AWARD NUMBER: W81XWH-14-1-0163

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

PRINCIPAL INVESTIGATOR: James A. Martin, PhD

CONTRACTING ORGANIZATION: University of Iowa
Iowa City, Iowa 52242

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Fort Detrick, Maryland 21702-5012

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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.
Twenty-six rabbits underwent sham or ACLT surgery and were euthanized at 4 or 8 weeks post-op, completing live animal work for Major Task 1. Histologic processing was completed and safranin-O-stained sections were scanned. All rabbits that underwent surgery survived to the scheduled endpoints without apparent complications. However, the 10 rabbits euthanized at 4 weeks (5 sham and 5 ACLT) all had joint effusions, indicating an inflammatory reaction to AAV. Effusions were not evident in our initial one-week pilot study designed to detect such responses. A review of AAV titers indicated that the preparation used in the 4-week study was ~2-fold more concentrated than that used in the earlier study, suggesting that the effusions were provoked by viral overload. Given these events it would appear prudent to better define the maximum tolerated dose of AAV before beginning Major Task 2, which calls for treatment after OA progression is already underway.
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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:

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 (completed, late October 2014);
  - Subtask 2: Injection of AAV-GFP or AAV-GFPLub intra-articularly. 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, completed, June 2016 ); 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 12 weeks post-op (n = 40) (months 4-10, 11/15/14 – 6/14/11, completed, June 2016);
  - 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, 75% 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: The live animal phase of Major Task 1 was completed. Histologic processing of tissues was completed. 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, 40% complete); 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 19-36, 3/15/16 – 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

Major Task 1: Twenty-six rabbits underwent sham or ACLT surgery and were treated at time 0 bringing the total number of animals to 40 (Subtask 2 completed). The animals were euthanized and drawer testing was performed (Subtask 3 completed). Joint tissues were processed for histology. Safranin-O-stained sections were scanned and images prepared for automated Mankin scoring. Additional sections were stained with antibodies to GFP or lubricin. Lubricin staining was quantified (Subtask 5).

2) Specific Objectives

Complete live animal work and histologic processing of tissues for Major Task 1.

3) Major findings

All rabbits that underwent surgery survived to the scheduled endpoints without apparent complications. However, the 10 rabbits euthanized at 4 weeks (5 sham and 5 ACLT) all had joint effusions, indicating an inflammatory reaction to AAV. This was surprising given that effusions were not evident in our initial one-week pilot study designed to detect such responses. A review of AAV titers indicated that the preparation used in the 4-week study was ~ 2-fold more concentrated than that used in the earlier study, suggesting that the effusions were provoked by viral overload. Given these events it would appear prudent to better define the maximum tolerated dose of AAV before beginning Major Task 2, which calls for treatment after OA progression is already underway.

Although we showed that GFP and GFPLub genes were expressed in joint tissues at 1 week post-AAV injection, immunostaining failed to detect expression in cartilage or synovia at 8 weeks after injection. This result indicated a disappointingly short duration of transgene expression. Despite the absence of GFP we found an increase in lubricin in GFPLub- versus in GFP-treated cartilage, which suggests that GFPLub
transduction led to higher endogenous expression. Although the mechanism of this effect is unclear, the increased abundance of native lubricin may have therapeutic value.

4) Other achievements.

We have begun to explore non-viral methods of gene delivery to articular cartilage in an internally-funded project. This strategy involves treatment with un-complexed plasmid DNA. In the example shown below, we suppressed the expression of MMP-13 in damaged cartilage with an expression plasmid encoding the microRNA miR200c (Figure). This simple delivery method could be used to transfec t chondrocytes with lubricin-encoding plasmids, which is much less likely to elicit an inflammatory response than AAV and more likely to be approved for human use.

![B-actin and MMP-13 expression](image)

**Figure.** Transfection of chondrocytes in cartilage with plasmid DNA. Osteochondral explants were impacted to simulate joint injury, and then treated with empty vector (pSil-4.1), or miR200c-encoding plasmid (pSil-200c). MMP-13 expression was notably suppressed in 3 of the 4 specimens treated with 200c.

**What opportunities for training and professional development has the project provided?**

Dr. Marc Brouillette has gained valuable practical experience in designing and implementing the drawer testing device. The skills he has gained in the process enhance his career prospects as a biomedical engineer.

**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?**

Complete data analysis for Major Task 1 and perform surgeries and treatments on all Major Task 2 rabbits.

4. IMPACT:

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

The delayed inflammatory response to AAV that we observed will be noteworthy to others contemplating intra-articular gene therapies.
What was the impact on other disciplines?

The results justify efforts by pharmacologists to develop non-viral methods of gene delivery.

