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PRINCIPAL INVESTIGATOR: Dongsheng Duan

CONTRACTING ORGANIZATION: University of Missouri System
Columbia, MO 65211-3020

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Dongsheng Duan; Craig Emter; Yi Lai; Hsiao Tung Yang; Chady Hakim; Yongping Yue

E-Mail: duand@missouri.edu

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13. SUPPLEMENTARY NOTES

14. ABSTRACT Duchenne muscular dystrophy (DMD) is a life threatening disease affecting all muscles in the body. An important therapeutic goal of DMD gene therapy is to deliver a therapeutic gene to all muscles in the body. The overarching goal of this project is to achieve systemic AAV micro-dystrophin gene therapy in young adult affected dogs. In the last funding period, we proposed to incorporate the CpG-free feature to our vector to further diminish the untoward immune response. In this funding period, we demonstrated that elimination of CpG from AAV ITR does not affect therapeutic efficacy but it reduces packaging efficiency. We also showed that CpG-free microgenes are functional. However, removal of hinge3 resulted in better protection against eccentric contraction. We further showed that the CK8 promoter is highly effective in driving muscle-specific expression. Based on these results, we develop a novel XP49 vector. In this vector, a CpG-free codon-optimized, hinge3-deleted human microgene is expressed from the CK8 promoter. We will move forward with this vector for dog studies and future human trials. In the last funding period, we showed that systemic delivery of a canine micro-dystrophin AAV vector is safe in young adult affected dogs. We now further extended this result and demonstrated robust expression for 12 months. Importantly, we observed amelioration of muscle pathology and improvement of muscle force. In addition, we have developed a novel noninvasive assay to evaluate whole body mobility in dogs. Finally, we published three review papers on the current status of AAV DMD gene therapy.

15. SUBJECT TERMS
Duchenne muscular dystrophy, DMD, dystrophin, micro-dystrophin, adeno-associated virus, AAV, muscle, gene therapy, systemic gene delivery, canine model

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1. Introduction

Duchenne muscular dystrophy (DMD) is a life threatening disease affecting approximately one in 5,000 newborn boys. It is caused by dystrophin deficiency. Currently there is no effective therapy. Adeno-associated virus (AAV)-mediated micro-dystrophin gene therapy has resulted in unprecedented success in treating mouse models of DMD by systemic delivery. We now propose to develop systemic AAV micro-dystrophin gene therapy in the canine model.

2. Keywords

Duchenne muscular dystrophy, DMD, dystrophin, micro-dystrophin, adeno-associated virus, AAV, muscle, gene therapy, systemic gene delivery, canine model

3. Accomplishments

Major goal. We have two specific aims. Our first aim is to design and validate a new, low immunogenic human ΔR2-15/ΔR18-19/ΔR20-23/ΔC microgene AAV vector in adult dystrophic dogs by direct local injection. Our second aim is to test systemic gene therapy in young adult DMD dogs with the newly developed microgene AAV vector.

Accomplishments.

Aim 1. In the last report, we proposed to improve our original design of AAV human micro-dystrophin construct in light of a recent report suggesting that elimination of the CpG motif in the vector can significantly diminish immune response in AAV gene therapy (Faust et al., 2013). We proposed two studies including (1) removing the CpG motif from inverted terminal repeats (ITRs), the essential viral packaging and replication signal of the AAV vector and (2) removing the CpG motif from the human micro-dystrophin gene. We have now successfully finished both studies.

Aim 1.1. Generation and evaluation of AAV vectors that carry the CpG-free ITRs. Each AAV vector has two ITRs. **Figure 1** shows the sequence structure of the original 5' and 3'-ITRs (top panels) and our modified CpG-free 5' and 3'-ITRs (bottom panels). To eliminate the CpG motif in the 5' ITR, we changed 20 nucleotides including 8 in the A/A' arm, 4 in the B/B' arm and 8 in the C/C' arm. To eliminate the CpG motif in the 3' ITR, we changed 22 nucleotides including 8 in the A/A' arm, 8 in the B/B' arm and 6 in the C/C' arm.

Since the ITR is essential for AAV packaging and AAV genome replication during vector production, it is possible that our CpG-free modification may inactivate ITR and completely abolish viral vector packaging. To determine whether our modified construct can still support AAV production, we carried out three independent rounds of production. As shown in **Figure 2**, indeed the vector yield from CpG-free ITR was significantly reduced (~3.5-fold lower). Nevertheless, our modification did not eliminate AAV production.

Next we examined the CpG-free AAV vector by transmission electron microscopy. The ITR CpG-free AAV particles showed similar structure as that of the original un-modified AAV particle (**Figure 3**). In light of inefficient packaging (as reflected by the low yield), we thought that we might see more empty particles in the ITR CpG-free vector preparation. Surprisingly, there was no difference compared to that of the un-modified vector (**Figure 3**).

To determine the functionality of the ITR CpG-free vector, we performed local injection at the tibialis anterior muscle in 10-m-old mdx mice. One side of the muscle received the ITR CpG-free

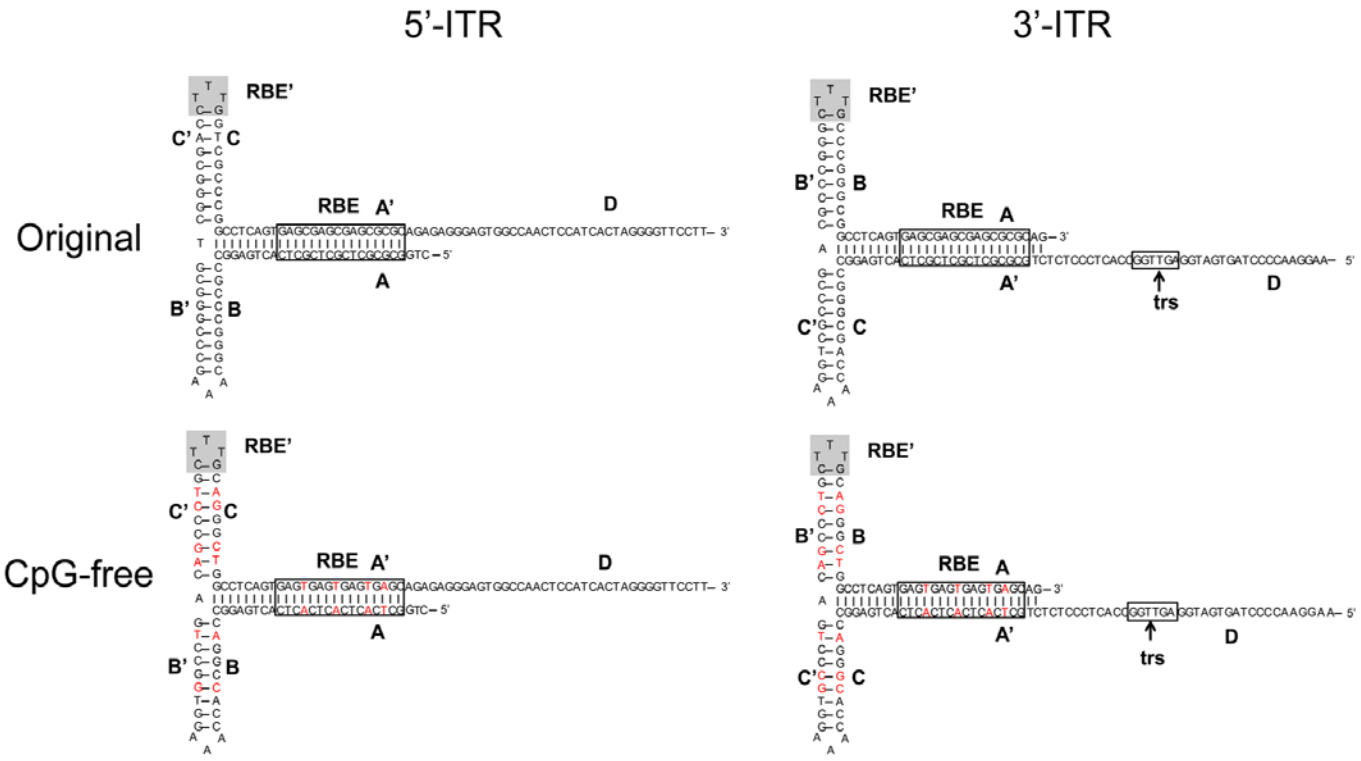


Figure 1. Structural illustration of the original and the CpG-free ITR. The capital letters A, B, C and D mark different region of the ITR. RBE stands for the Rep-binding element and trs stands for the terminal resolution site. CpG changes are marked in red.

Figure 2. Quantitative evaluation of the viral yield from the vector carrying the original ITR and the vector carrying the modified CpG-free ITR. **, p<0.001

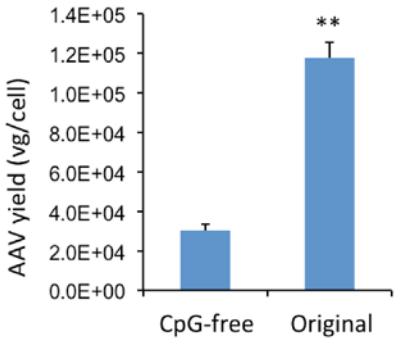
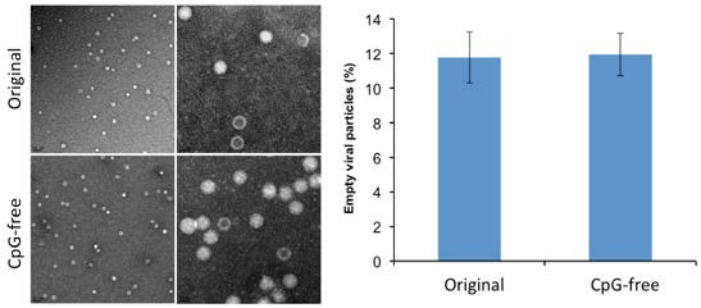


Figure 3. Evaluation of the unmodified AAV particles and AAV particles that carry the CpG-free ITR by transmission electron microscopy. Left panels, low power and high power transmission EM photomicrographs. Right panel, percentage of empty particle in the vector preparation.



vector and the contralateral side received the un-modified vector. Two months after injection, we examined muscle histology and muscle function. On immunostaining and histology, we did not see much difference between two vectors (**Figure 4**).

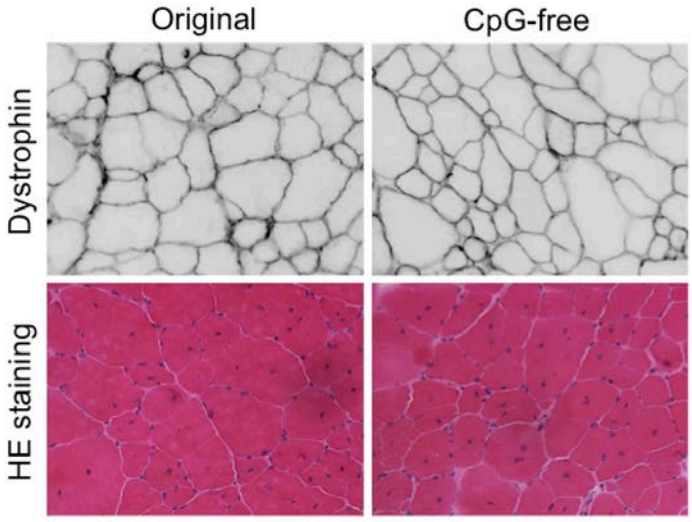
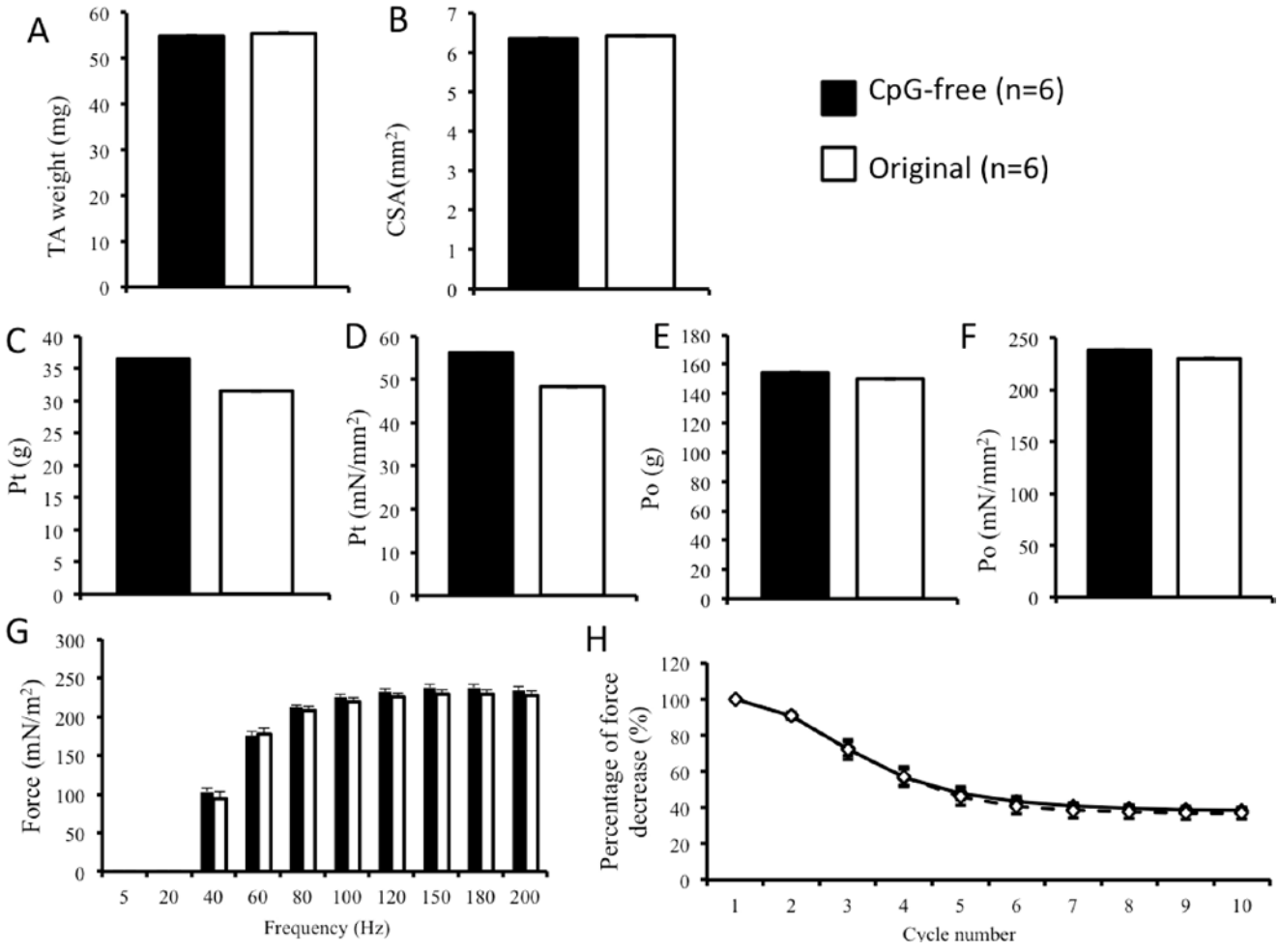


Figure 4. Dystrophin immunostaining (top panels) and HE staining from mdx muscle that had been injected with the wild type ITR micro-dystrophin AAV vector and the CpG-free ITR micro-dystrophin AAV vector.

To further compare the two vectors, we evaluated TA muscle weight, cross-sectional area (CSA), absolute twitch force (Pt in g), specific twitch force (Pt in mN/mm^2), absolute tetanic force (Po in g), specific tetanic force (Po in mN/mm^2), force-frequency relationship and percentage of the force drop follow 10 cycles of eccentric contraction (**Figure 5**, below). No difference was noticed between the modified ITR CpG-free vector and the original unmodified vector. Our data suggest that elimination of the CpG islands from the ITR does not affect the therapeutic efficacy of the vector.



Aim 1. 2. Generation and evaluation of CpG-free human micro-dystrophin. In our original DOD application, we proposed to generate a human version of our published canine ΔR2-15/ΔR18-19/ΔR20-23/ΔC microgene (**Figure 6**, right) (Shin et al., 2013). In this proposed construct, we will express the codon-optimized human ΔR2-15/ΔR18-19/ΔR20-23/ΔC microgene from the muscle-specific SpC5-12 promoter. As stated above, we have decided to further improve our vector by making it CpG-free in year 2 of the project. During this genetic engineering process, we realized that we could further enhance our construct by making additional changes. (1) We decided to add a Dys-2 epitope at the end of the microgene. In our original plan, the microgene was ended at the syntrophin/dystrobrevin binding site. A drawback of this design is that there is no antibody that can recognize the syntrophin/dystrobrevin binding site. This makes it challenging to determine whether we have a full-length micro-dystrophin protein. Dys-2 is a short peptide in the wild-type full-length dystrophin. It can be recognized by the Dys-2 monoclonal antibody. Addition of Dys-2 epitope will now allow us to definitively confirm that our micro-dystrophin is intact when expressed in muscle. (2) We decided to remove all CpG islands from the codon-optimized human microgene to reduce the immunity of the vector. (3) A new paper published by Liang et al caught our attention (Liang et al., 2015). In this study, the authors found that patients who have hinge 3 missing in their dystrophin show less severe disease. In reviewing the literature, we found another clinical study by (Carsana et al., 2005) that also showed that in-frame deletion of hinge 3 is associated with milder clinical presentation. Unfortunately, no study has directly compared the pros and cons of hinge 3 in experimental animals. To generate the most functional microgene, we decided to compare the therapeutic efficacy of micro-dystrophins with or without hinge 3. To this end, we engineered two different CpG-free, codon-optimized human microgenes. We named them XP16 (with hinge 3) and XP23 (without hinge 3) (**Figure 7**, below).

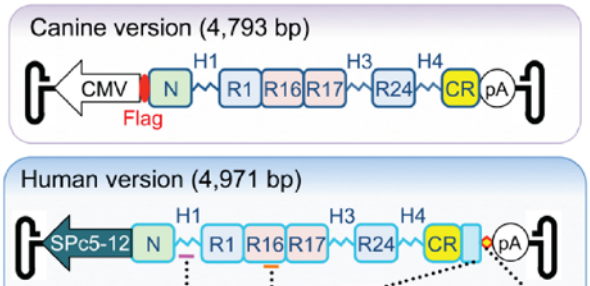
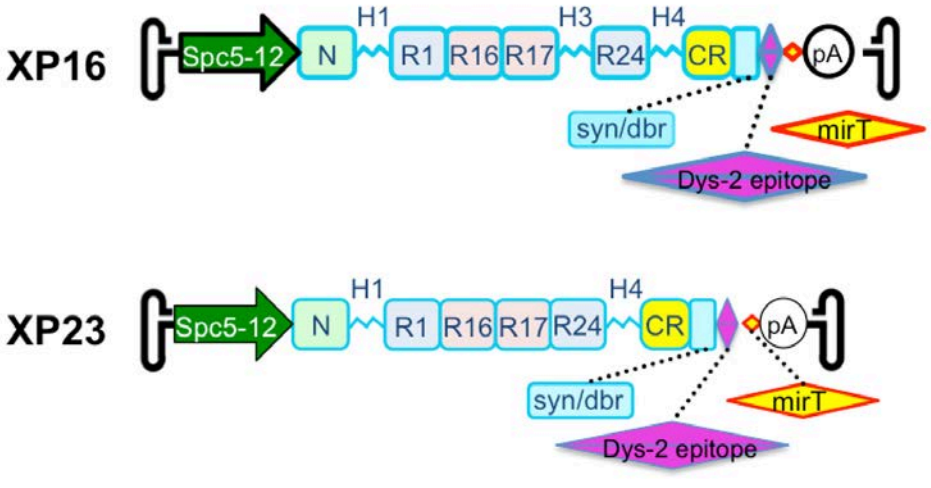
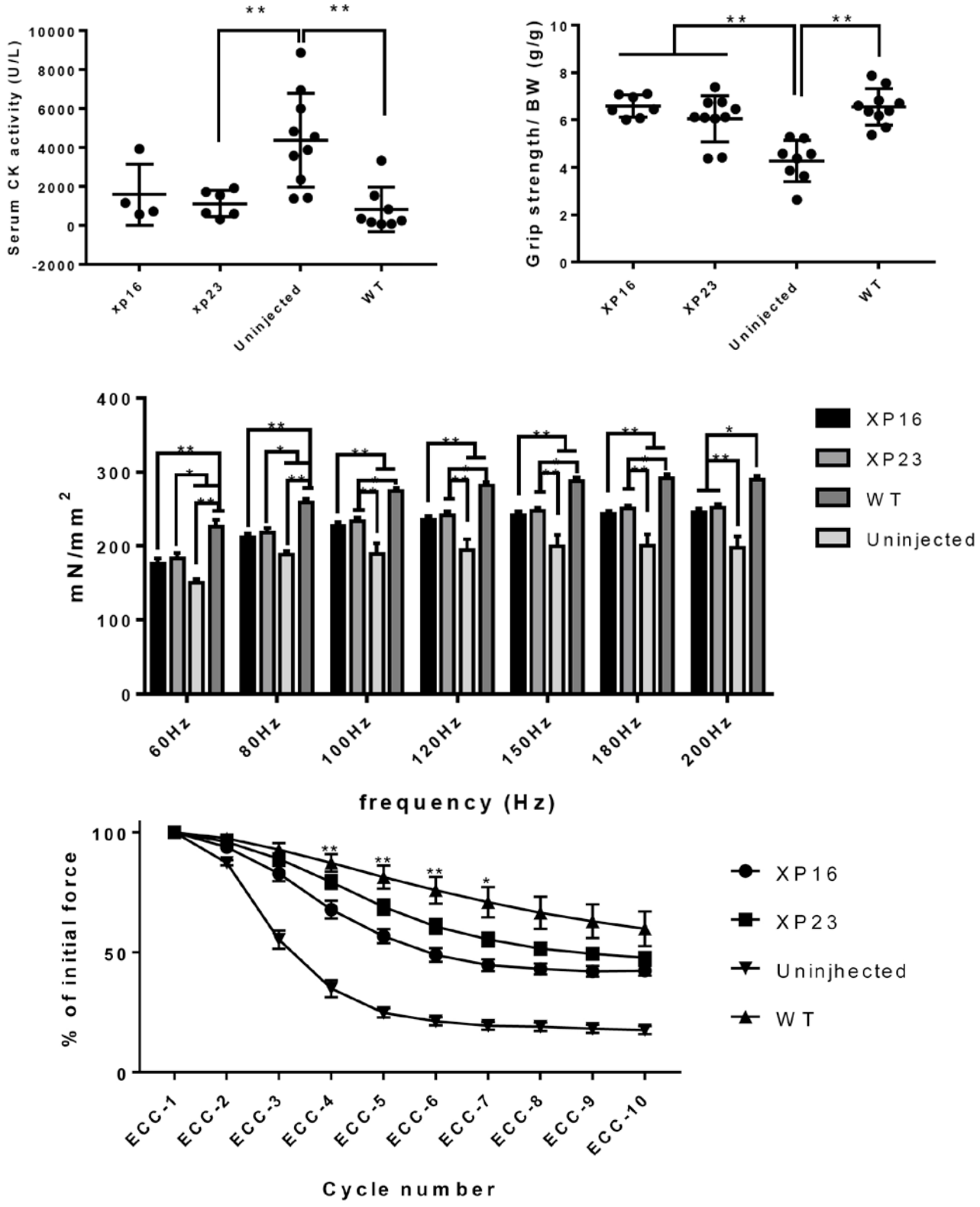


Figure 6. Schematic outline of the canine (top) and human (bottom) version microgene cassette. mirT, mir142-3p target sequence; syn/dbr, syntrophin and dystrobrevin binding site.

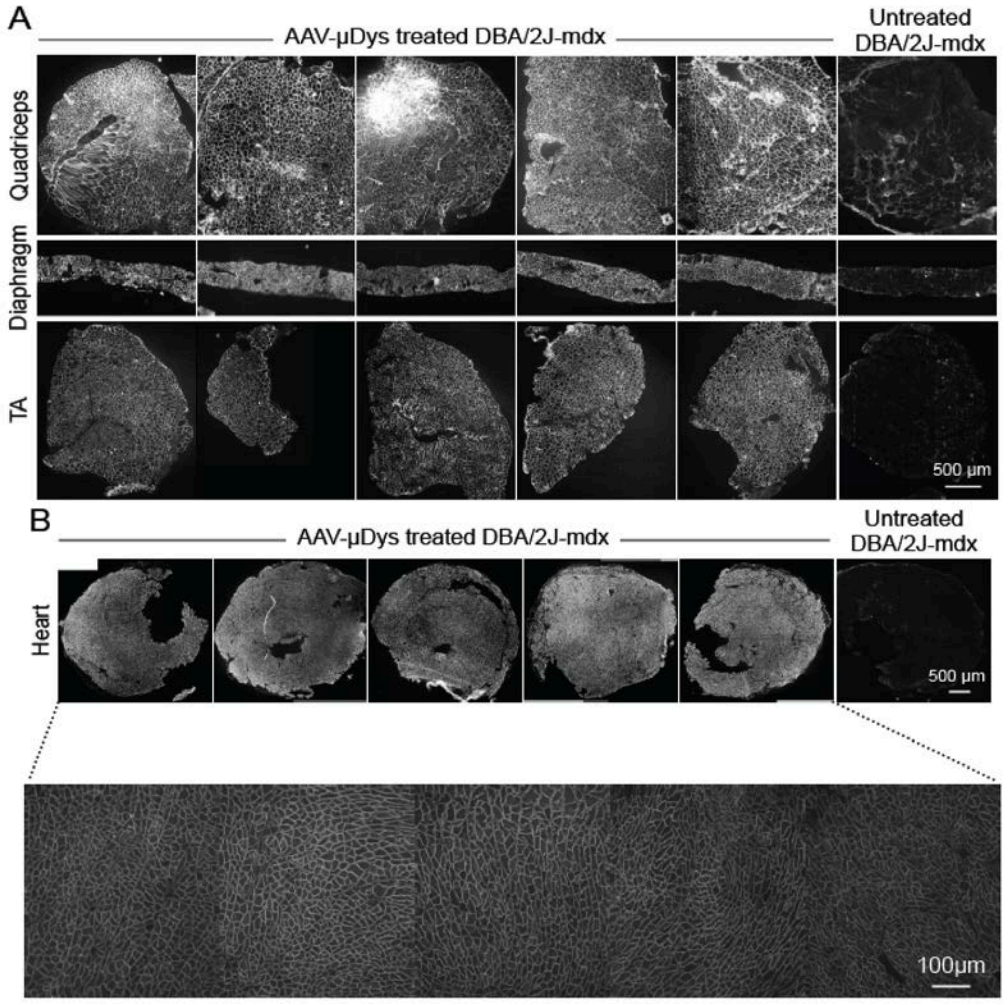


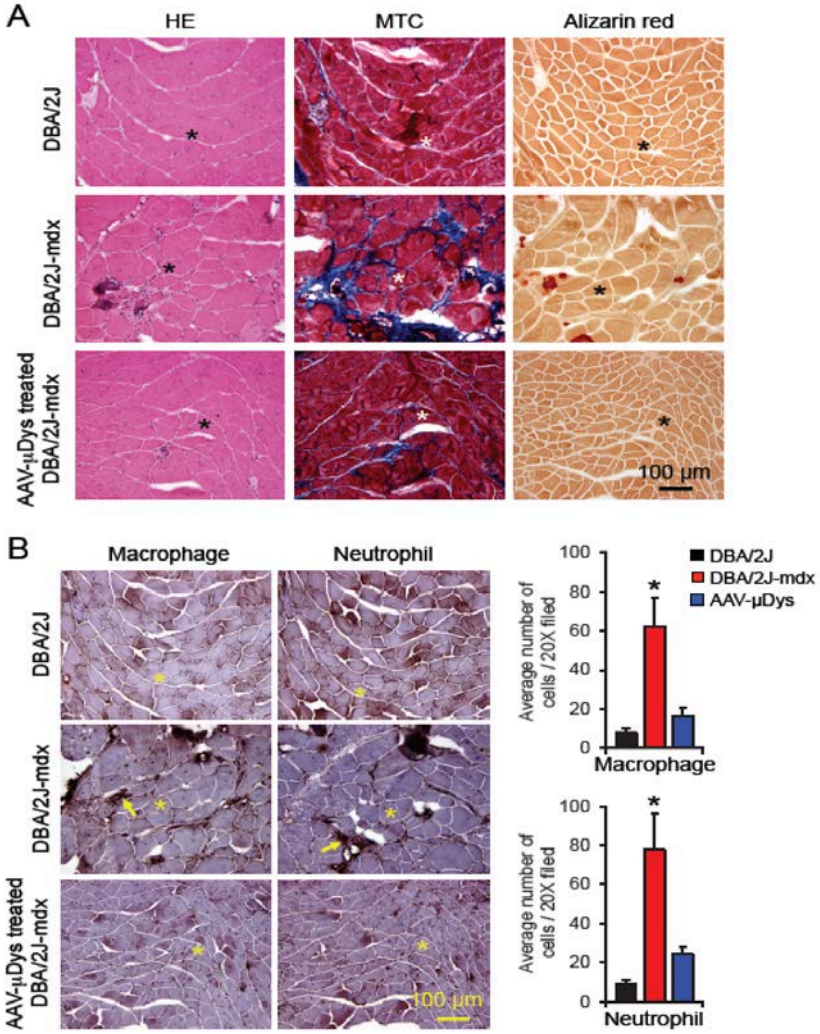
To compare the therapeutic efficacy of XP16 and XP23, we performed systemic delivery in 10-week-old mdx mice. Seven mice received XP16. Ten mice received XP23. We also included 9 untreated mdx mice and 7 wild type BL10 mice as controls. At 24 weeks after gene transfer, we measured the serum creatine kinase (CK) level, grip strength, force-frequency relationship and eccentric contraction profile (**Figure 8**, below).



We found that both XP16 and XP23 were equally effective in improving grip strength and tetanic force (at the frequency of 60, 80, 100, 120, 150, 180 and 200 Hz). While both constructs reduced the serum CK level, the statistical significance was only achieved with XP23. In eccentric contraction, both constructs protected against muscle damage. However, XP23 consistently outperformed XP16. In summary, our results are in line with clinical observations suggesting that a hinge3-free micro-dystrophin is more effective in protecting dystrophic muscle in adult mdx mice.

Aim 1.3. Evaluation of the CK8 promoter. We originally proposed to use the synthetic SPc5-12 promoter (Li et al., 1999). However, this promoter has many CpG and is difficult to work with in AAV cloning (likely due to its GC-rich nature). Recently, a superior muscle-specific promoter called CK8 promoter became available from Hauschka lab. This lab has focused on developing muscle-specific promoters for more than three decades and has generated the best muscle-specific promoter for the field (Bengtsson et al., 2016; Himeda et al., 2011). For this reason, we tested the CK8 promoter. Following systemic delivery of a CK8 driven micro-dystrophin AAV vector in a highly severe DBA2/J-mdx model (Coley et al., 2016; Fukada et al., 2010), we observed super-strong expression in all muscles and the heart (**Figure 9**, below). Treatment significantly improved muscle histology (HE staining), minimized fibrosis (MTC staining), eliminated calcification (Alizarin red staining), and diminished inflammation (macrophage and neutrophil immunostaining) (**Figure 10**, next page top panels). Importantly, muscle function was nearly normalized (**Figure 11**, next page bottom panels).




