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TITLE: Development of a Novel AAV Vector Capsid Optimized for OA Gene Therapy

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CONTRACTING ORGANIZATION: University of Florida, Gainesville, FL

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14. ABSTRACT

Osteoarthritis (OA) is a chronic, degenerative, often crippling disease that primarily affects large weight-bearing joints. OA is most commonly linked to wear and tear of the joints from old age. In younger people, though, it arises from a significant joint injury, one that damages the bones and cartilage (post-traumatic osteoarthritis; PTOA). Indeed, OA one of the primary causes of disability among active duty soldiers & veterans. Despite the constant claims in the media, there is no drug or dietary supplement capable of slowing joint destruction in OA and although some drugs can ease OA joint pain, they can't stop the erosive biological processes.

We have been working to develop a gene-based therapy for OA. Using a harmless virus, we can deliver the genes for these naturally-occurring anti-arthritic proteins to the cells and tissues in diseased joints. Following a single injection, the diseased joints continually make their own medicine for well over a year.

This experimental treatment has worked exceptionally well, first in small animals and then in the joints of horses similar in size to human knees that are also highly prone to OA. Following detailed toxicology studies this treatment was approved by the FDA for safety testing in humans with knee OA. In the current project we will use a highly advanced, state-of-the-art system developed by a member of our research team, to develop a new, custom-made AAV vector, optimized specifically for use in gene therapy for human OA. This system has been used to generate vectors with dramatically enhanced potency in treatments for blindness, cancer and liver disease. Based the successes in these applications, we expect to generate a delivery system for human OA 10-100x more effective than our current version. This new vector will be invisible to childhood antibodies, easier to manufacture and most importantly will be safe for use in humans with joint disease. The reagents developed in this project will help to advance OA gene therapy from the proof-of-concept stage to a therapy that is commercially viable. One attractive for development by the pharmaceutical industry that will ultimately result in an improved treatments for service members and civilians with OA.

15. SUBJECT TERMS
None listed

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INTRODUCTION:

In the current project we will use a highly advanced, state-of-the-art system developed by a member of our research team, to develop a new, custom-made AAV vector, optimized specifically for use in gene therapy for human OA. This system has been used to generate vectors with dramatically enhanced potency in treatments for blindness, cancer and liver disease. Based the successes in these applications, we expect to generate a delivery system for human OA 10-100x more effective than our current version. This new vector will be invisible to childhood antibodies, easier to manufacture and most importantly will be safe for use in humans with joint disease. The reagents developed in this project will help to advance OA gene therapy from the proof-of-concept stage to a therapy that is commercially viable. One attractive for development by the pharmaceutical industry that will ultimately result in an improved treatments for service members and civilians with OA.

1. KEYWORDS:

Osteoarthritis (OA)
Gene Therapy
Equine
Adeno-Associated Virus (AAV)
Interleukin-1 Receptor Antagonist (IL-1Ra)
Post-traumatic OA (PTOA)
Self-complimentary AAV (scAAV)
Cartilage
Synovium
Gene Transfer
Large animal model
Pharmacokinetics
Biodistribution
Toxicology
Directed Evolution (DE)
AAV Capsid Variant
AAV Combinatorial Capsid Library

2. ACCOMPLISHMENTS:

What were the major goals of the project?

Specific Aim 1: Using directed evolution screening of highly complex AAV capsid libraries, identify novel variants with enhanced tropism for human articular tissues in OA joints.	**Timeline**	
Major Task 1: Submit documentation and obtain required approvals; generate reagent libraries for use in Aims 1 and 2.	Months	
Subtask 1: Submit documents for ACURO approval	1-3	Dr. Ghivizzani
Subtask 2: Submit documents for HRPO approval	1-3	Dr. Ghivizzani
Milestone #1 Obtain ACURO and HRPO Approvals	2-4	Dr. Ghivizzani
Subtask 3: Expand combinatorial libraries for selection protocols in explants and equine joints	1-4	Dr. Zolotukhin
Subtask 4: Generate vector barcode library with IL-1Ra/GFP reporter	1-6	Dr. Palmer
Major Task 2: Perform Directed Evolution screening in equine OA joints		
Subtask 1: Acquire animals; register with ACS and complete health examinations	4-14	Dr. Biedrzycki
Subtask 2: Deliver combinatorial libraries in OA forelimb joints in equine model; euthanize animals and harvest synovium and cartilage	4-14	Dr. Biedrzycki
Subtask 3: Isolate synovial fibroblasts and chondrocytes; seed into culture; amplify AAV genomes; isolate episomal DNA.	4-14	Dr. Ghivizzani
Subtask 4: PCR variant capsid coding sequences; clone sub-library into AAV genome	4-14	Dr. Zolotukhin
Subtask 5: Perform Next- Generation Sequencing of variant sub-library; analyze sequence data using bioinformatics	4-14	Dr. Zolotukhin
Subtask 6: Package variant sub-library into virions, purify and characterize. (repeat Subtasks 2-6 as necessary)	4-14	Dr. Zolotukhin
Major Task 3: Perform Directed Evolution screening in equine joints with naturally occurring disease		
Subtask 1: Acquire OA tissues, process, seed into culture; infect with combinatorial library	4-14	Dr. Ghivizzani
Subtask 2: Isolate synovial fibroblasts and chondrocytes; seed into culture; amplify AAV genomes; isolate episomal DNA.	4-14	Dr. Ghivizzani
Subtask 4: PCR variant capsid coding sequences; clone sub-library into	4-14	Dr. Zolotukhin

