AWARD NUMBER:  W81XWH-14-1-0163

TITLE:  Intra-Articular Lubricin Gene Therepy for Post-Traumatic Arthritis

PRINCIPAL INVESTIGATOR:  James A. Martin, PhD

RECIPIENT:  University of Iowa

                         Iowa City, Iowa 52242

REPORT DATE:  September 2015

TYPE OF REPORT:  Annual Report

PREPARED FOR:  U.S. Army Medical Research and Materiel Command
                  Fort Detrick, Maryland  21702-5012

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                        Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.
Mankin scoring of safranin-O stained sections showed that there was significant pathology in both treatment groups. However, changes in the LubGFP group tended to be less severe than in the GFP group. Although there was significant overlap at the low end of the scale, the highest scores (9 or greater) were found in the GFP group, where full-thickness lesions were commonly seen. A t-Test indicated a less than significant p value of 0.08 for the group difference. However, the study, which included 7 animals per group, was slightly underpowered; sample size estimates based on the data indicated that 2-3 more animals per group will be needed to reject the null hypothesis. This confirmed the original estimate that a total of 10 animals per group will provide sufficient power. In any case the results thus far are encouraging and we see no reason to alter the treatment protocol going forward. We also confirmed that transgene expression was sustained for 8 weeks in joints injected with AAV-LubGFP immediately after ACLT.
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1. INTRODUCTION:

This project focuses on the role of cartilage lubricant failure in PTOA associated with ligamentous/meniscal injuries. Up to 60% of patients who undergo anterior cruciate ligament (ACL) replacement surgery develop symptomatic knee osteoarthritis within 15 years. The need to provide treatment options is urgent, as refinements in surgical procedures over the last few decades have not substantially lowered this risk. There are strong reasons to suspect that cartilage lubrication may be a productive treatment option. Because the half-life of lubricin in the injured human joint is likely to be less than 2-3 weeks, achieving sustained lubrication would require multiple intra-articular injections. Moreover, the slow onset of OA in humans suggests that such treatments might have to be continued for months or years. We propose a gene therapy approach to overcome these problems. We sub-cloned a full length version of the human proteoglycan 4 (PRG4) gene encoding lubricin C (LubC) in adeno-associated virus (AAV) vectors and retroviral vectors and have begun to assess intra-articular PRG4 gene therapy as a means to mitigate OA in ACL-deficient joints in the rabbit.

2. KEYWORDS: Provide a brief list of keywords (limit to 20 words).

ACL replacement, post-traumatic OA, PRG4, lubricin, gene therapy, adeno-associated virus

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Specific Aim 1: Determine the effects of PRG4 gene therapy on the progression of OA in a rabbit ACL transection model

- Major Task 1: Test the effects of GFPLub transduction on cartilage degeneration at 4 and 12 weeks after ACL transection (ACLT) (months 0-22, 8/15/14 – 6/14/16)
- Subtask 1: Preparation of AAV-GFP and AAV-GFPLub constructs (months 0-3; completed, late October 2014);
- Subtask 2: Injection of AAV-GFP or AAV-GFPLub intra-articularly 7 days pre-surgery. Perform rabbit ACLT or sham surgery on 20 rabbits each, each split evenly among receiving AAV-GFP or AAV-GFPLub (months 4-10, 11/15/14 – 6/14/15, 35% complete); 14 of 40 rabbits underwent ACLT surgery and were treated with AAV-GFP or AAV-GFPLub and were euthanized
- Subtask 3: Drawer test immediately post-euthanasia at 4 weeks post-op (n = 20) or 12 weeks post-op (n = 20) (months 4-10, 11/15/14 – 6/14/15, 40% complete); the device was built and programmed, its function validated. The device was used successfully to test laxity in 14 rabbits with ACLT by Marc Brouillette, who was added to the protocol
• Subtask 4: Confocal microscopy to confirm GFPLub expression (months 4-10, 11/15/14 – 6/14/15, **35% complete**); 14 rabbits were imaged.

• Subtask 5: Safranin-O histology, automated Mankin score for OA, Lubricin/GFP immunohistochemistry, Lubricin turnover from synovial fluid harvested at euthanasia (immunoblots) (months 11-16, 6/15/15 – 12/14/15, **30% complete**); Safranin-O stained sections were digitally scanned in preparation for scoring.

• Subtask 6: Statistical Analysis (month 17, 1/15/16 – 2/14/16, 0% complete);

Milestone Achieved: Results will be gathered and analyzed for statistical significance and write-ups begun on histological, confocal, and joint stability data (months 18-22, 2/15/16 – 7/14/16, **10% complete**); No significant treatment-related differences in joint laxity were observed in the first set of 14 rabbits

• Local IACUC Approval (Pre-award, completed April 2014), Marc Brouillette added; and

• ACURO Approval (Pre-award, completed July 2014)

Specific Aim 2: Determine the effects of PRG4 gene therapy after OA has already begun to develop

• Major Task 2: Test the effects of delayed PRG4 gene therapy on cartilage degeneration at 16 weeks post ACLT; (months 20-36, 4/15/16 – 8/14/17);

• Subtask 1: Gene therapy (AAV-GFP or AAV-GFPLub, evenly split among ACLT or sham surgeries) will be initiated 8 weeks after ACL transection (20) or sham (20) surgery and its effects on the subsequent progression of OA will be evaluated at 16 weeks post ACLT (months 20-26, 4/15/16 – 11/14/16, 0% complete);

• Subtask 2: Drawer test immediately post-euthanasia (months 20-26, 4/15/16 – 11/14/16, 0% complete);

• Subtask 3: Confocal microscopy to confirm GFPLub expression (months 20-26; 4/15/16 – 11/14/16, 0% complete);

• Subtask 4: Safranin-O histology, automated Mankin score for OA, Lubricin/GFP immunohistochemistry, Lubricin turnover from synovial fluid harvested at euthanasia (immunoblots) (months 26-30, 10/15/16 – 3/14/17, 0% complete);

• Subtask 5: Statistical analysis (month 31, 3/14/17 – 4/14/17, 0% complete);

Milestone Achieved: Results will be gathered and analyzed for statistical significance and write-ups begun on histological, confocal, and joint stability data (months 32-36, 4/15/17 – 8/14/17, 0% complete);

• Local IACUC Approval (Pre-award, completed April 2014); and

• ACURO Approval (Pre-award, completed July 2014)
What was accomplished under these goals?