A
HE
MTC
Alizarin red
DBA/2J
DBA/2J-mdx
AAV-µDys treated DBA/2J-mdx
100 µm
B
Macrophage
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AAV-µDys
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Macrophage
Neutrophil
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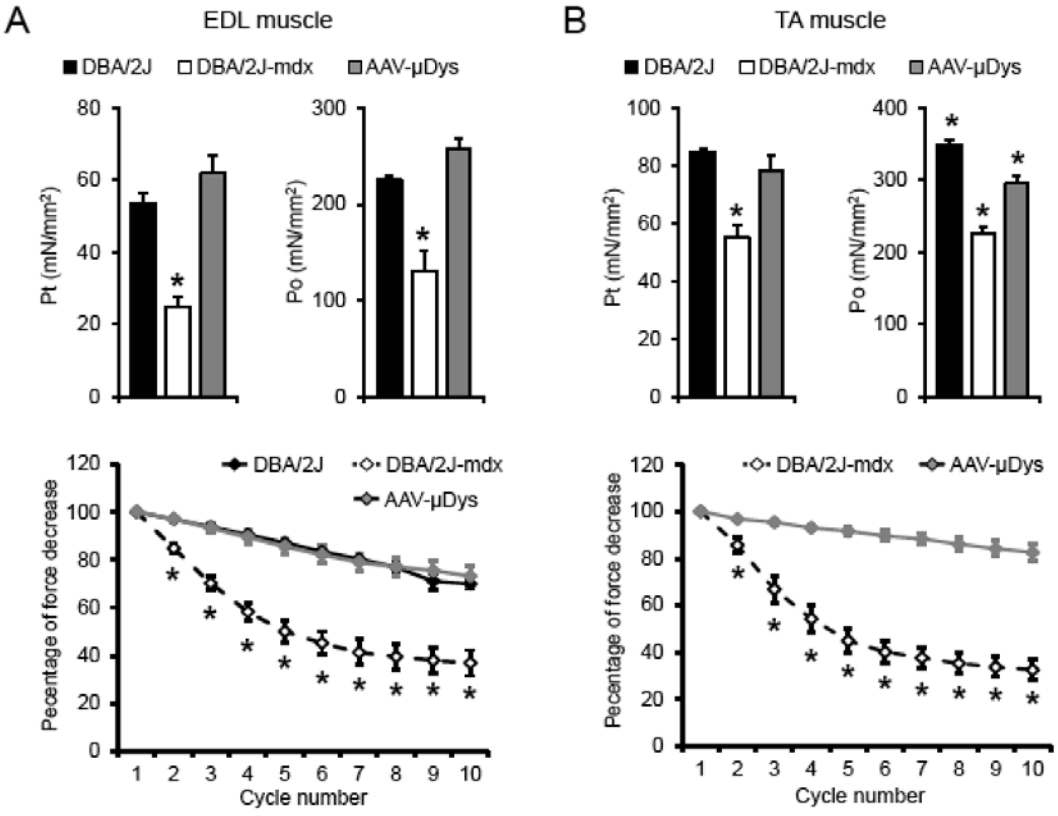
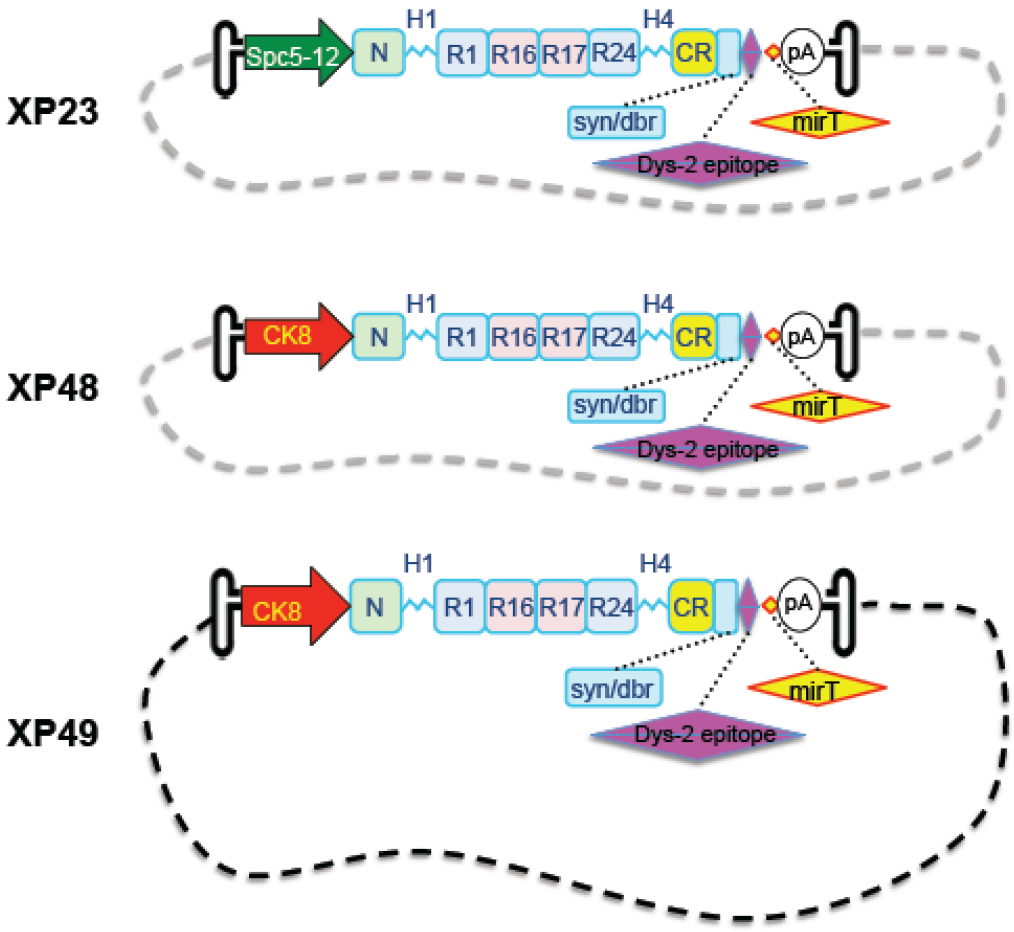

A
EDL muscle
B
TA muscle
DBA/2J
DBA/2J-mdx
AAV-µDys
Pt (mN/mm²)
Po (mN/mm²)
Pecentage of force decrease
Cycle number

Aim 1.4. Finalization of the design of the CpG-free human micro-dystrophin AAV vector for systemic delivery in affected dogs. Based on the results of XP16 and XP23 comparison study, we decided to remove hinge 3 from our originally proposed design.

Based on the evaluation of the CK8 promoter, we decided to use the CK8 promoter, instead of the SpC5-12 promoter. With this in mind, we generated XP48 (**Figure 12**, below).

The AAV packaging plasmids described above (XP16, XP23 and XP48) all have a relatively small backbone (<4kb). On evaluating the stocks using a highly sensitive TaqMan PCR protocol, we noticed a very low level (<1%) of contamination of the backbone DNA in the viral stock. This is thought to be caused by so-called "reverse packaging". For the mouse study, <1% contamination may not be a big problem. However, when we inject up to 10^{15} to 10^{16} vg particles per subject in large mammals, the amount of contaminating backbone becomes a safety concern. Increasing the size of the backbone to >5kb will prevent "reverse packaging" because this will make the backbone exceed AAV packaging capacity. To this end, we further modified XP48 and generated XP49 that is on a much larger (6kb) backbone (**Figure 12**, below).

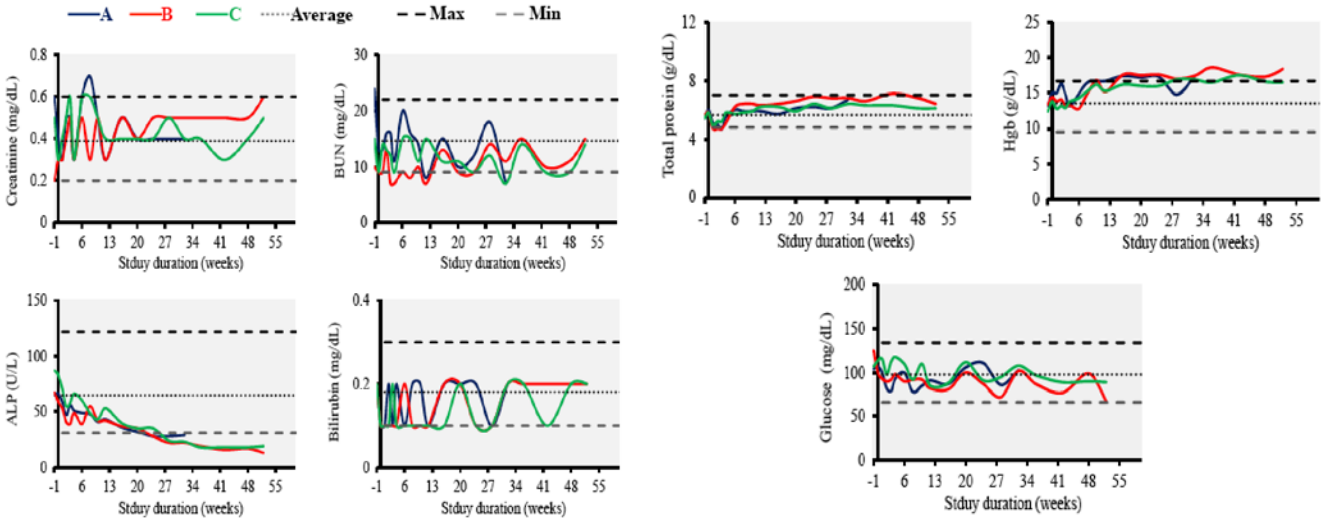


In summary, we have made great progress in Aim1. Specifically, (1) we have obtained all regulatory approval for our animal studies, (2) we have prepared vectors for mouse studies, (3) we have tested human micro-dystrophin AAV vector in two independent studies in mdx mice including (a) comparison of CpG-free ITR and wild type ITR (**Figures 1 to 5**), and (b) comparison of hinge3-free and hinge3-containing micro-dystrophin (**Figures 6 to 8**), (4) we have tested the CK8 micro-

dystrophin AAV vector in the highly severe DBA/2J-mdx mice (**Figures 9 to 11**), (5) we have generated AAV vector for local injection in affected dogs, (6) we have generated affected dogs and demonstrated in these dogs that inclusion of muscle-specific promoter and miR142-3p target site significantly attenuated immune response associated with AAV micro-dystrophin injection in dystrophic dog muscle (described in the last progress report), and (7) most importantly, we have developed a AAV vector much superior than we originally proposed (**Figure 12**). We will now use this improved vector (XP49) for systemic injection in affected dogs. We envision to carry this vector forward to a phase I human trial in the future.

Aim 2. Our eventual goal is to establish the proof-of-principle that systemic AAV micro-dystrophin gene therapy is doable in a large dystrophic mammal.

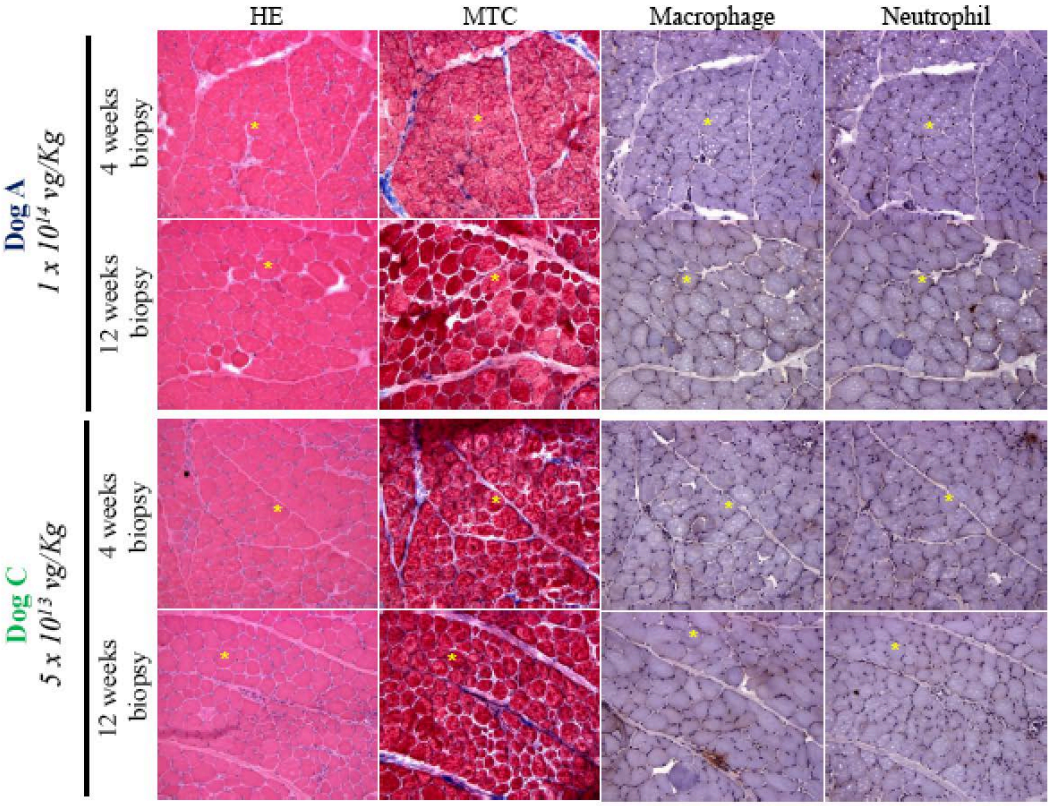
Aim 2.1. Evaluation of long-term systemic AAV micro-dystrophin expression. In the last progress report, we described our initial test of systemic AAV micro-dystrophin delivery in affected dogs (Yue et al., 2015). Our report is the first convincing demonstration that systemic gene therapy is feasible in a young adult large mammal that suffers from Duchenne muscular dystrophy. As the first report, we only followed AAV injected dogs for four months. Since DMD is a lifelong disease, it is important to determine whether we can achieve long-term micro-dystrophin expression. To this end, we performed additional study in three more dogs (named dog A, B and C in Figures 13 to 16). Consistent with our report in 2015 (Yue et al., 2015), the blood profiles of all three treated dogs were within the normal range suggesting there is no major safety concern (**Figure 13**, below).

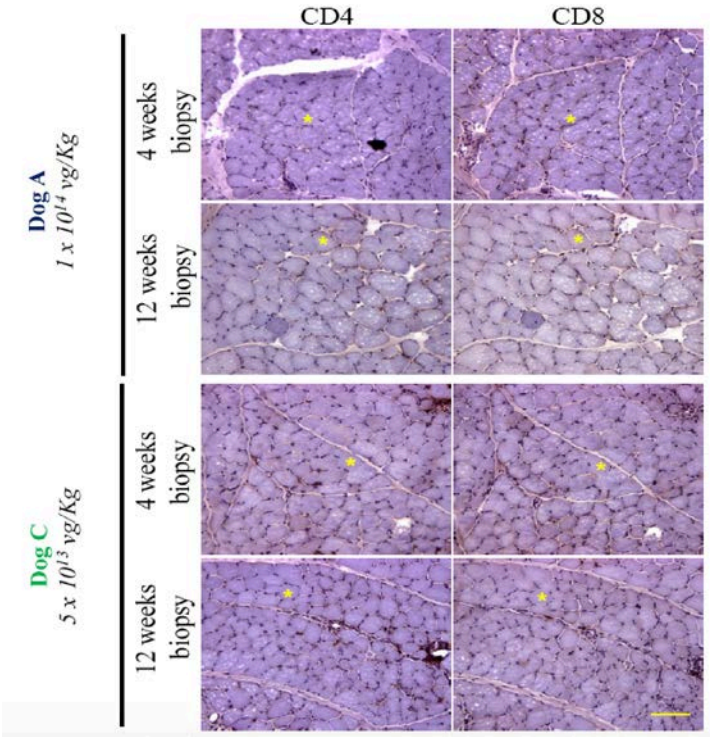


Next we examined muscle histology on the biopsied tissues (**Figure 14**, next page top panel). On HE staining, muscle appeared normal although we indeed noticed a few infiltrating mononuclear cells in the interstitial region. Nevertheless, mononuclear cell infiltration was limited to small areas. We suspect that they may come from muscle damage that happened before our AAV gene therapy. On Masson trichrome staining, we did not see apparent fibrosis. A little bit of fiber tissue was seen in the interstitial region where we expect to see the facia. Immunohistochemistry staining for macrophage and neutrophil revealed minimal inflammation.

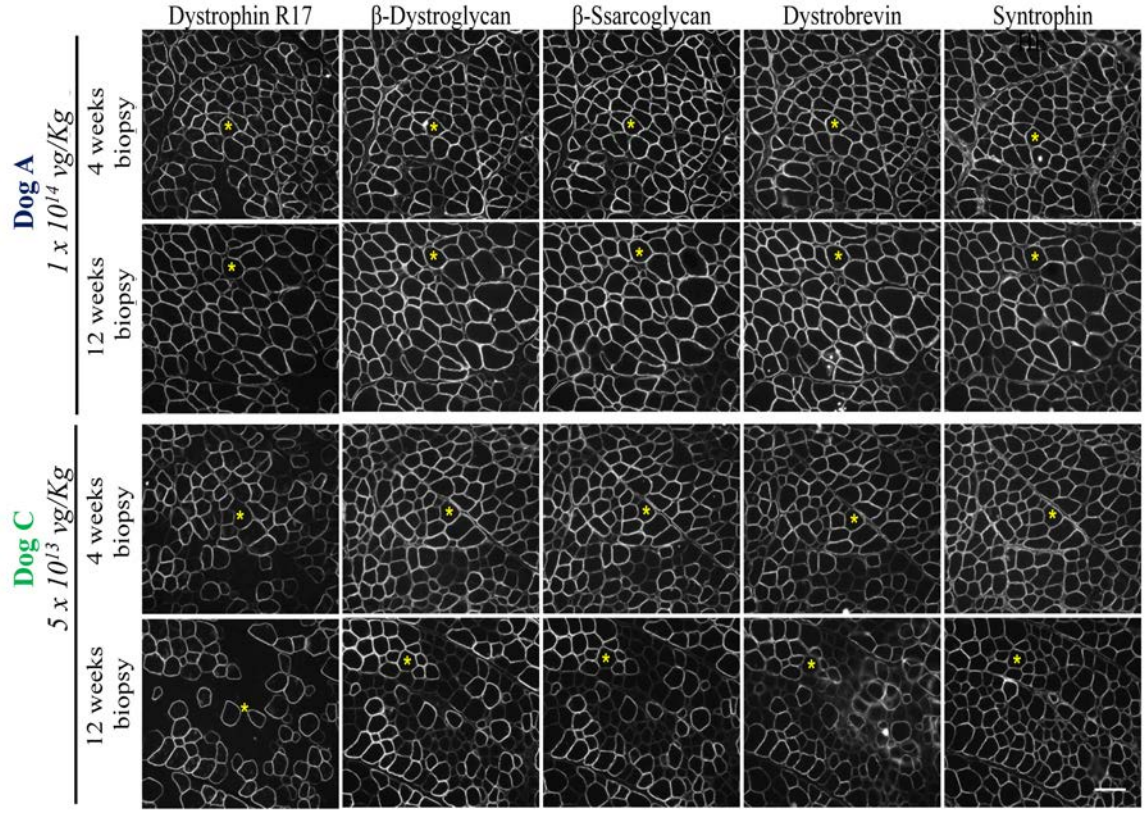
Since the T cell response is a major barrier for AAV micro-dystrophin gene therapy, we next evaluated CD4+ and CD8+ T cells by immunohistochemistry staining (**Figure 15**, next page bottom

panel). We did not detect massive infiltration of CD4+ and CD8+ T cells suggesting there was minimal immune reaction.

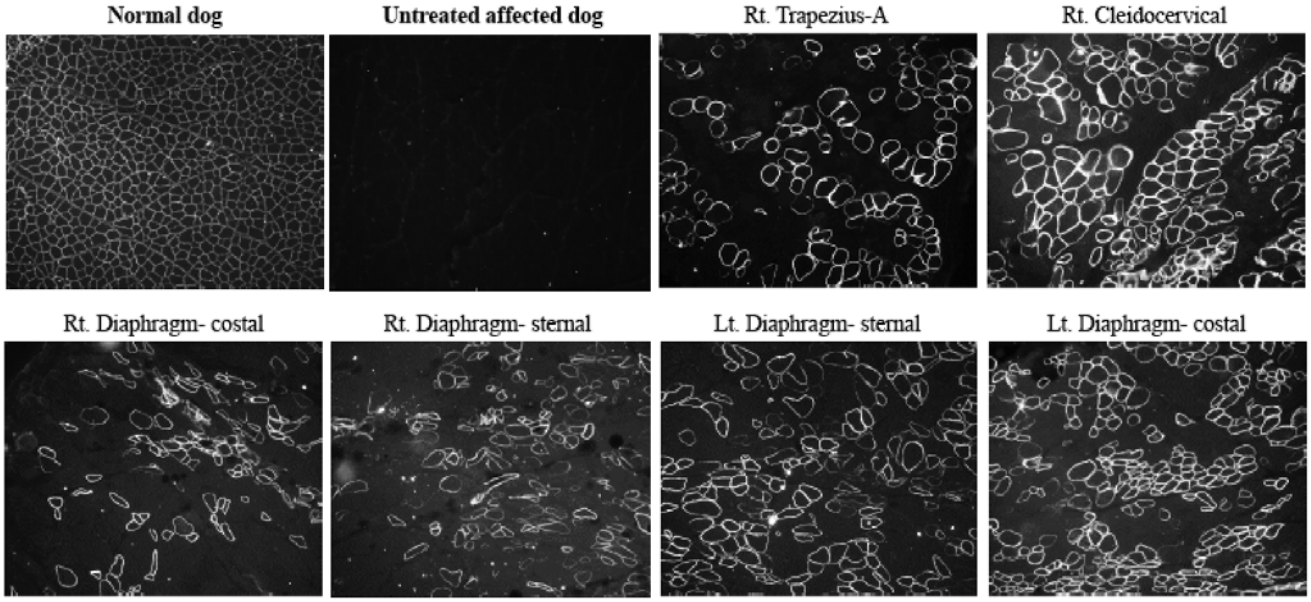


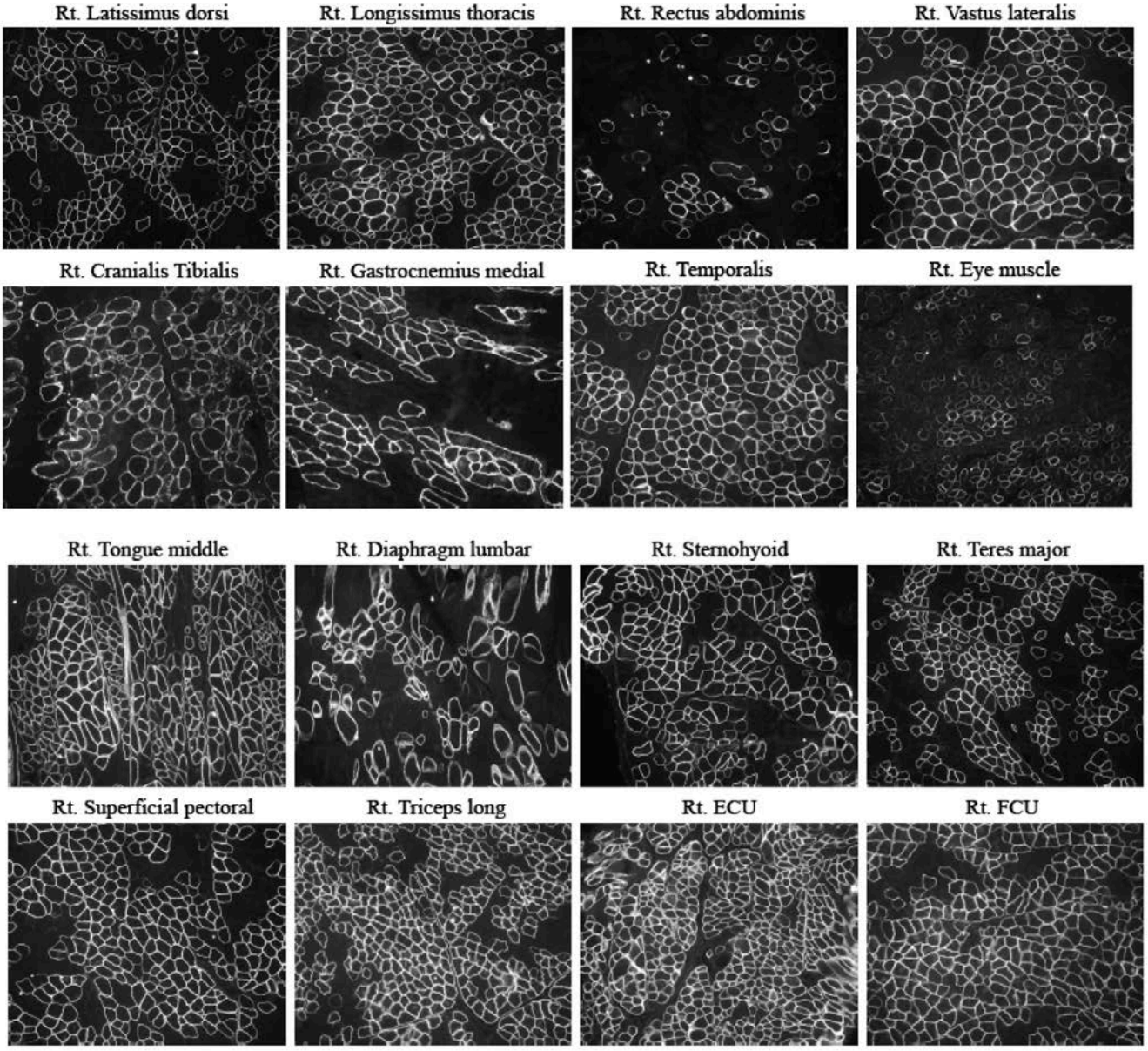


Next, we examined the dystrophin-associated glycoprotein complex by immunostaining (**Figure 16**, below). Micro-dystrophin expression (detected by an antibody against dystrophin spectrin-like repeat 17, R17) successfully restored β-dystroglyan, β-sarcoglycan, dystrobrevin and syntrophin.

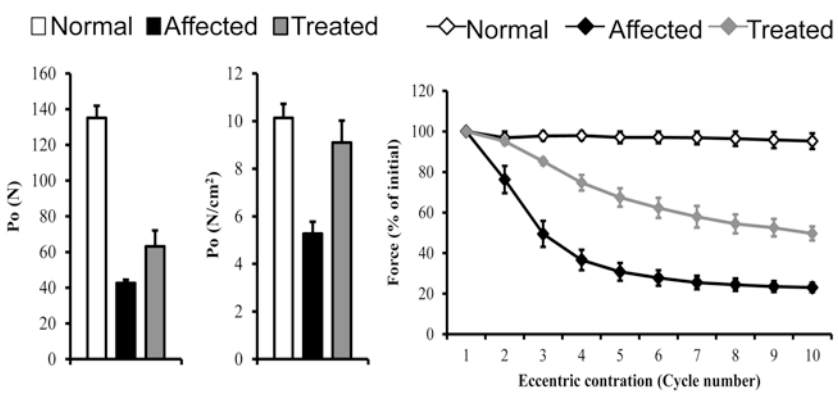


To confirm bodywide transduction, we euthanized one dog at 8 months after AAV injection. Dystrophin immunostaining photos from various muscles are shown in **Figure 17** (below and next page). We indeed achieved efficient whole body muscle micro-dystrophin expression.

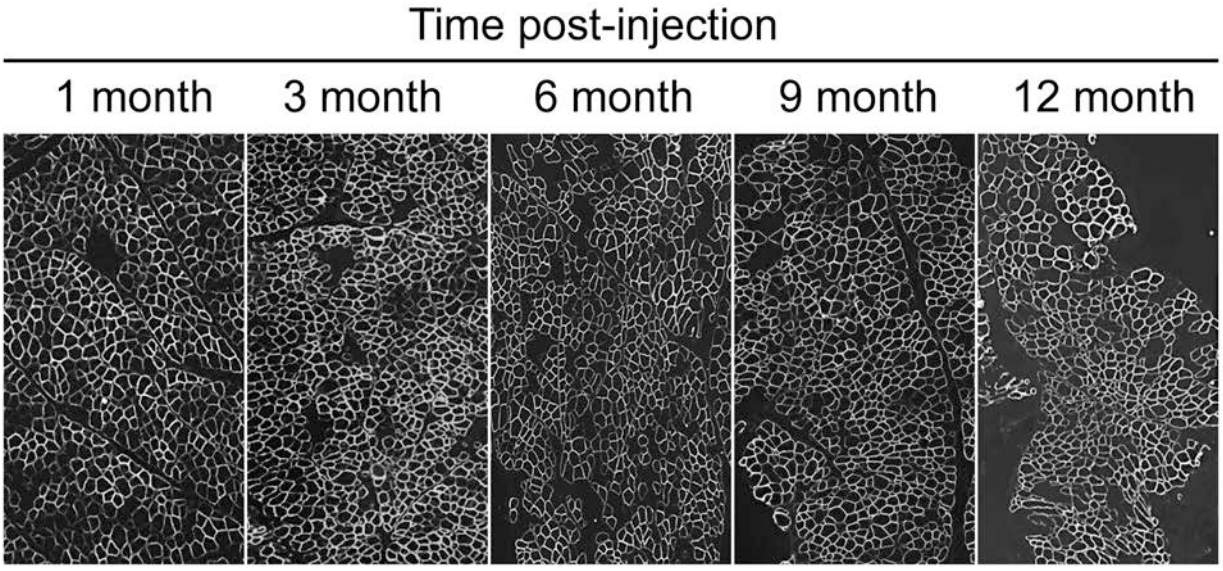




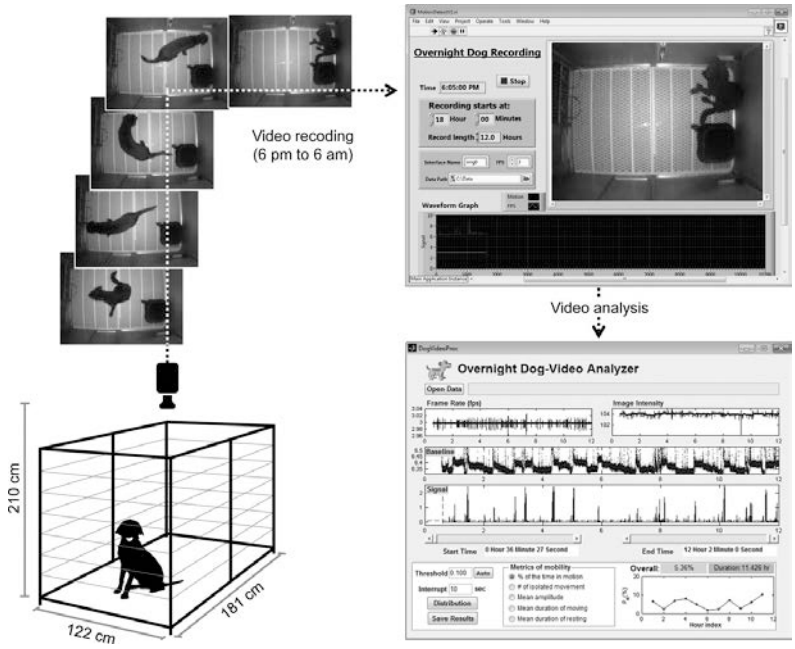
At the time of necropsy, we also performed in situ muscle force assay on the ECU muscle (both left and right side) (**Figure 18**, right). Although we only have N=2 treated muscles, the absolute muscle force (Po in N) was clearly improved. Muscle cross-sectional area normalized specific force (Po in N/cm^2) almost reached the level of normal dogs. On eccentric contraction, we also detected clear improvement. The force drop following cycles of eccentric contraction was greatly attenuated in the treated dog.



We have now kept these dogs for 12 months. Periodic biopsy revealed persistent robust micro-dystrophin expression for at least 12 months (**Figure 19**, below). We will continually monitor treated dogs till the end of the study.



Aim 2.2. Development of a novel non-invasive method to evaluate the overall activity of dogs. Issues related to outcome measurements have greatly and negatively impacted drug development in the field of DMD. This has caught significant attention in recent FDA review of the read-through drug Ataluren and exon-skipping drugs (Eteplirsen and Drisapersen). The situation is even worse in regards to the dog model. So far, there is no effective method to objectively evaluate whole-body mobility in a dog. Since a robust outcome measurement is essential to the success of our project, we decided to explore new non-invasive approaches that can objectively quantify dog mobility. Specifically, we developed a robust automatic video capturing/processing system to quantify dog mobility at night (**Figure 20**, below).



Using this system, we captured and analyzed dog movement at night. **Figure 21** (below) shows representative data from a normal and an affected dog.

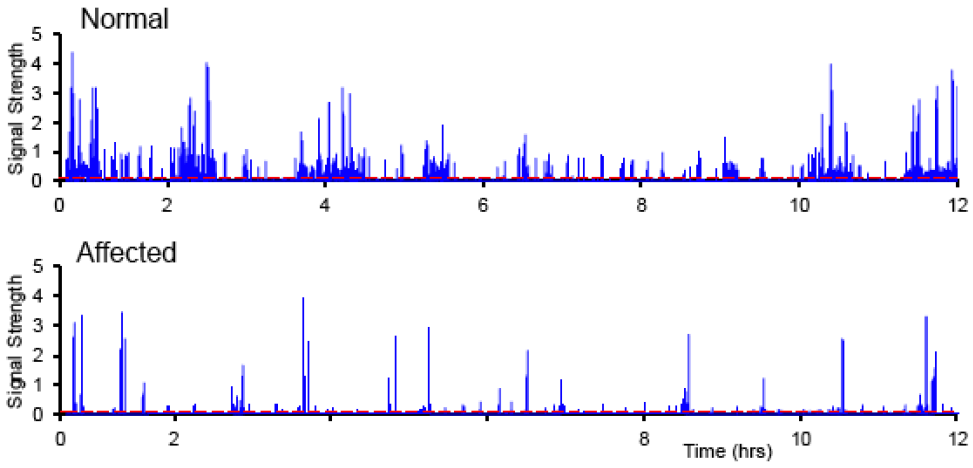
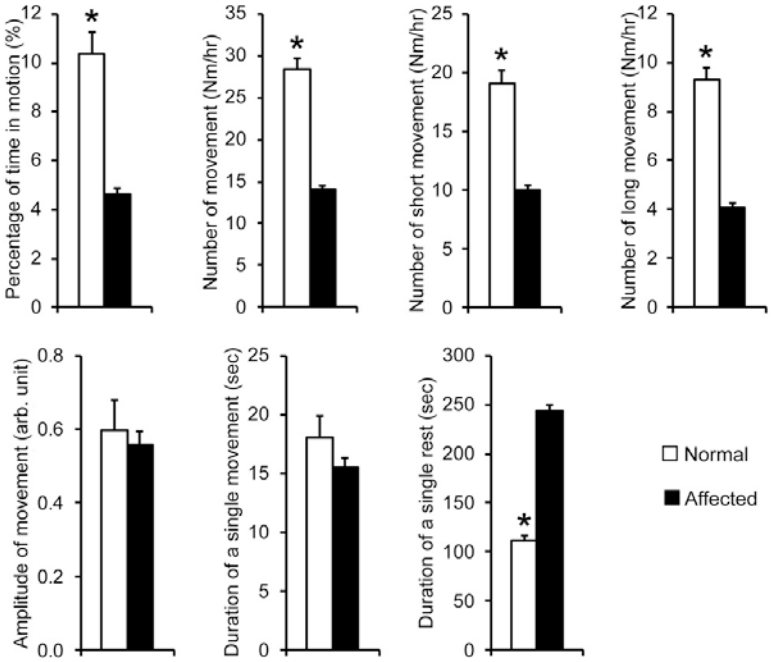


Figure 22 (below) shows population data from 12 normal (age 14.7±1.9 months) and 22 affected dogs (age 15.4±1.24 months). Throughout the night, normal dogs were in motion 10.4±0.9% of the time while affected dogs were in motion 4.6±0.2% of the time ($p<0.0001$). In other words, normal dogs moved approximately 75 min and affected dogs moved approximately 33 min during 12-hr recording. On average, normal dogs moved 28.3 ± 1.3 times per hour while affected dogs only moved 14.1 ± 0.4 times per hour ($p<0.00001$). Additional analysis showed that normal dogs not only made significantly more short movements but also made significantly more long movements ($p<0.00001$). Interestingly, the average amplitude of movement was similar between normal (0.60 ± 0.08) and affected (0.56 ± 0.04) dogs ($p=0.68$). Further, there was no significant difference in the average duration of movement between normal and affected dogs. In contrast to the duration of movement, the average duration of rest in affected dogs (243.1 ± 0.6 sec) was significantly longer than that of normal dogs (111.5 ± 4.5 sec) ($p<0.00001$). Collectively, we have established this overnight activity monitoring as an excellent outcome measurement for studying whole body activity in adult

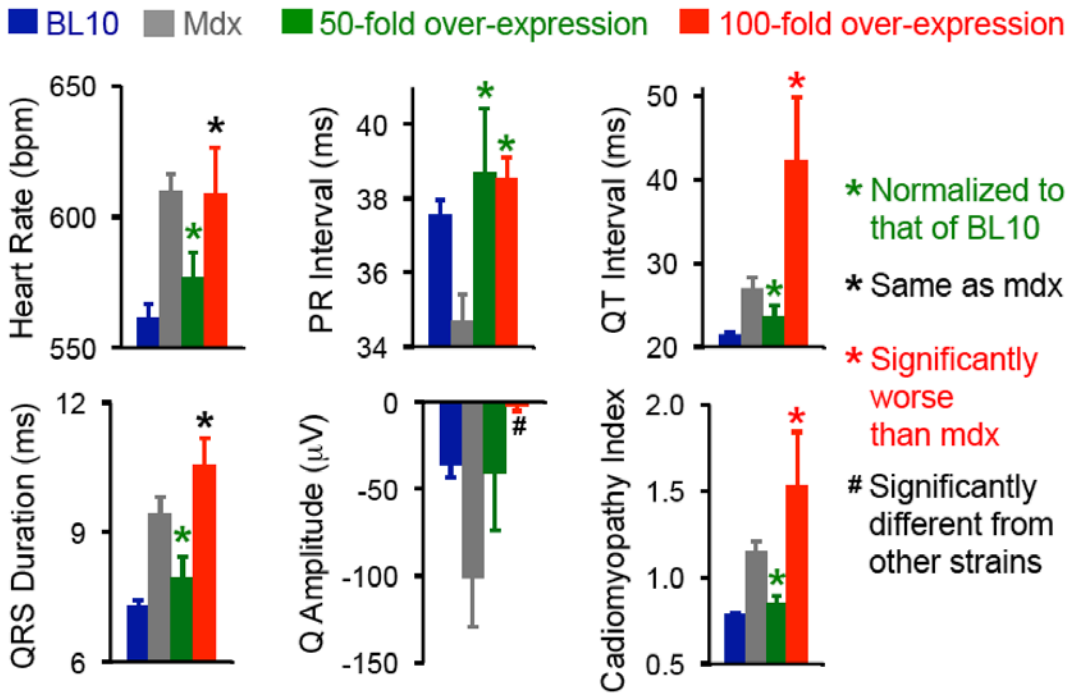


dogs. We expect this assay to greatly enhance our ability to study the functional outcome of systemic AAV micro-dystrophin gene therapy in affected dogs. We will include this assay in our future studies.

