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CONTRACTING ORGANIZATION: Massachusetts General Hospital

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14. ABSTRACT

Scientific objective: Our goal is to test a novel method of gene therapy that uses vesicles or "bubble-like" structures to carry the therapeutic gene and protect it from damage by the recipient's immune system.
Rationale: One form of gene therapy involves the use of a tool known as an adeno-associated viral (AAV) vector to deliver or carry the therapeutic gene to the muscle fibers. Sometimes a person's immune system can make gene therapy difficult by attacking the AAV vector, thereby reducing the delivery of the AAV vector. A method that enhances delivery of the AAV vector to muscle tissue and that is resistant to the immune system is needed.
Topic Area: Myotonic dystrophy; **Area of Encouragement**: Development and/or testing of novel and/or innovative treatments, including those utilizing gene editing or silencing.

15. SUBJECT TERMS
Myotonic dystrophy; gene therapy; AAV vectors; extracellular vesicles; *in vivo* imaging; CRISPR; gene editing.

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1. Introduction: Our long-term goal is to develop targeted molecular therapies for myotonic dystrophy. Recently, a new technology has become available that enables corrective changes in the DNA of genes that can have therapeutic benefit. One of these approaches is called CRISPR/Cas9 and has shown promise for treatment of DM1 using cells in a dish and a mouse model. To make this therapeutic approach viable for DM1 patients will require use of a gene therapy tool known as an adeno-associated viral (AAV) vector to deliver, or carry, the CRISPR/Cas9 gene to muscle fibers. Sometimes a person's immune system can make gene therapy difficult by attacking the AAV vector, thereby reducing the delivery of the therapeutic gene. This project will use a novel method to increase the efficiency of AAV gene therapy in muscle tissue of DM1 mice by the use of small vesicles or "bubble-like" structures that contain the AAV and serve to protect the AAV and its cargo from the immune system. Development of AAV-containing vesicles that carry CRISPR/Cas9 for the treatment of DM1 will be a new venture.

2. Key words: Myotonic dystrophy; gene therapy; AAV vectors; extracellular vesicles; *in vivo* imaging; CRISPR; gene editing.

3. Accomplishments:

Major goal (task) 1: Generate plasmid DNA constructs for production of AAV vectors.

Major activities: We generated a plasmid DNA construct containing the reporter gene luciferase, the muscle specific promoter/enhancer element CK8 (Bengtsson, et al., 2017), and inverted terminal repeat (ITR) elements that are required for production of AAV vectors (Figure 1). Restriction enzyme digests of purified plasmid DNA confirmed that the luciferase gene, CK8 promoter, and ITR regions were intact (Figure 1).

Significant results or key outcomes:

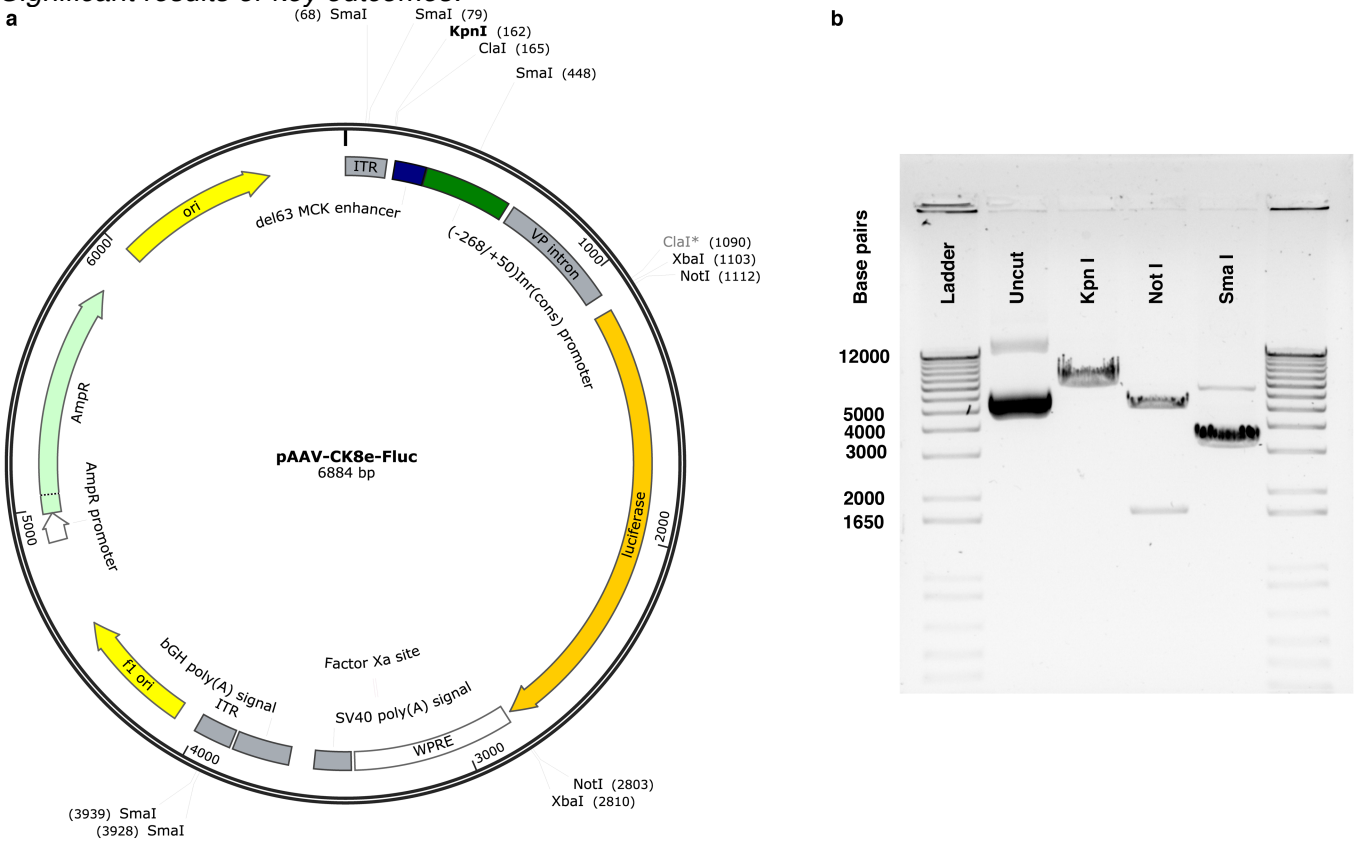

Figure 1. Muscle-specific luciferase plasmid DNA. a) Plasmid map showing the location of the luciferase gene, upstream CK8 promoter (del63 MCK enhancer/-268/+50InrCons promoter), and ITR elements required for AAV vector production. b) Gel electrophoresis of uncut plasmid DNA and plasmid DNA treated with restriction enzymes Kpn I, Not I, and Sma I produced band patterns of expected number and size.

Major goal (task) 2: Produce standard and exo-AAV vectors and test vector activity in DM1 mice.

Major activities: We prepared plasmid DNA that the Massachusetts General Hospital (MGH) Vector Core then used to produce standard AAV vectors. Using the tissue culture medium collected from AAV producer cells immediately prior to cell lysis, we isolated exo-AAV vectors by serial centrifugation. The MGH Vector Core measured concentration of the standard AAV and exo-AAV vectors using qPCR. The initial vector preparations were complicated by cell detachment, resulting in low yields. After switching to lower passage AAV producer cells, detachment was reduced and yields improved to some extent.

We obtained local IACUC approval before the funding period began and ACURO approval during Month 2. Based on the vector quantification values, we tested standard AAV and exo-AAV vectors for gene expression in muscle tissue of DM1 mice after intramuscular or intravenous (tail vein) injection.

Significant results or key outcomes:

We used Vector Core titers to administer a target dose of 1 x 10^{11} genome copies by tail vein injection. One week later, luciferase activity by *in vivo* bioluminescence imaging was undetectable (not shown).

Next we tested standard AAV vector vs. exo-AAV vector by intramuscular (IM) injection of tibialis anterior (TA) muscles (Figure 2).

