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

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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 existing vectors. 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. If successful, the reagents developed in this project will help advance OA gene therapy from "experimental" to commercially viable, raising its potential for clinical development by the pharmaceutical industry and ultimately lead to improved OA treatment for service members and civilians alike.

15. SUBJECT TERMS

NONE LISTED

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER**	**19a. NAME OF RESPONSIBLE PERSON** USAMRDC
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INTRODUCTION: Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

In the current project we will use a highly advanced, state-of-the-art system for directed evolution (DE) screening to identify and validate novel AAV vector capsids optimized specifically for therapeutic gene delivery in human OA. This system, which employs highly-complex libraries of combinatorial capsid variants derived from AAV2, AAV3 and AAV5 has been used to generate novel AAV vectors with dramatically enhanced potency in treatments for blindness, cancer and liver disease. Based on the successes in these applications, we expect to generate a gene delivery vehicle(s) for human OA 10-100x more effective than existing AAV vector systems. We expect to identify a panel of vector capsids that will: i) transduce cells in human OA joints with maximum efficiency, ii) package and propagate with high yield, and iii) evade pre-existing neutralizing antibodies from natural infection and prior gene therapeutics. Optimizing the AAV capsid will lower treatment dose to both improve safety and reduce vector production costs. In these respects we expect this project will provide enabling vector technology that will advance OA gene therapy to commercial viability and clinical development by the pharmaceutical industry, ultimately resulting in an improved treatment modality for the broad population of service members and civilians affected by OA.

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

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?

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: UF IACUC and DoD ACURO approval was obtained for all animal work. UF IRB and HRPO approval was obtained for the use of discarded human articular tissues in explant culture.

Due to the complexity of the project, and the diverse procedures and biologic agents required, including human tissues, (and their xenogenic transplantation), multiple viral vector systems, large and small animals and disease models, the volume of documentation, subsequent rounds of review, revision and re-review by the State of Florida, the DEA, as well as the UF EH&S and Biosafety Committees, the IRB and IACUC followed by ACURO and HARPO, equired considerably longer than anticipated and delayed work by several months.

We have generated a panel of barcoded scAAV vector reporters for use in functional testing of select capsid variants enriched during DE selection. The scAAV vector construct was engineered to contain an IL-1Ra/GFP dual reporter cassette in which the cDNAs for IL-1Ra and GFP are linked in-frame by a 2A self-cleaving peptide sequence, enabling stoichiometric co-expression of both gene products in transduced cells. To accomodate the increased length of the bivalent coding sequence in the "half-size" scAAV genome, the redundant bovine growth hormone Poly A sequence was deleted from the 3' end of the expression casette.

To generate a panel of barcoded reporter vectors a series of 20 oligonucleotide cassettes engineered with unique 6 nt barcode sequences were inserted individually between the terminal stop codon of the GFP coding sequence and the SV40 poly A signal of the scAAV reporter. To identify the vector genomes packaged by individual variants, each variant of interest will be paired with a scAAV reporter vector containing a unique barcode identifier which can be distinguished by targeted sequencing reactions or PCR using appropriate primer pairs.

To evaluate selected capsid variants for vector function the corresponding coding sequence for each will be inserted into a Rep2-Cap AAV helper plasmid and used to package a linked barcoded scAAV reporter vector for characterization of intra-articular gene transfer and expression. Following delivery in vivo, quantification of IL-1Ra levels in synovial fluids or conditioned medium, will provide an index of therapeutic protein expression cumulatively in the joint tissues. Coordinate analysis of GFP fluorescence among the cells resident in the articular tissues will reveal the nature of the cell population(s) transduced by each variant; the number, locations, phenotypes, density and tissue distribution following intra-articular delivery. The combined readouts will provide a comprehensive profile of intra-articular gene transfer and expression mediated by each variant, including preferential targeting of cell types or tissues, and the proclivity of individual variants to migrate from the joint to engage cells at extra-articular locations.

The goal of this project is to identify a novel AAV capsid(s) optimized for therapeutic gene delivery in large human joints, (knees, hips) commonly affected by OA. From prior experience with DE in vivo. screening should be performed under selection conditions that duplicate the intended use of the optimized capsid to the greatest extent possible. As our goal is to develop a vector for intra-articular delivery in large human joints with OA, one arm of the DE screening process will be performed in the forelimb joints of horses,which are similar in size, function and tissue composition to the human knee, and likewise are highly prone to OA onset secondary to trauma or excessive loading. While the equine model closely

mimics the scale and intra-articular environment of the human OA knee, it also presents unique technical and logistical challenges.