***Additional accomplishments that have benefited from this grant*.**

Accomplishment benefit from this grant 1. Because our goal is to develop systemic AAV gene therapy to treat DMD, we published three review articles on this topic. Specifically, (1) we provided a comprehensive perspective on AAV capsid modification for DMD gene therapy (Nance and Duan, 2015), (2) we reviewed gene therapy for muscular dystrophy associated cardiomyopathy (Yue et al., 2016a), (3) we reviewed the current status of DMD gene therapy (Duan, 2016). We also published a lay educational article on the canine DMD model (http://atlasofscience.org/understanding-the-dog-model-of-duchenne-muscular-dystrophy/).

Accomplishment benefit from this grant 2. A potential outcome of systemic AAV gene therapy is supra-physiological expression of the therapeutic micro-dystrophin gene. While it has been accepted that dystrophin over-expression is not toxic, we have found that 50-fold over-expression was tolerated and effectively ameliorated electrophysiological deficiency in mdx mice. However, 100-fold over-expression of dystrophin causes cardiac toxicity (Yue et al., 2016b). Specifically, 100-fold over-expression did not mitigate tachycardia, nor did it correct QRS prolongation. Importantly, 100-fold over-expression significantly worsened QT interval and cardiomyopathy index (**Figure 23**, below).



Training and professional development opportunities. Nothing to report.

Dissemination of the results. Above mentioned studies and review articles have been either published in peer-reviewed scientific journals or presented in academic conferences.

Plan for the next reporting period.

Aim 1. We have developed a highly potent human micro-dystrophin vector for future clinical translation (XP49, see Figure 12). This new vector contains a number of favorable features that are missing in our original design. Studies in mouse models of DMD suggest that it is highly competent in ameliorating muscle disease and improving muscle force in dystrophic mice. Studies in affected dogs suggest that our new low-immunogenic design significantly reduced immune response. We will now focus on systemic delivery of XP49 in affected dogs.

Aim 2. We have provided the first ever proof-of-principle for systemic AAV micro-dystrophin gene therapy in an affected young adult large mammal. Importantly, we have further extended our initial observations using an existing vector and demonstrated robust expression for up to 12 months after a single intravenous injection in young adult dystrophic dogs (Figure 19). Preliminary studies showed that micro-dystrophin expression restored dystrophin-associated glycoprotein complex, reduced muscle pathology and enhanced muscle function (Figures 14 to 18). With the development of this new vector (XP49, see Figure 12), we will start large-scale AAV production and purification and conduct systemic delivery studies in affected dogs.

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4. Impact. Nothing to report.

5. Changes/Problems.

Changes in approach and reasons for change.

We originally proposed to conduct a comprehensive local injection to validate our micro-dystrophin vector. In light of the additional workload associated with the development of the more effective new vector (XP49) and also in light of exciting results from systemic delivery (Figures 13 to 19), we now propose to focus on systemic injection with the new vector XP49.

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

We do not anticipate significant technical hurdles. We have many years of experience in breeding affected dogs and producing and purifying high quality AAV-vectors. Encouraging data obtained thus far strongly supports the strategy to move forward with systemic injections using our new XP49 vector. We are highly optimistic that this study will generate critical data for subsequent IND application and a phase I human trial.

Changes that had a significant impact on expenditures.

Nothing to report.

Significant changes in use or care of human subjects, vertebrate animal, biohazards and/or selected agents.

Nothing to report.

6. Products

6.1. Peer-reviewed publications (a total of 5) (All 5 publications have federal support)

1) Hakim CH, Peters AA, Feng F, Yao G, **Duan D.** *Night activity reduction is a signature physiological biomarker for Duchenne muscular dystrophy dogs.* **Journal of Neuromuscular Diseases**. 2(4):397-407, **2015**.
"This work was supported in part by the Department of Defense, Duchenne Muscular Dystrophy Research Program (DMDRP), Congressionally Directed Medical Research Programs under Award No. W81XWH-14-1-0302." "Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the Department of Defense."

2) Nance ME and **Duan D.** *Perspective on adeno-associated virus (AAV) capsid modification for Duchenne muscular dystrophy gene therapy.* **Human Gene Therapy** 26(12):786-800, **2015**.
"This work was supported in part by the Department of Defense, Duchenne Muscular Dystrophy Research Program (DMDRP), Congressionally Directed Medical Research Programs under Award No. W81XWH-14-1-0302." "Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the Department of Defense."

3) Yue Y, Binalsheikh IM, Leach SB, Domeier TL, **Duan D**. *Prospect of gene therapy for cardiomyopathy in hereditary muscular dystrophy*. **Expert Opinion on Orphan Drugs** 4(2):169-183, **2016**.
"This work was supported in part by the Department of Defense, Duchenne Muscular Dystrophy Research Program (DMDRP), Congressionally Directed Medical Research Programs under Award No. W81XWH-14-1-0302." "Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the Department of Defense."

4) **Duan D.** *Dystrophin gene replacement and gene repair therapy for Duchenne muscular dystrophy in 2016.* **Human Gene Therapy Clinical Development**. 27(1):9-18, **2016**.
"This work was supported in part by the Department of Defense, Duchenne Muscular Dystrophy Research Program (DMDRP), Congressionally Directed Medical Research Programs under Award No. W81XWH-14-1-0302." "Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the Department of Defense."

5) Yue Y, Wasala NB, Bostick B, **Duan D.** *100-fold but not 50-fold dystrophin overexpression aggravates electrocardiographic defects in the mdx model of Duchenne muscular dystrophy.* **Molecular Therapy-Methods & Clinical Development**. 3:16045, **2016**
"This work was supported in part by the Department of Defense, Duchenne Muscular Dystrophy Research Program (DMDRP), Congressionally Directed Medical Research Programs under Award No. W81XWH-14-1-0302." "Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the Department of Defense."

6.2. Educational lay publications (a total of 1)

1) McGreevy JW, **Duan D.** *Understanding the dog model of Duchenne muscular dystrophy.* **Atlas of Science** web site posted on 04/13/16 http://atlasofscience.org/understanding-the-dog-model-of-duchenne-muscular-dystrophy/

6.3. Conference presentations (a total of 9)

1) Chady Hakim, Xiufang Pan, Kasun Kodippili, Thais Blessa, Hsiao T Yang, Gary Yao, Stacey Leach, Craig Emter, Yongping Yue, Keqing Zhang, Sean X Duan, Nalinda Wasala, Gregory Jenkins, Charles R. Legg, Joel S. Schneider, Jeffrey S Chamberlain, and Dongsheng Duan. **Intravenous delivery of a novel micro-dystrophin vector prevented muscle deterioration in young adult canine Duchenne muscular dystrophy dogs** 2016 *19th Annual Meeting of the American Society of Gene & Cell Therapy*. Washington, DC May 4-7, 2016 **(selected for oral presentation)**

2) Chady Hakim, Xiufang Pan, Kasun Kodippili, Thais Blessa, Hsiao T Yang, Gary Yao, Stacey Leach, Craig Emter, Yongping Yue, Keqing Zhang, Sean X Duan, Nalinda Wasala, Gregory Jenkins, Charles R. Legg, Joel S. Schneider, Nora Yang, Jeffrey S Chamberlain, and Dongsheng Duan. **A single intravenous injection of a novel AAV micro-dystrophin vector resulted in extended amelioration of muscle disease in the canine model of Duchenne muscular dystrophy.** 2016 *16th International Workshop on Parvoviruses*. Ajaccio, Corsica, June 19-June 23, 2016 **(selected for oral presentation)**

3) Chady H Hakim, Nalinda B. Wasala, Xiufang Pan, Kasun Kodippili, Yongping Yue, Keqing Zhang, Gang Yao, Joel S. Schneider , Nora Yang , Jeffrey Chamberlain, Dongsheng Duan. **A 5-repeat micro-dystrophin gene ameliorated dystrophic phenotype in the severe DBA/mdx model of Duchenne muscular dystrophy** *2016 16th International Workshop on Parvoviruses*. Ajaccio, Corsica, June 19-June 23, 2016.

4) Xiufang Pan, Nalinda B Wasala, Chady H Hakim, Yongping Yue, Keqing Zhang, John Hu, Dongsheng Duan. **Comparison of 4-repeat and 5-repeat micro-dystrophins in dystrophin deficient mice.** *2016 New Directions in Biology and Disease of Skeletal Muscle Conference*. Orlando, Florida, June 29-July 2, 2016.

5) Nalinda B. Wasala, Yi Lai, Jinhong Shin, Junling Zhao, Yongping Yue, Dongsheng Duan. **Genomic removal of a therapeutic mini-dystrophin gene from adult mice elicits a Duchenne muscular dystrophy-like phenotype.** *2016 New Directions in Biology and Disease of Skeletal Muscle Conference*. Orlando, Florida, June 29-July 2, 2016.

6) Chady H Hakim, Nalinda B. Wasala, Xiufang Pan, Kasun Kodippili, Yongping Yue, Keqing Zhang, Gang Yao, Joel S. Schneider , Nora Yang , Jeffrey Chamberlain, Dongsheng Duan. **A 5-repeat micro-dystrophin gene ameliorated dystrophic phenotype in the severe DBA/mdx model of Duchenne muscular dystrophy.** *2016 New Directions in Biology and Disease of Skeletal Muscle Conference*. Orlando, Florida, June 29-July 2, 2016.

7) Michael Nance, Yongping Yue, Dennis Discher , Dongsheng Duan. **Development of novel adeno-associated virus that are resistant to macrophage phagocytosis.** *2016 New Directions in Biology and Disease of Skeletal Muscle Conference*. Orlando, Florida, June 29-July 2, 2016.

8) Chady Hakim, Xiufang Pan, Kasun Kodippili, Thais Blessa, Hsiao T Yang, Gary Yao, Stacey Leach, Craig Emter, Yongping Yue, Keqing Zhang, Sean X Duan, Nalinda Wasala, Gregory Jenkins, Charles R. Legg, Joel S. Schneider, Nora Yang, Jeffrey S Chamberlain, and Dongsheng Duan. **A single intravenous injection of a novel AAV micro-dystrophin vector resulted in extended amelioration of muscle disease in the canine model of Duchenne muscular dystrophy.** *2016 New Directions in Biology and Disease of Skeletal Muscle Conference*. Orlando, Florida, June 29-July 2, 2016. **(selected for oral presentation)**

9) Dongsheng Duan. **Duchenne muscular dystrophy gene therapy.** *Clinical and Translational Medicine 2016, 5(Supp 1):A16. A One Health overview, facilitating advances in comparative medicine and translational research*. Kansas City, MO August 28-29, 2016.

7. Participants/collaborating organizations:

What individuals have worked on the project?

Name: Dongsheng Duan – "No change"

Name: Craig Emter
Project Role: Co-I
Research Identifier: not applicable
Nearest person month worked: 1
Contribution to the Project: Dr. Emter has performed cardiac echocardiography.

Name: Yi Lai – "No change"

Name: Hsiao Tung "Steve" Yang
Project Role: Research Professor
Researcher Identifier: not applicable
Nearest person month worked: 2
Contribution to the Project: Dr. Yang has helped with AAV delivery, biopsy, necropsy and dog function assay

Name: Yongping Yue – "No change"

Changes in the active other support of the PI and key personnel since the last reporting period.

Dongsheng Duan, PI

Previous/active grants that have closed:
Exploring the possibility of SERCA2a therapy in DMD dogs

10% effort, Duan, PI
Parent Project Muscular Dystrophy
01/01/2015-12/31/2015
This is a small exploratory grant to test SERCA2a therapy in affected dogs.

A pilot study on systemic delivery of an AAV-9 five-repeat micro-dystrophin vector in juvenile DMD dogs
10% effort, Duan, PI
Solid Ventures
06/01/2015-08/31/2016
To establish the proof-of-principle for systemic gene therapy of Duchenne muscular dystrophy using an AAV-9 5-repeat micro-dystrophin vector in the canine model.

A pilot study to evaluate dual AAV mini dystrophin vectors in DMD
3% effort, Duan, PI
Hope for Javier
06/01/2015-05/31/2016
We propose to engineer the third-generation dual AAV minigene vectors to express a larger minidystrophin protein. We further propose to test the third-generation vectors by local injection in one infected dog to generate preliminary data for future NIH application.

AAV gene therapy for Krabbe disease
Steve LeVine, PI
3% effort, Duan, Co-I
KCALSI-Kansas City Area Life Sciences Institute
08/01/2015-07/31/2016
As Co-Investigator, I will construct, produce and purify high quality AAV.galactosylceramidase vectors for Dr. LeVine to test in cellular and animal models of Krabbe disease.

Design low-immunogenic Cas9 for gene repair in the mdx model of DMD
1% effort, Duan, PI
Hope for Javier
10/01/2015-09/30/2016
The goal of this project is to design and evaluate a low-immunogenic Cas9 AAV vector in the hope of applying it for CRISPR/Cas9-mediated gene repair therapy for DMD.

New/active:
Maximum feasible dose study in a canine model of Duchenne muscular dystrophy
10% effort, Duan, PI
Solid Biosciences
07/01/2016-01/14/2017

$80,000 (direct costs)
This study will assess the expression, localization and bio-distribution of canine SGT-001.

R16/17-independent nNOS anchoring mechanism
10% effort, Duan, PI
NIH/NIAMS (R21 AR067985)
04/01/2016-03/31/2018
The goal is to identify the dog nNOS-binding domain and develop relevant gene delivery vectors.

CRISPR/Cas9-based gene editing for the correction of Duchenne muscular dystrophy
Gersbach, PI – Duke University
10% effort, Duan, CO-I
NIH/NIAMS (R01 AR069085)
04/01/2016-03/31/2021
The Duan lab will perform in vivo gene delivery and functional outcome measurements in mice treated by AAV-CRISPR gene repair vectors and if needed will also assist with the production of recombinant AAV vectors.

A pilot study to evaluate long-term safety and efficacy of AAV-9 5Rc micro-dystrophin therapy
5% effort, Duan, PI
Solid Biosciences
06/01/2016-05/31/2019
The overarching goal of this project is to determine whether systemic AAV-9 micro-dystrophin gene therapy can yield long-term (up to 4 years after injection) microgene expression without causing serious adverse events (SAEs).

Treatment of Duchenne muscular dystrophy with the muscle calcium pump
20% effort, Duan, PI
NIH/NIAMS (R01 AR070517)
7/01/2016-08/31/2021
Elevation of cytosolic calcium is a pivotal pathogenic event in Duchenne muscular dystrophy (DMD). We found that sarco/endoplasmic reticulum calcium ATPase 2a (SERCA2a) therapy can reduce muscle disease and improve muscle function in the mouse DMD model. In the proposed study, we will test whether this therapy can treat symptomatic DMD dogs and our results will lay the foundation for a future clinical trial.

Evaluation of the human version second-generation AAV micro-dysrophin vector in adult dystrophic dogs
5% effort, Duan, PI
Jesse's Journey Foundation
07/01/2014-06/30/2017

This is a supplementary grant to the DOD grant. The goal is to develop novel AAV micro-dystrophin vector.

Treating Duchenne cardiomyopathy in the mouse model by gene repair
10% effort, Duan, PI
Department of Defense W81XWH-16-1-0221
08/01/2016-07/31/2019
We propose to test this "permanent exon skipping" therapy to the treatment of Duchenne cardiomyopathy in an authentic mouse model. Our study will open the door to the eventual application of CRISPR/Cas9 therapy in human patients in the future.

Craig Emter, Co-PI

Previous/active grants that have closed:
Saxagliptin Attenuates Cardiac Hypertrophy and Remodeling Induced by Hypertrophic Stimuli
40% effort, Emter, PI
Bristol Myers-Squibb/Astrazeneca
01/01/2013-09/30/2015
Major Goals: To investigate the role of cGMP signaling in the development of cardiac remodeling in heart failure.
No overlap

New/active:
Translational swine model for the study of HFpEF
10% effort, Emter, PI
University of Missouri Research Board
01/01/2015-12/31/2016
Major goals: Pilot clinical and translational studies for developing an obese and diabetic ossabaw swine model of HFpEF.
No overlap

Coronary Dysfunction, BK Channels, & Exercise in Heart Failure
33% effort, Emter, PI
NIH/NHLBI, R01 HL112998
5/1/14-4/30/2019
Major Goals: The goal of this project is to determine the role of the coronary vascular BK_{Ca} channel in the development of heart failure with preserved ejection fraction.

Regulation of Work Capacity in Cardiac Myocytes
McDonald, PI
5% effort, Emter, Co-I
NIH/NHLBI, R01 HL57852-14
3/1/12-3/1/2017
Major Goals: The goal of this project is to investigate the determinants of power output in myocardium.

Pathological mechanisms of sympathetic-mediated cerebrovascular vasoconstriction in heart failure with preserved ejection fraction
5% effort, Emter/Olver, Co-PI's
University of Missouri, Internal
College of Veterinary Medicine COR Faculty Research Program
1/1/16-12/31/16
Major goals: Pilot clinical and translational studies for examining sympathetic nervous system contributions to developing heart failure in a mini-swine model of HFpEF
Role: PI

Mechanisms of sympathetic-mediated cerebrovascular vasoconstriction in heart failure with preserved ejection fraction
AHA Postdoctoral Fellowship (Olver, PI)
0% effort, Emter, Supervising PI
American Heart Association
1/1/2016-12/31/2017
Major goals: Salary support for research career development.

Pathological Mechanisms of Sympathetic-mediated Cerebrovascular Vasoconstriction as a Function of Menopause in Heart Failure with Preserved Ejection Fraction
Olver, PI
0% effort, Emter, Supervising PI
University of Missouri, Internal
MU Interdisciplinary Center on Aging - Research Enrichment and Dissemination (READ) Small Grants Program
1/22/16-12/31/18
Major goals: Pilot clinical and translational studies for examining sympathetic nervous system contributions to developing heart failure in a mini-swine model of HFpEF

Hsiao Tung Yang

Previous/active grants that have closed:
Central and peripheral control mechanisms of hypertension in rat model with peripheral arterial insufficiency
10% effort, Yang, PI
College of Veterinary Medicine faculty research grant, Research Dean's Office, University of Missouri
01/01/2014-12/31/2015
The main goal of this project is to obtain sufficient preliminary data to identify the central and peripheral control mechanisms of hypersympathetic state in animal model of peripheral arterial insufficiency.
There is no overlap with the proposed DOD project.

New/active:
Solid GT Fellowship Award
50% effort, Yang, PI
Solid GT
05/01/2016-04/30/2017
The goal is to support the position of Dr. Hsiao-tung Yang, assisting Dr. Dongsheng Duan in his work helping Solid GT's efforts in gene therapy for Duchenne Muscular Dystrophy. Dr. Yang will be responsible for program management, program execution and study report delivery.

Yi Lai
No change in active other support from previous submission.

Organizations that have involved as partners.

Organization Name: Jesse's Journey
Location of Organization: Canada
Partner's contribution to the project: Financial support

8. Special reporting requirements: None

9. Appendices:

Peer-reviewed publications
Educational lay publication

Journal of Neuromuscular Diseases 2 (2015) 397–407
DOI 10.3233/JND-150114
IOS Press

Research Report

Night Activity Reduction is a Signature Physiological Biomarker for Duchenne Muscular Dystrophy Dogs

Chady H. Hakim[a], Austin A. Peters[a], Feng Feng[b], Gang Yao[c] and Dongsheng Duan[a,*]
[a]*Department of Molecular Microbiology and Immunology, School of Medicine, University of Missouri, Columbia, MO, USA*
[b]*Department of Electrical and Computer Engineering, College of Engineering, University of Missouri, Columbia, MO, USA*
[c]*Department of Bioengineering, College of Engineering, University of Missouri, Columbia, MO, USA*

Abstract.
Background: Duchenne muscular dystrophy (DMD) is an X-linked lethal muscle disease. Dystrophic dogs are excellent models to test novel therapies for DMD. However, the use of the dog model has been hindered by the lack of an effective method to evaluate whole-body mobility. We recently showed that night activity is a good indicator of dog mobility. However, our published method relies on frame-by-frame manual processing of a 12-hour video for each dog. This labor-intensive and time-consuming approach makes it unrealistic to use this assay as a routine outcome measurement.
Objective: To solve this problem, we developed an automatic video-capturing/imaging processing system. The new system reduces the data analysis time over 1,000 fold and also provides a more detailed activity profile of the dog.
Methods: Using the new system, we analyzed more than120 twelve-hour recordings from 12 normal and 22 affected dogs.
Results: We observed similar activity profiles during repeated recording of the same dog. Throughout the night, normal dogs were in motion $10.4 \pm 0.9\%$ of the time while affected dogs were in motion $4.6 \pm 0.2\%$ of the time ($p < 0.0001$). Further, normal dogs made significantly more movements ($p < 0.0001$) while affected dogs rested significantly longer ($p < 0.0001$) during the period of recording (from 6 pm to 6 am next day). Importantly, statistical significance persisted irrespective of the coat color, gender and mutation type.
Conclusions: Our results suggest that night activity reduction is a robust, quantitative physiological biomarker for dystrophic dogs. The new system may be applicable to study mobility in other species.

Keywords: Duchenne muscular dystrophy, DMD, dystrophin, dog, canine model, biomarker, night activity, mobility, muscle function

INTRODUCTION

Duchenne muscular dystrophy (DMD) is a devastating muscle-wasting disease that affects boys and young men and leads to premature death [1]. DMD is caused by the loss of a subsarcolemmal cytoskeletal protein called dystrophin. Dystrophin is an essential component of the dystrophin-associated glycoprotein (DGC) complex that preserves the integrity of the muscle cell membrane. In the absence of dystrophin, the cell membrane becomes fragile and cannot sustain shearing stress generated during muscle contraction. This results in membrane damage and myofiber degeneration. Eventually the dead muscle cells are replaced by connective tissue and affected individuals lose their mobility.

*Correspondence to: Dongsheng Duan, Ph.D., Professor, Department of Molecular Microbiology and Immunology, One Hospital Dr., Columbia, 65212 MO, USA. Tel.: +1 573 884 9584; Fax: +1 573 882 4287; E-mail: duand@missouri.edu.

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Current treatments for DMD are limited to corticosteroids and symptom management [2]. Tremendous progress has been made over the last decade in the development of novel pharmacologic and genetic therapies for DMD [3–5]. Most of these experimental treatments show promising efficacy in dystrophin-null mdx mice. However, mouse results often translate poorly to DMD patients due to the lack of the dystrophic phenotype in mdx mice, differences in immune responses between mice and humans, and/or the failure to scale-up from mice to large mammals [6]. Dystrophin-deficient dogs show characteristic symptoms of muscular dystrophy and dogs also have the scale-up advantage [7]. Results from studies performed in the canine DMD model may better inform the design of human trials.

Dystrophin deficiency has been described in more than 20 different dog breeds. Experimental DMD dog colonies have also been established in many places around the world [7]. However, there are limited tools to study muscle function in dogs. We recently developed an *in situ* protocol to evaluate contractility of a single dog muscle [8]. Kornegay and colleagues reported an assay for measuring hindlimb force in dogs [9]. Yet, there is no method to objectively study whole body mobility in dogs. To address this issue, we recently tested the hypothesis that spontaneous movement at night can serve as a physiological biomarker to objectively evaluate dog mobility. As a pilot study, we recorded night activity in a litter of three affected and four normal dogs [10]. We then converted the video to images (one frame every three seconds) and determined dog movement by manually quantifying differences between neighboring frames. This pilot study, though labor-intensive and time-consuming, has provided the critical preliminary data supporting our hypothesis that night activity is a useful endpoint for studying dog mobility.

Several questions were not answered in our pilot study. Specifically, it was not clear whether reproducible data could be obtained from the same dog on different days, whether dogs of different kindreds could yield consistent results, and whether reduced activity is a common finding in all dystrophic dogs irrespective of the gender and genotype type. The pilot study also had several inherent constraints such as the small sample size, cumbersome method and limited output parameters. To address all these issues, we developed a computer-based system that can automatically record and analyze spontaneous movements at night in canines. The new system allows quantification of dog mobility using a comprehensive set of metrics. The results from the new system confirm and expand our initial observation [10]. Together these data suggest that night activity is a robust whole body mobility biomarker for dogs. The new system will greatly facilitate ongoing and future translational studies in the canine DMD model [11].

MATERIALS AND METHODS

Animals

Animal housing and experiments were approved by the Animal Care and Use Committee of the University of Missouri and were performed in accordance with guidelines of the National Institutes of Health and Department of Defense. All experimental dogs were on a mixed genetic background of golden retriever, Labrador retriever, beagle, and Welsh corgi. There are three different types of dystrophin gene mutations in affected dogs including type G (intron 6 point mutation identical to that of golden retriever muscular dystrophy dogs), type L (intron 19 insertion identical to that of Labrador retriever muscular dystrophy dogs) and type W (intron 13 insertion identical to that of Welsh corgi muscular dystrophy dogs) [12–15]. Experimental dogs were generated by in house artificial insemination. Specifically, fresh or frozen semen from an affected dog (genotype YG, YL, YW) were injected into the uterus of a carrier (genotype XG, XL, XW) twice at 48 h and 72 h post-ovulation. The dog coat color was determined according to Wikipedia (https://en.wikipedia.org/wiki/Coat_%28dog%29).
The genotype was determined by polymerase chain reaction as we previously described [12, 13]. The diagnosis was further confirmed by the significantly elevated serum creatine kinase (CK) level in affected dogs. Night activity in 12 normal male (mean age, 14.8 ± 1.9 months; range, 8.0 to 22.9 months), 11 affected male (mean age, 18.2 ± 1.3 months; range, 9.7 to 23.3 months) and 11 affected female (mean age, 12.6 ± 1.8 months; range, 6.8 to 19.9 months) dogs were recorded and analyzed. These dogs were from 27 different litters. All experimental dogs were housed in specific-pathogen free animal care facilities and kept under a 12-hour light/12-hour dark cycle.

Video recording equipment

A low-light charge-coupled device (CCD) camera (PC164C, Supercircuits Inc., Austin, TX) was used for overnight video recording. The video camera was equipped with an infrared lens (Part #12VM412ASIR,

Tamron, Commack, NY) and a near infrared (NIR) light source (Part # IR045, Clover electronics USA, Cerritos, CA).

Video recording conditions

The dog was housed individually in a kennel (181 cm × 122 cm) with an elevated floor (Fig. 1A). The video camera was centered and secured to the ceiling on top of the kennel through a custom-made mounting bracket. Rubber dampers were added to the bracket to isolate any vibration from the ceiling to the camera. The camera and light source were positioned at 210 cm above the kennel floor to ensure a full view and even illumination of the cage floor (Fig. 1A). Spontaneous movement of the dog was recorded during the dark cycle from 6 pm to 6 am next day. During this period, there was no interference from environment cues or animal caregivers. Temperature and humidity were controlled and monitored during the recording period. The dog had free accessed to water but food was removed from the kennel.

Video recording

The CCD camera was controlled by the Overnight Dog Recording software, a program developed in Labview (National Instrument Inc., Austin, TX) (Fig. 1A). The program automatically started the recording system at 5:30 pm to warm up the hardware. Each session of recording was started at 6 pm and continued for 12 hrs. The frame rate was set to 3 frames per second. The recorded video was streamed to the hard drive of the control computer. The motion signal S(t) was defined as the signal difference between every two consecutive images. It was computed according to the following formula

$$S(t) = \frac{\sum_{i,j} |I(i, j, t + \Delta t) - I(i, j, t)|}{N_{i,j}} \qquad (1)$$

where $I(i, j, t)$ is the image pixel value at the pixel location (i, j) and acquired at the time point t. Δt is the time interval between two consecutive image frames. $I(i, j, t + \Delta t)$ is the image pixel value at the pixel location (i,j) and acquired at the time point t+Δt. $N_{i,j}$ is the total number of pixels in the image and it is equal to 640 × 480 in our system. If the dog does not move, the two images should be the same and I(i,j,t) will be equal to $I(i, j, t + \Delta t)$. The motion signal S(t) will be zero. When the dog moves, the difference of the dog position results in a change of the image pixel value between $I(i, j, t)$ and $I(i, j, t + \Delta t)$. The change of the image pixel values produces a S(t) value to reflect the movement.

Processing of the recorded video

The recorded S(t) was analyzed by the *Overnight Dog-Video Analyzer*, a software program developed in Matlab (Mathworks Inc., Natick, MA) (Fig. 1A). Ideally, the baseline should be zero when the dog is not moving. However the recorded baseline always fluctuated and drifted with time due to various noises from the video recording system and environment (such as air ventilation, building vibration and thermal noise of the electronics). To correct the baseline drift, the signal baseline was extracted by removing all motion-induced signal peaks from the raw signal. The missing baseline segments corresponding to the removed motion signals were reconstructed using a piecewise cubic interpolation algorithm. The resulting baseline was further smoothed by applying a 7th order Savitzky-Golay filter followed by a median filter, both with a window size of 1.1 sec (33 data points). This reconstructed baseline was then subtracted from the raw signal to create a flat baseline. Finally, a motion threshold was applied to remove noisy spikes that were not considered as true motion signals (Fig. 1B). Signals that are above this threshold were considered as motion spikes.

Definition of the movement and rest

The processed motion signals showed three types of motion spike(s) including a single motion spike, a stretch of continuous non-interrupted spikes and a stretch of spikes with $\leq$10 sec interruptions between neighbouring spikes (Fig. 1C). The 10 sec value was chosen based on a pilot study. Each identifiable type of spike (or spikes) was defined as a single movement. The interval between two consecutive movements was defined as rest. By definition, a rest is always longer than 10 sec.

Motion quantification metrics

A series of metrics were used to comprehensively evaluate dog night activity. These included the percentage of time in motion (%; the percentage of the time the dog was in motion during the entire 12-hour recording period), amplitude of the movement (arbitrary unit; relative signal strength of a motion spike), number of movement per hour (Nm/hr), average duration of a single movement (sec) and average duration of a single rest (sec). Based on the duration, a movement was

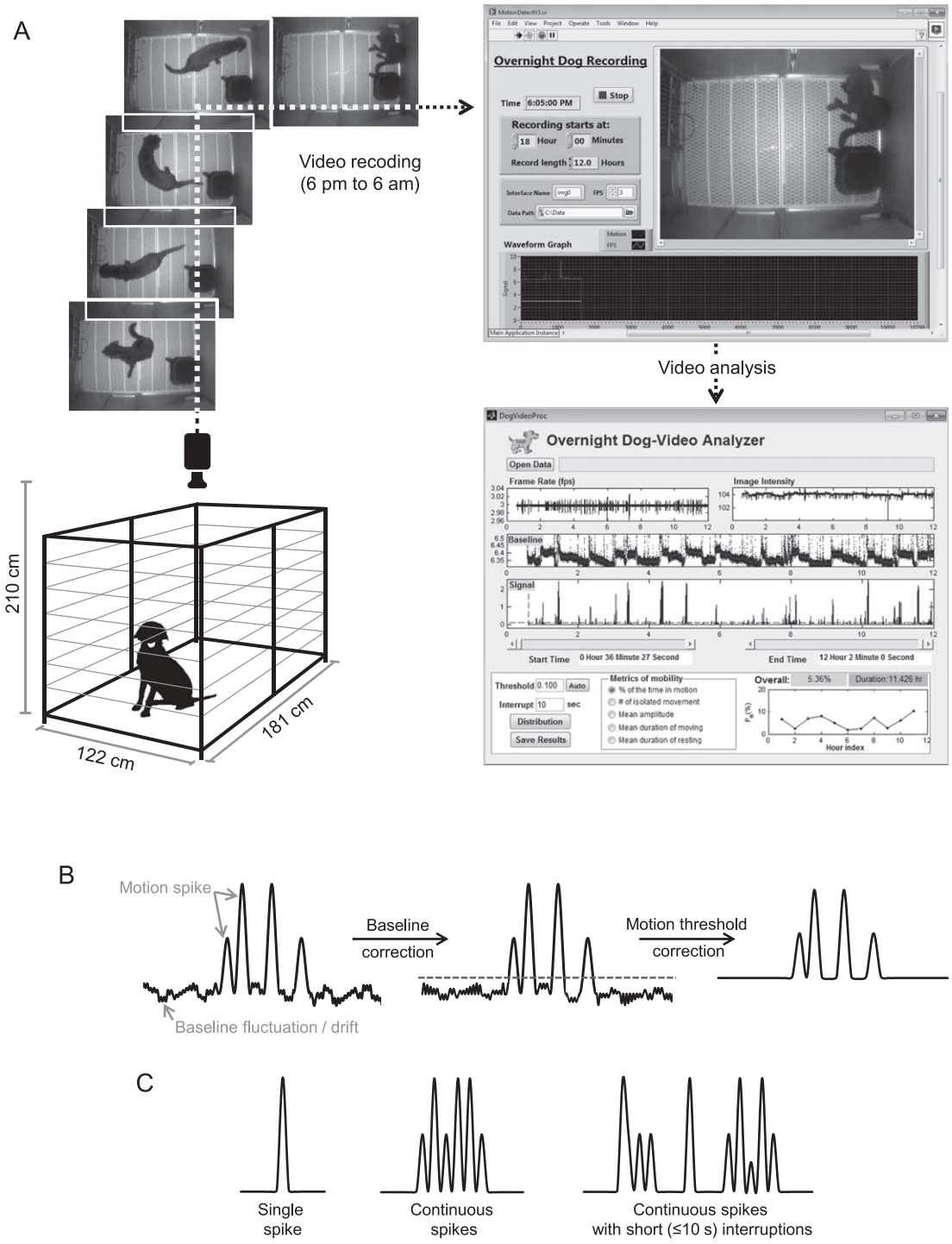

Fig. 1. Dog night activity evaluation system. A, Overview of the system. The experimental dog was housed in the recording kennel. The CCD camera was installed to the ceiling allowing full visualization of the entire floor. Dog activity was recorded from 6 pm to 6 am next day by the "overnight dog recording" system. The signals were sent to the "overnight dog-video analyzer" for activity evaluation. B, Video processing outline. First, the baseline drift of the raw motion signals was corrected. Then noisy spikes were eliminated by the motion threshold (gray dotted line). C, A cartoon illustration of three types of motion spike(s): a single isolated spike, a stretch of continuous spikes and a stretch of spikes with short ($\leq$10 s) interruptions.

further divided into short-duration movement (≤10s) and long-duration movement (>10s). To gain more detailed understanding on the movement, we further quantify the frequency of short and long-duration movements per hour and expressed these parameters as "number of short movement/hour" and "number of long movement/hour", respectively.

