

AWARD NUMBER: W81XWH-16-1-0221

TITLE: Treating Duchenne Cardiomyopathy in the mouse Model by Gene Repair

PRINCIPAL INVESTIGATOR: Dongsheng Duan

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

REPORT DATE: August 2019

TYPE OF REPORT: Annual

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

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE August 2019		2. REPORT TYPE Annual		3. DATES COVERED 1 Aug 2018 - 31 July 2019	
4. TITLE AND SUBTITLE Treating Duchenne Cardiomyopathy in the Mouse Model by Gene Repair				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-16-1-0221	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Dongsheng Duan, Nalinda Wasala, Yongping Yue, Gary Yao, Yi Lai, Charles Gersbach, Adrienne Pittman, Matthew Gemberling E-Mail: duand@missouri.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Missouri System 316 University Hall Columbia, MO 65211-3020				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Development Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT The goal of this project is to test adeno-associated virus (AAV) CRISPR (clustered regularly interspaced palindromic repeat) gene editing therapy for Duchenne cardiomyopathy in the mdx model. In the previous funding period, we performed AAV CRISPR therapy in young adult male mdx mice. We observed widespread dystrophin restoration in the heart on immunostaining. However, we did not detect dystrophin expression in skeletal muscle. Further cardiac dystrophin restoration is too low to restore hemodynamic function. In the last funding period, we discovered the underlying mechanism that is responsible for the low CRISPR efficiency in our original study. To determine whether long-term CRISPR therapy can improve cardiac function, we performed the study in female mdx mice because female mice show more severe cardiac disease than male mice. Based on the finding, we also changed our strategy. Our study showed that the new strategy can enhance CRISPR editing in the heart and improve heart function. However, in our last year's study, we did not evaluate whether our new strategy can work in skeletal muscle. In the current funding period, we performed the study in male mice using our new strategy. We used male mice because they show more severe skeletal muscle disease than female mice. Similar to what we saw in female mice, we found our new strategy also significantly enhanced CRISPR editing in skeletal muscle. Specifically, we observed significantly higher dystrophin restoration than what we saw in our original study. Further, we observed reduction in skeletal muscle fibrosis and increase in muscle force. Our results in male mice confirmed and extended our study in female mice. To further understand whether local dystrophic micro-environment underlies our observation, we performed local injection study. In sharp contrast to our finding in systemic injection, the new strategy yielded similar gene editing efficiency as the original strategy. Collectively, these results suggest that our new strategy is a valid approach to achieve long-term systemic (but not local) CRISPR therapy in both skeletal muscle and heart.					
15. SUBJECT TERMS Duchene muscular dystrophy, DMD, dystrophin, Adeno-associated virus, AAV, Cardiomyopathy, gene editing, clustered regularly interspaced palindromic repeat (CRISPR), CRISPR-associated endonuclease 9 (Cas9), heart					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU Unclassified	18. NUMBER OF PAGES 140	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U Unclassified	b. ABSTRACT U Unclassified	c. THIS PAGE U Unclassified			19b. TELEPHONE NUMBER (include area code)

Table of Contents

	<u>Page</u>
1. Introduction.....	4
2. Keywords.....	4
3. Accomplishments.....	4-16
4. Impact.....	16
5. Changes/Problems.....	17
6. Products.....	17-18
7. Participants & Other Collaborating Organizations.....	19-34
8. Special Reporting Requirements.....	34
9. Appendices.....	35-140
6.1. Peer-reviewed publications (3)	
6.2. Non-Peer-reviewed publications (4)	

1. Introduction

Duchenne muscular dystrophy (DMD) is a progressive muscle wasting disease caused by the loss of dystrophin, an essential sub-sarcolemmal cytoskeleton protein in muscle. Repair of the mutated dystrophin gene offers the opportunity to express a near full-length dystrophin protein. We have recently published proof-of-principle data suggesting that gene editing with clustered regularly interspaced palindromic repeat (CRISPR)/ CRISPR-associated endonuclease 9 (Cas9) by adeno-associated virus (AAV) may ameliorate skeletal muscle disease. We now propose to evaluate AAV CRISPR gene therapy to treat DMD-associated cardiomyopathy, a leading cause of morbidity and mortality in DMD patients.

2. Keywords

Duchenne muscular dystrophy, DMD, dystrophin, adeno-associated virus, AAV, cardiomyopathy, gene editing, clustered regularly interspaced palindromic repeat (CRISPR), CRISPR-associated endonuclease 9 (Cas9), heart

3. Accomplishments

Major goals in the original proposal. We have three specific aims in our original proposal. Our first aim is to test if AAV CRISPR therapy can prevent Duchenne cardiomyopathy in neonatal mdx mice. Our second aim is to test if AAV CRISPR therapy can treat early stage Duchenne cardiomyopathy in young adult mdx mice. Our third aim is to test if AAV CRISPR therapy can treat symptomatic late-stage Duchenne cardiomyopathy in aged mdx mice.

Revised goals based on the results of the first funding period. We have two specific aims. Our first aim is to evaluate whether body weight reduction is a safety concern in young adult mdx mice following systemic AAV CRISPR delivery. Our second aim is to identify the mechanism underlying poor dystrophin restoration in long-term systemic AAV CRISPR therapy and to explore novel strategies to improve the level of dystrophin restoration in chronic systemic AAV CRISPR therapy in the mdx model of Duchenne muscular dystrophy.

Accomplishments.

Increasing the gRNA AAV vector dosage resulted in significant improvement in dystrophin restoration in male mdx mice and improved skeletal muscle function.

DMD is a chronic disease. A desirable therapy for DMD requires persistent, ideally lifelong, dystrophin restoration in both skeletal and cardiac muscle. To address whether a single intravenous AAV CRISPR therapy can result in sustained disease amelioration, we treated 6-week-old mdx mice, a commonly used mouse model for Duchenne muscular dystrophy. Therapeutic effect was evaluated at the molecular, histological and functional levels when mice reached 18 months of age. Surprisingly, nominal dystrophin was restored in skeletal muscle. Cardiac dystrophin was restored but histology and hemodynamics were not improved (*reported in Year 1 progress report*). To understand the molecular mechanism under this unexpected finding, we evaluated Cas9 expression at the transcript and protein levels and we also evaluated the Cas9 AAV vector genome and gRNA vector genome. Surprisingly,

we found gRNA AAV vector was selectively depleted. To determine whether selective gRNA vector depletion is indeed responsible for poor editing in our original study, we performed a new study using a higher dose of the gRNA vector in female mdx mice. We used female mice because they show more severe cardiac disease than male mice. In particular, only female mice show classic dilated cardiomyopathy as seen in human patients (Bostick B, Yue Y, Duan D. *Gender influences cardiac function in the mdx model of Duchenne cardiomyopathy*. Muscle & Nerve 42(4):600-603, 2010.). Our study in female mice show that increasing the gRNA vector dosage is an effective approach to achieve long-term systemic CRISPR editing in the heart and to improve heart function (*reported in Year 2 progress report*).

Skeletal muscle disease is also a critical concern in DMD. To determine whether increasing the gRNA vector dosage can also improve CRISPR editing in skeletal muscle, we performed the study in male mice. We used male mice here because they show more severe skeletal muscle disease than female mice (Hakim CH, Duan D. *Gender differences in contractile and passive properties of mdx extensor digitorum longus muscle*. Muscle & Nerve 45(2):250-256, 2012). Similar to what we did in the previous study, we co-injected the Cas9 AAV vector and the gRNA AAV vector via the tail vein to 6-week-old mdx mice. Skeletal muscle function was examined by invasive ex vivo EDL force assay at 18 months of the age. Tissues were harvested following the muscle function assay and examined for dystrophin expression, dystrophin gene editing and off-target editing.

Table 1 (below) shows the age, body weight and AAV vector dose in our previous studies and the new study. In our original study, the gRNA AAV vector does is 3.63E+12 vg/mouse (1.61E+14 vg/kg). In the female mouse study with the new strategy, the gRNA AAV vector does is 3.00E+12 vg/mouse (1.82E+14 vg/kg), hence, an approximately 10-fold increase. In the current study in male mice, the gRNA AAV vector does is 3.00E+12 vg/mouse (1.36E+15 vg/kg).

Table 1. Mouse and AAV vector summary

	n	Sex	Age at injection (m)	Body weight at injection (g)	AAV vector (vg/mouse)		AAV vector (vg/kg)	
					Cas9	gRNA	Cas9	gRNA
Original study	6	male	1.54 ± 0.02	23 ± 0.4	7.20E+12	3.63E+12	3.19E+14	1.61E+14
New strategy in female	8	female	1.48 ± 0.05	16 ± 0.7	1.00E+13	3.00E+13	6.07E+14	1.82E+15
New strategy in male (new)	6	male	1.56 ± 0.07	22 ± 0.6	1.00E+13	3.00E+13	4.61E+14	1.38E+15

First, we examined dystrophin expression in skeletal muscle and heart (**Figures 1, 2, 3**).

Figure 1 shows dystrophin expression examined by high power immunostaining and quantification (see next page).

Figure 2 shows dystrophin expression examined by western blot in both heart and skeletal muscle (see next page).

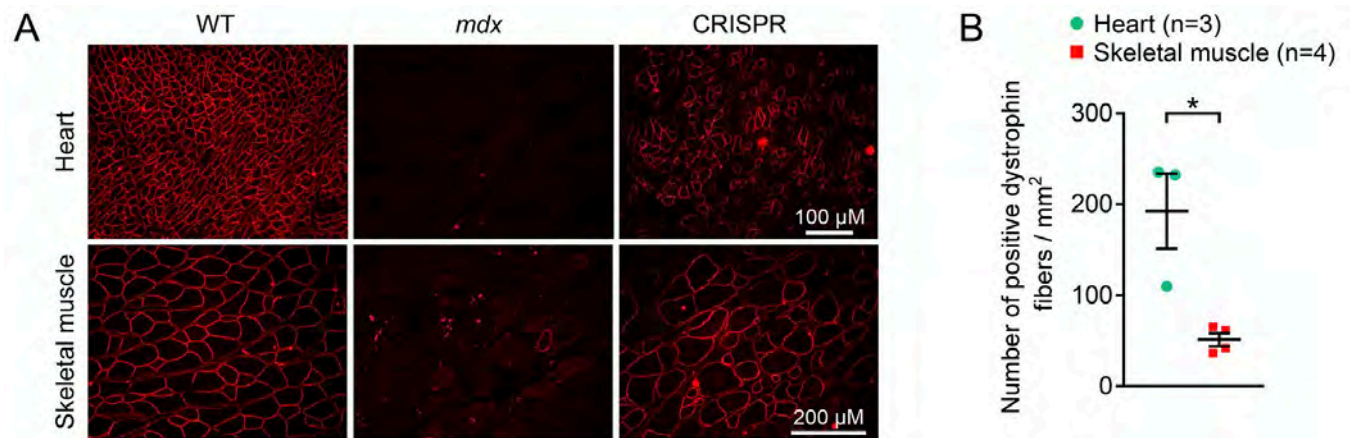


Figure 1. Examination of dystrophin expression by immunofluorescence staining. Systemic AAV-9 CRISPR therapy was performed in 6-week-old female *mdx* mice at the dose of 1×10^{13} vg /mouse and 3×10^{13} vg/mouse for the Cas9 and gRNA vectors, respectively. Mice were evaluated at 18 months of age. **A**, Representative dystrophin immunostaining photomicrographs from wild type (WT), *mdx* and CRISPR-treated *mdx* mice. **B**, Quantification of dystrophin positive myofibers.

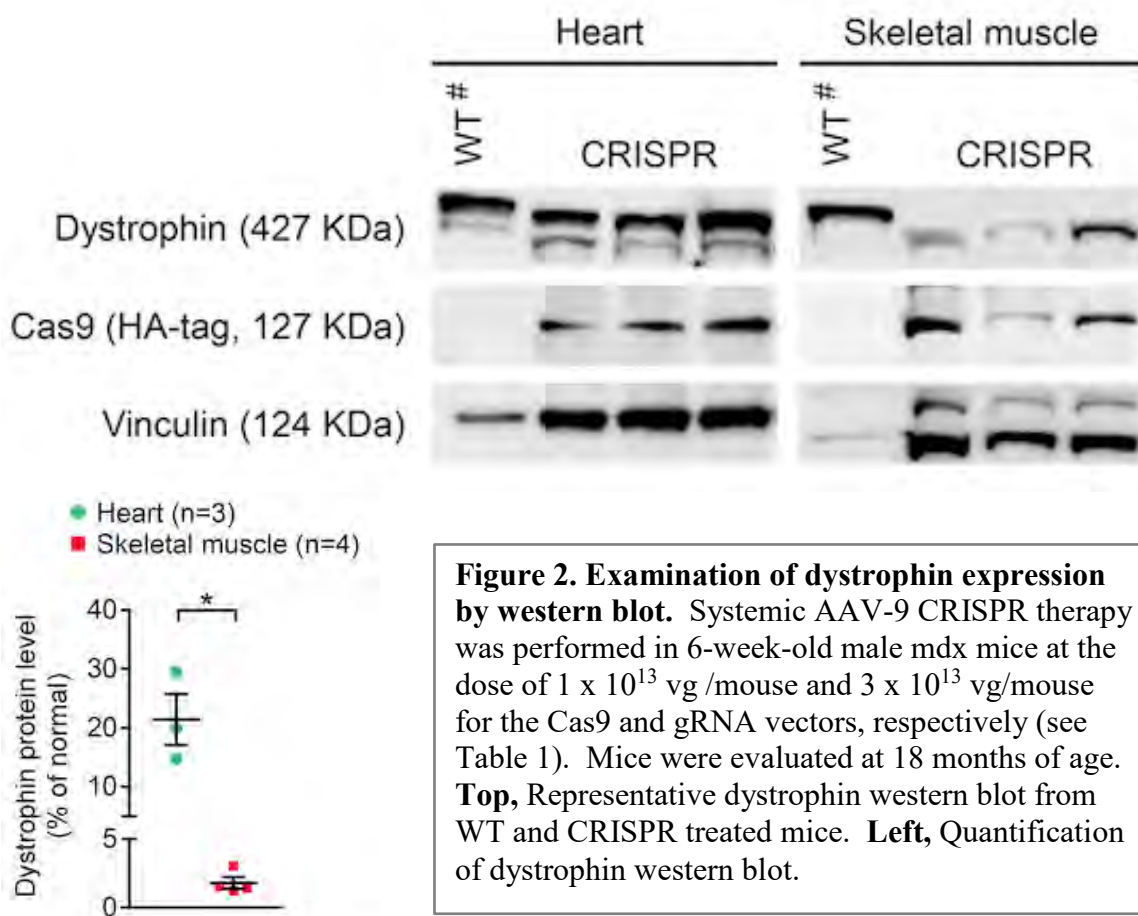


Figure 2. Examination of dystrophin expression by western blot. Systemic AAV-9 CRISPR therapy was performed in 6-week-old male *mdx* mice at the dose of 1×10^{13} vg /mouse and 3×10^{13} vg/mouse for the Cas9 and gRNA vectors, respectively (see Table 1). Mice were evaluated at 18 months of age. **Top**, Representative dystrophin western blot from WT and CRISPR treated mice. **Left**, Quantification of dystrophin western blot.

Figure 3 shows dystrophin expression examined by low power immunostaining (**see next page**). We not only got wide-spread dystrophin expression in the heart but also wide-spread dystrophin expression in skeletal muscle all over the body.

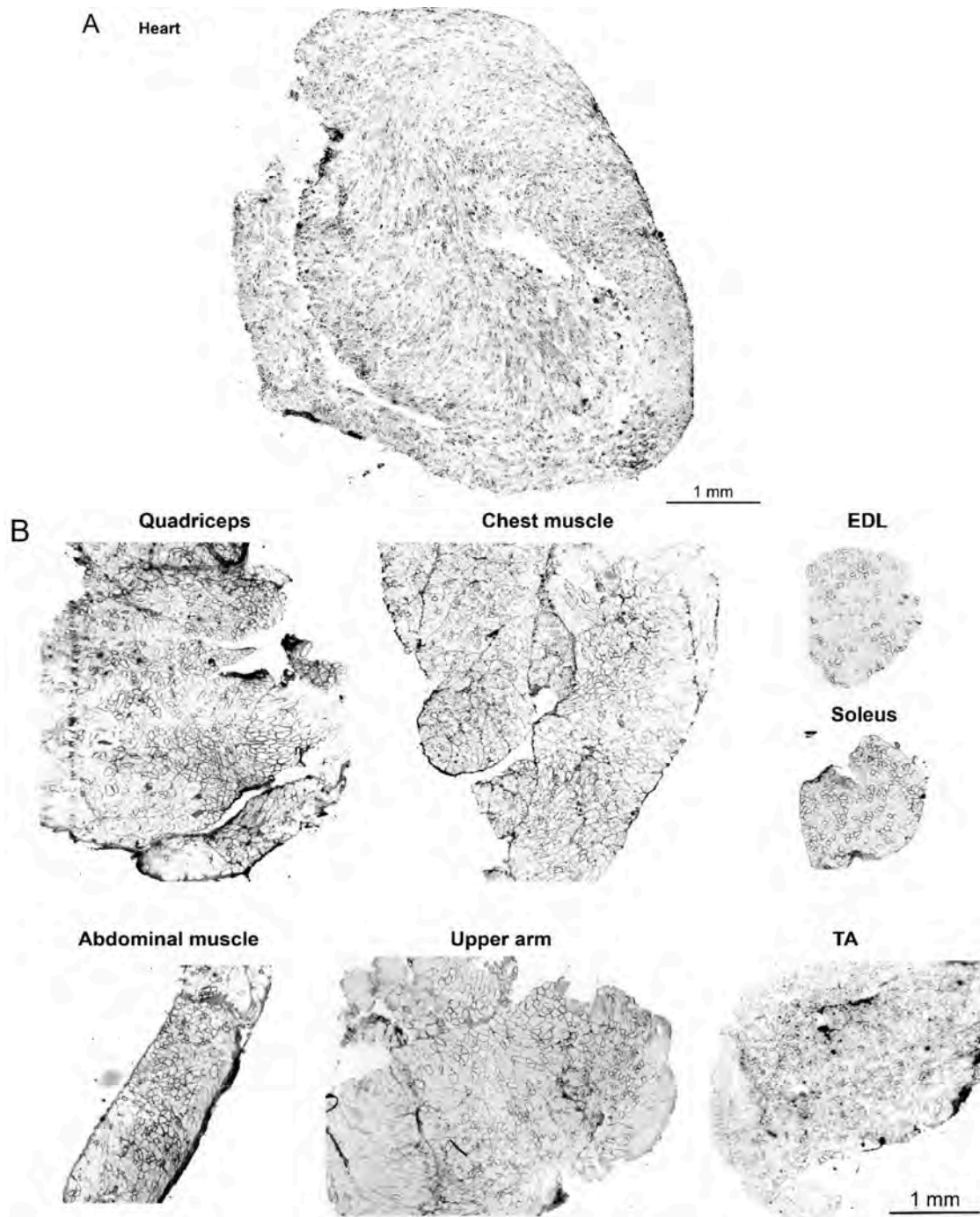


Figure 3. Representative dystrophin immunofluorescence staining in the new study. A, Representative full-view dystrophin staining images of the heart. **B,** Representative low-power dystrophin staining images from the quadriceps, chest muscle, extensor digitorum longus (EDL), soleus, abdominal muscle, upper arm muscle, and tibialis anterior (TA).

Next, we quantified the amount of total dystrophin transcripts, edited and unedited dystrophin transcripts.

Figure 4 shows dystrophin transcript quantification results. We detected edited transcripts in both heart and skeletal muscle. Notable, ~80% and ~40% of the dystrophin transcripts were edited in the heart and skeletal muscle, respectively. Unedited mdx dystrophin transcripts undergo non-sense mediated decay. Edited transcripts are stable. Hence, we expect an increase in total dystrophin transcripts. Encouragingly, total dystrophin transcript levels were greatly increased in the heart ($p<0.05$). A trend of increase was seen in skeletal muscle.

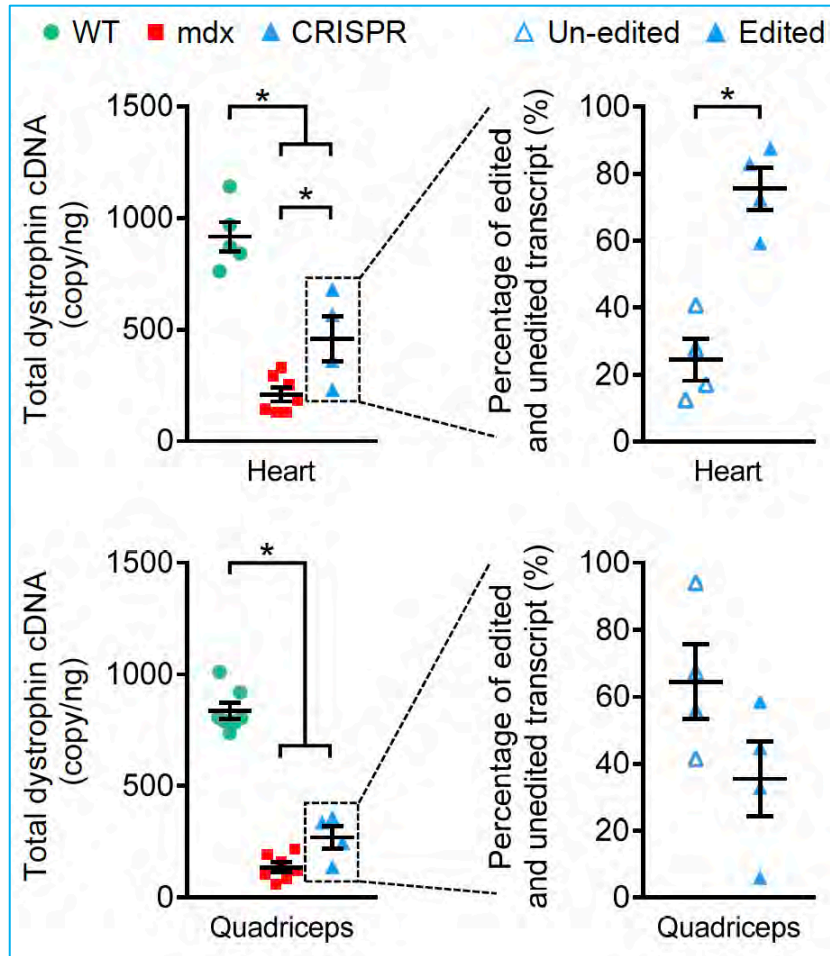


Figure 4. Quantification of dystrophin transcript restoration in the heart and skeletal muscle. Top right, total dystrophin transcript in the heart. Top left, the percentage of the edited and unedited dystrophin transcript in the heart of CRISPR treated male mice. Bottom right, total dystrophin transcript in skeletal muscle. Bottom left, the percentage of the edited and unedited dystrophin transcript in skeletal muscle of CRISPR treated male mice. Asterisk, statistically significant ($p<0.05$).

Dystrophin transcript quantification results (**Figure 4**) are consistent with dystrophin protein expression results (**Figures 1 to 3**). Together, these results suggest that increasing the quantity of the gRNA AAV vector at the time of dosing has successfully enhanced editing in male mdx mice too.

Next, we examined Cas9 expression. **Figure 5** shows Cas9 expression at the protein and transcript level, Cas9 AAV vector genome copy number quantification in skeletal muscle and the heart. Similar to what we seen in female mice (Year 2 progress report), no significant differences were seen in either Cas9 cDNA and Cas9 protein level between heart and skeletal muscle.

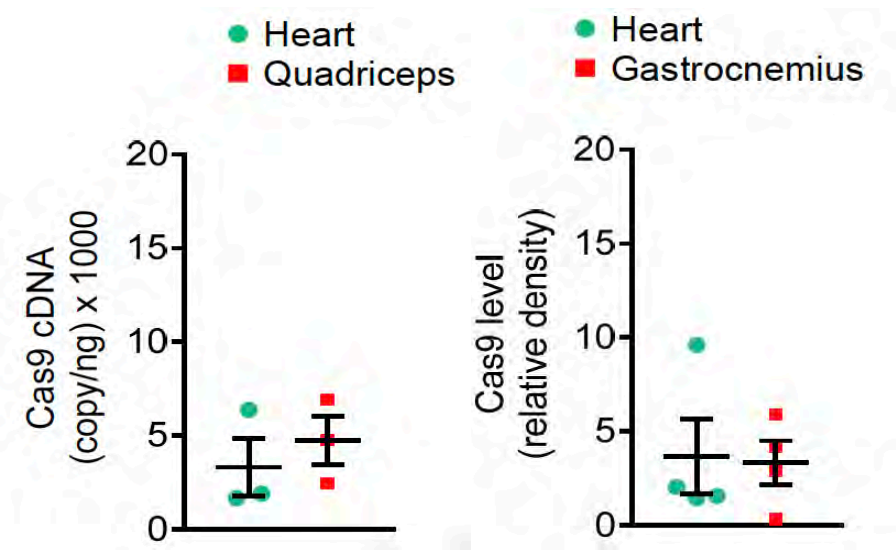


Figure 5. Evaluation of Cas9 protein and Cas9 transcript. The Cas9 cDNA copy number was determined by digital droplet PCR. The Cas9 protein level was examined by quantitative western blot.

Next, we examined AAV vector genome copy number for both Cas9 vector and gRNA vector in both heart and skeletal muscle. **Figure 6** shows results. More AAV vector genome was detected in the heart than in skeletal muscle for both Cas9 vector and gRNA vector.

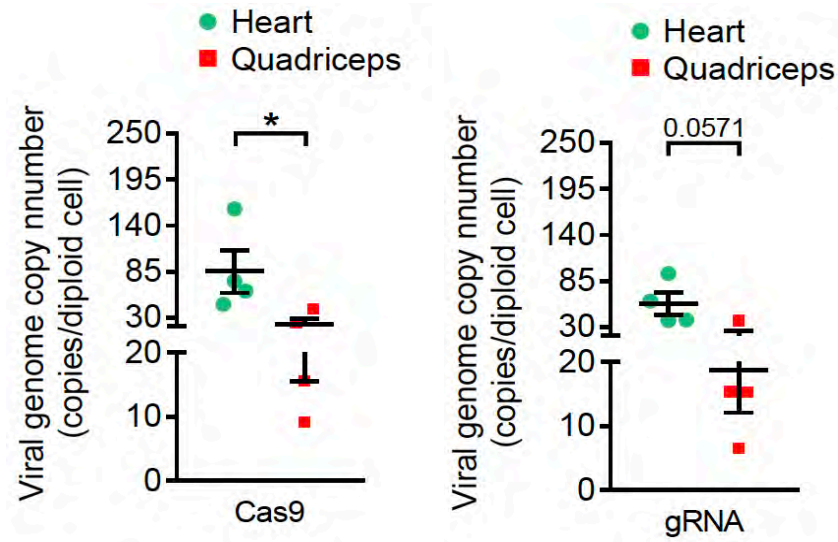


Figure 6. Evaluation of genome copy of the Cas9 vector and gRNA vector. The Cas9 AAV genome copy number was determined by TaqMan PCR. Asterisk, statistically significant ($p < 0.05$).

To further extend the finding, we quantified AAV vector genome copy number for both Cas9 vector and gRNA vector in additional tissues. **Figure 7** shows results. The liver has the highest amount of the vector genome follow by the heart.

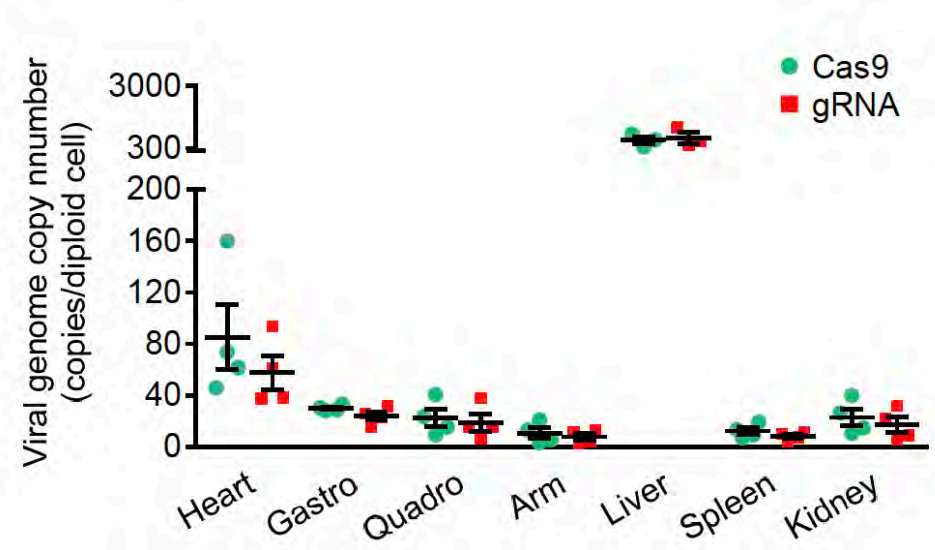


Figure 7. Evaluation of genome copy of the Cas9 vector and gRNA vector in additional tissues. The Cas9 AAV genome copy number was determined by TaqMan PCR. Asterisk, statistically

Next, we calculated the ratio of Cas9 vector to gRNA vector in various tissues. **Figure 8** shows results. In all the tissues we examined, the ratios were between 1 to 1.5.

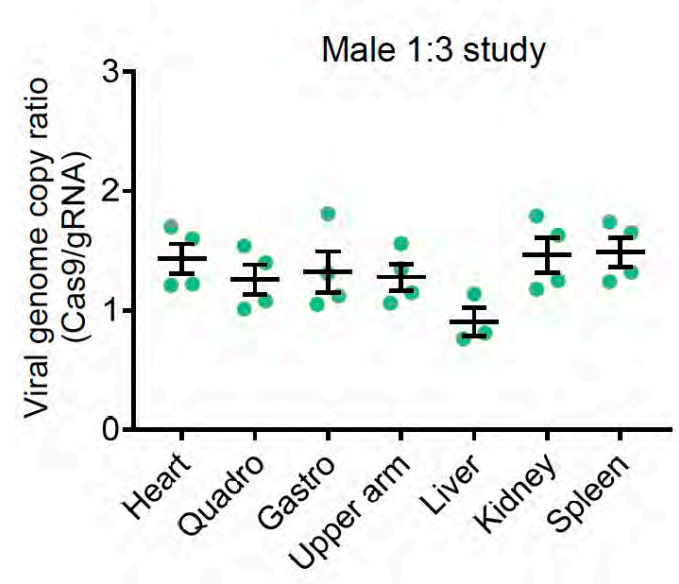


Figure 8. The Cas9 to gRNA vector genome copy number ratio. The Cas9 to gRNA vector genome copy number ratios were calculated for the heart, various skeletal muscle (quadriceps, gastrocnemius, upper arm muscle), liver, kidney and spleen.

Off-target editing is an important concern in CRISPR therapy. We quantified on-target editing efficiency and the off-target cutting on computer predicted top off-target side by deep sequencing. Our data suggests that there was no off-target editing in our new study (**Figure 9**).

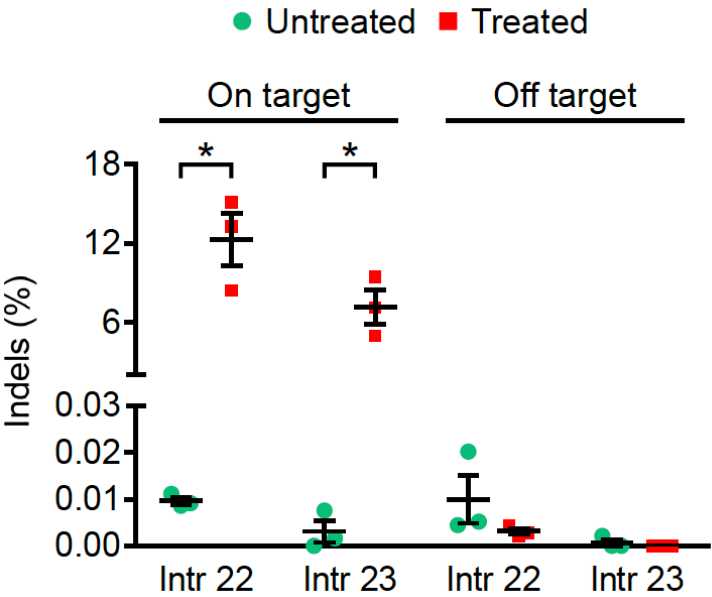
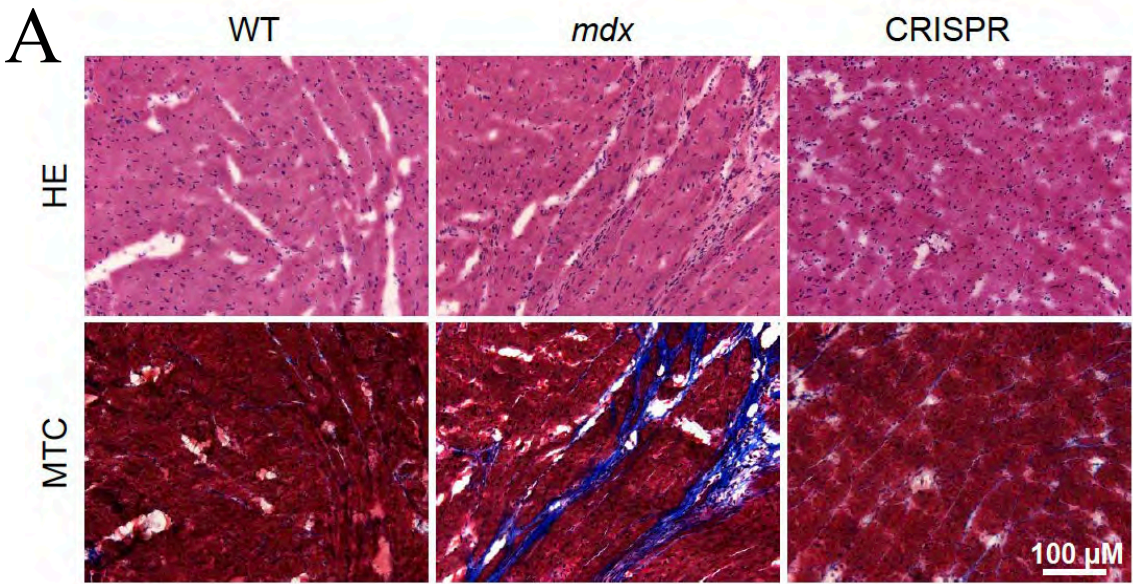


Figure 9. Quantification of indels by deep sequencing. Deep sequencing was performed on both the target sites and predicted off-target sites in untreated and CRISPR treated mdx mice. Significant on-target editing was observed for both gRNAs only in CRISPR treated mdx mice. Off-target editing was not detected with either gRNA. Asterisk, statistically significant ($p < 0.05$).

Next, we evaluated whether treating male mdx mice with our optimized strategy reduced muscle disease. We first evaluated fibrosis by Masson trichrome staining. **Figure 10** shows results.



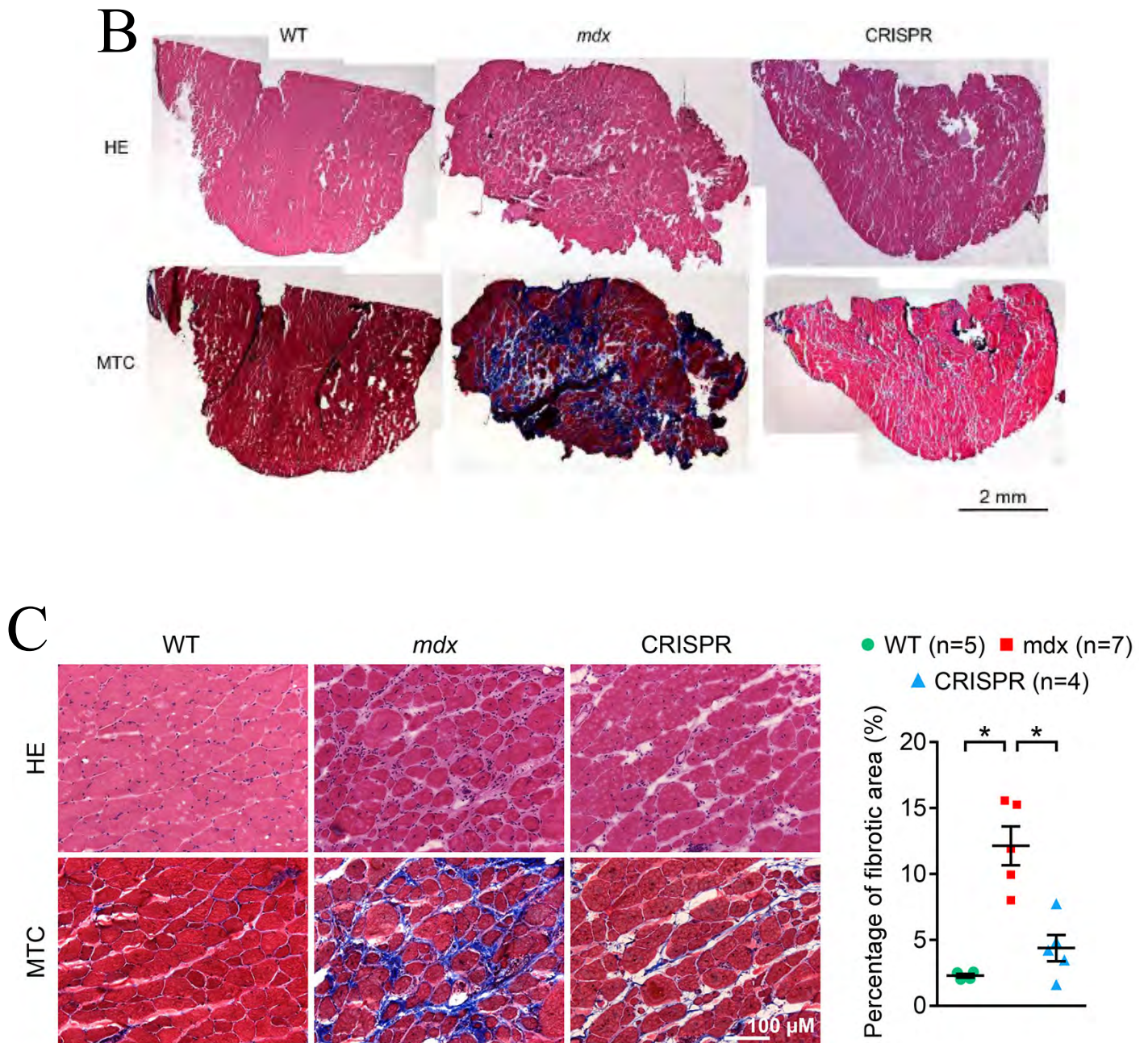


Figure 10. Evaluation of heart and skeletal muscle histology and fibrosis. **A**, Representative high-power images of HE and MTC staining of the heart from normal, untreated *mdx* and CRISPR treated *mdx* mice. **B**, Representative low-power images of HE and MTC staining of skeletal muscle from normal, untreated *mdx* and CRISPR treated *mdx* mice. **C, Left panel**, Representative high-power images of HE and MTC staining of skeletal muscle from normal, untreated *mdx* and CRISPR treated *mdx* mice. **Right panel**, Quantification of skeletal muscle fibrosis.

As planned, we then evaluated anatomic properties and muscle force in the extensor digitorum longus muscle in treated mice. **Table 2** shows anatomic properties. **Figure 11** shows muscle contractility.

Table 2. Anatomic properties

Strain	n	EDL weight	Lo	CSA
WT	7	12.62 ± 0.29	13.16 ± 0.05	2.06 ± 0.05
mdx	12	16.13 ± 0.64 #	13.99 ± 0.11 #	2.48 ± 0.09 #
CRISPR	6	12.14 ± 0.85	13.40 ± 0.15	1.95 ± 0.14

Significantly different from BL10 and CRISPR

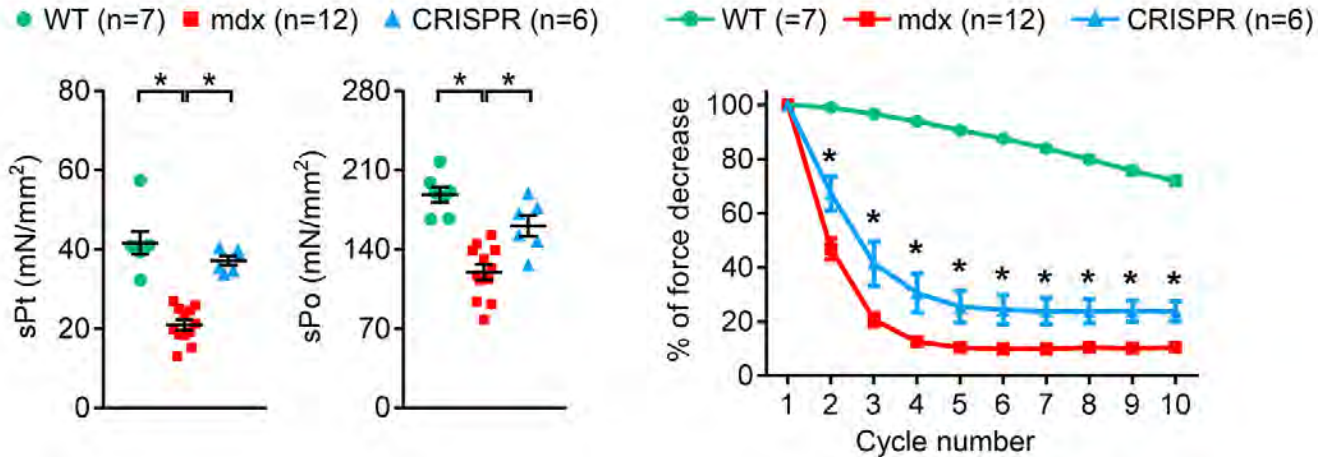


Figure 11. Evaluation of muscle contractility. Left panel, Specific twitch tensions; Middle panel, Specific tetanic tensions; Right panel, Eccentric contraction profiles.

In summary, we show that by increasing gRNA vector dosage at the time of vector administration, we achieved a more balanced ratio of the Cas9 vector to gRNA vector. As a result, we observed significant improvement in on-target editing. Dystrophin expression was observed in the heart and skeletal muscle throughout the body by immunostaining and western blot. Most importantly, muscle fibrosis was significantly reduced by CRISPR therapy. Pseudo-hypertrophy was prevented in skeletal muscle. Both twitch and tetanic forces were significantly enhanced reaching the level of wild type mice. CRISPR therapy also significantly prevented eccentric contraction induced muscle force drop. Collectively, the results from male mice corroborated our findings in female mice and suggest that systemic CRISPR therapy for DMD requires high dose of gRNA vector delivery.

Increasing the gRNA vector level did not enhance gene editing following intramuscular injection.

To determine whether the vector ratio affected the outcome of intramuscular CRISPR therapy, we injected the Cas9 and gRNA vectors at different ratios to the tibialis anterior muscle of 6-week-old mdx mice (**Table 3**). No appreciable dystrophin restoration was detected when only the Cas9 or gRNA vector was injected (**Figures 12- 14**). Co-injection of both vectors at the dose of 1×10^{11} vg/vector resulted in ~30% dystrophin positive myofibers. Interestingly, tripling the gRNA vector yielded similar levels of dystrophin restoration (**Figures 12-14**). Vector genome quantification did not reveal preferential loss of the gRNA vector (**Figure 15**).