To our knowledge DE screening has never been attempted in an animal model of comparable scale. In order to place appropriate selective pressure, to isolate variants with the greatest "fitness" the target cells must be infected at low copy number. In order to sufficiently screen or "oversample" a highly complex library, requires the processing and analysis of large volumes of tissue. In an effort to streamline the DE process, while increasing the likelihood of a successful screen we first explored a novel screening approach with the potential to dramatically accelerate the selection process and increase the likelihood of a successful screen.

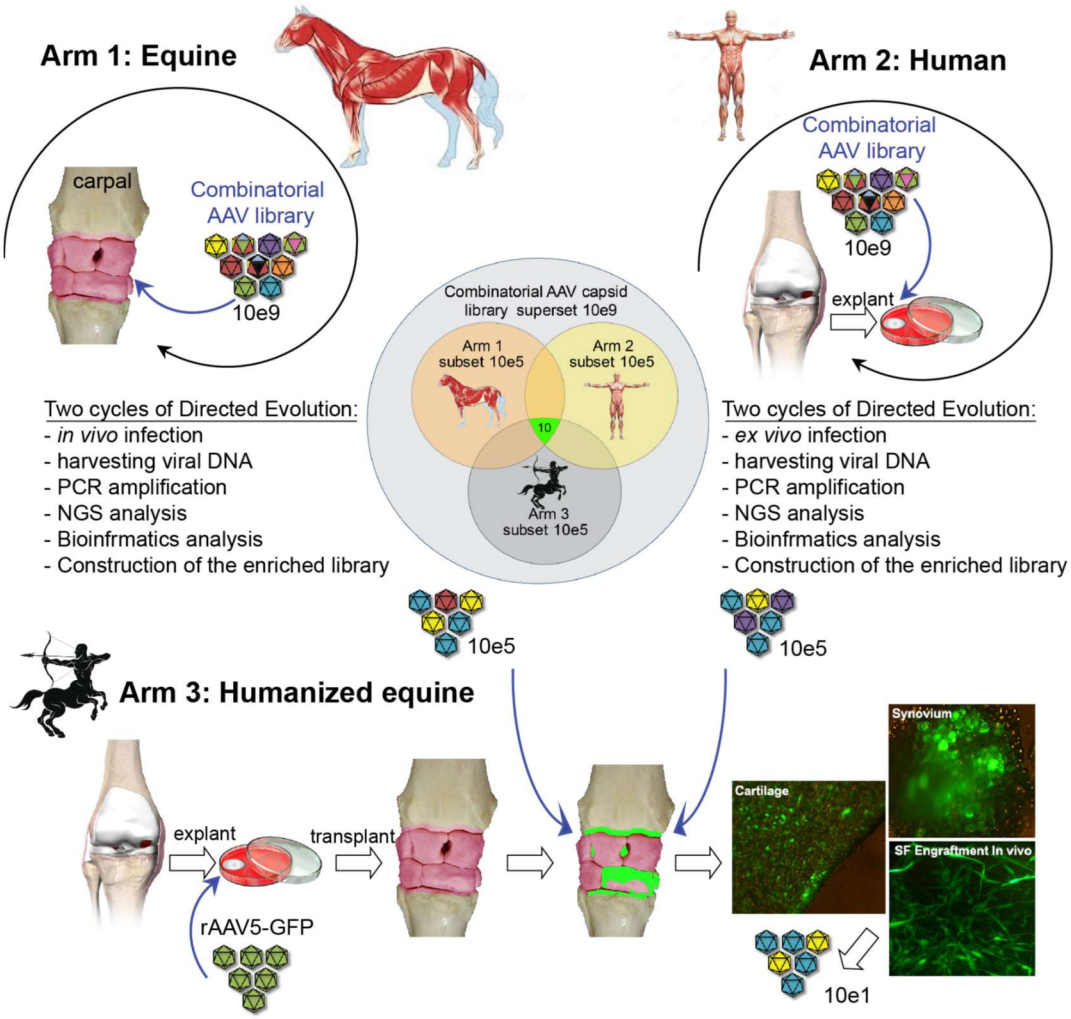
To facilitate the recovery of a rare population of functional genomes from the equine articular tissues, a novel fluorescently tagged capsid library was generated in which the *rep* gene of the AAV2 background genome was replaced by the coding sequence for mCherry. In this manner cells infected and functionally transduced by a variant capsid would express the reporter and fluoresce RED, pinpointing their exact locations within the large expanse of synovium or deep within the ECM of the articular cartilage. Regions of tissue enriched for fluorescent cells can be micro-dissected, the cells recovered and the fluorecent subpopulation isolated by FACS. resulting in selective recovery of vector genomes encoding functional capsids, whose sequences could be readily determined by NextGen sequencing.

