AWARD NUMBER: W81XWH-19-1-0515

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

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REPORT DATE: September 2023

TYPE OF REPORT: Annual

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

REPORT DOCUMENTATION PAGE		*Form Approved OMB No. 0704-0188*
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1. REPORT DATE September 2023	**2. REPORT TYPE** Annual	**3. DATES COVERED** 15Aug2022-14Aug2023
4. TITLE AND SUBTITLE Development of a Novel AAV Vector Capsid Optimized for OA Gene Therapy		**5a. CONTRACT NUMBER** W81XWH-19-1-0515 **5b. GRANT NUMBER** 12712000 / PR180272 **5c. PROGRAM ELEMENT NUMBER**
6. AUTHOR(S) Steven Ghivizzani E-Mail: ghivisc@ortho.ufl.edu		**5d. PROJECT NUMBER** 0011331694-0001 **5e. TASK NUMBER** **5f. WORK UNIT NUMBER**
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Florida 207 Grinter Hall Gainesville, FL 32611-0001		**8. PERFORMING ORGANIZATION REPORT NUMBER** W81XWH-19-1-0515 Year 4
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Development Command Fort Detrick, Maryland 21702-5012		**10. SPONSOR/MONITOR'S ACRONYM(S)** **11. SPONSOR/MONITOR'S REPORT NUMBER(S)**
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited		
13. SUPPLEMENTARY NOTES		

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 OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON** USAMRDC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**	Unclassified	14	**19b. TELEPHONE NUMBER** *(include area code)*
Unclassified	Unclassified	Unclassified			

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1. **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.

2. **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

3. 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		
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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
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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.

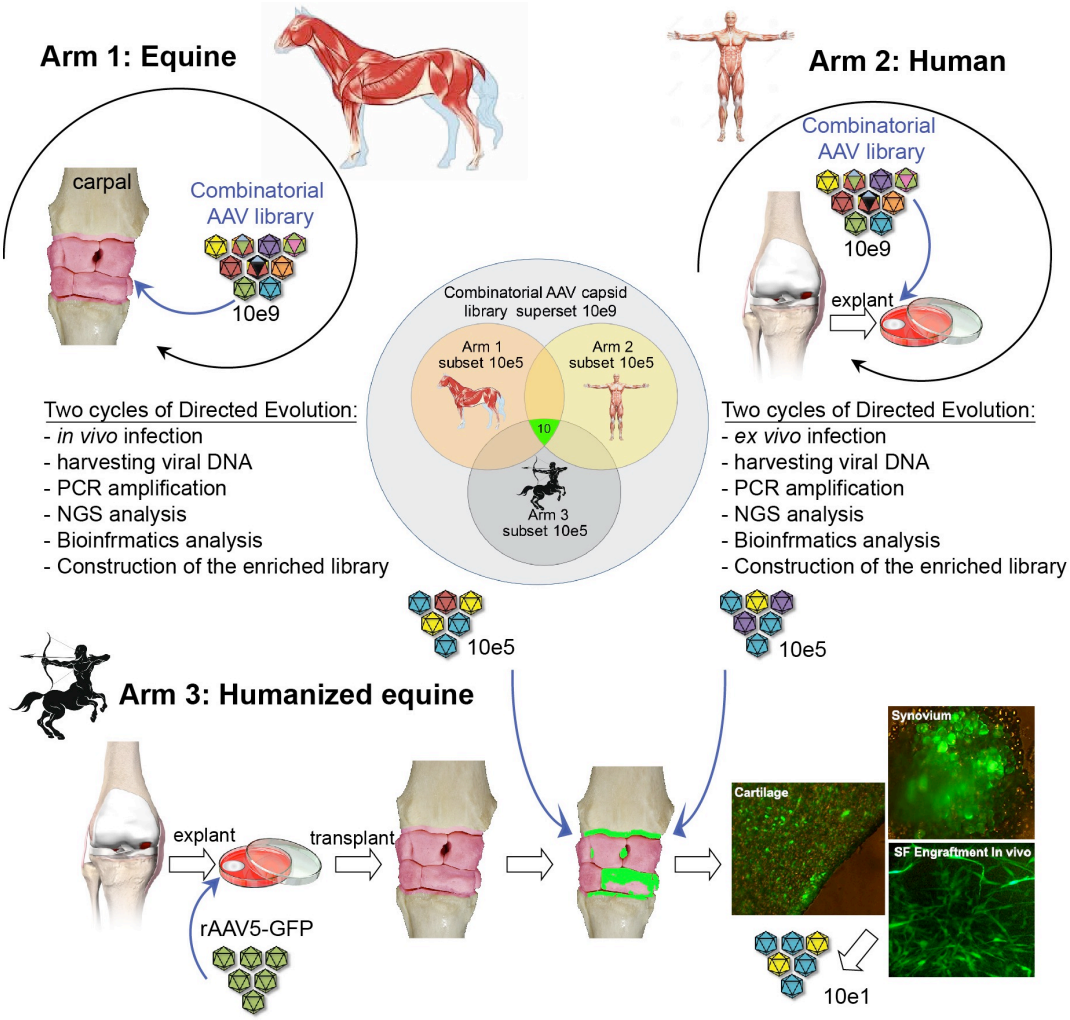
To our knowledge DE screening has never been attempted in an animal model of comparable scale. In order to place appropriate selective pressure, to isolate variants with the greatest "fitness" the target cells must be infected at low copy number. In order to sufficiently screen or "oversample" a highly 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 primers specific to each round of selection and species the amplified capsid sequences from the equine joint tissues were prepared then combined with those from the human explants for Next Generation Sequencing (NGS) in the same run.