Table 3. Vector ratios used in the intramuscular injection study

Vector ratio (Cas9:gRNA)	n	Cas9 vector (vg/muscle)	gRNA vector (vg/muscle)
Un-injected	6	0.00E+00	0.00E+00
(0:1)	4	0.00E+00	1.00E+11
(1:0)	4	1.00E+11	0.00E+00
(1:1)	4	1.00E+11	1.00E+11
(1:3)	4	1.00E+11	3.00E+11

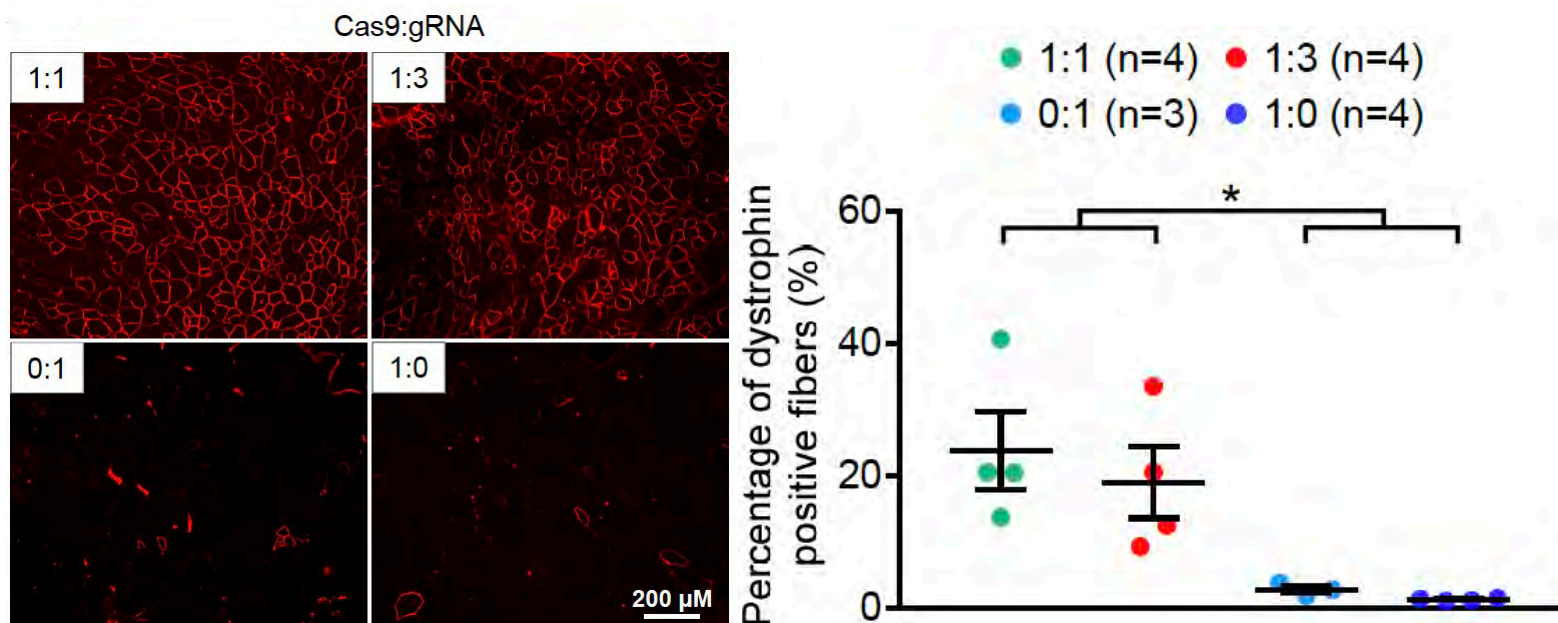


Figure 12. Evaluation of dystrophin expression by immunofluorescence staining. Left panel, Representative immunostaining photomicrographs; Right panel, Quantification of dystrophin positive cells. Asterisk, Significantly different.

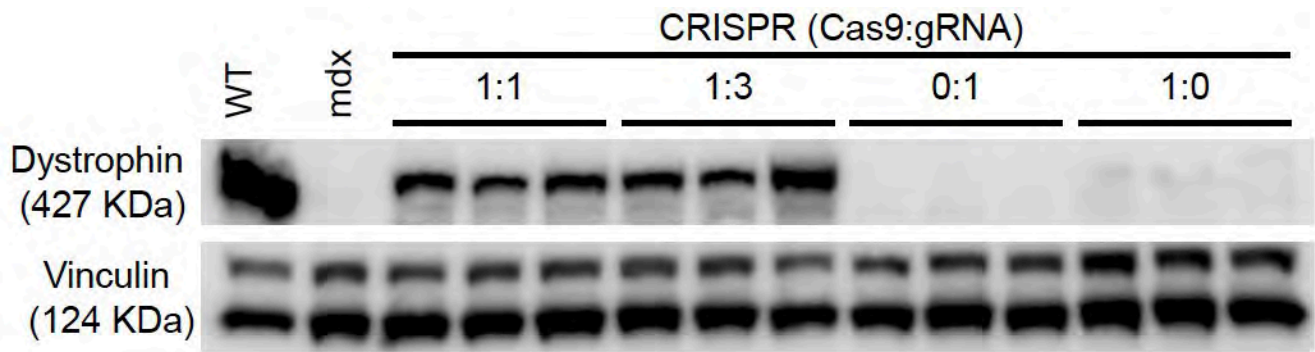


Figure 13. Evaluation of dystrophin expression by western blot. Representative western blot photomicrographs; Right panel, Quantification of dystrophin level in western blot. Asterisk, Significantly different.

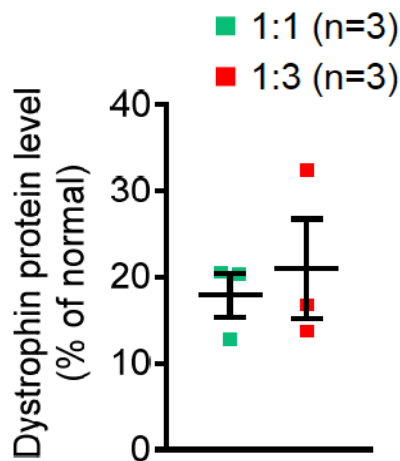
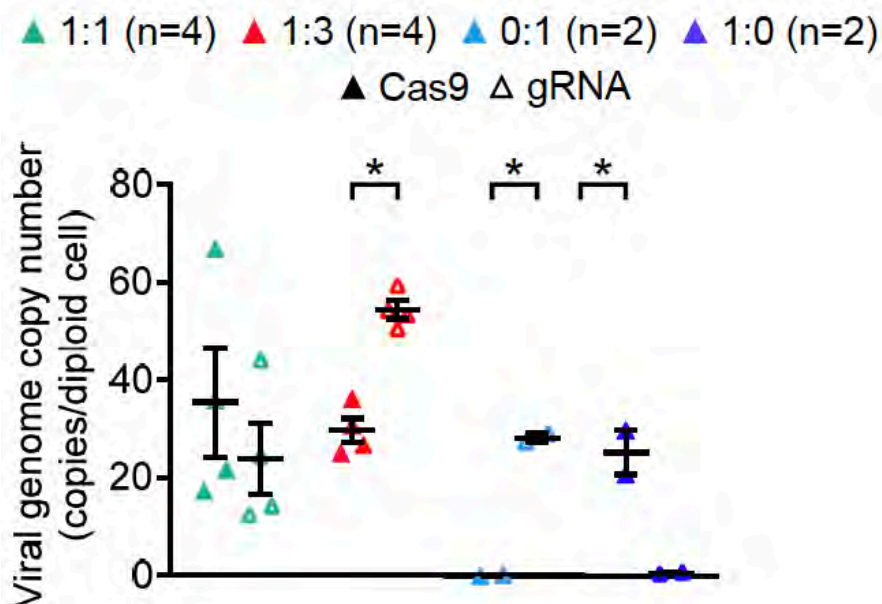


Figure 14 (left). Evaluation of dystrophin expression by quantitative western blot. Quantification of dystrophin level in western blot. Asterisk, Significantly different.

Figure 15 (below). Quantification of the vector genome copy number following local intramuscular injection. Asterisk, Significantly different.



Additional accomplishments that have benefited from this grant.

Accomplishment benefit from this grant 1. In this funding period, we published our study on the impact of gRNA vector and Cas9 vector ratio on systemic and local CRISPR editing in the mouse DMD model. We show that by optimizing gRNA vector dosage, we can achieve life-long CRISPR editing, dystrophin restoration, muscle and heart function improvement in mdx mice (Hakim et al 2018 JCI Insight).

Accomplishment benefit from this grant 2. In this funding period, we published our study on long-term therapeutic benefit in mdx mice. We also showed unintended genome modification at the target site. Further we show Cas9 is immunogenic (Nelson et al 2019 Nature Medicine).

Accomplishment benefit from this grant 3. During this funding period, we published a peer-reviewed review article on the translational future of CRISPR editing therapy for Duchenne muscular dystrophy in the canine model (Wasala et al 2019 Hum. Gene Ther).

Accomplishment benefit from this grant 4. During this funding period, we published a co-authored article on the mouse model of DMD. Using the right mouse model is critical for developing CRISPR therapy for DMD (Gordish-Dressman et al 2018).

Accomplishment benefit from this grant 5. During this funding period, I was invited to write a commentary article on CRISPR therapy in canine DMD model (Duan 2018 Nature Biomedical Engineering).

Accomplishment benefit from this grant 6. During this funding period, we published a method article on how to conduct systemic AAV delivery in animal models for DMD gene therapy.

Accomplishment benefit from this grant 7. During this funding period, I wrote a chapter on the considerations for preclinical DMD gene therapy. In this Chapter, I discussed critical issues related to preclinical gene therapy for DMD including gene replacement therapy and CRISPR gene repair therapy.

Training and professional development opportunities. Nothing to report.

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

Plan for the next reporting period.

1. DMD is a disease due to failed muscle regeneration. It has been suggested that if we don't correct gene mutation in muscle stem cells, we may not get long lasting therapy in human patients. We have now initiated a study to determine whether muscle stem cells can be edited by AAV vector. For the no cost extension period, we will wrap up the stem cell editing study.

4. Impact. Nothing to report.

5. Changes/Problems.

Changes in approach and reasons for change.

Nothing to report.

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

Nothing to report.

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

- 1) Hakim CH, Wasala NB, Nelson CE, Wasala LP, Yue Y, Louderman JA, Lessa TB, Zhang K, Jenkins GJ, Nance ME, Pan X, Kodippili K, Yang NN, Chen S-J, Gersbach CA, **Duan D**. *AAV CRISPR editing rescues cardiac and muscle function for 18 months*. **JCI Insight** 3(23): e124297, **2018**
- 2) Wasala NB, Hakim CH, Chen S-J, Yang NN, **Duan D**. *Questions answered and unanswered by the first CRISPR editing study in the canine model of Duchenne muscular dystrophy*. **Human Gene Therapy** 30(5):535-543, **2019**.
- 3) Nelson CE, Wu Y, Gemberling MP, Oliver ML, Waller MA, Bohning JD, Robinson-Hamm JN, Bulaklak K, Castellanos Rivera RM, Collier JH, Asokan A, **Gersbach CA**. *Long-term evaluation of AAV-CRISPR genome editing for Duchenne muscular dystrophy*. **Nature Medicine** 25(3):427-432

6.2. Non-peer-reviewed publications

- 1) Gordish-Dressman H, Willmann R, Dalle Pazzi L, Kreibich A, van Putten M, Heydemann A, Bogdanik L, Lutz C, Davies K, Demonbruen AR, **Duan D**, Elsey D, Fukada SI, Girgenrath M, Patrick Gonzalez J, Grounds MD, Nichols A, Partridge T, Passini M, Sanarica F, Schnell FJ, Wells DJ, Yokota T, Young CS, Zhong Z, Spurney C, Spencer M, De Luca A, Nagaraju K, Aartsma-Rus A. *"Of Mice and Measures": A Project to Improve How We Advance Duchenne Muscular Dystrophy Therapies to the Clinic (First Workshop Report: Examining current findings and opportunities around the emerging D2.B10-Dmd^{mdx}/J (D2/mdx) model in context of the classic C57BL/10ScSn-Dmd^{mdx}/J (B10/mdx))*. **Journal of Neuromuscular Diseases** 5(4):407-417, **2018**.

- 2) **Duan D.** *CRISPR alleviates muscular dystrophy in dogs.* **Nature Biomedical Engineering**, 2(11):795-796, **2018**
- 3) Wasala LP, Hakim CH, Yue Y, Yang NN, **Duan D.** *Systemic delivery of adeno-associated viral vectors in mice and dogs* **Methods in Molecular Biology** 1937:281-294, **2019**
- 4) **Duan D.** *Considerations on preclinical muscle gene therapy studies.* **Muscle Gene Therapy** 2nd edition (Publisher: Springer.) Duan D and Mendel JR (Ed.) **2019.** p291-326

6.3. Conference presentations

- 1) **Duan D.** Gene replacement and gene repair therapy for Duchenne muscular dystrophy. October 3, 2018 **Wake Forest University.** Winston-Salem, NC. (Oral Presentation, no abstract available)
- 2) **Duan D.** Long-term outcome in AAV9 micro-dystrophin treated DMD dogs and AAV9 CRISPR treated mdx mice. November 9, 2018 16th Action Duchenne Conference. Birmingham, England (Oral Presentation, no abstract available)
- 3) **Duan D.** Editing out muscular dystrophy with CRISPR. November 13, 2018 Gene Editing, Innovation and Impact in Missouri. Danforth Center, St Louis, MO (Oral Presentation, no abstract available)
- 4) **Duan D.** Duchenne cardiomyopathy gene therapy. February 4, 2019 Department of Medicine Research Seminar. University of Missouri. School of Medicine. Columbia, MO (Oral Presentation, no abstract available)
- 5) **Duan D.** AAV9 CRISPR editing therapy for DMD in muscle and muscle stem cells. March 14 2019 Nationwide Children's Hospital. Columbus, OH (Oral Presentation, no abstract available)
- 6) **Duan D.** CRISPR editing therapy for Duchenne muscular dystrophy. March 25 2019 Seminar in Translational Neuroscience-a forum for collaboration, Interdisciplinary Neuroscience and Pathology Graduate Program, University of Missouri. School of Medicine. Columbia, MO. (Oral Presentation, no abstract available)
- 7) **Duan D.** Gene therapy for heart disease in Duchenne muscular dystrophy. March 29, 2019 Department of Child Health Seminar. University of Missouri. School of Medicine. Columbia, MO (Oral Presentation, no abstract available)
- 8) **Duan D.** Duchenne muscular dystrophy:CRISPR/Cas9 treatment. June 20-22, 2019 Recent advances in rare diseases (RARD2019). Bogota, Columbia (Oral Presentation, no abstract available)

6.4. Inventions, patent applications, and/or licenses

A non-provisional, PCT application no. PCT/US2019/028635 was filed 4/23/19. The title of the application is "Improved CRISPR therapy".

7. Participants & Other Collaborating Organizations

What individuals have worked on the project?

Name: Dongsheng Duan

Project Role: PI

Research Identifier: ORCID #: 0000-0003-4109-1132

Nearest person month worked: 1.2

Contribution to project: Dr. Duan has overseen all aspects of the project.

Name: Yi Lai

Project Role: Associate Research Professor

Research Identifier: ORCID #:0000-0001-7303-8665

Nearest person month worked: 1.2

Contribution to project: Dr. Lai will offer scientific and technical assistance in this project.

Name: Nalinda Wasala

Project Role: Postdoc

Research Identifier: N/A

Nearest person month worked: 6

Contribution to project: Dr. Wasala will be responsible for function assays, histopathology assays and biochemical analysis and assist with AAV preparation and purification.

Name: Adrienne Pittman

Project Role: Research Staff

Researcher Identifier: N/A- Not Key

Nearest person month worked: 2.4 calendar months

Contribution to Project: Processing of tissues and preparation of materials and reagents

Funding Support: Levo Therapeutics (5UG3AR075336), Allen Family Foundation, Sarepta Therapeutics (5R21NS103007)

Name: Matthew Gemberling

Project Role: Postdoc

Researcher Identifier: N/A-Not Key

Nearest person month worked: 3 calendar months

Contribution to Project: Analysis of tissue DNA and RNA

Funding Support: Levo Therapeutic TDH Grant (5R33DA041878)

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

See following other support.

Other Support – Progress Report YR 3
Duan, Dongsheng, PI

Previous Active Grants That Have Closed

Pilot study of OPG analog 6 (OPG-6) in the mdx model of Duchenne muscular dystrophy

1% effort, PI

Ryan's Quest

David Schultz

PO Box 2544, Hamilton, NJ 08690

03/01/2018-12/31/2018 (ncte)

\$21,985 (direct costs)

To study muscle function rescue in the diaphragm and EDL muscle following OPG-6 therapy.

(There is no scientific/budget overlap with the current proposal)

Evaluation of osteoprotegerin (OPG) in the mdx model of Duchenne muscular dystrophy

5% effort, PI

Ryan's Quest

David Schultz

PO Box 2544, Hamilton, NJ 08690

10/15/2017-12/31/2018 (ncte)

\$83,385 (direct costs)

To validate muscle protection effect of osteoprotegerin (OPG) in 25-day-old mdx mice.

(There is no scientific/budget overlap with the current proposal)

Cardiac and skeletal muscle function evaluation and tissue banking of aged carrier DMD dogs

0.5% effort, PI

Parent Project Muscular Dystrophy

Elizabeth Habeeb-Louks

401 Hackensack Avenue, 9th Floor, Hackensack, NJ 07601

04/01/2018-09/30/2019

\$51,148 (direct costs)

We propose to conduct a function-histology correlation study in aged carrier dogs.

(There is no scientific/budget overlap with the current proposal)

Current Research Support

Whole body single AAV microgene therapy in canine DMD

20% effort, PI

NIH, NINDS R01 NS090634

National Institutes of Health; 6701 Rockledge Drive; Suite 1040, MSC 7710; Bethesda, MD 20817

09/01/2015-07/31/2020

\$406,687 (direct costs/current year)

In this study, we will test whether a newly developed canine Y731F AAV-9 micro-dystrophin vector gene therapy can lead to clinically meaningful improvement in dystrophic dogs.

Specific aim 1 is to test regional therapy in the hope of applying it to improve life quality in late-stage patients and aim 2 is to test systemic therapy in the hope of achieving bodywide improvement in young patients.

(There is no scientific/budget overlap with the current proposal).

CRISPR/Cas9-based gene editing for the correction of Duchenne muscular dystrophy

10% effort, Co-PI (PI: Charles Gersbach)

Duke University, NIH (R01 AR069085-01A1)

Charles Gersbach, Ph.D.; Duke University, 2353C CIEMAS Box 90281; Durham, NC 27708-0281

04/01/2016-03/31/2021

\$70,691 (total direct costs/current year)

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.

Specific aim: To test CRISPR/Cas9 gene therapy to treat muscle disease in mdx mice and hDMD mice.

(There is no scientific/budget overlap with the current proposal)

Treatment of Duchenne muscular dystrophy with the muscle calcium pump

20% effort, PI

NIH/NIAMS (R01 AR070517-01)

National Institutes of Health; 6701 Rockledge Drive, Suite 1040, MSC 7710; Bethesda, MD 20817

07/01/2016-08/31/2021

\$406,296 (direct costs/current year)

Goal: 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.

The specific aims are: (1) to test whether regional AAV SERCA2a therapy can ameliorate limb muscle disease and improve function and (2) to test whether systemic AAV SERCA2a therapy can lead to bodywide improvement in affected dogs.

(There is no scientific/budget overlap with the current proposal.)

A pilot study to evaluate long-term safety and efficacy of AAV-9 5Rc micro-dystrophin therapy

2% effort (during ncte), PI

Solid Biosciences

Joel Schneider, Ph.D.; 101 Main Street; Cambridge, MA 02142

06/01/2016-10/31/2019 ncte requested to 04/30/2020

\$100,000 (direct costs/current year)

The overarching goal of this project is to determine whether systemic AAV-9 5Rc micro-dystrophin gene therapy can yield long-term (up to 4 years after injection) microgene expression without causing serious adverse events (SAEs).

(There is no scientific/budget overlap with the current proposal.)

Evaluation of Montelukast as a potential therapy for Duchenne muscular dystrophy in the murine model

3% effort, PI

Duchenne UK

Fiona Lawrence; 11 Bedford Road; London, W4 1 JD; GBR United Kingdom

03/01/2017-02/28/2020

\$60,000 (direct costs/current year)

We propose to evaluate safety and therapeutic efficacy of Montelukast in mdx mice, the most commonly used mouse model for DMD.

(There is no scientific/budget overlap with the current proposal)

Evaluation of Montelukast as a potential therapy for Duchenne muscular dystrophy in the murine model

0% effort, PI

Michael's Cause

Robert Capolongo; PO Box 120323; Staten Island, NY 10312

03/01/2017-02/28/2020

\$16,666 (direct costs/current year)

We propose to evaluate safety and therapeutic efficacy of Montelukast in mdx mice, the most commonly used mouse model for DMD. This is a supplementary grant to the Duchenne UK grant.

(There is no scientific/budget overlap with the current proposal)

Evaluation of Montelukast as a potential therapy for Duchenne muscular dystrophy in the murine model

0% effort, PI

Ryan's Quest

David Shultz; PO Box 2544; Hamilton, NJ 08690

03/01/2017-02/28/2020

\$16,666 (direct costs/current year)

We propose to evaluate safety and therapeutic efficacy of Montelukast in mdx mice, the most commonly used mouse model for DMD. This is a supplementary grant to the Duchenne UK grant.

(There is no scientific/budget overlap with the current proposal)

Evaluation of Montelukast as a potential therapy for Duchenne muscular dystrophy in the murine model

0% effort, PI

Rally for Ryan

Marty Karlin; 2623 Evercrest Court; Naperville, IL 60564

03/01/2017-02/28/2020

\$16,666 (direct costs/current year)

We propose to evaluate safety and therapeutic efficacy of Montelukast in mdx mice, the most commonly used mouse model for DMD. This is a supplementary grant to the Duchenne UK grant.

(There is no scientific/budget overlap with the current proposal)

DMD gene therapy in the canine model by intramuscular sarcolipin knockdown

5% effort, PI

Jesse's Journey: The Foundation for Gene & Cell Therapy

Lisa Hoffman; PO Box 51 Station B; London, ON N6A 4V3; CANADA

08/01/2017-07/31/2020

\$131,515 CAN

The major goal of this study is to demonstrate that sarcolipin (SLN) knockdown improves the carco/endoplasmic reticulum calcium ATPase (SERCA) function and ameliorate the muscle disease in a dog model of Duchenne muscular dystrophy.

(There is no scientific/budget overlap with the current proposal)

Pilot study to evaluate protein CRISPR therapy in the DMD mouse model

0% effort, PI

Hubrecht Institute

Niels Geijsen

Utrecht University, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands

10/01/2017-09/30/2021

\$10,000 (direct costs)

In this pilot study, the Geijsen lab and the Duan lab will analyze the functional improvement of a mouse model of Duchenne muscular dystrophy upon Dystrophin gene repair by the iTOP-mediated introduction of Cas9 and sgRNA to skeletal muscle fibers and satellite cells of (DMD) to test DMD CRISPR therapy.

(There is no scientific/budget overlap with the current proposal)

Extension of the maximum feasible dose study in a canine DMD model

2.5% effort, PI

Solid Biosciences

Joel Schneider

161 First Street, Cambridge, MA 02142

04/01/2018-10/31/2019 requested ncte to 04/30/2020

\$135,147 (total direct costs)

To determine the long-term safety and efficacy of systemic delivery of high dose SGT-001c in young adult affected dogs.

(There is no scientific/budget overlap with the current proposal)

Skeletal Muscle Glucose Uptake: Exercise and Insulin

3% effort, Co-I

R01 DK071771 (Gregory Cartee, PI)

NIH University of Michigan (Duan subcontract)

Khaled J. Eid, MSF – Contract Administrator Lead

Sponsored Programs – Office of Contract Administration (OCA), 5065 Wolverine Tower, 3003 S. State, St., Ann Arbor, MI 48109-1287

05/05/2018-04/30/2021

\$17,287 (direct costs)

Duan lab will be in charge of recombinant adeno-associated viral vector (AAV) production, purification and characterization. The AAV vector will be used by Dr. Cartee's lab to perform in vivo study in experimental subjects. Dr. Duan will also participate in the design of AAV related studies, data analysis and troubleshooting.

(There is no scientific/budget overlap with the current proposal)

New Active Grants

Establishment of the Δ 52-54 DMD mouse model

1% effort, PI

Solid Biosciences

Joel Schneider

161 First Street, Cambridge, MA 02142

09/01/2018-02/29/2020 (in ncte)

\$50,000 (direct costs)

To establish the new Δ 52-54 mouse model at MU; to characterize muscle disease of Δ 52-54 mice at 3 months of age, and to perform explorative study to evaluate alternative CRISPR therapy in Δ 52-54 mice.

(There is no scientific/budget overlap with the current proposal)

Aging, calorie restriction and insulin sensitivity

4% effort, PI, Subaward

2R01 AG010026-25 (Gregory Cartee, PI)

NIH University of Michigan (Duan, PI, Subaward)

Khaled J. Eid, MSF – Contract Administrator Lead

Sponsored Programs – Office of Contract Administration (OCA), 5065 Wolverine Tower, 3003 S. State, St., Ann Arbor, MI 48109-1287

05/01/2019-02/28/2023

\$24,902 (direct costs)

Duan lab will be in charge of recombinant adeno-associated viral vector (AAV) production, purification and characterization. The AAV vector will be used by Dr. Cartee's lab to perform in vivo study in experimental subjects. Dr. Duan will also participate in the design of AAV related studies, data analysis and troubleshooting.

(There is no scientific/budget overlap with the current proposal)

To test the effect of EDG-5506 on the muscle function of micro-dystrophin transgenic mice

2% effort, Co-I

Edgewise Therapeutics, Inc. (Lai, PI)

Laura Hunter – Business Operations Manager

3415 Colorado Avenue

Boulder, CO 80303

06/01/2019-03/31/2020

\$67,636 (direct costs)

The scope of this contract is to test the effect of EDG-5506, a compound from Edgewise, on the muscle function of micro-dystrophin transgenic mice.

(There is no scientific/budget overlap with the current proposal)

Grants Funded and Closed Since Last Progress Report

Solid micro-dystrophin optimization

5% effort, Co-I (Lai, PI)

Solid Biosciences, LLC

Kenneth S. Casado, J.D. Contracts Manager

161 First Street, 3rd Floor, Cambridge, MA 02142

12/07/2017-08/31/2019

\$110,689 (direct costs)

(1) To engineer new adeno-associated virus (AAV) micro-dystrophin vectors by incorporating our recently discovered membrane-binding domains (MBD)s, and (2) to identify the best vector by examining therapeutic efficacy of new AAV micro-dystrophin vectors in mouse models of Duchenne muscular dystrophy (DMD).

(There is no scientific/budget overlap with the current proposal)

Dystrophin structure and heart function

5% effort, PI

Parent Project Muscular Dystrophy

Elizabeth Habeeb-Louks

401 Hackensack Avenue, 9th Floor, Hackensack, NJ 07601

09/01/2018-08/31/2019

\$100,000 (direct costs)

In this study, we will study therapeutic potential of two dystrophin domains that have been implicated in DMD cardiomyopathy in patients. Partial bridge funding.

(There is no scientific/budget overlap with the current proposal)

Other Support – Progress Report YR3
Gersbach, Charles A., Co-I

Previous Active Grants That Have Closed

Title: Reprogrammed autologous T cells to promote transplant tolerance

Time Commitments: 0.45 academic month (or 3.75% calendar)

Supporting Agency: Element Genomics, Inc (Gersbach, PI)

Point of Contact:

John Oxaal, CEO
300 West Duke Street
Durham, NC 27701

Performance Period: 04/13/18-04/12/19

Level of Funding: \$52,415 direct costs for current budget year

Goals/Aims: This project will provide proof-of-concept that human T cells that have been epigenetically-reprogrammed are immunosuppressive in vitro and have a T regulatory cell phenotype.

Overlap: None

Title: Deciphering the regulation of gene expression in the etiology of LOAD

Time Commitments: 1.20 academic (10% calendar) (Dr. Gersbach's effort never started on this project)

Supporting Agency: NIH 1R01AG057522-01 (Chiba-Falek)

Point of Contact:

Bradley C. Wise, Program Official
301496-9350

Performance Period: 09/01/17-08/31/22

Level of Funding: direct costs for current budget year

The objective of this proposal is to determine causal variants within GWAS loci associated with late onset Alzheimer's disease

Overlap: None

Current Research Support

Title: Investigating Autophagy in GSD-1a

Time Commitments: 0.3 summer months

Supporting Agency: NIH, 1R01DK105434 (Koeberl)

Point of Contact:

Grants and Contracts Specialist
Ellen W. Leschek
lescheke@extra.niddk.nih.gov
301-402-8291

Performance Period: 12/15/15-11/30/19

Level of Funding: \$229,500 direct costs for current budget year

Goals/Aims: The goal of this project is to develop a gene therapy for glycogen storage disorder type 1a

Overlap: None

Title: In Vivo Epigenome Editing with CRISPR-Based Histone Acetyltransferase Transgenic Mice

Time Commitments: 0.45 academic/0 summer months

Supporting Agency: NIH, 1R33DA041878 (West and Gersbach)

Point of Contact:

Grants and Contracts Specialist

Cheryl A. Nathaniel
nathanic@mail.nih.gov
202-526-0108

Performance Period: 05/01/18-04/30/21

Level of Funding: \$250,000 direct costs for current budget year

Goals/Aims: We will generate novel Cre/loxP-conditional CRISPR/Cas9-based transgenic mouse strains in order to use epigenome editing to test the functional consequences of chromatin modifications for the long-lasting behavioral plasticities induced by psychostimulant drugs of abuse.

Overlap: None

Title: CRISPR/Cas9-Based Gene Editing for the Correction of Duchenne Muscular Dystrophy

Time Commitments: 0.32 academic/0.57 summer months

Supporting Agency: NIH, R01AR069085 (Gersbach, PI)

Point of Contact:

Grants Management Specialist
Steve Austin
austins2@mail.nih.gov
301-594-3504

Performance Period: 4/1/16-3/31/21

Level of Funding: \$274,560 direct costs for current budget year

Goals/Aims: The overall objective of this research proposal is to develop methods to restore dystrophin expression via targeted genome editing in vivo.

Overlap: None, although there is some conceptual overlap between R01AR06985 and the University of Missouri (USAMRMC) funded work, the proposed experiments are distinct. In particular, the USAMRMC W81XWH1610221 funded work is supporting the proof-of-principle studies used in the R01AR069085 proposal as preliminary data.

Title: Epigenome Editing Technologies to Control Diverse Biological Functions

Time Commitments: .53 academic and 0.65 summer months

Supporting Agency: Allen Frontiers Group (Gersbach)

Point of Contact:

Kathy Richmond, Program Officer
kathrynr@alleninstitute.org
206-548-8491

Performance Period: 8/1/17-7/31/20

Level of Funding: \$486,609 direct costs for current budget period

The objective of this proposal is to optimize and apply epigenome editing tools to probing mechanisms of neuronal differentiation and neurological behaviors including learning and memory.

Overlap—None

Title: Systemic Inflammation in Microphysiological Models of Muscle and Vascular Disease

Time Commitments: 0.41 academic 0.18 summer months

Supporting Agency: 1UG3-TR002142 NIH (Truskey)

Point of Contact:

Danilo A. Tagle, Program Officer
tagled@ninds.nih.gov
301-496-5745

Performance Period: 8/1/17-6/30/19

Level of Funding: \$750,000 direct costs for current budget period

Our goal in this project is to develop clinically relevant hMPS disease models to examine rheumatoid arthritis (RA) risk for muscle dysfunction and atherosclerosis and the role of exercise in attenuating disease-associated inflammation.

Overlap—None

Title: Collaborative Research: Adapting Cas9 Protein from CRISPR as a Structural Unit for Molecular Assembly

Time Commitments: 0.25 summer effort

Supporting Agency: 1709527 – NSF (LaBean and Gersbach)

Point of Contact:

Aleksandr Simonian, Program Official

703-292-2191

Performance Period: 7/1/17-6/30/20

Level of Funding \$45,845 direct costs

Design, clone, express, and purify dCas9 fusion proteins bearing binding domains for various materials including gold nanoparticles and carbon nanotubes for testing (at the LaBean lab) the assembly of functional electronic and photonic nano-devices. Design, clone, express, and purify dCas9 fusion proteins bearing functional enzymatic domains for testing (at the LaBean lab) the assembly of multienzyme cascades.

Overlap--None

Title: Treating Duchenne Cardiomyopathy in the Mouse Model by Gene Repair

Time Commitments: 0.05 academic (or 0.4% calendar)*minimum effort covered by Duke

Supporting Agency: University of Missouri (Prime award USAMRMC W81XWH1610221)

Point of Contact:

Craig David, Director, OSPA

115 Business Loop 70W

Mizzou North Room 501

Columbia, MO 65211

Performance Period: 8/1/16-7/31/19

Level of Funding: \$60,000 direct costs for current budget year

Overlap: None, although there is some conceptual overlap between R01AR06985 and the University of Missouri (USAMRMC) funded work, the proposed experiments are distinct. In particular, the USAMRMC W81XWH1610221 funded work is supporting the proof-of-principle studies used in the R01AR069085 proposal as preliminary data.

Title: Regulatory Mechanisms of CD4+ T Cell Differentiation

Time Commitments: 0.61 academic 0.3 summer

Supporting Agency: NIH 1UM1HG009428 (Reddy, Gersbach, Ciofani, Crawford, Co-PI)

Point of Contact:

Michael J. Pazin, Program Official

301-435-7859

Performance Period: 08/21/17-06/30/21

Level of Funding: \$417,678 direct costs for current budget year

The objective of this ENCODE Functional Characterization Center grant is to annotate the function of gene regulatory elements involved in mouse T cell differentiation with high-throughput reporter assays and CRISPR screens

Overlap: None

Title: Enhancing Neuronal Reprogramming with Epigenome Editing

Time Commitments: 0.46 academic

Supporting Agency: NIH 1R21NS103007 (Gersbach)

Point of Contact:

Robert D. Riddle, Program Official

301-402-3315

Performance Period: 04/01/18-03/31/23

Level of Funding: \$150,000 direct costs for current budget year

We will use CRISPR/Cas9-based epigenetic modifiers to remodel endogenous chromatin at genes encoding master regulatory factors to direct differentiation of human pluripotent cells and reprogram human fibroblasts to induced neurons

Overlap: None

Title: An organotypic model recapitulating colon cancer microenvironment and metastasis

Time Commitments: 0.35 academic 0.10 summer

Supporting Agency: Cleveland Clinic Lerner College of Medicine (Prime NIH U01CA214300 (Shen))

Point of Contact:

Richard Cole, Assistant Director, Grants & Contracts

216-444-5223

Performance Period: 09/01/2018-08/31/2019

Level of Funding: \$115,152 direct costs for current budget year

To extend their organotypic colorectal cancer (CRC) model to incorporate cytokine-producing stroma cells and use their recently developed CRC blastocyst injection model and metastasis model to validate conclusions drawn from the tissue engineering model.

Overlap: None

Title: Epigenome editing by CRISPR/Cas9 in the PWS imprinted domain

Time Commitments: 0.38 academic

Supporting Agency: Levo Therapeutics, Inc (Jiang and Gersbach)

Point of Contact:

Sara Cotter

5215 Old Orchard Rd, Suite 450

Skokie, IL 60077

Performance Period: 04/13/18-04/12/19

Level of Funding: \$206,861 direct costs for current budget year

The goal is to use CRISPR-based screens to determine the mechanism of epigenetic regulation of imprinting at the Prader Willi Syndrome locus.

Overlap: None

New Active Grants

Title: Engineering Technologies to Determine Causal Relationships Between Chromatin Structure and Gene Regulation

Time Commitments: 0.50 summer

Supporting Agency: NSF EFMA-1830957 (Gersbach)

Point of Contact:

Denise B. Robinson

Email: drobinso@nsf.gov

Performance Period: 09/01/18-08/31/22

Level of Funding: \$318,602 direct costs for current budget year

The objective of this proposal is to establish the capability to accurately predict, quantitatively monitor, and deterministically program epigenetic states to generate improved disease models.

Overlap: None

Title: Combinatorial Biomimetic Systems for High-Throughput Manipulation of Genome-Edited Dystrophic Muscle Stem Cells

Time Commitments: 0.09 academic

Supporting Agency: Cornell University NIH 1R21AR072265 (Cosgrove-Gersbach)

Point of Contact:

Unknown at this time

Performance Period: 07/01/18-06/30/20

Level of Funding: \$15,000 direct costs for current budget year

The goal of this proposal is to use biomaterial design to enhance genome editing of muscle stem cells.

Overlap: None

Title: Epigenomic Reprogramming in Patient Derived Models of Colorectal Cancer

Time Commitments: 0.36 academic

Supporting Agency: NIH 1U01-CA217514 (Shen)

Point of Contact:

Jacquelyn Saval

Email: savalj@mail.nih.gov

Phone: 240-276-6312

Performance Period: 07/01/18-06/30/23

Level of Funding: \$480,125 direct costs for current budget year

This study will systematically characterize the epigenetic reprogramming between different patient-derived cancer models and use novel epigenetic editing tools to identify epigenomic drivers that contribute to the phenotypic differences between the models.

Overlap: None

Title: Evolving High Potency AAV Vectors for Neuromuscular Genome Editing

Time Commitments: 0.77 academic 0.3 summer

Supporting Agency: NIH1UG3-AR075336 (Asokan)

Point of Contact:

Steve Austin

Email: steve.auston@nih.gov

Phone: 301-594-3504

Performance Period: 09/01/18-08/31/23

Level of Funding: \$ 175,322 direct costs for current budget year

The goal of this study is to identify gene delivery vectors, based on adeno-associated virus (AAV), with enhanced targeting of skeletal muscle, and in particular the stem cells of skeletal muscle

Overlap: None

Title: Genome Editing with Engineered Vectors to Correct Neurofibromatosis Type I

Time Commitments: 0.59 academic

Supporting Agency: Gilbert Family Foundation (Gersbach)

Point of Contact:

Kathy Russell

Email: klrussell411@gmail.com

Performance Period: 12/01/18-11/30/21

Level of Funding: \$575,527 direct costs for current budget year

The overall goal of this proposal is to develop a transformative approach to treating Neurofibromatosis Type I (NF1) by targeted genome editing of the causal underlying mutation with engineered viral vectors

Overlap: None

Title: Thwarting Influenza with RNA-powered Modulators (ThIRM)

Time Commitments: 0.27 academic

Supporting Agency: Georgia Institute of Technology (DARPA)

Point of Contact:

Susan K Shean

Email: susan.shean@darpa.mil

Phone: 703-526-4128

Performance Period: 04/01/19-3/31/23

Level of Funding: \$ 643,856 direct costs for current budget year

The objective of this proposal is to establish the capability to accurately predict, quantitatively monitor, and deterministically program epigenetic states to generate improved disease models. To do so, we will generate new molecular tools for monitoring changes to epigenetic states using FRET-based nanosensors.

Overlap: None

Title: Identification and application of cis-regulatory enhancer elements for muscle stem cell function and muscle regeneration in aging

Time Commitments: 0.27 academic

Supporting Agency: American Federation for Aging Research (Diao, Yarui)

Point of Contact:

Unknown at this time

Performance Period: 07/01/19-06/30/20

Level of Funding: \$ 92,593 direct costs for current budget year

The major goal of this project is to test the hypothesis that enhancers control satellite cell regeneration activity and muscle function during aging, and the "rejuvenation" cis-regulatory elements can be utilized to facilitate muscle homeostasis and regeneration in the aging process

Overlap: None

Title: Quantifying the genetic diversity of human regulatory element activity

Time Commitments: 0.38 academic 0.15 summer

Supporting Agency: NIH 1R01HG010741-01 (Reddy, Crawford, Gersbach, Allen)

Point of Contact:

Lisa A. Oken

Email: loken@mail.nih.gov

Phone: 301-594-5250

Performance Period: 07/29/19-05/31/23

Level of Funding: \$486,124 direct costs for current budget year

The objective of this proposal is to develop a platform of empirical and statistical methods to reliably and systematically determine the regulatory mechanisms underlying human traits and diseases

Overlap: None

Title: Microphysiological Human Tissue Systems for Monitoring of Genome Editing Outcomes

Time Commitments: .75 academic

Supporting Agency: NIH 1U01EB028901-01 (Gersbach, Bursac, Truskey)

Point of Contact:

Angelos Bacas

Email: ab329b@nih.gov

Phone: 301-451-4785

Performance Period: 09/01/19-08/31/23

Level of Funding: \$415,000 direct costs for current budget year

The goal of this project is to develop a platform to systemically address the most significant challenges to realizing the transformative potential of genome editing therapies in human tissues.

Overlap: None

Title: Epigenome Editing Technologies for Treating Diverse Disease

Time Commitments: 0.73 academic

Supporting Agency: NIH 1U01AI146356-01 (Gersbach)

Point of Contact:

Shaun W Gratton

Email: shaun.gratton@nih.gov

Phone: 240-627-3594

Performance Period: 07/08/19-06/30/23

Level of Funding: \$ 282,000 direct costs for current budget year

We will engineer and validate new epigenome editing tools in vivo in a set of pilot experiments in mouse models of neuromuscular disease encompassing a representative set of epigenomic states

Overlap: None

Title: Systemic Inflammation in Microphysiological Models of Muscle and Vascular Disease

Time Commitments: 0.45 academic

Supporting Agency: NIH 3UH3TR002142-03S1 (Truskey)

Point of Contact:

Jenelle D. Wiggins

Email: jenell.wiggins@nih.gov

Phone: 301-435-0843

Performance Period: 09/16/19-06/30/20

Level of Funding: \$155,849 direct costs for current budget year

Overlap: None

Title: Nonhuman Primate Testing Center for Evaluation of Somatic

Time Commitments: 0 .14 academic

Supporting Agency: University of Davis NIH 3U42OD0270964-01S1 (Asokan)

Point of Contact:

Unknown at this time

Performance Period: 09/01/19-03-31-20

Level of Funding: \$ direct costs for current budget year

Overlap: None

Other Support – Progress Report YR3
Yao, Gang, Co-I

Previous/active grants that have closed

Title: Developing Pupillary Light Reflex Technologies for Early Screening of Neurodevelopmental Disorders

Level of Effort: 10% effort, PI

Funding Agency: National Science Foundation

Project Goals and Specific Aims: The goal of this project is to develop a new imaging system and data analysis methodology for investigating pupillary light reflex in infants and extracting neurological information specific to autonomic nervous system.

Performance Period: 07/15/2015 to 06/30/2019

Level of Funding: \$203,688 total direct costs

Point of contact at the agency: Alexander Leonessa, 201 Wilson Boulevard, Arlington, Virginia 22230, USA

Overlap/complement with the current proposal: None

Title: Optical polarization tractography for cervical lesion detection

Level of Effort: 5% effort, Co-PI

Funding Agency: University of Missouri, MU Coulter Partnership

Project Goals and Specific Aims: The goal of this project is to investigate the potential of using optical polarization tractography to detect and differentiate cervical lesions.

Performance Period: 07/01/2017 to 08/31/2018

Level of Funding: \$22,000

Point of contact at the agency: Cynthia Helphingstine, 235 Agricultural Engineering Building, University of Missouri

Overlap/complement with the current proposal: None

Title: Photon migration method for characterizing fiber formation in meat analogs

Level of Effort: 2% effort, PI

Funding Agency: Nestle/Purina

Project Goals and Specific Aims: The goal of this project is to help Nestle Purina to develop a imaging system to measure fiber formation in meat analogs

Performance Period: 07/16/2018-12/31/2018

Level of Funding: \$8,982

Point of contact at the agency: Robert Miller, Robert.Miller@RD.Nestle.com, 314-982-1356

Overlap/complement with the current proposal: None

Current Research Support

Title: A pilot study to evaluate long-term safety and efficacy of AAV-9 5Rc micro-dystrophin therapy

Level of Effort: 2% effort during ncte, Co-I; Duan (PI)

Funding Agency: Solid Biosciences

Project Goals and Specific Aims: The overarching goal of this project is to determine whether systemic AAV-9 5Rc micro-dystrophin gene therapy can yield long-term (up to 4 years after injection) microgene expression without causing serious adverse events (SAEs).

Performance Period: 06/01/2016-10/31/2019 ncte requested to 04/30/2020

Level of Funding: \$100,000 (direct costs/current year)

Point of contact at the agency: Joel Schneider, Ph.D.; 101 Main Street; Cambridge, MA 02142

Overlap/complement with the current proposal: None

Title: Extension of the maximum feasible dose study in a canine DMD model

Level of Effort: 5% effort, Co-I (Duan, PI)

Funding Agency: Solid Biosciences

Project Goals and Specific Aims: To determine the long-term safety and efficacy of systemic delivery of high dose SGT-001c in young adult affected dogs.

