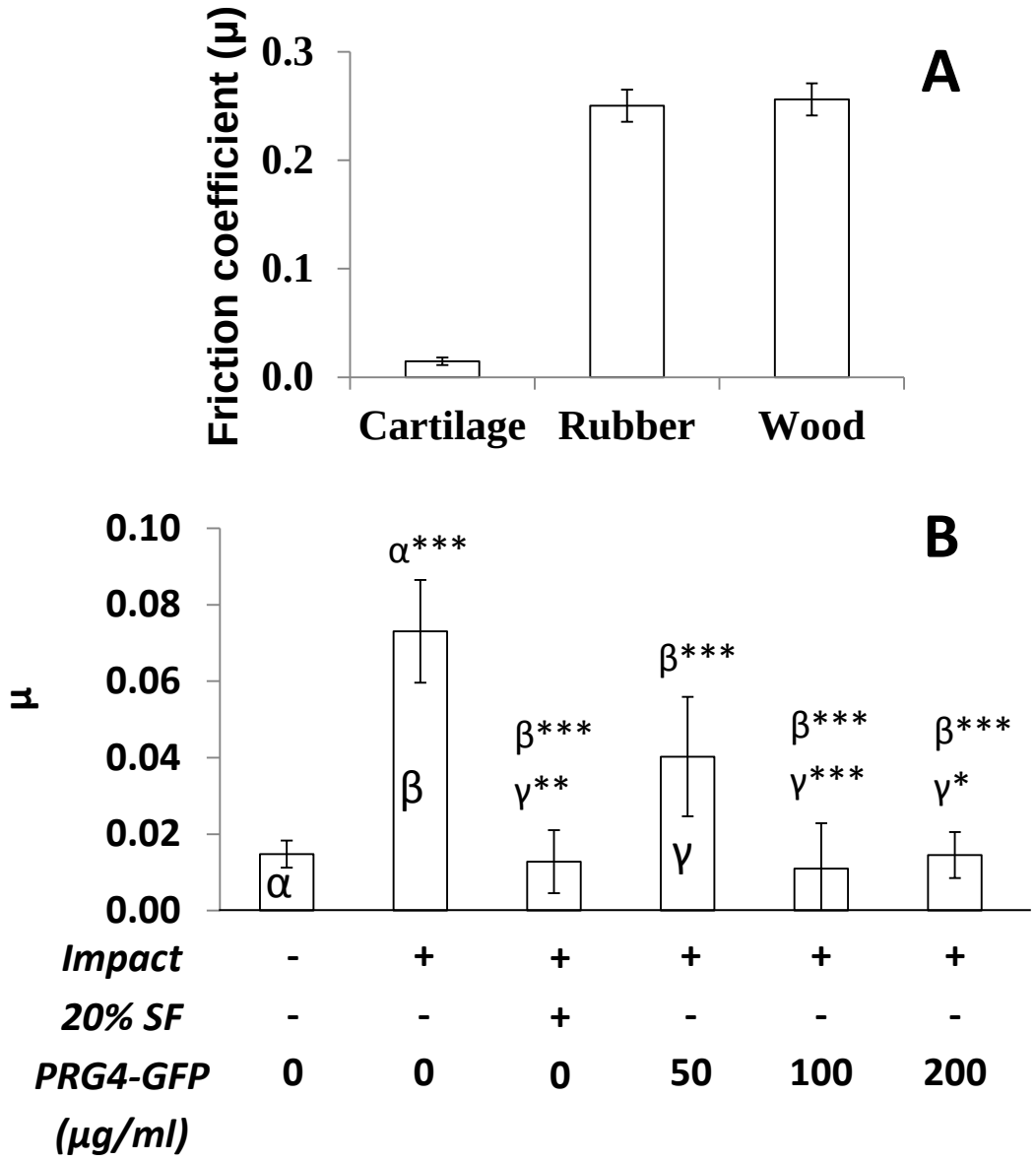

Figure 4.2 Friction coefficient (μ). (A) Several materials which was identified the friction coefficient (μ) were tested to validate the friction device (n = 4). The result was similar to literature. In particular, the coefficient of articular cartilage closed to zero (0.015 ± 0.004). (B) PRG4-GFP and synovial fluid (SF) showed a dramatic effect on reducing coefficient. Compared to impacted cartilage (0.073 ± 0.013), the coefficient in groups of PRG 4-GFP ranged from 100 and 200 μg/ml and 20% SF was significantly low (less than 0.015) without any difference in intact tissue. N = 4-10, *p<0.05, **p<0.01, and ***p<0.001.