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 is of high interest.

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 small pieces and placed into multi-well dishes for culture as tissue explants. As primary cells cultured in monolayer rapidly de-differentiate, by infecting each cell type with AAV in its native ECM and 3 dimensional context, the natural repertoire of surface antigens should be better preserved.

Following infection with equal genome copies of each mutant 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, indicative of insufficient selection pressure from excessive viral dose. In subsequent screening rounds, the same procedures were used but the dose of virus was reduced by 50-fold. 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. A portion of each AAV preparation was reserved for DNA isolation and analysis genomes from this second round indicates strong recovery in 5 of 6 samples.

As mentioned above using barcoded primers specific to each round of selection and species, the amplified capsid sequences from both rounds of the equine screen were prepared then combined with those from selection rounds 1 and 2 in the human explants for Next Generation Sequencing (NGS).

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

Complex libraries engineered with combinatorial mutations in variable regions (VRs) 2-9 of the capsid coding sequences of AAV2, AAV3 and AAV5 were screened in two iterative rounds in equine OA joints and in human articular tissues in explant culture, as previously described. Afterward, next generation sequencing (NGS) was performed on the AAV genomes recovered in each round using barcoded primers specific to each experimental condition and AAV genome. The sequence data from the AAV2 library (discussed in detail below) provided surprisingly consistent results in both the equine joints and human articular explants, identifying common VR-5 and VR-7 variant motifs. The data from the AAV3 and AAV5 libraries was considerably more heterogeneous in both contexts; additional rounds of screening will be required to sort these out.