Performance Period: 04/01/2018-10/31/2019 requested ncte to 04/30/2020

Level of Funding: \$135,147 (total direct costs)

Point of contact at the agency: Joel Schneider, Ph.D.; 161 First Street; Cambridge, MA 02142

Overlap/complement with the current proposal: None

Other Support – Progress Report YR3

Lai, Yi, Associate Research Professor – Key Personnel

Previous Active Grants That Have Closed

Solid micro-dystrophin optimization

50% effort, PI

Solid Biosciences

Ilan Ganot

161 First Street, 3rd Floor

Cambridge, MA 02142

12/07/2017-08/31/2019

\$236,944 (total direct costs)

Project objective. (1) To engineer new adeno-associated virus (AAV) micro-dystrophin vectors by incorporating our recently discovered membrane-binding domains (MBD)s, and (2) to identify the best vector by examining therapeutic efficacy of new AAV micro-dystrophin vectors in mouse models of Duchenne muscular dystrophy (DMD).

(There is no scientific/budget overlap with the current proposal)

Current Research Support

To test the effect of EDG-5506 on the muscle function of micro-dystrophin transgenic mice

2.5% effort (Phase I); 10% effort (Phase II), PI

Edgewise Therapeutics, Inc.

Laura Hunter – Business Operations Manager

3415 Colorado Avenue

Boulder, CO 80303

06/01/2019-03/31/2020

\$67,636 (direct costs)

The scope of this contract is to test the effect of EDGE-5506, a compound from Edgewise Therapeutics, Inc. on the muscle function of micro-dystrophin transgenic mice.

(There is no scientific/budget overlap with the current proposal)

What other organizations were involved as partners?

Organization Name: Duke University

Location of Organization: Durham, NC

Partner's contribution to the project: Dr. Gersbach's lab will perform droplet PCR to quantify genome editing and RNA correction and in vivo quantification of off-target effect in the study of Duchenne cardiomyopathy gene editing therapy in the mouse model for DMD.

Dr. Gersbach's lab is involved in the study, design and quantification of on-target and off-target genome editing and data analysis in the current funding period. The results of these collaborative efforts are combined in the accomplishment section.

8. Special Reporting Requirements

N/A

AAV CRISPR editing rescues cardiac and muscle function for 18 months in dystrophic mice

Chady H. Hakim, ... , Charles A. Gersbach, Dongsheng Duan

JCI Insight. 2018;3(23):e124297. <https://doi.org/10.1172/jci.insight.124297>.

Research Article

Therapeutics

Adeno-associated virus–mediated (AAV-mediated) CRISPR editing is a revolutionary approach for treating inherited diseases. Sustained, often life-long mutation correction is required for treating these diseases. Unfortunately, this has never been demonstrated with AAV CRISPR therapy. We addressed this question in the mdx model of Duchenne muscular dystrophy (DMD). DMD is caused by dystrophin gene mutation. Dystrophin deficiency leads to ambulation loss and cardiomyopathy. We treated 6-week-old mice intravenously and evaluated disease rescue at 18 months. Surprisingly, nominal dystrophin was restored in skeletal muscle. Cardiac dystrophin was restored, but histology and hemodynamics were not improved. To determine the underlying mechanism, we evaluated components of the CRISPR-editing machinery. Intriguingly, we found disproportional guide RNA (gRNA) vector depletion. To test whether this is responsible for the poor outcome, we increased the gRNA vector dose and repeated the study. This strategy significantly increased dystrophin restoration and reduced fibrosis in all striated muscles at 18 months. Importantly, skeletal muscle function and cardiac hemodynamics were significantly enhanced. Interestingly, we did not see selective depletion of the gRNA vector after intramuscular injection. Our results suggest that gRNA vector loss is a unique barrier for systemic AAV CRISPR therapy. This can be circumvented by vector dose optimization.

Find the latest version:

<http://jci.me/124297/pdf>



AAV CRISPR editing rescues cardiac and muscle function for 18 months in dystrophic mice

Chady H. Hakim,^{1,2} Nalinda B. Wasala,¹ Christopher E. Nelson,^{3,4} Lakmini P. Wasala,¹ Yongping Yue,¹ Jacqueline A. Louderman,¹ Thais B. Lessa,¹ Aihua Dai,¹ Keqing Zhang,¹ Gregory J. Jenkins,¹ Michael E. Nance,¹ Xiufang Pan,¹ Kasun Kodippili,¹ N. Nora Yang,² Shi-jie Chen,^{5,6} Charles A. Gersbach,^{3,4,7} and Dongsheng Duan^{1,8,9,10}

Authorship note: CHH and NBW contributed equally to this work.

Conflict of interest: DD is a member of the scientific advisory board of and an equity holder in Solid Biosciences LLC. DD and YY are inventors on patents that were licensed to Solid Biosciences LLC (US patent 7,892,824; Chinese patent ZL 200880002602.3; Japanese patent 5575486; Hong Kong patent HK1143326; German patent 602008028126.4; Great Britain patent GB2125006; Irish patent IE2125006; French patent FR2125006). The Duan lab has received research support unrelated to this project from Solid Biosciences LLC. CHH, DD, NBW, and YY have filed a patent application related to the findings described in this paper (US provisional application 62/661,391). CAG has filed patent applications related to genome editing for Duchenne muscular dystrophy (international patent application PCT/US2013/038536; US provisional applications 61/839127, 61/904911, 62/260,712, 62/332,297, 62/363,888). CAG is an advisor to and receives research support from Sarepta Therapeutics Inc. CAG and CEN are inventors on patent applications related to genome editing (international patent application PCT/US14/41190; US provisional applications 61/803254, 61/981575, 62/082,315, 62/113,569, 62/230,931, 62/195,680, 62/209,466, 61/719,276). Solid Biosciences LLC has optioned disclosures 6UMC049 and 17UMC006, which include patent application PCT/US2017/038418.

License: Copyright 2018, American Society for Clinical Investigation.

Submitted: August 20, 2018

Accepted: October 31, 2018

Published: December 6, 2018

Reference information:

JCI Insight. 2018;3(23):e124297.

<https://doi.org/10.1172/jci.insight.124297>

insight.124297.

¹Department of Molecular Microbiology and Immunology, School of Medicine, University of Missouri, Columbia, Missouri, USA. ²National Center for Advancing Translational Sciences, NIH, Rockville, Maryland, USA. ³Department of Biomedical Engineering and ⁴Center for Genomic and Computational Biology, Duke University, Durham, North Carolina, USA.

⁵Department of Physics and ⁶Department of Biochemistry, University of Missouri, Columbia, Missouri, USA. ⁷Department of Orthopaedic Surgery, Duke University Medical Center, Durham, North Carolina, USA. ⁸Department of Neurology, School of Medicine, ⁹Department of Bioengineering, and ¹⁰Department of Biomedical Sciences, College of Veterinary Medicine, University of Missouri, Columbia, Missouri, USA.

Adeno-associated virus-mediated (AAV-mediated) CRISPR editing is a revolutionary approach for treating inherited diseases. Sustained, often life-long mutation correction is required for treating these diseases. Unfortunately, this has never been demonstrated with AAV CRISPR therapy. We addressed this question in the mdx model of Duchenne muscular dystrophy (DMD). DMD is caused by dystrophin gene mutation. Dystrophin deficiency leads to ambulation loss and cardiomyopathy. We treated 6-week-old mice intravenously and evaluated disease rescue at 18 months. Surprisingly, nominal dystrophin was restored in skeletal muscle. Cardiac dystrophin was restored, but histology and hemodynamics were not improved. To determine the underlying mechanism, we evaluated components of the CRISPR-editing machinery. Intriguingly, we found disproportional guide RNA (gRNA) vector depletion. To test whether this is responsible for the poor outcome, we increased the gRNA vector dose and repeated the study. This strategy significantly increased dystrophin restoration and reduced fibrosis in all striated muscles at 18 months. Importantly, skeletal muscle function and cardiac hemodynamics were significantly enhanced. Interestingly, we did not see selective depletion of the gRNA vector after intramuscular injection. Our results suggest that gRNA vector loss is a unique barrier for systemic AAV CRISPR therapy. This can be circumvented by vector dose optimization.

Introduction

CRISPR/Cas9-mediated genome editing is an emerging and revolutionary approach to treat human diseases. In this technology, the Cas9 endonuclease is directed to the intended location by the guide RNA (gRNA) to create a double-strand break. Subsequent DNA repair results in religation of the broken ends and genomic modifications at the target site. Duchenne muscular dystrophy (DMD) is a progressive degenerative disease affecting skeletal muscle and the heart in approximately 250–300,000 boys and young men worldwide. DMD is caused by out-of-frame mutations in the dystrophin gene. Recent studies suggest that delivery of CRISPR genome-editing tools by adeno-associated virus (AAV) can reframe the mutated dystrophin gene and restore dystrophin expression in DMD patient cells in vitro and in short-term mouse studies in vivo (1–11).

DMD is a chronic disease. A desirable therapy for DMD requires persistent, ideally lifelong, dystrophin restoration in both skeletal and cardiac muscle. Despite the encouraging proof-of-principle results from short-term studies (1–3, 7, 10), it is unclear whether a one-time systemic AAV CRISPR therapy can lead to long-term dystrophin restoration and disease amelioration. To address this critical issue, we injected AAV-9 CRISPR vectors via the tail vein to 6-week-old mdx mice, a widely used DMD model, and examined dystrophin expression and disease rescue at 18 months of age. The mdx mouse carries a nonsense

mutation in exon 23 of the dystrophin gene (12). This mutation can be removed by codelivery of an AAV-Cas9 vector that expresses *Staphylococcus aureus* Cas9 and an AAV-gRNA vector that directs Cas9 to introns 22 and 23 (2). AAV-9 is a well-established AAV serotype for robust skeletal muscle and cardiac transduction in mdx mice (13, 14). We first used a method that has shown success in short-term studies (7). To our surprise, this method yielded nominal CRISPR editing in skeletal muscle, limited dystrophin restoration in the heart, and no improvement in cardiac histology and hemodynamic function. Subsequent analysis showed similar levels of Cas9 expression in the heart and skeletal muscle. Interestingly, vector genome quantification showed a disproportionate reduction of the gRNA vector by a large margin. To determine whether depletion of the gRNA vector contributed to the poor outcome, we repeated the study with an increased dose of the gRNA vector. This modification not only significantly enhanced dystrophin expression in the heart, but also resulted in body-wide dystrophin restoration in skeletal muscle. Importantly, it reduced fibrosis in the heart and skeletal muscle, significantly increased skeletal muscle force, and enhanced cardiac hemodynamic function. Additional studies suggest that vector dose alteration had minimal effect on dystrophin restoration on local AAV CRISPR therapy. These results identify gRNA vector depletion as a hurdle for systemic AAV CRISPR therapy. Increasing gRNA vector dose is necessary to achieve sustained skeletal muscle and heart rescue in DMD.

Results

Loss of the gRNA vector limited long-term systemic AAV CRISPR therapy in mdx mice. Effective DMD treatment requires persistent dystrophin restoration and functional improvements in both skeletal and cardiac muscle. To determine whether systemic AAV CRISPR therapy can lead to long-lasting protection for DMD, we coinjected the Cas9 and gRNA vectors into six 6-week-old male mdx mice at the dose of 7.2×10^{12} viral genome particles (vg)/mouse and 3.6×10^{12} vg /mouse, respectively, via the tail vein (study 1 in Supplemental Table 1; supplemental material available online with this article; <https://doi.org/10.1172/jci.insight.124297DS1>). One mouse was harvested at 8 months of age to evaluate dystrophin restoration. By immunostaining and Western blot, robust dystrophin expression was detected in the heart but not in skeletal muscle (Supplemental Figure 1). Remaining mice were harvested at 18 months of age. Likewise, dystrophin expression was barely detected in skeletal muscle (Figure 1, A–D; Supplemental Figure 1B; and Supplemental Figure 2), but widespread dystrophin expression was found throughout the entire heart cross-section by immunostaining (Figure 1A and Supplemental Figure 2A). On Western blot, cardiac dystrophin reached approximately 5% of the WT level (Figure 1, C and D; Supplemental Figure 1B; Supplemental Figure 3; and Supplemental Figure 4). Consistent with immunostaining and Western blot results, nominal levels of the edited dystrophin transcript were detected in skeletal muscle (Figure 1E and Supplemental Figure 5). No difference was noted in the total dystrophin transcript levels between treated and untreated mdx mice (Supplemental Figure 6). We next examined whether cardiac dystrophin expression improved heart structure and function (Figure 1F and Supplemental Tables 2–4). Significant improvement was detected in ECG but not in other cardiac endpoints (Figure 1F and Supplemental Tables 2–4). To determine whether long-term Cas9 expression increased off-target editing, we performed deep sequencing on both target sites and predicted off-target sites. Despite the high levels of indel formation at the target sites, we did not find significant editing in the predicted top off-target sites (Figure 1G). Histological examination of major internal organs also showed no obvious abnormality (Supplemental Figure 7).

To understand the mechanism(s) underlying poor dystrophin restoration, we quantified the levels of Cas9 expression and vector genome persistence at 18 months after treatment (Figure 1, H and I, and Supplemental Figures 8–10). Similar levels of Cas9 expression were observed at the cDNA and protein levels between the heart and skeletal muscle, although the Cas9 vector genome in the heart was significantly higher than that in skeletal muscle (Figure 1, H and I, and Supplemental Figure 8). Most of the AAV vector genome was found in the liver (Supplemental Figure 9) (13, 15–17). Interestingly, the gRNA vector genome was significantly depleted compared with that of the Cas9 vector genome in every tissue examined (Figure 1I and Supplemental Figures 9 and 10). The Cas9 vector-to-gRNA vector genome copy ratio reached approximately 12–14 in muscles and internal organs (Supplemental Figure 10). This was approximately 6- to 7-fold higher than the expectation based on the vector dose at the time of AAV injection (Supplemental Table 1).

Increasing the gRNA vector dose resulted in long-lasting dystrophin restoration and function improvement in striated muscles throughout the body. With recognition of the preferential gRNA vector depletion, we repeated the study using 1×10^{13} vg/mouse of the Cas9 vector and 3×10^{13} vg/mouse of the gRNA vector in 6-week-old

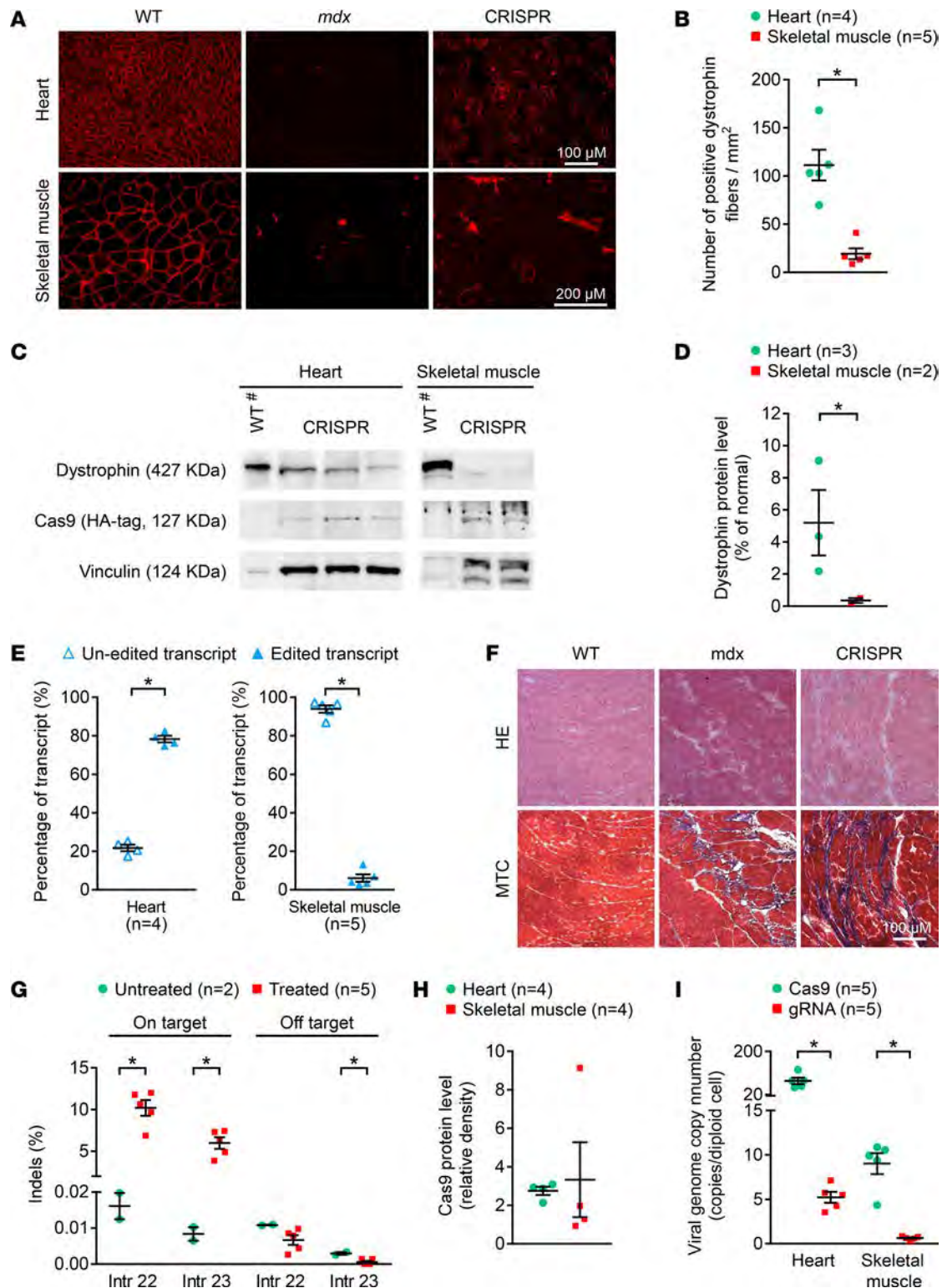


Figure 1. Long-term systemic AAV CRISPR therapy in *mdx* mice is associated with disproportional depletion of the gRNA vector genome. Systemic AAV-9 CRISPR therapy was performed in 6-week-old male *mdx* mice at the dose of 7.2×10^{12} vg/mouse and 3.6×10^{12} vg/mouse for the Cas9 and gRNA vectors, respectively. Mice were evaluated at 18 months of age. **(A)** Representative dystrophin immunostaining photomicrographs from WT, *mdx*, and CRISPR-treated *mdx* mice. Scale bar: 100 μ m (top); 200 μ m (bottom). **(B)** Quantification of dystrophin-positive myofibers. **(C)** Representative dystrophin Western blot from WT and CRISPR-treated mice. **(D)** Quantification of dystrophin Western blot. **(E)** Quantification of edited and unedited dystrophin transcripts. **(F)** Representative H&E and Masson trichrome (MTC) staining of the heart. Scale bar: 100 μ m. **(G)** Deep sequencing quantification of indels in untreated and CRISPR-treated *mdx* mice. **(H)** Quantification of Cas9 protein expression. **(I)** Quantification of the AAV genome copy number. For **B**, **E**, and **H**, statistical analyses were performed using Mann-Whitney test. For **D**, unpaired *t* test was used. For **G** and **I**, multiple *t* tests was used for statistical analysis. **P* < 0.05. #Loading in this lane was at one-sixth the volume of that in other lanes. The quadriceps or gastrocnemius muscle was used to generate the skeletal muscle data shown in the figure.

mdx mice (studies 2 and 3 in Supplemental Table 1). We first repeated the study in male mdx mice (study 2 in Supplemental Table 1). At the 18 months of age, we observed widespread dystrophin expression by immunostaining in both the hearts and skeletal muscle in treated mice (Figure 2A and Supplemental Figure 11). On Western blot, dystrophin reached approximately 20% in the heart and approximately 2% in skeletal muscle (Figure 2, C and D). Consistent with these results, we detected a significant increase of the total dystrophin transcript in the hearts of treated mice (Figure 2E). 80% of these transcripts were corrected by CRISPR editing (Supplemental Figure 12). In contrast to what we saw in study 1 (Figure 1E), we found that approximately 30% of dystrophin transcripts were edited in skeletal muscle in study 2 (Supplemental Figure 12). The patterns of the Cas9 vector genome distribution and Cas9 expression in heart and skeletal muscle were similar to what we saw in study 1 (Figure 1H and Supplemental Figures 8 and 13). There was also no detectable off-target editing at the computer-predicted off-target sites for these gRNAs (Supplemental Figure 14). Encouragingly, the Cas9 vector-to-gRNA vector genome copy number ratio was now close to 1 in every tissue we examined (Figure 2F and Supplemental Figure 10B). Next, we evaluated muscle histology and skeletal muscle strength (Figure 2, G–J; Supplemental Figure 15; and Supplemental Table 5). Fibrosis was reduced in both skeletal muscle and the heart (Figure 2, G and H, and Supplemental Figures 15 and 16). Pathologic muscle hypertrophy in mdx mice was mitigated (Supplemental Table 5). Specific twitch and tetanic forces were significantly enhanced (Figure 2I). CRISPR therapy also significantly improved resistance to eccentric contraction-induced force drop (Figure 2J).

To validate the findings in male mdx mice, we performed study 3 using female mdx mice (study 3 in Supplemental Table 1). Similar to what we saw in male mice in study 2 (Figure 2, A–D, and Supplemental Figure 11), body-wide dystrophin expression was observed in all striated muscles in CRISPR-treated female mdx mice at 18 months of age (Figure 3, A–D, and Supplemental Figure 17). Quantification of dystrophin Western blot showed approximately 9% expression in the heart and approximately 4% expression in skeletal muscle (Figure 3D and Supplemental Figure 4). These values are significantly higher than what we observed in study 1 (Figure 1D and Supplemental Figure 4). Treatment significantly increased the total dystrophin transcript level in the heart, of which approximately 80% was edited (Figure 3E). In skeletal muscle, edited transcripts accounted for half of the total dystrophin transcripts (Figure 3E). Similar patterns of Cas9 expression, Cas9 vector genome, and on/off-target editing were found in CRISPR-treated female mdx mice (Supplemental Figures 18 and 19). Consistent with the findings in male mice in study 2 (Figure 2F and Supplemental Figure 10B), the copy number of the Cas9 and gRNA vector reached similar levels in every tissue examined (Figure 3F and Supplemental Figure 10C). By histological examination, we detected a clear reduction of muscle and heart fibrosis (Figure 3, G and H, and Supplemental Figures 20 and 21). Cardiac anatomy was unremarkable, but improvement was found in ECG (Supplemental Tables 6 and 7). Failure to enhance heart pump function was a major concern of study 1 (Supplemental Table 4). We found a significant improvement of the end-systolic volume and ejection fraction in CRISPR-treated mice (Figure 3I). A trend of improvement was also observed in other hemodynamic parameters (Supplemental Table 8).

Manipulation of the vector dose did not influence dystrophin restoration in mdx muscle in local CRISPR therapy. To determine whether the vector ratio affected the outcome of intramuscular CRISPR therapy, we injected the Cas9 and gRNA vectors at different ratios into the tibialis anterior muscles of 6-week-old mdx mice (Supplemental Table 9). No appreciable dystrophin restoration was detected when only the Cas9 or gRNA vector was injected (Figure 4, A and B, and Supplemental Figure 22). Coinjection of both vectors at the dose of 1×10^{11} vg/vector resulted in approximately 20% dystrophin-positive myofibers. Interestingly, tripling the gRNA vector yielded similar levels of dystrophin restoration (Figure 4, A and B, and Supplemental Figure 22). Western blot confirmed immunostaining results (Figure 4, C and D). Vector genome quantification did not reveal preferential loss of the gRNA vector (Figure 4E).

Discussion

Targeted correction of mutated genes with AAV CRISPR is being actively pursued in preclinical studies to treat inherited diseases that affect multiple tissues/organs, such as DMD (18–20). A therapy that can result in sustained disease amelioration with a one-time administration would be highly preferred. Despite the need, long-term protection from AAV CRISPR therapy has never been demonstrated. Here, we tested whether a single intravenous AAV-9 CRISPR therapy in young mdx mice can result in long-lasting disease rescue in the commonly used mdx model. We initially hypothesized that a high amount of the Cas9 vector might result in more efficient cutting of the mutated genome and, hence, better disease rescue (7). Surprisingly, we observed

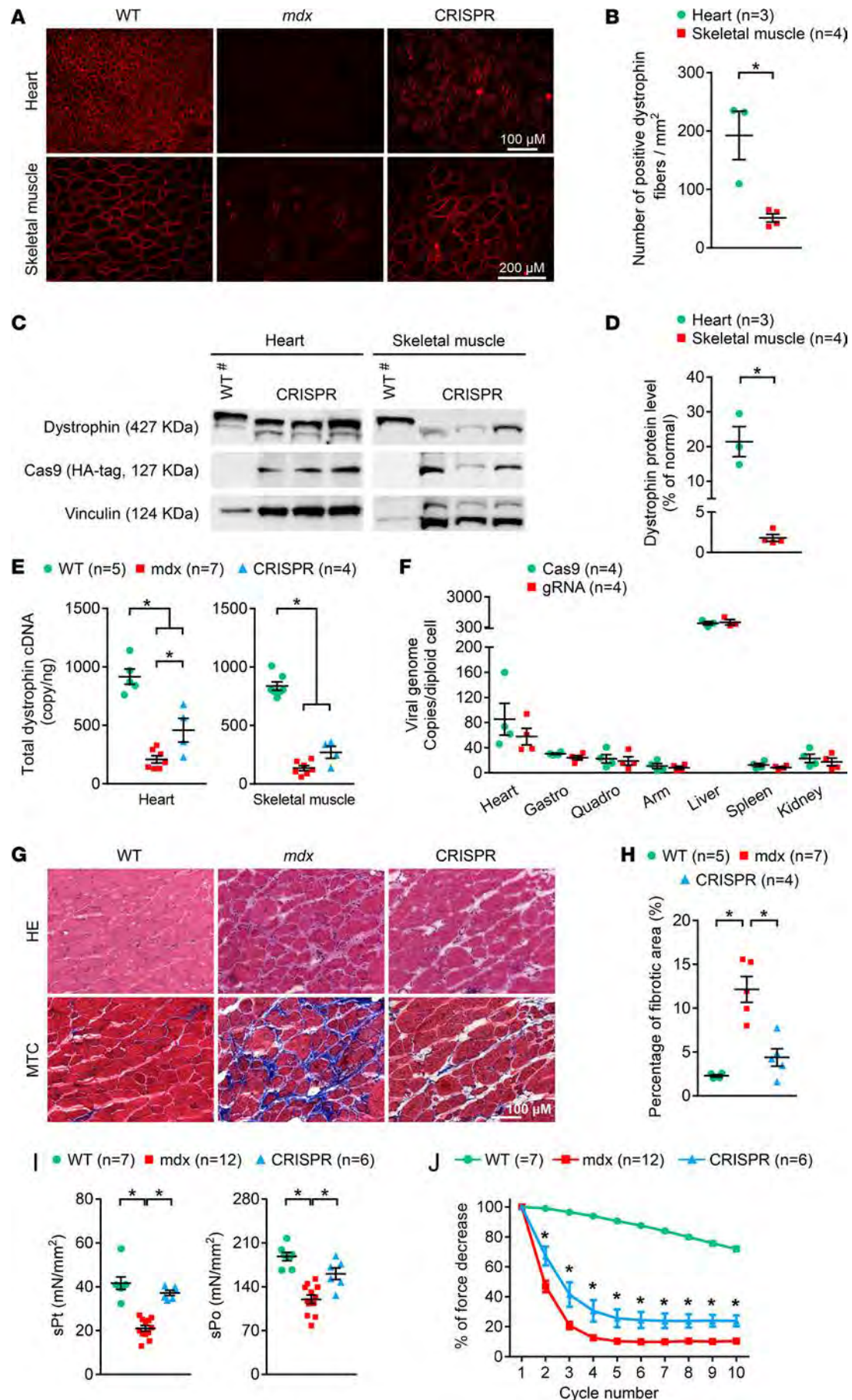


Figure 2. Systemic AAV CRISPR therapy resulted in sustained skeletal muscle function improvement in mdx mice. Systemic AAV-9 CRISPR therapy was performed in 6-week-old male mdx mice at the dose of 1×10^{13} vg/mouse and 3×10^{13} vg/mouse for the Cas9 and gRNA vectors, respectively. Mice were evaluated at 18 months of age. (A) Representative dystrophin immunostaining photomicrographs from WT, mdx and CRISPR-treated mdx mice. Scale bar: 100 μ m (top); 200 μ m (bottom). (B) Quantification of dystrophin-positive myofibers. (C) Representative dystrophin Western blot from WT and CRISPR-treated mice. (D) Quantification of dystrophin Western blot. (E) Quantification of total dystrophin transcripts. (F) Quantification of the AAV genome copy number. (G) Representative skeletal muscle H&E and Masson trichrome (MTC) staining. Scale bar: 100 μ m. (H) Quantification of fibrosis in skeletal muscle. (I) Specific twitch (sPt) and tetanic force (sPo). (J) Eccentric contraction profile. Statistical analyses were done using following tests: B and D, 2-tailed Student's *t* test; F, multiple *t* tests (statistical analysis was only performed between the Cas9 data and the gRNA data in each organs; we did not compare data from different organs); E, H, and I, 1-way ANOVA; and J, 2-way ANOVA. **P* < 0.05. #Loading in this lane was at one-fourth the volume of that in other lanes. The quadriceps or gastrocnemius muscle was used to generate the skeletal muscle data shown in the figure.

poor dystrophin restoration and minimal attenuation of dystrophic signs at 18 months of age (Figure 1, A–D; Supplemental Figures 1–4; and Supplemental Tables 2–4). Intriguingly, we also detected disproportional depletion of the gRNA vector genome (Figure 1I and Supplemental Figures 9 and 10). To determine whether the gRNA vector loss underlay the poor outcome, we repeated the study with increased amount of the gRNA vector in a larger cohort of mice. In humans, dystrophin deficiency affects both males and females (21). In mdx mice, males display more severe skeletal muscle disease, while females show more severe cardiomyopathy (22, 23). To more stringently test the treatment effect, we included males to study skeletal muscle rescue and females to study cardiac rescue in new experiments (studies 2 and 3 in Supplemental Table 1). In contrast to what we saw in our first study (study 1 in Supplemental Table 1), the revised dose regimen significantly increased dystrophin expression (Figure 2, A–D; Figure 3, A–D; and Supplemental Figures 4, 11, and 17). Importantly, improved dystrophin restoration greatly mitigated muscle fibrosis and resulted in physiologically significant enhancement in skeletal muscle and heart function (Figure 2, G–J; Figure 3, G–I; Supplemental Figures 15, 16, 20, and 21; and Supplemental Tables 5, 7, and 8).

Short-term dual AAV CRISPR therapy has been performed in a variety of animal models by many laboratories. In these studies, often equal amounts of the Cas9 and gRNA vectors are coinjected and therapeutic effect is examined a few weeks later to establish proof-of-principle. In the context of DMD, a 1:1 or 2:1 ratio of the Cas9 to gRNA vector has been shown to induce dystrophin expression in skeletal and cardiac muscle after systemic delivery in newborn and young mice (1–3, 7, 10, 11). We used a dose similar to those published before ($\geq 2 \times 10^{12}$ vg/vector/adult mouse) at a Cas9-to-gRNA vector ratio of 2:1 (Supplemental Table 1). Given the need for long-term therapy, we examined dystrophin expression at 8 and 18 months of age. Unexpectedly, we were only able to detect expression of the edited dystrophin protein in the heart at a level insufficient to reduce myocardial fibrosis or enhance blood pumping function (Figure 1; Supplemental Figures 1, 2, and 4; and Supplemental Tables 2–4). Minimal dystrophin restoration was found in skeletal muscle (Figure 1, A–D, and Supplemental Figures 1 and 2).

To better understand this observation, we quantified the dystrophin transcript, Cas9 expression, and the vector genome copy number. Similar to the protein results, edited transcripts were only found in the heart (Figure 1E). However, the total dystrophin transcript level was not increased, suggesting that the limited editing did not overcome nonsense-mediated decay in mdx mice (Supplemental Figure 6) (24). Despite the detection of significantly more Cas9 vectors in the heart than in skeletal muscle (Supplemental Figure 8), no significant difference was noticed in regards to Cas9 expression between the heart and skeletal muscle (Figure 1H and Supplemental Figure 8). This result was consistent with reports that the cytomegalovirus promoter has different activity in different cell types (25). Importantly, this result excluded the Cas9 level as the cause of poor skeletal muscle editing. Next, we quantified the amount of the AAV vector genome. The copy number of the Cas9 vector was significantly higher than that of the gRNA vector in all tissues (Figure 1I and Supplemental Figure 9). Remarkably, it greatly exceeded the 2-fold dose difference at the time of AAV injection (Supplemental Figure 10 and Supplemental Table 1). The gRNA vector genome copy number per myonucleus was 0.33 ± 0.04 in upper arm muscles, 0.63 ± 0.09 in quadriceps, and 0.77 ± 0.16 in gastrocnemius (Supplemental Figure 9). Hence, a large proportion of skeletal myonuclei had no gRNA vector. This correlated well with the editing failure seen in skeletal muscle (Figure 1E). The reason for the preferential depletion of the gRNA vector genome is unclear, but it may likely relate to the hairpin structure in this vector. The cruciform configuration is known to compromise DNA stability (26).

The discovery of the selective gRNA genome loss suggests that the gRNA vector dose may represent an important limiting factor for dual AAV CRISPR therapy. To investigate this possibility, we conducted two new studies using a higher dose of the gRNA AAV vector. Since our first study revealed an approximately 6- to 7-fold greater loss of the gRNA vector than expected, we increased the gRNA AAV dose by 6-fold

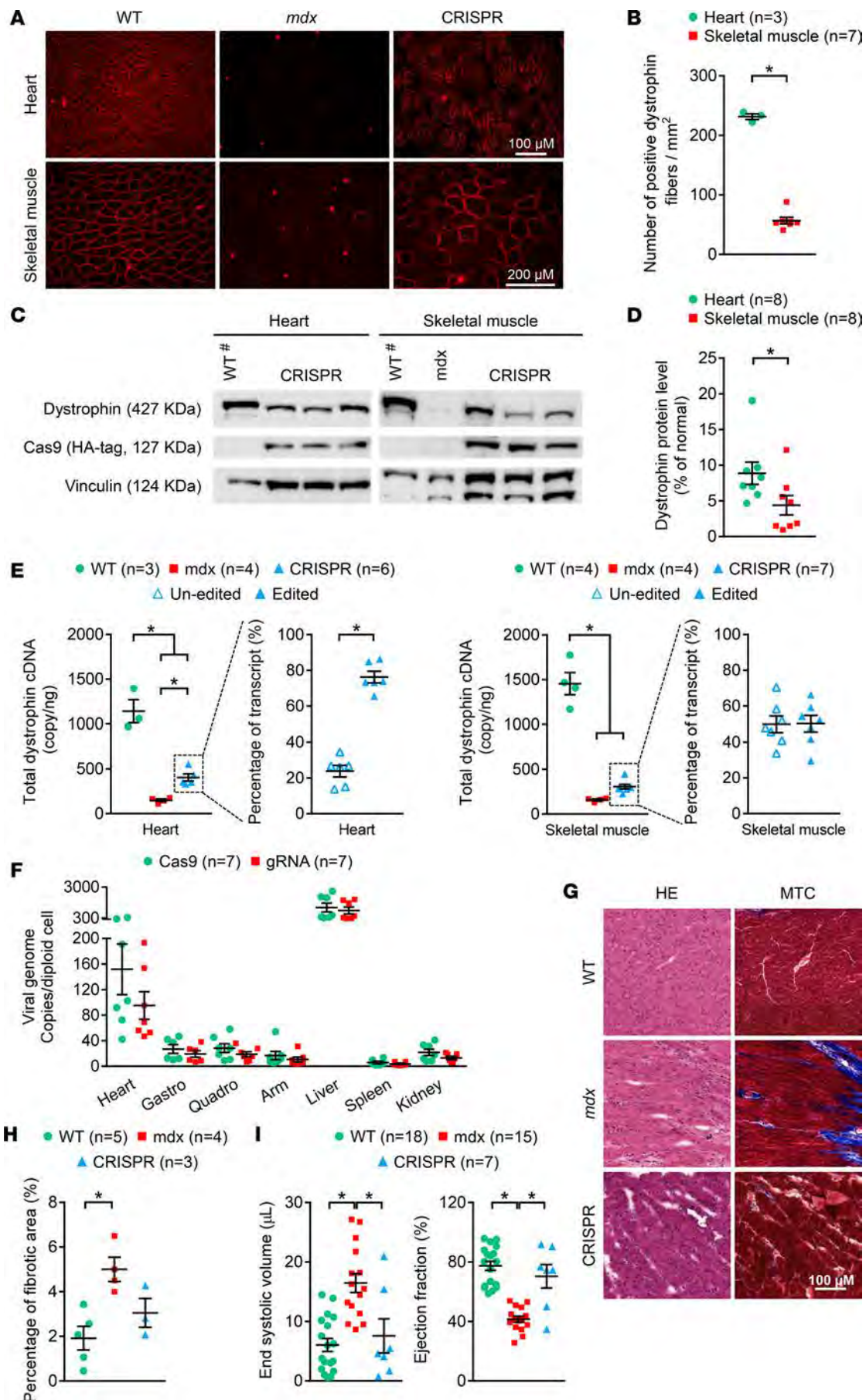


Figure 3. Systemic AAV CRISPR therapy resulted in cardiac function improvement in mdx mice for 18 months. Systemic AAV-9 CRISPR therapy was performed in 6-week-old female mdx mice at the dose of 1×10^{13} vg/mouse and 3×10^{13} vg/mouse for the Cas9 and gRNA vectors, respectively. Mice were evaluated at 18 months of age. (A) Representative dystrophin immunostaining photomicrographs from WT, mdx, and CRISPR-treated mdx mice. Scale bar: 100 μ m (top); 200 μ m (bottom). (B) Quantification of dystrophin-positive myofibers. (C) Representative dystrophin Western blot from WT and CRISPR-treated mice. (D) Quantification of dystrophin Western blot. (E) Quantification of total, edited, and unedited dystrophin transcripts. (F) Quantification of the AAV genome copy number. (G) Representative heart H&E and Masson trichrome (MTC) staining. Scale bar: 100 μ m. (H) Quantification of fibrosis in the heart. (I) Quantitative evaluation of the end-systolic volume and ejection fraction by cardiac catheterization. Statistical analyses were done using following tests: B and E (2-group analyses), Mann-Whitney test; D, 2-tailed Student's *t* test; F, multiple *t* tests (statistical analysis was only performed between the Cas9 data and the gRNA data in each organs; we did not compare data from different organs); and H and I, 1-way ANOVA. **P* < 0.05. *Loading in this lane was at one-fourth the volume of that in other lanes. The quadriceps or gastrocnemius muscle was used to generate the skeletal muscle data shown in the figure.

in new studies to reach a final Cas9-to-gRNA vector ratio of 1:3 (Supplemental Table 1). Consistent with the first study, similar levels of Cas9 protein and transcript were observed in the heart and skeletal muscle in new studies (Supplemental Figures 13 and 18). Further, the gRNA vector remained less than expected. However, the ratio of the Cas9-to-gRNA vector was now balanced in tissues harvested at 18 months of age (Figure 2F, Figure 3F, and Supplemental Figure 10). The gRNA vector genome copy number per myonucleus now reached approximately 10–30 in skeletal muscle (Figure 2F and Figure 3F).

The benefit of the new dosing regime was clearly revealed by dystrophin mRNA quantification, immunostaining, and Western blot. In the heart, the total dystrophin transcript level was significantly increased compared with that in untreated mdx mice, suggesting an effective inhibition of nonsense-mediated decay (Figure 2E and Figure 3E). Compared with that of study 1 (Figure 1, A–D, and Supplemental Figure 4), the dystrophin protein level was doubled or more than doubled in terms of the dystrophin-positive cell number in heart cross-sections and the total dystrophin level in heart lysates (Figure 2, A–D; Figure 3, A–D; and Supplemental Figure 4). In skeletal muscle, dystrophin expression increased from barely detectable in study 1 (Figure 1, A–D, and Supplemental Figures 1 and 2) to body-wide restoration (Supplemental Figure 11B and Supplemental Figure 17B), reaching approximately 2%–5% on Western blot (Figure 2, C and D, and Figure 3, C and D) in studies 2 and 3.

Fibrosis is a major pathologic presentation of dystrophic muscle. Consistent with the observed increase in dystrophin expression, we detected a clear reduction of muscle fibrosis in both skeletal and cardiac muscle in studies 2 and 3 (Figure 2, G and H; Figure 3, G and H; and Supplemental Figures 15, 16, 20, and 21). A critical goal of DMD gene therapy is to restore muscle and heart function, because only functional rescue can be directly translated to life quality improvement. To more stringently evaluate physiological changes associated with the new dose regime, we intentionally assayed skeletal muscle force in treated male mice, despite the facts that (a) male mdx mice display more severe skeletal muscle force deficits than female mdx mice (22) and (b) the dystrophin level on Western blot was relatively lower in skeletal muscle of treated male mice than that of treated female mice (Figure 2, C and D, and Figure 3, C and D). For similar reasons, we also intentionally assayed heart function in treated female mice (Figure 2, B and D, and Figure 3, B and D) (23). The extensor digitorum longus (EDL) muscle is one of most representative muscles commonly used for measuring skeletal muscle function in mdx mice (27, 28). We found that the modified AAV CRISPR therapy significantly mitigated pathological muscle hypertrophy and significantly increased specific twitch and tetanic forces (Figure 2, I and J, and Supplemental Table 5). Importantly, it significantly mitigated the rapid force drop induced by eccentric contraction, a characteristic feature of mdx mice (Figure 2J). To study heart function, we measured cardiac electrophysiology and hemodynamics by ECG and closed chest cardiac catheter assay, respectively (29). Compared with untreated mdx mice, CRISPR-treated mice showed improvement in most ECG parameters (Supplemental Table 7). On hemodynamic evaluation, a significant reduction of the end-systolic volume was detected, suggesting a prevention of pathological chamber size dilation (Figure 3I). CRISPR therapy also significantly enhanced the heart pump function, as reflected on ejection fraction measurement (Figure 3I). In addition, all other hemodynamic parameters showed a trend of improvement (Supplemental Table 8). This is in sharp contrast with what we saw in study 1, where the values of all hemodynamic parameters were essentially identical between CRISPR-treated and untreated mice (Supplemental Table 4). Collectively, by increasing the gRNA vector dose, we have achieved disease amelioration for 18 months with a single intravenous AAV CRISPR therapy.