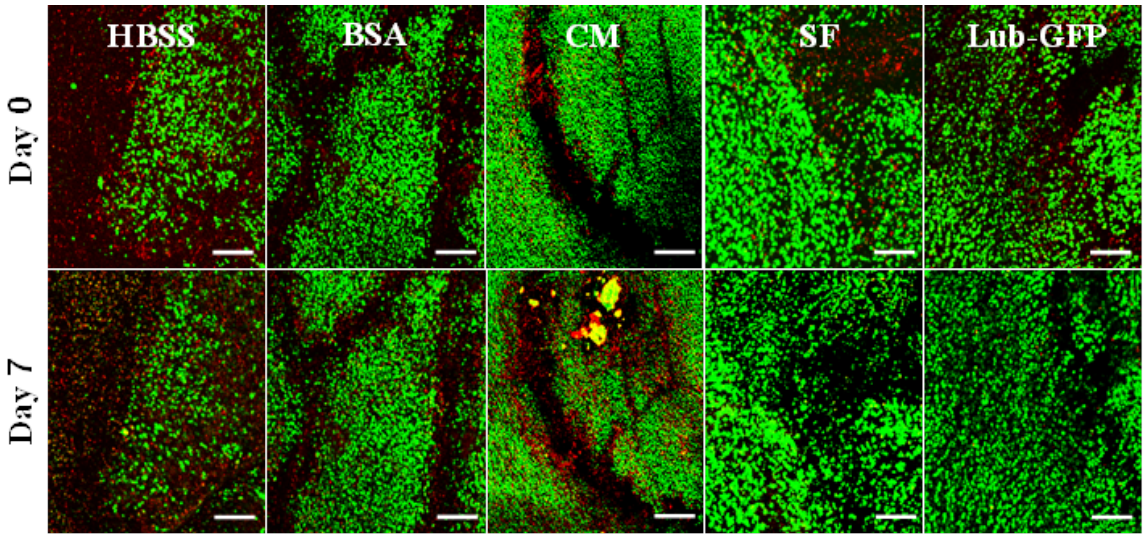

Figure 4.3 Cytoprotective effect of PRG4-GFP on chondrocytes in shear-loaded cartilage. (A) Confocal images show live cells (green) and dead cells (red) on the surface of explants at day 0 (post-impact) and day 7. Microscopically there was apparent cell death in only HBSS during the culture. CM; conditioned medium, SF; 20% bovine synovium fluid, Scale bars = 250 μm.

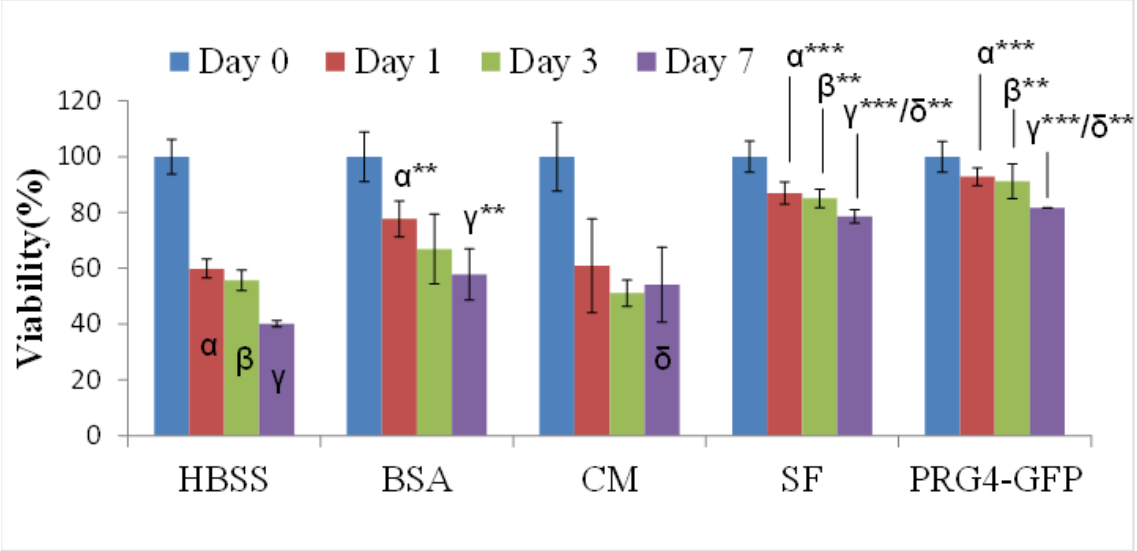

Figure 4.4 Cell viability was calculated using QCIP™ and then normalized by an initial viability (day 0). The viability was significantly higher in the 20% SF (synovial fluid)- and 100 μg/ml PRG4-GFP-treated groups than in the no treatment (HBSS) or 0.3 μM BSA (bovine serum albumin)-treated groups or Conditioned Medium group (CM) (n = 3, **p<0.01, and ***p<0.001).

CHAPTER 5

TRANSDUCTION OF JOINT CELLS *IN VIVO* BY AAV-PRG4-GFP

5.1 Background and significance

Gene therapy is an experimental technique that uses genes to treat or prevent disease. This technique inserts a gene into a patient's cells without resorting to drugs or surgery. Gene therapy is introduced in the therapeutic delivery of nucleic acid polymers into a patient's cells as a drug to treat disease. Nowadays, gene therapy could be a way to fix a genetic problem by expressing proteins, interfering with protein expressions, or possibly correcting genetic mutations.(114, 115)

The most common form uses DNA that encodes a functional, therapeutic gene to replace a mutated gene. The polymer molecule is packaged within a "vector", which carries the molecule inside cells. Researchers are testing several approaches to gene therapy; replacing a mutated gene that causes disease with a healthy copy of the gene, inactivating a mutated gene that is functioning improperly, and introducing a new gene into the body to help fight a disease.(116, 117) For osteoarthritis gene therapy, many researches and studies suggest and introduce the candidate genes. There are candidate genes to prevent progress of OA; cartilage growth factors such as insulin-like growth factor I genes (IGF-1), transforming growth factorβ (TGFβ), bone morphogenetic protein (BMP); extra cellular matrix molecule such as cartilage oligomeric matrix protein (COMP), type II collagen; cytokine/cytokine antagonist such as interleukin 1 receptor

antagonist (IL-1Ra), interleukin 4 (IL-4); signaling molecule/transcription factor such as Smad, Sox-9; lubricant protein such as HA, lubricin. These proteins or molecules are to block the actions of interleukin-1 (IL-1) or promote the synthesis of cartilage matrix molecules or help to regeneration cartilage or resist to degeneration.(118, 119)