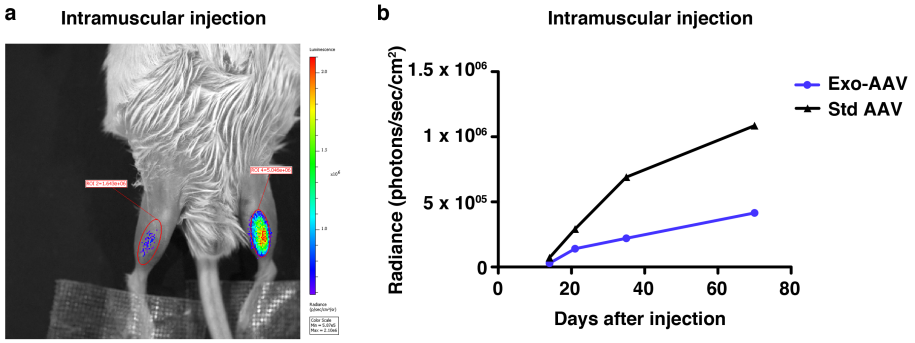

Figure 2. Intramuscular injection of AAV vectors. We used MGH Vector Core titers to administer a target dose of 5 x 10^{10} genome copies of either standard AAV or exo-AAV by intramuscular (IM) injection of TA muscles and monitored gene expression by serial *in vivo* bioluminescence imaging. a) A representative image of a mouse in the supine position at Day 70 after injection of vector is shown. b) Quantification of luciferase activity over time after IM injection. Error bars indicate mean ± s.e.m.

We repeated exo-AAV purification and tested it against standard AAV vector using Vector Core titers to determine the dose administered (Figure 3).

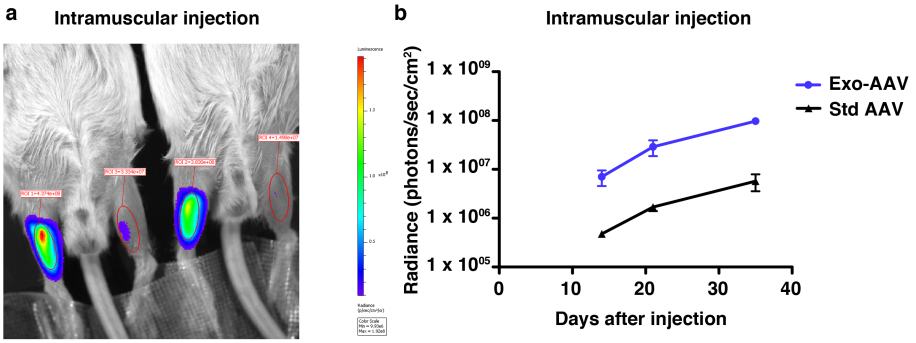

Figure 3. Intramuscular injection of AAV vectors. We used Vector Core titers to administer a target dose of 5 x 10^{10} genome copies of either standard AAV or exo-AAV by IM injection and monitored gene expression by *in vivo* bioluminescence imaging. a) Representative image of mice in the supine position at Day 35 after injection is shown. b) Quantification of luciferase activity over time after IM injection. Error bars indicate mean ± s.e.m.

Conclusion: The undetectable expression after the intravenous study and the low asymmetrical and inconsistent expression evident after intramuscular injections suggested that the MGH Vector Core titers were inaccurate.

Major goal (task) 3: Develop a protocol for AAV vector quantification.

Major activities: We developed a droplet digital PCR assay to quantify standard and exo-AAV vectors by modifying a previously published protocol (Lock, et al, 2014). The protocol uses a primer probe set that targets the BGH polyA sequence and is shared in all of our constructs. We tested the protocol by using serial dilutions of a known quantity of plasmid DNA containing the BGH target followed by quantification of standard and exo-AAV vectors (Figure 4). For comparison, we also quantified vectors using standard qPCR and found that ddPCR appears to be more accurate.

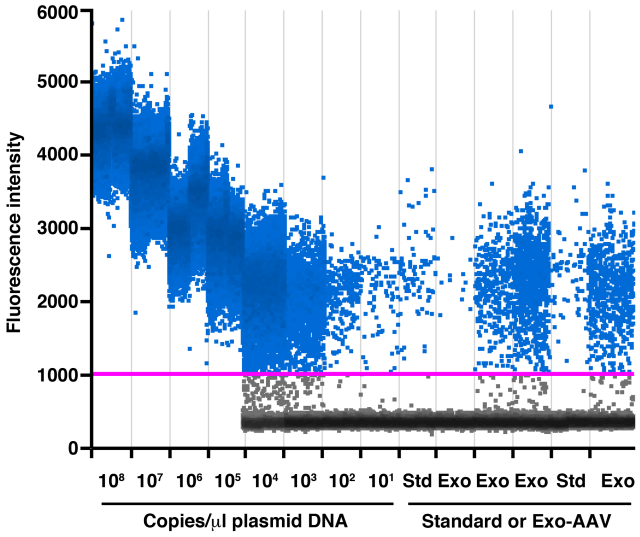

Figure 4. Quantification of AAV and exo-AAV vectors. We developed a droplet digital PCR (ddPCR) assay targeting the BGH polyA sequence. Using serial dilutions of a known quantity of plasmid DNA that was used to make the vectors, we measured copies/microliter of plasmid DNA. Representative droplet populations as shown. ddPCR quantification of vector titer found that the MGH Vector Core titers were between 70- and 1600-fold over-estimates, explaining the low and inconsistent gene expression evident in our early studies.