Statistical analysis

Data are presented as mean ± standard error of mean. Statistical difference was assessed with the Student's *t*-test followed by Bonferroni correction to correct familywise error caused by multiple two-group comparisons. The *p* value is presented in the figure legend.

RESULTS

Night activity analyzer is a robust system to evaluate dog mobility

To objectively evaluate dog mobility, we intentionally performed recording at night when there was neither human nor environment interference. To eliminate potential influences from changes in temperature and humidity, we also maintained a constant temperature (23.8 ± 0.2°C) and humidity (40.2 ± 1.5%) in the kennel.

To determine whether our newly developed software can consistently record and analyze dog activity, we performed repeated recording. Specifically, the same dog was recorded multiple times during an 11-day period (2 to 10 times, either daily or in different days). The percentage of time in motion was quantified for each recording and compared with the first night recording. Consistent activity was observed for both normal and affected dogs during repeated recording (Fig. 2). For normal dogs, the difference (compared with the result of the first night recording) was ≤28% when the same dog was recorded at different nights. For affected dogs, the difference (compared with the result of the first night recording) was ≤18% when the same dog was recorded at different nights.

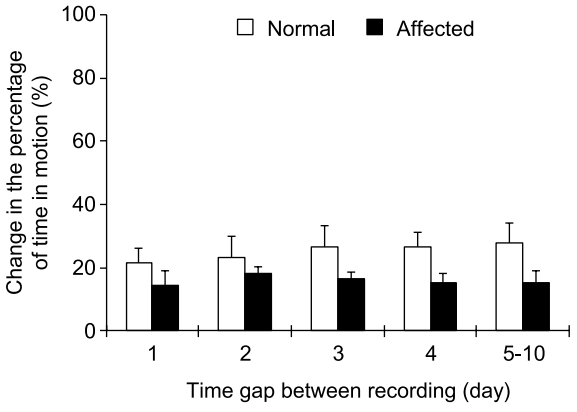

Fig. 2. Repeated recording yielded consistent results from the same dog. The dog was recorded at different nights (either continuously every night or at alternating nights). The results of subsequent recordings were compared to that of the first night. The graph shows repeated recording results from 11 normal dogs and 10 affected dogs.

Dystrophin deficiency significantly reduced night activity in affected dogs

To determine whether night activity can serve as a robust physiological biomarker for DMD dogs, we initiated a comprehensive study with a large cohort of crossbred dogs. These included 12 male normal dogs (age 14.7 ± 1.9 months) and 22 mixed-gender affected dogs (age 15.4 ± 1.24 months). They came from 27 different litters. Figure 3A showed representative processed 12-hr recordings from a normal and an affected dog. Throughout the night, normal dogs were in motion 10.4 ± 0.9% of the time while affected dogs were in motion only 4.6 ± 0.2% of the time ($p < 0.00001$) (Fig. 3B). In other words, normal dogs moved approximately 75 min and affected dogs moved approximately 33 min during 12-hr recording. On average, normal dogs moved 28.3 ± 1.3 times per hour while affected dogs only moved 14.1 ± 0.4 times per hour ($p < 0.00001$) (Fig. 3C). Additional analysis showed that normal dogs not only made significantly more short movements but also made significantly more long movements ($p < 0.00001$) (Fig. 3D and E). Interestingly, the average amplitude of movement was similar between normal (0.60 ± 0.08) and affected (0.56 ± 0.04) dogs ($p = 0.68$) (Fig. 3F). Further, there was no significant difference in the average duration of movement between normal and affected dogs (Fig. 3G). In contrast to the duration of movement, the average duration of rest in affected dogs (243.1 ± 0.6 sec) was significantly longer than that of normal dogs (111.5 ± 4.5 sec) ($p < 0.00001$) (Fig. 3H).

As a consequence of crossbreeding, our study dogs displayed different coat colors which produced different pixel intensities in the video. To determine whether the coat color influenced the outcome, we grouped normal and affected dogs based on their color. Affected dogs consistently showed reduced activity irrespective of the coat color (Supplementary Figure 1).

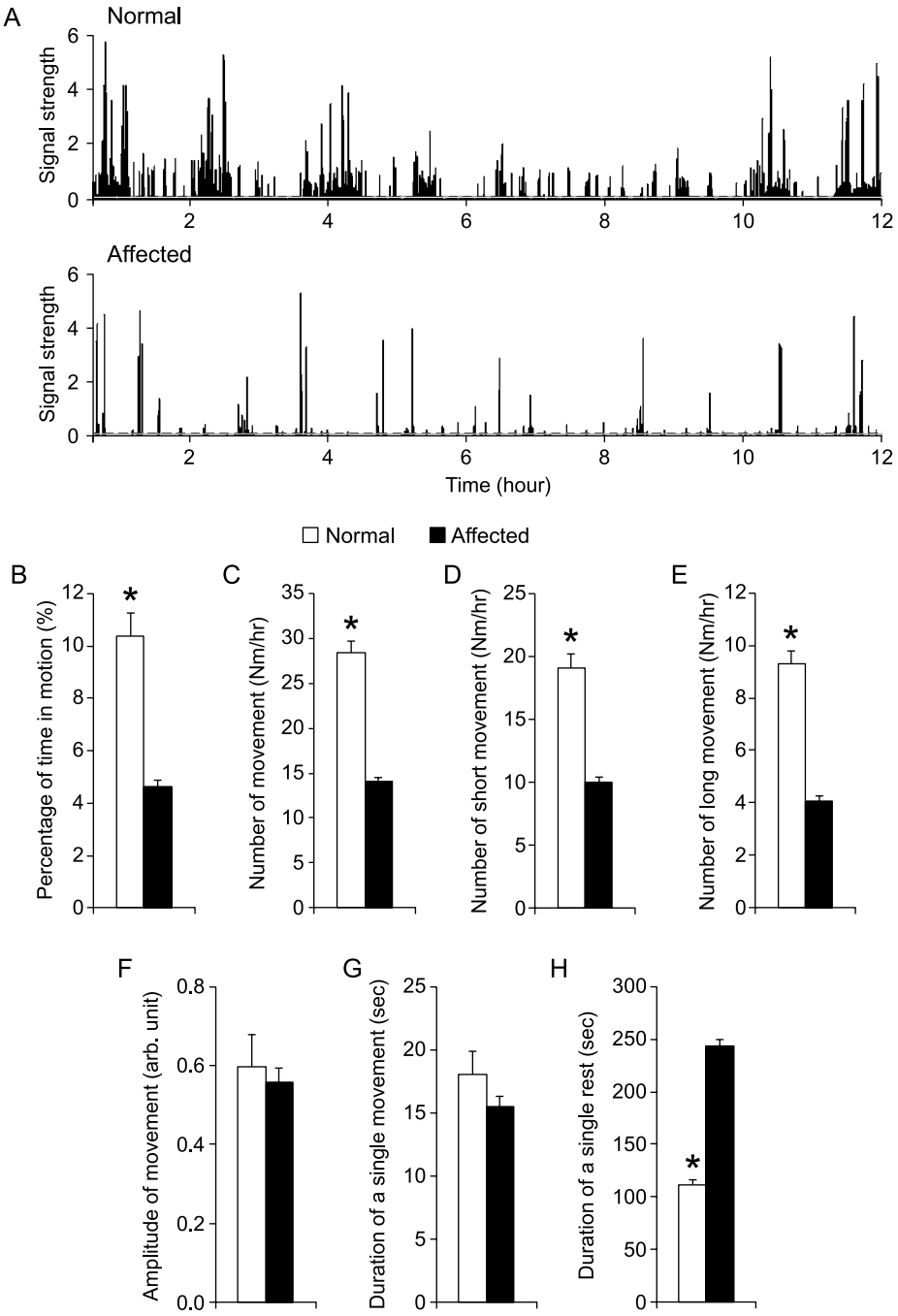

Fig. 3. Night activity was significantly reduced in affected dogs. A, Representative processed motion signals from a normal (top panel) and an affected dog (bottom panel). The normal dog showed more activity than the affected dog. Dotted line, motion threshold. B to H, A quantitative comparison of indicated night activity property between normal ($n = 12$) and affected ($n = 22$) dogs. Asterisk, $p < 0.0001$.

Gender had minimal impact on night activity

The normal group consisted of male dogs only while the affected group included both male and female dogs. To determine whether gender had confounded our initial analysis (Fig. 3), we removed female dogs from the affected group and re-analyzed the data (Fig. 4). Impressively, normal male and affected male comparison resulted in a pattern nearly identical to that of the initial analysis (Figs. 3 and 4).

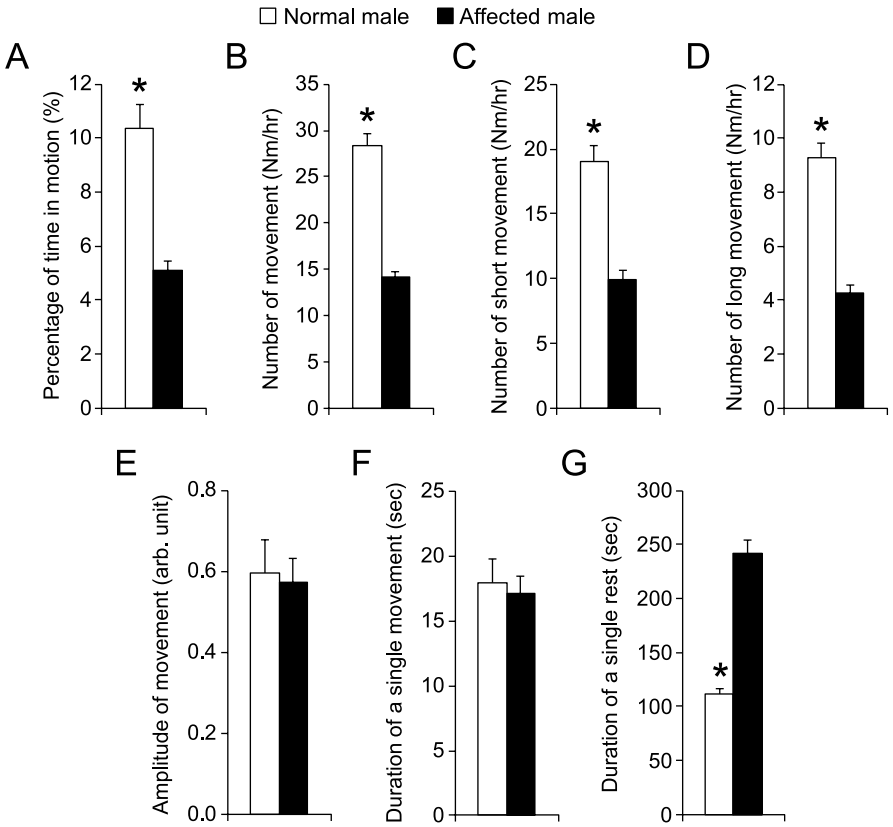

Fig. 4. Comparison of night activity between normal and affected male dogs. $n = 12$ for normal male, $n = 11$ for affected male. Asterisk, $p < 0.0001$.

To further characterize the potential influence of the gender, we compared results of male affected and female affected dogs (Fig. 5). Affected male dogs showed slightly more movement than affected female dogs (the percentage of time in motion was $5.1 \pm 0.4\%$ and $4.1 \pm 0.3\%$ for affected male and female, respectively; $p = 0.03$) (Fig. 5A). This appeared to have resulted from a slightly longer movement time in males (the average duration of a single movement was 17.1 ± 1.4 sec and 13.8 ± 0.8 sec for affected male and affected female, respectively; $p = 0.05$). No difference was noticed in other parameters between affected male dogs and affected female dogs.

Affected dogs showed reduced night activity irrespective of the mutation type

There are three different types of dystrophin gene mutations in our affected dogs including type G, L and W [13–15]. In our study, all affected males carried type G mutation in their X-chromosome. However, all affected females carried two different types of dystrophin gene mutations, one on each chromosome. Comparison between male and female affected dogs (Fig. 4) suggests that the type of gene mutation had minimal impact on night activity. To confirm this observation, we performed detailed analysis in female affected dogs. Among 11 affected females, six had the genotype of LW and four had GW. No difference was seen in any metrics between LW and GW affected dogs (Fig. 6).

DISCUSSION

In this study, we developed a robust automatic video capturing/processing system to quantify dog mobility at night. A large cohort of crossbred normal and DMD dogs were evaluated using this system. We found that normal dogs had significantly more movements and shorter rests. In contrast, affected dogs displayed a significantly reduced night activity irrespective of the coat color, gender and type of mutations in the dystrophin gene.

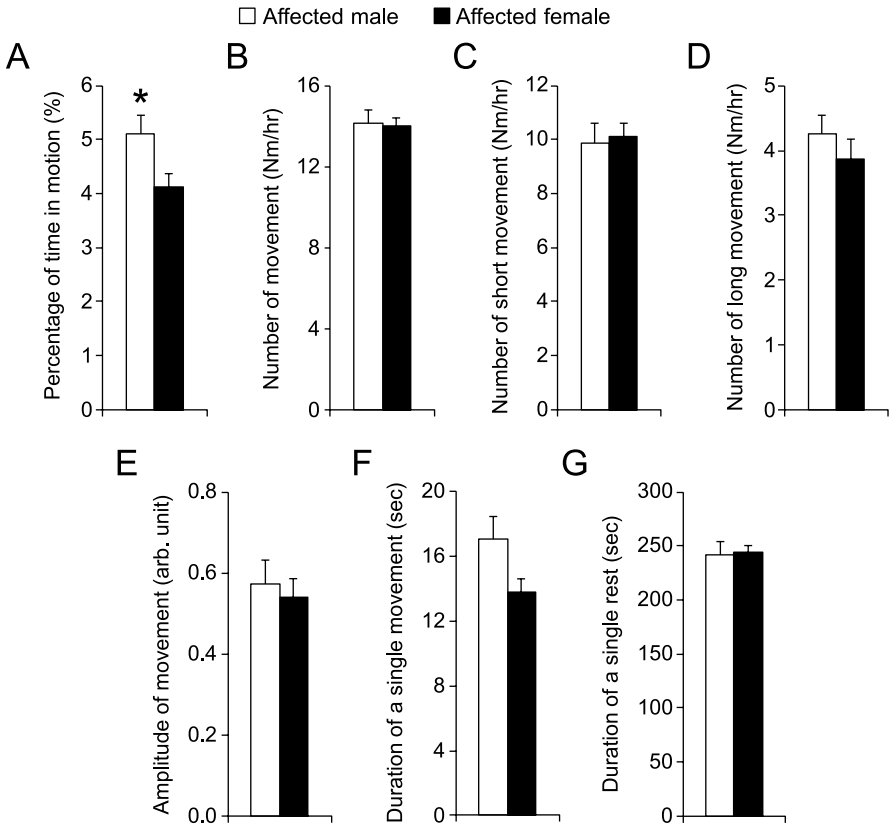

Fig. 5. Comparison of night activity between male and female affected male dogs. $n = 11$ for affected male, $n = 11$ for affected female. Asterisk, $p < 0.0001$.

Dystrophin-deficient dogs have been considered an excellent large animal model for DMD because of their striking clinical and pathological similarity to human patients. However, the research use of affected dogs has been limited by the lack of physiological assays that can reliably quantify muscle function. To address this issue, investigators have begun to adopt *in situ* protocols that are commonly used in rodent studies [8, 9, 16, 17]. While these methods allow detailed characterization of the contractile profile of a single muscle or a group of muscles, there are important limitations. First, these assays require the subject be anesthetized. Unfortunately, DMD patients and affected dogs are especially vulnerable to anesthesia-associated risks such as sudden cardiac arrest and rhabdomyolysis [18–21]. Second, these assays are often limited to limb muscle and cannot provide information on whole body mobility. Third, some of these assays (such as single muscle *in situ* force measurement) are terminal assays and can only be performed once in a subject before euthanization [8]. Non-invasive goniometry and accelerometry were developed recently to evaluate the range of motion of a joint and the gait pattern [10, 22–25]. These non-invasive assays had minimum risks and they can also be performed repeatedly in the same dog to monitor disease progression or responses to experimental therapy. However, the results obtained from kinematic studies could be influenced by training, environment, experiment conditions (such as the use of treats) and people involved in the study (caregivers and investigators). These interferences may introduce subjective bias. A truly objective assay would require evaluating dog mobility in its natural status (without the need of dog training and without interference from the environment and people). We reasoned that monitoring dog activity at night might accomplish this goal. We hypothesized that muscle degeneration and inflammation should compromise night movement in affected dogs. To test this hypothesis, we conducted in a pilot study and quantified night video recordings frame-by-frame manually [10]. To minimize the labor and the potential influence of the genetic background and age, we only evaluated three affected and four normal dogs from the same litter. Despite the small sample size, we

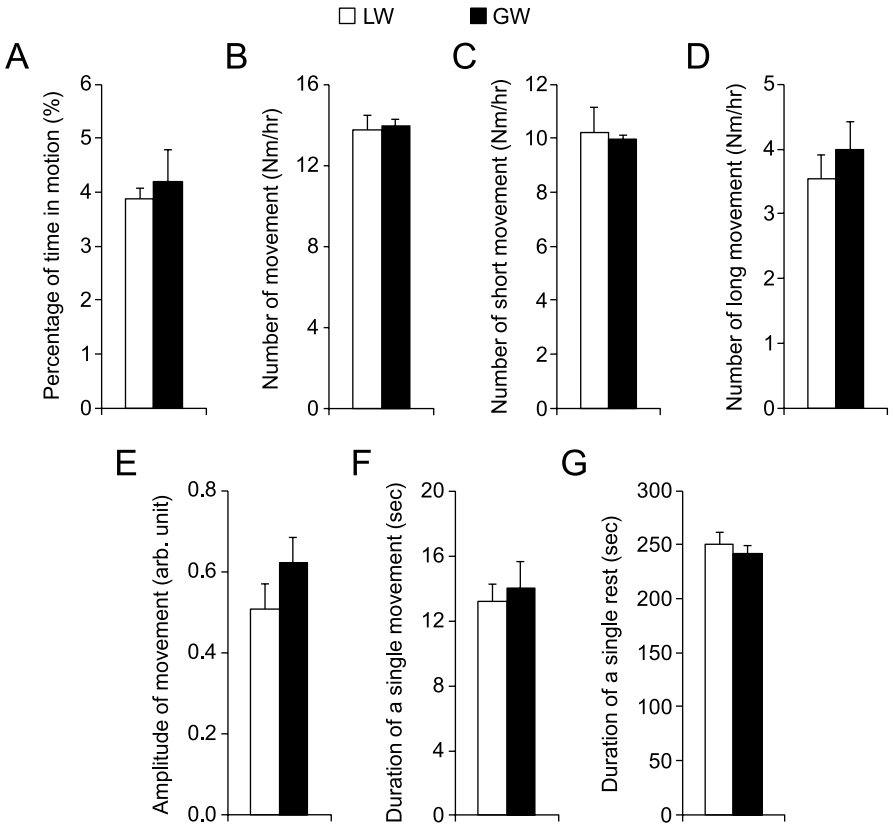

Fig. 6. Comparison of night activity in female affected dogs that had different mutations in the dystrophin gene. LW, affected female dogs with one X-chromosome carrying intron 19 insertion and another X-chromosome carrying intron 13 insertion. GW, affected female dogs with one X-chromosome carrying intron 6 point mutation and another X-chromosome carrying intron 13 insertion. Asterisk, $p < 0.0001$.

found that the relative movement of affected dogs was significantly reduced [10].

To confirm this preliminary finding, we decided to expand the study to a large cohort of adult dogs from different litters. In preparation for this large sample-size study and also to facilitate easy application of the protocol by other investigators in the future, we streamlined the video recording and analysis procedures. Instead of manual recording and analysis, we developed the *Overnight Dog Recording* software and the *Overnight Dog-Video Analyzer* to control the entire process of video recording and to perform subsequent video quantification (Fig. 1). These software programs greatly reduce the labor. Further, they yield a set of metrics for comprehensive analysis. We can now not only quantify overall movement (percent of time in motion and number of movements) during the entire 12-hour recording period but also perform a detailed analysis of each individual movement (such as the type, amplitude and duration of the movement). A robust reliable protocol should yield consistent results on repeated measurements. We tested the same dog at different days (Fig. 2). Despite some changes (especially for the normal dog), overall there were minimal day-to-day variations suggesting our new system is highly reliable.

The ultimate goal of our study is to determine whether the muscle health of the dog correlates with night activity. We studied 34 dogs from 27 litters of different kindreds. As expected for diurnal animals, dogs were in sleep (without movement) most part of the night (Fig. 3A and B). Consistent with the data of the pilot study [10], night mobility of affected dogs was significantly reduced (Fig. 3). This is reflected in the total amount of time in motion, number of movements and duration of the rest. Overall, the percent of time in motion and number of movements was reduced by 50% while the duration of the rest was doubled in affected dogs. Surprisingly, the amplitude and the duration of the movements were not altered in affected dogs. The exact reasons for these observations are not clear but they may relate to neuronal control of sleep (hence cannot be influenced by muscle health). Alternatively,

they may reflect inherent limitations of the software. For example, the current version of the software cannot discriminate a small-scale fast movement (such as the rapid wriggling of the dog tail) and a large-scale slow movement (such as when a dog moves its entire body slowly over a short distance).

DMD is a worldwide disease. It affects all human races. While the vast majority of DMD patients are male, female could also be affected if the dystrophin gene in both X-chromosomes is mutated or when random X-chromosome inactivation is skewed in a carrier [26–28]. Further, more than 7,000 different mutations have been reported in the dystrophin gene [29]. A robust assay for DMD should differentiate normal versus affected irrespective of their skin color, gender and the type of mutation the patient has. To this end, we analyzed on the impact of the coat color, sex and gene mutation (Figs. 4 to 6, Supplementary Figure 1). None of these factors significantly changed the outcome.

There are also several limitations. First, the loss of dystrophin also affects cognitive functions. It is possible that the observed differences may reflect a combined effect of dystrophin deficiency in muscle as well as in the central nerve system. Second, the muscle groups involved in different types of movement (such as short movement versus long movement, single spike versus continuous spikes) remain to be defined. Third, the results are obtained from a single colony. Additional studies are needed to see if similar findings exist in other dystrophic dog colonies. Fourth, we observed statistically significant differences between normal and affected dogs. However, our study did not establish the overnight activity as a valid surrogate end point. Future studies are needed to determine if a therapy that increases muscle force also results in improvement in overnight activity.

In summary, our results suggest that night activity reduction is a signature functional biomarker for DMD dogs. The newly developed video system is an easy-to-use, cost-effective and highly reliable platform to objectively study dog (and potentially other species) mobility. The night activity monitoring should be included in future studies to help assess disease progression and therapeutic intervention in the canine DMD model.

COMPETING FINANCIAL INTERESTS

D.D. is a member of the scientific advisory board for Solid GT, LLC, a venture company founded to advance gene therapy for DMD.

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SUPPLEMENTARY MATERIAL

The supplementary table and figure are available in the electronic version of this article: http://dx.doi.org/10.3233/JND-150114.

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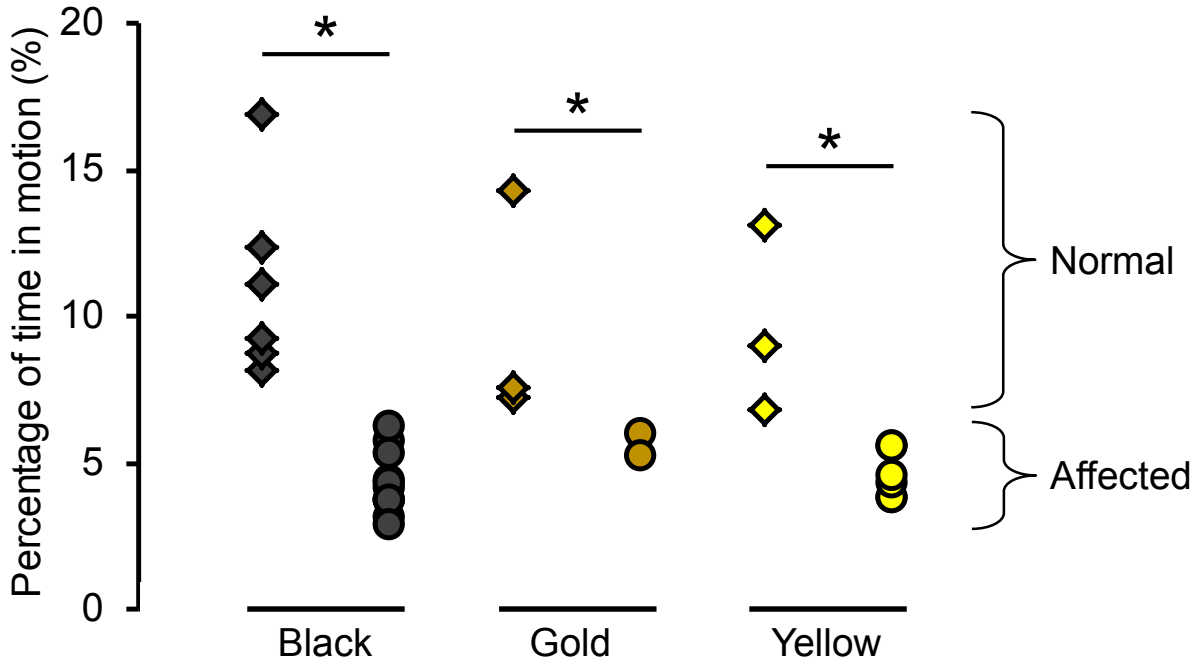
Supplementary Figure 1



REVIEW

Perspective on Adeno-Associated Virus Capsid Modification for Duchenne Muscular Dystrophy Gene Therapy

Michael E. Nance and Dongsheng Duan*

Department of Molecular Microbiology and Immunology, School of Medicine, University of Missouri, Columbia, Missouri.

Duchenne muscular dystrophy (DMD) is a X-linked, progressive childhood myopathy caused by mutations in the *dystrophin* gene, one of the largest genes in the genome. It is characterized by skeletal and cardiac muscle degeneration and dysfunction leading to cardiac and/or respiratory failure. Adeno-associated virus (AAV) is a highly promising gene therapy vector. AAV gene therapy has resulted in unprecedented clinical success for treating several inherited diseases. However, AAV gene therapy for DMD remains a significant challenge. Hurdles for AAV-mediated DMD gene therapy include the difficulty to package the full-length dystrophin coding sequence in an AAV vector, the necessity for whole-body gene delivery, the immune response to dystrophin and AAV capsid, and the species-specific barriers to translate from animal models to human patients. Capsid engineering aims at improving viral vector properties by rational design and/or forced evolution. In this review, we discuss how to use the state-of-the-art AAV capsid engineering technologies to overcome hurdles in AAV-based DMD gene therapy.

INTRODUCTION

DUCHENNE MUSCULAR DYSTROPHY (DMD) is the most common childhood muscle disease. It is characterized by progressive muscle weakness, loss of ambulation, and premature death caused by respiratory muscle and/or heart failure. DMD results from the loss of dystrophin, an essential cytoskeletal protein that protects muscle from contraction-induced injury (Fig. 1A). Soon after the discovery of the *dystrophin* gene,[1,2] it was postulated that expression of a functional *dystrophin* gene in muscle may provide a cure for this relentless disease.[3] Over the years, a number of nonviral and viral vectors have been explored to deliver the *dystrophin* gene. Currently, adeno-associated virus (AAV) stands out as the leading candidate vector.[4,5]

AAV is a dependent parvovirus with an ~5 kb single-stranded linear DNA genome.[6] Wild-type AAV has two major open reading frames (ORFs) flanked by two inverted terminal repeats (ITRs). The 5′ and 3′ ORFs encode replication and capsid proteins, respectively.[7] The ITR contains 145 nucleotides and serves as the AAV genome replication origin and packaging signal (Fig. 1B). In recombinant AAV, viral ORFs are replaced by the exogenous gene expression cassette, while the replication and capsid proteins are provided *in trans* (Figs. 1C and D). AAV has many appealing features as a gene therapy vector. For example, it has broad tissue tropism and high transduction efficiency. AAV can result in long-term persistent episomal expression.[8,9] In addition, wild-type AAV is not associated with any known human diseases, and recombinant AAV vectors have shown an excellent safety profile in many clinical trials.

Despite many appealing features, recombinant AAV vectors face several challenges for DMD gene therapy. First, AAV has a fairly limited packaging capacity. Two-thirds of the dystrophin coding sequence has to be removed in order to fit into an AAV particle. Second, the dystrophic muscle is an extremely hostile microenvironment for gene transfer (Fig. 1A). Myofiber degeneration and necrosis may result in the loss of the vector genome.[10,11] Inflammation in the muscle further intensifies the immune response. Third, muscle consists of ~40–50% the body mass and is spread

*Correspondence: Dr. Dongsheng Duan, Department of Molecular Microbiology and Immunology, University of Missouri School of Medicine, One Hospital Dr. M610G, MSB, Columbia, MO 65212. E-mail: duand@missouri.edu

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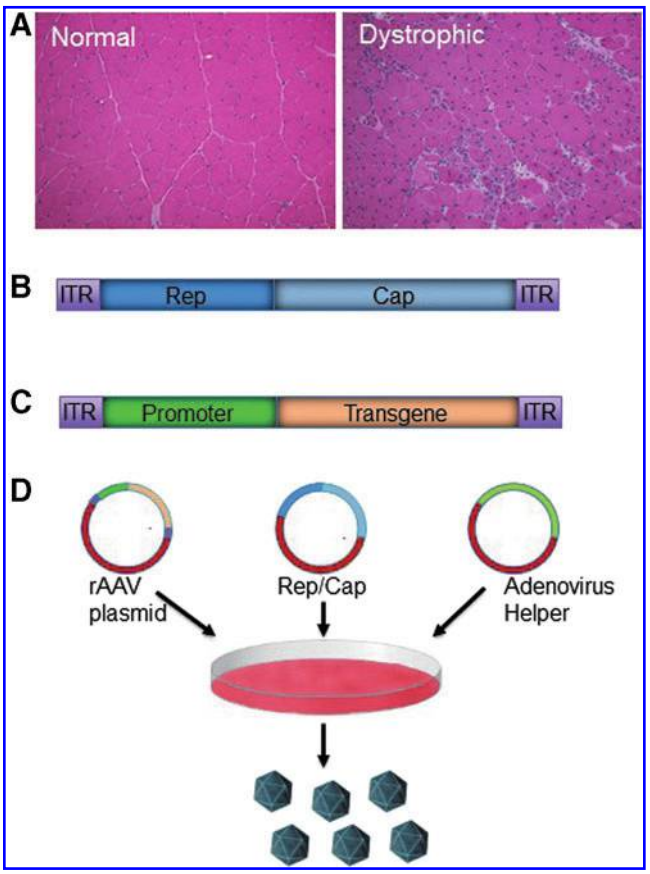

Figure 1. Adeno-associated viral vector (AAV) for Duchenne muscular dystrophy (DMD) gene therapy. The ultimate goal of AAV-mediated DMD gene therapy is to deliver a therapeutic gene using the gutless recombinant AAV (rAAV) to achieve bodywide, robust, and persistent gene transfer in highly inflammatory and degenerative dystrophic muscle and heart to improve muscle and heart function, life quality, and lifespan. **(A)** Representative histology images from normal (left panel) and dystrophic (right panel) muscle. Normal muscle has well-organized, uniform myofibers with peripherally placed nuclei. Dystrophic muscle shows myofiber disorganization, centrally localized nuclei, and abundant infiltration of inflammatory cells. **(B)** Schematic outline of the wild-type AAV genome. The wild-type AAV has an ~4.6–4.8 kb genome. It contains a *Rep* gene for viral replication and a *Cap* gene to generate viral capsid. Two inverted terminal repeats (ITRs) are positioned at the ends of the viral genome. ITR serves as the replication origin and packaging signal. **(C)** Schematic outline of the rAAV genome. The AAV vector is essentially gutted. A therapeutic expression cassette replaces the wild-type *Rep* and *Cap* genes. The only viral component in the rAAV genome is ITR. **(D)** The most commonly used AAV vector production method is triple-plasmid transfection in 293 cells. A *cis* rAAV plasmid carries the rAAV genome. A *trans* Rep/Cap plasmid expresses the replication and capsid proteins. Because the reproductive life cycle of AAV requires the help of adenovirus, an adenovirus helper plasmid is included in the transfection cocktail. AAV vector is purified using isopycnic ultracentrifugation and/or chromatography. Color images available online at www.liebertpub.com/hum

throughout the body. An effective gene therapy will require efficient systemic delivery to a variety of muscle groups, including the heart and diaphragm. Bodywide gene transfer brings in the issue of vector genome sequestration in nonmuscle tissues and off-target transduction. Fourth, 30–80% of the human population has preexisting serum antibodies to various AAV serotypes. There is also a high-level cross-reactivity among different AAV serotypes.[12] Capsids with distinctive serological properties will be needed to bypass these obstacles. Last but not least is the species-specific barrier. This not only refers to the scale-up of AAV production and delivery to large species (dogs, pigs, nonhuman primates, and humans), but also includes species-unique AAV transduction biology and possibly the differences in dystrophin structure and function among different species.