Although gene therapy is a promising treatment option for a number of diseases, the technique remains risky and is still under study to make sure that it will be safe and effective. Gene therapy is currently being tested for the treatment of diseases that have no other cures.(120-126)

In this study, we chose AAV for an enhanced gene therapy, utilizing *in vivo* expression of PRG4-GFP, and coating of cartilage surfaces in the stifle joints of mice and rabbits injected with AAV for the transfer of PRG4-GFP fusion gene (AAV-PRG4-GFP). AAV-PRG4-GFP transduction is a valuable new tool for evaluating the effects of long-term lubricant supplementation on PTOA in animal models.

5.2 Specific aims and hypotheses

Gene therapy holds great promise for the delivery of therapeutic peptides to the intra-articular space. The main advantage of gene therapy over other approaches is that constant levels of peptides can be sustained for months after a single treatment. We propose to use this advantage to deliver a steady supply of lubricin, a lubricant that has shown chondroprotective properties in ACL-deficient models and PTOA models. Analysis of recombinant proteins harvested from retroviral-transduced synoviocyte for *in vitro* friction test and chondrocyte

cultures indicate that the LubC product is appropriately glycosylated. Thus, we are now poised to move ahead with studies to assess intra-articular PRG4 gene therapy as a means to mitigate OA in ACL deficient joints. We hypothesize that ectopic expression of PRG4-GFP in the joint with infection of AAV-PRG4-GFP will protect cartilage from the degenerative effects of chronic joint instability.

We would like to assess the effects of PRG4 gene therapy on the progression of OA in the destabilization of a medial meniscus (DMM) mouse model and in a rabbit ACL transection model. Gene therapy will be started immediately after a complete ACL transection. Joint instability and osteoarthritis will be evaluated at 4 and 8 weeks post DMM or ACLT.

Specific Aim 1: AAV-PRG4-GFP transduction of joint cells *in vivo* for gene therapy

Hypothesis 1: Determine PRG4-GFP is expressed in PRG4 gene therapy in a mouse DMM model and in a rabbit ACL deficient model.

a. Verify AAV-PRG4-GFP infection in animal models through fluorescent imaging

b. Verify PRG4-GFP expression level confirm with immunohistochemistry

5.3 Materials and methods

5.3.1 *In vivo* feasibility test of PRG4-GFP

Six young adult male C57BL/6J mice (8 weeks-old) were obtained from the Jackson Laboratory (Bar Harbor, ME) and allowed to acclimate for 1 week. Animal study was performed according to a protocol approved by Institutional Animal Care and Use Committee (IACUC) at the University of Iowa. Under

anesthesia, 5 μl of 0.5 x 10^{10} v.g AAV-GFP (n = 3) or AAV-PRG4-GFP (n = 3) was injected into the intra-articular space of each right stifle joints using a Hamilton® syringe fitted with a 28 gauge needle (Hamilton co., Reno, NV). There was no injection in the contralateral stifle (left). After 2 weeks, all animals were euthanized with CO2 and stifles from both sides were isolated for confocal examination and DNA extraction.

5.3.2 Immunohistochemistry examination using anti-lubricin antibody and anti-GFP antibody

The femorotibial joints of mice that were killed 2 weeks following injection of AAV-PRG4-GFP were examined histologically. The joints were dissected and stored in a buffered neutral formalin solution (100 ml 37–40% formalin, 900 ml distilled water, 4 gram sodium phosphate monobasic, monohydrate, and 6.5 gram sodium phosphate dibasic, anhydrous [pH 7.4]). Decalcification was performed for 2 days at room temperature, using a decalcifying solution (100 ml concentrated formic acid, 80 ml concentrated HCl, 50 gm 1,3,5-trihydroxybenzene, and 800 ml distilled water). The specimens were embedded in paraffin, and sections were generated and stained with a 1 : 250 dilution of anti-lubricin antibody (Abcam Inc., Cambridge, MA) and anti-GFP antibody by peroxidase-linked goat anti-rabbit IgG. To receive more clear staining, Vectastain® ABC reagent (Vector Laboratories Inc., Burlingame, CA) was used.

5.3.3 μCT imaging in AAV-PRG4-GFP injected rabbit knees

The equilibrium partitioning of an ionic contrast-microcomputed tomography (EPIC- μCT) was performed for characterization morphological and compositional change after injection AAV on ACL ruptured rabbit knee models. The proximal end of each tibia was immersed in 2 ml of 30% Hexabrix 320 contrast agent (Covidien, Hazelwood, MO) and 70% ion-free phosphate buffer saline (PBS) at 37 °C for 30 min for equilibration of the agent. There was no difference in the average X-ray attenuation levels, thickness, volume or surface area determined for fresh and formalin-fixed cartilage at that time point. Proximal tibiae were scanned using a μCT 40 (Scanco Medical, Brüttisellen, Switzerland) at 45 kVp, 177 mA, 200 ms integration time, and a voxel size of 16 μm.