Regarding the AAV2 library, **Figure 1** (below) shows a summary of the NGS data from ~469,000 genomes of the mutant AAV2 library recovered after two sequential rounds of screening/selection in equine forelimb joints with naturally-occurring OA. The numerical positions and amino acid (AA) sequence of the wild type (wt) AAV2 capsid residues engineered to contain combinatorial substitution mutations are shown across the top. The variant AA positions of Variable Regions (VRs) 4 - 8, where the vast majority of the mutations in round 2 occur, are delineated with boxes. Underneath the header, the AA sequences of the capsid VRs encoded by the 56 most prevalent AAV genomes recovered from the joint tissues are shown in descending order of copy number and frequency of occurrence (i.e., % of the genome pool); dots indicate identity with the wt sequence. Each AA sequence in the list (seq. #s 1-56) reflects a distinct genome with a frequency of at least 0.10%. The copy number of each genome (**#Genomes**) and its frequency following selection round 2 (**RND2%**) are shown in the first two colums to the right of the sequence data as indicated. The adjacent two columns show the round 1 frequency of each sequence (**RND1%**) and its relative enrichment in round 2, shown as the Enrichment Factor; EF: (**RND2%/RND1%) - 1**. Variants with an EF $\geq$ 0.50 are shown in bold, and those with an EF $\geq$ 3.00 are underlined. Variants whose round 1 frequency was not meaningfully enriched in round 2 (EF $\leq$ 0.10) are highlighted in gray; those whose frequency declined are shown as negative numbers in red font.

Despite the high complexity of the AAV2 capsid library (>10^7 functional variants), a striking feature of the NGS data in both rounds 1 and 2 was the absence of genomes with substitution mutations in VR-8 (outlined by the dashed box). This was not entirely surprising as VR-8 contains the principle recognition domain for the AAV2 binding receptor, heparin sulfate proteoglycan (HSPG). The lack of variants with mutations in this region denotes strong selective pressure to maintain the native HSPG binding sequence, and points to HSPG as a key surface receptor for AAV2 binding and infection of articular tissues both in vivo and in explant culture.

Another conspicuous feature of the round 2 NGS data is the relative lack of genomes encoding VR-4 and VR-6 mutations, particularly in light of the round 1 data where in the horse 13 of the 40 most prevalent genomes contained multiple mutations in VR-4 and/or VR-6 and together accounted for > 4.5% of the round 1 pool. As is evident in Figure 1, these variants were essentially eliminated in selection round 2, and the frequencies of two the most prevalent VR-4 and VR-6 mutants in round 1 (seq. #s 23 and 40) dropped by $\geq$ 80%. Similar to VR-8, the rapid and widespread loss of variants with VR-4 and VR-6 mutations in selection round 2, implies that the native AA sequences in these regions also confer a functional advantage to the AAV capsid. The source of this selective pressure, though, is uncertain and could lie anywhere in the screening process, from viral replication and packaging to cell surface attachment and/or intracellular trafficking.

Analysis of the individual AA sequences of the round 2 capsid variants showed that (as in round 1) the wild type AAV2 capsid (sequence #1) was again the most prevalent after round 2. Importantly however, there was no amplification or enrichment of the wtAAV2 genome in round 2 vs round 1, as its frequency remained static at ~7% (EF 0.02). The steady-state maintenance of the wtAAV2 genome at high copy number confirms that the wt capsid is an effective vector for intra-articular gene transfer. However, numerous variants within the pool were highly enriched in round 2, some with EFs as high as 11.0 and 15.0, whose tropism for the joint tissues may be substantially greater.

Following the wtAAV2 genome (seq. #1) in order of prevalence, the next three variants in the round 2 pool (seq. #s 2, 3 and 4) each contained multiple overlapping substitution mutations solely in VR-5. In contrast to the wtAAV2 genome, the prevalence of variant #s 2, 3 and 4 increased ~ 2 - 3 fold (EF 1.92, 1.04 and

1.63, respectively) in the round 2 pool, such that their cumulative frequency increased from 6.5% in round 1 to 15.4% in round 2. Alignment of each variant motif on the native VR-5 sequence (Figure 2) shows that all are closely related and share at least 3 of the following substitutions: S492D, A493G, D494E, Y500F and T503P. A quick survey of the variant sequences in Figure 1, shows that the variant VR-5 motifs in seq. #s 2, 3 and 4, and their closely related derivatives, are highly recurrent in the round 2 genome pool, and appear in 38 of the following 52 variant genomes.