To examine the utility of the reporter in the context of the joint tissues, we peformed a series of pilot studies to assess reporter activity and sensitivity of detection first in monolayer culture, and then in the joints of rats in vivo. Despite ready detection of the mCherry reporter in synovial fibroblasts in monolayer culture, fluorescent activity from the rep promoter was too low to reliably discern against the background fluorescence of the ECM of the synovium and cartilage. Unfortunately the initial promise of the approach did not hold up in the context of the joint tissues.

Following this disappointing outcome, we initiated large scale production of the original AAV2, AAV3 and AAV5 libraries to generate quantities needed for the equine and explant screens. To permit amplification of selected capsid variants, the AAV genome and its encoded capsid protein must be physically coupled, i.e. the capsid must be linked to the genome it carries. To prevent decoupling, transfection of packaging cells must occur at low DNA copy (i.e. < one plasmid per cell), requiring large volumes of cells (~500, 150mm plates) to produce $>10^{13}$ vg per library.

b) Major activity 2: Perform Directed Evolution screening in equine OA joints with naturally occurring disease.

Specific Objective: 1: Acquire animals as needed; register each with ACS and coordinate health examinations. 2: Deliver combinatorial libraries to OA forelimb joints in equine model; euthanize animals and harvest synovium and cartilage 3: Isolate synovial fibroblasts and chondrocytes; seed into culture; infect with Ad5 virus to replicate/amplify AAV vector genome copy number;. 4: Isolate genomic DNAfor PCR amplication of variant capsid coding sequences; clone variant 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 AAV virions for subsequent rounds of screening as needed. (repeat Subtasks 2-6 as indicated by results)



Results: ongoing (one cycle completed). '
The COVID-19 pandemic imposed a prolonged delay in research progress work at UF involving animals was halted for the majority of 2020. Following approval for re-engagement of research activities, 2 suitable horses obtained from local facilities. Once production and characterization of the large-scale preps of the libraries was completed, we were able to initiate the DE screen in the equine model.

A horse with moderate, naturally-occurring OA in both MCP joints was selected for the initial screen. In our earlier work we've found that AAV vector doses in the range of 10^{12} -10^{13} genome copies provide robust expression of the IL-1Ra transgene. To provide selective pressure, while at the same time providing a sufficient number of virions to enable genome recovery, approximately 10^{10} genome copies from each of the three libraries were combined and delivered by intra-articular injection into each MCP joint. Three weeks later, allowing time for unbound virus to be cleared from the joint tissues, the animal was euthanized and large samples of synovium and cartilage were collected separately from all regions of both joints. The tissues were divided and ~half of each sample stored at -80°C. The remaining portions were enzymatically digested and the recovered cells (synovial fibroblasts and chondrocytes) seeded at high density into several multi-well dishes. After 48 hours, the cultures were infected at low MOI with Ad5 to amplify the AAV vector genomes. Following appearance of broad cytopathic effects, the viral genomes were purfied by Hirt extraction and the capsid coding sequences amplified by PCR for insertion into the Rep2-cap- vector plasmid. Preliminary PCR analysis indicates that genome recovery and amplification were both successful.

Using barcoded primer specific to each tissue type, round of selection and species the amplified capsid sequences equine joint tissues will be prepared individually then combined with those from the human explants for Next Generation Sequencing (NGS) in the same run.

Analysis of the AA sequences of the variants enriched from capsid from this round of selection will be compared with major activity 3 (human explant) to reveal the extent to which capsids are enriched and shared vs unique and inform decisions regarding the need for subsequent rounds of selection.