1) Major Activities

In the first year we performed studies to confirm that transgene expression was sustained for 8 weeks in joints injected with AAV-LubGFP immediately after ACLT, and to assess the effects of that treatment on the progression of OA. Rabbits treated with AAV-LubGFP or AAV-GFP were euthanized at 8 weeks post-op. Histologic sections were stained with safranin O and cartilage degeneration scored on the Mankin scale. Immunohistochemical analysis for lubricin expression was conducted.

2) Specific Objectives

To determine if a single injection of AAV-LubGFP is chondroprotective, and to confirm that transgene expression and lubricin coating on cartilage surfaces is maintained for at least 8 weeks after treatment.

3) Major findings

Mankin scoring of safranin-O stained sections showed that there was significant pathology in both treatment groups (Figure 1). However, changes in the LubGFP group tended to be less severe than in the GFP group. Although there was significant overlap at the low end of the scale, the highest scores (9 or greater) were found in the GFP group, where full-thickness lesions were commonly seen. A t-Test indicated a less than significant p value of 0.08 for the group difference. However, the study, which included 7 animals per group, was slightly underpowered; sample size estimates based on the data indicated that 2-3 more animals per group will be needed to reject the null hypothesis. This confirmed the original estimate that a total of 10 animals per group will provide sufficient power. In any case the results thus far are encouraging and we see no reason to alter the treatment protocol going forward.

Although we were able to detect LubGFP expression in intra-articular adipose tissue by confocal microscopy, attempts to image GFP in cartilage by this method failed due to intense yellow-green autofluorescence. To confirm gene transfer in cartilage we performed immunohistochemical analysis for lubricin on sagittal sections from weight-bearing surfaces of medial tibial plateaus. Sections were lightly stained with eosin after the immuno procedure and were scanned at 20x (Figure 2). Uninjured and untreated right legs were included as controls. As shown in the figure below, the left joints injected with AAV-LubGFP showed markedly more lubricin-positive chondrocytes than in the untreated right joints (Figure 3). Moreover, there was increased staining in the superficial matrix, indicating improved boundary lubrication. Although this is a promising result and it is tempting to conclude that expression increased largely because of viral gene transfer, it is possible that the increase was endogenous and related to the ACLT injury, as the antibody detects both native and transgenic lubricin. More definitive results are expected when AAV-GFP-treated ACLT controls are analyzed with this antibody, and when sections from AAV-LubGFP treated joints are stained for GFP, which should detect only the recombinant fusion protein. The immunostains will be quantified by image analysis (positive cells/cm2) and the
results paired with Mankin scores to determine if, as expected, there is a correlation between lubricin/LubGFP expression levels and OA progression.

Our initial 14 rabbit study has yielded a trove of data that supports our primary hypothesis and approach, but we are significantly behind the experimental schedule outlined in the original SOW due to bottlenecks in performing animal surgeries. This problem has been resolved by recent hiring of additional personnel in the SurgiCenter, and by the completion of other studies the center was conducting. We are now confident that the work described in the SOW can be completed by the end of the funding period.

4) Other Achievements

Nothing to Report.

Figure 1 Effects of gene therapy on OA progression at 8 weeks post-ACLT. Average Mankin scores (columns) are shown for the LubGFP and GFP groups (n = 7 per group). The left injured joints and right uninjured were analyzed. Error bars are standard deviations. The p value for the difference between the groups on the left side is indicated.

Figure 2. The image shows an immunostained tibia plateau section scanned at 20x. A close-up view of articular cartilage is shown in the inset. The dark spots in the enlarged image are chondrocytes expressing lubricin.
Figure 3. Immunohistochemical confirmation of increased lubricin expression associated with AAV-LubGFP transduction. Each set of 2 panels outlined in black show results for the left and right stifle joints from the same rabbit. The left joints were injected with AAV-LubGFP immediately after ACLT surgery. The right stifle joints were not injected with virus. The animals were euthanized after 8 weeks and the joints prepared for paraffin histology. Sections were stained with a monoclonal antibody to PRG4 (lubricin).
What opportunities for training and professional development has the project provided?


How were the results disseminated to communities of interest?

Nothing to Report.

What do you plan to do during the next reporting period to accomplish the goals?

Perform ACLT surgeries and treatments on all remaining rabbits (n = 66).

4. IMPACT: Distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?

Nothing to Report.
What was the impact on other disciplines?

Nothing to Report.

What was the impact on technology transfer?

Nothing to Report.

What was the impact on society beyond science and technology?

Nothing to Report.
5. **CHANGES/PROBLEMS:** The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, “Nothing to Report,” if applicable:

**Changes in approach and reasons for change**

| Nothing to Report. |

**Actual or anticipated problems or delays and actions or plans to resolve them**

| Nothing to Report. |

**Changes that had a significant impact on expenditures**

| Nothing to Report. |
Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

**Significant changes in use or care of human subjects**

Not Applicable, Nothing to Report.

**Significant changes in use or care of vertebrate animals.**

Nothing to Report.

**Significant changes in use of biohazards and/or select agents**

Not Applicable, Nothing to Report
6. **PRODUCTS:** Products resulting from the project during the reporting period.