Given the unexpected observation of gRNA vector depletion in systemically treated mice, we investigated the effect of the vector ratio on local injection. Surprisingly, increasing the Cas9-to-gRNA vector ratio from 1:1 to 1:3 did not enhance dystrophin expression (Figure 4, A–D). On vector genome quantification, we did not see preferential gRNA vector loss (Figure 4E). The Cas9-to-gRNA vector

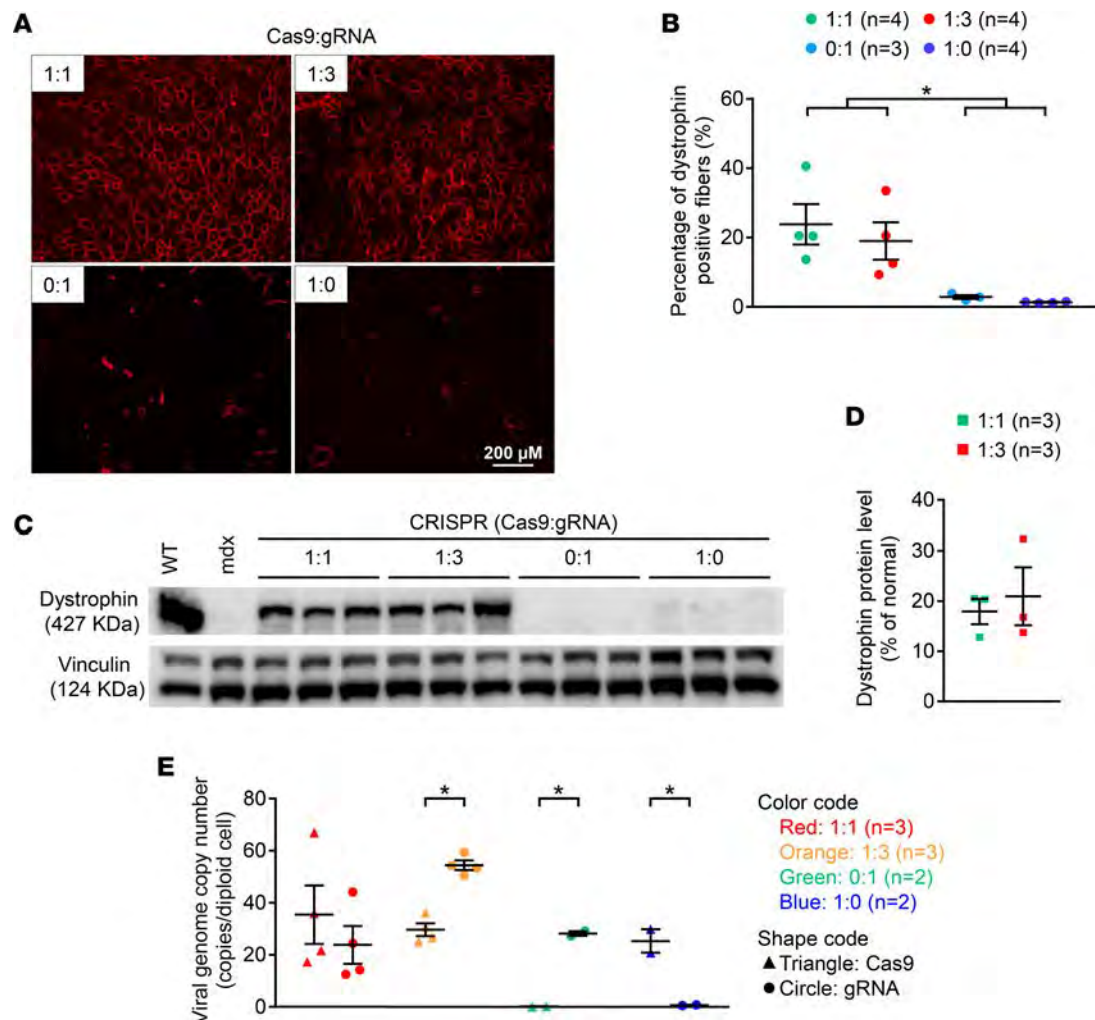


Figure 4. Increasing the gRNA AAV vector dose did not alter dystrophin expression following intramuscular AAV CRISPR therapy. The Cas9 and gRNA AAV vectors were coinjected to the tibialis anterior muscle of 6-week-old mdx mice at the Cas9 vector-to-gRNA vector ratio of 1:0 (1×10^{11} vg Cas9 only), 0:1 (1×10^{11} vg gRNA only), 1:1 (1×10^{11} vg Cas9 and 1×10^{11} vg gRNA), or 1:3 (1×10^{11} vg Cas9 and 3×10^{11} vg gRNA) and evaluated at 3 months of age. **(A)** Representative dystrophin immunostaining photomicrographs. Scale bar: 200 μ m. **(B)** Quantification of dystrophin-positive myofibers. **(C)** Representative dystrophin Western blot. **(D)** Quantification of dystrophin Western blot. **(E)** Quantification of the AAV genome copy number. Statistical analyses were done using following tests: **B**, 1-way ANOVA; **D** and **E**, Mann-Whitney test. * $P < 0.05$.

genome ratio in harvested muscle was proportional to that at injection (Figure 4E). To further validate these results, we quantified the AAV genome copy number in muscles harvested from our previously published local injection studies (2). Similarly, we did not see any evidence of selective gRNA vector degradation (Supplemental Figure 23). These results suggest that preferential gRNA vector depletion is a unique feature of systemic AAV CRISPR therapy. It should be pointed out that we observed selective depletion of the gRNA vector in long-term studies. Future short-term studies will reveal whether the loss of the gRNA vector is an acute effect following systemic delivery or a chronic process during long-term follow-up.

A frequent concern of CRISPR therapy is off-target editing. This is particularly worrisome for long-term CRISPR therapy because continuous expression of Cas9 may greatly increase the odds of cutting at unwanted sites. In all three long-term studies described in this manuscript, we did not detect significant off-target cutting at the top sites ranked by the computer algorithm (Figure 1G and Supplemental Figures 14 and 19). Our results agreed and extended findings of short-term studies (1–3, 7). Together, these data suggest that untoward indels at the locations predicted by the algorithm may not be a major risk for systemic AAV CRISPR therapy in dystrophic mice. Future studies are needed to determine whether long-term Cas9 expression can result in large deletions and complex rearrangements.

In summary, we have demonstrated for the first time to our knowledge that systemic AAV CRISPR editing can lead to long-term physiological benefits in a disease model. We also discovered a unique hurdle for long-term systemic AAV CRISPR therapy and developed a method to overcome it. Our findings open the door for using AAV CRISPR to treat any disease that requires systemic life-long mutation correction.

Methods

Study design. Our objective was to study long-term therapeutic benefits of AAV CRISPR therapy in a mouse DMD model. The sample size was determined based on previous experience. We did not use exclusion, randomization, or blinding approaches to assign animals to experimental group. Cardiac functional data from untreated normal and mdx mice also included those from our ongoing natural history study. All the data were included in the analysis. The primary endpoint was dystrophin expression. The secondary endpoints included vector genome copy number, Cas9 expression, on/off-target evaluation, and muscle and heart function. For each experiment, sample size reflected the number of independent biological replicates and is provided in related figures and tables.

Mice. Experimental mice were generated in house in a barrier facility using breeders purchased from The Jackson Laboratory. All mice were maintained in a specific pathogen-free animal care facility on a 12-hour-light (25 lux)/12-hour-dark cycle with access to food and water ad libitum.

AAV production and administration. The Cas9 and gRNA *cis*-plasmids have been published previously (2). AAV-9 vectors were generated by the transient transfection method and purified using CsCl ultracentrifugation, as we reported previously (30). Supplemental Figure 24 shows AAV titer titration results. The Cas9 and gRNA vectors were mixed thoroughly at the indicated quantity before injection. For systemic injection, AAV was delivered via the tail vein (Supplemental Table 1). For local injection, AAV was delivered directly to the tibialis anterior muscle (Supplemental Table 9).

AAV titration method. The AAV titer was determined by using SYBR green quantitative PCR. For the Cas9 AAV vector, we used a primer pair targeting bGHpA (forward, 5'-CGACTGTGCCTTCTAGTTGCC-3'; reverse, 5'-GACACCTACTCAGACAATGCGATG-3'). For the gRNA AAV vector, we used a primer pair targeting SV40pA (forward, 5'-AGCAATAGCATCACAAATTTACAA-3'; reverse, 5'-CCAGACATGATAAGATACATTGATGAGTT-3'). The following thermocycler program was used for amplification using 7900HT Fast-Real-Time PCR System (Applied Biosystems). Samples were denatured at 95°C for 2 minutes. This initial denaturation was followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. Each sample was replicated 6–8 times for quantification. Final viral titer was calculated against a 6-point plasmid standard series representing 1×10^6 vg/ μ l to 1×10^{11} vg/ μ l at $\log_{10}$ increments for each vector (Supplemental Figure 24).

Morphological studies. Tissues were harvested at the indicated time point in liquid nitrogen-cooled 2-methylbutane in the Tissue-Plus optimal cutting temperature compound (Scigen Scientific). General histology and fibrosis were examined by H&E and Masson trichrome staining, respectively. Dystrophin was detected with a rabbit polyclonal antibody against the C-terminal domain (catalog RB 9024, 1:200; Thermo Fisher Scientific). Slides were viewed using a Nikon E800 fluorescence microscope. Photomicrographs were taken with a Leica DFC7000 camera.

Western blot. Proteins from heart and skeletal muscle were extracted according to our published protocol (31). Dystrophin was detected with a rabbit polyclonal antibody against the C-terminal domain (catalog RB 9024, 1:500, Thermo Fisher Scientific). Cas9 was detected with a rat monoclonal antibody against the HA tag (catalog 1-867-423, 1:500, Roche). Vinculin was used as a loading control and was detected with a rabbit polyclonal antibody (catalog ab155120, 1:2000, Abcam). Signal was detected using Clarity Western ECL substrate (Bio-Rad) and visualized using the Li-COR Odyssey imaging system. In experiments shown in Supplemental Figure 4, we used infrared fluorescence dye-conjugated secondary antibodies (LI-COR Biotechnology). Densitometry quantification was performed using the Li-COR Image Studio version 5.0.21 software (<https://www.licor.com>). The relative intensity of the protein was normalized to the corresponding vinculin band in the same blot and further normalized to the WT control.

Vector genome copy number quantification. Genomic DNA was extracted from OCT-embedded tissues. DNA concentration was determined using the Qubit dsDNA high-sensitivity assay kit (Thermo Fisher Scientific). The vector genome copy number was quantified by TaqMan PCR using the TaqMan Universal PCR master mix (Thermo Fisher Scientific) and custom-designed primers and probes (Supplemental Table 10). The threshold cycle value of each reaction was converted to the vector genome copy number by measuring against the copy number standard curve of the known amount of the *cis*-plasmid.

Dystrophin transcript quantification. RNA was extracted from tissues preserved in RNA later (Thermo Fisher Scientific) using the RNeasy Fibrous Tissue kit (Qiagen). The cDNA was generated using the Superscript IV Kit (Thermo Fisher Scientific) and quantified using the Qubit ssDNA assay kit (Thermo Fisher Scientific). The dystrophin transcript was quantified by digital droplet PCR in the QX200 ddPCR system (Bio-Rad) using ddPCR supermix (Bio-Rad). Primers and probes for the junctions of exon 22–23, 22–24, and 24–25 are described in Supplemental Figure 5 and Supplemental Table 10. The data were reported as the transcript copy per ng of cDNA used in the reaction.

Analysis of on- and off-target gene editing. On- and off-target cutting was analyzed using DNA extracted from flash frozen livers according to our previously published protocol (2). Primers used for deep sequencing are listed in Supplemental Table 11. Analysis was performed with CRISPResso using a 5-bp window from the expected DSB (32).

Skeletal muscle function assay. EDL muscle function was evaluated ex vivo using our published protocols (27, 28). Muscle force was evaluated with a 305B dual-mode servomotor transducer using the Dynamic Muscle Control software (Aurora Scientific). Data were analyzed using the Dynamic Muscle Analysis (DMA) software (Aurora Scientific). The specific muscle force was calculated by dividing the absolute muscle force with the muscle cross-sectional area (CSA). Muscle CSA was calculated according to the following equation: $CSA = (\text{muscle mass})/(\text{muscle density} \times \text{length ratio} \times \text{optimal muscle length})$. Muscle density is 1.06 g/cm^3 (33). The length ratio is 0.44 for the EDL muscle (34, 35).

ECG and left ventricular hemodynamic assay. A 12-lead ECG assay was performed using a system from AD Instruments as previously published (36, 37). The ECG parameters were analyzed using ECG analysis module in Lab Chart software. The Q wave amplitude was determined using the lead I tracing. Other parameters were analyzed using the lead II tracing. The QTc interval was determined by correcting the QT interval with the heart rate as described by Mitchell et al. (38). The cardiomyopathy index was calculated by dividing the QT interval by the PQ segment (39). Left ventricular hemodynamics was evaluated with a closed chest approach using the Millar ultraminiature pressure–volume catheter (29, 37). Data were analyzed with the PVAN software (Millar Instruments). Detailed protocols are available at the Parent Project Muscular Dystrophy standard operating protocol web site (<https://www.parentprojectmd.org/research/for-researchers-industry/resources/standard-operating-procedures-for-duchenne-animal-models/>) (40).

Statistics. Data are presented as mean $\pm$ SEM. The sample size mentioned refers to the number of animals. For biochemical and molecular assays, 2–3 measurements were conducted in each assay for each animal. The average from these measurements was reported as the data for each animal. One-way ANOVA or two-way ANOVA with Tukey's multiple comparison was performed for statistical analysis for more than 2 group comparisons. Unpaired *t* test (2 tailed) or Mann-Whitney test was used for 2 group comparisons. The statistical method used for each analysis is indicated in the figure legends. All statistical analyses were performed using GraphPad PRISM software version 7.0. $P < 0.05$ was considered statistically significant.

Study approval. All animal experiments were approved by the University of Missouri animal care and use committee.

Author contributions

CHH, NBW, NNY, SC, CAG, and DD designed study. CHH, NBW, CEN, LPW, YY, JAL, TBL, AD, KZ, GJJ, MEN, XP, and KK performed research. CHH, NBW, LPW, and SC contributed new reagents or analytic tools. CHH, NBW, CEN, LPW, NNY, SC, CAG, and DD analyzed data. CHH, NBW, and DD wrote the paper. CHH, NBW, CEN, LPW, NNY, SC, CAG, and DD edited paper. All authors approved submission.

Acknowledgments

We thank Jim Wilson for providing the AAV-9 packaging plasmid. We thank Alex Hinken, Matt Burke, Taylor Klimt, Samantha Metzger, Courtney Harris, Mathew Pulcine, Cobi Bissell, Benjamin Schneider, Thomas Cassimatis, and Sean Duan for technical assistance. We thank the Bond Life Sciences Center at the University of Missouri for the use of the ddPCR machine. This work was supported by grants from the NIH (AR-69085 to CAG and DD; GM-063732 and GM-117059 to SC), the Intramural Research Program of the NIH National Center for Advancing Translational Sciences (to NNY and CHH), the Department of Defense (MD15-1-0469 to CAG; MD150133 to DD and CAG), Hope for Javier (to DD), the Jackson Freil DMD Research Fund (to DD), the Muscular Dystrophy Association (MDA277360 to CAG), and the Duke

Coulter Translational Partnership (to CAG). CEN was supported by a Hartwell Foundation Postdoctoral Fellowship. MEN is a predoctoral fellow supported by Hope for Javier.

Address correspondence to: Dongsheng Duan, Department of Molecular Microbiology and Immunology, The University of Missouri School of Medicine, One Hospital Drive M609, MSB, Columbia, Missouri 65212, USA. Phone: 573.884.9584; Email: duand@missouri.edu.

1. Long C, et al. Postnatal genome editing partially restores dystrophin expression in a mouse model of muscular dystrophy. *Science*. 2016;351(6271):400–403.
2. Nelson CE, et al. In vivo genome editing improves muscle function in a mouse model of Duchenne muscular dystrophy. *Science*. 2016;351(6271):403–407.
3. Tabebordbar M, et al. In vivo gene editing in dystrophic mouse muscle and muscle stem cells. *Science*. 2016;351(6271):407–411.
4. Ousterout DG, Kabadi AM, Thakore PI, Majoros WH, Reddy TE, Gersbach CA. Multiplex CRISPR/Cas9-based genome editing for correction of dystrophin mutations that cause Duchenne muscular dystrophy. *Nat Commun*. 2015;6:6244.
5. Wojtal D, et al. Spell Checking Nature: Versatility of CRISPR/Cas9 for developing treatments for inherited disorders. *Am J Hum Genet*. 2016;98(1):90–101.
6. Xu L, et al. CRISPR-mediated genome editing restores dystrophin expression and function in mdx mice. *Mol Ther*. 2016;24(3):564–569.
7. Bengtsson NE, et al. Muscle-specific CRISPR/Cas9 dystrophin gene editing ameliorates pathophysiology in a mouse model for Duchenne muscular dystrophy. *Nat Commun*. 2017;8:14454.
8. Young CS, et al. A single CRISPR-Cas9 deletion strategy that targets the majority of DMD patients restores dystrophin function in hiPSC-derived muscle cells. *Cell Stem Cell*. 2016;18(4):533–540.
9. Zhang Y, et al. CRISPR-Cpf1 correction of muscular dystrophy mutations in human cardiomyocytes and mice. *Sci Adv*. 2017;3(4):e1602814.
10. El Refaey M, et al. In vivo genome editing restores dystrophin expression and cardiac function in dystrophic mice. *Circ Res*. 2017;121(8):923–929.
11. Amosii L, et al. Single-cut genome editing restores dystrophin expression in a new mouse model of muscular dystrophy. *Sci Transl Med*. 2017;9(418).
12. Sicinski P, Geng Y, Ryder-Cook AS, Barnard EA, Darlison MG, Barnard PJ. The molecular basis of muscular dystrophy in the mdx mouse: a point mutation. *Science*. 1989;244(4912):1578–1580.
13. Hakim CH, et al. A five-repeat micro-dystrophin gene ameliorated dystrophic phenotype in the severe DBA/2J-mdx model of Duchenne muscular dystrophy. *Mol Ther Methods Clin Dev*. 2017;6:216–230.
14. Duan D. Systemic delivery of adeno-associated viral vectors. *Curr Opin Virol*. 2016;21:16–25.
15. Inagaki K, et al. Robust systemic transduction with AAV9 vectors in mice: efficient global cardiac gene transfer superior to that of AAV8. *Mol Ther*. 2006;14(1):45–53.
16. Zincarelli C, Soltys S, Rengo G, Rabinowitz JE. Analysis of AAV serotypes 1–9 mediated gene expression and tropism in mice after systemic injection. *Mol Ther*. 2008;16(6):1073–1080.
17. Ghosh A, Yue Y, Shin JH, Duan D. Systemic Trans-splicing adeno-associated viral delivery efficiently transduces the heart of adult mdx mouse, a model for Duchenne muscular dystrophy. *Hum Gene Ther*. 2009;20(11):1319–1328.
18. Cox DB, Platt RJ, Zhang F. Therapeutic genome editing: prospects and challenges. *Nat Med*. 2015;21(2):121–131.
19. Zhang Y, Long C, Bassel-Duby R, Olson EN. Myoediting: Toward prevention of muscular dystrophy by therapeutic genome editing. *Physiol Rev*. 2018;98(3):1205–1240.
20. Nelson CE, Robinson-Hamm JN, Gersbach CA. Genome engineering: a new approach to gene therapy for neuromuscular disorders. *Nat Rev Neurol*. 2017;13(11):647–661.
21. Ishizaki M, Kobayashi M, Adachi K, Matsumura T, Kimura E. Female dystrophinopathy: Review of current literature. *Neuromuscul Disord*. 2018;28(7):572–581.
22. Hakim CH, Duan D. Gender differences in contractile and passive properties of mdx extensor digitorum longus muscle. *Muscle Nerve*. 2012;45(2):250–256.
23. Bostick B, Yue Y, Duan D. Gender influences cardiac function in the mdx model of Duchenne cardiomyopathy. *Muscle Nerve*. 2010;42(4):600–603.
24. Kerr TP, Sewry CA, Robb SA, Roberts RG. Long mutant dystrophins and variable phenotypes: evasion of nonsense-mediated decay? *Hum Genet*. 2001;109(4):402–407.
25. Qin JY, et al. Systematic comparison of constitutive promoters and the doxycycline-inducible promoter. *PLoS One*. 2010;5(5):e10611.
26. Varani G. Exceptionally stable nucleic acid hairpins. *Annu Rev Biophys Biomol Struct*. 1995;24:379–404.
27. Hakim CH, Li D, Duan D. Monitoring murine skeletal muscle function for muscle gene therapy. *Methods Mol Biol*. 2011;709:75–89.
28. Hakim CH, Wasala NB, Duan D. Evaluation of muscle function of the extensor digitorum longus muscle ex vivo and tibialis anterior muscle in situ in mice. *J Vis Exp*. 2013;(72):e50183.
29. Bostick B, Yue Y, Duan D. Phenotyping cardiac gene therapy in mice. *Methods Mol Biol*. 2011;709:91–104.
30. Shin JH, Yue Y, Duan D. Recombinant adeno-associated viral vector production and purification. *Methods Mol Biol*. 2012;798:267–284.
31. Wasala NB, Shin JH, Lai Y, Yue Y, Montanaro F, Duan D. Cardiac-specific expression of Δ H2-R15 mini-dystrophin normalized all electrocardiogram abnormalities and the end-diastolic volume in a 23-month-old mouse model of Duchenne dilated cardiomyopathy. *Hum Gene Ther*. 2018;29(7):737–748.
32. Pinello L, et al. Analyzing CRISPR genome-editing experiments with CRISPResso. *Nat Biotechnol*. 2016;34(7):695–697.

33. Mendez J, Keys A. Density and composition of mammalian muscle. *Metabolism*. 1960;9(2):184–188.
34. Burkholder TJ, Fingado B, Baron S, Lieber RL. Relationship between muscle fiber types and sizes and muscle architectural properties in the mouse hindlimb. *J Morphol*. 1994;221(2):177–190.
35. Brooks SV, Faulkner JA. Contractile properties of skeletal muscles from young, adult and aged mice. *J Physiol (Lond)*. 1988;404:71–82.
36. Bostick B, Shin JH, Yue Y, Duan D. AAV-microdystrophin therapy improves cardiac performance in aged female mdx mice. *Mol Ther*. 2011;19(10):1826–1832.
37. Wasala NB, Bostick B, Yue Y, Duan D. Exclusive skeletal muscle correction does not modulate dystrophic heart disease in the aged mdx model of Duchenne cardiomyopathy. *Hum Mol Genet*. 2013;22(13):2634–2641.
38. Mitchell GF, Jeron A, Koren G. Measurement of heart rate and Q-T interval in the conscious mouse. *Am J Physiol*. 1998;274(3 Pt 2):H747–H751.
39. Nigro G, Comi LI, Politano L. Electrocardiographic evaluation of the P-type stage of dystrophic cardiomyopathy. *Cardiology*. 1984;3:45–58.
40. Duan D, et al. Standard operating procedures (SOPs) for evaluating the heart in preclinical studies of Duchenne muscular dystrophy. *J Cardiovasc Transl Res*. 2016;9(1):85–86.

Questions Answered and Unanswered by the First CRISPR Editing Study in a Canine Model of Duchenne Muscular Dystrophy

Nalinda B. Wasala,¹ Chady H. Hakim,^{1,2} Shi-Jie Chen,^{3,4}
N. Nora Yang,^{2,*} and Dongsheng Duan^{1,5,6,7,*}

Departments of ¹Molecular Microbiology and Immunology and ⁵Neurology, School of Medicine; Departments of ³Physics, ⁴Biochemistry, and ⁶Bioengineering; and ⁷Department of Biomedical Sciences, College of Veterinary Medicine, The University of Missouri, Columbia; and ²National Center for Advancing Translational Sciences, National Institutes of Health, Rockville, Maryland.

Clustered regularly interspaced short palindromic repeats (CRISPR) editing is being considered as a potential gene repair therapy to treat Duchenne muscular dystrophy, a dystrophin-deficient lethal muscle disease affecting all muscles in the body. A recent preliminary study from the Olson laboratory (Amoasii *et al.* Science 2018;362:89–91) showed robust dystrophin restoration in a canine Duchenne muscular dystrophy model following intramuscular or intravenous delivery of the CRISPR editing machinery by adeno-associated virus serotype 9. Despite the limitation of the small sample size, short study duration, and the lack of muscle function data, the Olson lab findings have provided important proof of principle for scaling up CRISPR therapy from rodents to large mammals. Future large-scale, long-term, and comprehensive studies are warranted to establish the safety and efficacy of CRISPR editing therapy in large mammals.

Keywords: CRISPR, DMD, dog, AAV, dystrophin, editing

CLUSTERED REGULARLY INTERSPACED short palindromic repeats (CRISPR) is a novel gene editing technology that became widely used about 5 years ago.^{1–3} Despite its recent debut, it is rapidly advancing from a research tool to a potential therapeutic modality. CRISPR technology is relatively easy to understand and administer. A small sequence-specific guide RNA (gRNA) is used to direct the CRISPR-associated (Cas) nuclease to an intended genomic location for site-specific modification. For therapeutic application, a vector, such as adeno-associated virus (AAV), is used to deliver the gRNA and Cas nuclease to the diseased cells. The disease-causing mutation can be either corrected or removed by CRISPR/Cas-mediated editing *in vivo*, holding the promise of providing one-time curative therapeutic outcomes for patients. Though highly promising, significant challenges remain in applying CRISPR technology in

human gene therapy. Creative strategies are needed to deliver the CRISPR/Cas system to the target cells efficiently and selectively, to achieve a sufficient level of editing for disease amelioration, to avoid and/or attenuate immune responses induced by the bacterial Cas protein and/or delivery vectors, and to prevent and/or minimize untoward off-target genomic alterations.⁴

Duchenne muscular dystrophy (DMD) is a relatively common X-linked inherited muscle disease caused by reading frame-aborting mutations in the *DMD* gene. The *DMD* gene is one of the largest genes in the body, and it encodes the 427 kD dystrophin protein. Dystrophin is a sub-sarcolemmal cytoskeletal protein, and it protects muscle from contraction-induced injuries. Muscle is essential for body movement, posture maintenance, respiration, and blood pumping. The absence of dystrophin leads to muscle degeneration, necrosis, inflammation, and fibrosis. As a consequence, DMD patients

*Correspondence: Dr. Dongsheng Duan, Department of Molecular Microbiology and Immunology, The University of Missouri School of Medicine, One Hospital Dr. M610G, MSB, Columbia, MO 65212. E-mail: duand@missouri.edu; or Dr. N. Nora Yang, National Center for Advancing Translational Sciences, National Institutes of Health, Rockville, MD 20850. E-mail: nora.yang@nih.gov

Table 1. CRISPR editing of DMD gene mutations from 2014 to 2018

Publication				Experiment details							In vivo editing outcome				
Year	Author	Ref. no.	Editing enzyme	Approach	Repair mechanism	Model	Age at injection	Study duration	Vector	Route	Dystrophin level (staining)	Dystrophin level (Western)	Histology	Muscle function	Heart function
In vitro editing	2015	Li <i>et al.</i>	12	SpHcCas9	1 cut with 1 gRNA	HDR and NHEJ	Patient iPSCs		Plasmid						
	2015	Ousterout <i>et al.</i>	11	SpCas9 and hSpCas9	1 cut with 1 gRNA and 2 cuts with 2 gRNAs	NHEJ	Patient myoblasts		Plasmid						
	2016	Young <i>et al.</i>	13	SpCas9	2 cuts with 2 gRNAs	NHEJ	Patient iPSCs		Plasmid						
	2016	Iyombe-Engembe <i>et al.</i>	19	SaCas9	2 cuts with 2 gRNAs	NHEJ	Patient myoblasts		Plasmid						
	2016	Wojtal <i>et al.</i>	18	SpCas9	1 cut with 1 gRNA	NHEJ	Patient myoblasts		Lentivirus						
	2016	Maggio <i>et al.</i>	17	SpCas9	2 cuts with 2 gRNAs	NHEJ	Patient myoblasts		Adenovirus						
	2017	Kyrchenko <i>et al.</i>	15	SpCas9	2 cuts with 2 gRNAs	NHEJ	Patient iPSCs		Plasmid						
	2017	Lattanzi <i>et al.</i>	16	SpCas9	2 cuts with 2 gRNAs	NHEJ	Patient myoblasts		Lentivirus						
	2017	Zhang <i>et al.</i>	14	LbCpf1	1 cut with 1 gRNA	HDR and NHEJ	Patient iPSCs		Plasmid						
	2018	Long <i>et al.</i>	21	SpCas9	1 cut with 1 gRNA	NHEJ	Patient iPSCs and EHM		Plasmid						
	2018	Duchêne <i>et al.</i>	20	SaCas9	2 cuts with 2 gRNAs	NHEJ	Patient myoblasts		Lentivirus						
Embryo editing	2014	Long <i>et al.</i>	10	SpCas9	1 cut with 1 gRNA	HDR and NHEJ	Mdx embryo		mRNA						
	2017	Zhang <i>et al.</i>	14	LbCpf1	1 cut with 1 gRNA	HDR and NHEJ	Mdx embryo		mRNA						

(continued)

Table 1. (Continued)

Publication			Experiment details							In vivo editing outcome						
Year	Author	Ref. no.	Editing enzyme	Approach	Repair mechanism	Model	Age at injection	Study duration	Vector	Route	Dystrophin level (staining)	Dystrophin level (Western)	Histology	Muscle function	Heart function	
In vivo editing in mice	2015	Xu <i>et al.</i>	25	SpCas9	2 cuts with 2 gRNAs	NHEJ	Mdx	P1–3 and 2 months old	3 weeks	Adenovirus	i.m.	ND	~50%	ND	ND	ND
	2016	Long <i>et al.</i>	23	SpCas9	2 cuts with 2 gRNAs	NHEJ	Mdx	P1–18	3–12 weeks	AAV	i.m., i.p., r.o.	~20–70%	ND	Improved	Grip force increased	ND
	2016	Nelson <i>et al.</i>	22	SaCas9	2 cuts with 2 gRNAs	NHEJ	Mdx	P2 and 1.5–2 months old	7 weeks to 6 months	AAV	i.m., i.p., i.v.	~67%	~8%	Improved	TA force increased	ND
	2016	Tabebordbar <i>et al.</i>	24	SaCas9	2 cuts with 2 gRNAs	NHEJ	Mdx	P3 and 1.5 months old	3–14 weeks	AAV	i.m., i.p., i.v.	ND	~0.03–12%	Improved	TA force increased	ND
	2017	Amasii <i>et al.</i>	28	SpCas9	1 cut with 1 gRNA	NHEJ	ΔEx50 mice	P4–12	3–8 weeks	AAV	i.m., i.p.	≤90%	~85%	Improved	Grip force increased	ND
In vivo editing in dogs	2017	Bengtsson <i>et al.</i>	27	SaCas9 and SpCas9	2 cuts with 2 gRNAs	HDR and NHEJ	Mdx4cv	2–12 weeks old	4–18 weeks	AAV	i.m., r.o.	41–45%	~0.8–23%	Improved	TA force increased	ND
	2017	El Rafeay <i>et al.</i>	26	SaCas9 and SpCas9	2 cuts with 2 gRNAs	NHEJ	Mdx and Mdx/Utr ^{+/−}	P1–3	4–10 weeks	AAV and adenovirus	i.p., i.v., r.o.	40%	~23%	Improved	ND	Muscle strip force increased
	2017	Young <i>et al.</i>	30	SpCas9	2 cuts with 2 gRNAs	NHEJ	ΔEx45 mice	12–18 weeks old	3–5 weeks	Plasmid	i.m.e.	ND	ND	ND	ND	ND
	2018	Ryu <i>et al.</i>	32	ABE	1 cut with 1 gRNA	NHEJ	Exon20 disrupted	7 weeks old	8 weeks	AAV	i.m.	17%	ND	ND	ND	ND
	2018	Duchêne <i>et al.</i>	20	SaCas9	2 cuts with 2 gRNAs	NHEJ	Δ52hDMD/mdx	24–5 weeks old	6 weeks	AAV	i.v.	ND	ND	ND	ND	ND
	2018	Hakim and Wasala <i>et al.</i>	29	SaCas9	2 cuts with 2 gRNAs	NHEJ	Mdx	6 weeks old	6 weeks to 17 months	AAV	i.m., i.v.	ND	2–20%	Improved	EDL force increased	ECG improved, hemodynamics enhanced
	2018	Koo <i>et al.</i>	31	CjCas9	1 cut with 1 gRNA	NHEJ	Exon23 disrupted	8 weeks old	7 weeks	AAV	i.m.	~26%	ND	ND	TA force increased	ND
	2018	Amasii <i>et al.</i>	34	SpCas9	1 cut with 1 gRNA	NHEJ	ΔEx50 dogs	1 month old	6–8 weeks	AAV	i.m., i.v.	ND	5–92%	Improved	ND	ND

AAV, adeno-associated virus; ABE, adenine base editors (engineered adenine deaminase and the Streptococcus pyogenes Cas9); Cas9, CRISPR associated protein 9; Cpfl1, CRISPR from *Prevotella* and *Francisella* 1; CjCas9, *Campylobacter jejuni* Cas9; DMD, Duchenne muscular dystrophy; EDL, extensor digitorum longus; EHM, engineered heart muscle; gRNA, guide RNA; HDR, homologous recombination; i.m., intramuscular injection; i.m.e., intramuscular electroporation; iPSCs, induced pluripotent stem cells; i.p., intraperitoneal injection; i.v., intravenous injection; LbCpf1, *Lachnospiraceae bacterium* Cpf1; ND, not done; NHEJ, non-homologous end joining; P1–3, postnatal days 1–3; P1–18, postnatal days 1–18; P2, postnatal day 2; P3, postnatal day 3; P4–12, postnatal days 4–12; r.o., retro-orbital injection; SaCas9, *Streptococcus aureus* Cas9; SpCas9, *Streptococcus pyogenes* Cas9; SpHcCas9/hSpCas9, human codon-optimized SpCas9; TA, tibialis anterior.

become wheelchair-bound in their early teens and die prematurely in their 20s or 30s from respiratory and/or heart failure. The human body has >600 muscles. These muscles consist of ~45% body mass and are distributed throughout the body. Given the large size of the gene, the presence of muscle throughout the body, and the highly inflamed nature of the dystrophic muscle, DMD is considered one of the most challenging monogenic diseases amenable to gene therapy. Over the last 40 years, numerous viral and non-viral vector-delivered gene therapy strategies have been tested in mouse models of DMD.^{5–7} Many of these strategies have resulted in remarkable levels of dystrophin restoration and function improvement in mouse muscles. Yet, none of these “mouse therapies” has directly led to equivalent results in human patients. Preclinical study in the symptomatic canine model may help bridge the gap between mice and humans.^{8,9}

CRISPR technology was first introduced for editing *DMD* gene mutations in 2014 to correct the germline DNA in *mdx* mice, a mouse DMD model (Table 1).¹⁰ Subsequently, a series of studies showed restoration of dystrophin expression in patient cells *in vitro* by co-transfection of the gRNA and *Streptococcus pyogenes* Cas9 (SpCas9) or *Lachnospiraceae* Cpf1 (a homolog of Cas9; Table 1).^{11–21} *In vivo* editing in postnatal animals was first achieved in 2016 in *mdx* mice by co-delivering the gRNA and *Staphylococcus aureus* Cas9 (SaCas9) or SpCas9 using adenovirus or AAV (Table 1).^{22–25} These studies revealed a moderate level of DNA editing ($\leq 10\%$). However, robust dystrophin expression and muscle function improvement were observed in newborn and young adult *mdx* mice following local or systemic AAV injection. These findings were subsequently validated by other laboratories in different mouse models using SaCas9, SpCas9, *Campylobacter jejuni* Cas9, and adenine-based editors (Table 1).^{20,26–32} The next logical step was to see if this success can be reproduced in the canine model.³³

This milestone has now been accomplished according to a recent study by Amoasii *et al.* (Fig. 1).³⁴ The authors packaged the gRNA and *SpCas9* in two AAV serotype 9 (AAV9) vectors and tested them in the Δ Ex50 canine DMD model (Fig. 1). In this model, a point mutation at the beginning of intron 50 of the *DMD* gene results in exon 50 skipping in the RNA transcript, reading-frame shift, and loss of dystrophin expression.³⁵ Dystrophin expression can be recovered by reframing or removing exon 51. In the Cas9 vector, the authors used the muscle specific CKe promoter. In the gRNA vector, three copies of the same gRNA were ex-

pressed using three different pol III promoters. This gRNA targets the *SpCas9* to the splicing acceptor site of exon 51. This genomic modification results in the skipping of exon 51 or reframing of the disrupted reading frame. The authors co-injected these two vectors into 1-month-old Δ Ex50 puppies either intramuscularly in the absence of immune suppression ($n=2$; 1.2×10^{13} vg/vector into the cranial tibialis muscle) or intravenously under immune suppression ($n=2$; 2×10^{13} vg/kg/vector in one puppy and 1×10^{14} vg/kg/vector in another puppy; Fig. 1).³⁴ The outcome of CRISPR editing was studied 6–8 weeks after injection. Specifically, the authors examined (1) gene editing on genomic DNA and cDNA by tracking indels by decomposition analysis and deep sequencing of the polymerase chain reaction product amplified from the target site; (2) dystrophin expression by immunostaining and Western blot; (3) dystrophin-associated glycoprotein complex restoration by β -dystroglycan immunostaining; (4) disease amelioration by qualitative hematoxylin and eosin (H&E) staining of muscle sections, developmental myosin heavy chain (a regeneration marker) expression, and serum creatine kinase levels; (5) safety by hematology, blood biochemistry, target site indel formation, and Cas9 expression in the testes, and deep sequencing of the predicted top off-target sites in muscle; (6) immune response by CD4+ and CD8+ T-cell immunostaining in the locally injected muscle.

DNA-level editing was similar to that seen in mouse studies (1–14%).^{22–24} Immunostaining revealed widespread dystrophin expression. Western blots showed dystrophin restoration to 3–92% of wild-type levels. Remarkably robust dystrophin expression was detected in the heart and diaphragm (~90% and 50% of normal, respectively, on Western blot) in the puppy that was systemically injected at the dose of 1×10^{14} vg/kg/vector. In the puppy that received the lower dose (2×10^{13} vg/kg/vector), dystrophin expression was consistently lower and more sparse. The authors also found some evidence suggesting a reduction of muscle damage in treated puppies. Importantly, in this short-term study, the authors did not detect T-cell response and germ cell editing. No safety concern was raised either.

The Amoasii *et al.* study is significant to the development of therapeutic CRISPR editing for DMD. First, this study demonstrated feasibility of systemic CRISPR editing in a diseased large mammal. Second, the level of dystrophin restoration, especially systemic delivery at the dose of 1×10^{14} vg/kg/vector, has reached the range considered to benefit patients according to mouse

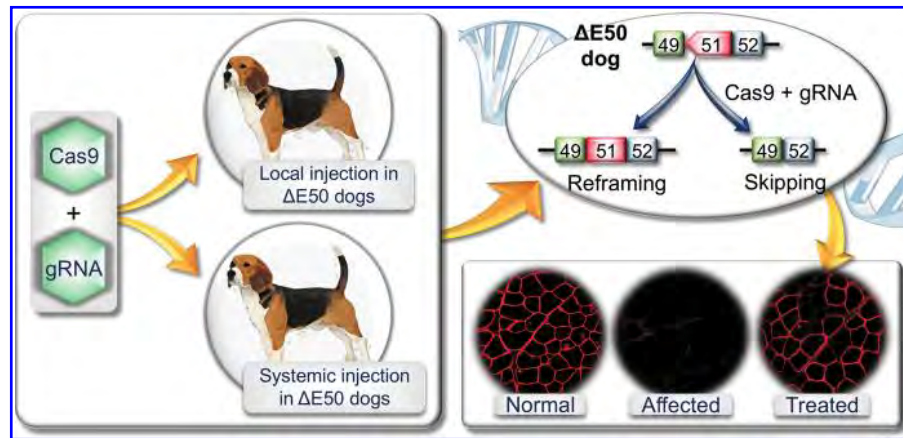


Figure 1. Schematic illustration of clustered regularly interspaced short palindromic repeats (CRISPR) editing in the $\Delta E50$ canine Duchenne muscular dystrophy model. An adeno-associated virus (AAV) CRISPR-associated protein 9 vector and an AAV guide RNA vector were co-delivered to $\Delta E50$ dogs by intramuscular or intravenous injection. In $\Delta E50$ dogs, exon 50 is skipped in the RNA transcript by a naturally existing point mutation. This leads to frame-shift and dystrophin deficiency. CRISPR therapy restores dystrophin expression by reframing or skipping exon 51. Please note: immunostaining images are for illustration purposes. They are not from the Amoasii *et al.* publication.

studies and clinical case reports.³⁶ This dose is well tolerated in newborns with spinal muscular atrophy type 1 and is in line with the dose used in ongoing systemic AAV micro-dystrophin trials in DMD patients.^{37,38} Third, the strategy described by the authors may be directly applicable to ~13% of DMD patients.³⁹

As a proof of principle, the findings reported by Amoasii *et al.* are encouraging. However, the implication of the study is considerably limited due to the sample size, age of injection, duration of the treatment, and methods used to characterize the treatment outcome. A sample size of two for local injection and one for each dose of systemic injection is too small for statistical analysis. Additional studies in more dogs are needed in order to validate the findings.

In the Amoasii *et al.* study, the affected dogs were treated at 1 month of age. This is analogous to ~1.5 years in humans. DMD patients are usually diagnosed between 3 and 5 years of age. Hence, the strategy described by Amoasii *et al.* might be suitable for treating neonatal patients identified through newborn screening. However, to meet the need of existing patients, the protocol has to be optimized in older affected dogs.

DMD is a chronic disease. DMD treatment requires continuous dystrophin expression.⁴⁰ It was recently shown that persistent (up to 18 months) dystrophin restoration and muscle and cardiac function improvement can be achieved in young adult *mdx* mice from a single systemic AAV CRISPR injection (Table 1).²⁹ Amoasii *et al.* terminated the study before treated puppies were weaned (2.5–3

months of age). At this age, affected puppies usually show mild clinical symptoms and signs.⁴¹ The short study duration makes it difficult to monitor clinical improvement, durability of gene editing, and potential untoward adverse events that arise later.

As discussed above, Amoasii *et al.* performed a panel of assays to study editing effect, dystrophin restoration, and disease amelioration. There is no doubt that all these studies are important. However, a more thorough analysis should be included in future studies to assess the consequences of AAV CRISPR delivery fully in dystrophic large mammals. A significant safety concern of *in vivo* CRISPR editing is off-target editing. The authors only quantified indel formation in the predicted off-target sites in muscle. Unbiased approaches such as the recently reported verification of *in vivo* off-targets (VIVO) method should provide a more comprehensive view on genome-wide off-target editing.⁴² Besides indels, other important genetic changes such as AAV vector genome integration and large chromosomal alterations (*e.g.*, large deletion, inversion, translocation, and duplications) should also be investigated. It is worth noting that a significant portion of systemically injected AAV vectors will end up in the liver. Hence, in addition to muscle, the liver should also be carefully examined for potential off-target genomic modification. To determine the safety, the authors also studied hematology and blood biochemistry. This can be further expanded to include the growth curve (body weight) and histology examination of major internal organs such as the liver, kidney, lung, and brain. Germline editing is an ethical/safety

concern.^{43,44} Amoasii *et al.* did not find Cas9 expression and target-site indel formation in the testes. Similar analysis in germ cell forming tissues (the testes and ovaries) should be included in future large-scale, long-term studies.

Immune responses have been at the center stage of gene therapy for decades.⁴⁵ These include innate, cellular, and humoral responses to the viral capsid, vector genome, and transgene product. The cellular immune response has been a major hurdle when intramuscular AAV delivery was initially tested in dog muscle. Robust cytotoxic immune rejection was evoked in normal adult dogs by AAV2, AAV6, and AAV9 that express the transgene from a ubiquitous promoter.^{46–48} In one study, cellular immunity was even detected in neonatal normal dogs.⁴⁶ Interestingly, nominal CD4+ and CD8+ T-cell infiltration was found in locally injected dogs by Amoasii *et al.* This suggests that the use of the tissue-specific promoter is a powerful approach to prevent untoward cellular immune responses. While this finding is encouraging, a generalized conclusion that the CRISPR/Cas system does not induce severe innate and/or cellular immune response will require more thorough examination. For instance, (1) some muscle-specific promoters (such as the desmin and SPc5-12 promoter) have been shown to drive expression in antigen-presenting cells (APCs),^{49,50} (2) some AAV capsids may transduce APCs more effectively, and (3) age and breed influence the activity and profile of the dog immune system.⁵¹ There is a high likelihood that the T-cell response may still be a major hurdle for future AAV CRISPR local injection studies in the canine DMD model if a different promoter, different AAV capsid, different age, or different breed is used.