5.3.4 Statistical analysis

Cell viability among tested groups was compared with the statistical analysis software package SPSS (IBM, Armonk, NY, USA). One-way analysis of variance (ANOVA) with the Tukey *post hoc* test was conducted to test all possible pairwise comparisons. The level of significance was set at $p<0.05$.

5.4 Results

5.4.1 *In vivo* feasibility test of PRG4-GFP

PRG4-GFP was injected into the murine knee joint to verify the potential use of *in vivo* environment. Green fluorescence was detected on the intra-articular cells including the articular cartilage (Figure 5.1A), synovium (Figure 5.1B) and intra-patellar fat pad (Figure 5.1C). In contrast, there was no detection

in the contralateral joints (Figure 5.1D). Finally, DNA isolated from mouse whole stifle joint was verified the infection of PRG4-GFP. Unlike GFP injection, PRG4-GFP gene was detected in all 3 PRG4-GFP-injected mice (right) (Figure 5.2). PRG4-GFP was examined histologically in a paraffin embedded cartilage section. AAV-PRG4-GFP injected knees showed an immune response to both GFP antibody and lubricin antibody (Figure 5.3C, 5.3D). Normal murine cartilage groups had no response to either antibody (Fig 5.3A, 5.3B).

5.4.2 *In vivo* test of PRG4-GFP in PTOA animal models

PRG4-GFP was injected into the mouse knee joint to verify its potential in the *in vivo* environment. AAV-PRG4-GFP injected in the destabilization of the medial meniscus (DMM) knees (5 μl of 0.5 x 10^{10} v.g) and ACL insufficiency rabbit knees (200 μl of 0.25 x 10^{10} v.g) showed minimal proteoglycan and lubricin loss compared to control GFP groups. The μCT imaging in AAV-PRG4-GFP injected rabbit knees showed PRG4-GFP is inhibiting degeneration in damaged tissue compared to AAV-GFP injected group (Figure 5.5). In load bearing cartilage region, a stable level of PG contents and PRG4-GFP signal were identified in AAV-PRG4-GFP injected knees. The AAV-PRG4-GFP injected left knees were strongly expressed with GFP antibody and lubricin antibody compare to AAV-GFP injection group or right knees (Figure 5.6).

5.5 Discussion and conclusions

Through molecular cloning, PRG4-GFP was inserted in the AAV vector. Injection of AAV-PRG4-GFP in mouse stifle joints (5 μl of 0.5 x 10^{10} v.g) resulted in persistent expression of the transgene by superficial and transitional

chondrocytes, as well as by synoviocytes, adipocytes, and meniscal cells (Figure 5.1). Depending on regions of AAV-PRG-GFP infection, expression levels and PRG4-GFP covered areas were varied. Cartilage surfaces in injected joints were positive for both GFP and lubricin, whereas control joints were largely negative for both targets through feasibility test of immunohistochemistry (Figure 5.3). We are convinced that PRG4-GFP gene therapy enhanced the surface lubricant layer on cartilage surfaces. If we find the proper infection concentration and more exact region to recover with lubricin, our approach will make it possible to refine the long-term therapeutic effects of lubricant supplementation on the development of PTOA in joint injury models.

Our studies are intended to provide a definitive assessment of the potential of PRG4/lubricin gene therapy to block the development of PTOA associated with mice DMM and ACL insufficiency in the rabbit. AAV-PRG4-GFP treatment groups show less proteoglycan loss and slow degeneration in damaged area (Figure 5.4, 5.5, 5.6). If PRG4-GFP gene therapy proves to be effective in the context of DMM and ACL transection, a natural follow-up to this investigation would be to apply the treatment to other joint injuries that might also benefit from improved joint lubrication. Moreover, our gene therapy will be enhanced if implemented with the previous well- known candidate gene. Thus, we are well-positioned to broaden the indications for PRG4-GFP therapy to encompass the majority of injuries that lead to PTOA. We suspect these results will fully predict what may occur in humans. However, positive findings in this proof-of-concept study will encourage us to further examine the long-term

effects of PRG4-GFP therapy in models that better approximate the slow, gradual pace of OA progression in humans.