- **Publications, conference papers, and presentations**
  - **Journal publications.**
    - Nothing to Report.
  - **Books or other non-periodical, one-time publications.**
    - Nothing to Report.
  - **Other publications, conference papers, and presentations.**
    - Nothing to Report.
  - **Website(s) or other Internet site(s)**
    - Nothing to Report.
- **Technologies or techniques**

  Nothing to Report.

- **Inventions, patent applications, and/or licenses**

  Nothing to Report.

- **Other Products**

  Data demonstrating that AAV-lubricin gene therapy is safe and mitigated PTOA in rabbits, a strong first step toward implementation in people.
### 7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

<table>
<thead>
<tr>
<th>Name:</th>
<th>James A. Martin, PhD</th>
</tr>
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<tbody>
<tr>
<td>Project Role:</td>
<td>Principal Investigator</td>
</tr>
<tr>
<td>Researcher Identifier</td>
<td>NA</td>
</tr>
<tr>
<td>Nearest person month worked:</td>
<td>1.2 person month (Calendar)</td>
</tr>
<tr>
<td>Contribution to Project:</td>
<td>Dr. Martin has directed all programmatic activities, including decision-making and conducting research group discussions on a weekly basis to assure progress is being made as planned.</td>
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<table>
<thead>
<tr>
<th>Name:</th>
<th>Douglas R. Pedersen, Ph.D.</th>
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<tbody>
<tr>
<td>Project Role:</td>
<td>Co-Investigator</td>
</tr>
<tr>
<td>Researcher Identifier</td>
<td>NA</td>
</tr>
<tr>
<td>Nearest person month worked:</td>
<td>1.44 person months (Calendar)</td>
</tr>
<tr>
<td>Contribution to Project:</td>
<td>Dr. Pedersen was responsible for discussing the design and operation of mechanical testing devices, and helped support the implementation of drawer testing for rabbit stifle joint ACL laxity. In the coming months, he will be helping with objectively automating analysis of images and histological specimens.</td>
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<tr>
<th>Name:</th>
<th>Hongjun Zheng, PhD</th>
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<tbody>
<tr>
<td>Project Role:</td>
<td>Co-Investigator</td>
</tr>
<tr>
<td>Researcher Identifier</td>
<td>NA</td>
</tr>
<tr>
<td>Nearest person month worked:</td>
<td>1.0 person months (Calendar)</td>
</tr>
<tr>
<td>Contribution to Project:</td>
<td>Dr. Zheng has been involved with producing and preliminarily testing the AAV-GFP and AAV-GFPLubricin constructs. Additionally, testing rabbit joint samples for GFP and Lubricin expression after there were difficulties imaging the GFP via confocal microscopy.</td>
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<thead>
<tr>
<th>Name:</th>
<th>Hyeonghun Choe, M.S.</th>
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<tbody>
<tr>
<td>Project Role:</td>
<td>Graduate Research Assistant (PhD Student until August 2015)</td>
</tr>
<tr>
<td>Researcher Identifier</td>
<td>NA</td>
</tr>
<tr>
<td>Nearest person month worked:</td>
<td>6 person months (Calendar) – figured based on equivalency of hours to full-time faculty member</td>
</tr>
<tr>
<td>Contribution to Project</td>
<td>Mr. Choe has provided preliminary testing with AAV-GFP and AAV-GFPLubricin constructs in preparation for and during injection of pilot animals; preparing for immunoblotting investigation of GFP and lubricin markers in rabbit joint synovial fluid.</td>
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<tr>
<td>Name:</td>
<td>Marc Brouillette, Ph.D.</td>
</tr>
<tr>
<td>Project Role:</td>
<td>Post-doctoral Research Fellow (late May 2015)</td>
</tr>
<tr>
<td>Researcher Identifier</td>
<td>NA</td>
</tr>
<tr>
<td>Nearest person month worked:</td>
<td>1.25 person month (Calendar) – figured based on equivalency of hours to full-time faculty member during graduate research assistantship and post-doc positions.</td>
</tr>
<tr>
<td>Contribution to Project:</td>
<td>Dr. Brouillette has provided support for the drawer testing device for measuring rabbit knee laxity post-ACLT. It was anticipated that the drawer testing device, which was an older device, would be operable after 5 years of not being used, but it was determined not to be. Dr. Brouillette has extensive experience writing operation software for such mechanical devices in my laboratory. His expertise was found to be required after grant start, and he is hoped to be included as a replacement for Hyeonghun Choe, post-graduation, in October 2015 as a post-doctoral research scholar to do immunohistochemistry on joint tissues and aid in the continuation of the drawer testing (troubleshooting, analyzing drawer testing data along with Dr. Pedersen). Please see Changes/Problems below.</td>
</tr>
<tr>
<td>Name:</td>
<td>Gail L. Kurriger, B.S.</td>
</tr>
<tr>
<td>Project Role:</td>
<td>Research Associate</td>
</tr>
<tr>
<td>Researcher Identifier</td>
<td>NA</td>
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<tr>
<td>Nearest person month worked:</td>
<td>1.2 person months (Calendar)</td>
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<tr>
<td>Contribution to Project:</td>
<td>Ms. Kurriger performed all rabbit joint dissections thus far and has performed all histological processing and sectioning and safranin-O staining. She performed some immunohistological preparations as well.</td>
</tr>
<tr>
<td>Name:</td>
<td>Jacob T. Roberts, B.S.</td>
</tr>
<tr>
<td>Project Role:</td>
<td>Research Assistant</td>
</tr>
<tr>
<td>Researcher Identifier</td>
<td>NA</td>
</tr>
<tr>
<td>Nearest person month worked:</td>
<td>2 person months (Calendar)</td>
</tr>
<tr>
<td>Contribution to Project:</td>
<td>Mr. Roberts scanned histological slides and did measurements on synovia for inflammation.</td>
</tr>
<tr>
<td>Name:</td>
<td>Douglas C. Fredericks, B.S.</td>
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<tr>
<td>Project Role:</td>
<td>Co-Investigator (Research Specialist – Animal Surgeon)</td>
</tr>
<tr>
<td>Researcher Identifier</td>
<td>NA</td>
</tr>
<tr>
<td>Nearest person month worked:</td>
<td>1.2 person months (Calendar)</td>
</tr>
<tr>
<td>Contribution to Project:</td>
<td>Mr. Fredericks is one of two animal surgeons and did preliminary animal protocol writing and interacted with the University of Iowa’s Office of Animal Resources for IACUC and ACURO approval, and helped perform the first batches of rabbit surgeries.</td>
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<tr>
<th>Name:</th>
<th>Emily B. Petersen, D.V.M.</th>
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<tr>
<td>Project Role:</td>
<td>Veterinarian</td>
</tr>
<tr>
<td>Researcher Identifier</td>
<td>NA</td>
</tr>
<tr>
<td>Nearest person month worked:</td>
<td>1 person month (Calendar) – no salary support from the present grant</td>
</tr>
<tr>
<td>Contribution to Project:</td>
<td>Dr. Pedersen is one of two animal surgeons and did preliminary animal protocol writing and interacted with the University of Iowa’s Office of Animal Resources for IACUC and ACURO approval, and helped perform the first batches of rabbit surgeries.</td>
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Funding Support: Dr. Petersen is funded through the Bone Healing Research Lab’s Animal Research Surgicenter.
Newly Funded Projects since W81XWH-14-1-0163 was negotiated:

**Joint Distraction Treatments of Intra-articular Fracture Induced Post-traumatic Osteoarthritis in a Large Animal Model**

W81XWH-15-1-0642

Sponsor Agency: US Department of Defense, Congressionally Directed Medical Research Program

Contract Specialist: Lance L. Nowell, 301-619-1357, lance.l.nowell.civ@mail.mil

820 Chandler Street

Fort Detrick, MD 21702-5014

Was under negotiations during Year 1 of the current project.

09/30/2015 – 09/29/2018, $1,058,085 total direct costs

Jessica E. Goetz, PhD (PI)

James A. Martin, PhD (Co-Investigator), 0.60 Calendar months

Douglas C. Fredericks, BS (Co-Investigator), 1.20 Calendar months

Post-traumatic osteoarthritis (PTOA) has been, and will continue to be a substantial problem for members of the military who sustain a joint injury as a result of combat or training duties, or as a result of motor vehicle crashes or recreational activities. In our previously funded work, we developed the Yucatan minipig as a large animal model of PTOA following an intra-articular fracture (IAF). As part of that work, the model was used to demonstrate short term effects of a promising biological treatment, and it is currently being used to investigate the ability of biological treatments to prevent the development of PTOA during long-term survival studies. Expanding upon this body of previous and ongoing work, we propose to investigate the effects of early joint distraction after IAF (a mechanical therapy) on the development of PTOA. Joint distraction has been shown to be clinically effective for relieving pain and restoring the native joint space in a few small series of patients with late-stage osteoarthritis. In this work, joint distraction will be applied at two much earlier time points before the onset of irreversible joint destruction, in order to halt the development of PTOA that is known to occur as a result of our previous work. OVERLAP: There is no overlap with the current work.

**Bioactive Gel for Cartilage Repair**

Sponsor Agency: CartilaJoint GenTech, LLC

Sponsor Contact: Michael Sinsheimer, CEO, msinsheimer@carolina.rr.com, 704-773-7652

1723 Beverly Drive

Charlotte, NC 28207

07/23/2015 – 04/30/2016, $54,982.60 Total Direct Costs

James A. Martin, PhD (PI), 0.24 Calendar months (no salary support)

Douglas C. Fredericks, BS (Co-Investigator), 0.24 Calendar months (no salary support)

Articular cartilage lesions caused by common forms of joint trauma contribute to the pathogenesis
of post-traumatic osteoarthritis (PTOA). Spontaneous healing of cartilage lesions is rare and a number of methods designed to overcome its limited regenerative capacity have been developed. Many strategies involve removing damaged cartilage and grafting chondrocytes or stem cells to fill the resulting defect. While patients derive some benefit from these interventions, the procedures carry substantial risks including morbidity associated with cartilage or bone marrow harvests, and the need to grow, or otherwise manipulate cells, \textit{ex vivo}. Moreover, some procedures, such as chondrocyte implantation, require two surgeries to harvest cartilage and graft the cells. To address these issues, we have developed a strategy that exploits the intrinsic regenerative powers of progenitor cells residing in cartilage. The strategy includes an interpenetrating network (IPN) fibrin (TisseelTM/hyaluronan, Gel-One hydrogel), which serves as a defect filler and conductor of cell migration. The potent progenitor cell chemotactic factor, stromal-derived factor-1 alpha (SDF-1 alpha), is dissolved in the IPN gel and promotes full cellularization of the defects. Polylactic co-glycolic acid (PLGA) microparticles that slowly release the chondrogenic factor transforming growth factor beta 3 (TGF-beta 3) are suspended in the IPN gel and stimulate progenitor cells to replace the original hydrogel with neo-cartilage that closely approximates the biomechanical and biochemical characteristics of the surrounding cartilage. Strategy is being tested in goats. OVERLAP: There is no overlap with the current work.