All three types of immune responses have been reported following high-dose systemic AAV delivery in neonatal large animals (reviewed by Duan³⁶). Of note are two recent reports showing a fatal innate immune response in nonhuman primates and piglets.^{52,53} In the Amoasii *et al.* study, both systemically injected puppies survived to the scheduled experiment termination date, suggesting acute immunological death might not be a major concern if CRISPR AAV vectors are delivered to affected puppies using an experimental design and study protocol identical to that described by Amoasii *et al.*³⁴ Several recent reports suggest that immune responses to the bacterial-originated Cas protein cannot be underestimated.^{54–56} Evaluation on anti-Cas9 T cells (by CD4, CD8, and Treg immunostaining, tetramer staining, INF- γ enzyme-linked immunosorbent spot, T-cell lineage, and functionality study and intracellular

cytokine staining), anti-Cas9 antibodies (by *in vitro* neutralizing assay for the neutralizing antibody and by enzyme-linked immunosorbent assay [ELISA] for the binding antibody), and serum cytokines and complement (by bead-based multiplex cytokine analysis and complement pathway ELISA assay) should be included in future studies.^{57,58}

The ultimate goal of CRISPR therapy is to mitigate dystrophic muscle disease. In this regard, the treatment effect should be quantified by morphometric analysis of muscle histology and physiological measurement of muscle function. Histopathology evaluation should include, but not be limited to, quantification of the percentage of centrally nucleated myofibers, analysis of myofiber size distribution, evaluation of macrophage and neutrophil infiltration, and qualitative (ideally quantitative) study of muscle fibrosis by Masson trichrome staining or picrosirius red staining (or by biochemical quantification of the collagen content in muscle). Similar assays have been routinely used to characterize morphological changes in dystrophic canine muscles in the literature.^{59–61}

The mouse and dog DMD models were established at about the same time.^{62,63} While a quite comprehensive list of methods has been available for studying muscle function in mice, methods to study dog muscle function remain underdeveloped until recently. Over the past few years, a number of new assays have been developed, including *in situ* evaluation of a single dog muscle contractility,⁶⁴ hind-limb muscle force measurement,⁶⁵ method to study sympatholysis and functional ischemia in canine muscle,⁶⁶ electrical impedance myography,⁶⁷ noninvasive gait analysis,^{68–70} and noninvasive whole-body activity assay.^{69,71} The standard operating procedures have been published for many of these protocols and are freely accessible on the Treat-NMD Neuromuscular Network Web site (www.treat-nmd.eu/research/preclinical/overview/) and Parent Project Muscular Dystrophy Web site (http://join.parentprojectmd.org/site/PageServer?pagename=Advance_researchers_sops).^{72,73}

Likely due to the limited sample size and the termination of the study at the pre-symptomatic age, the above-mentioned histological and physiological evaluations were not performed in the Amoasii *et al.* study. Inclusion of these assays in future studies will better inform the potential outcome when systemic CRISPR therapy moves to human patients.

There are hundreds of muscles in the body (~700 in a dog). Muscle disease in DMD patients is

shaped by the level and extent of dystrophic changes in all body muscles, in particular the heart, diaphragm, and major pelvic limb muscles (e.g., quadriceps) because dystrophy in these muscles leads directly to death or ambulation loss. Following systemic injection, Amoasii *et al.* performed H&E staining on three skeletal muscles, dystrophin immunostaining on the heart and six skeletal muscles, dystrophin Western blot on the heart and five skeletal muscles, and Cas9 Western blot on the heart (left ventricle, right ventricle, and septum) and 10 skeletal muscles. The authors also quantified level of developmental myosin heavy chain in two skeletal muscles and on-target indel formation on the heart (left ventricle, right ventricle, and septum) and four skeletal muscles. Although Amoasii *et al.* included the heart and diaphragm in all these assays, the total number of skeletal muscles examined remains low compared to how many muscles a dog has. It will be worthwhile to include more muscles in future studies to appreciate bodywide dystrophin restoration and disease amelioration better.

In summary, the findings reported by Amoasii *et al.* are encouraging because they provide the critical proof of principle for continuing to explore systemic CRISPR editing as a potential therapy for DMD.³³ The results of Amoasii *et al.* also suggest that the immune response to AAV9-mediated Cas9 expression may, under certain circumstances (as in the Amoasii *et al.* study), not be a severe concern as

has been speculated. Validating these observations with additional studies, as suggested in this article, should prove to be extremely advantageous in the development of CRISPR technology as a therapeutic modality. The Amoasii *et al.* study has provided a good starting point for experimentally testing AAV-mediated CRISPR therapy in the canine DMD model (or other large mammalian models of human diseases). Large-scale, comprehensive, long-term studies are needed to determine the risk–benefit ratio of systemic AAV CRISPR therapy for DMD before considering testing this promising therapy in patients.

ACKNOWLEDGMENTS

The authors thank the support from the National Institutes of Health (NIH; AR-69085 to D.D., GM-063732 and GM-117059 to S.C.), Department of Defense (MD150133 to D.D.), Hope for Javier (to D.D.), Jackson Freil DMD Research Fund (to D.D.), and Intramural Research Program of the NIH, NCATS (to N.N.Y. and C.H.H.).

AUTHOR DISCLOSURE

D.D. is a member of the scientific advisory board for Solid Biosciences and an equity holder of Solid Biosciences. The Duan lab has received research supports unrelated to CRISPR editing from Solid Biosciences. C.H.H., D.D., N.B.W. have filed a patent disclosure on systemic CRISPR therapy.

REFERENCES

1. Cong L, Ran FA, Cox D, et al. Multiplex genome engineering using CRISPR/Cas Systems. *Science* 2013;339:819–823.
2. Mali P, Yang LH, Esvelt KM, et al. RNA-guided human genome engineering via Cas9. *Science* 2013;339:823–826.
3. Jinek M, Chylinski K, Fonfara I, et al. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 2012;337:816–821.
4. Cox DB, Platt RJ, Zhang F. Therapeutic genome editing: prospects and challenges. *Nat Med* 2015; 21:121–131.
5. Abdul-Razak H, Malerba A, Dickson G. Advances in gene therapy for muscular dystrophies. *F1000Res* 2016;5.
6. Duan D. Dystrophin gene replacement and gene repair therapy for Duchenne muscular dystrophy in 2016. *Hum Gene Ther Clin Dev* 2016;27:9–18.
7. Chamberlain JR, Chamberlain JS. Progress toward gene therapy for Duchenne muscular dystrophy. *Mol Ther* 2017;25:1125–1131.
8. Duan D. Duchenne muscular dystrophy gene therapy in the canine model. *Hum Gene Ther Clin Dev* 2015;26:57–69.
9. McGreevy JW, Hakim CH, McIntosh MA, et al. Animal models of Duchenne muscular dystrophy: from basic mechanisms to gene therapy. *Dis Model Mech* 2015;8:195–213.
10. Long C, McAnally JR, Shelton JM, et al. Prevention of muscular dystrophy in mice by CRISPR/Cas9-mediated editing of germline DNA. *Science* 2014;345:1184–1188.
11. Ousterout DG, Kabadi AM, Thakore PI, et al. Multiplex CRISPR/Cas9-based genome editing for correction of dystrophin mutations that cause Duchenne muscular dystrophy. *Nat Commun* 2015;6:6244.
12. Li HL, Fujimoto N, Sasakawa N, et al. Precise correction of the dystrophin gene in Duchenne muscular dystrophy patient induced pluripotent stem cells by TALEN and CRISPR-Cas9. *Stem Cell Reports* 2015;4:143–154.
13. Young CS, Hicks MR, Ermolova NV, et al. A single CRISPR-Cas9 deletion strategy that targets the majority of DMD patients restores dystrophin function in hiPSC-derived muscle cells. *Cell Stem Cell* 2016;18:533–540.
14. Zhang Y, Long C, Li H, et al. CRISPR-Cpf1 correction of muscular dystrophy mutations in human cardiomyocytes and mice. *Sci Adv* 2017;3: e1602814.
15. Kyrychenko V, Kyrychenko S, Tiburcy M, et al. Functional correction of dystrophin actin binding

- domain mutations by genome editing. [JCI Insight](#) 2017;2:e95918.
16. Lattanzi A, Duguez S, Moiani A, et al. Correction of the exon 2 duplication in DMD myoblasts by a single CRISPR/Cas9 system. [Mol Ther Nucl Acids](#) 2017;7:11–19.
 17. Maggio I, Liu J, Janssen JM, et al. Adenoviral vectors encoding CRISPR/Cas9 multiplexes rescue dystrophin synthesis in unselected populations of DMD muscle cells. [Sci Rep](#) 2016;6:37051.
 18. Wojtal D, Kemaladewi DU, Malam Z, et al. Spell checking nature: versatility of CRISPR/Cas9 for developing treatments for inherited disorders. [Am J Hum Genet](#) 2016;98:90–101.
 19. Iyombe-Engembe JP, Ouellet DL, Barbeau X, et al. Efficient restoration of the dystrophin gene reading frame and protein structure in DMD myoblasts using the CinDel method. [Mol Ther Nucl Acids](#) 2016;5:e283.
 20. Duchêne BL, Cherif K, Iyombe-Engembe JP, et al. CRISPR-induced deletion with SaCas9 restores dystrophin expression in dystrophic models *in vitro* and *in vivo*. [Mol Ther](#) 2018;26:2604–2616.
 21. Long C, Li H, Tiburcy M, et al. Correction of diverse muscular dystrophy mutations in human engineered heart muscle by single-site genome editing. [Sci Adv](#) 2018;4:eaap9004.
 22. Nelson CE, Hakim CH, Ousterout DG, et al. *In vivo* genome editing improves muscle function in a mouse model of Duchenne muscular dystrophy. [Science](#) 2016;351:403–407.
 23. Long C, Amoasii L, Mireault AA, et al. Postnatal genome editing partially restores dystrophin expression in a mouse model of muscular dystrophy. [Science](#) 2016;351:400–403.
 24. Tabebordbar M, Zhu K, Cheng JK, et al. *In vivo* gene editing in dystrophic mouse muscle and muscle stem cells. [Science](#) 2016;351:407–411.
 25. Xu L, Park KH, Zhao L, et al. CRISPR-mediated genome editing restores dystrophin expression and function in mdx mice. [Mol Ther](#) 2016;24:564–549.
 26. El Refaey M, Xu L, Gao Y, et al. *In vivo* genome editing restores dystrophin expression and cardiac function in dystrophic mice. [Circ Res](#) 2017;121:923–929.
 27. Bengtsson NE, Hall JK, Odom GL, et al. Muscle-specific CRISPR/Cas9 dystrophin gene editing ameliorates pathophysiology in a mouse model for Duchenne muscular dystrophy. [Nat Commun](#) 2017;8:14454.
 28. Amoasii L, Long C, Li H, et al. Single-cut genome editing restores dystrophin expression in a new mouse model of muscular dystrophy. [Sci Transl Med](#) 2017;9:piaa08081.
 29. Hakim CH, Wasala NB, Nelson CE, et al. AAV CRISPR editing rescues cardiac and muscle function for 18 months in dystrophic mice. [JCI Insight](#) 2018;3.
 30. Young CS, Mokhonova E, Quinonez M, et al. Creation of a novel humanized dystrophic mouse model of Duchenne muscular dystrophy and application of a CRISPR/Cas9 gene editing therapy. [J Neuromusc Dis](#) 2017;4:139–145.
 31. Koo T, Lu-Nguyen NB, Malerba A, et al. Functional rescue of dystrophin deficiency in mice caused by frameshift mutations using *Campylobacter jejuni* Cas9. [Mol Ther](#) 2018;26:1529–1538.
 32. Ryu SM, Koo T, Kim K, et al. Adenine base editing in mouse embryos and an adult mouse model of Duchenne muscular dystrophy. [Nat Biotechnol](#) 2018;36:536–539.
 33. Duan D. CRISPR alleviates muscular dystrophy in dogs. [Nat Biomed Eng](#) 2018;2:795–796.
 34. Amoasii L, Hildyard JCW, Li H, et al. Gene editing restores dystrophin expression in a canine model of Duchenne muscular dystrophy. [Science](#) 2018;362:86–91.
 35. Walmsley GL, Arechavala-Gomez V, Fernandez-Fuente M, et al. A Duchenne muscular dystrophy gene hot spot mutation in dystrophin-deficient Cavalier King Charles Spaniels is amenable to exon 51 skipping. [PLoS One](#) 2010;5:e8647.
 36. Duan D. Systemic AAV micro-dystrophin gene therapy for Duchenne muscular dystrophy. [Mol Ther](#) 2018;26:2337–2356.
 37. Mendell JR, Al-Zaidy S, Shell R, et al. Single-dose gene-replacement therapy for spinal muscular atrophy. [N Engl J Med](#) 2017;377:1713–1722.
 38. Duan D. Micro-dystrophin gene therapy goes systemic in Duchenne muscular dystrophy patients. [Hum Gene Ther](#) 2018;29:733–736.
 39. Aartsma-Rus A, Fokkema I, Verschuuren J, et al. Theoretic applicability of antisense-mediated exon skipping for Duchenne muscular dystrophy mutations. [Hum Mutat](#) 2009;30:293–299.
 40. Wasala NB, Lai Y, Shin J-H, et al. Genomic removal of a therapeutic mini-dystrophin gene from adult mice elicits a Duchenne muscular dystrophy-like phenotype. [Hum Mol Genet](#) 2016;25:2633–2644.
 41. Kornegay JN. The golden retriever model of Duchenne muscular dystrophy. [Skelet Muscle](#) 2017;7:9.
 42. Akcakaya P, Bobbin ML, Guo JA, et al. *In vivo* CRISPR editing with no detectable genome-wide off-target mutations. [Nature](#) 2018;561:416–419.
 43. Cwik B. Designing ethical trials of germline gene editing. [New Engl J Med](#) 2017;377:1911–1913.
 44. Kohn DB, Porteus MH, Scharenberg AM. Ethical and regulatory aspects of genome editing. [Blood](#) 2016;127:2553–2560.
 45. Mingozi F, High KA. Overcoming the host immune response to adeno-associated virus gene delivery vectors: the race between clearance, tolerance, neutralization, and escape. [Annu Rev Virol](#) 2017;4:511–534.
 46. Yuasa K, Yoshimura M, Urasawa N, et al. Injection of a recombinant AAV serotype 2 into canine skeletal muscles evokes strong immune responses against transgene products. [Gene Ther](#) 2007;14:1249–1260.
 47. Wang Z, Allen JM, Riddell SR, et al. Immunity to adeno-associated virus-mediated gene transfer in a random-bred canine model of Duchenne muscular dystrophy. [Hum Gene Ther](#) 2007;18:18–26.
 48. Yue Y, Ghosh A, Long C, et al. A single intravenous injection of adeno-associated virus serotype-9 leads to whole body skeletal muscle transduction in dogs. [Mol Ther](#) 2008;16:1944–1952.
 49. Boisgerault F, Gross DA, Ferrand M, et al. Prolonged gene expression in muscle is achieved without active immune tolerance using MicroRNA 142.3p-regulated rAAV gene transfer. [Hum Gene Ther](#) 2013;24:393–405.
 50. Wang L, Dobrzynski E, Schlachterman A, et al. Systemic protein delivery by muscle-gene transfer is limited by a local immune response. [Blood](#) 2005;105:4226–4234.
 51. Day MJ. Immune system development in the dog and cat. [J Comp Pathol](#) 2007;137:S10–15.
 52. Hinderer C, Katz N, Buza EL, et al. Severe toxicity in nonhuman primates and piglets following high-dose intravenous administration of an adeno-associated virus vector expressing human SMN. [Hum Gene Ther](#) 2018;29:285–298.
 53. Hordeaux J, Wang Q, Katz N, et al. The neurotropic properties of AAV-PHP.B are limited to C57BL/6J mice. [Mol Ther](#) 2018;26:664–668.
 54. Chew WL, Tabebordbar M, Cheng JK, et al. A multifunctional AAV-CRISPR-Cas9 and its host response. [Nat Methods](#) 2016;13:868–874.
 55. Simhadri VL, McGill J, McMahon S, et al. Prevalence of pre-existing antibodies to CRISPR-associated nuclease Cas9 in the USA population. [Mol Ther Methods Clin Dev](#) 2018;10:105–112.
 56. Charlesworth CT, Deshpande PS, Dever DP, et al. Identification of pre-existing adaptive immunity to Cas9 proteins in humans. [bioRxiv](#) 2018 Jan 5 [Epub ahead of print]; DOI: 10.1101/243345.
 57. Calcedo R, Chichester JA, Wilson JM. Assessment of humoral, innate, and T-cell immune responses to adeno-associated virus vectors. [Hum Gene Ther Methods](#) 2018;29:86–95.
 58. Martino AT, Herzog RW, Anegón I, et al. Measuring immune responses to recombinant AAV gene transfer. [Methods Mol Biol](#) 2011;807:259–272.
 59. Shin J-H, Pan X, Hakim CH, et al. Microdystrophin ameliorates muscular dystrophy in the canine model of Duchenne muscular dystrophy. [Mol Ther](#) 2013;21:750–757.
 60. Smith BF, Yue Y, Woods PR, et al. An intronic LINE-1 element insertion in the dystrophin gene aborts dystrophin expression and results in Duchenne-like muscular dystrophy in the cori breed. [Lab Invest](#) 2011;91:216–231.

61. Kodippili K, Hakim CH, Pan X, et al. Dual AAV gene therapy for Duchenne muscular dystrophy with a 7-kb mini-dystrophin gene in the canine model. [Hum Gene Ther](#) 2018;29:299–311.
62. Sicinski P, Geng Y, Ryder-Cook AS, et al. The molecular basis of muscular dystrophy in the mdx mouse: a point mutation. [Science](#) 1989;244:1578–1580.
63. Cooper BJ, Winand NJ, Stedman H, et al. The homologue of the Duchenne locus is defective in X-linked muscular dystrophy of dogs. [Nature](#) 1988; 334:154–156.
64. Yang HT, Shin J-H, Hakim CH, et al. Dystrophin deficiency compromises force production of the extensor carpi ulnaris muscle in the canine model of Duchenne muscular dystrophy. [PLoS One](#) 2012;7: e44438.
65. Childers MK, Grange RW, Kornegay JN. *In vivo* canine muscle function assay. [J Vis Exp](#) 2011;(50).
66. Kodippili K, Hakim CH, Yang HT, et al. Nitric oxide dependent attenuation of norepinephrine-induced vasoconstriction is impaired in the canine model of Duchenne muscular dystrophy. [J Physiol](#) 2018; 596:5199–5266.
67. Hakim CH, Mijailovic A, Lessa TB, et al. Non-invasive evaluation of muscle disease in the canine model of Duchenne muscular dystrophy by electrical impedance myography. [PLoS One](#) 2017; 12:e0173557.
68. Jenkins GJ, Hakim CH, Yang NN, et al. Automatic characterization of stride parameters in canines with a single wearable inertial sensor. [PLoS One](#) 2018;13:e0198893.
69. Shin J-H, Greer B, Hakim CH, et al. Quantitative phenotyping of Duchenne muscular dystrophy dogs by comprehensive gait analysis and overnight activity monitoring. [PLoS One](#) 2013;8:e59875.
70. Barthelemy I, Barrey E, Aguilar P, et al. Longitudinal ambulatory measurements of gait abnormality in dystrophin-deficient dogs. [BMC Musculoskelet Disord](#) 2011;12:75.
71. Hakim CH, Peters AA, Feng F, et al. Night activity reduction is a signature physiological biomarker for Duchenne muscular dystrophy dogs. [J Neuromuscul Dis](#) 2015;2:397–407.
72. Duan D, Rafael-Fortney JA, Blain A, et al. Standard operating procedures (SOPs) for evaluating the heart in preclinical studies of Duchenne muscular dystrophy. [J Cardiovasc Transl Res](#) 2016;9:85–86.
73. Nagaraju K, Willmann R, Network T-N, et al. Developing standard procedures for murine and canine efficacy studies of DMD therapeutics: report of two expert workshops on “Pre-clinical testing for Duchenne dystrophy”: Washington DC, October 27th–28th 2007 and Zurich, June 30th–July 1st 2008. [Neuromuscul Disord](#) 2009; 19:502–506.

Received for publication November 19, 2018;
accepted after revision January 11, 2019.

Published online: January 15, 2019.

Long-term evaluation of AAV-CRISPR genome editing for Duchenne muscular dystrophy

Christopher E. Nelson^{1,2}, Yaoying Wu¹, Matthew P. Gemberling^{1,2}, Matthew L. Oliver¹,
Matthew A. Waller^{1,2}, Joel D. Bohning^{1,2}, Jacqueline N. Robinson-Hamm^{1,2}, Karen Bulaklak^{1,2},
Ruth M. Castellanos Rivera³, Joel H. Collier¹, Aravind Asokan^{4,5} and Charles A. Gersbach^{1,2,6*}

Duchenne muscular dystrophy (DMD) is a monogenic disorder and a candidate for therapeutic genome editing. There have been several recent reports of genome editing in pre-clinical models of Duchenne muscular dystrophy^{1–6}, however, the long-term persistence and safety of these genome editing approaches have not been addressed. Here we show that genome editing and dystrophin protein restoration is sustained in the *mdx* mouse model of Duchenne muscular dystrophy for 1 year after a single intravenous administration of an adeno-associated virus that encodes CRISPR (AAV-CRISPR). We also show that AAV-CRISPR is immunogenic when administered to adult mice⁷; however, humoral and cellular immune responses can be avoided by treating neonatal mice. Additionally, we describe unintended genome and transcript alterations induced by AAV-CRISPR that should be considered for the development of AAV-CRISPR as a therapeutic approach. This study shows the potential of AAV-CRISPR for permanent genome corrections and highlights aspects of host response and alternative genome editing outcomes that require further study.

Duchenne muscular dystrophy (DMD) is a debilitating and prematurely fatal genetic disease caused by mutations in the *DMD* gene leading to the absence of dystrophin^{8,9}. Despite recent clinical advancements^{10,11}, a curative approach remains to be found. Adeno-associated viruses (AAVs) are being used as a gene delivery vector for recently initiated DMD clinical trials and for two approved gene therapy products and have been tested in more than 100 clinical trials¹². Multiple groups are using AAVs to deliver genome-editing technologies to make permanent genetic modifications to treat disease, including the first human genome editing clinical trial using AAV that is currently underway using zinc finger nuclease technology^{13,14}. Genome editing has been used to repair the *DMD* gene by exon deletion^{1–6}, splice-site targeting¹⁵ or homology directed repair⁶ in mouse models of DMD and most recently in a canine model of DMD¹⁶. These studies show that genome editing restores dystrophin expression in mouse models of DMD, leading to an improvement in skeletal muscle function. The enthusiasm for a genome-editing strategy is based on the potential that a single administration can have life-long therapeutic benefits. However, published studies have focused on short-term restoration of dystrophin, typically assessed at 4–8 weeks after treatment. In this study, we treated mice with a dual-AAV system, consisting of one AAV encoding Cas9 and the other AAV encoding two guide

RNAs (gRNAs) that were designed to excise exon 23 from the *Dmd* gene in *mdx* mice. For viral packaging, we used the smaller 3.2-kb Cas9 derived from *Staphylococcus aureus* (SaCas9)¹⁷. We examined both AAV serotype 8 (AAV8) and 9 (AAV9) (Fig. 1a,b), which have differential tissue tropism for the heart, skeletal muscle, and liver in animal models that are not perfectly predictive of human tropism¹⁸. We examined adult and postnatal day 2 (P2) neonatal mice treated locally by intramuscular (i.m.) injection and systemically by intravenous facial-vein injection (FVI), respectively, for restoration of dystrophin expression (Fig. 1a,b). We adapted an unbiased deep-sequencing method for precise quantification of gene-editing efficiencies. Mice injected by i.m. injection as adults had a significant decrease in genome-editing levels over time (Fig. 1c and Extended Data Fig. 1). By contrast, systemically treated mice had a modest statistically significant increase in genome-editing levels over 1 year (Fig. 1d and Extended Data Fig. 1). The SaCas9 expression cassette was driven by a constitutive CMV promoter that is expressed in multiple muscle cell types, including striated muscle and muscle progenitors⁵. However, genome-editing events were also detected in other tissues, including liver, spleen, kidney and brain, as well as the testis at levels barely above the limit of detection (approximately 0.1%; Extended Data Fig. 2). Use of a myocyte-specific promoter could restrict editing to striated muscle nuclei⁶, but potentially at the cost of editing muscle progenitor cells. Analysis of *Dmd* mRNA transcripts by droplet digital PCR (ddPCR) showed the same trend as the genomic deletions with significant increases over time noted in cardiac muscles obtained from systemically treated mice (Fig. 1e,f). Sustained restoration of dystrophin was detected by immunofluorescence staining and western blot of cardiac and skeletal muscle from systemically treated mice for at least 1 year after a single administration (Fig. 1g,h and Extended Data Fig. 3). The restored dystrophin was slightly smaller in younger mice than in older mice, potentially owing to a smaller protein isoform produced at the early time point while nearly full-length dystrophin was detected at 1 year. Serum creatine kinase levels were reduced at 8 weeks after treatment in mice treated systemically as neonates, demonstrating protection from muscle damage by the restored dystrophin protein (Fig. 1i). Deep sequencing of the top ten predicted off-target sites showed no significant increase in off-target cutting after 1 year with slight activity above background noted for gRNA1 at off-target site 8 (gRNA1-OT8), as was previously identified following local administration⁴ (Supplementary Tables 1 and 2).

¹Department of Biomedical Engineering, Duke University, Durham, NC, USA. ²Center for Genomic and Computational Biology, Duke University, Durham, NC, USA. ³Gene Therapy Center, University of North Carolina Chapel Hill, Chapel Hill, NC, USA. ⁴Department of Surgery, Duke University Medical Center, Durham, NC, USA. ⁵Department of Molecular Genetics and Microbiology, Duke University Medical Center, Durham, NC, USA.

⁶Department of Orthopaedic Surgery, Duke University Medical Center, Durham, NC, USA. *e-mail: charles.gersbach@duke.edu

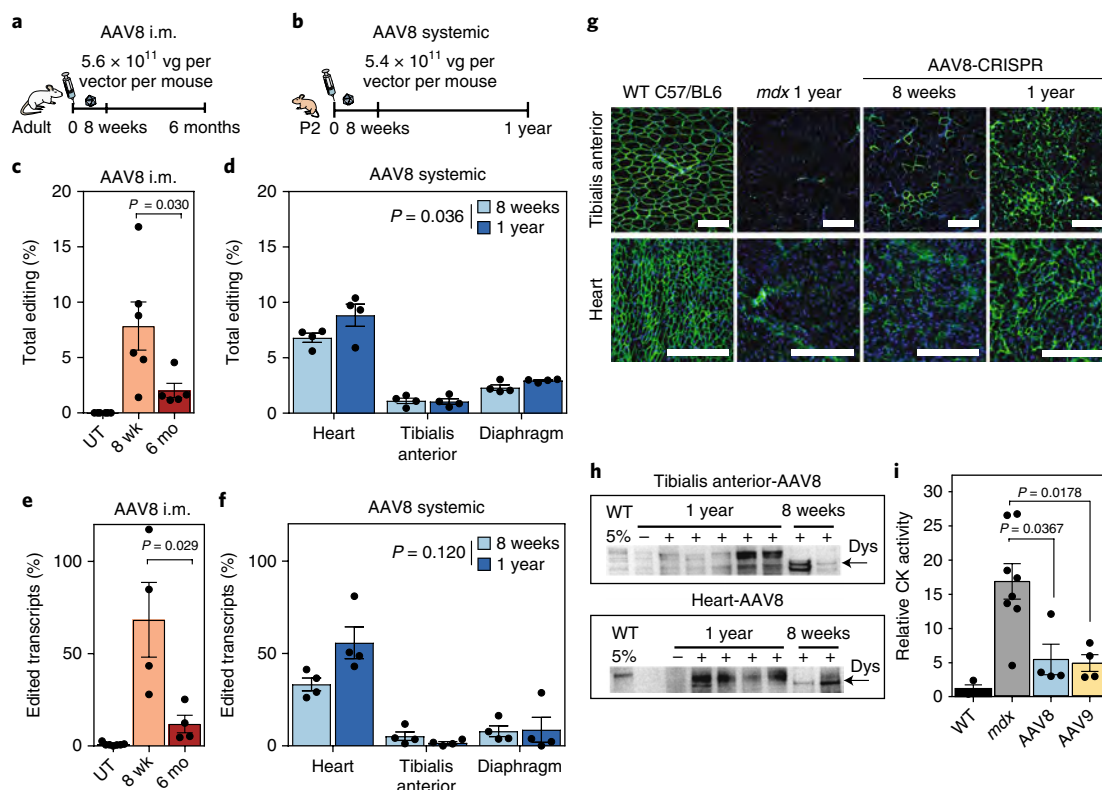


Fig. 1 | Genome editing is sustained for 1 year in neonatal mice treated by intravenous administration. **a,b**, Mice were treated as adults by AAV injection into the tibialis anterior (**a**) or systemically by FVI as neonates (**b**). vg, vector genomes. **c,d**, Quantification of total gene modification shows a significant decrease over 6 months following local administration (**c**; $n = 6$, 8 weeks; $n = 5$, 6 months; one-sided t -test) and a significant increase in neonates treated systemically (**d**; $n = 4$; two-way ANOVA). UT, untreated. 8 wk, 8 weeks; 6 mo, 6 months. **e,f**, ddPCR shows the same trend for deletion of exon 23 from the transcript for local injections (**e**; $n = 4$; one-sided t -test) and systemic injections (**f**; $n = 4$; two-way ANOVA). **g**, Dystrophin expression is sustained in cardiac muscle and skeletal muscle 1 year after systemic administration into neonates. Scale bars, 200 μ m. WT, wild-type mice. Histological images are available as Source data. **h**, Western blot confirms the presence of dystrophin (Dys) in skeletal and cardiac muscle. Full uncropped blots and are available as Source data. **i**, After 8 weeks, systemically treated neonatal mice show a significant decrease in creatine kinase (CK) ($n = 3$, wild-type mice; $n = 8$, untreated mice; $n = 4$, treated mice; one-way ANOVA with multiple comparisons correction). Data are mean $\pm$ s.e.m.

An important consideration to long-term therapeutic benefit of *in vivo* genome editing is the host response to bacteria-derived Cas9 proteins. In our study, a humoral immune response was detected against the SaCas9 protein in nearly all mice injected as adults ($n = 31$ out of 32 mice; Fig. 2a and Extended Data Fig. 4). By contrast, no humoral response against SaCas9 was detected in mice treated as neonates by FVI or intraperitoneal injections ($n = 0$ out of 19 mice; Fig. 2a). A cellular response was detected by restimulation with SaCas9 to produce interferon (IFN) γ -secreting T cells in mice treated as adults but not neonates, regardless of administration route (Fig. 2b and Extended Data Fig. 4). The *mdx* mouse model has an increased baseline number of infiltrating macrophages and neutrophils owing to muscle degeneration and inflammation¹⁹, which treatment with AAV-CRISPR has been shown to decrease⁴. Expression of FOXP3, granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-1 β , and IL-12 β decreased relative to untreated *mdx* mice after i.m. injection. However, IFN γ significantly increased approximately seven-fold after local injection relative to untreated *mdx* mice (Extended Data Fig. 4). By contrast, systemically treated adults and neonates showed no significant changes in these markers of inflammatory cell infiltration. Most AAV vector genomes remain episomal after cell entry and are stably maintained in non-dividing cells²⁰. In this study, AAV vectors persisted between 8 weeks and 1 year in cardiac muscle but were significantly lost in skeletal muscle after i.m. or FVI injection (Fig. 2c,d).

Regardless, expression of SaCas9 mRNA and both gRNAs is almost absent after 6 months or 1 year by either route of administration (Fig. 2e,f), which may be the result of promoter silencing²¹. The host response to AAV-CRISPR will need to be carefully considered for future clinical development, including pre-existing immunity in humans²². We have previously shown CRISPR-based gene silencing elicits a Cas9-dependent host response that resolves without intervention *in vivo*²³. Our data here indicates that a significant host response is avoided if AAV-CRISPR is administered at the neonatal stage. Although the P2 mice have an undeveloped immune system that can be exploited for antigen-specific tolerance including Cas9^{24–26}, it is not yet clear to what extent this approach applies in newborn humans. Other methods that could be explored to avoid anti-Cas9 immune response include transient immunosuppression for the length of vector expression, induction of immune tolerance²⁷, removal of T cell epitopes²⁸, the use of self-limiting/cleaving vectors, or other transient delivery vehicles including non-viral vectors²⁹.

The methods used to assess *in vivo* genome-editing efficiencies have typically been designed to quantify the frequency of expected genome-editing outcomes. Additionally, different methods often must be used to quantify the various possible editing outcomes. For example, PCR-based methods for deep sequencing can detect the formation of insertions and deletions (indel) after genome editing but cannot quantify gene deletions and do not capture larger structural changes that remove one or both primer sites. Previously, we used

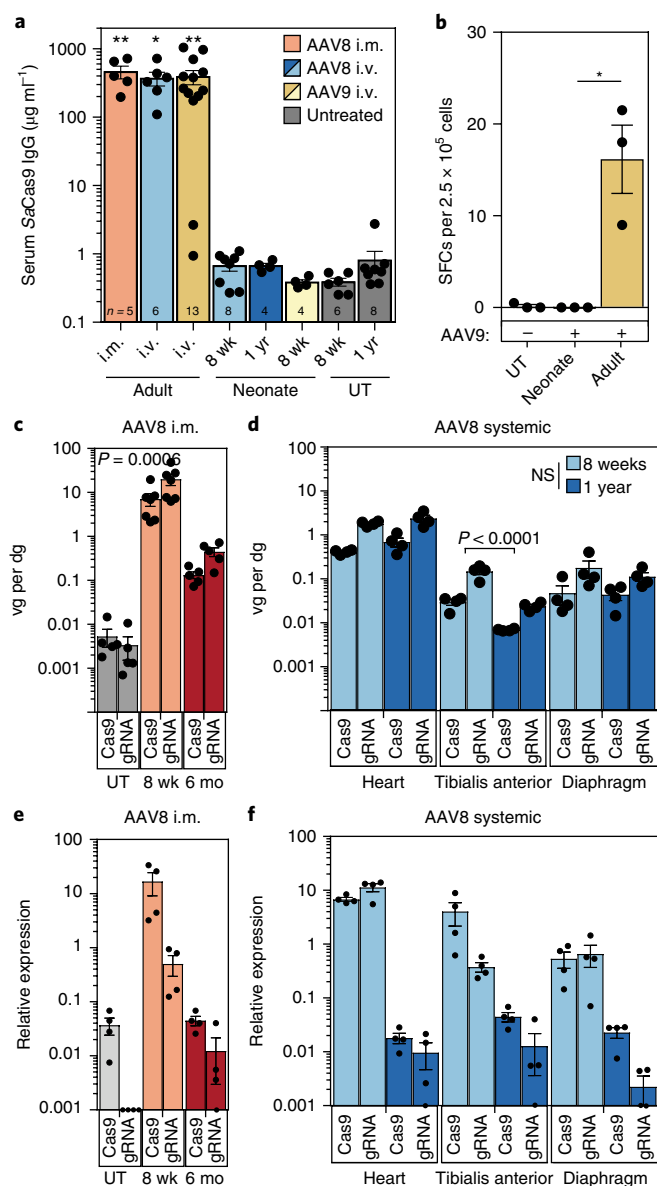


Fig. 2 | Host response to AAV-CRISPR for DMD. **a**, Antibodies against SaCas9 are detected in mice treated as adults but not in mice treated as neonates after 8 weeks or 1 year (one-way ANOVA with multiple comparisons $^*P < 0.05$, $^{**}P < 0.01$ compared with untreated mice at 8 weeks, n numbers as indicated). **b**, Mice injected as adults with an AAV encoding Cas9 have T cells that are stimulated by exposure to SaCas9 to produce IFN γ as shown by enzyme-linked immune absorbent spot (ELISpot, one-sided t -test, $^*P = 0.0246$, $n = 3$ mice per condition). SFCs, spot-forming colonies. **c,d**, A significant loss in total AAV vector genomes (vg) per diploid genome (dg) is detected in skeletal muscle following intramuscular injection and intravenous injection but not in cardiac muscle and diaphragm (two-way ANOVA with Tukey's multiple comparisons test, $n = 7$, i.m., 8 weeks; $n = 5$, i.m., 6 months, $n = 4$, all systemic groups). NS, not significant. **e,f**, Expression of both the Cas9 mRNA and gRNAs dissipates between the early and late time points. Skeletal muscle also shows lower gRNA expression than cardiac muscle (two-way ANOVA with Tukey's multiple comparisons test, $n = 4$, all groups). Data are mean $\pm$ s.e.m.

ddPCR to quantify genetic changes including deletions⁴; however, ddPCR requires separate priming strategies to amplify each gene-editing outcome, including unedited alleles and different editing events, and cannot detect unexpected events. To comprehensively

map all possible genome-editing outcomes with an unbiased approach, we adapted Illumina's Nextera-transposon-based library preparation method for unbiased sequencing³⁰. This method used a single genome-specific forward primer for target enrichment and a reverse primer specific for the DNA tag integrated by the transposon. In addition to genomic deletions, this method is sensitive to indel formation, inversion of exon 23 and surrounding introns and integration of the AAV genome (Fig. 3a). Using this method, we show that quantifiable and heterogeneous genome-editing events at the on-target *Dmd* locus occur, including deletions, inversions, indels, and AAV integrations in all treated mice (Fig. 3b and Extended Data Fig. 5). Importantly, no chromosomal translocations driven by off-target DNA cutting were detected in this experiment (estimated limit of detection of approximately 0.01%; Extended Data Fig. 5). The majority of deletion events were perfect deletions consistent with previous observations^{4,31}. We detected a low prevalence ($<0.5\%$) of large asymmetrical deletions (Fig. 3c and Supplementary Table 3), consistent with a previous report using long-read sequencing to monitor genome-editing outcomes in pluripotent cells in vitro³². However, our method cannot detect large deletions that remove both primer sites. The sequencing method used here is reproducible and matches indel quantification collected through a more standard next-generation sequencing method (Extended Data Fig. 6). We also applied this Nextera-based sequencing approach to cDNA of treated mice. This approach is sensitive to exon 23 removal and unexpected transcript changes including aberrant splicing (Fig. 3d and Extended Data Fig. 7). We detected removal of exon 23, changes in splicing including multi-exon skipping, putative circular RNA formation, and AAV splicing events (Fig. 3e and Supplementary Tables 4 and 5). Splicing events with the AAV vector genome contained canonical splice acceptors or donors (Extended Data Fig. 8 and Supplementary Table 6). Multi-exon skipping may lead to a partially functional or dysfunctional protein, depending on the change to the reading frame. The circular RNAs do not resemble a functional mRNA and will not be translated into protein and therefore are expected to have little biological importance. The relative enrichment of circular RNAs seen here may be caused by the stability of circular RNAs against exonuclease activity³³. Transcript isoforms that contain partial AAV genomes have an unknown biological effect. The levels of exon 23 excision determined by this sequencing method are comparable to the results obtained by ddPCR analysis for quantification of exon 23 removal (Extended Data Fig. 8). Sequencing of the cDNA isolated at different time points indicated that the transcript isoform levels are sustained over 1 year (Extended Data Fig. 7).

AAVs are being used extensively as a delivery vector for CRISPR-Cas9 in preclinical studies to treat inherited diseases including DMD^{1,34}. Although the safety of AAVs as a gene-delivery vehicle has been shown preclinically and through over 100 clinical trials, the potential genotoxicity of the combination of AAV and CRISPR requires further characterization. Here we adapted next-generation sequencing modalities to characterize unintended genome-editing events and AAV genome integrations. In this study, AAV typically integrated within the viral inverted terminal repeats (ITRs, 62%), resembling canonical integration³⁵; however, insertions within the viral genome were also detected (38%) (Fig. 4a). Insertions that occur internally within the vector genome may be the result of vector truncations from AAV packaging or from AAV genome insertion during DNA repair. Separately, a primer specific to the AAV vector genome was used in conjunction with the same transposon-specific primer to map genome-wide AAV vector episome integration into the mouse genome (Fig. 4b). This showed that the targeted site within the *Dmd* gene was the preferential location for integration in both neonatal liver and cardiac muscle. In tissues that were analyzed 8 weeks after systemic delivery in neonatal mice, 94 AAV integration sites were identified in the liver and 72 sites in cardiac muscle, with the majority of integration events

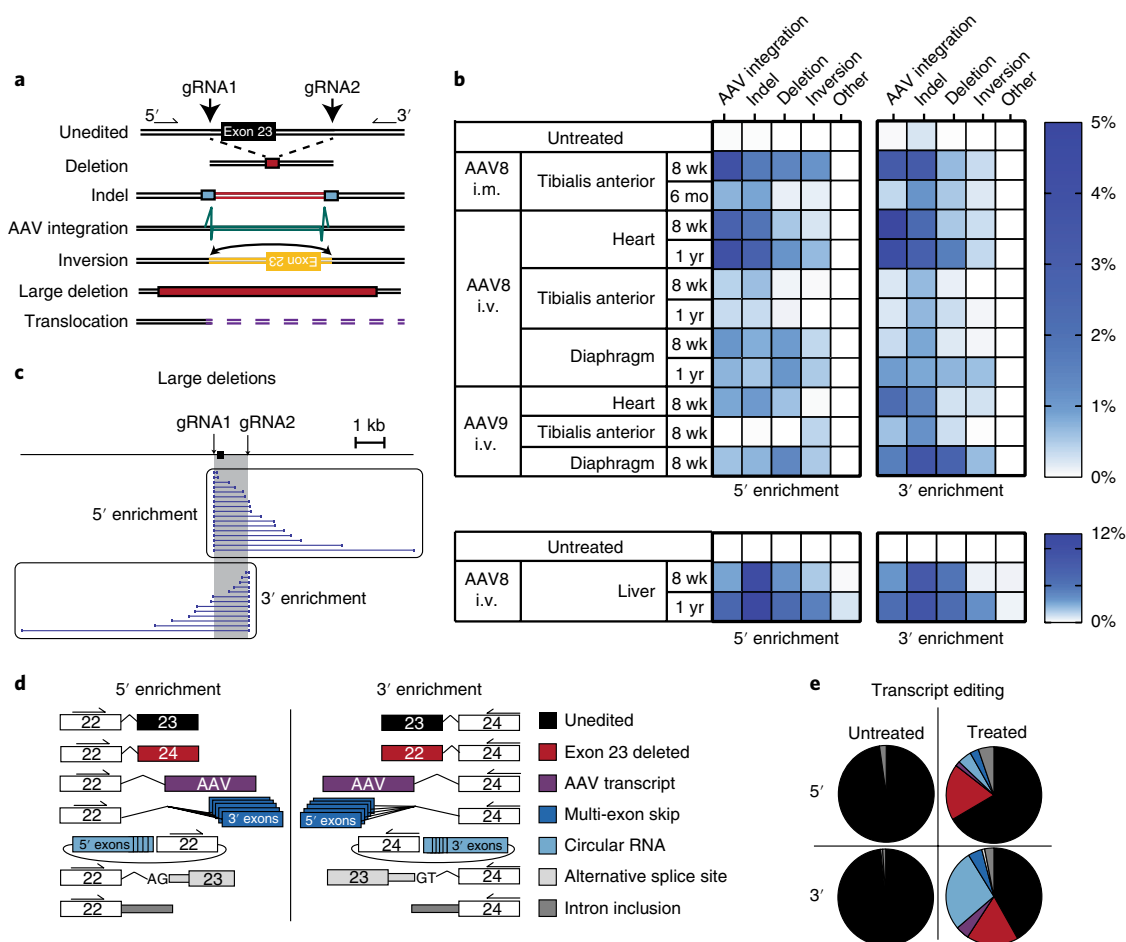


Fig. 3 | In vivo genome editing generates diverse on-target genome modifications including AAV integrations and aberrant splicing. **a**, Potential on-target genomic changes that resulted from targeted DNA cleavage are shown schematically. **b**, Unbiased analysis of the target site in multiple tissues after administration of AAV-CRISPR quantifies the level of each category of editing events. **c**, Large deletions around the target site were detected in the liver of mice treated with AAV8. **d**, Unbiased sequencing applied to the cDNA showed diverse transcript outcomes including aberrant splicing as shown schematically. **e**, Data from extracted cardiac muscle 8 weeks after administration show the proportion of transcript-editing events. **b, d**, Data were obtained from $n = 4$ individual mice for treated and untreated groups.

occurring within introns of genes consistent with previous observations, including several previously identified integration sites³⁶ (Fig. 4c and Supplementary Table 7). Several putative gRNA off-target sites were also identified by AAV integration (Fig. 4b,d), including a previously predicted off-target site in an intergenic region of chromosome 14 for which there was no detectable activity by conventional targeted deep sequencing in the same samples⁴ (Fig. 4c). This suggests that unbiased mapping of AAV integrations may be a more sensitive approach to determine the specificity of genome-editing reagents than typical methods.