The next 4 most prevalent variants (seq. #s 5 - 8) each contained 6-8 substitution mutations specifically in VR-7. Although the majority of the mutations in each variant occured at common residue positions (548-552 and 556) the AA substitutions were considerably more heterogeneous than the preceding VR-5 variants (seq. #s 2 - 4). Despite their relative prevalence in the round 2 genome pool (cumulatively ~3.8%), the respective frequencies of VR-7 variants #s 5 - 8 either remained static or showed a modest decline relative to round 1 (EF $\leq$ 0.10); none was meaningfully enriched. The ability of these specific VR-7 variants to persist within the pool at relatively high copy number suggests that each is functionally equivalent to the wt VR-7 domain and the wtAAV2 capsid broadly, and superior to the diverse populations of variants eliminated from the round 2 pool. The majority of the remaining VR-7 variants contained one of five single point mutations: N551K, I554S, S547A, Q545K, and Q545E, and though less prevalent than #s 5-8, followed the same pattern of enrichment.

The remaining variants in Figure 1 were primarily composed of one of the 3 dominant VR-5 variants or a close derivative, either: i) alone or ii) paired with with one of the prevalent VR-7 variants (shown in descending order of enrichment in Figure 3). All capsids containing variant VR-5 domains were enriched in round 2. The VR-7 variants, however, were only enriched when paired with a *variant* VR-5, but not the wt sequence. While the mutant VR-5 motifs appear to provide the AAV capsid with a marked selective advantage, it's unclear whether a paired variant VR-7 provides additional function or is simply a neutral passenger, but it doesn't appear to be inhibitory. Though not a factor in either selection paradigm, it's possible that the number and breadth of the substitutions in the more prevalent VR-7 variants could provide a variant VR-5 capsid with increased resistance (stealth) to pre-existing neutralizing antibodies from prior wt AAV2 or AAV2.5 infection.

Though the genome containing the mutant VR-7 motif in seq. #35 (Figures 1 & 2) declined in frequency in round 2 of selection in the equine joint, in the human explants it was the most prevalent of the VR-7 variants and thus may provide an advantage for infection of human articular tissues. In this respect the VR-7 motif in seq. #35 bears testing for its utility as a potential species-selective element.

Finally, variant sequence #11 which depicts a single Y444F substitution just upstream of VR-4, is an additional mutation of particular interest. The substitution of a phenylalanine (F) for the tyrosine (Y) at position 444 has been shown in a variety of cell types and tissues to inhibit phosphorylation of the AAV capsid by intracellular tyrosine kinases and thereby reduce subsequent ubiquitination following cellular entry, leading to increased capsid stability and transduction efficiency. The same effect has been associated with the Y500F substitution present in VR-5 variants #s 2-4. While the presence of the Y444F mutation alone did not enhance the round 2 frequency of the wt sequence in variant #11 (EF -0.21), when paired with the dominant VR-5 variants, the round 2 EF of each increased substantially (from 1.92 to 2.80 [seq #26], 1.04 to 2.29 [seq #19] and 1.23 to 1.75 [seq #50]). However, when paired with any VR-4, VR-6 or VR-7 variant, which alone do not provide a selective advantage in vivo (EF $\leq$ 0.10), addition of the Y444F didn't increase their prevalence in round 2 vs round 1 (e.g. the frequency of seq. #23 dropped by 80%).

The variant sequences shown in Figure 1 and their apparent impact on capsid prevalence (both positive and negative) with iterative rounds of selection in vivo and in explant culture, provide a roadmap for modification of the AAV2/AAV2.5 capsids to enhance their utility for therapeutic gene delivery intra-articularly. A list of candidate variants whose coding sequences have been submitted for DNA synthesis is shown in Figure 4. Upon return each will be inserted into the pACG2 packaging plasmid and using the bar-coded reporter vectors generated previously, each will be tested for packaging and transduction efficiency relative to AAV2 and AAV2.5, as well as resistance to capsid-targeted neutralizing antibodies.