Major goal (task) 4: Test standard AAV and exo-AAV vectors in DM1 mice using ddPCR vector quantification to determine dose.

Major activities: We repeated exo-AAV purification and tested it against standard AAV vector using ddPCR titers to determine the dose administered (Figure 5).

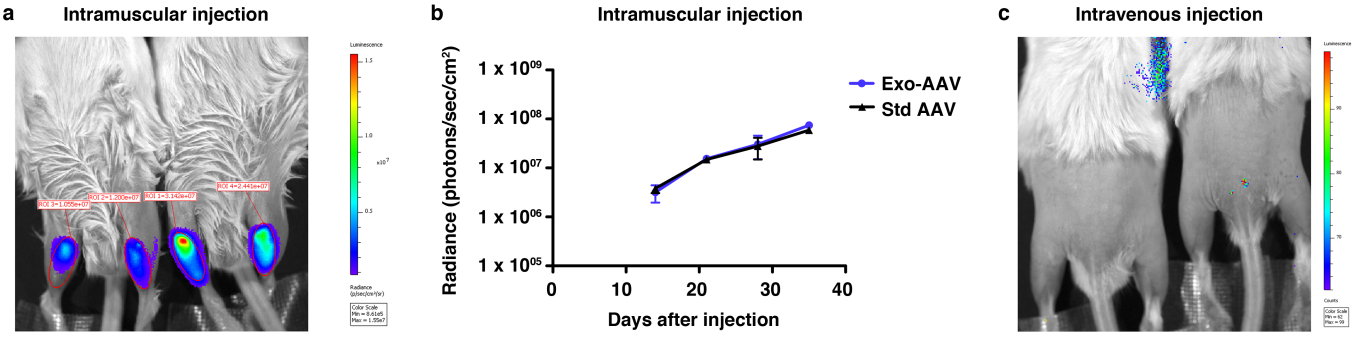

Figure 5. *In vivo* expression of AAV vectors. a) We injected tibialis anterior (TA) muscles of HSA^{LR} mice with exosome-associated AAV-CK8 luciferase (exo-AAV; right TA) or standard AAV-CK8-luciferase (std-AAV; left TA) and monitored gene expression by serial *in vivo* bioluminescence imaging. A representative image of mice in the supine position is shown. Luminescence is measured in regions of interest (ROIs) of the right and left TAs. The total dose of each vector injected was 6.5 x 10^9 genome copies in the left or right TA, as calculated by ddPCR, or 8.4 x 10^{11} genome copies of standard AAV in left TA and 4.5 x 10^{11} genome copies of exo-AAV in the right TA, as calculated by the MGH Vector Core. b) Quantification of luciferase activity over time after IM

injection. Error bars indicate mean ± s.e.m. c) We treated HSA^{LR} mice with undiluted exo-AAV-CK8-luciferase by tail vein injection and monitored luciferase activity by *in vivo* bioluminescence imaging. The dose administered was 2.46×10^{11} genome copies, as calculated by ddPCR, or 6.23×10^{12} genome copies, as calculated by the Vector Core. Luciferase activity was undetectable in all treated mice, supporting the accuracy of the ddPCR titer.

Conclusion: The low and symmetrical luciferase activity after IM injection supports the accuracy of the ddPCR titer in determining the dose to administer. As expected, our yield of exo-AAV correlates with the yield of standard AAV. Although vector production improved to some extent, the overall quantity and concentration has remained too low for study of gene expression in muscle tissue after systemic delivery. It will be necessary to find an alternative Vector Core to produce vector for these studies.

Major goal (task) 5: Identify PAM sequences in DM1 mice.

Major activities: We examined the *ACTA1* transgene in the HSA^{LR} mouse model and identified several candidate PAM sequences for SaCas9 or SpCas9 for editing the CTG repeat.

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

Preeti Kumari, PhD is a post-doctoral fellow working on the project.

How were the results disseminated to the communities of interest?