The biological properties (such as tropism and immunity) of the viral vectors are mainly determined by the viral capsid. The AAV capsid is composed of three overlapping capsid proteins called viral protein 1 (VP1), VP2, and VP3. The mature virion contains ~5 copies of VP1, ~5 copies of VP2, and ~50 copies of VP3 in a 1:1:10 ratio. Together, 60 copies of capsid proteins form a $T = 1$ icosahedral particle with 5-fold, 3-fold, and 2-fold axes of symmetry.[13] There are nine highly conserved regions (defined as regions A–I) in each AAV capsid protein. Region A is an α-helix (αA). Regions B–I are β-sheets. These β-sheets are arranged as a jelly-roll β-barrel. Between the β-sheets are intervening loops. These loops form much of the outer surface of an assembled AAV particle. Importantly, they are composed of variable amino acid sequences and hence are amenable to genetic manipulation.[14]

Capsid engineering refers to intentional modification of hypervariable loops (and possibly other regions) of the capsid to achieve a desired biological property. Currently, there are two major approaches: rational design and directed evolution. In rational design, existing knowledge of the capsid structure/function is used to guide genetic engineering to achieve predetermined outcomes. In directed evolution, investigators apply selective pressure(s) to a random capsid library to drive the evolution of the most adapted capsid. The beauty of directed evolution is that it does not require prior knowledge on the structure or function of the capsid. Directed evolution also has the advantage of integrating multiple selective pressures simultaneously to achieve desired features. Most importantly, directed evolution may result in unique capsid variants that are impossible to program using the rational design approach.

In this review, we summarize the state-of-the-art capsid engineering technologies and discuss the potential to apply these technologies to overcome major barriers in AAV-mediated DMD gene therapy. It should be noted that strategies based on

AAV genome engineering (such as the dual AAV vector) and expression cassette optimization (such as the use of a tissue-specific promoter, transgene codon optimization, and microRNA targeting sequence) have also been used to improve AAV vectors for DMD gene therapy. Since these mechanisms are not based on capsid modification, we opt to not include them in this review.

AAV CAPSID ENGINEERING BY RATIONAL DESIGN

Rational design depends on accruing insight into structure–function relationships between the primary amino acid sequence, assembled quaternary structure, and biological phenotype of the AAV capsid. Defined structures for 12 naturally occurring or modified AAV serotype/variants have been resolved in recent years using X-ray crystallography and/or cryo-electron microscopy. These include AAV-1,[15] AAV-2,[16–22] AAV-3B,[23,24] AAV-4, [25,26] AAV-5,[27–30] AAV-6,[31,32] AAV-7,[33] AAV-8,[34–38] AAV-9,[39–41] AAVrh32.33,[42] AAV-DJ,[43,44] and AAV-rh8.[172] The structural information of these AAV capsids as well as their interaction with respective cellular receptor/co-receptors have provided the necessary framework to logically design tailored vectors. Successful application of educated capsid design has been used to alter tissue tropism (targeting or de-targeting), avoid immune recognition, and improve postentry processing.

Engineering to enhance AAV uptake in muscle

Bodywide muscle delivery is a prominent challenge in developing a viable therapy for DMD, especially since muscle occupies such a large volume of the body. Further complicating the picture, significant amount of vectors can be sequestered by non-muscle tissues (such as the liver and spleen), hence reducing the effective dose in muscle. Consequently, an effective gene therapy for DMD will inevitably require a large quantity of AAV vectors (up to 10^{15}–10^{16} viral genome particles per patient).[45,46] This not only exposes the body to a large antigen load but also puts an increased demand on vector production. Controlling AAV tropism to favor skeletal and cardiac muscle could minimize some of these concerns.

Two strategies are often used to improve AAV uptake in muscle. In one approach, a muscle homing peptide is inserted on the surface of the capsid to facilitate the entry of AAV into muscle cells. In the second approach, enhanced muscle targeting is achieved by modulating AAV interaction with its natural receptors. In the discussion below, we illustrate both strategies.

Peptide insertion to improve muscle homing. Inserting a tissue-specific peptide ligand to the capsid is a convenient method to alter AAV tropism. When considering peptide insertion, there are two key questions: where should the peptide be inserted and what peptide(s) should be inserted? Ideally, insertion should not alter capsid assembly, vector genome packaging, and the overall yield of vector production. The insertion site should also facilitate proper peptide presentation such that the peptide can effectively interact with its receptor. For muscle gene therapy, apparently, a muscle targeting peptide would be preferred.

Identifying sites amenable to peptide insertion has been the focus of a considerable amount of research. During early years, this was achieved by the "hit-or-miss" approach or scanning mutagenesis. Yang and colleagues inserted a single chain CD34 antibody in front of VP1, VP2, or VP3 of AAV-2.[47] Fusion to the N-terminus of VP2, but not VP1 and VP3, resulted in particles capable of infecting previously nonpermissible CD34 cells.[47] It is currently unclear why this strategy did not work for VP1 and VP3. However, in terms of VP1, it may relate to the unique biological property of the VP1 N-terminal region. This region not only contains the nuclear localization signal[48] but also contains phospholipase A2 activity that is essential for AAV infectivity.[49,50] Some recent studies suggest that the VP1 N-terminal region undergoes structural changes in the endosome and this change is essential for viral trafficking to the nucleus.[51,52] To identify regions that are suitable for peptide insertion, Rabinowitz et al. and Wu et al. performed independent scanning mutagenesis across the entire capsid region of AAV-2.[53,54] They found many sites that can tolerate peptide insertion. For example, Wu et al. successfully enhanced AAV-2 infection in lung epithelial cells by inserting an expanded serpin receptor ligand (10 residues) at residue 138 of the AAV-2 capsid. Warrington et al. inserted larger peptides such as fractalkine chemokine (76 residues) and leptin (146 residues) at exactly the same location (residue 138 of the AAV-2 capsid). Surprisingly, these insertions abolished VP3 production and viral particle assembly.[55] Collectively, in addition to the location, the size and/or amino acid composition of the peptide may also play an important role.

The resolution of the parvovirus structure has opened the door to rationally select the peptide insertion site.[56–59] Girod et al. were the first to successfully use this approach.[60] They inserted an integrin ligand peptide (14 residues) to a putative loop region of AAV-2 and achieved efficient transduction in AAV-2 resistant cells that expressed the

integrin receptor.[60] Grifman et al. successfully targeted tumor cell lines by inserting an NGR peptide, which binds CD-13, a marker for tumor angiogenesis.[61] In another study, Shi et al. identified potential insertion sites on a computer-simulated AAV structure.[62] They successfully inserted the 15-residue luteinizing hormone receptor ligand into AAV-2 capsid and enhanced transduction of ovarian carcinoma cells.

With the availability of the AAV-2 atomic structure, it has become possible to precisely select a site for peptide insertion.[16,17] For example, residues 587 and 588 (AAV-2 numbering) are located within a flexible loop on the external surface of the AAV-2 particle.[63–73] Insertion at these two locations will likely allow good presentation of the peptide without substantially compromising virion assembly and stability. As the crystal structures of many AAV serotypes are now available, designing tailored peptide insertion in these serotypes may improve their transduction efficiency and specificity.[74,75]

A major goal of DMD gene therapy is to achieve efficient transduction of skeletal and cardiac muscles. Insertion of muscle-homing peptides may improve targeted delivery of AAV to muscle and at the same time limit uptake in other tissues (Table 1). Ideally, a muscle-targeting peptide should (1) bind with high affinity to a muscle-specific receptor, (2) disrupt binding to the natural AAV receptor/co-receptors (such as the heparan sulfate receptor for AAV-2) (Table 2), and (3) incorporate on the surface of the capsid without altering assembly and infectivity. High-affinity ligands may occur naturally (e.g., transferrin and α-bungarotoxin)[76] or may be isolated using phage biopanning (Table 2).[65,67,77–83] The success of *in vivo* biopanning depends on the delivery method (systemic versus local) and selection criteria. For DMD, systemic injection is a preferable selective pressure as a therapeutic vector will most likely be administered intravenously. Phage biopanning has been particularly successful for isolating peptides specific to skeletal and cardiac muscle. For example, Samoylova and Smith isolated a 7-residue muscle-targeting peptide (ASSLNIA) (Table 2).[78] Yu et al. inserted this peptide between residues 587 and 588 in AAV-2.[68] The modified vector displayed enhanced skeletal and cardiac muscle uptake.[68] Targeting the heart is particularly important in DMD as ~90% DMD patients develop cardiomyopathy. While intracardiac injection of AAV dystrophin constructs partially restores the dystrophin complex to the heart,[84] a cardiac muscle targeting peptide could provide a less invasive approach for cardiac delivery. To this end, Ying and colleagues applied *in vivo* biopanning on organotypic cultures of heart tissue and obtained a unique isolate (VNSTRLP) capable of myocardial targeting.[67]

Altering capsid structural motifs involved in receptor interactions. Ligand–receptor interaction is a major determinant of viral tissue tropism. In the case of AAV, the ligand refers to the special structural motifs on the surface of the capsid that interact with cellular receptor/co-receptors. The ligand is often called the "footprint."[20,85] An AAV footprint does not have to be a series of contiguous amino acid residues. More often, a footprint is composed of a cluster of three-dimensionally related amino acids. For example, the footprint of AAV-2 is made of basic residues R484, R487, K527, K532, R585, and R588.[73,86,87]

Cellular receptors for at least eight different AAV serotypes have been identified (Table 2). These receptors consist of various carbohydrates on the cell surface, in particular, N-linked α-2,3 sialic acid for AAV-1 and AAV-5,[88,89] heparan sulfate proteoglycan for AAV-2 and AAV-3,[90,91] O-linked α-2,3 sialic acid for AAV-4,[92] N-linked α-2,6 sialic acid for AAV-6,[88] and N-linked galactose for AAV-9[93,94] (Table 2).

Several studies have explored the possibility of modifying AAV tropism through the alteration of AAV footprint–cellular receptor interaction. The AAV-2 footprint interacts with its receptor heparan sulfate proteoglycan. Heparan sulfate proteoglycan binding correlates with liver transduction.

Table 1. *AAV receptor and co-receptor(s)*

Serotype	*Receptor*	*Co-receptor(s)*	*Reference*
AAV-1	N-linked α-2,3 sialic acid		Wu et al.[88]
AAV-2	Heparin sulfate proteoglycan	α5β1-integrin, hFGFR1, αVβ5-integrin, hHGFR, LamR	Qing et al.[166]; Summerford et al.[91]
AAV-3	Heparin sulfate proteoglycan	hFGFR, hHGFR, LamR	Handa et al.[90]; Lerch et al.;[168] Ling et al.[167]
AAV-4	O-linked α-2,3 sialic acid	Unknown	Kaludov et al.[92]
AAV-5	N-linked α-2,3 sialic acid	PDGFR	Di Pasquale et al.[169]; Walters et al.[89]
AAV-6	N-linked α-2,6 sialic acid	EGFR	Wu et al.[88]
AAV-7	Unknown	Unknown	
AAV-8	Unknown	LamR	Akache et al.[170]
AAV-9	Galactose	LamR	Akache et al.[170]; Bell et al.[94]; Shen et al.[93]

EGFR, epidermal growth factor receptor; hFGFR1, human fibroblast growth factor receptor 1; hHGFR, human hepatocyte growth factor receptor; LamR, 37/67kDa laminin receptor; PDGFR, platelet-derived growth factor receptor; AAV, adeno-associated virus.

Table 2. *Muscle-targeting peptides*

Reference	*Peptide name*	*Peptide sequence*	*Length*	*Comment*
Barry et al.[77]	Peptide 20.1	TPHSLYEDLKRQMMQLGRH L	20-mer	This peptide targets fibroblasts and myoblasts but not differentiated myotubes *in vitro*.
	Peptide T.1	TGGETSGIKKAPYASTTRNR	20-mer	This peptide targets differentiated myotubes.
	Peptide T.2	SHHGVAGVDLGGGADFKSI A	20-mer	This peptide targets differentiated myotubes.
Flint et al.[79]	Peptide (P5)	PYDQLRH	7-mer	This peptide targets C2C12 muscle cell line *in vitro*.
	Peptide (P6)	KAMHQMQ	7-mer	This peptide targets C2C12 muscle cell line *in vitro*.
	Peptide (P9)	YASINPM	7-mer	This peptide targets C2C12 muscle cell line *in vitro* and rat skeletal muscle *in vivo*.
		NPSQVKH	7-mer	This peptide targets rat laryngeal muscle *in vivo*.
Samoylova and Smith[78]; Yu et al.[68]		ASSLNIA	7-mer	This peptide targets C2C12 muscle cell *in vitro* and mouse skeletal and cardiac muscle *in vivo*.
Ghosh and Barry[80]	Peptide 12.51	TARGEHKEEELI	12-mer	This peptide targets C2C12 muscle cell line *in vitro*.
McGuire et al.[81]	Peptide PCM.1	WLSEAGPVVTVRALRGTGS W	20-mer	This peptide targets primary cardiomyocytes *in vitro* and murine heart *in vivo*.
Seow et al., 2010[171]	Peptide T9	SKTFNTHPQSTP	12-mer	This peptide targets C2C12 muscle cells *in vitro* and mdx heart and quadriceps muscle *in vivo*.
Ying et al.[67]		PSVSPRP and VNSTRLP	7-mers	This peptide targets rat heart slices *in vitro* and mouse heart *in vivo*.
		EGRVRPP and GTFSRAP	7-mers	This peptide targets rat cardiomyocytes *in vitro* and mouse heart *in vivo*.
Gao et al.[82]	Peptide M12	RRQPPRSISSHP	12-mer	This peptide targets C2C12 muscle cell line *in vitro* and skeletal and heart muscle *in vivo*.

Theoretically, alteration of the AAV-2 footprint should reduce heparan sulfate proteoglycan binding and hence decrease liver transduction. To test this hypothesis, Asokan and colleagues replaced a section of AAV-2 footprint (from residue 585–590, RGNRQA) with corresponding residues from AAV-8 (QQNTAP).[95] The chimeric capsids indeed showed reduced liver uptake. AAV-9 enters a cell through binding to N-linked galactose on the surface of the cell. This interaction is thought to underlie systemic transduction of AAV-9. The residues responsible for galactose binding in AAV-9 were identified recently.[41,96,97] Engraftment of these residues to AAV-2 (Q464V, A467P, D469N, I470M, R471A, D472V, S474G, Y500F, S501A, and D514N) indeed resulted in galactose binding and significantly improved systemic gene transfer efficiency of AAV-2.[96,97]

Another approach to modulate AAV interaction with its receptor is to conditionally hide the receptor binding residues. Judd et al. invented such a gating system for AAV-2.[98] To block the interaction of AAV-2 with its receptor, they inserted a short peptide between R585 and R588. They also engineered matrix metallo-protease (MMP) cleavage sites at the end of this short peptide. In the presence of MMP, the inserted peptide was removed and the heparan sulfate proteoglycan binding motif of AAV-2 was reconstituted allowing receptor recognition and viral uptake.[98] This type of capsid modification will be very useful for DMD gene therapy because inflammation is a prominent feature of DMD and MMP is highly expressed in inflamed tissues.

Rational modification to circumvent immune responses

As we alluded to earlier, immune responses constitute a critical barrier to DMD gene therapy.[12,99] Preexisting neutralizing antibodies may nullify AAV in the circulation. Capsid- or transgene product-specific T-cell responses can result in the elimination of AAV-transduced cells. While molecular mechanisms of the immune response remain to be fully elucidated, the immunological hurdle must be addressed in order to achieve successful DMD gene therapy. As the focus of this review is on capsid modifications, we will limit our discussion to capsid-related immune responses.

Alteration of neutralizing antibody binding epitopes to improve transduction in seropositive subjects. Preexisting neutralizing antibodies at levels as low as 1:2 are sufficient to attenuate AAV transduction in animal models.[100,101] A significant portion of the human population ($\sim$30–80%) has preexisting neutralizing antibodies to AAV.[12] This precludes a large percentage of patients from AAV therapy unless new vectors with enhanced antibody evasion become available. Several strategies have been developed to identify capsid epitopes that interact with neutralizing antibodies. Peptide display and scanning have been used to identify linear epitopes that reside in continuous amino acid residues.[102,103]

However, to fish out conformational epitopes that reside in noncontiguous amino acid residues, one has to use nondenatured intact viral particles.[103]

Capsid mutagenesis has been used to identify both linear and conformational neutralizing antibody binding motifs in AAV. For example, Huttner et al. applied the peptide insertion approach and found that AAV-2 residues 534, 573, and 587 are important for neutralization escaping.[104] Adachi et al. mapped the epitopes for AAV-1 and AAV-9 neutralization antibody binding to 452-QSGSAQ-457 and 453-GSGQN-457, respectively, by screening double-alanine mutants.[96] Antibody–capsid interactions can also be visualized with cryo-electron microscopy and three-dimensional image reconstruction.[37,105,106] High-resolution imaging shows that capsid epitopes tend to localize around the threefold protrusions and overlap with key regions involved in receptor recognition and tropism.[105] This information suggests that perhaps capsid engineering to improve tropism may simultaneously alter antibody recognition. In summary, delineating specific antibody recognition sites and targeted mutagenesis may yield new capsid variants that can be used in patients with preexisting immunity to existing serotypes.

Engineering AAV vectors to reduce cellular immune responses. The dystrophic muscle presents a formidable inflammatory environment for AAV gene therapy (Fig. 1A). Expression of major histocompatibility complexes (MHC) is upregulated in dystrophic muscles.[107] Cytokines and chemokines released from degenerative/necrotic myofibers may recruit more immune cells to muscle and further aggravate inflammation.

Both capsid-specific and dystrophin-specific cellular immune responses have been implicated in transduction loss in dystrophic muscle.[108–110] Capsid-specific T-cell responses depend on AAV endosomal escape and subsequent proteasome processing (Fig. 2).[111,112] Therefore, capsid modifications to de-target the proteasome may be a useful method to reduce capsid T-cell responses. Zhong et al. found that AAV capsid was degraded by the ubiquitin–proteasome system upon phosphorylation of surface-exposed tyrosine (Y) residues.[113,114] Replacement of these residues with phenylalanine (F) aborted phosphorylation.[115] Martino and colleagues evaluated immunological benefits of tyrosine mutated AAV-2.[116] They found that the mutant vector showed reduced MHC presentation and less killing of hepatocytes in a liver gene transfer study (Fig. 2).[116] It is currently unclear whether tyrosine mutants will fare better in dystrophic muscle in terms of the T-cell response. However, some tyrosine-mutated AAV serotypes have shown promise for intramuscular injection in mouse muscle (Y445F or Y731F AAV-6) or body-wide muscle delivery in newborn dogs (Y445F/Y731F AAV-1).[45,117] We recently found that Y731F AAV-9 resulted in robust local and systemic muscle gene transfer in adult DMD dogs.[46,118] It is possible that tyrosine-mutated AAV capsids may prove useful to DMD gene therapy. In addition to tyrosine, surface-exposed serine, threonine, and lysine residues are also targets for phosphorylation. Sen and colleagues showed reduced ubiquitination and enhanced hepatic transduction when these

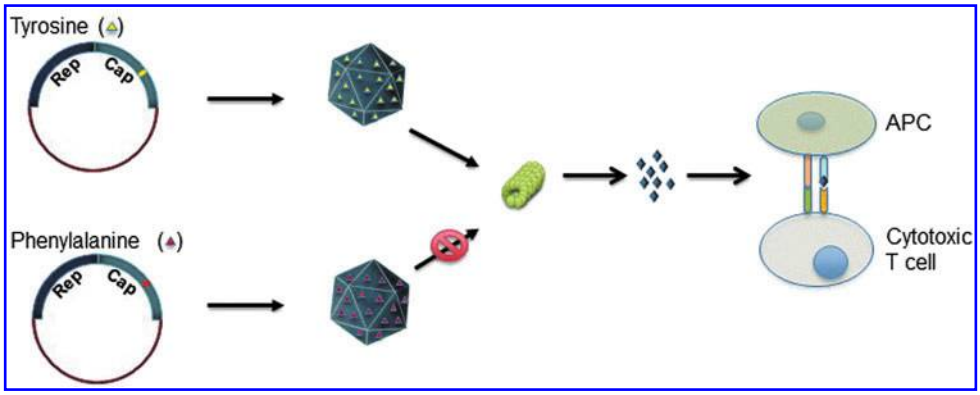

Figure 2. Engineering AAV vector by tyrosine substitution for proteasome evasion. Capsid-induced T-cell response is a major barrier for AAV-mediated DMD gene therapy. Viral capsids are degraded in the proteasome in the host cell cytoplasm. Degraded peptide fragments are transported into the endoplasmic reticulum and loaded into major histocompatibility complexes (MHC) class I molecules on the surface of antigen presenting cells (APC). The peptide–MHC I complex traffics to the cell membrane where peptide is recognized by the T-cell receptor leading to T-cell activation. The tyrosine residue on the surface of the viral capsid plays an important role in this process. Substitution of surface-exposed tyrosine by phenylalanine may reduce MHC presentation and T-cell immunity. Color images available online at www.liebertpub.com/hum

residues were substituted in AAV-1, 5, and 8.[119–122] Additional studies are needed to determine whether these modifications offer immunological advantages in inflamed dystrophic muscle.

Uptake by antigen-presenting cells (such as macrophages) is a premise for the induction of cellular immune responses.[123–125] Strategies that can minimize macrophage uptake may improve AAV transduction in dystrophic muscle. CD47 glycoprotein is a surface marker for "self" in all human cells. Interaction of CD47 with signal regulatory protein-α on macrophages prevents uptake of "self" cells by macrophages.[126] Recently, a 21-residue peptide was found to mediate this self-recognition.[127] Incorporation of this peptide to nanoparticles reduced phagocytic clearance and enhanced gene delivery.[127] It is foreseeable that insertion of this peptide to the surface loops of AAV capsids may reduce macrophage uptake and improve AAV transduction in DMD muscle.

AAV CAPSID ENGINEERING THROUGH DIRECTED EVOLUTION

Directed evolution harnesses natural selection to identify capsids with desired phenotypes. In this manner, it is possible to develop AAV vectors capable of coping with multiple selective pressures without any prior knowledge of the capsid structure.[128] Like natural selection, directed evolution requires a diverse starting population to increase the odds of identifying rare, desirable variants. This translates into the creation of a diverse capsid library. Theoretically, the more diverse the library, the more likely you will find a variant that can meet your need. A key technical challenge of directed evolution is the ability to retrieve the capsid coding sequence based on the phenotype of the capsid protein. For example, one may have identified an attractive neutralization escaping capsid variant. In order to package a therapeutic gene into this capsid variant, the exact coding sequence for this particular capsid variant must be fished out from millions of capsid coding sequences in the library. This could be quite challenging if not impossible. An ingenious solution is to have this capsid variant carrying its own coding sequence in its genome. Genomes can then be extracted from the capsids after selection. We will discuss various strategies used by different groups and a surprising but pleasant new discovery on capsid–genome correlation. The last step in directed evolution is to apply selective pressures to enrich capsid variants with desirable properties, and finally to isolate the favored capsid variant. We will discuss screening strategies that may help isolation of new AAV capsids for DMD gene therapy.

Methods for library creation

Below we summarized the most commonly used strategies for AAV capsid library generation. More information can be found in a recent review article by Kotterman and Schaffer.[128]

Error-prone polymerase chain reaction. Error-prone polymerase chain reaction (EP-PCR) is a relatively simple method to randomly mutate a DNA sequence. This is achieved by enhancing the inherent polymerase error rate. The Taq polymerase has an inherent cumulative error rate of $\sim 10^{-3}$ per nucleotide favoring A-to-G and T-to-C mutations yielding from 1 to 20 mutations per 1,000 bases.[129–131] This error rate may be further enhanced by incorporating high levels of Mn^{2+} or Mg^{2+} in the PCR or, alternatively, providing disproportionate amounts of deoxynucleotides. Mn^{2+} and Mg^{2+} help to stabilize noncomplementary strands, while disproportionate deoxynucleotides leads to unequal base pair incorporation. Additionally, nontraditional bases such as 8-oxo-GTP or dITP may be introduced to the PCR products.[132,133] Using these techniques, it is possible to generate an AAV capsid library with a complexity of approximately 10^7 variants.[134]

Mosaic capsids. The construction of mosaic capsids provides an alternative to EP-PCR for increasing the library diversity. To generate mosaic capsids, cultured cells are transfected with AAV helper plasmids expressing capsid genes from different serotypes, the adenovirus helper plasmid and a rAAV plasmid. This results in the incorporation of viral protein monomers from different serotypes during capsid assembling (Fig. 3A). The resulting viral capsids are mosaic capsids because they contain capsid units from several serotypes. Since not all capsid monomer combinations are viable,[135] the diversity is limited for this approach.

PCR-based *in vitro* recombination. PCR-based *in vitro* recombination methods create highly diverse chimeric libraries. It takes the advantage of high sequence similarity among different AAV serotypes to develop capsids of various combinations of the original parental sequences. Three most commonly used PCR strategies are staggered extension, overlap extension, and DNA shuffling.

Staggered extension PCR. Staggered extension PCR (StEP) provides a simple method to generate

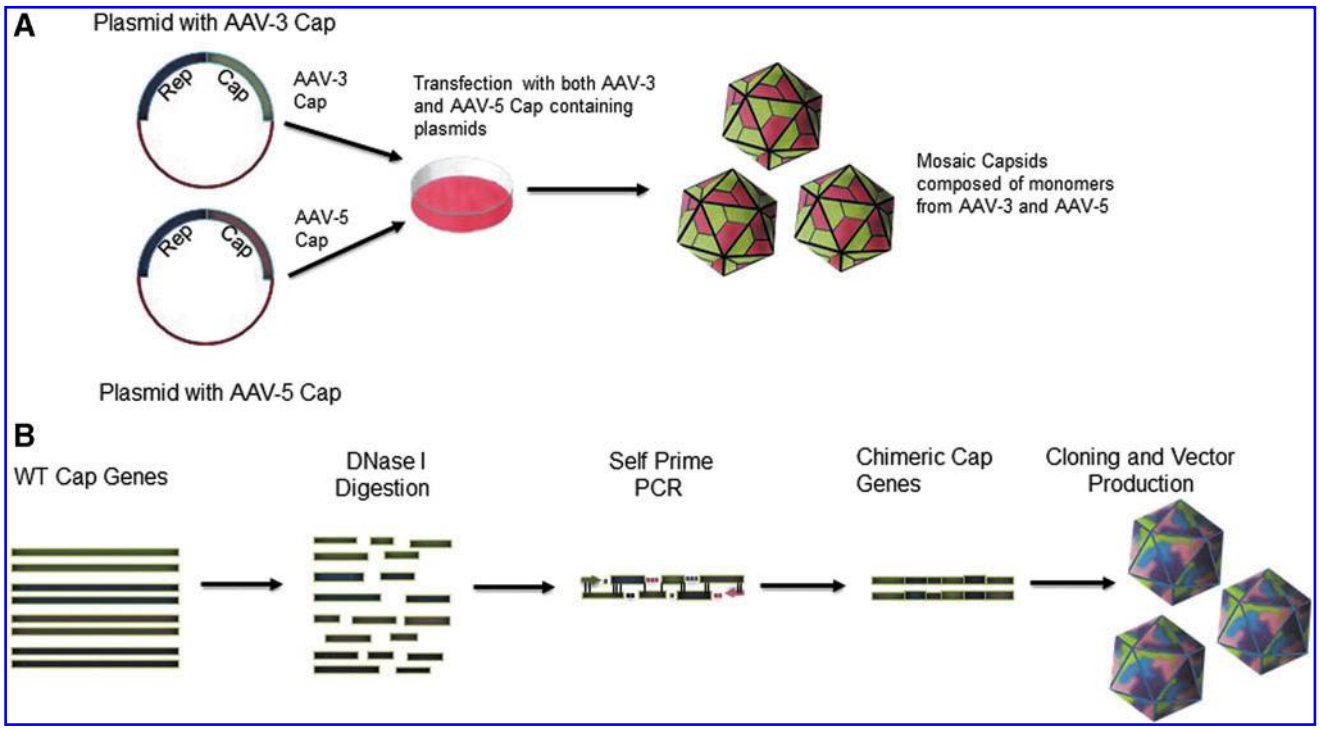

Figure 3. Generation of mosaic capsid library and DNA-shuffled capsid library. **(A)** Mosaic capsids are produced by co-transfection of the *Cap* gene from several AAV serotypes (in the example here, AAV-3 and AAV-5). During viral production, viral protein monomers from different serotypes are incorporated in the capsids to form mosaic capsids. **(B)** In DNA shuffling, AAV capsid genes from several serotypes are fragmented by DNase I. Homology between different capsid genes allows re-ligation through self-priming PCR. This haphazard re-assembly results in a library of chimeric capsids containing fragments from different serotypes. Color images available online at www.liebertpub.com/hum

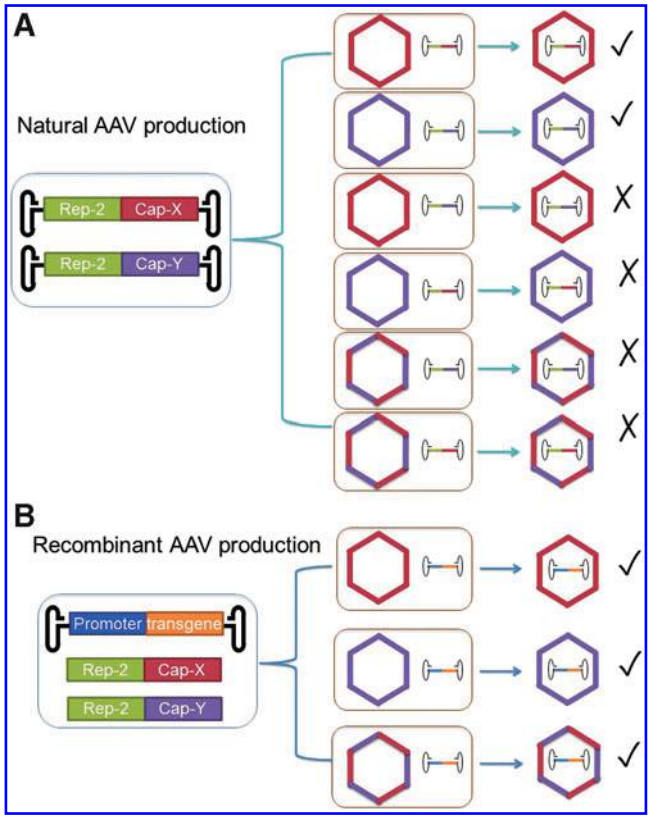

Figure 4. Capsid–genome correlation in AAV production. **(A)** Natural AAV production refers to AAV production using a *cis* plasmid that contains a wild-type-like AAV genome. In this genome, the *Rep* and *Cap* genes are flanked by the ITRs. In the presence of multiple different *cis* plasmids, the capsid produced by a particular *Cap* gene will package only the viral genome containing this *Cap* gene. There is a tight capsid–genome correlation. In the example here, two *cis* plasmids are used for AAV production, one carries the *Cap-X* gene (marked in red color) and the other carries the *Cap-Y* gene (marked in purple color). The Rep-2/Cap-X genome (green/red) will be packaged only inside capsids X (the red colored capsid). The Rep-2/Cap-Y genome (green/purple) will be packaged only inside capsids Y (the purple colored capsid). The Rep-2/Cap-X genome (green/red) will not be packaged inside capsids Y (the purple colored capsid), neither will the Rep-2/Cap-Y genome (green/purple) be packaged inside capsids X (the red colored capsid). In addition, this system will not generate chimeric viral particles in which the viral genome is packaged in chimeric capsids. **(B)** Recombinant AAV production refers to AAV production using a *cis* plasmid that contains a recombinant AAV genome (a genome in which a foreign expression cassette is flanked by the ITR). In recombinant AAV production, viral replication protein and capsid protein are produced from a *trans* plasmid that does not contain the ITR. In the example here, the recombinant AAV genome can be packaged in the capsid X, the capsid Y, or a mosaic capsid. The capsid–genome correlation is lost during recombinant AAV production. Color images available online at www.liebertpub.com/hum

chimeras by altering the annealing and extension phases in a standard PCR. By reducing the annealing and extension period, staggered extension produces incomplete PCR fragments, which, due to the high sequence homology of AAV capsids, are able to anneal and self-prime on other parent templates.[136] Through reiterative cycles of staggered extension, capsid sequences from various parent templates are linked together to form chimeras.[137,138]

Overlap extension PCR. This method is usually used to insert specific mutation at specific points in a sequence or to stitch smaller DNA fragments into a larger fragment. In overlap extension PCR, primers with a 5′ overhang complementary to the opposite parent template amplify parent templates yielding fragments with overlapping 5′ regions. Primer-less PCR combines the individual amplicons into a larger gene sequence based on the overlapping regions. Terminal flanking primers are then used to amplify the entire sequence.[139] This method takes advantage of homologous 5′ sequences between PCR products allowing parental templates to be sown together. In as few a 5–10 cycles of overlap extension followed by 30 cycles of flanking primer amplification, it is possible to fuse 2–7 parental capsid fragments.[140] Overlap extension PCR is particularly useful for inserting amino acids from one serotype into the capsid of another serotype.