**Direct Delivery of Bone Morphogenetic Protein-2 and Fibroblast Growth Factor-2 Plasmid Genes for Diabetic Fracture Healing in a Rabbit Model**

Sponsor Agency: American Orthopaedic Foot & Ankle Society
Contract Specialist: Joy Keller, MS, MSUS, Director of Research, jkeller@aofas.org, 847-430-5085
9400 West Higgins Road, Suite 220
Rosemont IL 60018

05/12/2015 – 10/31/2016, $20,000 Total Costs
Nathan A. Nicholson, MD (PI)
James A. Martin, PhD (Co-Investigator), 0.12 Calendar months (no salary support)
Douglas C. Fredericks, BS (Co-Investigator), 0.12 Calendar months (no salary support)

This is an animal study investigating the effects of diabetes mellitus on bone healing in a diabetic rabbit femur fracture model. Specific aims include 1)Determine if long-standing type 1 diabetes mellitus results in alterations in gene expression, architecture, and amount of new bone at regenerate sites and 2)Determine if nonviral gene delivery with bone morphogenetic protein-2 (BMP-2) and Fibroblast Growth Factor-2 (FGF-2) will attenuate the deleterious effects of Type 1 diabetes mellitus on healing of a bone defect site. OVERLAP: There is no overlap with the current work.

**Engineering Endogenous Cartilage Repair**

Sponsor Agency: Arthritis Foundation
Arthritis Foundation National Office
1330 W. Peachtree St., Suite 100
Atlanta, GA 30309
Estimated Start Date 02/01/2016-01/31/2019, $892,057 Total Direct Costs (under negotiations)
The overall goal of these studies is to test the feasibility of a novel method for \textit{in situ} progenitor cell-mediated cartilage regeneration in a large animal model. We propose to package the chondrogenic factor TGF\textbeta3 in biodegradable polylactic co-glycolic acid (PLGA) microparticles and suspend them in hydrogel before arthroscopic \textit{in vivo} delivery into a goat femoral condyle chondral defect. Specific Aims include: 1) Determine the effects of SDF/TGF on \textit{in situ} cartilage regeneration in experimental defects in the goat femoral condyle, and 2) Determine if early joint immobilization improves cartilage regeneration in defect sites. OVERLAP: There is no overlap with the current work.

\textbf{Evaluation of TriPore Putty in a Sheep Femoral Defect Model}

Sponsor Agency: Orthogem Limited
Sponsor Contact: Paul Markgraf, Phone 0044 115 950 5721, p.markgraf@orthogem.com
BioCity, Pennyfoot Street
Nottingham, NG1 1GF
09/01/2015 – 08/31/2016, $155,876.70 Total Direct Costs
Douglas C. Fredericks, BS (PI), 0.12 Calendar months

While autograft continues to be regarded as the de-facto “gold standard” for bone grafting, morbidity at the autograft harvest site and increased surgical cost to perform the autograft recovery procedure has led to new alternatives. The use of allograft tissue is now commonplace; however, allograft tissue is prone to therapeutic risks related to tissue sourcing and immunological sensitivity. As a result of limitations in the standard of care, there is significant interest in the use of synthetic bone graft substitutes that can provide the structural matrix of allograft or autograft, followed by progressive and orderly remodeling of the implant into normal bone. Synthetic grafts that inherently mimic the inorganic chemistry of natural bone provide an attractive alternative to naturally sourced tissues due to the ability to generate multiple product variants of consistent high quality with no disease transmission risks. These manufactured grafts have the potential to be combined with osteogenic sources such as autograft and/or rhBMP (per approved indications) to enhance graft integration and overall fusion rates. TriPore Putty is a line extension of the successful, CE-Marked and FDA cleared (K070132) cleared TriPore bone graft, with the difference that TriPore Putty contains the graft within an aqueous Poloxamer gel designed to facilitate intraoperative handling. The poloxamer gel is expected to be cleared from the implant site within a week of surgery and does not inhibit bone formation. The goals of the study are to determine the efficacy of the test articles relative to positive and negative controls on the following outcomes: quantity and quality of new bone formed (measured by histopathology) and host response (measured by histopathology). OVERLAP: There is no overlap with the current work.
Silhouette Lapine Posterolateral Fusion Model  
Sponsor Agency: Biostructures, LLC  
Sponsor Contact: Duraid Antone, Dantone@biostructures.net, 949-553-1717; or Natalie Adams, Natalie@biostructures.net, 949-553-1717, fax 949-553-0407  
1201 Dove Street, Suite 470  
Newport Beach, CA 92660  
09/01/2015 – 09/01/2016, $157,846 Total Direct Costs  
Douglas C. Fredericks, BS (PI), 1.2 Calendar months  

Silhouette (Strip and Putty) is a bone void filler device intended for the treatment of surgical and trauma related osseous defects of the skeletal system (i.e., extremities, pelvis & spine) that are not intrinsic to the stability of the bony structure. The device comprises biphasic (HA/βTCP) granules suspended in a porous type I collagen matrix that is chemically crosslinked and lyophilized to impart porosity, hydration capability and handling properties. The device is supplied in both a strip form that can be cut and shaped as desired by the surgeon, and a morsel form that facilitates gently packing into osseous defects. The implant is designed to be hydrated with bone marrow aspirate (BMA) in a 1:1 volume ratio and combined with autologous bone (1:1 ratio by volume) prior to use and following implantation, and provides a resorbable osteoconductive scaffold that guides bone regeneration during the healing process. The granulate component is intended to support osteoconductivity through controlled dissolution, bone bonding and resorption processes. The collagen matrix is a highly porous, interconnected scaffold and is intended to contain the uniformly dispersed biphasic granules during surgical application, and is designed for optimal hydration and handling properties. The Strip and Putty configurations are similar in composition but exhibit different handling properties to address the range of handling properties desired by the clinician. Based on minor differences in processing, the Putty is designed for greater flexibility and compressibility compared to the Strip, which exhibits greater stiffness and resiliency during intraoperative handling. The goals of the study are to determine the effect of the test article (Silhouette) in posterolateral fusion and determine volume of new bone formation relative to Vitoss on the following outcomes: fusion rate (measured by palpation, motion analysis, radiography, CT, and histopathology), quantity and quality of new bone formed (measured by radiography, CT and histomorphometry), quantity and quality of graft resorption (measured by radiography, CT and histomorphometry), and host response (measured by histopathology). OVERLAP: There is no overlap with the current work.