Nothing to report.

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

To achieve a yield of AAV vector sufficient for systemic delivery to skeletal muscle, we will obtain vectors from the University of Washington (UW) Vector Core that has 20 years experience producing vectors for gene delivery to skeletal muscle. Once we have sufficient AAV vector quantity, we will test standard AAV vs. exo-AAV vectors by tail vein injection and monitor gene expression by serial in vivo imaging. To test vector activity in the setting of neutralizing antibodies, as subset of mice will be pre-treated with pooled human immune globulins 24 hours prior to vector administration.

We will design and generate CRISPR/Cas9 constructs containing PAM sequences targeting the ACTA1 transgene for removal of the CTG repeat and test these constructs by intramuscular injection of DM1 mice.

4. Impact:

Nothing to report.

5. Changes/problems:

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

An unanticipated problem was the inability of the MGH Vector Core to produce sufficient AAV vectors for systemic delivery to skeletal muscle. To resolve this problem, we will obtain AAV vectors from the University of Washington (UW) Vector Core in Seattle, Washington, which has 20 years experience producing AAV vectors for systemic delivery to skeletal muscle tissue. The UW Vector Core supports efforts by investigators

in the U.S. and across the World related to gene therapy for muscular dystrophies: http://depts.washington.edu/mdcrc/vector_core.html.

6. Products:

Publications, conference papers, and presentations.

Nothing to report.

7. Participants and other collaborating organizations:

Which individuals have worked on the project?

Name: Thurman Wheeler
Project role: PI
Nearest person month worked: 2
Contribution to project: direct and supervise all aspects of this project, including experimental design and data interpretation. When needed, will assist with cloning, plasmid DNA preparation, vector purification, vector quantification, *in vivo* imaging, droplet digital PCR, genotyping, and management of the mouse colony.

Name: Ningyan Hu
Project role: Research technologist
Nearest person month worked: 3
Contribution to project: Cloning, plasmid DNA preparation, vector purification, vector quantification, *in vivo* imaging, droplet digital PCR, genotyping, and management of the mouse colony.

Name: Preeti Kumari
Project role: Post-doctoral fellow
Nearest person month worked: 3
Contribution to project: Cloning, plasmid DNA preparation, vector purification, vector quantification, *in vivo* imaging, and droplet digital PCR.

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

PI Wheeler
Previously active grant now closed:

Nothing to report.

Previously pending grants now active:
W81XWH-20-1-0293 06/01/20 - 05/31/23 2.4 CM
Department of Defense-Congressionally
Directed Medical Research Programs cumulative TC
PI: Thurman Wheeler
Extracellular RNA biomarkers of myotonic dystrophy
The major goals of this project are, 1) identify and characterize the extracellular RNA profile in myotonic dystrophy, and 2) determine the natural history of extracellular RNA biomarkers and quantitative measurements of muscle function in myotonic dystrophy.
Grant management specialist: Jamie Shortall
Email: jamie.a.shortall.civ@mail.mil

U.S. Army Medical Research Acquisition Activity
Fort Detrick, MD 21702-5014
Overlap: None

W81XWH-20-1-0492 | 06/15/20 - 06/14/22 | 1.8 CM
Department of Defense-Congressionally Directed Medical Research Programs | year
PI: Thurman Wheeler
Exosomes as therapeutic vehicles for Duchenne muscular dystrophy
The major goal of this project is to develop a novel approach to micro-dystrophin gene transfer therapy that is designed to avoid vector degradation by neutralizing antibodies.
Grant management specialist: Jamie Shortall
Email: jamie.a.shortall.civ@mail.mil
U.S. Army Medical Research Acquisition Activity
Fort Detrick, MD 21702-5014
Overlap: None

Pending:
R61NS117210 | 6/01/2021 - 05/31/2026 | 3.0 CM
NIH - NINDS | cumulative TC
PI: Thurman Wheeler
Extracellular RNA biomarkers of myotonic dystrophy type 1
The major goals of this project are, 1) develop and internally validate extracellular RNA biomarkers of myotonic dystrophy type 1, 2) establish feasibility and reliability of extracellular RNA biomarkers as predictors of disease severity in myotonic dystrophy, and 3) identify a lead candidate urine exRNA biomarker that will move forward for rigorous clinical and analytical validation studies. This is a multi-center collaborative study that includes investigators from the University of Pittsburgh and Wake Forest University.

Which other organizations were involved as partners?

None.