DNA shuffling. DNA shuffling involves the fragmentation of related genetic sequences by DNase I digestion and subsequent recombination based on sequence homology in a reassembly PCR.[141,142] In recursive cycles of DNA shuffling, selective pressure is applied to enrich the recombinant population that has desirable characteristics (Fig. 3B). To shuffle AAV capsids, parental capsid sequences are PCR amplified with primers containing unique restriction sites on the 5′ ends. The unique restriction sites are for cloning each capsid into a common backbone containing the wild-type ITRs and the AAV-2 replication gene. In a subsequent PCR, the capsid sequences are amplified, pooled, and digested briefly with DNaseI. Because of sequence homology, fragments are able to undergo template switching and self-priming, allowing the *in vitro* recombination of homologous sequences. Unique restriction sites allow the assembled capsid sequences to be cloned into the common plasmid backbone for virus production.[143]

Capsid–genome correlation

In order to retrieve the AAV genome that encodes the desired capsid, it is important that each capsid encapsulates only its own coding genome during packaging. Several strategies have been developed to address this issue. Schaffer and colleagues used limiting dilution.[144] Basically, they controlled the amount of plasmids used in transfection to achieve one proviral plasmid per cell. In this case, the AAV vector made in each cell will likely carry only its own genome. Alternatively, Muller and colleagues utilized a shuttle vector system by transfecting the capsid plasmid library with wild-type capsid.[65] Using a low multiplicity of infection, shuttle vectors theoretically deliver only one genome to each cell. Recently, the Weber laboratory encountered a surprising observation when comparing AAV production using natural or recombinant proviral AAV plasmids (Fig. 4).[145] Natural proviral AAV plasmids contain the 5′-ITR, replication gene, capsid gene, and 3′-ITR. Weber and colleagues found excellent capsid–genome correlation when they transfected a mixture of natural proviral AAV plasmids from different serotypes at the dose of 5,000–50,000 plasmids/cell. Essentially, 75–80% of capsids packaged only their own coding genome (Fig. 4A). This is surprising because there are hundreds of proviral plasmids in each cell and these plasmids encode different capsids.[145] In contrast to natural proviral AAV plasmids, recombinant AAV proviral plasmids contain the 5′-ITR, transgene expression cassette, and 3′-ITR. For the production of recombinant AAV, the AAV replication and capsid genes are provided *in trans* using ITR-deficient plasmids. When Nonnenmacher et al. transfected a recombinant AAV proviral plasmid with ITR-null capsid plasmids from different serotypes, the tight capsid–genome correlation was lost (Fig. 4B). It is currently unclear why a capsid packages only the "correct (its own)" genome in the case of natural AAV reproduction. Nevertheless, this new observation suggests that capsid–genome correlation may not be a big concern as previously thought. If the capsid library is made in natural AAV plasmids, one may not need to perform limiting dilution or to use shuttle vectors.

Evolution of novel capsids for DMD gene therapy

In natural selection, environmental pressure creates a selective advantage for phenotypes with higher fitness. Likewise, selective pressures applied to capsid libraries should force the survival of one or a few dominant capsids that can handle the specific selective pressure. Directed AAV evolution can be performed in cultured cells *in vitro* or in live animals *in vivo*. Selective pressure can also be applied to the capsid library before (e.g., preincubation of the library with antibodies or antisera) or after AAV is delivered to cells/animals.

Directed evolution to improve muscle tropism. Directed evolution is particularly well suited for creating AAV variants with enhanced tissue specificity. Simultaneous application of positive (e.g., muscle uptake) and negative (e.g., restricted liver uptake) pressures can dramatically improve targeting and de-targeting. Using a shuffled capsid library of AAV-1, 2, 3B, 4, 6, 7, 8, and 9, Yang and colleagues isolated a chimeric vector called AAV-M41.[146] AAV-M41 displayed enhanced cardiac tropism comparable to that of AAV-9, the so-called "cardiotropic" AAV. But in contrast to AAV-9, AAV-M41 resulted in minimum liver transduction.[146] The Asokan laboratory utilized EP-PCR to mutate AAV-9 capsids and screened for vectors with enhanced muscle transduction.[147] A resultant vector, AAV-9.45, displayed significantly increased transgene expression in the heart and skeletal muscle. Interestingly, AAV-9.45 was de-targeted from the liver, a feature similar to that of AAV-M41.

Directed evolution has also been used to identify novel capsids that can transduce difficult targets such as nonpermissive melanoma cells.[148] Failure to transduce muscle progenitor cells (such as satellite cells) by conventional AAV serotypes represents a major challenge in DMD gene therapy.[149] Directed evolution may yield capsids with enhanced transduction efficiency in satellite cells.

A major challenge in gene therapy is to reproduce animal study results in human patients.[150–152] The traditional approach is to confirm rodent results in large animal models before the initiation of human trials. Scaling up from small to large animals gives investigators a good chance to gauge the impact of the body size. However, this cannot address species-specific reactions. The xenograft model may provide a platform to test promising candidate vectors in human tissues or to identify viral capsids with enhanced tropism in human cells. Lisowski and colleagues screened a shuffled capsid library of AAV-1, 2, 3B, 4–6, 8, and 9 in a human liver xenograft model. In this model, human hepatocytes were engrafted in the liver of immune-deficient mice. After several rounds of *in vivo* selection, the authors isolated a chimeric vector called AAV-LK03. This vector showed preferred transduction of human but not mouse hepatocytes.[153] A human skeletal muscle xenograft model was developed by the Wagner laboratory recently.[154] This model recapitulated dystrophic muscle pathology. Future directed evolution in this model could potentially yield AAV capsids suitable for muscular dystrophy gene therapy.

Directed evolution to modify immune reactivity. Another important application of directed evolution is to develop immune-evasive vectors. By screening against human neutralizing sera, directed evolution has yielded vectors that are resistant to preexisting neutralizing antibodies. Schaffer's group was the first to test this strategy. Maheshri et al. included a neutralizing sera preincubation step before screening EP-PCR- and StEP-derived libraries.[155] This strategy led to the isolation of AAV-r2.4 and r2.15. Both showed improved transgene expression in the presence of the neutralizing antibody in *in vitro* and *in vivo* tests. In another study, Schaffer's lab used a library generated by DNA shuffling. They then selected against pooled intravenous immunoglobulin (IVIG). This led to the isolation of several vectors (AAV-cA2, cA3, cA4) that are highly resistant to IVIG challenge.[144] Similarly, Perabo and colleagues used preincubation with human sera to screen an EP-PCR capsid library for retained infectivity.[134] Capsids with mutations (R459G/K and N551D) showed increased resistance to human neutralizing sera. Neutralizing antibody screening can also be used in combination with tissue-selective pressures. For example, Grimm and colleagues developed a shuffled library from AAV-2, 4, 5, 8, and 9 and then screened this library on human hepatocytes and antisera.[156] A resultant vector, AAV-DJ, displayed enhanced liver tropism and resistance to neutralizing sera. This study suggests that combined use of multiple selective pressures may yield vectors with multiple desired features. So far, none of these immune evasive capsids has been tested in clinical trials. Future study with sera from DMD patients may reveal the utility of these capsids for DMD gene therapy.

CAPSID MODIFICATION TO INCREASE AAV PACKAGING CAPACITY FOR DMD GENE THERAPY

The *dystrophin* gene is one of the largest genes in the genome. The 2.4 mb gene carries 79 exons and an 11.2 kb protein coding sequence.[157] This greatly exceeds the packaging capacity of AAV. Dual- and tri-AAV vectors are currently under development to deliver a larger *dystrophin* gene via interviral genome recombination.[158,159] An alternative approach is to increase the payload of each individual viral particle. One study suggests that AAV-5, the phylogenetically most divergent AAV serotype, may package an 8.2 kb vector genome.[160] However, subsequent studies from several laboratories suggest that this is not the case.[161–163]

AAV belongs to the dependent genera of parvovirus. All parvovirus shares a similar structure. Interestingly, some members appear to have a more flexible capsid.[164] For example, human bocavirus-1 (a member of autonomous genera of parvovirus) carries a viral genome of 5,543 nucleotides. This is 18.5% larger than that of the AAV-2 genome (4,679 nucleotides). Yan et al. recently tested cross-genera packaging of an AAV genome in human bocavirus-1.[165] The chimeric AAV-2/human bocavirus-1 vector indeed packaged a larger genome than conventional AAV. It is possible that further interrogation along this line may lead to the development of oversize AAV vectors suitable for DMD gene therapy.

PERSPECTIVE ON AAV CAPSID ENGINEERING FOR DMD GENE THERAPY

Capsid engineering has contributed significantly to the development of better, more efficient, and specific viral vectors. As we have discussed throughout this review, there is a wide array of methodologies for altering the biological properties of AAV capsids. With the continual understanding of AAV capsid structure–function relationship, improved library generation, and creative screening methods, we may develop AAV vectors that better fit the needs of DMD gene therapy. In this regard, low-immunogenic, large-capacity, and human muscle-specific capsids will be particularly appealing. It is tempting to speculate that a more integrated approach, combining several rational and directed evolution approaches, may lead to the development of one or several AAV capsids that can meet the needs of DMD gene therapy.

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REVIEW

Prospect of gene therapy for cardiomyopathy in hereditary muscular dystrophy

Yongping Yue[a], Ibrahim M. Binalsheikh[b], Stacey B. Leach[c], Timothy L. Domeier[d] and Dongsheng Duan[a,e]

[a]Department of Molecular Microbiology and Immunology, School of Medicine, University of Missouri, Columbia, MO, USA; [b]Department of Child Health, School of Medicine, University of Missouri, Columbia, MO, USA; [c]Department of Veterinary Medicine and Surgery, College of Veterinary Medicine, University of Missouri, Columbia, MO, USA; [d]Department of Medical Pharmacology and Physiology, School of Medicine, University of Missouri, Columbia, MO, USA; [e]Department of Neurology, School of Medicine, University of Missouri, Columbia, MO, USA

ABSTRACT

***Introduction*:** Cardiac involvement is a common feature in muscular dystrophies. It presents as heart failure and/or arrhythmia. Traditionally, dystrophic cardiomyopathy is treated with symptom-relieving medications. Identification of disease-causing genes and investigation on pathogenic mechanisms have opened new opportunities to treat dystrophic cardiomyopathy with gene therapy. Replacing/repairing the mutated gene and/or targeting the pathogenic process/mechanisms using alternative genes may attenuate heart disease in muscular dystrophies.

***Areas covered*:** Duchenne muscular dystrophy is the most common muscular dystrophy. Duchenne cardiomyopathy has been the primary focus of ongoing dystrophic cardiomyopathy gene therapy studies. Here, we use Duchenne cardiomyopathy gene therapy to showcase recent developments and to outline the path forward. We also discuss gene therapy status for cardiomyopathy associated with limb-girdle and congenital muscular dystrophies, and myotonic dystrophy.

***Expert opinion*:** Gene therapy for dystrophic cardiomyopathy has taken a slow but steady path forward. Preclinical studies over the last decades have addressed many fundamental questions. Adeno-associated virus-mediated gene therapy has significantly improved the outcomes in rodent models of Duchenne and limb-girdle muscular dystrophies. Validation of these encouraging results in large animal models will pave the way for future human trials.

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AAV; adeno-associated virus; gene therapy; cardiomyopathy; Duchenne muscular dystrophy; DMD; dystrophin; capsid engineering; vector; capsid; muscular dystrophy; gene therapy; heart failure; limb-girdle muscular dystrophy; congenital muscular dystrophy; myotonic dystrophy

1. Clinical presentation, pathogenic mechanism, and therapeutic challenge of dystrophic cardiomyopathy

Dystrophic cardiomyopathy refers to cardiac manifestations of muscular dystrophies. Muscular dystrophies are a clinically, genetically, and biochemically heterogeneous group of disorders. They are characterized by progressive muscle wasting, force loss, and dystrophic muscle pathology.[1,2] Muscular dystrophies can be classified in many different ways, such as the age of onset (congenital/neonatal, adolescent, or adult), disease progression (rapid or slow), the muscle groups involved (such as limb-girdle, facioscapulohumeral, and oculopharyngeal, etc.), and the mode of inheritance (such as X-linked or autosomal, recessive or dominant). Some muscular dystrophies are named after people who discovered the disease (such as Duchenne muscular dystrophy (DMD), Becker muscular dystrophy (BMD), and Emery–Dreifuss muscular dystrophy, etc.). Despite the unique clinical features of each type of muscular dystrophy, cardiac involvement has been a common finding in most muscular dystrophies and often represents a major cause of morbidity and mortality.[3–7] Interestingly, the cardiac phenotype varies in different types of muscular dystrophies and even in different patients or disease stages of the same type of muscular dystrophy. Some present with dilated/hypertrophic/restrictive cardiomyopathy with eventual heart failure, while others exhibit conduction defects leading to arrhythmias and sudden cardiac death. In the case of DMD and BMD, MRI studies have revealed a unique pattern of subepicardial fibrosis predominantly in the left ventricular lateral wall.[8–10]

The pathogenic mechanisms of dystrophic cardiomyopathy are not completely understood.[11,12] However, it may at least involve destabilization of the cardiomyocyte membrane, or sarcolemma. Unlike other cells in the body, muscle cells undergo continuous calcium-regulated contraction/relaxation cycles. A consequence of this unique physiology is the repeated cycles of shrinking and expansion of the cell. This dynamic deformation process places

CONTACT Dongsheng Duan duand@missouri.edu Department of Molecular Microbiology and Immunology, School of Medicine, University of Missouri, One Hospital Dr. M610G, MSB, Columbia, MO, USA.

Article highlights

- Cardiomyopathy is a common complication in inherited muscular dystrophies.
- Gene therapy holds great promise to reduce heart-related morbidity and mortality in muscular dystrophies.
- AAV is the most effective cardiac gene delivery vector.
- Micro-dystrophin and sarcoglycan gene therapies have significantly improved the cardiac outcome in animal models of DMD and LGMD, respectively.
- Targeting pathogenic mechanisms with disease gene-independent gene therapy opens exciting new opportunities.
- Preclinical test in large animal models will pave the way to human trials.

This box summarizes key points contained in the article.

enormous stress on the sarcolemma. Such stress is especially problematic for cardiomyocytes because of the repetitive pumping activity of the heart. To relieve contraction-induced stress, muscle cells have evolved specialized trans-membrane protein complexes such as the dystrophin-associated glycoprotein complex (DGC) and the integrin complex. These protein complexes constitute physical connections between the cytoskeleton and the extracellular matrix. Mutations in the genes encoding the components of these complexes result in various forms of muscular dystrophies. Failure to maintain sarcolemmal integrity leads to membrane leakage, myocyte degeneration, necrosis, and eventual replacement by fibrofatty tissue. Clearly, strengthening the destabilized sarcolemma holds the key for treating dystrophic cardiomyopathy. Unfortunately, this cannot be achieved with conventional medical/surgical treatments.[13] Gene therapy, however, provides a great opportunity to address this therapeutic challenge.

2. Strategies to deliver a therapeutic gene to a dystrophic heart

Disease-causing genes for many muscular dystrophies have been discovered. The identification of the genetic underpinning makes it possible to treat dystrophic cardiomyopathy with gene therapy. The first step of gene therapy is delivery of a therapeutic gene to the heart. A number of viral and nonviral vectors have been tested.[14] So far, the most effective and least immunogenic vector is the adeno-associated virus (AAV). AAV is a 20-nm single-stranded DNA virus.[15] Recombinant AAV vectors contain no wild-type viral genes. The vector genome can be readily packaged into naturally existing or synthetic capsids to meet specific therapeutic needs. The nano-size AAV particle creates a packaging dilemma. The maximal carrying capacity of a single AAV particle is 5 kb.[16] This is too small for many genes required for muscular dystrophy gene therapy (such as the dystrophin gene and the dysferlin gene). To overcome this limitation, we and others have invented a series of dual and tri-AAV vectors.[17] The basic idea is to fragment a large therapeutic gene and package each segment into an AAV particle. The full-length gene is reconstituted by cellular recombination machinery after co-infection. These multi-vector strategies have made it possible to deliver the 6–8-kb mini-dystrophin gene and even the 12-kb full-length dystrophin coding sequence to dystrophin-deficient mdx mice, the most commonly used animal models for DMD.[18–22]

Over the years, a number of different strategies have been developed to achieve effective AAV gene transfer in dystrophic hearts. Early studies were mainly based on AAV-2 using invasive and complicated methods such as direct myocardial injection,[23] intracavity injection,[24] transcoronary perfusion,[25] and ex vivo coronary perfusion.[26] The identification and development of novel AAV capsids has opened the door to transduce dystrophic hearts with peripheral vein injections.[27–32] This simple method not only greatly reduces the risks associated invasive heart gene transfer but also allows simultaneous treatment of both cardiac and skeletal muscle disease in muscular dystrophy.

The tissue tropism of the AAV vector is largely determined by the viral capsids. Experimenting with natural and engineered AAV capsids has proven to be a fruitful approach in identifying cardiotropic AAV vectors. For example, a comparison of AAV-1 to AAV-9 revealed AAV-9 as the most potent vector for the mouse heart.[33] Indeed, AAV-9 results in robust, widespread myocardial transduction in mdx mice irrespective of the age and the route of delivery (intravenous or intra-arterial).[34–36] Directed evolution and cardiotropic peptide insertion have also yielded novel AAV variants with enhanced cardiac transduction in rodent models of limb-girdle muscular dystrophy (LGMD) 2F, an extremely rare type of muscular dystrophy caused by δ-sarcoglycan deficiency.[32,37,38]

3. Disease gene-specific gene therapy

3.1. Dystrophin-based Duchenne cardiomyopathy gene therapy

The dystrophin gene was the first muscular dystrophy-associated gene cloned by the positional cloning approach.[39] Its mutation leads to DMD. The 2.4-mb full-length dystrophin gene contains 79 exons and it

transcribes into a ~12-kb cDNA. The full-length dystrophin protein has four major functional domains, including the N-terminal, rod, cysteine-rich, and C-terminal domains. The N-terminal domain binds to cytosolic γ-actin. The rod domain consists of 24 spectrin-like repeats. Within the rod domain, there are several important subdomains, including one for γ-actin-binding, one for neuronal nitric oxide synthase (nNOS)-binding, and one for microtubule-binding.[40–43] The cysteine-rich domain links dystrophin to the extracellular matrix through dystroglycan, a transmembrane glycoprotein. The C-terminal domain binds to syntrophin and dystrobrevin.

The enormous size of the dystrophin gene presents a delivery challenge, because it is beyond the packaging capacity of most viral vectors. Interestingly, some naturally existing, internally deleted dystrophins (e.g. Δ17–48) are quite functional.[44] These mini-dystrophin genes are about 6–8-kb in length, and their expression in humans and animals has greatly mitigated skeletal muscle disease.[19–21,44–47] The therapeutic implication of mini-dystrophin in the heart has only been investigated in transgenic mice.[48] We expressed mini-dystrophin specifically in the heart of mdx mice. This cardiac-restricted expression completely corrected cardiac histopathology, improved exercise performance, and enhanced myocardial contractility.[48] Whether mini-dystrophin gene therapy can achieve similar effectiveness remains to be seen. In this regard, dual AAV vectors have been developed to express the mini-dystrophin gene.[18–21,49–51] Further, systemic injection of dual AAV vectors has been shown to transduce the myocardium at high efficiency in mdx mice.[52,53]

A single vector therapy would be more advantageous. To package dystrophin into AAV, highly abbreviated micro-dystrophin genes have been developed. The microgene is about 3.5–4 kb in length and contains ~30% of the dystrophin coding sequence. In contrast to mini-dystrophin, micro-dystrophin does not carry the C-terminal domain. Additionally, it has a shorter rod domain with only four to five spectrin-like repeats. AAV-mediated micro-dystrophin gene therapy has been extensively studied in various mouse models and more recently in the canine model.[47,54–59] Direct or systemic AAV microgene therapy significantly ameliorated skeletal muscle disease in dystrophic mice and dogs. The first study to evaluate the therapeutic effect of micro-dystrophin in the heart was performed by Yue et al.[24] In this study, an AAV-5 microgene vector was directly injected into the cardiac cavity of neonatal mdx mice. Micro-dystrophin restored the DGC complex in the heart and enhanced the membrane stability of cardiomyocytes.[24] In subsequent studies, newly developed AAV capsids (such as AAV-6 and AAV-9) were utilized to deliver micro-dystrophin to the heart through peripheral vein injection.[34–36,54,60–62] Of particular interest are studies by Bostick et al., in which an AAV-9 microgene vector was delivered to the heart of aged female mdx mice. This study is noteworthy because aged female mdx mice develop a cardiac phenotype nearly identical to that observed in dilated cardiomyopathy of human patients.[34,35,63,64] Despite the advanced heart disease in very old mice, surprisingly, cardiomyocytes were efficiently transduced.[34,35] The average lifespan of mdx mice is ~22 months.[65,66] In preterminal mdx mice (16–20 m old), microgene therapy reduced myocardial fibrosis, improved the electrocardiographic profile, and enhanced hemodynamic function.[34] In terminal-aged mdx mice (>21 m old), neither fibrosis nor hemodynamic function was improved.[35] However, some ECG parameters were partially corrected, and dobutamine stress-induced acute cardiac death was reduced.[35]

Expression of a full-length or near-full-length dystrophin protein may lead to a better recovery. This is feasible with tri-AAV vectors, but the efficiency is too low to be of practical use currently.[22] Editing the mutated RNA transcript or genome offers alternative approaches to reach this goal. Exon skipping is a potent method to achieve RNA-level editing. Briefly, antisense oligonucleotides (AONs) are delivered to modulate RNA splicing so that the mutated (and sometimes adjacent) exons are removed. The resulting mRNA, though abbreviated, is in-frame and can yield a near-full-length protein.[67] Several chemically distinctive classes of AONs have been developed, including 2-O-methylated phosphorothioated (2-OMePS), phosphorodiamidate morpholino oligomers (PMOs), peptide/polymer/nanoparticle–conjugated PMOs, and most recently tricycle-DNA (tcDNA). 2-OMePS and PMOs are currently in clinical trials.[68–73] However, these AONs cannot reach the heart.[74–76] Peptide/polymer/nanoparticle-conjugated PMOs can induce exon-skipping in the heart of mdx mice and improve heart function.[77–87] However, there are issues related to potential toxicity and immunogenicity.[88] The newly developed tcDNA represents the most advanced AON formulation.[89] Because of its unique pharmacological property, systemic delivery of tcDNA–AONs resulted in phenomenal uptake in many tissues, including the heart and brain. Treatment in mdx mice and more severe utrophin/dystrophin double knockout (u-dko) mice improved cardiac, respiratory, and behavioral functions.[89] Importantly, no overt toxicity was detected with tcDNA.[89] An alternative strategy to

deliver AONs is to use the AAV vector. AAV-9-mediated systemic AON delivery resulted in high, efficient dystrophin expression in the heart of u-dko mice.[90] More recently, two independent groups achieved long-term dystrophin restoration in the heart of the canine DMD model with AAV-6-mediated local exon-skipping.[91,92]

Compared to RNA editing with exon-skipping, targeted editing of the mutated dystrophin gene has just entered an exciting time due to recent development of highly versatile genome engineering tools such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and most importantly, the clustered regularly interspaced palindromic repeat (CRISPR)-associated endonuclease 9 (Cas9).[93] A series of elegant studies from the Gersbach laboratory has provided compelling proof-of-concept evidence in restoring dystrophin expression in cells from DMD patients using these new technologies.[94–96] It is highly anticipated that genome editing will soon be used to treat skeletal muscle disease and cardiomyopathy in animal models of DMD.[97]

3.2. Disease gene-based therapy for cardiomyopathy in other muscular dystrophies

3.2.1. Targeting disease gene to treat LGMD cardiomyopathy

LGMD refers to a group of muscle disorders with a wide range of clinical and genetic heterogeneity.[98,99] Based on the inheritance pattern, they are classified as autosomal dominant type 1 (LGMD1) and autosomal recessive type 2 (LGMD2). Each type of LGMD is further classified according to the time the disease gene was discovered. For LGMD1, the goal of the gene therapy is to decrease the expression of the mutated gene. This can be achieved with RNA interference (RNAi) to silence the mutated gene.[100,101] So far, only one study has tested gene therapy for dominant LGMD. LGMD1A is caused by myotilin gene mutation. Liu et al. targeted mutant myotilin with an AAV-6 microRNA vector.[102] The treatment significantly reduced the expression of the mutated myotilin protein and ameliorated skeletal muscle myopathy. Although LGMD1A patients do not exhibit cardiac abnormalities,[4] the RNAi approach described by Liu et al. may treat cardiac manifestations in other dominant myopathies such as lamin A/C gene mutation-induced LGMD1B and Emery–Dreifuss muscular dystrophy.

There has been significant progress in LGMD2 gene therapy. AAV-mediated gene therapy has been tested in animal models of at least seven different subtypes of LGMD2 (2A to 2F, and 2I). LGMD2A and 2B are caused by mutations in the calpain-3 gene and the dysferlin gene, respectively. According to Hermans, LGMD2A and 2B do not show cardiac manifestations.[4] However, cardiomyopathy has been seen in dysferlin-deficient mice, and there are also a few reports of cardiac involvement in some LGMD2B patients.[103–106] Three different approaches have been explored to express a functional dysferlin gene. These include delivering a minimized dysferlin gene with a single AAV vector, delivering a full-length dysferlin cDNA with dual AAV vectors, and exon-skipping or pre-mRNA trans-splicing to repair the defective dysferlin RNA transcript.[107–113] Defective membrane repair has been considered as the major pathogenic mechanism for LGMD2B. The in vitro membrane repair assay has been used as a surrogate endpoint to evaluate the therapeutic efficacy. Surprisingly, a recent study by Lostal et al. found that a correction of membrane repair as measured by the in vitro assay did not correlate with the correction of muscle pathology. The authors overexpressed myoferlin, a homolog of dysferlin, in dysferlin-null mice by the transgenic approach and they also expressed the mini-dysferlin gene in 4-week-old dysferlin-null mice. Neither transgenic overexpression of myoferlin nor AAV-mediated expression of mini-dysferlin improved muscle histology, although both corrected membrane repair deficits in vitro.[114]

LGMD2C to 2F are often referred to as sarcoglycanopathies, because they are caused by mutations in the sarcoglycan genes. The most common sarcoglycanopathy is α-sarcoglycan-deficient LGMD2D. Cardiac involvement is rare in LGMD2D.[6] LGMD2C, which is caused by mutations in the γ-sarcoglycan gene, usually exhibits mild cardiomyopathy. Deficiency of β-sarcoglycan and δ-sarcoglycan results in LGMD2E and LGMD2F, respectively. These two diseases are associated with dilated cardiomyopathy.[3,6] The molecular weights of sarcoglycans are in the range of 35–50 kD. The small size makes sarcoglycan genes perfect candidates for AAV delivery. Sarcoglycanopathies were among the first few inherited diseases proposed for AAV gene therapy.[115] Recently, AAV gene therapy for LGMD2C and 2D has entered into clinical trials.[116–118] Very few studies have explored AAV β-sarcoglycan gene transfer for treating LGMD2E.[119,120] However, therapeutic delivery of the δ-sarcoglycan gene by AAV has been tested extensively in the mouse and hamster models of LGMD2F. Systemic or direct myocardial delivery of the δ-sarcoglycan gene not only reduced histological lesions in the heart (such as myocardial necrosis, inflammation, calcification, and fibrosis) but also improved heart function and extended lifespan.[38,121–125] Collectively, these preclinical studies suggest that AAV δ-sarcoglycan gene transfer is an effective treatment for dilated cardiomyopathy in LGMD2F.

LGMD2I is caused by mutations in the fukutin-related protein (FKRP) gene. FKRP is located in the Golgi apparatus, and it is essential for post-translational glycosylation of α-dystroglycan, the protein that directly interacts with the extracellular matrix in the DGC complex. More than half of LGMD2I patients have cardiac abnormalities, and a quarter of them develop heart failure.[126] Gene therapy for LGMD2I has been hindered by the lack of a good animal model. Nonsense mutations and whole-gene deletions are embryonically lethal.[127] To overcome this hurdle, Lu and colleagues recently generated an FKRP L276I knock-in mouse.[128] This nonsense mutation model mimics the clinical phenotype of LGMD2I. To determine whether systemic delivery of the FKRP gene with AAV can protect the heart, Qiao et al. performed intraperitoneal injection in newborn FKRP L276I knock-in mice using an AAV-8 vector. Dobutamine-stressed echocardiography in 7-m-old treated mice showed significantly higher ejection fraction and fractional shortening than those of untreated mice.[128]

3.2.2. Targeting disease gene to treat heart disease in other muscular dystrophies

Dystroglycanopathies are a group of congenital muscular dystrophies (MDC). They are caused by mutations in the genes involved in the glycosylation pathway of α-dystroglycan.[129,130] Fukuyama muscular dystrophy, a dystroglycanopathy caused by retrotransposon insertion in the 3′-untranslated region of the fukutin gene, is associated with severe cardiomyopathy and congestive heart failure.[131,132] Blockade of pathogenic exon-trapping by a cocktail of AONs restored fukutin expression and α-dystroglycan glycosylation in the mouse model and human cells.[132] Whether this therapy can rescue heart function remains to be determined by future studies.

FRKP gene mutation not only causes LGMD2I but also causes MDC type 1C (MDC1C). Similar to LGMD2I, cardiac involvement is also a frequent finding in MDC1C patients.[133] A mouse model for MDC1C has been generated with FKRP P448L knock-in.[134] AAV-9 mediated FKRP expression normalized α-dystroglycan glycosylation in the heart of MDC1C mice. Unfortunately, cardiac function was not assessed due to mild heart disease at the age of euthanization (5 months).[134]

Myotonic dystrophy (DM), the second most common muscular dystrophy, is an autosomal dominant disease. It is caused by pathogenic RNA gain-of-function toxicity due to CTG (for DM1) or CCTG (for DM2) expansion. Cardiac conduction deficits (conduction block and arrhythmia) contribute significantly to the morbidity and mortality.[135] About 20 different mouse models have been developed to reveal various aspects of the disease.[136] Among these, tamoxifen-inducible EpA960 mice and tetracycline-inducible GFP-DMPK-$(CTG)_5$ mice are considered as good models to test cardiac interventions for DM.[137,138] The field of DM gene therapy has been particularly active in recent years. RNAi, ribozyme, AONs, and more recently, site-specific RNA endonuclease have all been explored for DM gene therapy.[139–144] However, most of these studies have not examined therapeutic efficacy in the heart. The in vivo proof-of-principle for reversing cardiac conduction defects has only been shown in GFP-DMPK-$(CTG)_5$ mice. In this model, administration of doxycycline induced myotonia and cardiac conduction abnormalities. Discontinuation of doxycycline dramatically reduced myotonic symptoms and conduction block in the heart.[137]

4. Expanding the armory of dystrophic cardiomyopathy gene therapy by targeting pathogenic mechanisms

4.1. Dystrophin-independent Duchenne cardiomyopathy gene therapy

4.1.1. Stabilization of cardiomyocyte membrane with endogenous cellular genes

Given that membrane weakening is a primary pathogenic mechanism, strategies that enhance sarcolemmal stability should theoretically ameliorate Duchenne cardiomyopathy. Utrophin is a dystrophin homolog.[145] Despite some differences,[43,146,147] utrophin shares significant structural and functional similarity to dystrophin and assembles the utrophin-associated glycoprotein complex (UGC). As is the case for dystrophin, micro-utrophin has been generated for AAV delivery.[40,148] More recently, AAV-mediated expression of jazz, an artificial zinc finger transcription factor, was found to activate the utrophin promoter and enhance utrophin expression.[149] So far, these utrophin-based strategies have only been shown to protect skeletal muscle. Their therapeutic efficacy in the heart remains to be tested experimentally. Several components of the DGC and UGC, including sarcoglycans, sarcospan, and nNOS, were recently shown to reduce the skeletal muscle phenotype in mdx mice.[66,150,151] Of these, only nNOS has been shown to treat Duchenne cardiomyopathy.[152] Specifically, Lai et al. delivered a PDZ domain-truncated version of the nNOS gene to the heart of 14-m-old mdx mice and examined the cardiac phenotype when mice reached 21 months of age. Supraphysiological ΔPDZ–nNOS expression significantly

reduced myocardial fibrosis, inflammation, and apoptosis. Importantly, treatment partially ameliorated ECG abnormalities and improved hemodynamic performance.[152]

Besides the DGC and UGC, the integrin complex (especially α7β1) is another membrane-crossing complex that stabilizes the sarcolemma.[153] Expression of the α7-integrin gene by AAV was recently shown to reduce limb muscle disease in mdx mice and extend the life span of u-dko mice.[154,155] The cardiac benefit of AAV-mediated α7-integrin expression remains to be demonstrated.

4.1.2. Treating Duchenne cardiomyopathy with calcium-regulating genes

Cytosolic calcium overload is a pivotal pathogenic event leading to muscle damage and force reduction in DMD.[156] Restoring calcium homeostasis holds great promise for treating Duchenne cardiomyopathy. The sarco/endoplasmic reticulum calcium ATPase (SERCA) is a calcium pump that removes calcium from the cytosol and transports it into the lumen of the sarcoplasmic reticulum (SR). SERCA accounts for ≥70% of calcium removal from the cytosol in muscle cells. SERCA2a is expressed in the heart and slow-twitch skeletal muscle. We found that SERCA2a expression is reduced in the heart of mdx mice by immunostaining.[157] When the AAV-9 SERCA2a vector was delivered to the heart of 12-m-old mdx mice, it increased myocardial SERCA2a expression and significantly improved cardiac electrophysiology.[157] Encouragingly, similar protection was observed when the AAV-9 SERCA2a vector was administrated to terminal-aged (22-m-old) mdx mice.[158]

4.1.3. Additional dystrophin-independent gene therapy strategies

Besides strengthening the sarcolemma and restoring calcium homeostasis, investigators have explored many other creative gene therapy strategies that are not dependent on dystrophin. These include AAV-mediated inhibition of the myostatin pathway, AAV-mediated overexpression of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), the cytotoxic T cell GalNAc transferase (Galgt2) and miR486, and AAV-mediated blocking of the nuclear factor kappa-light-chain-enhancer of activated B cell (NF-κB) signaling pathway.[159–165] However, most of these studies only demonstrated disease amelioration in skeletal muscle. Whether cardiac muscle can be protected is yet to be seen. Among these strategies, the myostatin inhibition approach is especially intriguing, because this approach aims at increasing the muscle mass. This raises two concerns: (a) muscle hypertrophy may increase stress on the sarcolemma and hence worsen muscle disease, and (b) myostatin inhibition may lead to hypertrophic cardiomyopathy. Indeed, conflicting results have been achieved depending on the models used. In animal models for DMD (mice and dogs), myostatin inhibition has consistently improved skeletal muscle pathology and function.[163,164,166–168] In a phase I trial, AAV-mediated regional expression of the myostatin antagonist follistatin improved walking distance in five out of six BMD patients.[169] However, the results of myostatin inhibition appear less promising in preclinical studies of some other muscular dystrophies such as LGMD2B, LGMD2C, LGMD2F, and congenital muscular dystrophy type 1A (MDC1A).[170–173] Cohn et al. examined whether myostatin deficiency can cause myocardial hypertrophy in normal C57BL/6 mice and mdx mice.[174] Surprisingly, myostatin elimination did not affect heart weight and heart weight/body weight ratio in either strain.[174] A major protective mechanism of myostatin inhibition is to reverse muscle fibrosis by inducing fibroblast apoptosis.[175] For reasons yet unknown, this mechanism appears to be deficient in the heart.[174] Collectively, there is a lack of clear evidence suggesting that myostatin blockade benefits a dystrophic heart. Myostatin inhibition-based gene therapy strategies have to be carefully weighted against potential undesirable side effects.[170–173,176]

4.2. Disease gene-independent gene therapy for cardiomyopathy in other muscular dystrophies

4.2.1. Disease gene-independent gene therapy for dilated cardiomyopathy in LGMD2E and 2F

MicroRNAs (miRs) are regulatory noncoding RNAs. Recent studies suggest that miRs play crucial roles in myocardial remodeling.[177] Sampaolesi and colleagues found that miR669 is downregulated in the heart of β-sarcoglycan-null LGMD2E mice.[178] In a subsequent study, they evaluated preventive miR gene therapy in β-sarcoglycan knockout mice.[179] After intraventricular delivery of an AAV-2 miR669a vector to neonates, they quantified survival, cardiac fibrosis, and function at the age of 18 months. AAV-injected mice showed less myocardial fibrosis, better heart function, and significantly better survival.[179]

Several disease gene-independent approaches have been tested to treat dilated cardiomyopathy in rodent models of LGMD2F.[25] Mitsugumin 53 (MG53) is a 53-kD membrane repair protein and also a ubiquitin E3 ligase.[180] Mice lacking MG53 show increased susceptibility to sarcolemmal injury and develop a slow but progressive myopathy.[181] He et al. introduced MG53 to neonatal and young adult LGMD2F hamsters with

AAV-8. Supraphysiological MG53 expression in the heart and limb muscle partially reduced the serum creatine kinase level, stabilized the sarcolemma, and slowed muscle degeneration and fibrosis. It also improved treadmill performance and heart function.[182] Since sarcolemmal disruption is a common pathogenic process, it is suggested that MG53 therapy may serve as a broadband therapeutic for a wide range of muscular dystrophies.[180] Unfortunately, there are some important safety concerns for long-term use. In one study, authors noticed elevation of hepatic enzymes due to leaky MG53 expression in the liver.[182] Most alarmingly, two recent studies found that the E3 ligase function of MG53 targets the muscle insulin receptor and insulin-receptor substrate 1 for degradation.[183,184] Transgenic overexpression of MG53 in striated muscle and heart resulted in metabolic syndrome and diabetic cardiomyopathy, respectively.[183,185]

Defects in SR calcium cycling play a pivotal role in the pathogenesis of inherited and acquired cardiomyopathy.[186] As eluded before, SERCA2a is the primary calcium pump in the heart. AAV-mediated SERCA2a overexpression ameliorates some cardiac manifestations in the mdx model of Duchenne cardiomyopathy.[157,158] The activity of SERCA2a is regulated by phospholamban. Unphosphorylated phospholamban inhibits SERCA2a activity but phosphorylated phospholamban does not. A single amino acid change (Ser16 Glu) locks phospholamban in a conformation that resembles the phosphorylated form. Hoshijima et al. delivered this pseudo-phosphorylated phospholamban to the heart of δ-sarcoglycan-deficient hamsters using AAV-2.[25] Chronic expression of pseudo-phosphorylated phospholamban markedly improved heart function in this LGMD2F-dilated cardiomyopathy model.[25]

Apoptosis has been implicated in the progression of heart failure. In particular, activation of apoptosis signal-regulating kinase 1 (ASK1) induces cardiomyocytes apoptosis. Hikoso et al. tested whether delivery of the dominant mutant form of ASK1 can reduce cardiomyopathy in the LGMD2F hamster model.[187] They delivered dominant mutant ASK1 by AAV-2 via transcoronary perfusion to 10-week-old affected hamsters. Evaluation at the age of 24 weeks revealed remarkable improvements of systolic and diastolic function as well as a reduction of chamber dilation and myocardial fibrosis.