Canine Ulna Defect dBMP Pilot (SOW 6)  
Rabbit Femur Core Defect dBMP Pilot (SOW 7)  
Segmental Defects in Rabbits (SOW 8)  
Sponsor Agency: Bioventus LLC  
Sponsor Contact: Howard Seeherman, VP BMP Program Development, 919-474-6700  
8 Saint Mary’s Street  
Boston, MA 02115  
06/01/2015 – 12/31/2015, $39,308 Total Direct Costs (SOW 6)  
10/22/15 – 10/22/15, $8,922.50 Total Direct Costs (SOW 7)  
10/22/15 – 10/22/15, $8,922.50 Total Direct Costs (SOW 8)  
Douglas C. Fredericks, BS (PI), 0.12 Calendar months  
SOW 6: This is a pilot project involving a canine ulnar defect model to assess dosing of a designer
BMP. The project goals are to perform bilateral 2 centimeter ulnar defects in 6 animals, each having 6 sets of radiographs (post-op, 2, 4, 6, 8, and 12 weeks) with lateral and A-P views to ensure adequate visualization of the defect site. All implants will be treated with the Bioventus Composite Matrix with either dBMP or rhBMP-2 protein. Radiograph visualization will be used for monitoring defect sites. Samples are examined histologically at Bioventus.

SOW 7: The objective is to identify dose(s) of BV265 that trigger little to no resorption of trabecular bone in the rabbit.

SOW 8: The objective is to compare the distribution of new bone arising from BV265/CM and BMP2/CM with BV26/granules+Surgicel and BMP2/granules+Surgicel. OVERLAP: There is no overlap with the current work.


Sponsor Agency: Bioventus LLC
Sponsor Contact: Eric Vanderploeg
8 Saint Mary’s St.
Boston, MA 02115
04/01/2014 – 03/01/2016, $99,928 Total Direct Costs
Douglas C. Fredericks, BS (PI), 0.12 Calendar months
The goal of the study is to determine the effectiveness of a designer BMP use with a collagen sponge in multiple bone sites. A dose curve will be established for each implantation site and an optimal dose determined. OVERLAP: There is no overlap with the current work.

Closed Projects since W81XWH-14-1-0163 was negotiated (some may have started and completed since that time):

A Clinically Realistic Large Animal Model of Intra-Articular Fracture
W81XWH-10-1-0864
Sponsor Agency: United States Department of Defense, Army Medical Research Acquisition Activity
Contract Specialist: Susan Dellinger, susan.dellinger@amedd.army.mil
US Army Medical Research Acquisition Activity
820 Chandler St.
Fort Detrick MD 21702-5014
09/15/2010 – 10/14/2014, $1,348,298 Total Costs
Jessica E. Goetz, PhD (PI)
James A. Martin, PhD (Investigator), 0.6 Calendar months, no Salary Support during NCE
Douglas C. Fredericks, BS (Investigator), 1.8 Calendar months, no salary support during NCE

The primary objective of this project is to develop a survival animal model of intra-articular fracture (IAF) in which all major pathophysiological attributes of corresponding human clinical injuries are realistically replicated, and in which post-traumatic osteoarthritis (OA) predictably develops. This animal model will facilitate translational research of orthopaedic treatment for IAFs by providing opportunities to test efficacy and safety of new treatment strategies prior to a human clinical trial. A pendulum-based impaction system for implementing an “offset impaction” technique in survival
animal surgery has been designed, built, and piloted for creating IAFs. The purpose of this work is to validate this modeling methodology by addressing the following three research questions: 1) Does the developed insult methodology allow constant introduction of experimental IAFs in the minipig hock in vivo, while ensuring pathophysiological realism? 2) Do the introduced fractures heal in a clinically relevant manner? and 3) Is the proposed animal IAF model capable of demonstrating the effects of biopharmaceutical interventions?

OVERLAP: There is no overlap with the current work.

**Orthogem in a Rabbit Posterolateral Fusion Model**
Sponsor Agency: Orthogem Limited
Contract Specialist: Paul Z. Markgraf, CEO
BioCity, Pennyfoot Street
Nottingham, NG1 1GF
Phone: 0044 115 950 5721
Fax: 0044 115 950 5921
p.markgraf@orthogem.com
09/01/2014 – 01/01/2015, $18,974 total direct costs [did not originally appear on latest support document that was submitted during award negotiations for the current project]

Douglas C. Fredericks, BS (PI), 0.12 Calendar months

The objective of this study was to evaluate the performance of Orthogem in rabbit posterolateral spine fusion (PLF) model. The goals of the study were to determine the effect of the Orthogem on the following outcomes: fusion rate (measured by palpation, motion analysis, and uCT), and quantity and quality of new bone formed (measured by radiography and CT).

OVERLAP: There is no overlap with the current work.