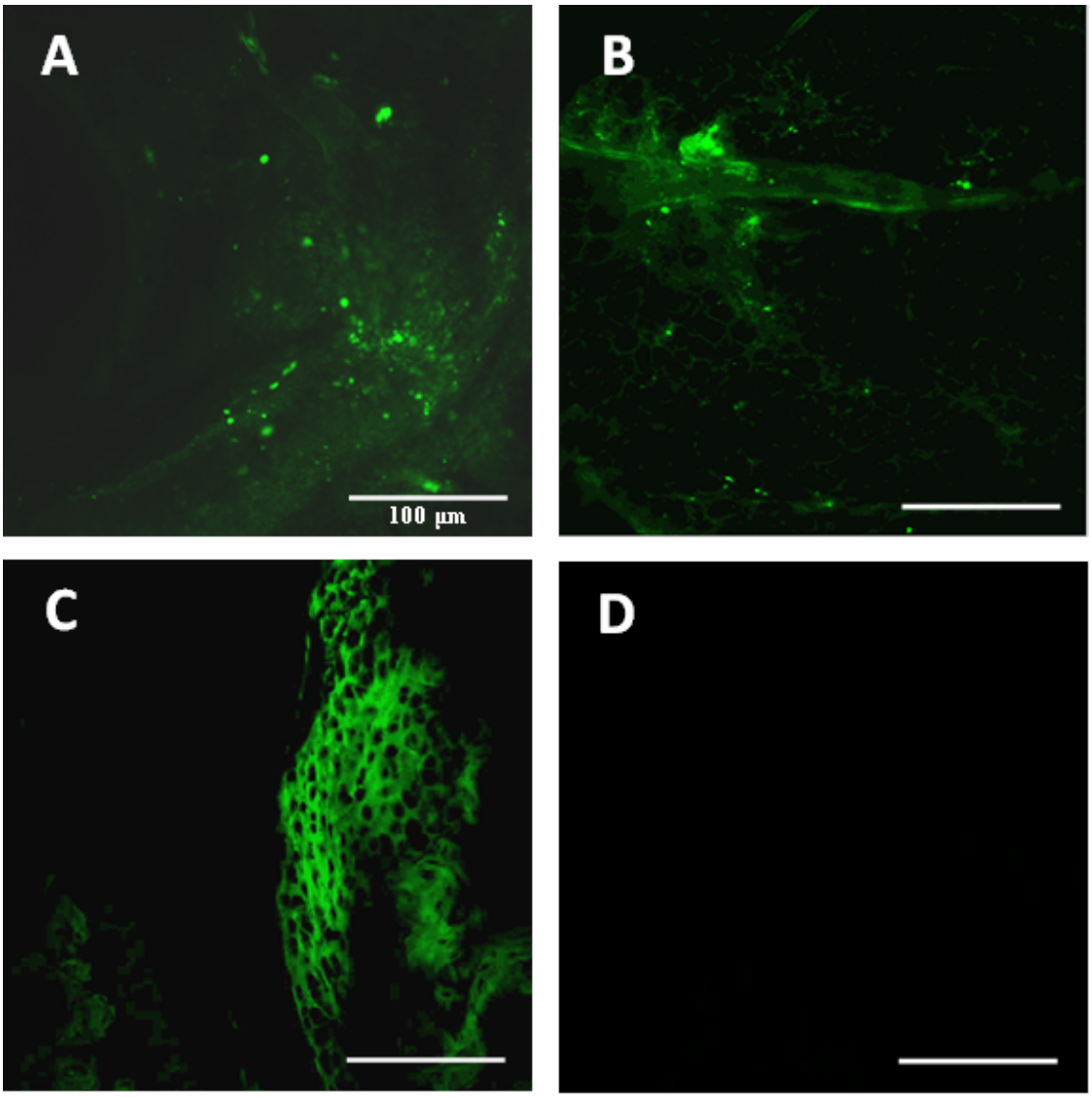

Figure 5.1 Ectopic expression of PRG4-GFP *in vivo*. Six mice had GFP (n = 3) or PRG4-GFP (n = 3) injection in right stifle and euthanized at 2 weeks. Confocal images show apple green fluorescence emitted from the articular cartilage (A), synovium (B), and infrapatellar fat pad (C) in mouse knee joint after 2.5 x 10^{10} v.g injection of AAV-PRG4-GFP. (D) contralateral joints

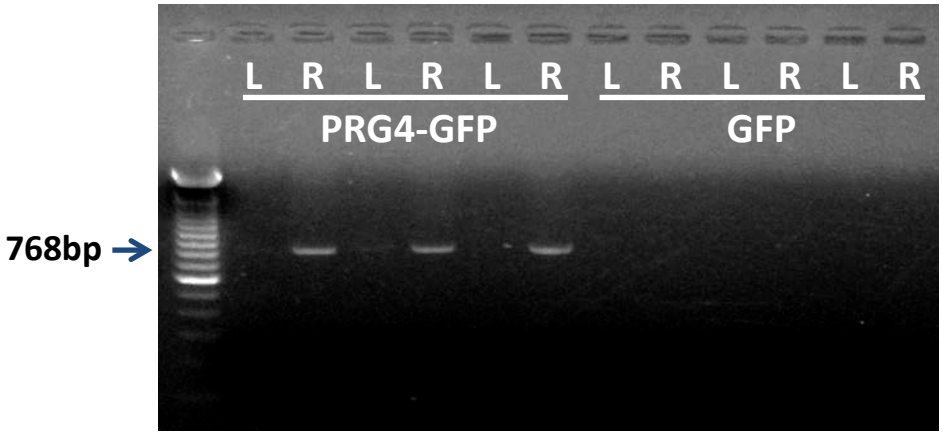

Figure 5.2 Confirmation of PRG4-GFP gene through PCR. PCR products were detected in only PRG4-GFP group (R; right), while the contralateral joint (L; left) had no PRG4-GFP construction gene.