In this study, the frequency of AAV integrations into the CRISPR-induced double-stranded break was higher than the intended deletion (Fig. 3b). AAV integration into targeted double-stranded breaks was reported more than a decade ago³⁵ and has also been applied as a therapeutic gene therapy approach¹⁴. AAVs can integrate into random breaks across the genome by non-homologous end-joining and can also be copied into target loci by homologous recombination without expression of nucleases³⁷. Preclinical reports of hepatocellular carcinoma caused by genotoxicity of the vector have been controversial and risks can be managed by vector design^{36,38–40}. AAVs are currently the gene delivery vehicle for more than 100 clinical trials targeting liver, skeletal muscle, cardiac muscle, central nervous system and other tissues with no reported adverse events

caused by genotoxicity of the vector. However, the induction of a novel DNA break by any genome-editing construct could potentially change the integration landscape and genotoxicity profile of the AAV (Fig. 4b–d). Additionally, each genome-engineering construct will have different genome-wide insertional mutagenesis profiles and should be carefully considered when developing vectors for therapeutic genome editing. Preclinical work can monitor *cis* activation of oncogenes and clonal expansion of AAV integration sites to reduce potential genotoxicity risks of genome-editing technologies delivered by AAVs³⁶, analogous to efforts to characterize lentiviral vector integration, which also has an excellent safety profile in human clinical trials.

Important future preclinical developments will be focused on increasing the overall editing efficiency and increasing the proportion of the intended gene modification by optimizing delivery and the gene-editing strategy. This study further establishes the feasibility of permanent gene correction as a therapeutic approach for DMD and potentially other diseases. Despite the presence of a host response to Cas9 and persistent unintended genome modifications, AAV-CRISPR was well-tolerated for 1 year with no sign of toxicity, although much larger studies are required to confirm the absence of genotoxicity risk. Moreover, the restoration of dystrophin expression was sustained over this period. New developments

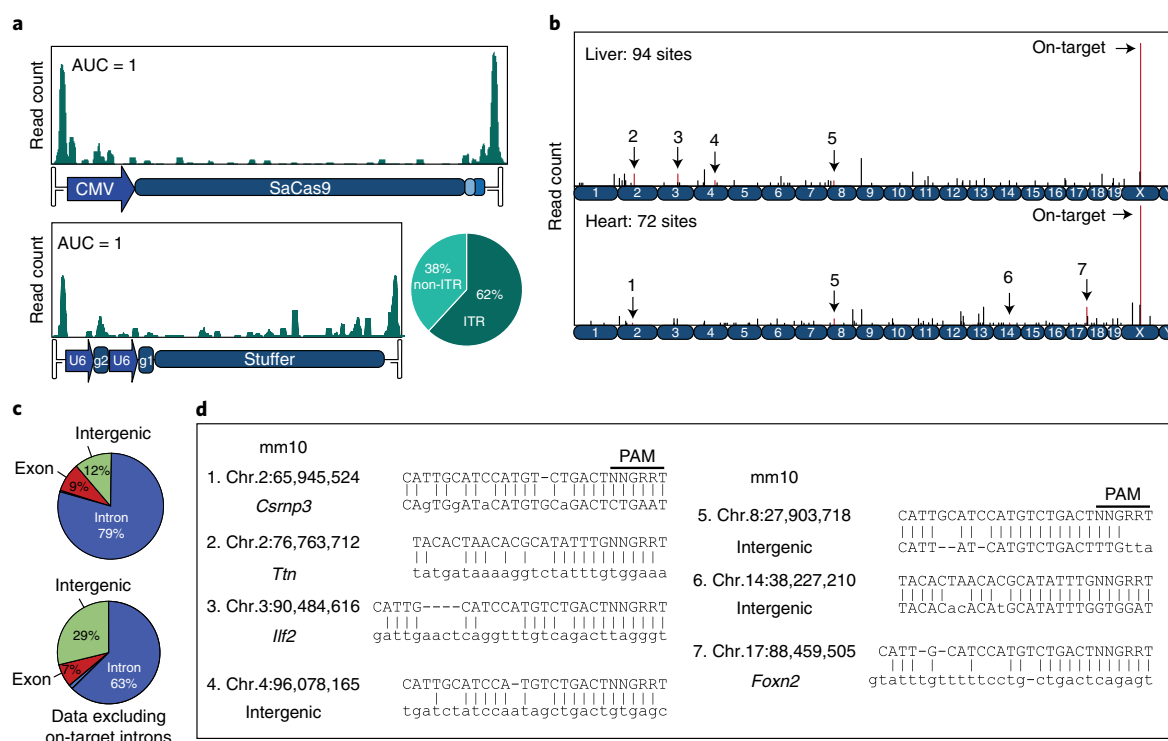


Fig. 4 | AAV integrations into the *Dmd* locus and genome-wide. a, AAV integrations were detected in the gRNA target sites. In total, 62% of integrations occurred within the ITRs and the remaining 38% occurred within the viral genome. AUC, area under the curve; g1, gRNA1; g2, gRNA2; stuffer, promoterless sequence included for AAV packaging. **b**, A similar approach was used to map AAV integrations genome-wide in both the liver and heart, 8 weeks after systemic administration in neonatal mice. The top hits include the two gRNA target sites flanking exon 23 of the *Dmd* gene, but numerous other AAV integration sites were detected. **c**, Genome-wide AAV integration sites were primarily located within the introns of genes. **d**, Possible sites of CRISPR off-target activity were identified by searching for sequences similar to the intended gRNA on-target site nearby the recovered genomic AAV integrations. PAM, protospacer adjacence motif.

to characterize safety and efficiency in larger animal models and mitigate the potential immune response will be crucial to translate this technology to treat genetic disease.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at <https://doi.org/10.1038/s41591-019-0344-3>.

Received: 7 August 2018; Accepted: 21 December 2018;

Published online: 18 February 2019

References

- Nelson, C. E., Robinson-Hamm, J. N. & Gersbach, C. A. Genome engineering: a new approach to gene therapy for neuromuscular disorders. *Nat. Rev. Neurol.* **13**, 647–661 (2017).
- Xu, L. et al. CRISPR-mediated genome editing restores dystrophin expression and function in *mdx* mice. *Mol. Ther.* **24**, 564–569 (2016).
- Long, C., Amoasii, L., Bassel-Duby, R. & Olson, E. N. Genome editing of monogenic neuromuscular diseases: a systematic review. *JAMA Neurol.* **73**, 1349–1355 (2016).
- Nelson, C. E. et al. In vivo genome editing improves muscle function in a mouse model of Duchenne muscular dystrophy. *Science* **351**, 403–407 (2016).
- Tabebordbar, M. et al. In vivo gene editing in dystrophic mouse muscle and muscle stem cells. *Science* **351**, 407–411 (2016).
- Bengtsson, N. E. et al. Muscle-specific CRISPR/Cas9 dystrophin gene editing ameliorates pathophysiology in a mouse model for Duchenne muscular dystrophy. *Nat. Commun.* **8**, 14454 (2017).
- Chew, W. L. et al. A multifunctional AAV-CRISPR–Cas9 and its host response. *Nat. Methods* **13**, 868–874 (2016).
- Flanigan, K. M. Duchenne and Becker muscular dystrophies. *Neurol. Clin.* **32**, 671–688 (2014).
- Hoffman, E. P., Brown, R. H. Jr. & Kunkel, L. M. Dystrophin: the protein product of the Duchenne muscular dystrophy locus. *Cell* **51**, 919–928 (1987).
- Chamberlain, J. R. & Chamberlain, J. S. Progress toward gene therapy for Duchenne muscular dystrophy. *Mol. Ther.* **25**, 1125–1131 (2017).
- Robinson-Hamm, J. N. & Gersbach, C. A. Gene therapies that restore dystrophin expression for the treatment of Duchenne muscular dystrophy. *Hum. Genet.* **135**, 1029–1040 (2016).
- Dunbar, C. E. et al. Gene therapy comes of age. *Science* **359**, eaan4672 (2018).
- Sharma, R. et al. In vivo genome editing of the albumin locus as a platform for protein replacement therapy. *Blood* **126**, 1777–1784 (2015).
- Laoharawee, K. et al. Dose-dependent prevention of metabolic and neurologic disease in murine MPS II by ZFN-mediated in vivo genome editing. *Mol. Ther.* **26**, 1127–1136 (2018).
- Amoasii, L. et al. Single-cut genome editing restores dystrophin expression in a new mouse model of muscular dystrophy. *Sci. Transl. Med.* **9**, eaan8081 (2017).
- Amoasii, L. et al. Gene editing restores dystrophin expression in a canine model of Duchenne muscular dystrophy. *Science* **362**, 86–91 (2018).
- Ran, F. A. et al. In vivo genome editing using *Staphylococcus aureus* Cas9. *Nature* **520**, 186–191 (2015).
- Kotterman, M. A. & Schaffer, D. V. Engineering adeno-associated viruses for clinical gene therapy. *Nat. Rev. Genet.* **15**, 445–451 (2014).
- Spencer, M. J., Montecino-Rodriguez, E., Dorshkind, K. & Tidball, J. G. Helper (CD4⁺) and cytotoxic (CD8⁺) T cells promote the pathology of dystrophin-deficient muscle. *Clin. Immunol.* **98**, 235–243 (2001).
- Kotterman, M. A., Chalberg, T. W. & Schaffer, D. V. Viral vectors for gene therapy: translational and clinical outlook. *Annu. Rev. Biomed. Eng.* **17**, 63–89 (2015).
- Brooks, A. R. et al. Transcriptional silencing is associated with extensive methylation of the CMV promoter following adenoviral gene delivery to muscle. *J. Gene Med.* **6**, 395–404 (2004).
- Charlesworth, C. T. et al. Identification of pre-existing adaptive immunity to Cas9 Proteins in humans. *Nat. Med.* <https://doi.org/10.1038/s41591-018-0326-x> (2019).
- Thakore, P. I. et al. RNA-guided transcriptional silencing in vivo with *S. aureus* CRISPR–Cas9 repressors. *Nat. Commun.* **9**, 1674 (2018).

24. Hu, C. & Lipshutz, G. S. AAV-based neonatal gene therapy for hemophilia A: long-term correction and avoidance of immune responses in mice. *Gene Ther.* **19**, 1166–1176 (2012).
25. Lee, E. K. et al. Long-term survival of the juvenile lethal arginase-deficient mouse with AAV gene therapy. *Mol. Ther.* **20**, 1844–1851 (2012).
26. Singh, K. et al. Efficient in vivo liver-directed gene editing using CRISPR/Cas9. *Mol. Ther.* **26**, 1241–1254 (2018).
27. Zhang, P. et al. Immunodominant liver-specific expression suppresses transgene-directed immune responses in murine Pompe disease. *Hum. Gene Ther.* **23**, 460–472 (2012).
28. Ferdosi, S. R. et al. Multifunctional CRISPR/Cas9 with engineered immunosilenced human T cell epitopes. Preprint at <https://www.biorxiv.org/content/early/2018/07/02/360198> (2018).
29. Nelson, C. E. & Gersbach, C. A. Engineering delivery vehicles for genome editing. *Annu. Rev. Chem. Biomol. Eng.* **7**, 637–662 (2016).
30. Giannoukos, G. et al. UDiTaTM, a genome editing detection method for indels and genome rearrangements. *BMC Genomics* **19**, 212 (2018).
31. Iyombe-Engembe, J. P. et al. Efficient restoration of the dystrophin gene reading frame and protein structure in DMD myoblasts using the CinDel method. *Mol. Ther. Nucleic Acids* **5**, e283 (2016).
32. Kosicki, M., Tomberg, K. & Bradley, A. Repair of double-strand breaks induced by CRISPR-Cas9 leads to large deletions and complex rearrangements. *Nat. Biotechnol.* **36**, 765–771 (2018).
33. Memczak, S. et al. Circular RNAs are a large class of animal RNAs with regulatory potency. *Nature* **495**, 333–338 (2013).
34. Cox, D. B., Platt, R. J. & Zhang, F. Therapeutic genome editing: prospects and challenges. *Nat. Med.* **21**, 121–131 (2015).
35. Miller, D. G., Petek, L. M. & Russell, D. W. Adeno-associated virus vectors integrate at chromosome breakage sites. *Nat. Genet.* **36**, 767–773 (2004).
36. Chandler, R. J. et al. Vector design influences hepatic genotoxicity after adeno-associated virus gene therapy. *J. Clin. Invest.* **125**, 870–880 (2015).
37. Barzel, A. et al. Promoterless gene targeting without nucleases ameliorates haemophilia B in mice. *Nature* **517**, 360–364 (2015).
38. Bell, P. et al. No evidence for tumorigenesis of AAV vectors in a large-scale study in mice. *Mol. Ther.* **12**, 299–306 (2005).
39. Donsante, A. et al. AAV vector integration sites in mouse hepatocellular carcinoma. *Science* **317**, 477 (2007).
40. Gil-Farina, I. et al. Recombinant AAV integration is not associated with hepatic genotoxicity in nonhuman primates and patients. *Mol. Ther.* **24**, 1100–1105 (2016).

Acknowledgements

This work has been supported by Sarepta Therapeutics, the Allen Distinguished Investigator Program through The Paul G. Allen Frontiers Group, the Muscular Dystrophy Association (grant MDA277360), a Duke–Coulter Translational Partnership Grant, a Duke/UNC–Chapel Hill CTSA Consortium Collaborative Translational Research Award, NIH grant R01AR069085, an NIH Director's New Innovator Award (DP2OD008586) and the Office of the Assistant Secretary of Defense for Health Affairs, through the Duchenne Muscular Dystrophy Research Program under awards W81XWH-15-1-0469 and W81XWH-16-1-0221. C.E.N. was supported by a Hartwell Foundation Postdoctoral Fellowship and the NIH Pathway to Independence Award (K99EB023979). J.N.R.-H. was supported by a National Science Foundation Graduate Research Fellowship and American Heart Association Predoctoral Fellowship (17PRE33350013).

Author contributions

C.E.N. designed and conducted experiments, Y.W. provided expertise on assays to measure immune responses including ELISpot, M.P.G. conducted experiments and mouse procedures, M.L.O. purified proteins and performed histological analyses, M.A.W. completed qPCR experiments, J.D.B. performed histological analyses, J.N.R.-H. performed creatine kinase assays, K.B. performed mouse procedures, R.M.C.R. produced recombinant AAV, A.A., J.H.C. and C.A.G. designed experiments, C.E.N. and C.A.G. wrote and revised the manuscript.

Competing interests

J.N.R.-H., C.E.N. and C.A.G. have filed patent applications related to genome editing for Duchenne muscular dystrophy. C.A.G. is an advisor for Sarepta Therapeutics, and a co-founder of and advisor for Element Genomics and Locus Biosciences. A.A. is a co-founder of and advisor for StrideBio.

Additional information

Extended data is available for this paper at <https://doi.org/10.1038/s41591-019-0344-3>.

Supplementary information is available for this paper at <https://doi.org/10.1038/s41591-019-0344-3>.

Reprints and permissions information is available at www.nature.com/reprints.

Correspondence and requests for materials should be addressed to C.A.G.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

© The Author(s), under exclusive licence to Springer Nature America, Inc. 2019

Methods

AAV preparation. SaCas9- and gRNA-containing AAV constructs were generated as previously described⁴. In brief, a SaCas9 expression plasmid with a CMV promoter containing ITRs and a plasmid containing two gRNA expression cassettes driven by the human U6 polIII promoter were used to prepare recombinant AAV8 and AAV9. ITRs were confirmed by SmaI digestion before AAV production. Multiple batches of AAV8 and AAV9 were produced and titers measured by qPCR as previously described.

In vivo administration of AAV-CRISPR. All experiments involving animals were conducted with strict adherence to the guidelines for the care and use of laboratory animals of the National Institute of Health (NIH). All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at Duke University. The mouse strain C57BL/10ScSn-Dmdmdx/J (*mdx*) was obtained from Jackson laboratories. C57BL/10 mice were used as a wild-type control. Adult 8-week-old male mice were administered locally into the tibialis anterior muscle with 30 μ l of 5.6×10^{11} vector genomes per vector per mouse. Adult 8-week-old mice were administered intravenously with 200- μ l injections of 2.7×10^{12} vector genomes per vector per mouse. Two-day-old (P2) neonatal mice were administered intravenously through the facial vein⁴¹ with 5.4×10^{11} vector genomes per vector per mouse. At set time points (Fig. 1a), mice were euthanized and the organs collected for experiments involving multiple skeletal muscles, cardiac muscles, other organs and serum.

Genomic DNA extraction and next-generation sequencing. Genomic DNA was extracted from various mouse tissues at defined time points by digestion in proteinase K and ALT buffer at 56 °C overnight while shaking. DNA was further extracted with the DNeasy kit (Qiagen). Endpoint PCR to confirm deletion was performed with AccuPrime Polymerase (Invitrogen) using primers DMDin22F and DMDin23R (Supplementary Table 8). Indel formation was detected by next-generation sequencing. Amplicons were produced by PCR using AccuPrime Polymerase (Invitrogen) and a series of primers for each locus (Supplementary Table 8). A second short-cycle PCR was used to add Illumina flowcell-binding sequences and experiment-specific barcodes (Supplementary Table 8). The resulting PCR products were sequenced with 150-bp paired-end reads on an Miseq instrument (Illumina). Indel analysis was performed using a local distribution of CRISPResso Pooled⁴² using a 5-bp window and standard settings.

Transposon-mediated target enrichment and sequencing. Using a Nextera Tn5 transposon (Illumina), 100 ng to 1 μ g genomic DNA was tagged following the manufacturer's instructions, except the transposon was diluted 1:8 from specifications to encourage large fragment size (Extended Data Fig. 9). To enrich the targeted sequence, a single PCR reaction using a genome specific primer (DMDin22-Nextera-F or DMDin23-Nextera-R) was used paired with a reverse primer specific for the tag sequence inserted by the transposon (Nextera-R) for 25 cycles. Amplicons were purified with Ampure beads (Beckman Coulter) at 1.8 $\times$. A short 10-cycle PCR was used to add experimental barcodes and Illumina adapter sequences. Amplicons were gel-purified selecting the fragment size shown in Extended Data Fig. 9. Sequencing was conducted on an Illumina Miseq using v.2 chemistry and 2 $\times$ 150-cycle paired-end reads. Analysis was performed by aligning amplicons to the targeted locus and discarding misprimed sequences. Targeted amplicons ranged from 5 to 40% depending on the primer used (Extended Data Fig. 9). Reads were then aligned to the expected products, including deletions, inversions, AAV integrations and genome-wide translocations. Alignments to the AAV genome (Fig. 4a) used the NeedlemanWunsch algorithm with a GapOpenValue of 10. Some reads within the AAV ITRs were ambiguous and were randomly assigned to one of the two ITRs for alignment. This Nextera-based method is expected to reduce PCR-related bias from amplicon size; however, some bias may remain for the transposon selectivity³⁰.

Transcript evaluation and sequencing. RNA was extracted using the Qiagen Universal kit. Subsequently, 1 μ g was used to perform First-strand cDNA synthesis using the Vilo kit (ThermoFisher) according to the manufacturer's instructions. cDNA was diluted 1:4 in ultrapure water, aliquoted and stored for further analysis. For transposon-based sequencing, second-strand cDNA synthesis was performed using Klenow fragment DNA polymerase (NEB). cDNA was treated with the Nextera Tn5 transposon at 1:8 the recommended concentration. Enrichment of the target transcript was performed by PCR using transcript-specific primers (Ex22-Nextera-F or Ex24-Nextera-R) and a constant reverse primer specific to the tag inserted by the transposon (Nextera-R). Amplicons were purified by Ampure beads at 1.8 $\times$ and a second 10-cycle PCR was used to add adapters and barcodes. Reads were aligned to predicted amplicons and mis-aligned reads were discarded. Reads were then aligned to expected products and unexpected products were identified and quantified. qPCR was conducted using QuantaBio PerfeCTa SYBR Green SuperMix using the primers listed in Supplementary Table 8.

Western blot. Frozen muscle biopsies were disrupted with mortar and pestle and suspended in RIPA buffer (Sigma) with a proteinase inhibitor cocktail (Roche) and incubated for 30 min on ice with intermittent vortexing. Samples were

centrifuged at 16,000g for 30 min at 4 °C and the supernatant was isolated and quantified with a bicinchoninic acid assay (Pierce). Protein isolates were mixed with NuPAGE loading buffer (Invitrogen) with 5% β -mercaptoethanol and boiled at 100 °C for 10 min. Samples were flash-frozen in liquid nitrogen and stored at -80 °C. Subsequently, 25 μ g of total protein per lane was loaded into a 10 well 4–12% NuPAGE Bis-Tris gel (Invitrogen) with MES buffer (Invitrogen) and electrophoresed for 30 min at 200 V. Proteins were transferred to a nitrocellulose membrane for 1 h at 400 mA at 4 °C in transfer buffer containing 1 $\times$ Tris-glycine, 10% methanol and 0.01% SDS. The blot was blocked overnight in tris-buffered saline with 0.1% Tween 20 (TBST) with 5% milk at 4 °C. The blot was probed with MANDYS8 (1:200, Sigma D8168) and rabbit anti-GAPDH (1:5,000, Cell Signaling 2118S). The blot was washed with TBST and probed with mouse or rabbit horseradish peroxidase-conjugated secondary antibodies (Sigma) for 30 min in 5% milk-TBST. Blots were visualized using Western-C ECL substrate (Biorad) on a ChemiDoc chemiluminescent system (Biorad). The full blots are shown in the Source data associated with this paper.

Immunofluorescence staining. Skeletal and cardiac muscles were dissected and embedded in OCT using liquid nitrogen-cooled isopentane and stored at -80 °C. Subsequently, 10 μ m sections were cut onto pretreated histological slides using a cryostat (Leica). Slides were washed in PBS and blocked in PBS supplemented with 5% FBS and 5% goat serum with 0.5% Triton X-100. Dystrophin was detected with MANDYS8 (1:200, Sigma D8168) in blocking buffer overnight at 4 °C. Slides were washed 3 $\times$ with PBS for 10 min and the secondary antibody was applied with DAPI (1:5000) for 30 min at room temperature. Slides were washed and mounted with ProLong Gold Antifade Mountant (Invitrogen) and imaged with an inverted microscope (Leica).

Creatine kinase assay. Serum creatine kinase was measured using a Liquid Creatine Kinase Reagent set (Pointe Scientific) following the manufacturer's instructions. In brief, 5 μ l of serum was diluted into 20 μ l of sterile PBS and incubated with reagent for 2 min and measured by absorbance every minute for three readings at 37 °C using a nanodrop spectrophotometer set for 340 nm readings. Calculations for total creatine kinase in U l⁻¹ were made according to the manufacturer's instructions and plotted relative to levels in serum samples from wild-type mice.

Recombinant SaCas9 production. A plasmid containing an IPTG-inducible bacterial SaCas9 expression cassette was transformed into Rosetta 2 cells (Millipore EMD) and plated on LB plates with 50 μ gml⁻¹ kanamycin and 30 μ gml⁻¹ chloramphenicol. Colonies were selected and grown in a starter culture overnight then in a 1 l culture for 4–6 h until the optical density at 600 nm reached 0.6–0.8. The temperature was reduced to 18 °C and induced with 0.2 mM IPTG and incubated for 12–16 h overnight. Cells were isolated and resuspended to 6 mlg⁻¹ in lysis buffer containing EDTA-free protease inhibitor cocktail (Roche), 1 mM PMSE and 1 mgml⁻¹ lysozyme (Sigma) and kept at 4 °C for the remainder of the protocol. Cells were sonicated 10 s on and 10 s off for 15 min. Cell debris was pelleted by centrifugation for 30 min at 16,000g at 4 °C. Protein was isolated with Ni-NTA agarose beads (Qiagen) following the manufacturer's instructions. DNA was removed with Sepharose (Sigma) collecting the protein flow-through. The protein was dialyzed overnight at 4 °C with 10 kDa MWCO dialysis tubing. Proteins were concentrated with Vivaspin 20 50 kDa MWCO spin filters (GE Healthcare). Samples were aliquoted, flash-frozen and stored at -80 °C. Proteins were analyzed by protein gel and western blot (see Source data).

Antibody ELISA. Antibodies against SaCas9 were detected by adapting previously published protocols^{7,43}. In brief, recombinant SaCas9 protein was diluted in 1 $\times$ coating buffer (KPL) and used to coat a 96-well Nunc MaxiSorp plate with 0.5 μ g of protein per well. Proteins were incubated overnight at 4 °C to adsorb to the plate. Plates were washed three times 5 min each with 1 $\times$ wash buffer (KPL). Plates were blocked with 1% BSA blocking solution (KPL) for 1 h at room temperature. Standard curves for IgG were generated using an anti-SaCas9 antibody (Diagenode C15200230). Serum samples were added in dilutions ranging from 1:40 to 1:20,000 and plates were incubated for 5 h at 4 °C with shaking. Plates were washed three times 5 min each and 100 μ l of blocking solution containing goat anti-mouse IgG (Sigma 1:4,000) was added to each well and incubated at 1 h at room temperature. Plates were washed four times 5 min each and 100 μ l of ABTS ELISA HRP substrate (KPL) was added to each well. Optical density at 410 nm was measured with a plate reader.

T cell ELISpot. T cell ELISpots were performed as previously described⁴⁴. In brief, splenocytes from AAV9-SaCas9-injected mice were isolated and purified using Lympholyte M (Cederlane). Then, 250,000 cells were mixed with either Cas9 protein (Applied Biological Materials) or cell medium only as a negative control, and subsequently plated in a 96-well ELISpot plate (Millipore, MSIPS4510) in 100 μ l per well. Stimulation was performed with 0.02 μ gml⁻¹ of Cas9 protein at 37 °C in a humidified incubator, 7% (v/v) CO₂, for 40 h. Mouse IFN γ ELISpot pairs (3321-3-250 and 3321-6-250) and streptavidin-alkaline phosphatase (3310-10) were purchased from Mabtech. Spots were developed using substrate Sigmafast

BCIP/NBT (Sigma, B5655). Plates were shipped to Zellnet Consulting and enumerated using a Zeiss KS ELISpot system. Full plates images are shown in Extended Data Fig. 4.

Statistical methods and reproducibility. Single comparisons were carried out using a MannWhitney *U*-test with *P* values reported in the figures (Figs. 1c,e and 2c,e). Multiple comparisons were made using Kruskal–Wallis test (Fig. 1i). Two-way ANOVA with post-hoc Tukey test was used to evaluate systemically treated groups with *P* values reported in the figures (Figs. 1d,f and 2d,f). qPCR for gene expression was evaluated by *t*-test with Holm–Bonferroni multiple comparison correction (Extended Data Fig. 4). All plotted dots are independent biological replicates (individual mice).

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

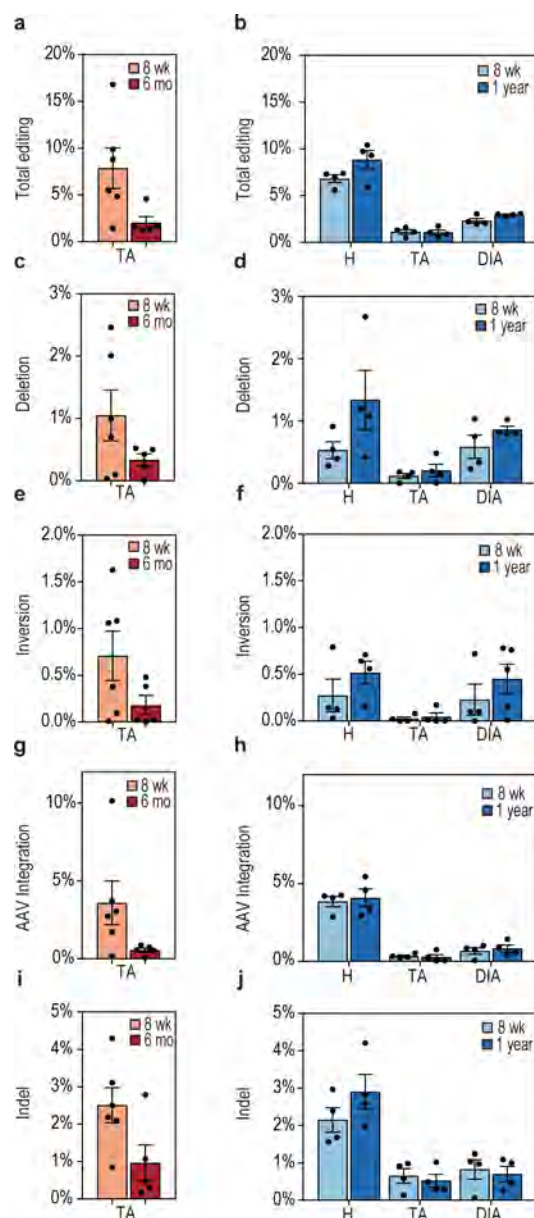
Data availability

All custom code for reproducing Figs. 1c,d, 3b,d, and 4a–d have been made available online (<https://github.com/chrisnelsonlab/CRISPR-Tn5/>). All sequencing data used in this study have been deposited in the National Center for

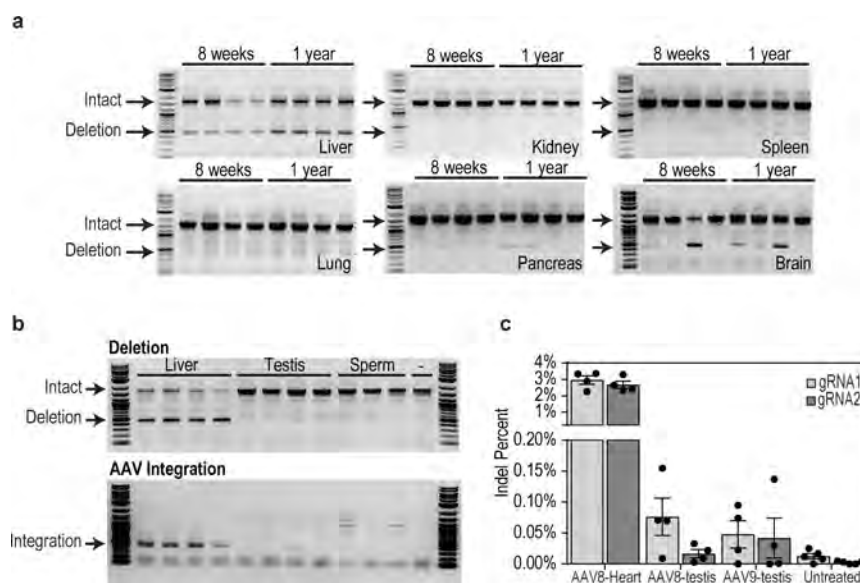
Biotechnology Information Sequence Read Archive (SRA) database (SRP157083). Full uncropped gels are included as Source data. All other relevant raw data are available from the corresponding author on request.

References

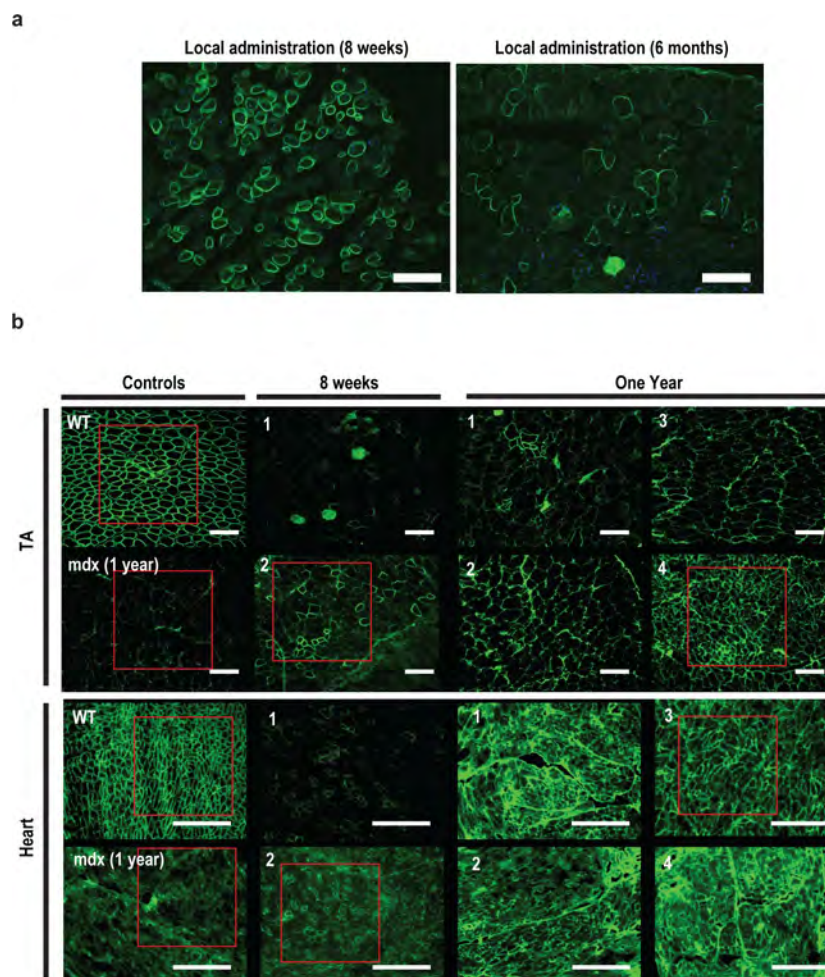
41. Gombash Lampe, S. E., Kaspar, B. K. & Foust, K. D. Intravenous injections in neonatal mice. *J. Vis. Exp.* **93**, e52037 (2014).
42. Pinello, L. et al. Analyzing CRISPR genome-editing experiments with CRISPResso. *Nat. Biotechnol.* **34**, 695–697 (2016).
43. Wang, D. et al. Adenovirus-mediated somatic genome editing of *Pten* by CRISPR/Cas9 in mouse liver in spite of Cas9-specific immune responses. *Hum. Gene Ther.* **26**, 432–442 (2015).
44. Chen, J. et al. The use of self-adjuncting nanofiber vaccines to elicit high-affinity B cell responses to peptide antigens without inflammation. *Biomaterials* **34**, 8776–8785 (2013).
45. Zincarelli, C. et al. Analysis of AAV serotypes 1–9 mediated gene expression and tropism in mice after systemic injection. *Mol. Ther.* **16**, 1073–1080 (2008).
46. Crooks, G. E., Hon, G., Chandonia, J. M. & Brenner, S. E. WebLogo: a sequence logo generator. *Genome Res.* **14**, 1188–1190 (2004).



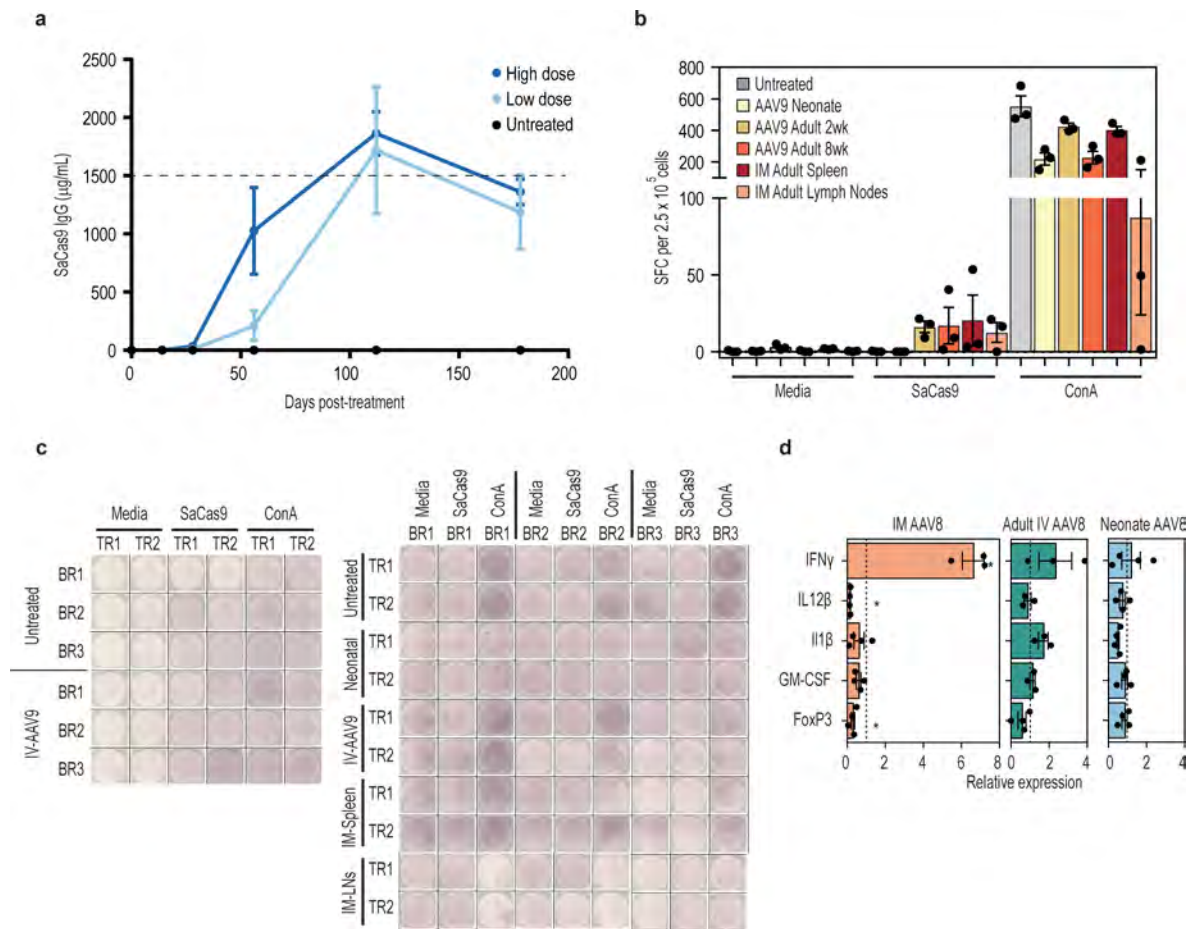
Extended Data Fig. 1 | Illumina Nextera-based unidirectional sequencing shows diverse genome changes including deletions, AAV integration, inversions and indel formation. **a**, Total editing for local administration. **b**, Total editing for systemic administration in neonates. **c,d**, Deletion frequency. **e,f**, Inversion frequency. **g,h**, AAV integration frequency. **i,j**, Indel frequency. Data are mean $\pm$ s.e.m. Locally injected mice, $n = 6$; systemically injected mice, $n = 4$.



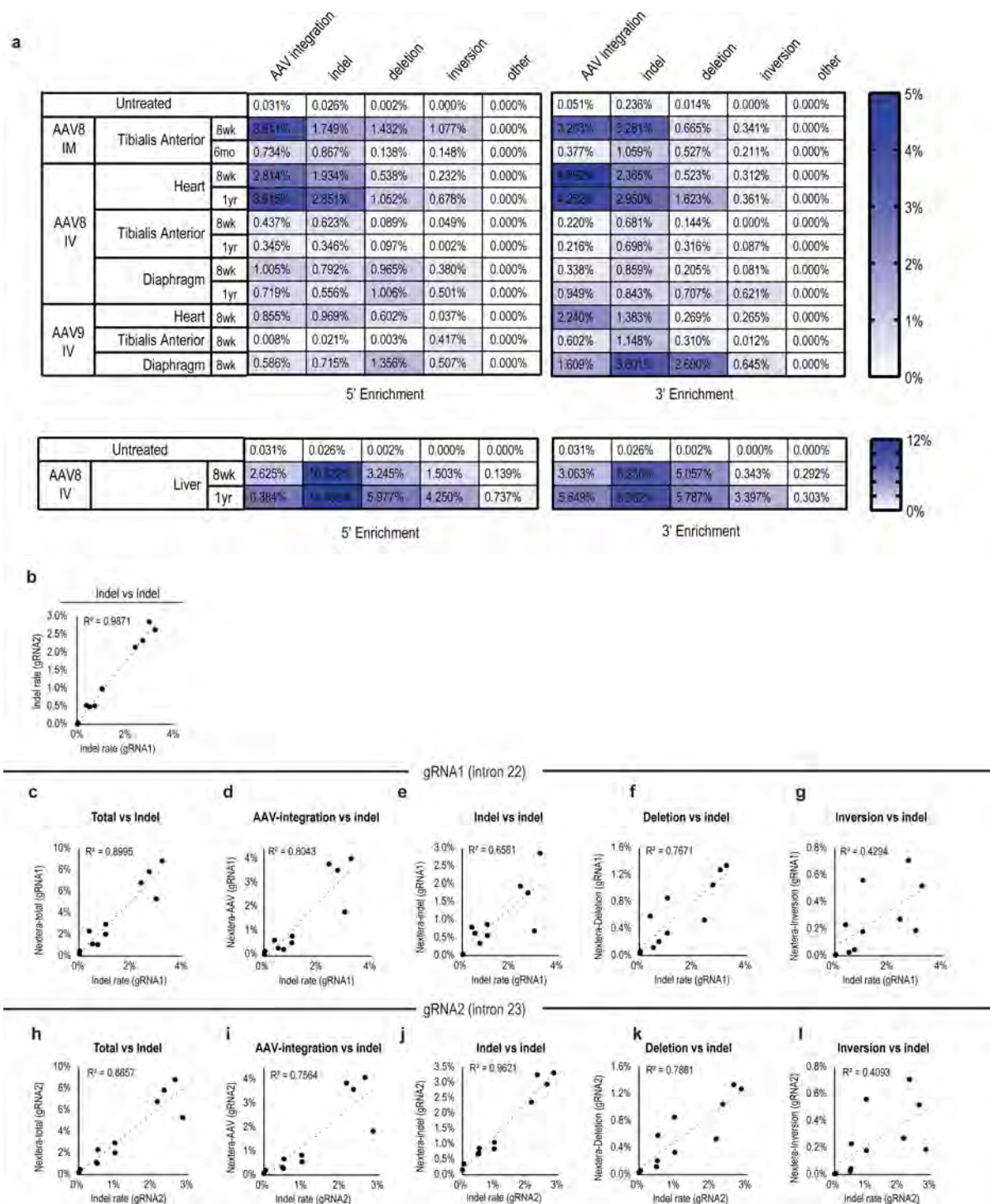
Extended Data Fig. 2 | On-target genome editing activity in somatic and germline tissues. a, Deletion PCR across the dystrophin locus shows Cas9 activity in multiple somatic tissues, including the liver, spleen, lung, pancreas and brain, which all show evidence of targeted gene deletion. There is no detectable deletion in the kidney samples. Cas9 expression is driven by a constitutive CMV promoter. This result is consistent with AAV8 tissue tropism⁴⁵. The off-target tissue-editing experiment was conducted once. **b**, Deletion PCR of genomic DNA from the testis is mostly negative and undetectable in sperm. AAV integration was only detected in one testis sample and no sperm samples. The germline experiment was conducted once. **c**, Deep sequencing of testis DNA indicates low levels of indel formation for both AAV8- and AAV9-injected mice that were injected as neonates. Data are mean $\pm$ s.e.m. $n = 4$ mice in all treated groups; $n = 5$ untreated mice.