4.2.2. Disease gene-independent gene therapy for cardiomyopathy in other muscular dystrophies

Merosin (laminin α2) is an extracellular matrix protein. Deficiency in merosin leads to MDC1A. Although MDC1A patients usually do not have clinically significant cardiomyopathy,[4] cardiac involvement has been documented in atypical patients and laminin α2-null dy/dy mice.[188–191] Agrin is also an extracellular matrix protein, but it has no structural similarity to laminin α2. Interestingly, AAV-1 mediated systemic expression of a miniature version of agrin greatly reduced myocardial fibrosis in dy/dy mice.[192]

LGMD2I and MDC1C are caused by mutations in the FKRP gene, and both diseases display prominent cardiac manifestations. FKRP knock-in mice L276I and P448L have been developed to model LGMD2I and MDC1C, respectively.[128,134] The pathway of α-dystroglycan glycosylation involves a series of glycosyltransferases. Like-acetylglucosaminyltransferase (LARGE) acts downstream of FKRP. Activation of a downstream enzyme presumably should correct the disease phenotype caused by upstream enzyme deficiency. Vannoy indeed found that AAV-mediated LARGE overexpression not only reduced myopathy in LARGE-deficient MDC mice but also improved α-dystroglycan glycosylation in the heart and skeletal muscle of FKRB P448L knock-in mice.[193]

5. Expert opinion

The cloning of the dystrophin gene in 1986 started a flood of discoveries on genes whose mutations cause various forms of muscular dystrophies.[39] All of a sudden, it appears that we may cure many muscular dystrophies and their associated cardiomyopathies by either fixing the mutated gene or introducing a functional copy of the normal gene. While conceptually straightforward, the journey thus far has turned out to be long and winding. Research in dystrophic cardiomyopathy and its gene therapy has made significant progress in the last decade.[194–196] Several fundamental issues have been addressed. These include the establishment of a large collection of animal models to test experimental gene therapy in various forms of dystrophic cardiomyopathy, the development of non-invasive AAV delivery methods to efficiently transduce the heart, and the expansion of therapeutic schemes from simply delivering a functional cDNA to dystrophic muscle to the modulation of the RNA/DNA structure and expression using a variety of coding and noncoding sequences, even oligonucleotides. Some critical parameters for dystrophic cardiomyopathy gene therapy have also been clarified. For example, studies in the mdx model of Duchenne cardiomyopathy have provided compelling evidence that we may achieve a near-wild-type protection by treating half of the cardiomyocytes instead of every single cell.[63,197] On the

other hand, debates on whether treating skeletal muscle disease will alleviate or aggravate cardiomyopathy have settled down on the conclusion that both should be treated either together or separately.[76,198,199]

There is no doubt that Duchenne cardiomyopathy gene therapy has led the way for the entire field. First, a number of models have been generated for Duchenne cardiomyopathy gene therapy studies such as aged mdx mice, Cmah/mdx mice, u-dko mice, myoD/dystrophin double-knockout mice, and telomerase RNA/dystrophin double-null mdx/mTR mice.[63,64,200–204] Importantly, most of these rodent models are commercially available.[205] In terms of large animal models, besides the commonly used golden retriever muscular dystrophy dogs (GRMDs), additional dog models have been identified and colonies established.[206–209] Second, we have successfully treated the cardiac phenotype in symptomatic u-dko mice and aged mdx mice using micro-dystrophin and exon-skipping.[34,89] We even achieved widespread myocardial AAV gene transfer and some ECG improvements in terminal-stage mdx mice.[35] For scaling up, efficient myocardial transduction has been achieved in newborn dogs and adult affected dogs with systemic and percutaneous transendocardial AAV delivery.[57,91,92,210,211] Third, many previously underappreciated disease targets (such as nNOS and SERCA2a) and revolutionary technologies (such as tcDNA, ZENs, TALENs, and CRISPR/Cas9) are now on the horizon for Duchenne cardiomyopathy gene therapy. Despite this substantial progress, we still do not have answers to a lot of important questions. For example, it is not clear whether supraphysiological dystrophin expression in the heart is toxic, whether there are heart-specific domain(s) in the dystrophin gene that should be included in micro-dystrophin, and whether cardiotropic features of some existing AAV serotypes can cross the species boundary and result in efficient heart transduction in humans. For this last point, some recent developments in the generation of the xenograft model using dystrophic human muscle and forced evolution of human tissue tropic AAV capsids may provide some hints.[32,212,213] It should be noted that emerging new technologies such as genome editing with CRISPR/Cas9 not only brings in new hopes, they are also accompanied with new questions such as potential toxicity from off-target editing.

There is a long to-do list for the field of dystrophic cardiomyopathy gene therapy. Some of these may include (1) continued development and characterization of large animal models for dystrophic cardiomyopathy. In light of recent success in creating rat, pig, and monkey models using the CRISPR/Cas9 technology, model generation may no longer represent a formidable barrier as it was before [214]; (2) thorough evaluation of the most promising gene therapy strategies in large animal models.[215] Lack of solid large animal data has been an important factor limiting the translation of rodent study results to human patients. In this regard, there is an urgent need to thoroughly evaluate therapeutic efficacy in large mammals. For example, treating heart disease with tcDNA exon-skipping and AAV micro-dystrophin gene therapy in dystrophin-deficient dogs [216]; (3) establishment of cardiac-specific biomarkers that can be used to monitor disease progression and responses to gene therapy in animal models of dystrophic cardiomyopathy; (4) investigations of gene therapy for cardiac manifestations in muscular dystrophies other than DMD and LGMD2F. For many of these muscular dystrophies, gene therapy strategies have been developed for treating skeletal muscle myopathy. We need to test if similar approaches can attenuate cardiac disease.

In summary, gene therapy for dystrophic cardiomyopathy has taken a slow but steady path toward preclinical and eventually clinical studies. These efforts will undoubtedly be complicated by issues related to vector manufacturing, host immune responses, and the lack of enough patients for large-scale clinical trials due to the relatively low incidence of the disease. Nevertheless, we already have a solid foundation. The future of dystrophic cardiomyopathy gene therapy is very bright.

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Dystrophin Gene Replacement and Gene Repair Therapy for Duchenne Muscular Dystrophy in 2016: An Interview

Dongsheng Duan*

Department of Molecular Microbiology and Immunology & Department of Neurology, School of Medicine, and Department of Bioengineering, The University of Missouri, Columbia, Missouri.

After years of relentless efforts, gene therapy has now begun to deliver its therapeutic promise in several diseases. A number of gene therapy products have received regulatory approval in Europe and Asia. Duchenne muscular dystrophy (DMD) is an X-linked inherited lethal muscle disease. It is caused by mutations in the *dystrophin* gene. Replacing and/or repairing the mutated *dystrophin* gene holds great promises to treated DMD at the genetic level. Last several years have evidenced significant developments in preclinical experimentations in murine and canine models of DMD. There has been a strong interest in moving these promising findings to clinical trials. In light of rapid progress in this field, the Parent Project Muscular Dystrophy (PPMD) recently interviewed me on the current status of DMD gene therapy and readiness for clinical trials. Here I summarized the interview with PPMD.

Parent Project Muscular Dystrophy (PPMD): What is gene therapy?

Dr. Dongsheng Duan: Gene therapy refers to therapies that use nucleic acids as the "drug" to treat and/or prevent inherited or acquired diseases. Nucleic acids can be DNA, RNA, or oligonucleotides. Nucleic acids can be naked or incapsidated in a viral or nonviral carrier.

Gene therapy can be classified as either disease gene-dependent or disease gene-independent therapies. In the former, treatment aims at the gene that encodes the protein (in the case of Duchenne, this would be dystrophin). The mutated gene can be repaired or replaced. In the case of dominant mutation, the mutated gene can be silenced. Disease gene-independent therapies take advantage of disease-modifying genes that either are functional substitutes of the diseased gene or are genes that intervening downstream pathogenic processes (in the case of Duchenne, utrophin and follistatin are examples of gene-independent therapies). Disease gene-independent therapies also involve strategies that target noncoding region of the genome (such as microRNA therapy).

PPMD: Can you define the key terminology used in gene therapy—such as transgene, serotype, and vector?

Dr. Duan: A transgene means the gene that is being transferred. In the context of gene therapy, it usually refers to the gene that is used for therapy. For example, Duchenne muscular dystrophy (DMD) is caused by mutations in the *dystrophin* gene. A functional *dystrophin* gene can thus be transferred to diseased muscle cells to treat DMD. Here the transgene is the normal dystrophin gene.

In the context of gene therapy, a vector means the vehicle that is used to transport the nucleic acid "drug" to target cells. Gene therapy vectors are classified as viral vectors (meaning they are derived from a virus) or nonviral vectors (meaning they are not derived from a virus). Some of the most commonly used viral vectors include adeno-associated virus (AAV), adenovirus, retrovirus, and lentivirus.

The serotype refers to the serologically distinguishable feature of a virus. When a virus infects our body, the body will generate a unique set of antibodies against the invading virus. These antibodies can be detected in the serum. A virus can thus be classified into different types according to the antibodies detected in the serum. The serotype is often used to classify different members of the same family virus. For example, the family of AAV virus has different serotypes such as AAV serotype-1, 2, 8, and 9 (abbreviated as AAV-1, 2, 8, and 9).

*Correspondence: Dr. Dongsheng Duan, Department of Molecular Microbiology and Immunology, One Hospital Drive, Columbia, MO 65212. E-mail: duand@missouri.edu

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PPMD: How is a gene therapy vector delivered to the cells where it is needed and what does it do once there?

Dr. Duan: A gene therapy vector can be delivered to our cells either *ex vivo* or *in vivo*. In *ex vivo* delivery, investigators first isolate the target cells (e.g., bone marrow stem cells) from the body. They then mix the target cells and the vector in a container outside the body (e.g., in a petri dish) to allow the vector to get into the cells. The cells that carry the vector are then isolated and put back to the body. *In vivo* delivery refers to directly deliver the vector to the body. This can be achieved either locally to a specific location (e.g., via intramuscular injection to a muscle) or systemically to whole body (e.g., via intravenous injection).

After a vector is delivered to a tissue, the vector will enter the cell through receptors and coreceptors that are located on the surface of the cell. Once inside the cell, the vector will release the therapeutic gene it carries into the nucleus. In a typical AAV vector, the therapeutic gene is in a single-stranded DNA format. This format cannot direct cells to make the protein. In order to express the protein, the incoming AAV genome has to be converted to a double-stranded transcription-competent DNA molecule. The vast majority of the AAV genome is converted into a double-stranded sealed circle. A very tiny fraction of AAV may integrate into the chromosome. Most often, it does not cause a safety concern.[1] However, in a retroviral or lentiviral vector, the vector genome enters the cell as an RNA molecule. The RNA molecule is subsequently reverse transcribed into a DNA molecule and integrates into the chromosome. The integration of a retroviral vector in the human genome has been shown to cause leukemia in several clinical trials.[2,3] A new generation of retro/lentiviral vectors has been developed to minimize this safety concern.[4]

PPMD: There appears to be considerable progress recently in developing gene therapy for several genetic disorders. Can you give us some insights into that progress in eye and blood diseases?

Dr. Duan: Over the last few years, gene therapy has begun to deliver its therapeutic promise in several diseases. One example is AAV-2-mediated gene therapy for Leber's congenital amaurosis 2 (LCA2). This is a rare inherited retinal degenerative disease. Affected children lose their vision because of mutations in a gene called *Rep65*. Investigators in the United States and United Kingdom put a normal *Rep65* gene in an AAV-2 vector and then injected the vector into the eye of patients with LCA2. Treated patients were able to regain their vision. Some patients still maintain their improved vision at eight years after gene therapy.[5]

Another major breakthrough is AAV-8-mediated gene therapy for hemophilia B. Hemophilia B is caused by mutations in a gene that encodes coagulation factor IX. To treat hemophilia B, scientists packaged a normal *factor IX* gene in an AAV-8 vector and injected intravenously to patients with severe hemophilia B. Factor IX produced from the AAV-8 vector significantly improved clinical outcomes without causing serious side effects. Therapeutic effect has maintained for more than four years in treated patients.[6,7]

PPMD: Have any gene therapy products received regulatory approval?

Dr. Duan: Several gene therapy products have been approved by regulatory agencies. In 2003, China approved the first gene therapy product called *Gendicine.*[8] This is an adenovirus vector for cancer therapy. In 2005, China approved *Oncorin,* another adenoviral vector for cancer gene therapy.[9] In 2007, the Philippines approved *Rexin-G,* a retroviral vector for cancer gene therapy.[10] In 2011, Russia approved *Neovasculgen*, a nonviral vector for treating peripheral arterial disease.[11,12] In 2012, European Medical Agency approved *Glybera*, an AAV-1 vector for treating a rare genetic disease called lipoprotein lipase deficiency.[13,14] On October 27, 2015, FDA approved *Imlygic*, an oncolytic herpes virus vector. This is the first commercial gene therapy product approved in the United States.[15,16] On October 10, 2015, a biotech company called Spark Therapeutics announced the results of its phase III trial on an AAV-2 vector for treating a form of childhood blindness. There were no serious adverse events. Treated patients showed significant vision improvement. Spark Therapeutics will seek regulatory approval from FDA in 2016 to market their gene therapy product. If successful, this will become the first AAV gene therapy approved by FDA.[5]

PPMD: What's the rationale for gene therapy in Duchenne? How does a gene delivered via gene therapy help ameliorate the progression of DMD? What's the potential impact for Duchenne patients?

Dr. Duan: The fundamental problem in DMD is the absence of dystrophin, an essential muscle protein. This is caused by mutations in the *dystrophin* gene.[17,18] Basically, mutations abort dystrophin production. Delivery of a new functional *dystrophin* gene or repair of the mutated *dystro-*

phin gene should restore dystrophin production in muscle and prevent muscle cells from dying. Gene therapy is expected to stop or slow down the progression of muscular dystrophy, improve life quality, and extend the life span of affected boys. If gene therapy is applied early enough, it may "cure" the disease.

PPMD: Gene therapy was tried years ago in muscular dystrophy, but suffered some setbacks from clinical trials in other diseases? Are we in better shape now and why?

Dr. Duan: Soon after the discovery of the *dystrophin* gene, scientists had begun to test gene therapy. Early studies used plasmid (nonviral vector), retrovirus, and adenovirus. These were performed in cultured muscle cells and dystrophin-deficient mdx mice. During this period, adenovirus delivery of a *mini-dystrophin* gene (which is derived from a very mild Becker patient) was at the forefront (see Note).[19] Unfortunately, adenovirus induces strong cellular immune responses and the mini-dystrophin produced from the adenovirus vector did not last long. In 1998, the entire field of gene therapy was put on hold because of the death of 18-year-old Jesse Gelsinger, who died from an adenovirus gene therapy trial for an inherited liver disease.[20]

Several gene therapy studies have been conducted in muscular dystrophy patients since that time. The first clinical trial for muscular dystrophy was published in 2004. This trial used a nonviral plasmid vector called *Myodys*.[21] It delivers a full-length dystrophin coding sequence. Investigators injected the plasmid directly into a muscle in DMD patients. Unfortunately, therapy yielded minimal dystrophin expression.[22]

The clinical trial of AAV gene therapy for muscular dystrophy was initially proposed in 2000 to treat limb girdle muscular dystrophy.[23] However, the first AAV gene therapy for muscular dystrophy was not reported until 2009.[24] In this study, Dr. Mendell and colleagues injected an AAV-1 vector that carried the *alpha-sarcoglycan* gene to the muscle of patients with limb girdle muscular dystrophy 2D and observed persistent expression of the therapeutic alpha-sarcoglycan in injected muscle for 6 months.[24] Another AAV trial was reported in 2010 for DMD.[25] This trial used an engineered AAV-2.5 vector that carried a highly minimized synthetic *dystrophin* gene. Unfortunately, dystrophin expression was barely observed in injected muscle. Detailed investigations suggest that the lack of expression was barely because of the immune response. The immune response to a gene therapy product first caught the attention in 2006 when an AAV-2 vector that carried *factor IX* gene was delivered to the liver of patients with hemophilia B.[26] Investigators initially observed a therapeutic level factor IX production in the blood. But it did not last because treated liver cells were rejected by the immune system a few weeks later. It is now clear that the gene delivery vehicle (AAV virus capsid), cargo (transgene), or the protein produced from the therapeutic transgene can all illicit immune responses. To achieve long-term persistent gene therapy, we need to overcome the immune response barrier.

The invention of antisense oligonucleotide (AON)-mediated exon skipping opens the door to repair the messenger RNA, the molecule that translates the language of the gene (DNA) into a protein.[27] In exon skipping, the mutated part of dystrophin is skipped and a shortened version of dystrophin is produced. The first exon skipping trial on DMD patients was published in 2007.[28] In that trial, AONs were directly injected into patient's muscle. Since then, there has been significant progress in exon skipping. Several trials have been conducted in Europe and the United States to achieve systemic exon skipping. The major hurdles in current exon skipping include its low efficiency, transient nature, and failure to treat the heart. The US Food and Drug Administration (FDA) recently reviewed the new drug application (NDA) for two exon-skipping drugs, one from BioMarin Pharmaceutical (Kyndrisa; drisapersen) and the other from Sarepta Therapeutics (eteplirsen; AVI-4658). Both drugs aim at skipping exon 51 which could benefit ~13% DMD patients. On January 14, 2016, the FDA issued a complete response on BiomaMarin's NDA application and stated the FDA could not approve the NDA in its present form (www.drugs.com/history/kyndrisa.html). According to a FDA briefing document published on November 24, 2015 (www.fda.gov/downloads/advisorycommittees/committeesmeetingmaterials/drugs/peripheralandcentralnervoussystemdrugsadvisorycommittee/ucm473737.pdf), the major issues are the lack of clinical efficacy, failure to show increased dystrophin expression by western blot, and some concerns on the safety (such as renal toxicity). On January 22, 2016, the FDA published a briefing document on Sarepta's NDA application for eteplirsen (www.fda.gov/downloads/advisorycommittees/committeesmeetingmaterials/drugs/peripheralandcentralnervoussystemdrugsadvisorycommittee/ucm481911.pdf). Significant concerns were raised by the FDA on clinical efficacy and dystrophin levels but not on the safety of the drug. Sarepta has since submitted four-year clinical effective data. According to a news release from

Sarepta[29], the FDA will further review the data and reach a conclusion on whether eteplirsen will be approved, conditionally approved, or not approved by May 2, 2016.

Looking forward, the field is in a much stronger position than it was ever before. For example, we have identified major hurdles in exon skipping and AAV gene therapy, and we have also developed novel strategies to overcome these hurdles. In terms of exon skipping, new AONs with superior chemical properties (such as tricycle-DNA AONs) have greatly improved correction in deep muscles such as the diaphragm and the heart without causing toxicity in mouse models of DMD.[30] Methods have also been developed to use AAV to deliver AONs for long-term widespread correction. In terms of AAV gene therapy, transient immune suppression protocols (before, at the time of, or after gene delivery) have been developed and have shown success in the hemophilia B trial and in dog DMD models. Novel AAV capsids with improved properties are also being engineered to meet the specific needs of DMD gene therapy.[31]

PPMD: Which Duchenne patients could potentially benefit from gene therapy? Early versus late-stage boys? Ambulatory versus nonambulatory?

Dr. Duan: Broadly speaking, gene therapy has the potential to benefit every DMD patient. Systemic bodywide gene therapy in early stage boys (especially before they lose large amount of muscle) may prevent muscle from deterioration and dramatically change the disease course. Clinical observations in mild Becker patients suggest that a successful gene therapy may allow ambulation to the age of 60s.[19] For late-stage boys, the goal of gene therapy is to improve life quality. Localized gene therapy in limb muscles may improve their function for holding and grasping and allow use of a keyboard. Cardiac gene therapy may also improve the heart function of late-stage boys.

PPMD: What attributes of the dystrophin gene and protein make it amenable to gene therapy (e.g., size of the gene, spectrin repeat region, proof-of-concept from large Becker muscular dystrophy deletions)?

Dr. Duan: The *dystrophin* gene is one of the largest genes in the genome. It has a size of 2.4 mb (mega base) and is beyond the carrying capacity of any viral vector.[32] A gene is composed of protein-coding exons and nonprotein coding introns. Dystrophin has 79 exons. The protein-coding region (also called cDNA) of dystrophin has a size of 11.2 kb (kilo base).[33] The dystrophin protein has a size of 427 kD (kilo Dalton).[34] Ideally, delivery of a full-length dystrophin cDNA will yield the production of a full-length dystrophin protein and the maximum protection of muscle. This can be achieved with a nonviral vector (such as a plasmid), gutted adenovirus, and tri-AAV vectors.[21,35,36] Currently, these strategies are not ready for clinical development because of issues related to delivery efficiency, the immune response, vector purification, and so on.

The full-length dystrophin protein can be divided into four domains. These are the N-terminal, rod, cysteine-rich, and C-terminal domains. The rod domain can be further divided into 24 spectrin-like repeats and 4 hinges (1 hinge sits between the N-terminal domain and the rod domain, 1 hinge sits between the rod domain and the cysteine-rich domain, and the other 2 hinges intervene spectrin-like repeats). It is now clear that not all the domains are absolutely required for muscle protection. Studies in mildly affected Becker patients suggest that deletion of a fairly large piece of the rod domain is not associated with major deleterious consequences to muscle function.

Knowledge learned from Becker patients inspired scientists to develop an abbreviated/truncated *dystrophin* gene for DMD gene therapy. There are two major classes of abbreviated *dystrophin* genes.[37] One is called the *mini-dystrophin* gene and the other is called the *microdystrophin* gene. The *mini-dystrophin* gene (minigene) is about 6 to 8 kb in size and it results in the production of a mini-dystrophin protein that is about the half size of the full-length protein (see Note). Based on clinical observations in Becker patients, there is a high likelihood that minigene therapy will improve muscle health in DMD patients.[19,38–41] The *microdystrophin* gene (microgene) is about 3.5 to 4 kb in size and it results in the production of a microdystrophin protein that is about one-third the size of the full-length protein (see note). In addition to the truncation in the rod domain, the C-terminal domain is also deleted in the microgene. Although studies in mouse and dog models of DMD suggest that the microgene can ameliorate muscle disease and improve muscle force, no human precedent has been identified for the super-small microgene.[42,43] We will not know whether the microgene can treat DMD patients until a clinical trial is conducted. The beauty of the microgene is that it can fit into the AAV vector, which has a maximal packaging capacity of 5 kb. To deliver a mini-dystrophin gene with AAV, the gene has to be split into two pieces and separately delivered by two independent AAV vectors (the dual-AAV

approach); using this approach would increase the complexity of therapy development.[44]

PPMD: Can you briefly describe what exon skipping is and how it compares and contrasts with gene therapy as a potential treatment for Duchenne?

Dr. Duan: Three different gene therapy methods can be used to restore dystrophin expression. These are gene repair, exon skipping, and *dystrophin* gene replacement. Gene repair strategies can be used either to fix the mutation and recover a full-length *dystrophin* gene, or covert a Duchenne mutation into a Becker mutation. For the former, it requires homologous recombination (which is very inefficient in mature muscle cells) and a template of the normal sequence. Further, it may work only for patients with small mutations (such as point mutation and small deletions). To covert a Duchenne into a Becker, the mutated region (and sometimes its surrounding regions) is removed and remaining parts are ligated together. This will yield a dystrophin protein with a slightly reduced size but still functional.

Exon skipping is another repair strategy, but it does not repair the mutated gene. The mutated gene generates mutated RNA molecules. In exon skipping, the mutated region is removed by AONs from the RNA molecule while it is being processed inside the cell. Because the mutated gene will continually generate mutated RNAs, one has to continually deliver AONs to the cells in order to achieve long-term therapy. In other words, one may consider exon skipping as a "transient" gene repair therapy.

Gene replacement therapy has the longest history. It is often referred to as "gene therapy." In this case, the original mutated *dystrophin* gene remains in the genome. A normal copy of the dystrophin gene or an engineered synthetic *dystrophin* gene is delivered to muscle to produce a functional dystrophin protein. As long as the therapeutic transgene persists in the body, it should continually produce dystrophin. Because of the packaging limitations of viral vectors, most gene replacement therapies are aimed at delivering the abbreviated versions of the *dystrophin* gene.

Compared with the naturally existing *mini-dystrophin* genes (in Becker patients) and the shortened *dystrophin* gene/RNA generated by gene repair/exon skipping, a synthetic *dystrophin* gene may have some advantages. For example, scientists may use molecular engineering techniques to generate synthetic *dystrophin* genes that are structurally and functionally superior. Further, viral vectors can be engineered to produce much more dystrophin than a cell can produce with its own gene. Nevertheless, *dystrophin* gene replacement therapy also has a drawback. The endogenous *dystrophin* gene expresses the physiological amount of dystrophin at selected tissues at defined times. These specificities are usually lost in gene replacement therapy.

PPMD: We've heard of recent progress in gene editing using CRISPR/Cas9 technology—there was even an article on gene editing in a recent *New Yorker*. Can you briefly describe the gene editing approach; how is it similar and different from gene therapy?

Dr. Duan: Gene editing is another term for gene repair. Traditionally, gene editing has been very inefficient because of the lack of a good gene-editing tool. The CRISPR/Cas9 technology is a newly developed gene-editing system that originates from the bacterial defense mechanism. The CRISPR/Cas9 technology allows scientists to cut the genome at the desired locations with a guider RNA that has a sequence complementary to the DNA target. Using the CRISPR/Cas9 technology, Gersbach and colleagues have successfully restored dystrophin expression in muscle cells isolated from DMD patients.[45] Recently, Cohn and colleagues also demonstrated correction of a duplication mutation in muscle cells from patients.[46] An important concern is whether what have been achieved in cultured cells in a petri dish can be replicated in a live muscle. To this end, several groups have reported exciting new development demonstrating that it is feasible to perform CRISPR/Cas9 therapy in a live muscle in an intact mdx mouse.[47–49] Importantly, CRISPR/Cas9 treatment significantly reduced dystrophic pathology and improved muscle contractility. Despite these encouraging progresses, we have to realize that the technology itself is still at its infant stage. There are a lot of hurdles before it can be tested in human patients. Some of these include the immune response to the bacterial-derived Cas9 protein and off-target cutting.

PPMD: What are your latest findings in delivering a microdystrophin systemically in an animal model?

Dr. Duan: DMD affects all muscles in the body. A big challenge of DMD gene therapy is to treat all muscles in the body. Such whole-body therapy was shown possible in rodents more than 10 years ago.[50,51] However, the body size of a mouse is approximately 1000-fold smaller than that of a boy. It has been daunting to try to scale-up from a mouse to a lager mammal (such as a dog that has a body size

closer to a boy). We initially demonstrated the feasibility of systemic delivery in newborn dogs.[52–54] But this did not work well in neonatal affected dogs because of unexpected side effects.[43,55] Recently, we have finally accomplished the scale-up of systemic AAV delivery to juvenile DMD dogs and published the results in October 2015 in *Human Molecular Genetics*.[56] We achieved efficient AAV delivery of either a marker gene or a therapeutic *microdystrophin* gene to every muscle in the body of several young muscular dystrophy dogs. No toxicity was observed, and microdystrophin-treated muscles showed fewer lesions on histology examination.[56]

PPMD: Since DMD muscles undergo degeneration and regeneration, and satellite or other muscle precursor cells will be incorporated into fibers attempting regeneration, does your approach deliver dystrophin to these precursor cells?

Dr. Duan: Treating muscle precursor cells (or muscle stem cells) has been an important goal of DMD gene therapy. However, directly delivering an AAV microdystrophin vector to muscle stem cells may have limited effect. This is because AAV mainly exists as episomal circular molecules in a cell. As a stem cell begins to divide, AAV vectors will be diluted and eventually lost in progeny cells after many rounds of cell division.

This problem can be overcome by delivering a therapeutic *dystrophin* gene with an integrating virus such as lentivirus. A tiny fraction of AAV vectors may also integrate into the genome but the integration efficiency is much lower than that of a retrovirus or lentivirus.

Another solution is to use AAV to deliver gene repair tools to muscle stem cells. In this case, the repaired *dystrophin* gene will persist for good in daughter cells.[48]

PPMD: You used young dogs in your study. What do you see for a therapeutic window for gene therapy in DMD? That is, what age range do you see benefiting from the gene therapy approach?

Dr. Duan: As a first step toward systemic AAV *microdystrophin* gene therapy in a large mammal, we intentionally used young dogs that are 2 to 3 months of age. There are several considerations. The first is the amount of AAV vectors needed for the therapy. The amount of vectors that can treat one adult dog is sufficient to treat several young dogs. Although the industrial-scale AAV production is being developed, it is beyond the budget limit of an academic lab. The second is the age. We choose an age when affected dogs just begin to show symptoms. This roughly correlates to 2 to 4 years of age in affected boys when they begin to show delay in their motor milestones and are diagnosed. At this stage, muscle damage is mild and early intervention may yield the best effect.

With this being said, we don't think age will be a limitation for systemic AAV gene therapy. Our group and Chamberlain laboratories have shown that systemic therapy in aged mdx mice (≥18 months old; this corresponds to ≥60 years of age in humans) can still improve skeletal muscle and heart function.[57–59]

PPMD: Are you evaluating respiratory muscles, like the diaphragm, and the heart in your studies? Can these muscles benefit from gene therapy?

Dr. Duan: We achieved good gene transfer in the heart and respiratory muscles (including the diaphragm, intercostal muscle, and abdominal muscle). Based on our previous studies in the mouse model, we believe these muscles will benefit from the therapy.

PPMD: You now have established proof-of-concept for a microdystrophin delivered systemically in dogs; how do you see that progressing toward trials?

Dr. Duan: This is a critical milestone in the eventual application of bodywide gene therapy in Duchenne patients. Bodywide AAV delivery has been demonstrated in the rodent models of muscular dystrophy since 2004. However, systemic gene transfer has never been achieved in an adult subject of a large mammal. The enormous amount of vectors needed for each animal ($>10^{15}$ particles) not only implies a huge cost in vector production but also represents a significant safety concern. Scale-up AAV production may amplify contaminations that are negligible in small-scale ($<10^{13}$ particles) preparations. Importantly, unexpected inflammatory and/or immune response to the infusion of trillions of viral particles may lead to fatal complications as demonstrated in the tragic death of Jesse Gelsinger in a 1998 clinical trial.[20] On top of these, the ongoing massive myofiber necrosis and inflammation in adult dystrophic dogs may further worsen untoward immune responses. The excellent safety profile we saw in our study suggests that above-mentioned issues are likely manageable.

In our study, we tested only three dogs (one received a reporter gene AAV vector and two received the microdystrophin AAV vector). There is a need to expand the study to see if the success can be reproduced in a large number of dystrophic dogs.

DMD is a life-long disease. However, the longest time point in our study was four months. It is thus important to conduct long-term study to see if there are delayed immune responses or unexpected toxicity. In our study, we observed microdystrophin expression in 5% to 60% of muscle cells in different muscles. Additional studies are needed to further improve gene transfer efficiency to eventually achieve near-saturated dystrophin expression in the majority of muscles in the body. It should also be noted that in our study we evaluated only muscle histology; further studies are needed to see if systemic AAV gene therapy can improve muscle function. Last but not least, in our study we tested only one version of microdystrophin. Although this is so far the only microdystrophin that has been shown to provide physiological benefits in a large mammal, the improvement in muscle force is limited. Additional studies are needed to engineer more potent microdystrophins.