AAV genome		
Subtask 5: Perform Next- Generation Sequencing of variant sub-library; analyze sequence data using bioinformatics	4-14	Dr. Zolotukhin
Subtask 6: Package variant sub-library into virions, purify and characterize. (repeat Subtasks 2-6 as necessary)	4-14	Dr. Zolotukhin
Major Task 4: Perform Directed Evolution screening in Humanized equine OA joints		
Subtask 1: Modify synovial fibroblasts and chondrocytes in explant tissues to express GFP transgene, isolate cells, prepare for delivery	9-18	Dr. Ghivizzani
Subtask 2: Implant GFP+ articular cells to OA equine forelimb joint; Inject combinatorial libraries; euthanize animal and harvest synovium and cartilage	9-18	Dr. Biedrzycki
Subtask 3: Isolate synovial fibroblasts and chondrocytes; seed into culture; amplify AAV genomes; isolate episomal DNA.	9-18	Dr. Ghivizzani
Subtask 4: PCR variant capsid coding sequences; clone sub-library into AAV genome	9-18	Dr. Zolotukhin
Subtask 5: Perform Next- Generation Sequencing of variant sub-library; analyze sequence data using bioinformatics	9-18	Dr. Zolotukhin
Subtask 6: Package variant sub-library into virions, purify and characterize. (repeat Subtasks 2-6 as necessary)	9-18	Dr. Zolotukhin
Milestone #2: Identify up to 40 variants as candidates for characterization as capsid vectors	17-18	Dr. Zolotukhin Dr. Ghivizzani
Specific Aim 2: Characterize candidate AAV capsid variants for efficiency of packaging, transgene delivery & expression and evasion of neutralizing antibody in articular explants and in vivo.		
Major Task 5: Functional Assessment of Candidate Capsids in Tissue Explants		
Subtask 1:Clone variant capsids into AAV helper plasmid, pair with barcoded reporter vector and package into AAV vectors, characterize titer and packaging efficiency	17-20	Dr. Zolotukhin
Subtask 2: Characterize each vector preparation for efficiency of gene transfer in synovial and cartilage explants	18-21	Dr. Ghivizzani
Subtask 3:Characterize candidate vectors for immune escape	18-21	Dr. Ghivizzani
Milestone #3: Identify up to 9 variants as candidates for characterization in vivo in large animal model	24	Dr. Zolotukhin Dr. Ghivizzani

Major Task 6: Functional Assessment of Candidate Capsids In Vivo in Large Animal Model.		
Subtask 1 Generate large-scale preparations of up to 9 candidate vectors + AAV2.5 as vector control; Characterize titer, packaging efficiency	24-28	Dr. Zolotukhin Dr. Ghivizzani
Subtask 2: Acquire animals; register with ACS and complete health	24-28	Dr. Biedrzycki
examinations		
Subtask 3: Inject vector preparations in equine joints; collect blood & urine; aspirate synovial fluids over 10 weeks. Euthanize animals, collect heart liver lung spleen samples, harvest synovium & cartilage,	25-30	Dr. Biedrzycki
Subtask 3: Measure IL-1Ra content in biological fluids, Analyze joint tissues for GFP expression, PCR analysis of tissue samples for AAV barcoded genomes.	28-30	Dr. Ghivizzani
Milestone #3: Identify 2 Optimized Capsids for Biodistribution and Toxicology Testing	30	Dr. Ghivizzani Dr. Zolotukhin
Specific Aim 3: Establish formal toxicology and biodistribution profiles for intra-articular IL-Ra gene transfer using optimized AAV capsids.		
Major Task 7: Biodistribution Studies in Large Animal Model		
Subtask 1 Generate large-scale preparations of 2 optimized vectors containing cDNA for human IL-1Ra for use in safety tests	30-32	Dr. Zolotukhin Dr. Ghivizzani
Subtask 2: Acquire animals; register with ACS and complete health examinations	29-30	Dr. Biedrzycki
Subtask 3: Deliver each optimized vector containing human IL-1Ra cassette into OA forelimb joint of 3 animals. Collect blood, urine, aspirate synovial fluids. Euthanize, necropsy and procure tissues for analysis.	30-34	Dr. Biedrzycki Ms. Erger-Coleman
Subtask 4: Analyze equine tissues for AAV genomes by qPCR, measure IL-1Ra content in blood and urine, measure AAV capsid antibody	32-36	Ms. Erger-Coleman
Major Task 8: Biodistribution and Toxicology Studies in Rodent Model		
Subtask 1: Acquire rats, inject mono-iodoacetate (MIA) to induce OA model and inject test AAV capsid vector in stifle joint, collect blood, monitor weight.	30-31	Ms. Erger-Coleman
Subtask 2: Euthanize animals, collect blood and 26 tissues for analysis.	31-34	Ms. Erger-Coleman
Subtask 3: Perform pathology and biodistribution analyses of collected tissues: Submit blood for clinical blood count and serum chemistries and tissue samples for pathologic analyses. Isolate genomic DNA from tissue samples and perform qPCR for vector genomes; perform enzyme linked immunosorbent assay for adeno-associated virus antibody	32-36	Dr. Biedrzycki Ms. Erger-Coleman