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

Changes in approach and reasons for change

Our studies suggest that AAV may instigate joint inflammation and effusion, which could obscure transgene effects. Thus, before proceeding with Major Task 2 we would like to identify a maximum tolerated dose for AAV. In this case, a tolerated dose is defined as a level that does not lead to joint effusion. We propose to test 3 different levels including the two used previously (0.7 and $1.4 \times 10^{13}$ viral particles/ml) and a lower dose ($0.35 \times 10^{13}$ viral particles/ml). A set of controls will be injected with vehicle only.

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

We propose that work on Major Task 2 be delayed pending a pilot experiment to establish the maximum tolerated dose of AAV.

Changes that had a significant impact on expenditures

If the maximum tolerated dose experiments are to be performed, this would require the addition of 12 rabbits to the protocol and project budget.

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

Significant changes in use or care of vertebrate animals.

Nothing to report

Significant changes in use of biohazards and/or select agents

Nothing to report

6. PRODUCTS:

Publications, conference papers, and presentations.
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**

Nothing to report

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### 7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

**What individuals have worked on the project?**

<table>
<thead>
<tr>
<th>Name</th>
<th>Project Role</th>
<th>Researcher Identifier</th>
<th>Nearest person month worked</th>
<th>Contribution to Project</th>
</tr>
</thead>
<tbody>
<tr>
<td>James A. Martin, PhD (NO CHANGE)</td>
<td>Principal Investigator</td>
<td>NA</td>
<td>1.2 person month (Calendar)</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. He has analyzed histological/ immunohistological staining.</td>
</tr>
<tr>
<td>Douglas R. Pedersen, Ph.D. (NO CHANGE)</td>
<td>Co-Investigator</td>
<td>NA</td>
<td>1.44 person months (Calendar)</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>
</tr>
</tbody>
</table>
Name: Marc Brouillette, Ph.D.
Project Role: Post-doctoral Research Fellow
Researcher Identifier NA
Nearest person month worked: 6.5 person months (Calendar)
Contribution to Project: 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 now included 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).

Name: Gail L. Kurriger, B.S. (NO CHANGE)
Project Role: Research Associate
Researcher Identifier NA
Nearest person month worked: 1.2 person months (Calendar)
Contribution to Project: Ms. Kurriger performed all rabbit joint dissections thus far and has performed all histological processing and sectioning and safranin-O staining and immunohistochemistry for GFP and Lubricin.

Name: Douglas C. Fredericks, B.S. (NO CHANGE)
Project Role: Co-Investigator (Research Specialist – Animal Surgeon)
Researcher Identifier NA
Nearest person month worked: 1.2 person months (Calendar)
Contribution to Project: Mr. Fredericks is one of the 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 rabbit surgeries and joint injections.

Name: Cheng Zhou, M.S.
Project Role: Graduate Research Assistant (started Oct 2015)
Researcher Identifier NA
Nearest person month worked: 1.4 person months (Calendar)
Contribution to Project: In October 2015, Cheng Zhou replaced Hyeong Choe and took over his duties. He will take over responsibility of immunoblot analyses and perform image analysis to quantify synovial inflammation and immunostains.

Name: Emily B. Petersen, D.V.M.
Project Role: Veterinarian
Researcher Identifier NA
Nearest person month worked: 1.2 person months (Calendar) – no salary support from the present grant
Contribution to Project: 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.

Funding Support: Dr. Petersen is funded through the Bone Healing Research Lab’s Animal Research Surgicenter.
Name: Keli McLaughlin  
Project Role: Veterinary Technician  
Researcher Identifier: NA  
Nearest person month worked: 1.2 person months (Calendar) – no salary support from the present grant  
Contribution to Project: Ms. McLaughlin is one of three veterinary technicians, and helped support the rabbit surgeries and aided with animal welfare checks and data entry.  
Funding Support: Ms. McLaughlin is funded through the Bone Healing Research Lab’s Animal Research Surgicenter.

Name: Amanda Wiebold  
Project Role: Veterinary Technician  
Researcher Identifier: NA  
Nearest person month worked: 1.2 person months (Calendar) – no salary support from the present grant  
Contribution to Project: Ms. Wiebold is one of three veterinary technicians, and helped support the rabbit surgeries and aided with animal welfare checks and data entry.  
Funding Support: Ms. Wiebold is funded through the Bone Healing Research Lab’s Animal Research Surgicenter.