**A: Rabbit Posterolateral Fusion Model BMP/Carrier Screen (SOW 4)**

**B: Rabbit Radius Defect BMP/Carrier Screen (SOW 3)**

**C: Rabbit PLF End Organ Pathology (SOW 5)**

**D: Rabbit PLF Model/BMP/Carrier Screen (SOW 2)**

**E: Rabbit Radius Defect BMP/Carrier Screen (SOW 1)**

Sponsor Agency: Bioventus, LLC
Sponsor Contact: Howard Seeherman, VP BMP Program Development, 919-474-6700
8 Saint Mary’s Street
Boston, MA 02115

Douglas C. Fredericks, BS (PI), 0.12 Calendar months (each)

A: 08/14/2014 – 11/06/2014, $37,261 Total Costs
B: 08/14/2014 – 10/09/2014, $13,952 Total Costs
C: 11/01/2014 – 12/01/2014, $22,186 Total Costs
D: 05/22/2014 – 08/22/2014, $26,755 Total Costs
E: 04/08/2014 – 04/08/2015, $35,666 Total Costs

The goals of the above studies were to determine the effects of the product on bone regeneration in a rabbit posterolateral fusion model. Bone healing was evaluated by radiographs, regenerate bone area, mechanical testing, and histologic scoring.

OVERLAP: There is no overlap with the current work.
A Radiographic and Mechanical Assessment of a TCP (GO)/Poloxamer with rhBMP-7 in a Canine Pancarpal Arthrodesis Model: A Dose Response Study
Sponsor Agency: Olympus Biotech America
Sponsor Agency Contracts Manager: Loan Lacy
Olympus Biotech Corporation
35 South Street
Hopkinton, MA 01748
04/18/2013 - 04/18/2014, $273,221 Annual Direct Costs
Douglas C. Fredericks, BS (PI), 1.2 Calendar months
The goals of the study were to determine the effect of the test article (rhBMP-7 in combination with TCP [G0]/Poloxamer) relative to ICBG on the following outcomes: union rate (measured by palpation, tensile testing, radiography, and MDCT), quantity of new bone formed (measured by radiography), antibody development (rhBMP-7 antibody levels in serum), and systemic levels of rhBMP-7.
OVERLAP: There is no overlap with the current work.

Cartilage-Specific MRI Assessment of KineSpring Performance
Prospective, Multi-Center, Pilot Study to Evaluate Symptom Relief in Patients with Medial Knee Osteoarthritis (OA) Treated with the KineSpring® Knee Implant for Load Reduction
Moximed SOAR US Pilot IDE Study
Sponsor Agency: Moximed, Inc.
Sponsor Agency Contact: Kevin Sidow, President and CEO, Moximed, Inc.
26460 Corporate Ave, Suite 100 Hayward, California, 94545
12/12/2012 – 12/12/2015 $146,095 Total Costs
Annunziato Amendola (PI)
Douglas R. Pedersen (Co-Principal Investigator), 0.12 Calendar months
The primary objective of this pilot study is to demonstrate safety and effectiveness of the KineSpring in patients with primarily unicompartmental medial knee osteoarthritis through 24 months postoperative follow-up.
OVERLAP: There is no overlap with the proposed work.
What other organizations were involved as partners?

Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: Not Applicable.

QUAD CHARTS: Please see Quad Chart below on page 26.

Intra-Articular Lubricin Gene Therapy for Post-Traumatic Arthritis
OR130365
W81XWH-14-1-0163 Year 1, Technical Progress Report
PI: James A. Martin, PhD Org: University of Iowa Award Amount: $727,955 Total Award ($493,233.73 Direct)

Study/Product Aim(s)

• Specific Aim 1: Determine the effects of lubricin gene therapy on the progression of OA in a rabbit ACL transection (ACLT) model (months 0-22)
  • Subtasks 1-6 (see SOW or annual technical report), milestones
• Specific Aim 2: Determine the effects of lubricin gene therapy after OA has already begun to develop (months 20-36)
  • Subtasks 1-5 (see SOW or annual technical report), milestones

Approach

Mankin scoring of tibial plateau cartilage was completed on 14 rabbits with ACLTs, including 7 treated with AAV-LubGFP and 7 with AAV-GFP. Lubricin immunohistochemistry was performed on the same rabbits. GFP immunohistochemistry is in process.

Goals/Milestones

CY14-15, CY15-16 Goals – Prepare AAV constructs & ACLT rabbits
✓ Preparation of AAV-GFP and AAV-GFPLub constructs;
✓ Pilot tests of intra-articular injection of AAV-GFPLub;
✓ Complete survival study animals;

CY16-17 Goals – Test delayed PRG4 gene therapy on cartilage degeneration at 16 weeks post ACLT
✓ Complete ACLT or sham surgeries, apply gene therapy after 8 weeks, euthanize rabbits at 16 weeks

Comments/Challenges/Issues/Concerns

Y1 spending was slightly lower than projected, but this was because of delays in the analysis of the first set of animals and in scheduling surgeries. Now that these issues have been resolved we will be spending more in Y2 on animals and their surgical costs.

Budget Expenditure to Date: Projected Expenditure: Y1 $235,334 total.
Actual Expenditure: Y1 8/15/14 – 8/14/15, $194,490 (includes addition of Marc Brouillette salary/fringe, financials done retroactively during Y2).

Accomplishments: Confirmed high levels of lubricin in cartilage at 8 weeks after ACLT in the LubGFP-transduced joints. Found that Mankin scores were elevated above normal in both treatment groups, but was highest in the GFP group, consistent with a chondroprotective effect of LubGFP.

Timeline and Cost

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Updated: Y1 (previous report 10-26-15), 3-6-16; purple indicates current location in time with respect to each aim’s timeline
Feasibility of intra-articular adeno-associated virus-mediated proteoglycan-4 gene therapy to prevent osteoarthritis

Hyeong Hun Choe
University of Iowa

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This dissertation is available at Iowa Research Online: http://ir.uiowa.edu/etd/1836

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

by

Hyeong Hun Choe

A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Biomedical Engineering in the Graduate College of The University of Iowa

August 2015

Thesis Supervisor: Associate Professor James A. Martin
CERTIFICATE OF APPROVAL

PH.D. THESIS

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

Hyeong Hun Choe

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

Thesis Committee:

James A. Martin, Thesis Supervisor
Tae-Hong Lim
Douglas Pedersen
Nicole M. Grosland
Aliasger K. Salem
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.