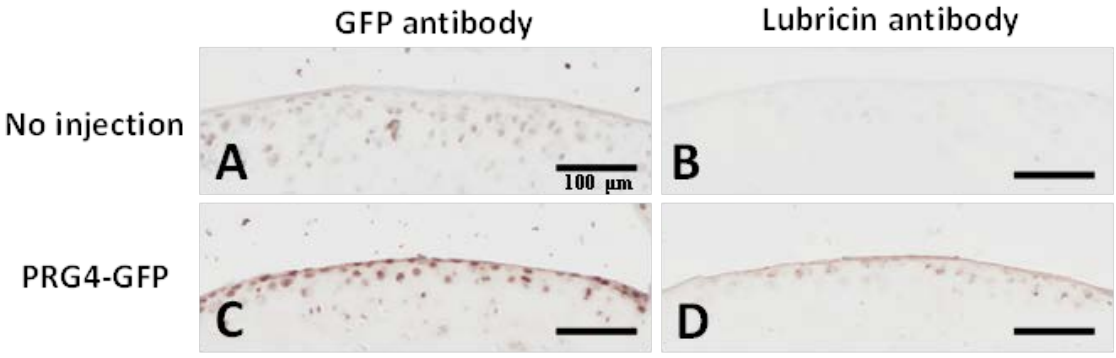

Figure 5.3 Immunohistochemistry with GFP antibody and lubricin antibody *in vivo*. Paraffin-embedded cartilage sections were stained with GFP (A and C) and lubricin (B and D) antibodies. The antibodies were only expressed positively in PRG4-GFP group (C and D) compared with normal cartilage (no injection) (A and B).

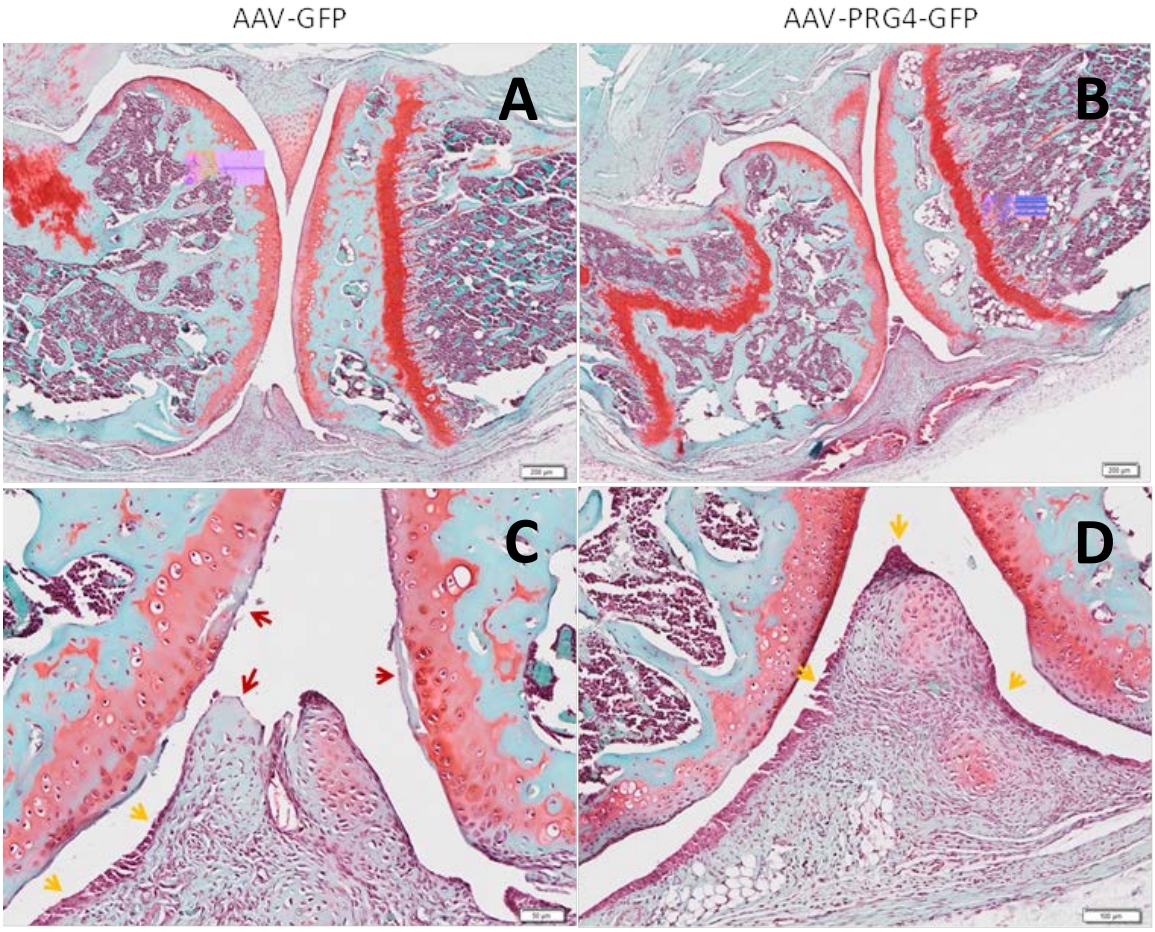

Figure 5.4 Representative histologic sections safranin O staining for measuring PG contents. (A, C) AAV-GFP injection in DMM mouse (B, D) AAV-PRG4-GFP injection in DMM mouse (n = 2-4). DMM: Destabilization of the medial meniscus