Milestone #4 Deliver final report on the biosafety of local gene delivery via 2 synthetic AAV capsids optimized for the treatment of osteoarthritis.	36	Ms. Erger-Coleman

What was accomplished under these goals?

What was accomplished under these goals?

a) Major Activity 1: Submit documentation and obtain required approvals; generate reagent libraries for use in Aims 1 and 2.
Specific Objective: 1) Obtain IACUC and ACURO approvals. 2) Obtain UF IRB and HRPO approvals. 3) Expand Combinatorial libraries for selection protocols in explants and equine joints. 4) Generate vector barcode library with IL-1Ra/GFP reporter.
Results: We have successfully obtained UF IACUC and DoD ACURO approval for all animal work. Despite submission in April of all requested documents including IRB approval letter, full IRB protocol and Informed Consent forms we still have not received approval from HRPO for the use of discarded human articular tissues in explant culture.

We have generated the IL-1Ra/GFP reporter cassette and 40/40 barcoded constructs have been generated. In addition, a novel, fluorescently tagged capsid library was generated to facilitate identification of regions in the equine joint transduced by variant capsids against the large volume background tissue. In this library, the *rep* gene on the AAV genome has been replaced by the coding sequence for mCherry, such that cells *functionally* transduced by a variant capsid will express the reporter gene and fluoresce RED. Prior to use in the joints of horses, we have recently initiated pilot studies in rats to assess the utility of the reporter in the context of the joint tissues. If reporter activity is readily visible in both cartilage and synovium, it would be extremely valuable to the directed evolution screening as we could locate within the huge volume of capsular and cartilaginous tissues, the specific locations of cells infected and transduced by variant capsids. Regions of tissue containing hotspots of fluorescence can be micro-dissected, and following enzymatic digestion of the ECM, the RED cells can be isolated by FACS. This will result in a pure population of cells containing AAV genomes with coding sequences for variant capsids which can be readily determined by NextGen sequencing.

b) Major activity 2: Perform Directed Evolution screening in equine OA joints with naturally occurring disease.
Specific Objective: 1: Acquire animals; register with ACS and complete health examinations. 2: Deliver combinatorial libraries in OA forelimb joints in equine model; euthanize animals and harvest synovium and cartilage 3: Isolate synovial fibroblasts and chondrocytes; seed into culture; amplify AAV genomes; isolate episomal DNA. 4: PCR variant capsid coding sequences; clone sub-library into AAV genome. 5: Perform Next- Generation Sequencing of variant sub-library; analyze sequence data using bioinformatics. 6: Package variant sub-library into virions, purify and characterize. (repeat Subtasks 2-6 as necessary)
Results: Due to concerns over the COVID-19 pandemic, research work at UF involving animals was halted in for several months in 2020. Following approval for re-engagement of research activities, two suitable horses were located from local donors and are now available for use in these studies. We have spent of this past reporting period focusing our available vector barcoded libraries on our screening of human explants and performing DE screening in these explants (see major activity 3). However, a significant amount of time was spent during this last year making more of our vector library stocks to inject into horses. Since horse joints are large, and an equivalent size to the human knee, a large amount of viral stocks are needed (excess of 10^12 viral genomes). We now have enough to inject into our two animals and have coordinated with our veterinarian, Adam Biedrzycki, to inject these animals the week of 9/20/2021. We have much experience with both the animal work and the DE screening and expect to get great data from this major activity.

c) **Major activity 3:** Perform Directed Evolution screening in human explant tissues collected from OA joints during total knee replacement surgeries.
Specific Objective: 1: Acquire OA tissues, process, seed into culture; infect with combinatorial library. 2: Isolate synovial fibroblasts and chondrocytes; seed into culture; amplify AAV genomes; isolate episomal DNA. 3: PCR variant capsid coding sequences; clone sub-library into AAV genome. 4: Perform Next- Generation Sequencing of variant sub-library; analyze sequence data using bioinformatics. 5: Package variant sub-library into virions, purify and characterize. (Repeat Subtasks 2-5 as necessary)
Results: Ongoing

This major activity is nearly completed. Over the last study period we were able to get ~10 human resected knee samples to be used in a DE screening. Using directed evolution screening of a highly complex AAV capsid library we are identifying novel variants with enhanced tropism for human articular tissues in OA joints. Starting from a library of >10^9 functional AAV capsid variants, a multi-armed Directed Evolution screening approach in human explants was used to isolate novel capsids optimized for enhanced gene delivery to the OA joint. Next Generation Sequencing and bioinformatics was used to monitor the diversity of library after each round of screening and aid in the selection of candidates for functional testing. So far, we have begun screening on 8 tissues.