Completion of my project could not be accomplished without the support of my colleagues, Lei Ding, Dongrim Seol, Keewoong Jang, Babara J. Laughlin, Abigail D. Smith, Yin Yu, Cheng Zhou, John F. Bierman, Gail L. Kurriger, Hongjun Zheng, Marc J. Brouillette, and Prem S. Ramakrishnan.

I extend thanks to my parents as well. I will never forget their countless support and grace, and unceasing prayer for me.

Finally, to my caring, loving, and supportive wife, Anna Chung: my deepest gratitude. Your encouragement despite difficult times is much appreciated. It was a great comfort and relief to know that you were willing to provide management of our household while I completed my work. I give thanks again to my parents and my wife: I’m struggling to find the right words to express my gratitude for all of your unending support, constant sacrifices, and unconditional love.
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. 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.

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. 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. Lubricin supplementation via intra-articular injection mitigates cartilage degeneration in ACL transection models, indicating that its loss contributes to OA progression. However, multiple injections are required for efficacy due to lubricin’s relatively short half-life in vivo (Figure 2.4).

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. There are several factors related to lubricating condition in the superficial zone (Figure 2.1, 2.2). 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. 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. 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.

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-dipalmitoylphosphatidylcholine (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 \textit{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 Schematic 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)
<table>
<thead>
<tr>
<th>Friction coefficient</th>
<th>Joint</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.014-0.024</td>
<td>Human ankle</td>
<td>(71)</td>
</tr>
<tr>
<td>0.005-0.01</td>
<td>Canine ankle</td>
<td>(72)</td>
</tr>
<tr>
<td>0.02-0.042</td>
<td>Human hip</td>
<td>(72)</td>
</tr>
<tr>
<td>0.007</td>
<td>Canine hip</td>
<td>(73, 74)</td>
</tr>
<tr>
<td>0.005-0.02</td>
<td>Human knee</td>
<td>(71)</td>
</tr>
<tr>
<td>0.075-0.13</td>
<td>Guinea pig knee</td>
<td>(72)</td>
</tr>
<tr>
<td>0.0009-0.0029</td>
<td>Murine knee</td>
<td>(72)</td>
</tr>
<tr>
<td>0.01-0.07</td>
<td>Bovine knee</td>
<td>(75, 76)</td>
</tr>
</tbody>
</table>

Table 2.1 Experimental friction coefficient friction values of intact joints. The friction coefficient was obtained with pendulum systems or custom-built apparatus at different intact joints.
<table>
<thead>
<tr>
<th>Friction coefficient</th>
<th>Contact</th>
<th>Lubricants</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.003-0.1</td>
<td>Glass</td>
<td>Synovial Fluid</td>
<td>(77)</td>
</tr>
<tr>
<td>0.0014-0.07</td>
<td>Glass</td>
<td>Synovial Fluid</td>
<td>(78)</td>
</tr>
<tr>
<td>0.1-0.9</td>
<td>Glass</td>
<td>Synovial Fluid</td>
<td>(79, 80)</td>
</tr>
<tr>
<td>0.01-0.1</td>
<td>Glass</td>
<td>Synovial Fluid</td>
<td>(81)</td>
</tr>
<tr>
<td>0.02-0.2</td>
<td>Metal</td>
<td>Saline</td>
<td>(82)</td>
</tr>
<tr>
<td>0.003-0.35</td>
<td>Metal</td>
<td>Synovial Fluid</td>
<td>(83, 84)</td>
</tr>
<tr>
<td>0.075-0.3</td>
<td>Cartilage</td>
<td>Saline, HA</td>
<td>(85)</td>
</tr>
<tr>
<td>0.024-0.055</td>
<td>Cartilage</td>
<td>Saline</td>
<td>(86, 87)</td>
</tr>
</tbody>
</table>

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.
<table>
<thead>
<tr>
<th>Constituent</th>
<th>Concentration (mg/ml)</th>
<th>Normal</th>
<th>OA</th>
<th>Acute Injury</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyaluronic acid (HA)</td>
<td></td>
<td>1-4</td>
<td>0.7-1.1</td>
<td>0.4-1.0</td>
</tr>
<tr>
<td>Lubricin</td>
<td></td>
<td>0.05-0.35</td>
<td>0.25-0.75</td>
<td>0.02-0.1</td>
</tr>
<tr>
<td>Phospholipids</td>
<td></td>
<td>0.1</td>
<td>0.2-0.3</td>
<td>0.02-0.08</td>
</tr>
<tr>
<td>Albumin/Globulins</td>
<td></td>
<td>15-25</td>
<td>29-39</td>
<td>&gt;25</td>
</tr>
<tr>
<td>Cells ($10^6$ WBC/ml)</td>
<td>&lt;0.2</td>
<td>&lt;2</td>
<td>2-100</td>
<td></td>
</tr>
</tbody>
</table>

**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% O₂/CO₂. After two days in culture the osteochondral plugs were injured by 7 J/cm² 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 10⁵) were seeded in 6-well plates and were infected with or without 2 x 10⁵ 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 10⁵ 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 x 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 retroviral-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.
<table>
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<th>AAV-PRG4-GFP</th>
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**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 stifles 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/cm² 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 = μN; 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 (QCIPTM).(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/cm² 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™ 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™.

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/cm² 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 ($\mu$). (A) Several materials which was identified the friction coefficient ($\mu$) 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 $\mu$g/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 QCIPTM 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.\(^\text{(114, 115)}\)

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.\(^\text{(116, 117)}\) 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 factor\(\beta\) (TGF\(\beta\)), 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 $\textit{in vivo}$ 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 $\textit{in vitro}$ 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. 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 $1 \times 10^6$ 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 $1 \times 10^6$ 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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