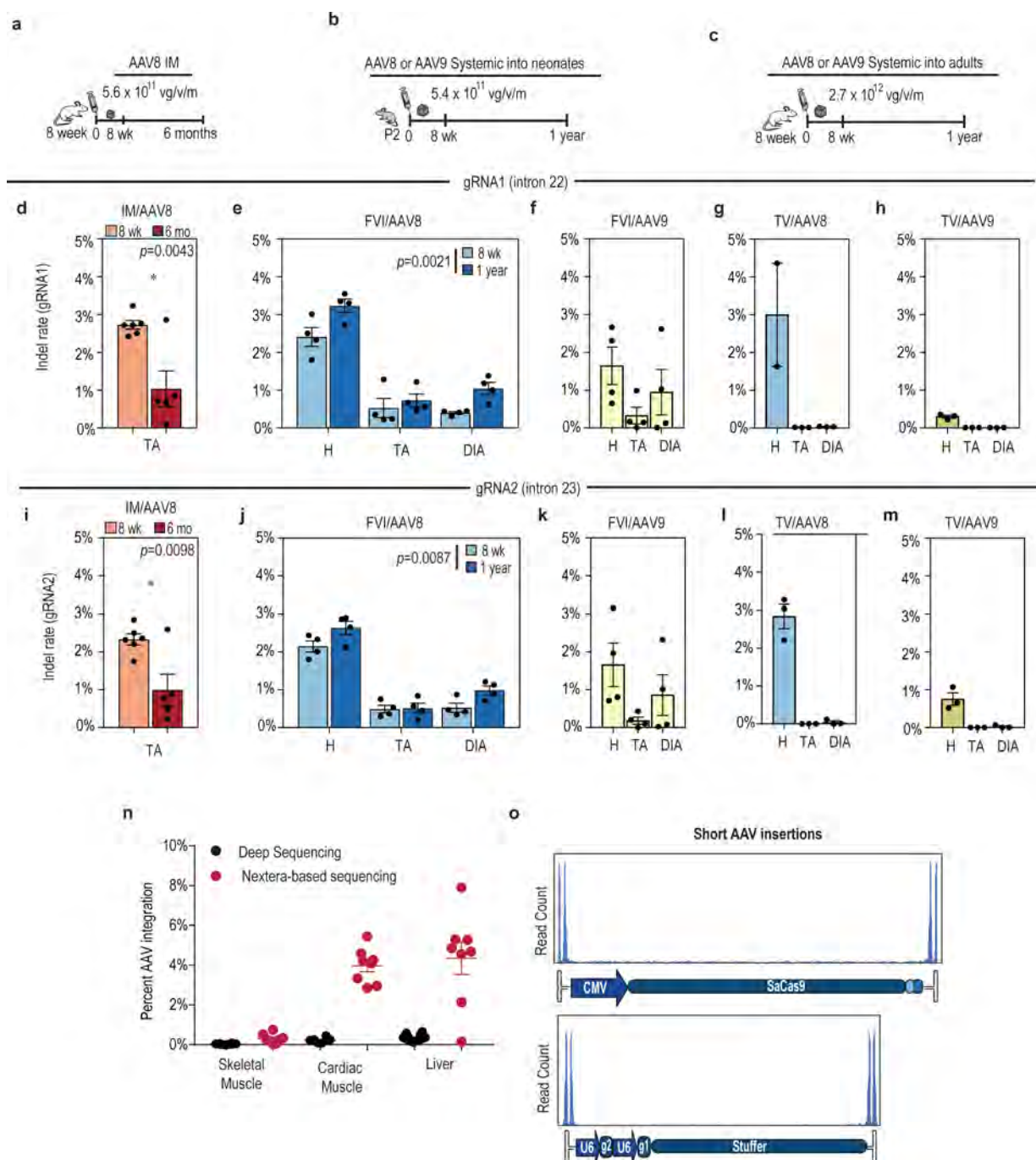
Extended Data Fig. 3 | Complete panel of histology shows a decrease in dystrophin staining following local administration and an increase in dystrophin at the 1-year time point in systemically treated mice. **a**, The histology images indicate a reduction in dystrophin after local injections, consistent with genomic and transcript data. **b**, Systemic samples show increased dystrophin expression after 1 year. Increased background at the 1-year time point may be a result of fibrosis in the tissue at the later time point. Representative images shown in Fig. 1 are highlighted in red. Scale bars, 200 μ m. Dystrophin-restoration experiments were conducted once for each treatment group.



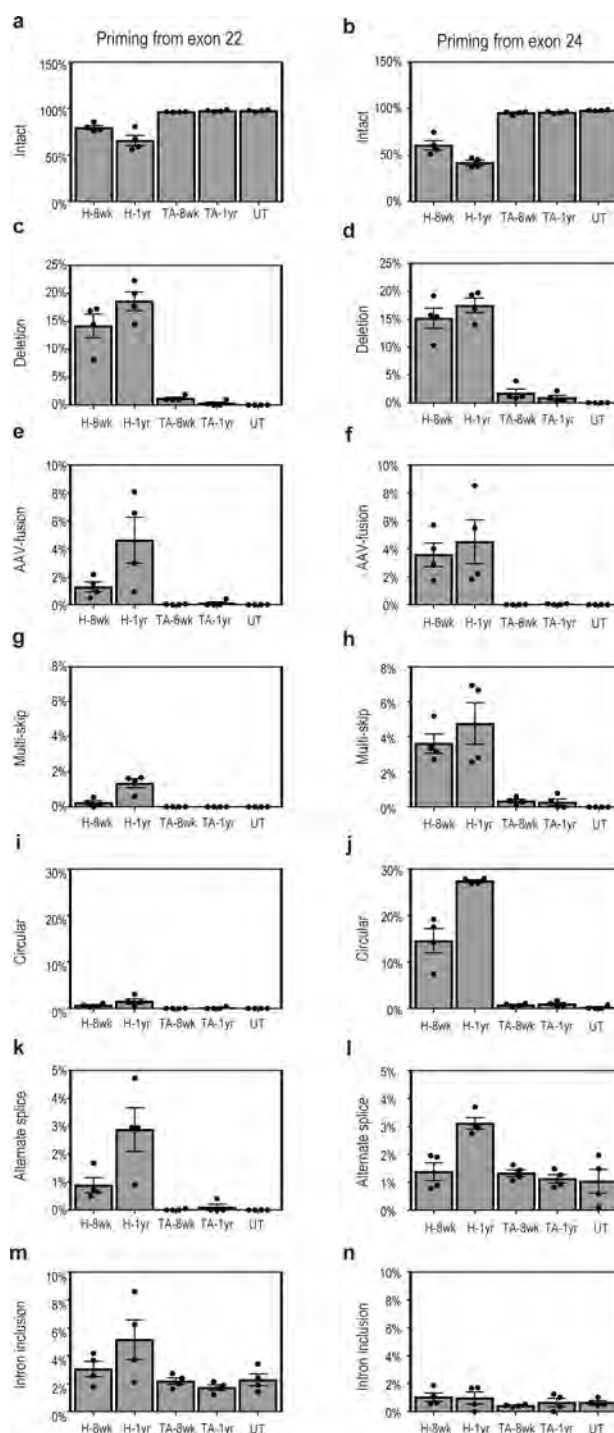
Extended Data Fig. 4 | AAV delivery of SaCas9 elicits humoral and cellular immune responses. **a**, Mice were administered with an AAV carrying a deactivated, nuclease-null SaCas9 transcriptional repressor (dSaCas9-KRAB) from a related study²³. Serum was collected at 4, 8, 16 and 26 weeks. The IgG response invariably developed by 8 weeks in all tested mice and continued to increase until the 16-week time point. Data are mean $\pm$ s.e.m. ($n=4$ individual mice). The dotted line indicates the end of the linear range of the standard. **b**, ELISpot shows T cell responses in treated adults but not neonates regardless of administration route. Spot-forming cells (SFCs) were detected in mice injected with AAV9-SaCas9 in adults at 2 weeks and 8 weeks after systemic administration, as well as 8 weeks after intramuscular injection. SFCs were not detected in mice treated as neonates. **c**, Complete image panel of ELISpot data. Two separate plates are shown. Data are mean $\pm$ s.e.m. $n=3$ individual mice, biological replicates (BR) were used with two technical replicates (TR) as individual isolates from each mouse. **d**, Mice administered locally show increased IFN γ and reduced FOXP3 and IL-12 β expression, whereas mice administered systemically as adults or neonates show no significant changes. Statistics calculated compared to untreated, t -test with Holm-Bonferroni multiple comparisons correction was used. $n=4$, IM-AAV8; $n=3$, IV-AAV8; $n=4$, Neonate-AAV8.



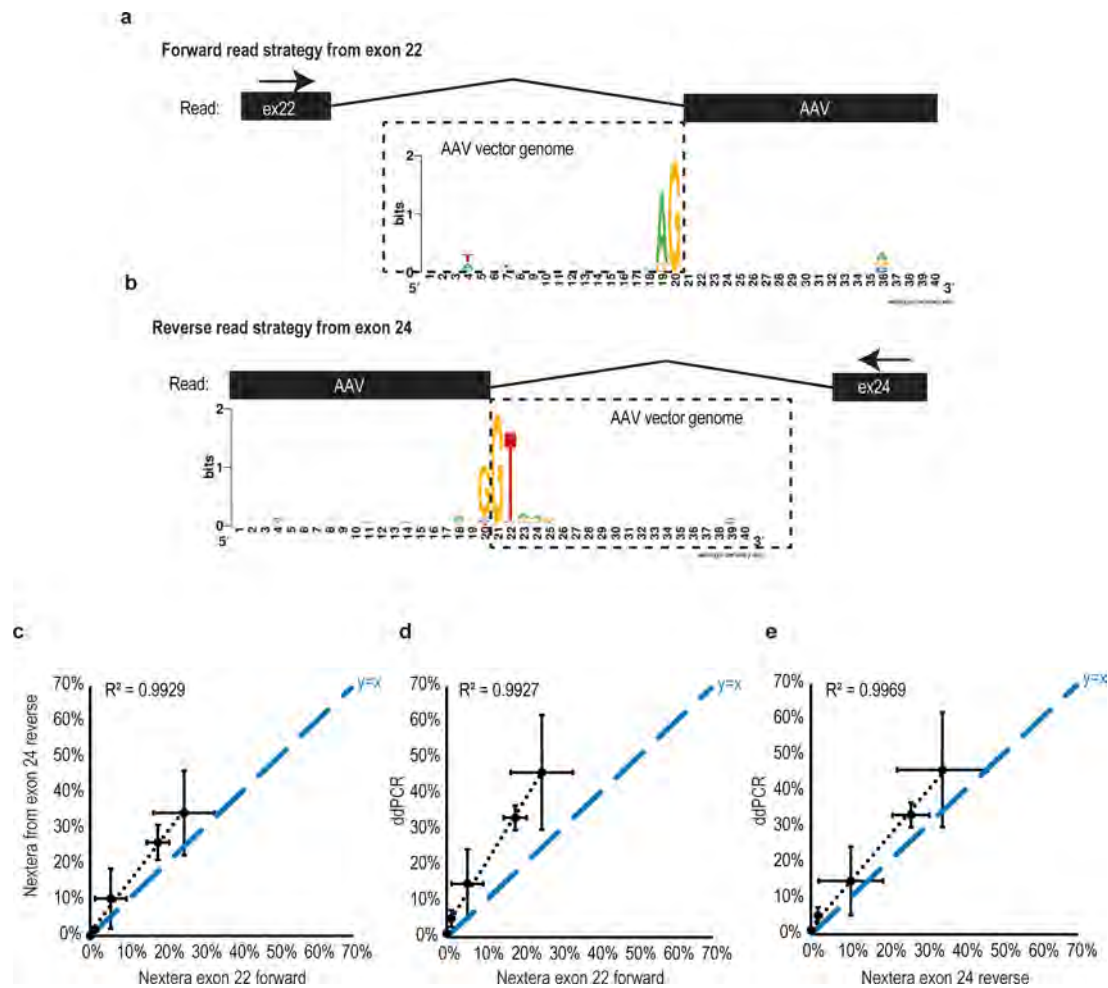
Extended Data Fig. 5 | Complete quantitative and reproducibility data for the Illumina Nextera-based unidirectional sequencing measures. Data associated with Fig. 3b. **a**, Quantitative data for genome-editing measurements are an average of $n = 4$ individual mice. Skeletal and cardiac muscle are shown on a separate scale from liver samples. **b**, Comparison of deep sequencing for both gRNAs. **c–g**, Comparison of indel rates for gRNA1 to identify alternate modifications. **h–l**, Comparison of indel rates of gRNA2 with alternate modifications. Rare events have poorer correlations. An estimated limit of detection is given based on the inversions detected that range between 0.1% and 0.2%. The limit of detection could be decreased with more input DNA and increased number of reads to detect more rare events, possibly including translocations.



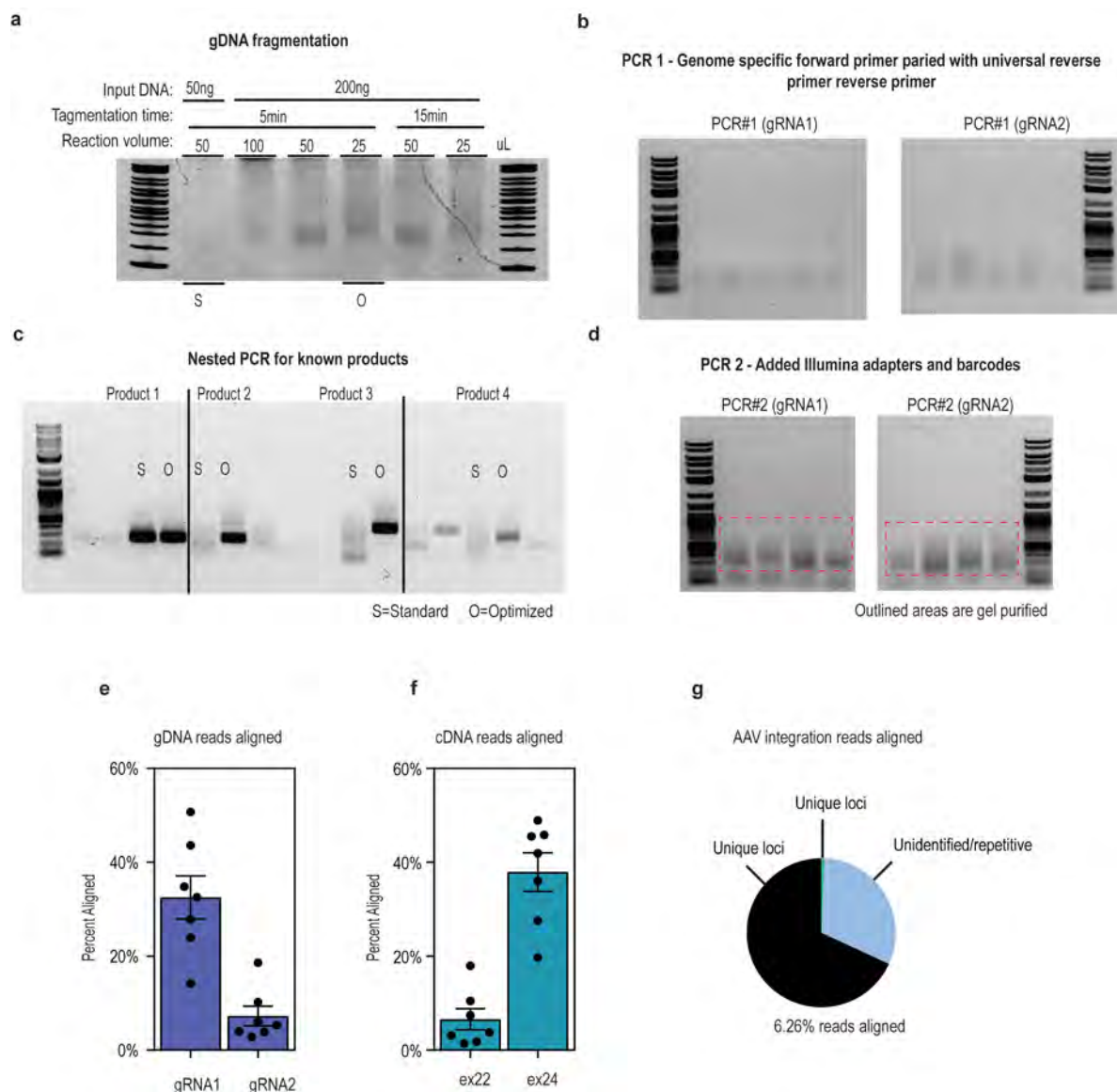
Extended Data Fig. 6 | Deep sequencing of target loci for gRNA1 and gRNA2 in multiple tissues and treatment routes show indel formation and short AAV insertions. **a**, Mice treated as 8-week-old adults by injection into the tibialis anterior were euthanized and tissues were collected at 8 weeks and 6 months after a single administration. **b**, Systemic administration in neonates by FVI of AAV8 or AAV9 was followed by analysis at 8 weeks and 1 year. **c**, Systemic administration in adults by tail-vein injection was followed by tissue collection at 12 weeks after the administration. **d**, Local administration with AAV8 ($n=6$, $n=5$, two-tailed t -test). **e**, Systemic administration with AAV8 in neonates ($n=4$, two-way ANOVA). **f**, Systemic administration in neonates with AAV9 ($n=4$). **g**, Tail-vein administration in adults with AAV8 ($n=3$). **h**, Tail-vein administration in adults with AAV9 ($n=3$). **i–m**, The same administration at the gRNA2 loci. **n**, Small AAV insertions were detected by deep sequencing for insertions that range from 10 to 45 bp in length. These insertions account for a small subset of integrations detected by Nextera-based sequencing. Nextera-based sequencing shows a higher detection rate of AAV genome insertions ($n=8$). **o**, Short AAV insertions detected by indel sequencing are almost exclusively located within the ITR regions of the AAV vector genome. Data are mean $\pm$ s.e.m.



Extended Data Fig. 7 | Illumina Nextera-based unidirectional sequencing of cDNA shows transcript changes over time in systemically administered neonates. There are notable differences in the number of circular RNA events and multi-skipping events when sequencing from the forward or reverse direction, which indicates that alternative splicing may be preferred in reverse direction. a-b) intact unedited transcripts, c-d) exon 23 deleted transcripts, e-f) AAV-fusion transcripts, g-h) transcripts with multiple skipped exons, i-j) circular transcripts, k-l) transcripts with alternative splicing, m-n) and transcripts with intron inclusion. Data are mean $\pm$ s.e.m. ($n=4$ for all samples).



Extended Data Fig. 8 | Nextera-based sequencing reveals dystrophin-AAV transcript fusions and is a reproducible method. **a**, Web logo map of nucleotide preference for splicing of dystrophin transcript to AAV vector genome shows canonical splicing is preferred. The forward read strategy priming from exon 22 shows that dystrophin-AAV splice fusions prefer an AG as the canonical splice acceptor. The sequencing read is shown as a black box. **b**, Similarly, the reverse priming strategy shows the preference for the canonical GT splice donor before the AAV-dystrophin fusion. The dotted-line box is not in the sequencing read so the AG or GT are revealed by alignment with the vector genome. Web logo maps were generated with the online tool⁴⁶ at <https://weblogo.berkeley.edu>. **c**, Deletions as measured by the Nextera method show a higher estimation by the reverse-sequencing method than the forward-sequencing method. **d,e**, ddPCR measures higher levels of gene deletion than either Nextera-based strategy. All comparisons were consistent ($R^2 > 0.99$). The right Nextera-based method had an order of magnitude higher read count and there is potential bias for transposon recombination. $y = x$ is plotted as a blue dotted line. Data are mean $\pm$ s.e.m., all data are from $n = 4$ mice.



Extended Data Fig. 9 | Optimization of the Nextera-based sequencing method. **a**, Multiple conditions were tested to find an optimized protocol. The standard method from the manufacturer's protocol is shown as 'S' and the optimized condition that we identified as 'O'. **b**, To test whether the random tagmentation works, a nested PCR was performed after the first PCR. The nested PCR used primer-binding sites of known amplicon size to detect the presence of expected products, including the unmodified target locus and the intended deletion. Only the optimized condition revealed all four predicted amplicons (5' and 3' enrichment for both products). We suspect that the standard condition generates fragments that are too short and lose the test-primer-binding site. By contrast, the optimized protocol generates longer DNA fragments that maintain the primer-binding site and would be better suited for unbiased sequencing. Optimization was performed only once. **c**, Gel showing amplicons after the first PCR. **d**, Gel showing amplicons after adding barcodes and adapters. Bands were purified within the outlined box. Gel images are representative of each sample analysed by deep sequencing ($n=3$ independent experiments). **e**, Each method shows varying quantities of reads aligned to the reference because of mispriming. gDNA reads show that the gRNA1-intron22 strategy had a higher percentage of reads aligned to the genome ($n=7$). **f**, The cDNA method shows a higher percentage of aligned reads for the exon 24 reverse priming strategy ($n=7$). **g**, Sequencing out of the AAV shows 6.26% of reads aligned to the reference with the majority of aligned reads consisting entirely of AAV vector episomes with no novel junctions, represented in black. In blue are reads that did not align to the mouse reference genome or were within repetitive regions that made identification impossible. Green labels indicate the 0.04% of reads that aligned to unique loci within the mouse genome. These reads are listed in detail in Supplemental Table 1, the majority of the reads are in the targeted *Dmd* locus. Data are mean $\pm$ s.e.m.

Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see [Authors & Referees](#) and the [Editorial Policy Checklist](#).

Statistical parameters

When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

n/a Confirmed

- ☐ ☒ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- ☐ ☒ An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- ☐ ☒ The statistical test(s) used AND whether they are one- or two-sided
Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- ☒ ☐ A description of all covariates tested
- ☐ ☒ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- ☒ ☐ A full description of the statistics including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- ☒ ☐ For null hypothesis testing, the test statistic (e.g. F , t , r) with confidence intervals, effect sizes, degrees of freedom and P value noted
Give P values as exact values whenever suitable.
- ☒ ☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- ☒ ☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- ☒ ☐ Estimates of effect sizes (e.g. Cohen's d , Pearson's r), indicating how they were calculated
- ☐ ☒ Clearly defined error bars
State explicitly what error bars represent (e.g. SD, SE, CI)

Our web collection on [statistics for biologists](#) may be useful.

Software and code

Policy information about [availability of computer code](#)

Data collection

Sequencing data was collected by illumina sequencing.

Data analysis

Targeted deep sequencing was analyzed through online software (CRISPResso available from <http://github.com/lucapinello/CRISPResso>). Further interpretation was made using a custom matlab pipeline. Nextera-based sequencing and AAV integration sequencing used custom matlab code to align all reads the genome and then to a list of the expected products. For genome-wide AAV integration, 3' ends of the reads were aligned to the mm10 genome using BLAT. Matlab-based code can be made available upon request and has been uploaded to <https://github.com/chrisnelsonlab/CRISPR-Nextera/>.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research [guidelines for submitting code & software](#) for further information.

Data

Policy information about [availability of data](#)

All manuscripts must include a [data availability statement](#). This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

Raw sequencing data has been deposited in the National Center for Biotechnology Information Sequence Read Archive (SRA) database (SRP157083). All other raw and interpreted data is either available in the supplemental information or upon request. Figure 3 and Figure 4 have data derived from SRP157083.

Field-specific reporting

Please select the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/authors/policies/ReportingSummary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	Sample size was determined based on our previous work for statistical significance. For biomolecular assays, n=4 was used as a minimum.
Data exclusions	Sequencing data - reads that did not align to the reference genome or expected products were excluded.
Replication	Multiple mice were examined for each endpoint. All findings can be reproduced with the same methodology in the multiple mice.
Randomization	Randomization was not used in this study. Mice were allocated into groups by cage or by litter in the case of neonatal injections. Covariates are not expected to affect dystrophin restoration in the mdx mouse. To help control for variation, all samples for each individual assay were processed by the same person.
Blinding	Single blinding was used for CK assay to eliminate potential bias in completing readings. Blinding was not necessary for other assays as bias cannot alter the outcome and all raw data is presented (i.e. gels/sequencing).

Reporting for specific materials, systems and methods

Materials & experimental systems

n/a	Involved in the study
☐	☒ Unique biological materials
☐	☒ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology
☐	☒ Animals and other organisms
☒	☐ Human research participants

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Unique biological materials

Policy information about [availability of materials](#)

Obtaining unique materials	Recombinant AAV used in this study can be produced commercially. Production of the AAV transfer plasmids is described in the methods and similar plasmids are available for purchased through Addgene. Specific plasmids used in this study can be made available upon request.
----------------------------	---

Antibodies

Antibodies used	SaCas9 antibody (Supplier: diagenode, catalog number: C15200230, clone name: S. aureus CRISPR/Cas9 monoclonal antibody,
-----------------	---

Dilution: 1:4000 WB).

Mandys8 (Supplier: Sigma, Catalog number D8168, clone name: Mandys8, Dilution: 1:200 WB and IF)

Validation

Both antibodies have validation on their website available below. Mandys8 has an extensive record. The SaCas9 antibody was tested in our lab by IF in cell culture and western blot from bacterial lysate and transfected cell culture recreating the validation listed on the diagenode website (see Supplemental Figure 20).

SaCas9 (<https://www.diagenode.com/en/p/s-aureus-crispr-cas9-monoclonal-antibody>)

Mandys8 (<https://www.sigmaaldrich.com/catalog/product/sigma/d8168?lang=en®ion=US>)

Animals and other organisms

Policy information about [studies involving animals](#); [ARRIVE guidelines](#) recommended for reporting animal research

Laboratory animals

The study involved laboratory animals.

(1) Species: Mouse

(2) C57/Bl6 wild type mice and mdx model of DMD

(3) Males were used owing to the X-linked nature of the gene but females were also used as breeding pairs or when gender was not determined when injecting neonatal mice.

(4) Mice from two days of birth to one year are reported

Wild animals

The study did not involve wild animals

Field-collected samples

The study did not involve field-collected samples

Review

“Of Mice and Measures”: A Project to Improve How We Advance Duchenne Muscular Dystrophy Therapies to the Clinic

First Workshop Report: Examining current
findings and opportunities around the emerging
D2.B10-*Dmd*^{mdx}/J (D2/*mdx*) model in context
of the classic C57BL/10ScSn-*Dmd*^{mdx}/J (B110/*mdx*)

Heather Gordish-Dressman^a, Raffaella Willmann^b, Laura Dalle Pazzi^c, Arati Kreibich^c,
Maaike van Putten^d, Ahlke Heydemann^e, Laurent Bogdanik^f, Cathleen Lutz^f, Kay Davies^g,
Alexis R. Demonbruen^h, Dongsheng Duanⁱ, David Elsey^j, So-ichiro Fukada^k,
Mahasweta Girgenrath^l, J. Patrick Gonzalez^m, Miranda D. Groundsⁿ, Andy Nichols^o,
Terry Partridge^a, Marco Passini^p, Francesca Sanarica^q, Frederick J. Schnell^p, Dominic J. Wells^f,
Toshifumi Yokota^s, Courtney S. Young^t, Zhong Zhong^u, Christopher Spurney^a, Melissa Spencer^t,
Annamaria De Luca^q, Kanneboyina Nagaraju^{v,1} and Annemieke Aartsma-Rus^{d,1,*}

^aChildren’s National Medical Center, Washington, DC, USA

^bBiozentrum, University of Basel, Basel, Switzerland

^cCharley’s Fund, New York, NY, USA

^dDepartment of Human Genetics, Leiden University Medical Center, the Netherlands

^eDepartment of Physiology and Biophysics, University of Illinois at Chicago, Chicago, IL, USA

^fThe Jackson Laboratory, Bar Harbor, ME, USA

^gDepartment of Physiology, University of Oxford, Anatomy and Genetics, Oxford, UK

^hCenter for Genetic Medicine, Northwestern University Feinberg School of Medicine, Chicago, IL, USA

ⁱDepartment of Molecular Microbiology and Immunology, Department of Neurology, Department of Biomedical Sciences and Department of Bioengineering, University of Missouri, Columbia, MO, USA

^jSummit Therapeutics, Abingdon, Oxfordshire, UK

¹Shared last authors.

*Correspondence to: Annemieke Aartsma-Rus, Department of Human Genetics, Leiden University Medical Center, the Netherlands. E-mail: a.m.rus@lumc.nl.

^kLaboratory of Molecular and Cellular Physiology, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan

^lRare Disease Research Unit, Pfizer, CA, USA

^mSolid Biosciences, Cambridge, MA, USA

ⁿSchool of Human Science, the University of Western Australia, Perth, Australia

^oCatabasis Pharmaceuticals, Cambridge, MA, USA

^pSarepta Therapeutics, Inc. Cambridge, MA, USA

^qDepartment of Pharmacy and Drug Sciences, Unit of Pharmacology, University of Bari “Aldo Moro”, Italy

^rDepartment of Comparative Biomedical Sciences, Royal Veterinary College, London, UK

^sDepartment of Medical Genetics, University of Alberta, Canada

^tDepartment of Neurology, Molecular Biology Institute, Center for Duchenne Muscular Dystrophy at UCLA, University of California, Los Angeles, CA, USA

^uWave Life Sciences, Cambridge, MA, USA

^vSchool of Pharmacy and Pharmaceutical Sciences, Binghamton University, New York, USA

Abstract. A new line of dystrophic *mdx* mice on the DBA/2J (D2) background has emerged as a candidate to study the efficacy of therapeutic approaches for Duchenne muscular dystrophy (DMD). These mice harbor genetic polymorphisms that appear to increase the severity of the dystropathology, with disease modifiers that also occur in DMD patients, making them attractive for efficacy studies and drug development. This workshop aimed at collecting and consolidating available data on the pathological features and the natural history of these new D2/*mdx* mice, for comparison with classic *mdx* mice and controls, and to identify gaps in information and their potential value. The overall aim is to establish guidance on how to best use the D2/*mdx* mouse model in preclinical studies.

Keywords: Duchenne muscular dystrophy, DMD, DBA/2J, *mdx*

BACKGROUND

Duchenne muscular dystrophy (DMD) is a severely progressive muscle-wasting disease, caused by mutations in the *DMD* gene, which abolish dystrophin production. Lacking this protein, muscle fibers are sensitive to contraction-induced damage, resulting in chronic inflammation, impaired regeneration and replacement of muscle tissue by fibrotic and adipose tissues. Patients become wheelchair-dependent around the age of 12, require assisted ventilation around the age of 20 and often die prematurely in the 2nd to 4th decade of life due to cardiac or respiratory complications. Currently, many therapeutic approaches are in preclinical and clinical development, and aim to restore the missing protein, to improve regeneration and muscle mass and/or reduce inflammation and fibrosis. However, thus far translating findings from preclinical models to patients is challenging, where findings in mouse models are often not fully recapitulated in DMD patients.

As we consider the body of existing data on two key (DMD) mouse models – the classic C57BL/10ScSn-*Dmd*^{*mdx*}/J (B110/*mdx*) [1] and the promising new

D2.B10-*Dmd*^{*mdx*}/J (D2/*mdx*) model [2, 3] – a need exists to clarify the field’s present understanding of best practices in preclinical research using these two models, in particular when to use which model and which outcome measures to select, depending on the objectives. Charley’s Fund, a foundation dedicated to accelerating the development of life-saving treatments for DMD, engaged leading experts from both academia and industry to establish a collaborative effort to address this issue. The founding objectives set forth included: 1) help establish a grounding perspective on the present state of the data; 2) align on best practices for selecting the best model for various types of interventions and appropriate outcome measures to assess therapeutic effects covering muscle function and pathology; 3) ensure that natural history of the new D2/*mdx* model is collected without gaps; and 4) help establish more rigorous selection criteria designed to improve decisions on when to graduate a therapy to human clinical trials.

It was acknowledged that success in achieving these objectives would require significant, ongoing work from the parties involved. As a key grounding step, however, a 1.5-day key stakeholder meeting

entitled “Of Mice and Measures: A Project to Improve How We Advance DMD Therapies to the Clinic” was organized in Paris, France on 2–3 October 2017 to discuss the available science around this topic and prioritize action steps to move the field forward to improved therapeutic testing.

PRE-WORKSHOP PREPARATION AND DATA COLLECTION

Charley’s Fund engaged several leading experts to facilitate this project in 2016, creating a Scientific Organizing Committee responsible for directing the project, including this workshop, in early 2017. This committee included Charley’s Fund personnel, academic and industry individuals who directly perform or oversee preclinical testing in their own laboratories, and a statistician experienced in handling complex datasets. Over the course of nearly a year leading up to the workshop itself, the members of the committee convened frequently by phone and email.

The goals of this project required the collection and organization of functional, histological and biomarker data on the B110/*mdx*¹ and D2/*mdx* murine models from several different sources. D2/*mdx* mice that were backcrossed for 5 generations (3) and intercrossed for more than 9 generations were used for most of the studies. In order to allow consolidation and comparison of data from different sources, a data collection template was developed and sent to each contributor. This template described the format of the data required, standardized the units of collection as much as possible, and provided directions to ensure that each contributor provided all necessary information.

Over a period of 3 months, data templates were populated by 10 contributors out of the several groups contacted by Charley’s Fund that work with D2/*mdx*. These included seven academic groups, two private industry groups, and one non-profit organization. The data included over 230 different outcome parameters collected on 650 individual mice and yielding nearly 18,000 individual data points for comparison (see Table 1).

Working groups were organized to assess the outcomes according to a few specialized areas and develop findings to be presented for discussion at the workshop. These working groups included genet-

ics, natural history of disease, histological markers, biomarkers, behavior and skeletal muscle strength outcomes, respiratory assessments, cardiac assessments, and therapeutic responses. Multiple rounds of analysis and discussion were done within each working group prior to the October 2017 meeting.

Based on the goals set out at the beginning of the project, several different statistical analyses were planned. These included an observational description of the data to assess the present state of the information, comparisons between the classic B110/*mdx* and the new D2/*mdx* model to define outcomes most sensitive to the disease model, and comparisons between therapeutic interventions to define outcomes most useful in the evaluation of future therapies. This ambitious project benefitted from the overall coordination of the processes by dedicated people from Charley’s Fund, the organized way in which the data were collected, and having the statistical analysis for each working group performed by the same statistician.

Many statistical models were used in the analysis of these data and they are not discussed in detail here, however, a few overarching principles were followed. Descriptive statistics were presented collectively and stratified by mouse strain and by data source. For some outcomes collected on mice over a wide range of ages, descriptive statistics were also stratified by age. For inferential statistics, normality of all continuous outcomes was assessed before analysis and data transformations were applied where applicable. If the normality assumption could not be met, non-parametric statistical tests were used. All analyses of repeated assessments made on the same mouse accounted for the dependence of the assessments using mixed effects linear regression models.

Several general observations were apparent from the start.

1. Many outcomes were collected from only one source, making the assessment of inter-laboratory reliability not yet possible for those outcomes. Many laboratories focus on one particular area of assessment, thus the outcomes submitted by each source primarily involved one body system and these differed between sources.
2. Several outcomes were assessed by multiple sources but different methodologies were used for collection. This was particularly true with muscle strength assessments, where data collection was done *in vivo* and *ex vivo* by different sources.
3. For behavioral outcomes the variability was higher, indicating the need to consider

¹ Of 650 individual mice, 29 *mdx* mice had a C57BL/6 background.

Table 1
Working groups and number of data points collected for this study

Class of readout	Readout	Data Data	Age range (weeks)	Strain measured				Contributors
				B110/ <i>mdx</i>	B110/ <i>wt</i>	D2/ <i>mdx</i>	D2/ <i>wt</i>	
Natural History	Lean and fat mass	394	6–12			*	*	Pfizer
	Body weight	2800	4–65	*	*	*	*	Aartsma-Rus, De Luca, Duan, Jax, Nagaraju, Pfizer, Sarepta, Spencer
	Tissue weights (excluding heart)	2135	4–65	*	*	*	*	Aartsma-Rus, De Luca, Duan, Nagaraju, Pfizer
	Tibia length	18	23–30			*	*	Duan
Functional	Wheel (voluntary and exhaustion)	318	8–58	*	*	*	*	De Luca, Nagaraju
	Open field (all assessments) ¹	376	8–65		*	*	*	Jax, Nagaraju, Spencer
	Grip strength (incl. normalized & hang test)	4142	4–65	*	*	*	*	Aartsma-Rus, De Luca, Jax, Nagaraju, Sarepta, Spencer
	Rotarod	612	6–65	*	*	*	*	Jax, Nagaraju, Sarepta
	Hindlimb sonography ²	72	28	*	*	*	*	De Luca
	Force (all muscles, all conditions)	1816	8–65	*	*	*	*	De Luca, Duan, Jax, Nagaraju, Pfizer
Cardiac	Functional assessments ³	761	16–58			*	*	De Luca, Duan, Nagaraju
	Size/weight assessments	288	4–65			*	*	Aartsma-Rus, Duan, Nagaraju
Histology	All assessments ⁴	1240	8–78			*	*	Aartsma-Rus, Duan, Jax, Nagaraju, Yokata
Respiratory	All assessments	1174	4–34			*	*	Aartsma-Rus, Spencer
Therapeutics	Gene expression in response to exon skipping	71	13	*		*		Aartsma-Rus
Biomarkers	CK	574	8–64	*	*	*	*	Aartsma-Rus, De Luca, Jax, Nagaraju
	LDH	76	9–64	*	*	*	*	De Luca
	Serum biomarkers ⁵	37	13–36			*	*	Duan, Nagaraju
	Dystrophin level	12	13	*		*		Aartsma-Rus
	Gene expression ⁶	939	10–34	*	*	*	*	Aartsma-Rus, Jax

¹Includes open field distance run, Digiscan measurements, and # of rearings. ²Includes volume and % vascularization. ³Shortening fraction, ejection fraction, stroke volume, and cardiac output also measured in B110 mice. ⁴Fiber area, fibrosis area, and % calcification also measured in B110 mice. ⁵Includes WBCs, macrophages, and neutrophils. ⁶Gene expression values from 12 genes in two different tissues (gastrocnemius and diaphragm).

operator-related issues and the need to follow standard operating procedures.

- The age of the mice from different sources varied considerably making direct comparisons between sources problematic.
- Data were not generated specifically for this project. Rather it was collected by each source from its own research studies. Thus, the majority of data collected were from mice included as part of drug studies but untreated with any active agent. This included both mice that had received a vehicle or sham treatment and mice that were completely untreated. The similarity of these two groups is unknown.

Given all of these observations, we generated descriptive statistics for all outcomes and performed

inferential statistical analysis where appropriate. The working groups generated presentations which included the results, notable findings and discussion points; these were presented to the group of key stakeholders at the workshop. After each working group presentation, a description of known issues, identification of additional questions, and conclusions based on the data presented were discussed in depth. The highlights of each working group are described below.

GENETICS OF THE D2/*mdx* MODEL

The D2/*mdx* carries the *mdx* mutation on the DBA/2J genetic background, which poses several advantages over the B110/*mdx* model. Several aspects

of the pathology match DMD patients' pathology, like the increased EBD-membrane permeability, fibrosis and TGF- β signaling, the impaired satellite cell activation and reduced regeneration [3]. Full genotyping of the D2/*mdx* background is still pending. However, it is already known that polymorphisms on the genes for latent TGF- β -binding protein 4 (*Ltbp4*) and annexin A6 (*Anxa6*) [4–6] respectively account for 40% and 7% of the phenotype in a mouse model for limb girdle muscular dystrophy type 2C (*Sgcg*) mice on a DBA/2J genetic background. The other 50% is unknown. The *Ltbp4* and *Anxa6* polymorphisms act in concert to modify muscle repair [7] and are thought to underlie the increased fibrosis and impaired regeneration in the D2/*mdx* model, respectively, while a variation in the *Abcc6* gene is responsible for the observed calcifications. Notably, the *Ltbp4* gene is also a modifier of human muscular dystrophy [8]. During breeding, genetic drift needs to be taken into account. The Jackson Laboratory advises refreshing in-house colonies every third generation.

While some natural history studies have been initiated with these mice, a lot of additional work is required to obtain a detailed picture of the natural history of disease. Furthermore, to determine the usefulness of the D2/*mdx* model for DMD therapeutic developments, the translatability of outcomes from mouse to human needs to be established.

It is important to be aware that wild type DBA/2J mice often have cardiac fibrosis and calcification, which poses challenges when using the D2/*mdx* model to study cardiac pathology, because it is not clear to which extent these are related to the lack of dystrophin or due to the DBA/2J background.

NATURAL HISTORY OF D2/*mdx* MICE

Life spans for animal models with dystrophin loss have been compared [9]. For DMD patients, life span has improved considerably in the last decade, due to improved pulmonary and cardiac care. For the D2/*mdx* life span, data up to 60 weeks of age are available and more is being collected.

Data on body weight of D2/*mdx* mice were collected from eight different laboratories. Body weights of D2/*mdx* mice were clearly lower than for B110/*mdx* and always lower than the wild type DBA/2J mice, in contrast with B110/*mdx* mice that tended to weigh more than their wild type strain (C57Bl/10Scsn) at all ages (Fig. 1). There was a difference of up to 10

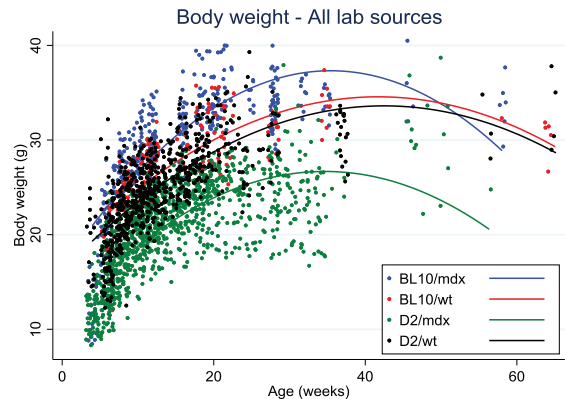


Fig. 1. Body weight distribution over age in B110/*mdx* (blue line), B110/wt (red line), D2/*mdx* (green line), D2/wt (black line). Data were collected from 8 labs.

grams between the body weights of D2/*mdx* from different sources that was seen with mixed genders as well as within males only. This variability may be due to husbandry, genetic drift or transportation stress if mice were purchased.

Skeletal muscle atrophy was observed in D2/*mdx* mice compared to DBA/2J wild types, which increased with age and was more apparent in bigger muscles than in small ones. D2/*mdx* mice had less fat and lean mass than DBA/2J wild types. Interestingly, spleen weight was higher in *mdx* as compared to wild type for both strains up to 30 weeks. Increased spleen weight probably reflects chronic muscle inflammation and muscle degeneration in these mice. Normalized heart weights were larger from a young age onwards in D2/*mdx* mice compared to DBA/2J wild type, and differences became more pronounced with increasing age. Functional heart deficits appeared earlier in D2/*mdx* than in B110/*mdx* and could possibly relate to the calcification process observed in all DBA/2J mice.

Serum creatine kinase (CK) levels decreased with age in both *mdx* mouse strains, similarly to what is observed in DMD patients [10]. This age-dependent decrease needs to be taken into account when CK is used to monitor the efficacy of a therapy that helps maintain muscle mass.

The forelimb grip strength normalized to body weight was higher in D2/*mdx* compared with B110/*mdx*. This may be due to the lower body weight observed in D2/*mdx* mice. The values of normalized grip strength tended to remain stable with age, and the difference to wild type was evident only for the B110 background. In DMD children, the loss of grip strength is more prominent than in *mdx* mice.

By contrast, the specific force, measured on the EDL muscle, was decreased in both *mdx* strains as compared to wild type, and B110/*mdx* specific force remains relatively constant with age although significantly lower than their wild type counterparts.

In summary, a certain variability was detected by comparing measurements from different labs, which may, next to husbandry conditions, be due to microbiota composition and nutritional aspects, instrument calibration and the genetic drift that occurs in the in-house colonies. In addition, the age range of D2/*mdx* mice likely to be influenced by treatments could not yet be defined.

HISTOLOGICAL MEASURES

Histology data were in general difficult to compare because labs collected different data and because the muscle pathology tended to be localized. The available data revealed that D2/*mdx* mice had fewer centronucleated myofibers than B16/*mdx* between 10–80 weeks of ages and that revertant fibers increased with age in B16/*mdx* but not in D2/*mdx* mice [11]. Both these parameters indicate less regeneration in D2/*mdx* compared with B16/*mdx* muscles and this was further supported by the D2/*mdx* mice having fewer eMHC positive fibers and BrdU positive nuclei. Terry Partridge presented data from his lab on BrdU incorporation as a measure of myogenic activity and showed that muscles D2/*mdx* began, like the original B110/*mdx*, to degenerate at 3 weeks of age, but did not start regenerating significantly over the subsequent two weeks, while B110/*mdx* had replaced 20–25% of muscle in the same period.