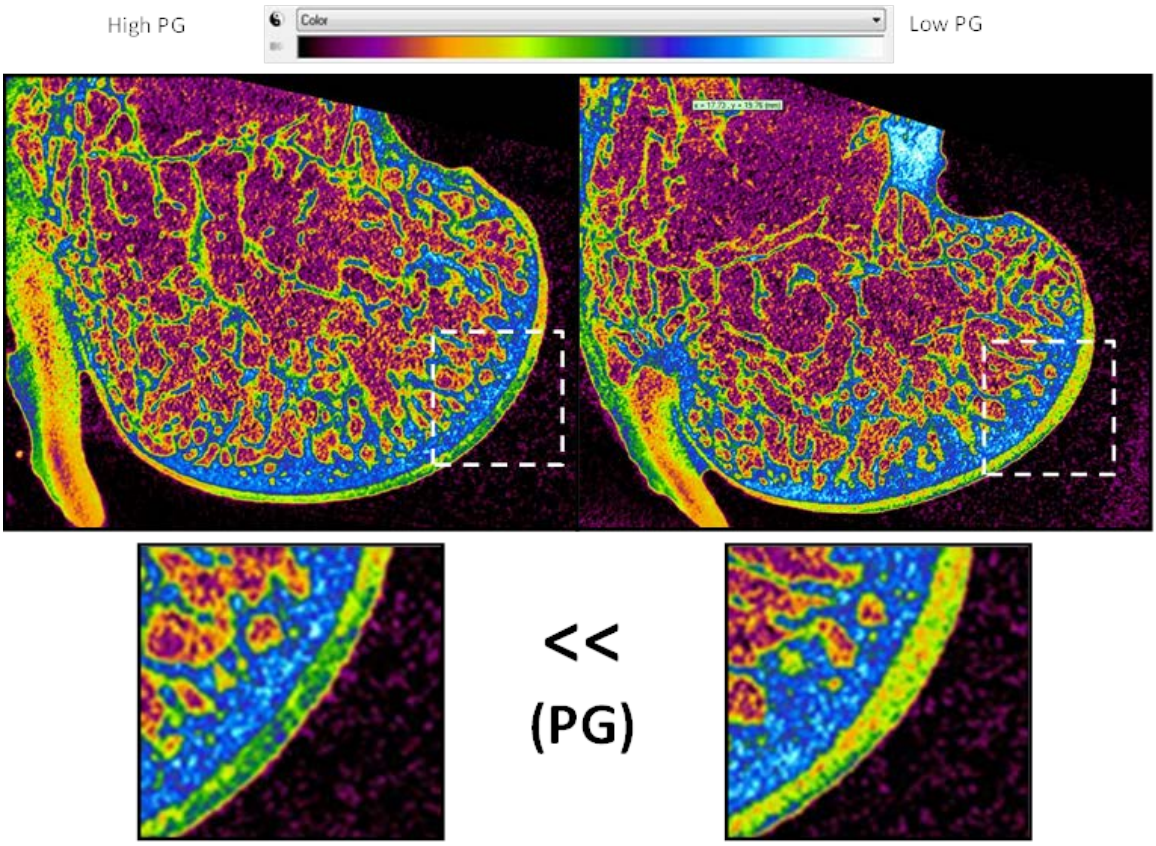

Figure 5.5 Representative histologic EPIC-μCT image in ACL transection rabbit model. (A) AAV-GFP injection in ACLT rabbit (B) AAV-PRG4-GFP injection in ACLT rabbit (n = 6) In load bearing cartilage region, a stable PG contents level and PRG4-GFP signal were identified in AAV-PRG4-GFP injected knees. ACLT: anterior cruciate ligament transection

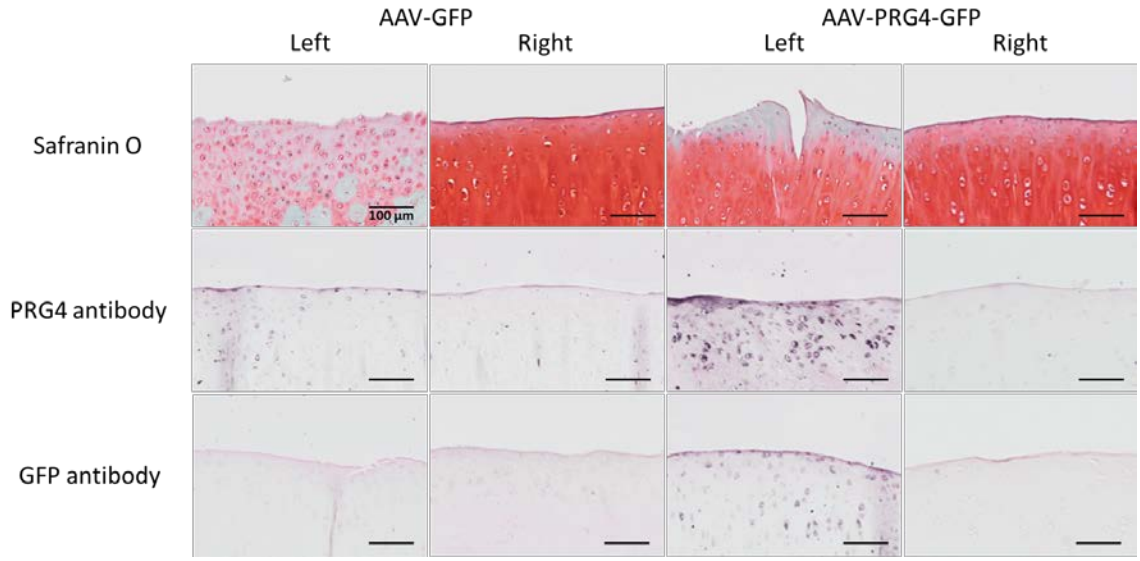

Figure 5.6 Representative immunohistochemistry image in ACL transection rabbit model with GFP antibody and lubricin antibody. AAV-GFP or AAV-PRG4-GFP were injected in ACLT left knees. Right knees are no injection control group. Paraffin-embedded cartilage sections were stained with GFP and lubricin antibodies. ACLT rabbit injected with AAV-GFP (n = 7), ACLT rabbit injected with AAV-PRG4-GFP (n = 7), ACLT: anterior cruciate ligament transection