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 synovium and cartilage from endstage OA joints, remove debris and cut intact portions into ~3 mm dia pieces, seed into culture; infect with pooled capsid libraries. 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

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. More importantly the articular pathologies associated with OA (cartilage erosion, osteophytes, synovitis, effusion) meaningfully alter the landscape and physiology of the resident cells, all of which can have a profound impact on therapeutic gene delivery and expression.

We've found that both of the major cell types within the joint (chondrocytes and synovial fibroblasts) are receptive to AAV transduction in vivo, and are similarly capable of long-term maintanence and expression AAV vector genomes. 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 a 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 the size of 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, identification of variants with enhanced tropism for either or both tissues are of high interest.

Over the prior study period multiple samples of synovium and cartilage were obtained from clinical patients undergoing total knee arthroplasty. Following collection from the OR the tissue samples of both synovium and cartilage were then cut into multiple small pieces and placed into multi-well dishes for culture as tissue explants. As primary cells cultured in monolayer rapidly de-differentiate, by maintaining each cell type in its native ECM and 3 dimensional context, the natural repertoire of surface antigens is better preserved.

Following infection with an equal mixture of genome copies of each library, the explants were washed and incubated for 7 days with daily change of medium. The explants of each type were pooled, enzymatically digested and the recovered cells were seeded into multi-well plates at high density. Following infection with Ad5 to amplify the AAV genomes, the viral DNAs were purified by Hirt extraction and the capsid coding sequences were amplified by PCR. Preliminary analysis however, showed an exceptionally high number of genome copies in the respective samples, inidcative of insufficient selection pressure from excessive viral dose. In the subsequent subsequent screening round the same procedures were used by the dose of virus was reduced by 50-fold. In this case however, following AAV amplication with Ad5, the replicated AAV virions were precipitated from the culture medium and cell lysates, then purified over iodixanol gradient. The recovered variants were then used to infect synovial and cartilage explants from new donors. PCR analysis of the genomes from this second round indicates strong recovery in 5 of 6 samples.

As mentioned above using barcoded primers specific to each tissue type, round of selection and species, the amplified capsid sequences from equine screen will be prepared individually then combined with those from both rounds of screening in the human explants for Next Generation Sequencing (NGS) in the same run.

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

1

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 coordinate health exams. 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 progress with directed evolution (DE) screening. Using multiple human samples we've completed two rounds of selection in explants and one round in the equine joint. The goal for this work is to 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. Analysis of the impending NGS data will aid in the interpretation of the enrichment and inform the need for additional rounds in either context. If the results are inconsistent between the equine and human explant screens, the "Humanized" equine OA model will combine the critical enrichment/selection features of the equine OA joints to clarify the subgroup of variants for functional testing. During the upcoming reporting period we will finish these final rounds of selection and begin the second Aim of the study to characterize candidate AAV variants for their efficiency of packaging, transgene delivery and expression and evasion of neutralizing antibodies in articular explant and in vivo.

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

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

We have no plans to modify or change the experimental approach. The overall strategy is technically sound, some refinement of specific methodologies will occur as indicated by experimental results, but the overall research plan will remain the same.

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

Over the last few years, a series of unforeseen circumstances beyond our control has arisen to delay research progress significantly. The COVID-19 outbreak caused a huge setback- the university shut down, the loss of research animals and housing, the halt of elective surgical procedures at UF Health. Beyond these, lingering supply-chain issues, limited availability of reagents and extended backorders have led to additional delays. Thankfully over the last year or so, the majority of these logistical impediments have resolved and no longer impose a significant hardship.

Further, as Dr. Zolotukhin and his research group are from Ukraine, the Russian invasion in early 2022, served to impose an additional set of delays, as various members returned home to aid family members. Dr. Mamchur a post-doc in the group even left to enlist in the Ukrainian military.

At this point, as it appears that the worst of these global disasters is behind us, the plan going forward is to re-double our efforts and push the project as far as we can in the time allowed. In this regard we will petition to extend the closing date for additional year to provide the time needed to successfully complete the work and identify a vector that we can move into clinical testing.

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

No significant changes.

Significant changes in use or care of vertebrate animals.

No significant changes.

Significant changes in use of biohazards and/or select agents

No significant changes.

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**

Although we anticipate filing an invention disclosure(s) describing novel AAV capsid variants with enhanced tropism for articular tissues, we are unable to at this time.

- **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
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?

No additional partners.

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