Name: Amie Pluskowski  
Project Role: Veterinary Technician  
Researcher Identifier: NA  
Nearest person month worked: 1.2 person months (Calendar) – no salary support from the present grant  
Contribution to Project: Ms. Pluskowski is one of three veterinary technicians, and helped support the rabbit surgeries and aided with animal welfare checks and data entry.  
Funding Support: Ms. Pluskowski is funded through the Bone Healing Research Lab’s Animal Research Surgicenter.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Newly Funded Projects during Year 2, not previously listed (8/15/15 – 8/14/16):

Extended Date from what was reported on Year 1 report: **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)

Fully negotiated during Year 2: **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
James A. Martin, PhD (PI), 1.2 Calendar months
Douglas C. Fredericks, BS, 0.6 Calendar months
Emily B. Petersen, DVM, 0.6 Calendar months

Previously listed: **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

Extended Date from what was reported on Year 1 report: **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 – 12/01/2016, $235,019 Total Direct Costs (includes non-competitive renewal)
Douglas C. Fredericks, BS (PI), 1.2 Calendar months

Projects that closed during Year 2 that were not previously reported as having closed (8/15/15 – 8/14/16):

**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 – 06/01/2016, $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)

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

**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 – 12/22/15, $8,922.50 Total Direct Costs (SOW 7)
10/22/15 – 12/22/15, $8,922.50 Total Direct Costs (SOW 8)
Douglas C. Fredericks, BS (PI), 0.12 Calendar months
**What other organizations were involved as partners?**

Nothing to Report

**8. SPECIAL REPORTING REQUIREMENTS**

Please see page 13 for the Quad Chart for Year 2.

**9. APPENDICES:**

Nothing to Report
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

Major Task 1: Twenty six rabbits underwent surgery (sham or ACLT) and treatment (AAV-GFP or AAV-GFPLub) (Table), bringing the total number for Subtask 2 to 40. The animals were euthanized and drawer tests were performed (Subtask 3). Joint tissues were processed for histology. Safranin-O-stained sections were scanned and images prepared for automated Mankin scoring. Additional sections were stained with antibodies to GFP or lubricin and lubricin staining was quantified (Subtask 5).

Table: Animal procedures performed in Year 2. Surgeries and treatments were performed on 26 rabbits to complete Major Task 1 (n = 40 total). Survival times were as indicated.

<table>
<thead>
<tr>
<th>Rabbit</th>
<th>Surgery + Treatment</th>
<th>Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>5665</td>
<td>Sham + AAV-GFPLub</td>
<td>8 weeks</td>
</tr>
<tr>
<td>5666</td>
<td>Sham + AAV-GFP</td>
<td>8 weeks</td>
</tr>
<tr>
<td>5667</td>
<td>ACLT + AAV-GFP</td>
<td>8 weeks</td>
</tr>
<tr>
<td>5668</td>
<td>ACLT + AAV-GFP</td>
<td>8 weeks</td>
</tr>
<tr>
<td>5669</td>
<td>Sham + AAV-GFPLub</td>
<td>8 weeks</td>
</tr>
<tr>
<td>5670</td>
<td>Sham + AAV-GFP</td>
<td>8 weeks</td>
</tr>
<tr>
<td>5671</td>
<td>ACLT + AAV-GFPLub</td>
<td>8 weeks</td>
</tr>
<tr>
<td>5672</td>
<td>Sham + AAV-GFP</td>
<td>8 weeks</td>
</tr>
<tr>
<td>5673</td>
<td>ACLT + AAV-GFP</td>
<td>4 weeks</td>
</tr>
<tr>
<td>5674</td>
<td>ACLT + AAV-GFP</td>
<td>4 weeks</td>
</tr>
<tr>
<td>5675</td>
<td>ACLT + AAV-GFPLub</td>
<td>4 weeks</td>
</tr>
<tr>
<td>5676</td>
<td>ACLT + AAV-GFP</td>
<td>4 weeks</td>
</tr>
<tr>
<td>5677</td>
<td>ACLT + AAV-GFPLub</td>
<td>4 weeks</td>
</tr>
</tbody>
</table>

Estimatem: Live animal work and histologic processing for Major Task 1 were completed.

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 8 weeks post ACLT
- Complete ACLT or sham surgeries, apply gene therapy after 4 weeks, euthanize rabbits at 8 weeks

Comments/Challenges/Issues/Concerns

Y2 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 Y3 on animals and their surgical costs.

Budget Expenditure to Date: Projected Expenditure: Y2 $242,310 total. Actual Expenditure: Y2 8/15/15 – 8/14/16, $220,899 (includes addition of Brouillette, Zhou, and Choe salary/fringe, financials done retroactively during Y2. Y3 financials will include some small retroactive salary adjustment due to fiscal year increases that were mistakenly left off).

Updated: Y2Q3 (12-12-16) purple indicates current location in time with respect to each aim’s timeline