CHAPTER 6

DISCUSSION AND CONCLUSIONS

The recombinant PRG4-GFP fusion protein produced by transduced synoviocytes in monolayer culture was of the expected molecular weight, appropriately glycosylated, and found to bind to cartilage surfaces. The friction coefficient of explant cartilage decreased significantly with the addition of PRG4-GFP to the culture medium. We measured the friction coefficient from an average of 3 back-and-forth cycles. However, some researchers report that repetitive motion friction increases the friction coefficient.(84) Thus, more lengthy friction tests are needed to acquire a more comprehensive understanding of the effects of PRG4-GFP on friction. On the other hand, PRG4-GFP functioned similarly to SF in terms of protecting chondrocytes from shear load-induced death, which supports the hypothesis that PRG4-GFP lowers friction. These findings confirmed that the insertion of the GFP sequence in PRG4, while helpful for tracking purposes, did not significantly disrupt boundary lubricant function.

Injection of AAV-PRG4-GFP in mouse stifle joints resulted in persistent (up to 4 weeks post-injection) expression of the transgene by superficial and transitional chondrocytes, as well as by synoviocytes, adipocytes, and meniscus cells. Immunohistochemistry showed that cartilage surfaces in injected joints were positive for both GFP and lubricin, whereas control joints were largely

negative for both targets. These results indicate that PRG4-GFP gene therapy enhanced the surface lubricant layer on cartilage surfaces.

Injection of AAV-PRG4-GFP in rabbit ACL deficient joints also resulted in a stable expression of PRG4-GFP by chondrocytes and synoviocytes on the articular surface. Immunohistochemistry showed that cartilage surfaces in injected joints were positive for both GFP and lubricin as in previous mice experiments. The μCT imaging in load bearing region on cartilage showed a stable level of PG contents, blocking PG loss, and PRG4-GFP signal. These results indicate that PRG4-GFP gene therapy enhanced the surface lubricant layer in animal models. A longer period of the experiment, approximately more than 8 weeks, would be required to verify long-term effects. Although we followed mice for only 4 weeks (rabbits for 8 weeks), we suspect that PRG4-GFP expression in post-mitotic cells like chondrocytes may persist for a year or more post-infection.(127) If that is the case, our approach has a favorable potential to test the long-term therapeutic effects of lubricant supplementation on the development of PTOA in joint injury models. Moreover, a quantification a method of therapeutic effects on cartilage surface needs to be verified for the expression of PRG4-GFP.

The studies proposed here are intended to provide a definitive assessment of the potential of PRG4/lubricin gene therapy to forestall the development of PTOA associated with ACL insufficiency in the rabbit. We do not know that these results will fully predict what may occur in humans. Rather, positive findings in this proof-of-concept study will encourage us to further examine the

long-term effects of PRG4-GFP therapy in models that better approximate the slow, gradual pace of OA progression in humans.

If PRG4-GFP gene therapy proves to be effective in the context of ACL transection, a natural follow-up to this investigation is to apply the treatment to other joint injuries and damages that would benefit from improved joint lubrication. These may include isolated meniscal injuries, combined ACL and meniscal injuries, and intraarticular fractures. Animal models for all of these injuries have been developed in our laboratories as part of the NIH program project and relevant Department of Defense-funded projects. Therefore, we are well-positioned to broaden the indications for PRG4-GFP therapy to encompass the majority of injuries that lead to PTOA.

Having overcome the most significant technical obstacles in preliminary studies we don't expect to encounter substantial difficulties in executing the proposed experiments. However, concerns regarding the adequacy of PRG4-GFP expression levels in the joint must be addressed. Our pilot studies showed that the concentration of PRG4-GFP secreted into 10 ml of culture medium by 1 x 10^6 transduced synovial fibroblasts in 2 days (0.3 μM) was sufficient to prevent the effects of injurious shear loading on explants. Since we are planning on introducing 10^{10} viral particles in the joint it is not unreasonable to expect that 1 x 10^6 cells or even more will be transduced. Thus we are confident that expression levels will be adequate to effectively lubricate joint surfaces, especially in the small confines of the rabbit joint. In any event we will carefully

monitor whether PRG4-GFP is in fact coating joint surfaces at every end point. For this reason, optimized volume of AAV-PRG4-GFP is needed.

It is important to note that we do not expect many chondrocytes to be infected by AAV due to the impenetrability of the cartilage extracellular matrix. More accessible cells in the synovial membrane and fat pad are more likely to be transduced. By secreting lubricin into joint fluid bathing cartilage surfaces, these cells contribute extensively to the lubricin bound to the cartilage surfaces. Thus, we expect that the secretion of PRG4-GFP by non-cartilage cells will be chondroprotective.

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