We are screening the variant libraries for AAV capsids capable of highly efficient transduction of the cells in both cartilage and synovial tissues Therapeutic gene delivery in the context of human OA is distinct from other gene therapy applications; the anatomy and tissues, their volume, vasculature, ECM and cell populations are highly specialized for skeletal support and joint articulation. More importantly the articular pathologies associated with OA (cartilage erosion, osteophytes, subchondral bone sclerosis, synovitis, joint effusion) dramatically alter the landscape and can have a profound impact on gene delivery and transgene expression patterns. Upon intra-articular injection, the fluid vehicle and rAAV vector particles are deposited into the viscous synovial fluid following joint articulation are physically mixed and dispersed in the joint space to engage: i) the synovium, a thin, villous, highly cellular, tissue that lines the internal surfaces of the fibrous capsule, and ii) the articular cartilage, composed of dense ECM of collagen II and hydrated proteoglycans, and populated by chondrocytes at low density. We've found that both cell types (chondrocytes and synovial fibroblasts) are receptive to rAAV transduction in vivo, and are similarly capable of maintaining the episomal AAV genome and providing sustained expression of an IL-1Ra transgene. Although the loss of articular cartilage matrix is its characteristic pathology, OA is a disease of the entire joint with significant signaling cross-talk between the cartilage and synovium. In this respect, the goal of gene-based therapy for OA is to raise the steady state IL-1Ra content in the synovial fluid for distribution throughout the joint to inhibit IL-1 signaling in all articular tissues. As small proteins synthesized within the joint experience rapid turn- over, elevating the steady state IL-1Ra content in the synovial fluid of a joint proportional to the human knee requires a substantial population of genetically modified cells. In this regard, the efficacy of treatment is tied directly to the efficiency of gene transfer mediated by the vector. As we have no basis to exclude cells from either synovium or cartilage as targets for gene delivery, we are screening the variant libraries for AAV capsids capable of highly efficient transduction of the cells in both tissues.

Starting from three highly complex combinatorial capsid libraries constructed from the capsid coding sequences for AAV2, AAV3 and AAV5, we have performed sequential rounds of selection in human synovium and human cartilage in explant tissues to isolate AAV capsids optimized for enhanced gene delivery to human synovial fibroblasts and chondrocytes in the context of the OA joint. To identify capsid variants that recognize surface receptors preferentially expressed by human articular cells, we are performing a screen in cartilage and synovial tissue explants discarded from total knee replacement surgeries. Unlike monolayer, in vitro culture of chondrocytes and synovial fibroblasts in their native ECM in a 3 dimensional context preserves their natural repertoire of surface antigens. NGS and bioinformatics analysis are being used to monitor the diversity of library after each round, and the selection pressures driving its evolution.

DoD sample preparation protocols
Protocol to isolate cells from human cartilage and synovium resected knee:

Day 0:
1. Rinse tissue sample with ~200 mL of PBS
2. Shave cartilage using scalpel, then mince by scissors; cut synovium on 5mm size parts by scissors
3. Add AAV library ~5E+11 per cartilage pieces or synovium, incubate 24h

Day 1:
1. Aspirate medium, rinse twice with 1xPBS
2. Add 10 ml of 1X Collagenase/Hyaluronidase (Stemcell, Catalog # 07912) in Dulbecco's Modified Eagle's Medium (DMEM) per cartilage or synovium sample
3. Incubate for 4-6 hours
4. Inactivate digestions by 20 ml of complete medium

Day 3:
1. Replace medium by fresh complete medium; change medium each 3-4 days

Day 20:
1. Split cells from 6 wells plate (cartilage) or 10cm plate (synovium) into T75 flask.

Day 27:
1. Infect cells by wild type Ad5, 100 ul per 80% confluent T75 flask.

Day 30-31:
1. Collect almost completely lysed by Ad5 cells, spin down cells/debris.
2. Add to supernatant PEG/NaCl for AAV precipitation.
3. Spin down medium/PEG/NaCl after 1h/4C. Resuspend in AAV lysis buffer, combine with cell precipitate resuspended in AAV lysis buffer.

Day 32-33:
1. Isolate AAV using standard iodixanol protocol (Zolotukhin et al.)

Day 34-35:
1. Titer AAV into iodixanol by qPCR.
2. Combine iodixanol from different patient samples
3. Filter iodixanol through 0.2um filter.
4. Sample is ready for the next Directed evolution cycle.