PPMD: You have a grant from PPMD to develop a gene therapy approach to deliver the sarco/endoplasmic reticulum calcium ATPase (*SERCA*) gene; can you tell us what the rationale is there and what progress you have made?

Dr. Duan: In DMD, a pivotal event downstream of dystrophin deficiency is the elevation of calcium in the cytoplasm of muscle cells. Elevated cytosolic calcium triggers proteolysis and muscle cell death.[60] Strategies that can reduce calcium overload in muscle cells will restore calcium homeostasis and reduce muscle disease. SERCA is the calcium pump that removes cytosolic calcium in muscle. With the funding from PPMD, we have tested whether AAV delivery of the *SERCA* gene can treat muscular dystrophy in animal models. We found that intravenous injection of the AAV SERCA vector to mdx mice significantly improved skeletal muscle force and heart function. Our next step is to test this highly promising therapy in affected dogs.

Duchenne patients do not have dystrophin in their body. Dystrophin generated by gene repair or gene replacement therapy could be considered as a foreign molecule by our immune system and hence mount an immune response to reject cells that contain the newly generated dystrophin protein. This will not be a concern for *SERCA* gene therapy because SERCA already exists in patient body.

PPMD: What preclinical steps need to be taken before an investigational new drug (IND) application can be filed for a gene therapy trial with systemic delivery of dystrophin?

Dr. Duan: A number of IND-enabling studies are needed before an IND can be issued. These include toxicity studies in small and large animals, generation of good manufacturing practices (GMP)-quality AAV vectors, pharmacokinetic and pharmacodynamics studies, and randomized blinded studies with sufficient sample size to confirm and validate systemic AAV therapy in dogs. It should also be noted that, in our study, we have used a canine *microdystrophin* gene. For clinical trial we need to develop a human-version *microdystrophin* gene.

PPMD: Delivering a gene therapy vector to all muscles affected in Duchenne has been one of the key challenges in developing this potential therapy. Can you tell us how research has progressed from the single-muscle injections that have been done in clinical trials toward systemic delivery?

Dr. Duan: Single-muscle injection is the foundation for systemic delivery. When gene therapy was initially tested in mdx mice, investigators performed single-muscle injection. The first attempt to achieve systemic delivery involved co-administration of vessel-perfusing agents such as histamine. The identification of new AAV serotypes that can escape from the vasculature and reach muscle cells has opened the door to "true" systemic delivery. Initial tests were performed in dystrophic mice and hamsters and then in neonatal dogs. Our study now suggests that systemic delivery can also be achieved in juvenile dystrophic dogs.

Single-muscle injection has been used in most of the muscular dystrophy clinical trials to date. Only one study has tested systemic delivery in human patients with a neuromuscular disease. In this trial (by AveXis, a biotech firm in Dallas), Drs. Mendell and Kaspar and colleagues delivered an AAV-9 vector to infants who suffered from a severe form of spinal muscular atrophy. According to the report by Dr. Mendell on October 5, 2015, at the International Congress of the World Muscle Society, nine patients have received therapy. The therapy appears to be generally safe and well tolerated. Signs of clinical improvement have also been noted (http://avexis.com/data-ongoing-study-avxs-101-spinal-muscular-atrophy-type-1-presented-world-muscle-congress/).

PPMD: Prior clinical trials of gene therapy in Duchenne have encountered immunological reactions that have impaired efficacy. What is being done to address that issue?

Dr. Duan: In our study in dystrophic dogs, we found that a five-week transient immune sup-

pression seemed to have made the trick. In hemophilia B trial and spinal muscular dystrophy trials, transient application of large dosage of steroids was found to be effective. Another important aspect is to screen patients for preexisting immunity to the viral vector and the therapeutic gene product. In our study, we screen affected dogs for the preexisting neutralization antibody to AAV-9 and we only used dogs that were seronegative for AAV-9 (meaning these dogs have never been exposed to AAV-9). It should be noted that additional efforts are needed before we can completely solve the problem of the immune response. In this regard, several new strategies that are being tested in laboratories (such as plasmapheresis and AAV capsid engineering) have shown promise.

PPMD: In scaling up to do clinical trials in Duchenne, vector production may be a limitation. How is the problem of having sufficient vector to do clinical trials and, later, to treat large numbers of Duchenne patients being addressed?

Dr. Duan: Vector manufacture has been recognized as a key bottleneck to scale-up of systemic AAV therapy in human patients.[61,62] Classic AAV production protocol requires transient transfection of three different plasmids to HEK 293 cells that are cultured in a petri dish. AAV is then purified from culture medium and cell lysate using ultracentrifugation. Numerous strategies have been tested to scale-up AAV production and purification. Some examples include the use of the infection approach with the baculovirus-based system or herpes virus-based system, and producer cell lines. Cell culture has also been expanded from the petri dish to roller bottles, cell factors, and bioreactors. Chromatography-based purification strategy has also been developed for different AAV serotypes. Most importantly, robust vector characterization and analytical quality control protocols and standards have been developed or are being developed and validated.

PPMD: What about commercial partners that would be needed to bring a therapy through regulatory approval and to market? Can you tell us about your partnership with SOLID GT and how that may move gene therapy toward clinical trials in Duchenne?

Dr. Duan: Industry investment is essential to bring an experimental vector into a gene therapy product. Funding from biopharmaceutical partners will offset the high cost of clinical studies. Solid GT is a subsidiary of Solid Biosciences and was started by parents of a boy with DMD. As stated in the company's website (http://solidbio.com/gt/), it "is dedicated to the development of durable disease-modifying interventions for Duchenne Muscular Dystrophy through gene therapy." Solid GT is currently pursuing systemic AAV *microdystrophin* gene therapy. According to the company's website "Solid GT is conducting a number of key studies that will enable us to enter the clinic within two years." "These studies include efficacy, safety and dose ranging assessments" in mdx mice and dystrophic dogs. We have been involved in the animal studies. The results so far are very promising. Besides animal studies, Solid GT is also working with academic and corporate partners to refine and scale-up AAV manufacturing technology, and is addressing a number of other key aspects of this program, in preparation for upcoming human clinical trials. On November 3, 2015, Solid GT announced that it has raised $42.5 million in series B financing to advance gene therapy for DMD (www.businesswire.com/news/home/20151103006362/en/Solid-GT-Raises-42.5-Million-Series-Financing).

NOTE

Early on, all truncated dystrophins are called mini-dystrophin. In 2002, Dr. Jeff Chamberlain coined the term "micro-dystrophin" to refer to the abbreviated dystrophins that are about one-third the size of the full-length dystrophin protein. Micro-dystrophin does not contain a complete C-terminal domain. The micro-dystrophin genes are 3.5 to 4-kb in size and can fit into a single AAV vector. The original term "mini-dystrophin" is now reserved for the abbreviated dystrophins that are at least half the size of the full-length dystrophin protein. Mini-dystrophin often contains the complete C-terminal domain. The minidystrophin genes are 6 to 8-kb in size and cannot fit into a single AAV vector. Dual AAV vectors are required to deliver the mini-dystrophin gene.

The micro-dystrophin gene is often abbreviated as the microgene. The mini-dystrophin gene is often abbreviated as the minigene. "Micro-dystrophin" and "mini-dystrophin" are also spelled as "microdystrophin"and "minidystrophin", respectively.

For this historic reason, some of the early versions of the microgene (these that were published before 2002) have been called the minigene. For example the Δ3849, Δ3990 and Δ4173 minigenes developed in Dr. Xiao Xiao's laboratory are actually microgenes.

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AUTHOR DISCLOSURE

D.D. is a member of the scientific advisory board and an equity holder of Solid GT, a subsidiary of Solid Biosciences.

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ARTICLE

100-fold but not 50-fold dystrophin overexpression aggravates electrocardiographic defects in the mdx model of Duchenne muscular dystrophy

Yongping Yue[1], Nalinda B Wasala[1], Brian Bostick[1] and Dongsheng Duan[1,2,3]

Dystrophin gene replacement holds the promise of treating Duchenne muscular dystrophy. Supraphysiological expression is a concern for all gene therapy studies. In the case of Duchenne muscular dystrophy, Chamberlain and colleagues found that 50-fold overexpression did not cause deleterious side effect in skeletal muscle. To determine whether excessive dystrophin expression in the heart is safe, we studied two lines of transgenic mdx mice that selectively expressed a therapeutic *minidystrophin* gene in the heart at 50-fold and 100-fold of the normal levels. In the line with 50-fold overexpression, minidystrophin showed sarcolemmal localization and electrocardiogram abnormalities were corrected. However, in the line with 100-fold overexpression, we not only detected sarcolemmal minidystrophin expression but also observed accumulation of minidystrophin vesicles in the sarcoplasm. Excessive minidystrophin expression did not correct tachycardia, a characteristic feature of Duchenne muscular dystrophy. Importantly, several electrocardiogram parameters (QT interval, QRS duration and the cardiomyopathy index) became worse than that of mdx mice. Our data suggests that the mouse heart can tolerate 50-fold minidystrophin overexpression, but 100-fold overexpression leads to cardiac toxicity.

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INTRODUCTION

Duchenne muscular dystrophy (DMD) is the most common childhood lethal muscle disease caused by dystrophin deficiency. This X-linked disease mainly affects boys and young men. Introducing a functional dystrophin gene back to muscle by gene therapy holds a great promise to treat DMD. Ideally, for gene therapy one would like to express the full-length gene or the full-length cDNA from the endogenous promoter. This will allow for spatially and temporally regulated expression of a full-length protein to meet developmental and physiological needs of muscle. Unfortunately, the *dystrophin* gene is one of the largest genes in the mammalian genome. It greatly exceeds the packaging limit of most viral gene delivery vectors. This not only excludes the use of the full-length gene or cDNA as the therapeutic gene but also excludes the use of the endogenous dystrophin promoter in the expression cassette. To overcome these hurdles, investigators are forced to express a synthetic *mini/micro dystrophin* gene from a constitutive promoter (either ubiquitous or muscle-specific). A likely consequence of this approach is unchecked expression and the production of excessive amount of dystrophin. From the safety standpoint, it is essential to determine whether supraphysiological expression of a therapeutic mini/micro dystrophin gene can lead to deleterious side effects.

Despite intensive research and exciting progresses in the field of dystrophin gene replacement therapy, so far only one study has examined potential toxicity of dystrophin overexpression. Cox *et al.* generated a strain of full-length dystrophin overexpression transgenic mice on the background of dystrophin-null mdx mice.[1] The authors found that the dystrophin level in transgenic mice was 50-fold higher than that of normal mice. Despite excessive amount of dystrophin, surprisingly, skeletal muscle morphology and force were completely normal. This study suggests that skeletal muscle can tolerate supraphysiological levels of dystrophin.[1]

Heart disease is a leading cause of morbidity and mortality in DMD. To treat DMD heart disease, we need to deliver a functional dystrophin gene to the heart. It is thus important to determine whether supraphysiological dystrophin expression in the heart is safe. To address this critical question, we developed cardiac transgenic mdx mice that selectively overexpressed the *ΔH2-R19 minidystrophin* gene in the heart.[2] This minidystrophin gene has previously being shown to protect both skeletal muscle and the heart in mdx mice.[2–4] In a line of 50-fold overexpression, we observed the expected benefits of the *minidystrophin* gene.[2] However, cardiac toxicity was detected in a line that showed

[1]Department of Molecular Microbiology and Immunology, School of Medicine, The University of Missouri, Columbia, Missouri, USA; [2]Department of Neurology, School of Medicine, The University of Missouri, Columbia, Missouri, USA; [3]Department of Bioengineering, The University of Missouri, Columbia, Missouri, USA.
Correspondence: D Duan (duand@missouri.edu)
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100-fold overexpression. Our results suggest that dystrophin overexpression in the heart is likely safe as long as it does not exceed 50-fold of the wild type level.

RESULTS

Generation of cardiac ΔH2-R19 minidystrophin overexpression transgenic mdx mice

To achieve heart specific overexpression of the therapeutic *ΔH2-R19 minidystrophin* gene, we used the 5.5 kb murine α-myosin heavy chain (αMHC) promoter.[5–7] This is the most commonly used promoter for cardiac transgenic studies and it drives transgene expression throughout the entire heart. Importantly, depending on the copy number, one can achieve a broad range of gene expression including supraphysiological expression with this promoter.[8–24] We obtained a total of 10 founder lines and nine lines were backcrossed to the congenic background of mdx mice.[2] In this study, we focused on lines 26 and 29.

Characterization of cardiac minidystrophin overexpressing transgenic mice

To determine the copy number of the minidystrophin gene in lines 26 and 29, we performed Southern blot. The 3.4 kb diagnostic band was detected in both lines (Figure 1a). On quantification, lines 26 and 29 contained 328.7 ± 11.5 and 5.3 ± 0.2 copies of the ΔH2-R19 minidystrophin gene, respectively (Figure 1b).

Next we examined the protein level by western blot. Both lines yielded the expected 220 kDa ΔH2-R19 minidystrophin band (Figure 1c). When compared with the level of full-length dystrophin in normal BL10 mice, the minidystrophin protein in lines 26 and 29 were 102.6 ± 4.3 and 50.8 ± 2.3 fold higher than that of normal mice, respectively (Figure 1d).

On immunofluorescence staining, ΔH2-R19 minidystrophin showed the expected sarcolemmal localization in the heart of line 29 mice (Figure 1e). However, minidystrophin was detected in both sarcolemma and sarcoplasm in cardiomyocytes of line 26 (Figure 1e, Supplementary Figure S1). On high magnification, sarcoplasmic minidystrophin staining displayed as small vesicles (Figure 1e).

Evaluation of heart histology and ECG in cardiac minidystrophin overexpression transgenic mice

On hematoxylin/eosin staining and Masson trichrome staining, heart histology of transgenic mice was indistinguishable from that of normal mice (see Supplementary Figure S2). To evaluate physiological consequences of minidystrophin overexpression, we performed 12 lead electrocardiogram (ECG) in 6-m-old mice. A characteristic change in DMD patients and mdx mice is tachycardia.[2,25–28] The heart

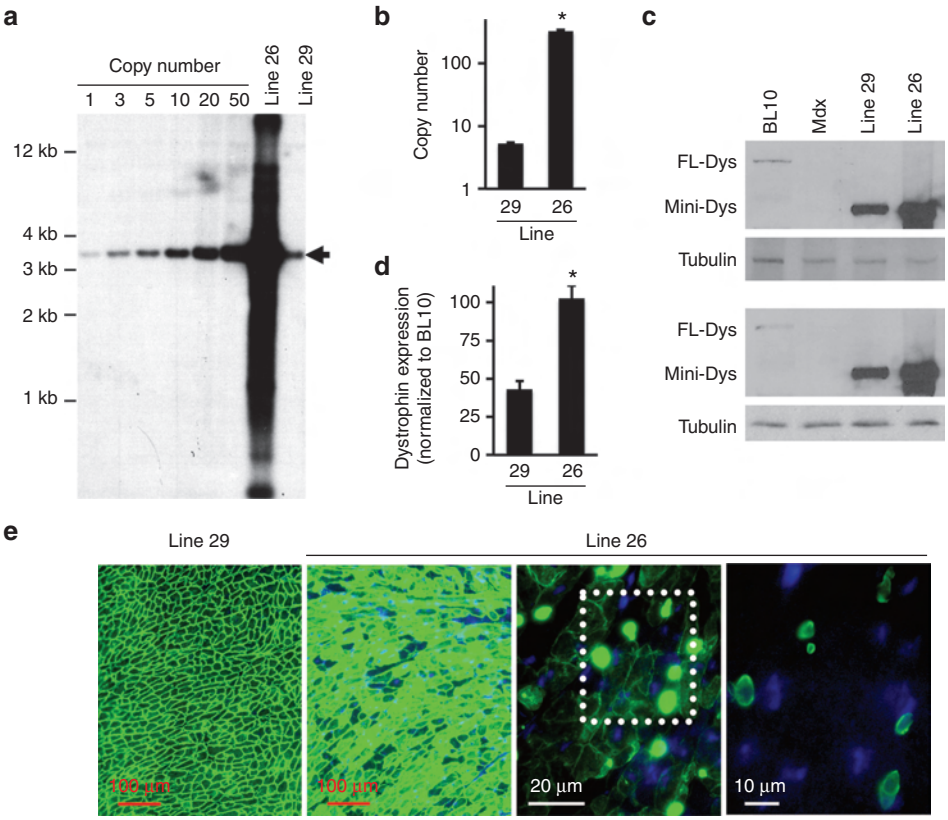

Figure 1 Transgenic overexpression of a therapeutic minidystrophin gene in the heart of mdx mice. (**a**) A representative Southern blot photomicrograph. Arrow, the 3.4 kb diagnostic band for transgenic mice. (**b**) Quantification of the minidystrophin gene copy number in transgenic mice. (**c**) Two representative dystrophin western blots of the heart of BL10, mdx and transgenic mice. (**d**) Quantification of minidystrophin expression. The level of expression was normalized to the loading control and BL10 control. (**e**) Representative dystrophin immunofluorescence staining from the heart of transgenic lines 26 and 29. The left panel (low-power images of line 29 heart) and the second to the left panel (low-power images of line 26 heart) had the identical exposure conditions. The middle panel of line 26 images shows a short-exposure, high-power photomicrograph. Excessively expressed dystrophin forms inclusion body inside cardiomyocytes. The right panel of line 26 images is an enlarged view of the boxed region of the middle panel and it was taken with a much reduced exposure time. The cytosolic dystrophin inclusion bodies appear as vesicles. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (blue color). Asterisk, significantly different from the other group.

rate was normalized in line 29 but not in line 26 (Figure 2). In fact, line 26 showed the same heart rate as that of mdx mice. The only ECG abnormality that was corrected in both lines was the PR interval (Figure 2b). When compared with BL10, mdx had a longer QT interval and QRS duration. These defects were completely corrected in line 29. Surprisingly, both parameters got worse in line 26. They were even significantly longer than those of mdx mice (Figure 2b). The Q-wave amplitude showed a peculiar trend. Line 26 was significantly shallower than all other strains. The cardiomyopathy index was used to evaluate overall electrophysiology in the heart. It was normalized in line 29 but was significantly higher than that of mdx in line 26.

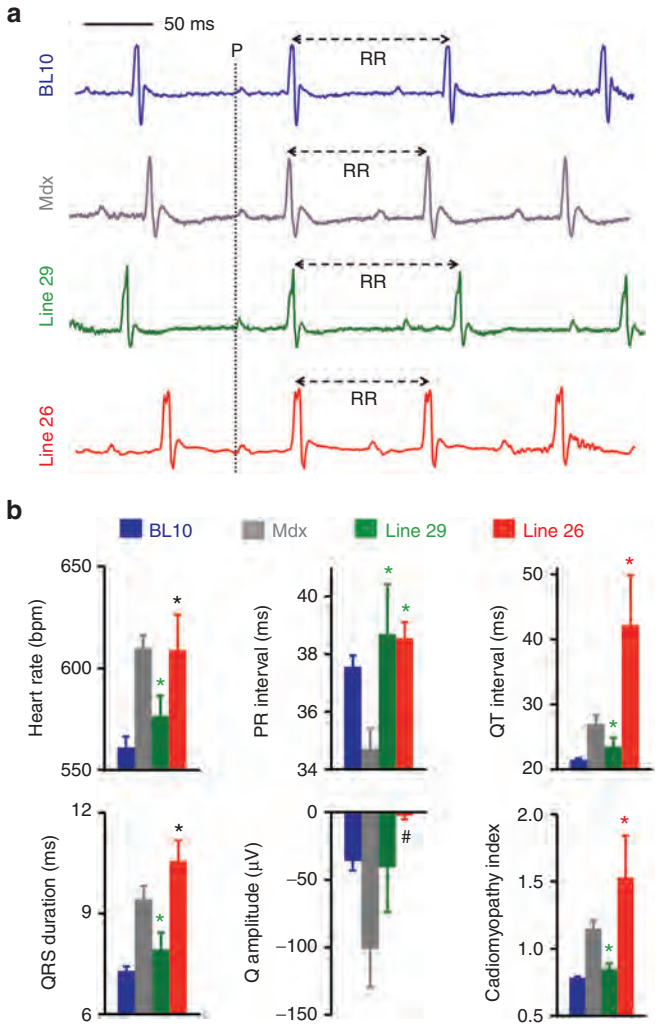

Figure 2 100-fold overexpression of the therapeutic ΔH2-R19 minidystrophin gene in the heart of transgenic line 26 worsened electrocardiogram (ECG) defects seen in mdx mice. (**a**) Representative ECG tracing from BL10, mdx, transgenic line 29 (50-fold overexpression) and line 26 (100-fold overexpression) mice. The dotted vertical line indicates the starting position of the P-wave. Respiratory rate (RR) duration (time between two neighboring heart beats) is clearly reduced in mdx mice and line 26, suggesting the presence of tachycardia in these two stains. In line 29, RR duration is similar to that of BL10 mice. (**b**) Quantitative comparison of the ECG profile from BL10, mdx, line 29, and line 26. Sample size: $n = 10$ for BL10 mice, $n = 9$ for mdx mice, $n = 17$ for line 29, and $n = 13$ for line 26. Green asterisk, results from transgenic mice are normalized to that of BL10 mice; black asterisk, results from transgenic mice are similar to that of mdx mice; red asterisk, results from transgenic mice are significantly worse than that of mdx mice; Pound sign, results from Line 26 mice are significantly different from all other lines (BL10, mdx and Line 29).

DISCUSSION

In this study, we examined the consequences of supraphysiological level minidystrophin expression in the heart of mdx mice. We found that 50-fold overexpression ameliorated ECG defects. In contrast, 100-fold overexpression not only failed to improve the outcome of the most of the ECG parameters but also aggravated abnormalities in several measures (such as the QT interval, QRS duration and cardiomyopathy index). Our results suggest that the murine heart has a quite impressive tolerance to dystrophin overexpression (up to 50-fold). However, when the level of expression becomes excessively high (*e.g.*, 100-fold), it will lead to cardiac toxicity. Specifically, overexpressed dystrophin formed aberrant cytosolic dystrophin vesicles and aggravated ECG abnormalities.

There has been significant progress in the development of DMD gene replacement therapy over the last few years. In particular, adeno-associated virus (AAV)-mediated *microdystrophin* gene delivery has yielded highly promising efficacy data in the murine and canine models.[29,30] Several clinical trials are currently in planning.[31] Despite these advances, one critical question remains incompletely answered. Specifically, how much dystrophin is too much? In other words, will dystrophin overexpression cause a problem? This is highly relevant because dystrophin is expressed from a constitutive promoter (either ubiquitous or muscle-specific) in all gene replacement therapy vectors and AAV is a long lasting virus.[32] To address this question, the Chamberlain laboratory, Seattle, WA studied transgenic mdx mice that had 50-fold dystrophin overexpression. Strikingly, no structural or functional abnormalities were found in skeletal muscle suggesting supraphysiological dystrophin expression is safe in skeletal muscle.[1]

Cardiac complications greatly compromise the life quality of DMD patients. A significant portion of patients dies from heart failure or sudden cardiac death. Hence, an effective DMD gene replacement therapy requires efficient delivery of a therapeutic dystrophin gene to the heart. Overexpression-induced cardiotoxicity is well documented in the literature.[11,14–16,20,33] For example, two fold overexpression of the green fluorescence protein, six fold overexpression of the adenosine receptor and 27-fold overexpression of myosin light chain 1 result in dilated cardiomyopathy.[14–16] Interestingly, depending on the protein being overexpressed, the heart seems to show different levels of tolerance. For example, 16-fold overexpression of myosin light chain 1 is not toxic.[14] Unfortunately, the tolerant range for dystrophin has never been determined. To address this unmet need, we generated cardiac dystrophin overexpressing mice. Since future clinical trials will likely use the abbreviated *dystrophin* gene, we overexpressed a therapeutic *minidystrophin* gene. Consistent with the finding of Cox *et al.*,[1] we did not detect any toxicity in the line with 50-fold minidystrophin overexpression. However, there was clear evidence of toxicity by the ECG assay when expression reached 100-fold of the normal. Based on these findings, we conclude that the heart has a relatively high safety margin for dystrophin overexpression. More specifically, 50-fold minidystrophin overexpression is not toxic to the mouse heart.

Several groups have tested AAV-mediated *dystrophin* gene replacement therapy for Duchenne cardiomyopathy in the mouse model.[25,34–40] Despite widespread expression throughout the entire heart, in none of these studies, AAV-mediated expression exceeded 10-fold of the normal dystrophin level. We have achieved cardiac AAV transduction in dogs and more recently demonstrated efficient

AAV micro-dystrophin expression in the heart of DMD dogs.[29,41–43] Two independent groups have also demonstrated AAV-mediated exon-skipping in dystrophic dog hearts.[44,45] Yet, it is still a great challenge to obtain saturated myocardial AAV transduction in the heart of a large mammal. We believe that with the current AAV technology, supraphysiologic dystrophin overexpression may not constitute a serious concern for Duchenne cardiomyopathy gene therapy. However, the development of novel AAV capsids, expression cassette and/or gene delivery methods may lead to significantly much higher transduction efficiency in the future.[46–48] The maximal tolerable dystrophin level described in our study will serve as an important reference to guide future studies.

The toxicity seen in the line of 100-fold overexpression suggests that a level higher than 50-fold may also cause harmful changes in skeletal muscle. Indeed, Harper *et al.* observed similar dystrophin aggregation vesicles in the quadriceps muscle of a microdystrophin transgenic line that specifically overexpressed the *ΔR2-R21+H3 microgene* in skeletal muscle.[3] On quantification of centrally located myonuclei of 6-m-old mice, the authors found ≤ 1%, 64% and 52% in BL10 (normal control), mdx, and ΔR2-R21+H3 microgene overexpression transgenic mice, respectively. Interestingly, in another line that expressed the *ΔR4-R23*, a structurally similar microgene (both microgenes have 4 repeats and 1 hinge), the percent of central nucleation was <1%. Although the authors did not quantify the level of overexpression, a rough evaluation based on the western blots in the paper suggests that the lines ΔR2-R21+H3 and ΔR4-R23 had a dystrophin level of ~90-fold and ~10-fold of BL10, respectively.

It is currently unclear how 100-fold dystrophin overexpression resulted in cardiac toxicity. We suspect that it may likely relate to the accumulation of excessive amount of dystrophin inclusion bodies in the cytosol. However, we believe the heart may tolerate limited levels of cytosolic dystrophin expression. In support of this notion, we did notice some dystrophin staining in the cytoplasm of cardiomyocytes in transgenic line 29 mice. Chamberlain also observed similar cytosolic dystrophin expression in the heart of their full-length dystrophin transgenic mice.[1] Besides the dystrophin level, it is also possible that the toxicity seen in line 26 may relate to the positional effect of transgene integration. Insertion may have either activated or shut down expression of other important cellular protein(s) and consequently result in toxicity. Nevertheless, the data of the current study as well as that of Cox *et al.* suggest that moderate dystrophin overexpression is relatively safe in muscle.[1] Tight control of dystrophin expression may not be necessary in DMD gene therapy.

MATERIALS AND METHODS

Experimental animals

All animal experiments were approved by the institutional animal care and use committee and were in accordance with NIH guidelines. All experimental mice were housed in a specific pathogen-free facility and kept under a 12 hours light (25 lux)/12 hours dark cycle with free access to food and water. C57Bl/10SnJ (BL10) and dystrophin-deficient C57Bl/10ScSn-*Dmd*^{mdx}/J (*mdx*) mice were purchased from the Jackson Laboratory (Bar Harbor, ME).

The cardiac specific minidystrophin transgenic mice were generated at the University of Missouri transgenic core. The expression cassette consists of the αMHC promoter (a gift from Dr Jeffrey Robbins, Division of Molecular Cardiovascular Biology, Cincinnati Children's Hospital Research Foundation, Cincinnati, OH), the *ΔH2-R19 minidystrophin* gene (a gift from Dr Jeffrey Chamberlain at the University of Washington, Seattle, WA) and the bovine growth hormone polyadenylation signal.[3,5,7] A total of 10 founders were identified and nine founders were backcrossed with mdx mice for 5–7 generations.[2] In this study, we evaluated incipient congenic mice from lines 26 and 29. Only male mice were used in the study. The ECG assay was performed in 6-m-old male mice.

Southern blot

Genomic DNA was extracted from the tail using a previously described high salt precipitation protocol.[49] A 414-bp BamH I (exon 4)/EcoR V (exon 7) double digested DNA fragment was used as the template for the Southern probe. Tail genomic DNA was digested with BamH I. After transfer to a nylon membrane, the blot was hybridized with a ^{32}P-labelled probe according to a previously published protocol.[49] The diagnostic band migrated at 3.4 kb. For the copy number control, the plasmid used for making transgenic mice was digested with BamH I and loaded on the same gel.

Immunostaining

Dystrophin immunofluorescence staining was performed essentially as we described before using a mouse monoclonal antibody against the C-terminal domain of dystrophin (Dys-2; 1:30; Vector Laboratories, Burlingame, CA).[36,50] Slides were viewed using a Nikon E800 fluorescence microscope. Photomicrographs were taken with a Qimage REtiga 1,300 camera (Burnaby, BC, Canada).[51]

Western blot

Membrane protein enriched microsomal preparation was extracted from the heart according to our published protocol.[52–54] 50 µg protein was separated on a 6% sodium dodecyl sulfate-polyacrylamide gel. After electrophoresis, protein was transferred to a polyvinylidene fluoride membrane. The membrane was probed with the Dys-2 antibody (1:100). For the loading control, we used the α-tubulin antibody (1:3,000; clone B-5-1-2; Sigma, St Louis, MO). Western blot quantification was performed using ImageJ (http://rsbweb.nih.gov/ij/). The relative intensity of the respective protein band was normalized to the corresponding loading control in the same blot. To determine the relative expression level of minidystrophin, the relative band intensity of transgenic mice was further normalized to that of full-length dystrophin in BL10 mice in the same blot. We would like to point out that the full-length dystrophin protein might transfer to the membrane less efficiently than the minidystrophin protein during western analysis due to the large size of the full-length protein (427 kDa). To overcome this technical problem, we have conducted overnight transfer in western blot analysis. Furthermore, we confirmed efficient transfer by the lack of Coomassie blue staining of the polyacrylamide gel after transfer. Despite these efforts, we cannot completely exclude the possibility of incomplete transfer of trivial amount of full-length protein. There is still a very small likelihood that we may have underestimated the quantity of full-length dystrophin. Hence, the relative overexpression of minidystrophin could be slightly lower.

ECG

A 12-lead ECG assay was performed with an ECG recording system from AD Instruments (Model MLA0112S; Colorado Springs, CO) as described in the *Standard Operating Procedures (SOP's) for Duchenne Animal Models-Cardiac Protocols* (http://www.parentprojectmd.org/site/PageServer?pagename=Advance_researchers_sops).[54,55] Briefly, cardiac electric activity signals were processed with a single channel bioamplifier (Model ML132; AD Instruments). Averaged value from at least 1 minute continuous recording was used for ECG analysis by LabChart software (AD Instruments). The amplitude of the Q-wave was analyzed using the lead I tracing. The remaining ECG parameters were analyzed using lead II tracing results. The cardiomyopathy index is determined by dividing the QT interval by the PQ segment.

Statistical analysis

Data are presented as mean ± SEM. Statistical analysis was performed with the Prism software (GraphPad, San Diego, CA). Statistical significance between two groups was determined by the Student's *t*-test. Statistical significance among different groups was determined by one-way analysis of variance followed by Tukey's *post hoc* analysis. Difference was considered statistically significant when $P < 0.05$.

CONFLICT OF INTEREST

D.D. is a member of the scientific advisory board for and an equity holder of Solid GT, a subsidiary of Solid Biosciences.

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Supplementary Information accompanies this paper on the *Molecular Therapy—Methods & Clinical Development* website (http://www.nature.com/mtm)

Understanding the dog model of Duchenne muscular dystrophy

Animal models are indispensible in the development of novel therapies. A clear understanding of the model itself is essential before the model is used in research. This means we need to know every aspect of the model including its strengths and weaknesses. Without such information, it will be impossible to make precise and correct interpretations of the animal study results. The commentary "*Early loss of ambulation is not a representative clinical feature in Duchenne muscular dystrophy dogs*: remarks on the article of Barthelemy et. al." provides an example on why accurate characterization of Duchenne muscular dystrophy (DMD) dogs is important.

DMD is a severe childhood lethal muscle disease that mainly affects boys. The disease is caused by genetic mutations in a gene called dystrophin. Patients show problems with walking and stair climbing around 2 to 5 years of age and are bound to a wheelchair in their teenage years. DMD is a worldwide disease and patients can come from any race and any country. Much like humans, dogs can also be affected by DMD. Mutations in the dystrophin gene have been found in various breeds. Since the first DMD dog was found in the golden retriever breed, most researchers around the world have been using the progeny from this dog in their studies. This brings two problems. First, DMD can be caused by thousands of different types of mutations, but the golden retriever muscular dystrophy dog (GRMD) only has one type of mutation. So studies based on GRMD cannot reflect other types of mutations. Second, to generate more DMD dogs for research purposes, scientists have used a method called inbreeding. Which means all DMD dogs generated by this method will be close relatives. These dogs will carry genetic traits unique to the family but may be uncommon in the entire dog population. Sometimes, the traits unique to the family can alter disease manifestations. Studies performed in this family will thus not be representative of the general population.

Like in humans, DMD will eventually cause the dogs to lose their ability to walk. If DMD boys lose their ability to walk at teenage, when should DMD dogs loss their walk ability? If we take 80 years as the average life span of a man and 10 years as the average life span of a dog, we may guess DMD dogs will likely loss their ability to walk around 1.25 years of age. To determine when DMD dogs lose their walk ability, Barthelemy et al studied an inbred GRMD dog colony. Interestingly, they found that about a quarter of the DMD dogs in their colony lose their walk ability at about 6 months of age, much earlier than expected. To determine whether this is the case for the entire DMD dog population, Duan et al examined several different breeds of DMD dogs, including GRMD dogs that were housed in other locations. In contrast to the results of Barthelemy et al, the study by Duan et al did not find early loss of walking ability as a general feature of DMD dogs. It is currently unclear why the colony studied by Barthelemy et al showed this unique feature. It could be caused by inbreeding and/or different environment these dogs were raised. Nevertheless, it is important to know the difference. For example, if all the dogs studied by Barthelemy et al remain ambulant at 8 months of age after receiving an experimental drug, it may suggest this drug is

effective. But we cannot draw this conclusion if we get the same results on DMD dogs from other colonies.

Joe W. McGreevy, Dongsheng Duan
Department of Molecular Microbiology and Immunology, School of Medicine, University of Missouri, USA

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