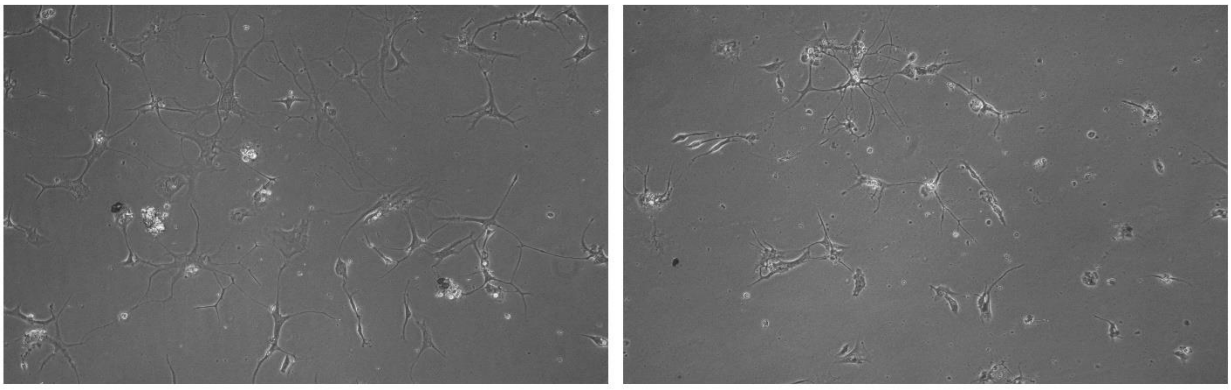

Figure 1: Representative Day 21 images of chondrocytes isolated from osteoarthritic human resected knee tissues. Explants of tissues were infected with appropriate library and tissues were harvested and cultured prior to identification of capsid variants.

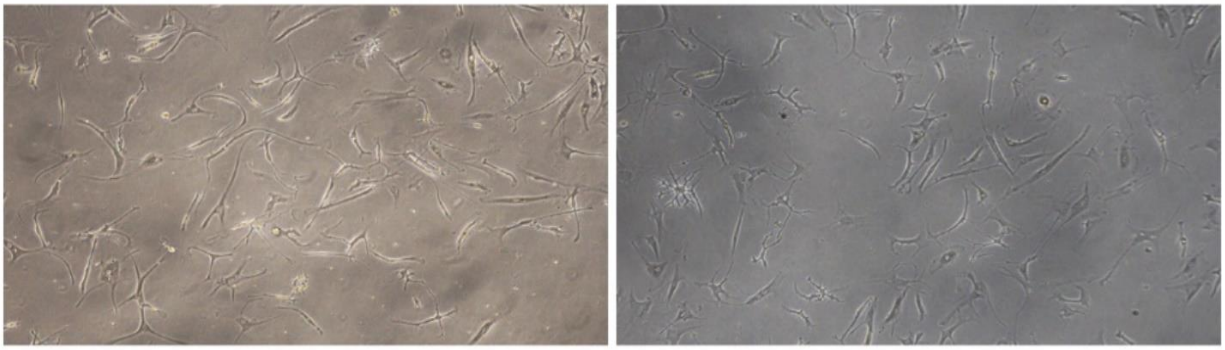

Figure 2: Representative Day 21 images of synovial cells isolated from osteoarthritic human resected knee tissues. Explants of tissues were infected with appropriate library and tissues were harvested and cultured prior to identification of capsid variants

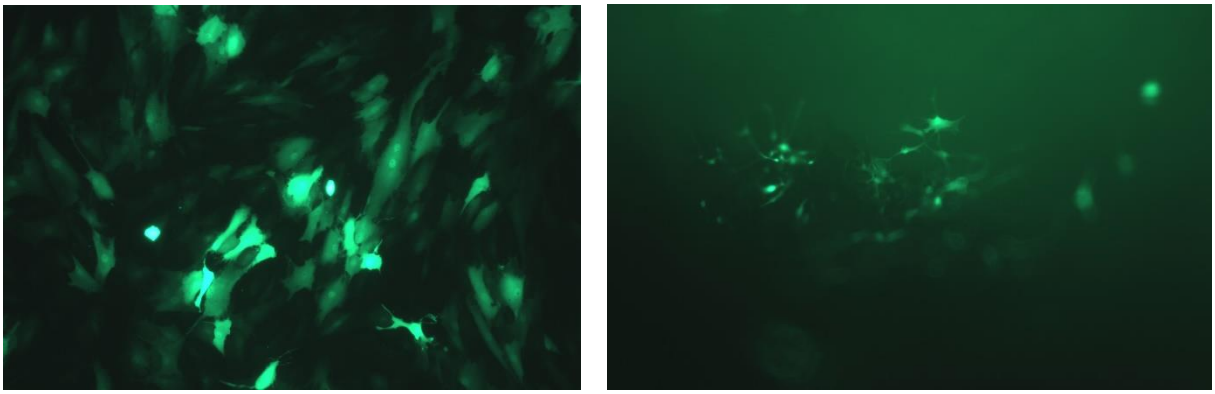

Figure 3: Different magnifications of ~day 30 culture chondrocytes post transduction with Ad5-GFP.

d) **Major activity 4:** Perform Directed Evolution screening in Humanized equine OA joints
Specific Objective: 1: Modify synovial fibroblasts and chondrocytes in explant tissues to express GFP transgene, isolate cells, and prepare for delivery. 2: Implant GFP+ articular cells to OA equine forelimb joint; Inject combinatorial libraries; euthanize animal and harvest synovium and cartilage. 3: Isolate synovial fibroblasts and chondrocytes; seed into culture; amplify AAV genomes; isolate episomal DNA. 4: PCR variant capsid coding sequences; clone sub-library into AAV genome. 5: Perform Next-Generation Sequencing of variant sub-library; analyze sequence data using bioinformatics. 6: Package variant sub-library into virions, purify and characterize. (Repeat Subtasks 2-6 as necessary). 7: Identify up to 40 variants as candidates for characterization as capsid vectors
Results: Not yet started

e) **Major activity 5:** Functional Assessment of Candidate Capsids in Tissue Explants
Specific Objective: 1: Clone variant capsids into AAV helper plasmid, pair with barcoded reporter vector and package into AAV vectors, characterize titer and packaging efficiency. 2: Characterize each vector preparation for efficiency of gene transfer in synovial and cartilage explants. 3: Characterize candidate vectors for immune escape. 4: Identify up to 9 variants as candidates for characterization in vivo in large animal model.
Results: Not yet started

f) **Major activity 6:** Functional Assessment of Candidate Capsids In Vivo in Large Animal Model.

Specific Objective: 1: Generate large-scale preparations of up to 9 candidate vectors + AAV2.5 as vector control; Characterize titer, packaging efficiency. 2: Acquire animals; register with ACS and complete health. 3: Inject vector preparations in equine joints; collect blood & urine; aspirate synovial fluids over 10 weeks. Euthanize animals, collect heart liver lung spleen samples, harvest synovium & cartilage. 4. Identify 2 Optimized Capsids for Biodistribution and Toxicology Testing.
Results: Not yet started

g) Major activity 7: Biodistribution Studies in Large Animal Model
Specific Objective: 1. Generate large-scale preparations of 2 optimized vectors containing cDNA for human IL-1Ra for use in safety tests. 2: Acquire animals; register with ACS and complete health examinations. 3: Deliver each optimized vector containing human IL-1Ra cassette into OA forelimb joint of 3 animals. Collect blood, urine, aspirate synovial fluids. Euthanize, necropsy and procure tissues for analysis. 4: Analyze equine tissues for AAV genomes by qPCR, measure IL-1Ra content in blood and urine, measure AAV capsid antibody.
Results: Not yet started

h) Major activity 8: Biodistribution and Toxicology Studies in Rodent Model
Specific Objective: 1: Acquire rats, inject mono-iodoacetate (MIA) to induce OA model and inject test AAV capsid vector in stifle joint, collect blood, monitor weight. 2: Euthanize animals, collect blood and 26 tissues for analysis. 3: Perform pathology and biodistribution analyses of collected tissues: Submit blood for clinical blood count and serum chemistries and tissue samples for pathologic analyses. Isolate genomic DNA from tissue samples and perform qPCR for vector genomes; perform enzyme linked immunosorbent assay for adeno-associated virus antibody. 4: Deliver final report on the biosafety of local gene delivery via 2 synthetic AAV capsids optimized for the treatment of osteoarthritis.
Results: Not yet started

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

Nothing to Report

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?

During the current reporting period we made a good deal of progress performing our directed evolution (DE) screening in 8 human resected knee samples. Our next step is to complete the Directed Evolution screening and perform the next generation sequencing and bioinformatic analysis and identify the variants receptive to both tissues. Additionally, to simulate the scale and treatment conditions of the OA knee we will perform one arm of the DE screen in equine forelimb joints with naturally- occurring OA. In this context we will select for capsids with the ability to negotiate the disease environment, (i.e. avoid the physical and biological barriers inherent to the large OA joint), penetrate the local ECM and diffuse through the respective tissue matrices of the synovium and cartilage to

infect the resident cells. We have two horses in house for these experiments and they are scheduled to be injected the week of 9/20/2021. Upon completion of the screening in humans, and horses we can begin our humanized screen, which will aid in the interpretation of the enrichment data from the human explants, and better inform the selection of variant capsid and motifs for testing in Aim 2. The "Humanized" equine OA model combines the critical enrichment/selection features of the equine OA model (A: Environment: appropriate scale and disease context) with those of the human explants Arm 2 (B: Targeting: interaction with human surface receptors). In this hybrid model, the chondrocytes and synovial fibroblasts in human explant tissues will be genetically modified to express GFP, which will serve as a cytological marker. After enzymatic digestion of the tissues, the recovered cells are injected into an equine forelimb joint with naturally-occurring OA, where they adhere and engraft in the local tissues. Equal amounts of virus from the enriched libraries from the human explants and equine screen will then be injected into the joint. After sufficient time for viral attachment and entry into the target cells, the animal is euthanized. GFP+ (human) and GFP- (equine) cells within the tissues will be isolated, seeded into culture and the viral genomes within each population will be amplified, and the capsid genes cloned and sequenced. This model system will be used to the select for/identify the variant capsids from the respective screens that are most efficient for gene transfer to human articular cells in the context of a large OA joint.

During the upcoming reporting period we will also make headway into our second Aim of the study and begin our characterization of candidate AAV capsid variants for their efficiency of packaging, transgene delivery and expressiona dn evasion of neutralizing antibodies in articular explant and in vivo. From Aim 1 we expect to identify as many as 40 variant capsids as candidates for testing as vector capsids for therapeutic gene delivery in OA. The candidates will be comprised of full-length capsid sequences enriched during the screening protocols and engineered sequences that combine recurrent AA substitutions and/or variant motifs enriched among otherwise diverse capsids. The coding sequence of each candidate will be cloned into a helper plasmid and used to package a barcoded AAV vector genome with an expression cassette containing a CMV enhancer/promoter linked to tandem reporter genes for IL-1Ra and GFP. IL-1Ra is a secreted protein, whose levels in conditioned medium or synovial fluid are readily measured by commercially available ELISA and provides a quantifiable index of the total therapeutic transgene expression in a culture sample, or intra-articularly. GFP fluorescence permits visual assessment of the transduced cell population (phenotype(s), density and distribution) via fluorescence microscopy and/or quantitative analysis by flow cytometry. To identify the vector genomes packaged by the different capsids, each candidate will be paired with an AAV reporter vector containing a different 6 bp barcode identifier which can be distinguished by PCR using targeted primer pairs. Following testing in human articular explants, and then in vivo following intra-articular delivery into equine joints, the complementary readouts from the IL-1Ra and GFP reporters will be compiled to generate a comprehensive profile of each capsid's capacity to serve as a vector for AAV gene transfer to articular tissues. For each of the assays in this Aim, AAV2.5 capsid will be used as a positive control and a reference standard for assessment of the novel vectors.

4. IMPACT:

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?

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Nothing to Report

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to Report

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

We had numerous delays with this study due to the COVID-19 pandemic. First, the entire university was shut down for months and we were not allowed access to our laboratories. We had issues obtaining and housing animals (all resolved) as the animal facilities also completely shut down due to the pandemic. Acquiring human samples as the hospital shut-down elective procedures for a time was also an issue. Currently here in Florida, we are yet again facing the possibility of not being able to get human tissues in the face of the Delta surge as the hospitals are overloaded and there are no available beds. Beyond this, many of our reagents and supplies have been backordered as stocks are low or non-existent and they have been slow to come in as well. However, we are continuing with the study as planned and making due with what we have available.

Lastly, Dr. Andrew Smith, a co-I and the veterinarian on the study has left the University of Florida. We have found his replacement and are working with our contact at the DoD to replace him on the study. Dr. Adam Biedrycki is also an orthopaedic surgeon and an Associate Professor in Large Animal Sciences here at the University of Florida. His transition into the project will be seamless. He has experience performing all of the procedures on this study and is very eager to begin.

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

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

Nothing to Report

6. PRODUCTS:

- **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**

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Steven Ghivizzani
Project Role:	PI
Researcher Identifier:	ORCID ID: 0000-0001-5154-7581
Nearest Person Month Worked:	0.5
Contribution to Project:	Dr. Ghivizzani is responsible for the overall planning and coordination of all aspects of the equine research study. Together with Dr. Zolotukhin, he will be responsible for the overall planning, coordination and execution of all aspects of the project. He will contribute to the AAV library screening in vivo and explant and will be primarily be responsible for the functional testing in Aim 2. He will coordinate the equine studies with Dr. Smith and the Toxicology studies with Ms. Erger-Coleman. He will assist in the collection and analysis of tissues and fluids, contribute to evaluation of all diagnostic measures and interpretation of the data. He will assume primary responsibility for progress reports and publication.

Name:	Rachael Watson Levings (Name change from Rachael Watson)
Project Role:	Senior Scientist
Researcher Identifier:	ORCID ID: 0000-0003-0913-290X
Nearest Person Month Worked:	5
Contribution to Project:	Dr. Watson is trained in molecular and cellular biological techniques as well as gene transfer and its application to musculoskeletal diseases. She has worked extensively in the equine model system and will be the technical point person involved in candidate testing. She will coordinate day to day work between VetMed, orthopaedic surgery and Dr. Zolotukhin's group. She will assist with acquisition, culture and infections of the explant tissues, and will assist with library screening in the equine model. She will be primarily responsible for the in vitro testing and analysis of the data from the in vivo studies. She will assist with equine injections and the collection of biological fluids and tissues and will perform biological assays for IL-1Ra, and immune escape from AAV neutralizing antibody.

Name: Glyn Palmer
Project Role: Co-Investigator
Researcher Identifier: ORCID ID:
Nearest Person Month Worked: 0.1
Contribution to Project: Dr. Palmer is an Assistant Professor in the Department of Orthopaedics & Rehabilitation in the College of Medicine at the University of Florida. His areas of expertise lie in molecular & cellular biology, connective tissue biology & repair, gene transfer technologies and transcriptional reporter systems to track the cellular responses in vitro and in vivo. Dr. Palmer also has significant experience with recombinant vector systems. He will assist with the generation of the lentiviral vector, AAV barcoded reporter constructs, candidate vector packaging and testing, data analyses and manuscript preparation.

Name: E. Anthony Dacanay
Project Role: Technician
Researcher Identifier: ORCID ID: 0000-0002-0050-7106
Nearest Person Month Worked: 0.7
Contribution to Project: Mr. Dacanay is a Biological Scientist in the Orthopaedic Gene Therapy Laboratory. He will work under the direction of Dr. Ghivizzani. He is trained in molecular and cellular biology including DNA isolation and purification from tissue samples. He is also trained in the production and application of AAV vectors for use in gene therapy. Mr. Dacanay will contribute to all phases of the project. He will help prepare the viral vectors for injection into the animals, assist with the collection and analysis of blood urine and synovial fluid samples, and will assist with their distribution among the research team. He will also perform biological assays, including ELISA for IL-1Ra, and PCR for vector genome quantification. Mr. Dacanay is responsible for preparing DNA for viral production. He also helps prepare vectors for injection and assists with the collection and storage of animal fluids.

Name: Rachel Leitz
Project Role: Clinical Research Coordinator
Researcher Identifier: ORCID ID:
Nearest Person Month Worked: 0.1
Contribution to Project: Ms. Leitz is a Clinical Research Coordinator in the Department of Orthopaedics & Rehabilitation of the College of Medicine at the University of Florida. She has worked in the Adult Arthroplasty and Joint Reconstruction clinical service for the past 5 years. Ms. Leitz will serve as the liaison between the OR and members of Dr. Ghivizzani's lab with regard to tissue acquisition. Ms. Leitz will coordinate scheduling and obtain informed consent from each patient donor prior to surgery.

Name: Sergei Zolotukhin
Project Role: Co-Principal Investigator
Researcher Identifier: ORCID ID: 0000-0002-4877-3745
Nearest Person Month Worked: 2.4
Contribution to Project: Dr. Zolotukhin is a Professor in the Division of Cellular and Molecular Therapy, in the Department of Pediatrics at the University Of Florida College Of Medicine. He is an expert in the biology, design and construction of AAV vectors, as well as their application in gene-based therapies. He will serve as a coPrincipal Investigator (Co-PI) on the

project. He will collaborate with Dr. Ghivizzanni on scientific design, coordination of experiments, analyzing data, and manuscript preparation. Additionally, Dr. Zolotukhin will be responsible for all AAV combinatorial library production and screening and supervising Drs. Kondratov and a post-doctoral associate who will be hired once funding is in place. Dr. Zolotukhin will devote 2.4 calendar months effort per year to the project.

Name: Oleksandr Kondratov
Project Role: Post-doctoral Associate
Researcher Identifier: ORCID ID: 0000-0002-6390-7158
Nearest Person Month Worked: 6
Contribution to Project: Dr. Kondratov is in the Division of Cellular and Molecular Therapy, Department of Pediatrics. He has worked with Dr. Zolotukhin since 2016 and is an accomplished molecular biologist. He has extensive experience working with combinatorial AAV capsid libraries, and production of rAAV vectors. His main asset to this project are his unique skills as a software engineer, bioinformatics analyst, and biostatistician. Not only will he assist in the wet lab with AAV library production and directed evolution at every step of the process, but he'll also conduct the bioinformatics analysis in real time as the capsid screening/selection progresses in cycles.

Name: Anatolii Mamchur
Project Role: Post-doctoral Associate
Researcher Identifier: ORCID ID: 0000-0001-5569-7579

Nearest Person Month Worked: 6 months
Contribution to Project: Dr. Mamchur, PhD, a recently hired Postdoctorate Associate, has a 5 years postdoctoral experience working in leading laboratories in Sweden, and Israel. His main responsibility is developing approaches of culturing mesenchymal stem cells, tissue explants, directed evolution (DE) in chondrocytes, amplifying combinatorial capsid libraries, and isolating viral DNA after DE.

Name: Liudmyla Kondratova
Project Role: Technician
Researcher Identifier: ORCID ID:
Nearest Person Month Worked: 8
Contribution to Project: Ms. Kondratova is in the Division of Cellular and MolecularTherapy, Department of Pediatrics. Ms. Kondratova has worked with Dr. Zolotukhin since 2016 and is skilled in molecular biology has extensive experience with rAAV vectors. She will provide technical support in rAAV vector production and rAAV purification.

Name: Adam Biedrzycki
Project Role: Co-Investigator
Researcher Identifier: ORCID ID:
Nearest Person Month Worked: 5
Contribution to Project: Dr. Biedrzycki is an Assistant Professor in the University of Florida, College of Veterinary Medicine. He is the director of the surgical

translation and 3D printing research lab and an EBVS European specialist in equine surgery. He has taken over the research responsibilities of Andrew Smith, who has left the University of Florida. He will perform all injections and fluid aspirations and contribute to all phases of animal care, including animal acquisition. He will also assist with euthanization and necropsies and tissue dissection.

Name:	TBD
Project Role:	Animal Technician
Researcher Identifier:	ORCID ID: 0000-0003-1296-4192
Nearest Person Month Worked:	5
Contribution to Project:	

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

Nothing to Report

What other organizations were involved as partners?

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating PI and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to **https://ers.amedd.army.mil** for each unique award.

QUAD CHARTS: No changes. See Attached PDF.

9. APPENDICES: Nothing to Report