Fibrosis in D2/*mdx* was found to occur earlier in heart tissue (at 8 vs 36–40 weeks) and at higher levels in skeletal muscle tissue (for instance 20% vs 10% of total area at 10 weeks in the gastrocnemius) compared to B110/*mdx*. Calcification was more prominent in D2/*mdx* mice than in B110/*mdx* mice and both wild type strains. It affected skeletal muscles and heart to different extents and decreased in severity with age. The calcification was especially pronounced in the diaphragm of 8-week old D2/*mdx* mice, but reduced in area in adult (34 weeks) mice. This calcification at an early age (8 weeks) needs to be taken into account when measuring muscle function, but also when staining tissues, since it sequesters dyes and antibodies, resulting in very bright, aspecific staining of calcified areas. A longitudinal study and a better understanding of this pathological

event are needed. It was observed that calcification can be local and requires quantitation of the whole muscle cross-sectional area. Calcification does not occur in human dystrophic muscles, and over time fibrosis is replaced by adiposis in Duchenne muscles. In the first report describing D2/*mdx* mice, mice exhibit increased fat accumulation compared with B10/*mdx* mice [2]. However, no fat infiltration was observed in D2/*mdx* muscles in the frame of this work.

It is not known whether the reduced regeneration is due to an intrinsic problem of the satellite cells or to the elevated TGF β levels, or simply to decreased necrosis and increased fibrotic environment of the extracellular matrix. Increased levels of inflammation seen in D2/*mdx* [3] could also lead to impaired regeneration.

The use of Student's *t*-test to evaluate histological data and the tendency to consider histology as a quantitative measurement are questionable.

In general, it was noted that the picture of disease progression is not complete for D2/*mdx*: regeneration appears to be reduced in D2/*mdx* mice and fibrosis and calcification more prominent, especially at a young age (<12 weeks). At later time points the amount of fibrosis and calcification decreases, showing that regeneration does occur at some time. Clearly, further analysis is required to clarify the processes of necrosis, calcification, fibrosis and regeneration over time.

Miranda Grounds' group analysed the sciatic nerves innervating hind limb muscles, based on the hypothesis that the repeated cycles of myofibre necrosis and regeneration with associated disruption of neuromuscular junctions over many months, may result in denervation of myofibres with consequent changes in the sciatic nerve of dystrophic mice (it is noted that such changes become apparent in older normal mice after about 15 months of age (12)). Indeed, for male mice aged 13 months, the B110/*mdx* mice showed significant signs of denervation (tested as accumulation of S100 and tau protein in nerves), whereas this was neither seen in D2/*mdx* mice, nor in the wild type strains at this age. The simplest explanation is that the amount of ongoing necrosis of dystrophic muscles was much higher in the B110/*mdx*, compared with D2/*mdx*, mice. The new technology OPT (optical polarization tractography) allows to reconstruct organization of a whole fiber and may be useful to look deeper at all histological aspects, especially in mixed fast/slow muscles like the tibialis anterior [13, 14].

BIOMARKERS

D2/*mdx* mice showed higher inflammation than B110/*mdx* or wild type strains, as recorded by FACS analysis for total leucocyte and macrophage populations, inflammatory foci analysis and Cd68 qPCR, which decreased over time. The FACS analysis showed a CD3+ population in both D2/*mdx* and DBA/2J wild type at 10 weeks of age, but this population disappeared by 30 weeks of age. It was proposed that neutrophils should be quantified by peroxidase. Inflammation markers are relevant due to the current development of anti-inflammatory drugs, however, differences in mouse and human immune systems need to be considered, and the inflammatory process in D2/*mdx* is not yet fully understood.

Gene expression of fibrosis markers (*Tgf β* , connective tissue growth factor (*Ctgf*) and collagen) was increased in *mdx* compared to wild type and higher in D2/*mdx* than in B110/*mdx*, although not as high as one would expect based on histology. Levels were age dependent (values decreased in D2/*mdx* from 10 to 34 weeks) and muscle dependent, the diaphragm being more affected than the gastrocnemius. As mentioned, the time course of the fibrotic process, its interaction with calcification and the reason for the decline of TGF- β need to be better clarified in the D2/*mdx* mice.

Finally, CK values in blood were somewhat lower in D2/*mdx* than in B110/*mdx*, and tend to decrease with age, as also observed in DMD patients (Fig. 2). However, the variability is very high, and a range of sampling methods and protocols were used which precludes a direct comparison. It was suggested to test titin fragments in urine (mass-spectrometry, western blots, antibody stains) as an alternative marker for muscle pathology.

BEHAVIORAL ACTIVITIES AND SKELETAL MUSCLE FORCE MEASUREMENTS

The open field assessment records several parameters (total distance travelled, horizontal and vertical activity, number and time of movements, number of rearings and resting time) and showed consistent differences between *mdx* and wild type mice in both strains. However, the total distance travelled and the number of rearings was lower in D2/*mdx* than in B110/*mdx*. This strain-specific difference needs to be clarified and better assessed over time. Therefore, this

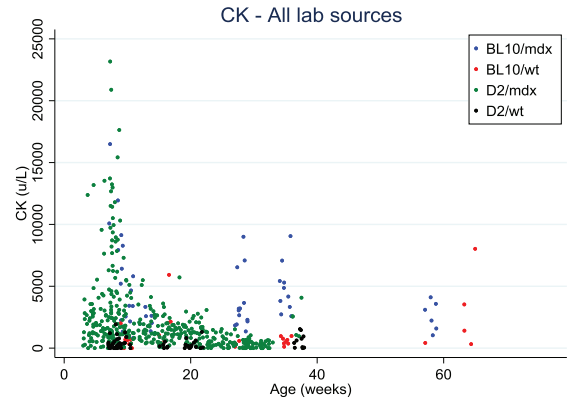


Fig. 2. Creatine kinase values distribution over age in B110/*mdx* (blue dots), B110/wt (red dots), D2/*mdx* (green dots), D2/wt (black dots). Data were collected from 4 labs.

assessment remains valuable for longitudinal, proof-of-concept studies but should be used in combination with behavioral, physiological and morphological measures in efficacy studies. The voluntary running wheel test and the exhaustion test with treadmill running showed differences in D2/*mdx* as compared to wild type, but there are still not enough data to support strain differences. The hanging wire test showed similar performances in *mdx* from both strains but a certain variability was present that could be partially explained by gender differences: male mice tend to perform less well than female mice and would be preferred in preclinical tests. Behavioral end points such as the hanging wire test are often influenced by many variables including sex, handling, time of testing etc.

As a general conclusion, fatigability tests are considered disease-relevant, given that in DMD patients, composite functional clinical readouts, such as 6-minute walk test or others, are accepted for approval of therapeutics. There are still a few issues that need further exploration, such as the progress of performance over time and possible correlations with reabsorption of calcifications and with fibrosis, the impact of exercise on pathology progression and the possible effects of drugs on the central nervous system with implications for volition and motivation. Furthermore, there is a clear need of consensus on best protocols to be used and on the appropriate method to acquire data: time-to-event representations on Kaplan-Meier diagrams may be more informative despite individual variability.

Grip strength declined with time for both *mdx* mouse strains as compared to wild type. However,

body weight increased in B110/*mdx* and decreased in D2/*mdx* mice over time making normalization to body weight a matter of debate, since it affects all normalizations of functional parameters. One option is to look at changes over time without normalization; another option, with a good sample size, is to adjust for the variant body weight and assess the functional parameter, if the variability is not too high. Therefore, although being a patient-relevant outcome, the grip strength is not recommended as primary outcome in mice, and should instead be used in combination with more direct muscle physiology assessments. Moreover, despite the high amount of data collected, the variability between labs and between operators was extremely high and will always represent an issue with this measurement. Grip strength remains an interesting parameter for longitudinal studies in mice, but ideally should be assessed by a single operator within an experiment.

Skeletal muscle force can also be measured directly with three methods: i) *In vitro/ex-vivo* method on isolated muscle, that assesses muscle performance without the interference of neural and vascular input; ii) *In situ* terminal method, that maintains physiological neural and vascular supply; iii) *In vivo* non terminal torque measurements, that record force from a group of muscles stimulated transdermally via the nerve and offers the advantages of maintaining physiological conditions, being minimally invasive and allowing multiple assessments in the same animal. For the *ex vivo* assays (i), *mdx* mice were weaker than wild type in both strains, and D2/*mdx* tended to be weaker than B110/*mdx* mice. EDL and diaphragm muscle force can be recommended as primary output, diaphragm data being useful for respiratory readouts. However, a parallel comparison of the two strains in the same laboratory over time is pivotal. For the *in situ* method (ii) on tibialis anterior, very limited data were available and no comparison between strains could be made. For the *in vivo* non-terminal torque assessment (iii), no standardized protocol is available; initial data suggest that D2/*mdx* were weaker than age-matched B110/*mdx*, but longitudinal studies will be needed. In general, muscle physiology assessments are considered valid as primary outcomes, they may detect drug effects that are not morphologically visible. However, further studies are needed to quantify the magnitude of strain difference, the age-dependent variations and the degree and progression of diaphragm impairment.

DIAPHRAGM AND RESPIRATORY PARAMETERS

Data pooling of respiratory function analyses was impossible due to the diversity in protocols used. In D2/*mdx* mice, the pulmonary function was reduced but the response to CO₂ challenge was not as strong as seen with B110/*mdx* mice, if the reduced weight of the D2/*mdx* strain was not accounted for. If the data were normalized, or if mice were tested at 32 weeks of age, this difference disappeared. This decline in function is relevant to DMD patients. However, the strain related differences need to be established. As described before, specific diaphragm force, measured by *ex vivo* analyses, decreased with age in B110/*mdx* and the drop in force was comparable in D2/*mdx* mice. This outcome is measured post-mortem and has a limited translatability, but is considered reproducible if standardized protocols are used.

The fibrosis and calcification of the diaphragm observed in D2/*mdx* mice were extensive and more pronounced than in other muscles: however, as in other muscles, the calcification decreased with age.

CARDIAC PARAMETERS

The most clinically relevant measurement on cardiac function is the shortening fraction, assessed by echocardiography in mice and patients; for mice, good standardized protocols (SOP: standardized operating procedure) exist and variability is small if these are followed. The ejection fraction is more difficult to measure clinically and in mice is a less reliable measure, most often calculated from M-mode measures [15, 16]. As measured using the TREAT-NMD/PPMD SOPs [17, 18], the shortening fraction was lower in D2/*mdx* than in its wild type counterpart aged 16–28 weeks, and this is normally seen in B110/*mdx* aged 36 to 52 weeks. However, the shortening fraction increased to normal levels at 52 weeks in the D2/*mdx* model [3]. The reason for this improvement in D2/*mdx* mice needs to be further elucidated, possibly heart wall thickness plays a role. Cardiac magnetic resonance (CMR) imaging was also used in the cardiac assessment of D2/*mdx* mice and demonstrated decreased function at 28 weeks of age [19]. Based on these measures, a mild cardiomyopathy occurs earlier in the D2/*mdx* compared to B110/*mdx* mice.

Other cardiac functional measures are: pressure/volume loops, (cardiac catheter assay) and ECG. The heart rate is increased in both *mdx* strains as compared to their wild type controls and is seen in patients with DMD. Fibrosis was evident in hearts of D2/*mdx* mice, as early at 8 weeks of age and, as expected, calcification was high in the 10-weeks-old heart of both *mdx* and wild type on DBA background, but absent on the B110 background. The etiology and significance of cardiac calcifications require further study. In summary, histological and biochemical assessments of fibrosis are recommended, but best methods need to be chosen. If possible, fibrosis quantification should be aligned with biomarkers or CMR of cardiac function or measurements of myocardial composition [19].

THERAPEUTIC INTERVENTIONS

In general, the D2/*mdx* mouse offers either a larger or smaller observational window to test effects of therapeutic interventions depending on the type of readout chosen. Therefore, which model is most relevant will differ based on the intervention that is studied.

Thus far, limited data are available on therapeutic interventions tested in the D2/*mdx* mouse model [20, 21]. A small pilot was performed comparing treatment with antisense oligonucleotides in D2/*mdx* and B110/*mdx* mice. There was no difference between B110/*mdx* and D2/*mdx* mice in exon skipping efficiency, suggesting that this model is suitable for future studies.

Natural history data are incomplete, the pathophysiology at early ages and the course of the processes of necrosis, regeneration, calcification and fibrosis need to be elucidated, which for now hampers designing optimal therapeutic intervention studies in the D2/*mdx* model. Such preclinical therapeutic studies should be run in parallel with vehicle-treated controls, and with a choice of outcomes not affected by intrinsic or operator-derived variability. Ideally, studies should be conducted in two independent labs. Treatments tested should target different pathways (for instance inflammation, muscle mass growth, dystrophin restoration, etc.).

RECOMMENDATIONS

From our efforts some points can be summarized:
1) Most reliable outcomes are muscle specific force

measures and open field; outcomes with caveats are grip strength and mouse fatigability, fibrosis and whole-body plethysmography with CO₂ challenge, cardiac outcomes; caution is needed with assays such as CK in blood and histological central nucleation. 2) Experimental conditions, especially mouse gender, power size per outcome, ages, vehicle use, best protocol but also husbandry and timing of measurements should be defined. 3) Blinded assessment is recommended especially when testing therapeutics in mice of the same genotype (undistinguishable by eyes) and especially for those methodologies which require a subjective role of the experimenter (i.e., behavioural tests, hand-made histology analysis, etc.). Additionally, data groups can be blinded for the statistical analysis. Existing TREAT-NMD standardized operating procedures can be selected and extended to the D2/*mdx* mouse model, others may need to be drafted ex novo.

It is recognized that a robust natural history data collection should represent a key achievement prior to testing therapies, and an appropriate location for a sharable database should be identified. In particular, more detailed longitudinal studies on the courses of necrosis/regeneration, calcification and fibrosis over time are needed; time points for histological data collection should be agreed upon (suggested: 2 wk, 4 wk, 6 wk, 12 wk, 6 mo, 1 year); cardiac pathology needs to be followed up after 24 weeks. For biomarkers, it seems more practical to choose clinically validated markers and test their preclinical translatability following treatment in mice. Furthermore, a minimum of criteria that need to be met for proof-of-concept versus preclinical studies should be defined.

As next steps, the following priorities were identified for the field and will be considered by the Organizing Committee. First, the existing TREAT-NMD SOPs need to be reviewed to assess their suitability for D2/*mdx* analysis and, if suitable, D2/*mdx* data need to be integrated. SOPs currently missing should be identified, and working groups to generate them should be established. Second, for the outcome measures identified as usable and reliable, data gaps should be filled. Ideally, the data should be collected in a concerted and independent experimental effort of laboratories using standardized and complementary approaches. Interested stakeholders will be needed for funding to support this time- and resource-intensive action. Such an effort will help to enable the compilation of a natural history database that hosts datasets for D2/*mdx* and B110/*mdx* mice to

benefit the scientific community (both academic and private) interested in translational research in DMD. Finally, considering the fact that even these expert groups collected data for outcomes in different ways, it is clear that the field needs to reach consensus on which outcomes to select and how to assess them. A white paper is under discussion to identify and define the best practices.

ACKNOWLEDGMENTS

A special thanks to the Scientific Organizing Committee: Annemieke Aartsma-Rus, Annamaria De Luca, Heather Gordish-Dressman, Kanneboyina Nagaraju, Melissa Spencer, Maaike van Putten, and Laura Dalle Pазze and Arati Kreibich of Charley's Fund for the coordination of all efforts before and during the workshop.

The workshop was sponsored by Charley's Fund in collaboration with the TREAT-NMD Alliance. Dr. Nagaraju is supported by National Institutes of Health grants # K26OD011171. Dongsheng Duan is supported by NIH (AR67985, AR69085, NS90634), DOD (MD150133), Jesse's Journey, Jackson Free! DMD Research Fund and Solid Biosciences.

DISCLOSURES

Dongsheng Duan is a member of the scientific advisory board for Solid Biosciences and an equity holder of Solid Biosciences. The Duan lab has received research support from Solid Biosciences. Annamaria De Luca has provided ad hoc consultancy in 2017 to Akashi Therapeutics and Grünenthal GmbH. Frederick J. Schnell and Marco A. Passini are paid employees of Sarepta Therapeutics Inc. Laurent Bogdanik and Cathleen Lutz are paid employees of The Jackson Laboratory. David Elsey is a paid employee of Summit Therapeutics plc. Mahasweta Girgenrath is a paid employee of Pfizer Inc. J. Patrick Gonzalez is a paid employee of Solid Biosciences. Andy Nichols is a paid employee of Catabasis Pharmaceuticals Inc. Zhong Zhong is a paid employee of Wave Life Sciences Ltd. Toshifumi Yokota has provided ad hoc consultancy in 2018 to Agada Biosciences.

REFERENCES

- [1] Bulfield G, Siller WG, Wight PA, Moore KJ. X chromosome-linked muscular dystrophy (mdx) in the mouse. *Proc Natl Acad Sci U S A*. 1984;81(4):1189-92.
- [2] Fukada S, Morikawa D, Yamamoto Y, Yoshida T, Sumie N, Yamaguchi M, et al. Genetic background affects properties of satellite cells and mdx phenotypes. *Am J Pathol*. 2010;176(5):2414-24.
- [3] Coley WD, Bogdanik L, Vila MC, Yu Q, Van Der Meulen JH, Rayavarapu S, et al. Effect of genetic background on the dystrophic phenotype in mdx mice. *Hum Mol Genet*. 2016;25(1):130-45.
- [4] Heydemann A, Ceco E, Lim JE, Hadhazy M, Ryder P, Moran JL, et al. Latent TGF-beta-binding protein 4 modifies muscular dystrophy in mice. *J Clin Invest*. 2009;119(12):3703-12.
- [5] Swaggart KA, Demonbreun AR, Vo AH, Swanson KE, Kim EY, Fahrenbach JP, et al. Annexin A6 modifies muscular dystrophy by mediating sarcolemmal repair. *Proc Natl Acad Sci U S A*. 2014;111(16):6004-9.
- [6] Swaggart KA, McNally EM. Modifiers of heart and muscle function: Where genetics meets physiology. *Exp Physiol*. 2014;99(4):621-6.
- [7] Quattrocchi M, Capote J, Ohiri JC, Warner JL, Vo AH, Earley JU, et al. Genetic modifiers of muscular dystrophy act on sarcolemmal resealing and recovery from injury. *PLoS Genet*. 2017;13(10):e1007070.
- [8] Flanigan KM, Ceco E, Lamar KM, Kaminoh Y, Dunn DM, Mendell JR, et al. LTBP4 genotype predicts age of ambulatory loss in Duchenne muscular dystrophy. *Ann Neurol*. 2013;73(4):481-8.
- [9] McGreevy JW, Hakim CH, McIntosh MA, Duan D. Animal models of Duchenne muscular dystrophy: From basic mechanisms to gene therapy. *Dis Model Mech*. 2015;8(3):195-213.
- [10] Hathout Y, Brody E, Clemens PR, Cripe L, DeLisle RK, Furlong P, et al. Large-scale serum protein biomarker discovery in Duchenne muscular dystrophy. *Proc Natl Acad Sci U S A*. 2015;112(23):7153-8.
- [11] Rodrigues M, Echigoya Y, Maruyama R, Lim KR, Fukada SI, Yokota T. Impaired regenerative capacity and lower revertant fibre expansion in dystrophin-deficient mdx muscles on DBA/2 background. *Sci Rep*. 2016;6:38371.
- [12] Krishnan VS, White Z, McMahon CD, Hodgetts SI, Fitzgerald M, Shavlakadze T, et al. A Neurogenic Perspective of Sarcopenia: Time Course Study of Sciatic Nerves From Aging Mice. *J Neuropathol Exp Neurol*. 2016;75(5):464-78.
- [13] Wang Y, Zhang K, Wasala NB, Duan D, Yao G. Optical polarization tractography revealed significant fiber disarray in skeletal muscles of a mouse model for Duchenne muscular dystrophy. *Biomed Opt Express*. 2015;6(2):347-52.
- [14] Wang Y, Zhang K, Duan D, Yao G. Heart structural remodeling in a mouse model of Duchenne cardiomyopathy revealed using optical polarization tractography [Invited]. *Biomed Opt Express*. 2017;8(3):1271-6.
- [15] Spurney CF, McCaffrey FM, Cnaan A, Morgenroth LP, Ghelani SJ, Gordish-Dressman H, et al. Feasibility and Reproducibility of Echocardiographic Measures in Children with Muscular Dystrophies. *J Am Soc Echocardiogr*. 2015;28(8):999-1008.
- [16] Soslow JH, Xu M, Slaughter JC, Stanley M, Crum K, Markham LW, et al. Evaluation of Echocardiographic Measures of Left Ventricular Function in Patients with Duchenne Muscular Dystrophy: Assessment of Reproducibility and Comparison to Cardiac Magnetic Resonance Imaging. *J Am Soc Echocardiogr*. 2016;29(10):983-91.
- [17] Available from: <http://www.treat-nmd.eu/research/preclinical/dmd-sops>.

- [18] Duan D, Rafael-Fortney JA, Blain A, Kass DA, McNally EM, Metzger JM, et al. Standard Operating Procedures (SOPs) for Evaluating the Heart in Preclinical Studies of Duchenne Muscular Dystrophy. *J Cardiovasc Transl Res.* 2016;9(1):85-6.
- [19] Vohra R, Batra A, Forbes SC, Vandenborne K, Walter GA. Magnetic Resonance Monitoring of Disease Progression in mdx Mice on Different Genetic Backgrounds. *Am J Pathol.* 2017;187(9):2060-70.
- [20] Ito T, Ogawa R, Uezumi A, Ohtani T, Watanabe Y, Tsujikawa K, et al. Imatinib attenuates severe mouse dystrophy and inhibits proliferation and fibrosis-marker expression in muscle mesenchymal progenitors. *Neuromuscul Disord.* 2013;23(4):349-56.
- [21] Takemoto Y, Inaba S, Zhang L, Baba K, Hagihara K, Fukada S. An herbal medicine, Go-sha-jinki-gan (GJG), increases muscle weight in severe muscle dystrophy model mice. *Clinical Nutrition Experimental.* 2017;16:13-23.

GENOME ENGINEERING

CRISPR alleviates muscular dystrophy in dogs

Widespread editing of the mutated *DMD* gene by CRISPR–Cas9, systemically delivered via an adeno-associated virus, restores dystrophin expression in a canine model of Duchenne muscular dystrophy.

Dongsheng Duan

Duchenne muscular dystrophy (DMD) — a lethal muscle-wasting disease affecting boys and young men — is caused by frame-shift mutations in *DMD*, the gene encoding the protein dystrophin¹. Thousands of mutations have been found in DMD patients, suggesting that individually tailored gene-editing strategies may be required to repair the specific mutation present in each patient. However, because most of the mutations involve deletions clustered into a mutation hot spot between exons 45 and 55 (which terminate the dystrophin open reading frame and dystrophin protein expression in ~13% of DMD patients), skipping or reframing exon 51 to restore dystrophin expression may be a solution for many of these patients². A number of recent studies have shown that the open reading frame of *DMD* can be restored by either deleting or reframing the out-of-frame exon(s) (Fig. 1a,b) via gene editing through clustered regularly interspaced short palindromic repeats (CRISPR). In these studies, dystrophin expression and function could be rescued in mouse models by co-delivering two adeno-associated virus (AAV) vectors, one expressing CRISPR associated protein 9 (Cas9) and the other expressing a guide RNA (gRNA)^{3–8}.

In particular, Eric Olson and colleagues reported in a study published in 2017 a strategy based on packaging the CRISPR editing machinery into a pair of AAV9 vectors, one expressing the *Streptococcus pyogenes* Cas9 (SpCas9) with a muscle-specific promoter and another expressing gRNA specific for the splicing acceptor site in exon 51. The approach effectively deleted exon 51, restored the dystrophin reading frame and ameliorated the symptoms of the disease in neonatal Δ Ex50 mice⁶ (mice with a *Dmd* mutation involving the loss of exon 50 and that can be corrected by skipping exon 51). To ensure sufficient gRNA expression, three gRNA expression cassettes were included to express the same gRNA from three different pol III promoters in the AAV9 gRNA vector. Despite the promising data in Δ Ex50 mice,

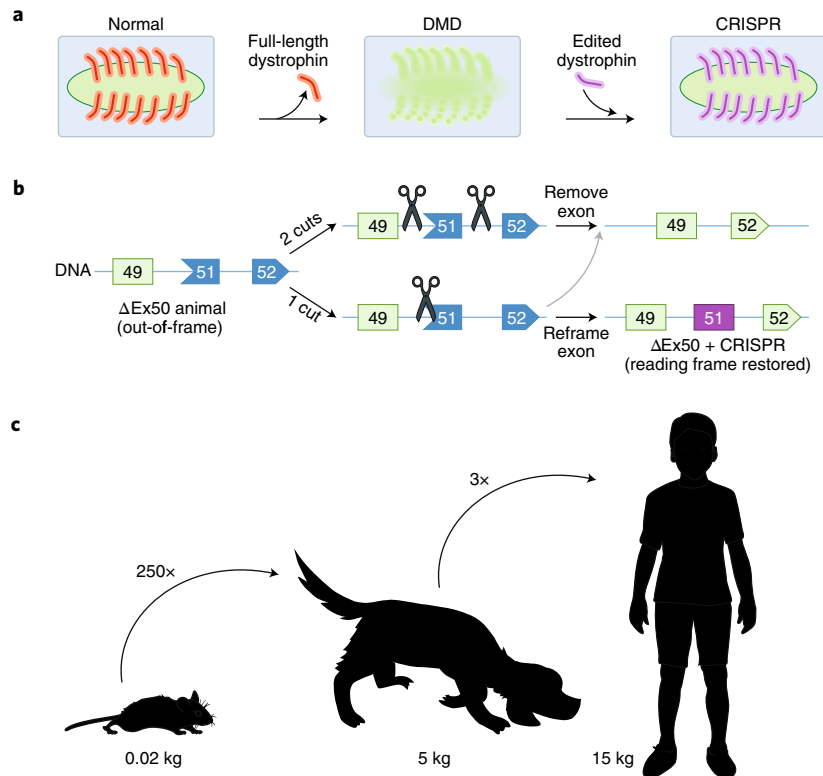


Fig. 1 | Scaling up body-wide gene editing via AAV-delivered CRISPR for restoring dystrophin levels in Duchenne muscular dystrophy. **a**, Dystrophin (red segments) is an essential muscle protein that links the extracellular matrix (grey) to the cytoskeleton (green) to stabilize the muscle-cell membrane (the dark green oval outline). Absence of dystrophin leads to muscle degeneration and necrosis. CRISPR-mediated editing can lead to the production of a modified, yet functional, dystrophin variant (purple segments) to restore the cross-membrane linkage and protect muscle from contraction-induced injury. **b**, Deletion of exon 50 disrupts the dystrophin open reading frame. Two different CRISPR editing approaches can be used to restore the reading frame. In the two-cut approach, exon 51 is removed by the incisions in adjacent introns, so that the resulting Δ 50–51 dystrophin is in-frame. In the one-cut approach, editing is made in exon 51 near its 5'-end; the reading frame is restored either by reframing or by removing exon 51. In terms of reframed dystrophin, only four residues located before or at the editing site are different from that of wild-type dystrophin. Exon removal is achieved by disrupting the splicing acceptor signal at the beginning of exon 51. **c**, Differences in body size and disease severity represent major challenges in the translation of body-wide muscle gene therapy from mouse models to human patients. Preclinical studies in symptomatic dystrophic dogs bridge the gap.

significant hurdles remain before CRISPR gene editing can be effectively applied to human patients, including the need to scale up treatment from symptomless mouse models (~700-fold smaller than a child) to

a disease-manifesting large mammal (~2 to 3-fold smaller than a child; Fig. 1c). There are animal models of DMD in dogs, pigs and non-human primates, but the canine model is the best studied of these⁹. Reporting in

Science, Eric Olson and colleagues now show that CRISPR-based deletion of exon 51 in *DMD* via intravenously delivered AAV vectors restored dystrophin expression in muscles throughout the body of the Δ Ex50 dogs¹⁰. The study provides insights into the translation of gene-editing technologies towards large mammals in terms of vector formulation and delivery strategies.

Olson and co-authors first show that intramuscular injection of the CRISPR-carrying viruses in 1-month-old Δ Ex50 puppies resulted in ~10% of the muscle cells' DNA being edited, with local dystrophin expression reaching 50–70% of the normal expression levels, and improved muscle histology assessed at 2.5 months of age. In this local injection study, the authors delivered 1.2×10^{13} viral genome (vg) particles of AAV9 vectors into the cranial tibialis muscle of two affected puppies in the absence of immune suppression. Intriguingly, the authors did not detect an increased infiltration of CD4⁺ and CD8⁺ T cells in the treated muscle, suggesting a lack of cellular immune response to the delivered vectors. Next, the authors tested intravenous injection of the CRISPR gene-editing vectors into another two 1-month-old Δ Ex50 puppies under immune suppression (one puppy at a dose of 2×10^{13} vg kg⁻¹ vector⁻¹ and another at 1×10^{14} vg kg⁻¹ vector⁻¹; these doses are in line with the vector doses currently in use in systemic AAV micro-dystrophin therapy trials¹¹) in the hope of treating all the affected muscles, especially the diaphragm and the heart, which can account for patient death in DMD but cannot be easily accessed for direct injection. At 3 months of age, approximately 3–8% and 5–10% of the DNA in muscles was edited in the puppies that received the lower dose and higher dose of AAV, respectively. Dystrophin expression, detected by immunostaining, reached 3–50% (low-dose vector) and 5–92% (high-dose vector) of the wild-type levels across muscle types. The authors also saw obvious histological improvement when treated and affected dogs were compared with the untreated yet affected dogs. More specifically, dystrophin expression reached 58% and 92% of the wild-type levels in the diaphragm and heart, respectively. No significant off-target edits were observed, including no editing on testes DNA, and no change in blood biochemistry and haematology when compared with untreated healthy and affected dogs. Taken together, the authors have demonstrated

that whole-body CRISPR editing is feasible in a large mammal, and suggest that the dystrophin levels observed in the puppy with the higher dose of vector may change the disease course if this level can be maintained¹¹. To put these findings into the context of human DMD patients, the authors tested the same exon 51-targeted CRISPR strategy in induced pluripotent stem cells (iPSCs) derived from a patient carrying a deletion-mutation region in exons 48–50. Similarly to the observations in mice and dogs, the iPSCs started expressing dystrophin.

Olson and colleagues' study is proof of principle of the successful scale-up of AAV CRISPR therapy. It also supports the ultimate objective of whether similar success can be readily achieved in human patients. Encouragingly, intravenous AAV9 delivery has resulted in remarkable clinical improvements in patients suffering from type-I spinal muscular atrophy (another fatal neuromuscular disease¹²). Hence, there is precedent for systemic AAV9 therapy in humans. However, this is not the only challenge. Many important questions remain to be addressed, including immune reaction to Cas9 and the viral vector, level and duration of dystrophin expression, changes in muscle and heart function, the ability to perform genome editing in muscle stem cells, and long-term safety and efficacy.

Cellular immune reaction was a major problem when AAV micro-dystrophin therapy was initially tested in the canine DMD model. As Cas9 is a bacterial protein, a vigorous immune response has been a concern for AAV CRISPR therapy in dystrophic dogs. Surprisingly, intramuscular injection of the Cas9 vector induced nominal T-cell infiltration even without administration of immunosuppressive drugs. Several factors may have contributed to this unexpected observation, including the use of a muscle-specific promoter, which probably minimized widespread antigen presentation. The age and breed of the dog may have also played a role¹³, as the immune system of neonatal dogs is not yet fully developed and is dominated by T helper type 2 rather than cytotoxic (T helper type 1) immunity. And genetic background may markedly influence immune-competence in dogs. Future studies in older and mixed-bred dogs affected by DMD may help delineate the immune consequences of AAV-mediated CRISPR therapy in the highly inflammatory milieu of the dystrophic muscle in large mammals.

DMD patients are usually diagnosed at about 3 to 5 years of age, when they begin to show symptoms. In reported mouse and dog studies^{8,10}, the therapy was introduced at the neonatal age, before apparent muscle disease commenced. Therefore, studies in older animals are needed to determine whether systemic CRISPR therapy can treat patients after diagnosis. As a chronic disease, efficacious treatment of DMD will require life-long dystrophin expression¹⁴. Because in these studies animals were euthanized less than two months after AAV delivery, it is unclear whether dystrophin expression would persist and whether safety issues would arise. In Olsen and colleagues' study, another limitation is the absence of data to verify whether heart and muscle function is restored, a question that could not be answered given the small sample size and the early termination of the study. Therefore, long-term studies in Δ Ex50 mice and dogs, as well as validation in other canine DMD models to account for the influence of genetic background¹³, are absolutely necessary before considering translation of the approach into human patients. Yet the demonstration of systemic AAV CRISPR therapy in a large mammal marks an important milestone towards the application of CRISPR technology to treat human genetic diseases. □

Dongsheng Duan

Departments of Molecular Microbiology and Immunology, Neurology, Bioengineering and Biomedical Sciences, University of Missouri, Columbia, MO, USA.

e-mail: duand@missouri.edu

Published online: 8 November 2018
<https://doi.org/10.1038/s41551-018-0320-0>

References

1. Kunkel, L. M. *Am. J. Hum. Genet.* **76**, 205–214 (2005).
2. Aartsma-Rus, A. et al. *Hum. Mutat.* **30**, 293–299 (2009).
3. Nelson, C. E. et al. *Science* **351**, 403–407 (2016).
4. Long, C. et al. *Science* **351**, 400–403 (2016).
5. Tabebordbar, M. et al. *Science* **351**, 407–411 (2016).
6. Amoasii, L. et al. *Sci. Transl. Med.* **9**, eaan8081 (2017).
7. Bengtsson, N. E. et al. *Nat. Commun.* **8**, 14454 (2017).
8. El Refaey, M. et al. *Circ. Res.* **121**, 923–929 (2017).
9. McGreevy, J. W., Hakim, C. H., McIntosh, M. A. & Duan, D. *Dis. Model. Mech.* **8**, 195–213 (2015).
10. Amoasii, L. et al. *Science* **362**, 86–91 (2018).
11. Duan, D. *Mol. Ther.* **26**, 2337–2356 (2018).
12. Mendell, J. R. et al. *N. Engl. J. Med.* **377**, 1713–1722 (2017).
13. Day, M. J. *J. Comp. Pathol.* **137**(Suppl. 1), S10–S15 (2007).
14. Wasala, N. B. et al. *Hum. Mol. Genet.* **25**, 2633–2644 (2016).

Competing interests

D.D. is a member of the scientific advisory board for Solid Biosciences and an equity holder of Solid Biosciences. D.D.'s lab has received research support from Solid Biosciences.



Chapter 18

Systemic Delivery of Adeno-Associated Viral Vectors in Mice and Dogs

Lakmini P. Wasala, Chady H. Hakim, Yongping Yue, N. Nora Yang, and Dongsheng Duan

Abstract

Many diseases affect multiple tissues and/or organ systems, or affect tissues that are broadly distributed. For these diseases, an effective gene therapy will require systemic delivery of the therapeutic vector to all affected locations. Adeno-associated virus (AAV) has been used as a gene therapy vector for decades in preclinical studies and human trials. These studies have shown outstanding safety and efficacy of the AAV vector for gene therapy. Recent studies have revealed yet another unique feature of the AAV vector. Specifically, AAV can lead to bodywide gene transfer following a single intravascular injection. Here we describe the protocols for effective systemic delivery of AAV in both neonatal and adult mice and dogs. We also share lessons we learned from systemic gene therapy in the murine and canine models of Duchenne muscular dystrophy.

Key words DMD, Systemic delivery, AAV, Neonatal mice, Neonatal dogs, Adult mice, Adult dogs

1 Introduction

Based on the delivery method, gene therapy can be divided into local therapy and systemic therapy. Local gene therapy refers to direct injection of a therapeutic vector to the diseased tissue in situ. Such an approach works extremely well for diseases that affect a confined region such as congenital blindness caused by mutations in the retinal gene. In this case, a simple one-time subretinal injection of an adeno-associated virus (AAV) vector can result in marvelous vision improvement [1]. As a matter of fact, a gene therapy drug (Luxturna) for treating an inherited form of blindness received Food and Drug Administration (FDA) approval on December 19, 2017, for commercial use [2]. However, many diseases affect multiple body systems or affect tissues that are widely distributed. An effective therapy for these diseases will require systemic delivery. In this case, a therapeutic vector is injected into the circulation to allow it to spread throughout the whole body.

Systemic delivery is much more challenging because the vector has to escape from the vasculature and still maintain its infectivity to transduce the target cells. Systemic delivery is also more risky due to the administration of a huge quantity of the vector ($\geq 10^{14}$ particles/kg) over a short period of time and inevitable spreading of the vector to untoward tissues.

A variety of viral and nonviral vector systems have been explored for systemic delivery (reviewed in Ref. [3]). Unfortunately, most of them have failed to result in robust, persistent bodywide gene transfer following intravascular delivery. Currently, AAV is the only vector that can lead to whole body systemic transduction at a level that can meet the needs of clinical gene therapy in human patients [4]. The exact molecular mechanisms underlying AAV-mediated systemic gene transfer remain to be elucidated. However, a number of factors may contribute to systemic AAV delivery, such as the small size of the viral particle (AAV is one of the smallest viruses), the reported transcytosis of certain AAV serotypes, and the unique viral capsid structure of certain AAV serotypes. The first successful systemic delivery in rodent (normal and diseased mice) was reported in 2004 by the Chamberlain lab [5]. The first systemic delivery in large mammals was performed in neonatal dogs in 2008 by the Duan lab [6]. The first systemic delivery in an adult diseased large mammal was performed in the canine model of Duchenne muscular dystrophy (DMD) in 2015 by the Duan lab [7]. The first systemic delivery in human patients was performed by the Mendell lab in 2017 for treating type I spinal muscular atrophy [4].

Over the last 11 years, we have conducted extensive preclinical systemic AAV delivery in normal mice and dogs, and murine and canine DMD models [3, 6–18]. In these studies, we tested both reporter and therapeutic vectors and explored a variety of AAV serotypes. Here we describe detailed protocols we have developed for systemic AAV delivery in mice and dogs.

2 Materials

2.1 Systemic Delivery of AAV to Neonatal Mice (Fig. 1a)

1. Personal protective equipment (PPE) including disposable gowns and/or dedicated scrubs, head cover, shoe covers, surgical mask, and gloves.
2. 30G Hamilton syringe (Hamilton, Reno, NV, USA).
3. PE10 tubing (Hamilton, Reno, NV, USA).
4. Wee Sight transilluminator vein finder (Philips, Omaha, NE, USA).
5. Food dye (Wilton, Darien, IL, USA) sterilized with 0.2 μm filter before use.

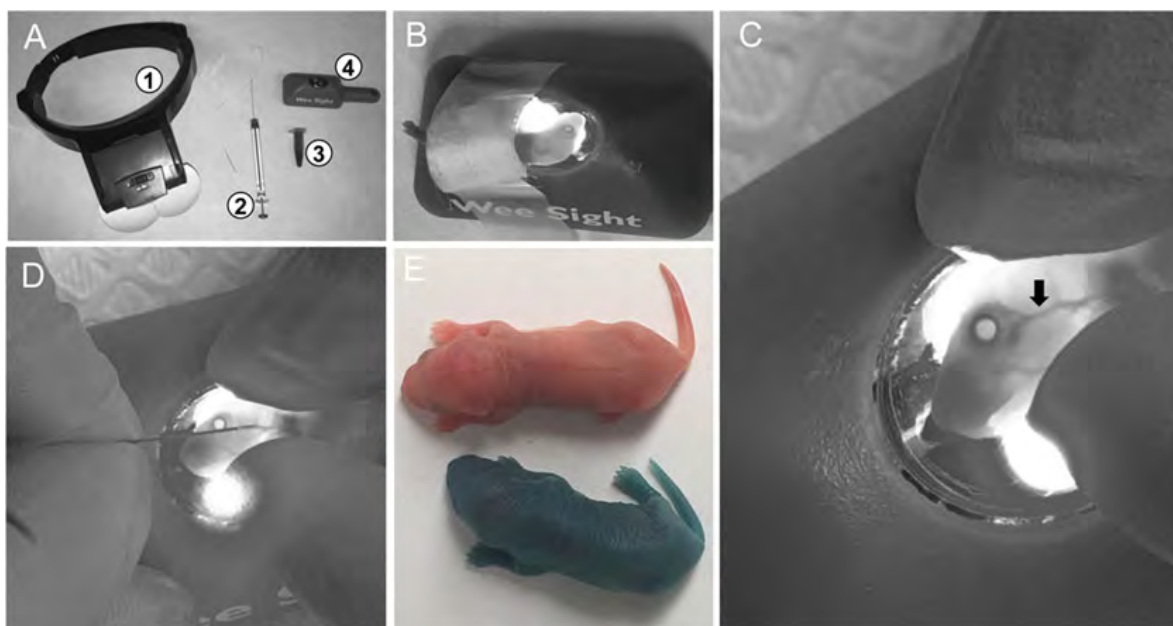


Fig. 1 Systemic injection in newborn mice via the facial vein. (a) Material required for the procedure: (1) loop headband magnifier, (2) Hamilton syringe with attached tubing and needle, (3) food dye, (4) Wee Sight transilluminator. (b) A newborn pup taped to the Wee Sight illuminator. (c) Position of the facial vein (arrow). (d) A photograph showing the placement of the injection needle in the facial vein. (e) Representative photographs showing an un-injected puppy (top) and an injected puppy (bottom)

6. Wet ice bucket.
7. Sterile gauge sponges (2 in. $\times$ 2 in.).
8. Medical tape (3 M, Maplewood, MN, USA).
9. Optivisor adjustable headband magnifier with a 2.5 $\times$ lens (Donegan Optical Company Inc., Lenexa, KS, USA).
10. Recombinant AAV vector (*see Note 1*).

2.2 Systemic Delivery of AAV to Adult Mice

1. Personal protective equipment (PPE) including disposable gowns and/or dedicated scrubs, head cover, shoe covers, surgical mask, and gloves.
2. Flat bottom rodent restrainer (Plas Labs Inc., Lansing, MI, USA).
3. BD Insulin syringe with a capacity of 100 μ L and a 30G $\times$ 0.5 in. needle.
4. Thermophore heating pad (Medwing, Columbia, SC, USA). Webcol alcohol prep pad (Covidien, Dublin, Republic of Ireland).
5. Digital scale.
6. Biosafety hood.
7. Recombinant AAV vector (*see Note 1*).

2.3 Systemic Delivery of an AAV Vector to Neonatal Dogs (Fig. 2a)

1. Personal protective equipment (PPE) including disposable gowns and/or dedicated scrubs, head cover, shoe covers, surgical mask, and gloves.
2. MyWeigh SCM2600BLACK iBalance tabletop digital scale (MyWeigh, Phoenix, Arizona, USA).
3. Digital thermometer (ReliOn Inc., Spokane Valley, WA, USA).
4. Pediatric stethoscope (3 M Littmann, St. Paul, MN, USA).
5. Masimo Rad-57 handheld pulse oximeter (DRE, Louisville, KY, USA).
6. Thermal blanket (100% cotton) (Elite Home Products Inc., Saddle Brook, NJ, USA).
7. Animal fine hair clipper.
8. Infusion set (23G $\times$ 3/4 in. butterfly needle attached to a 7.5" infusion line with Luer lock connector) (Greiner Bio-One, Kremsmünster, Austria).
9. Syringe with Luer-Lock Tip (3 mL) (Becton Dickinson company, Franklin Lakes, NJ, USA).
10. BD PosiFlush Pre-filled saline (0.9% sodium chloride) syringe for intravenous (IV) injection (Becton Dickinson company, Franklin Lakes, NJ, USA).
11. Sterile gauze sponges (2 in. $\times$ 2 in.).
12. ChloroPrep one step (2% Chlorhexidine gluconate and 70% Isopropyl alcohol) (CareFusion, Leawood, KS, USA).
13. Recombinant AAV vector (*see Note 1*).

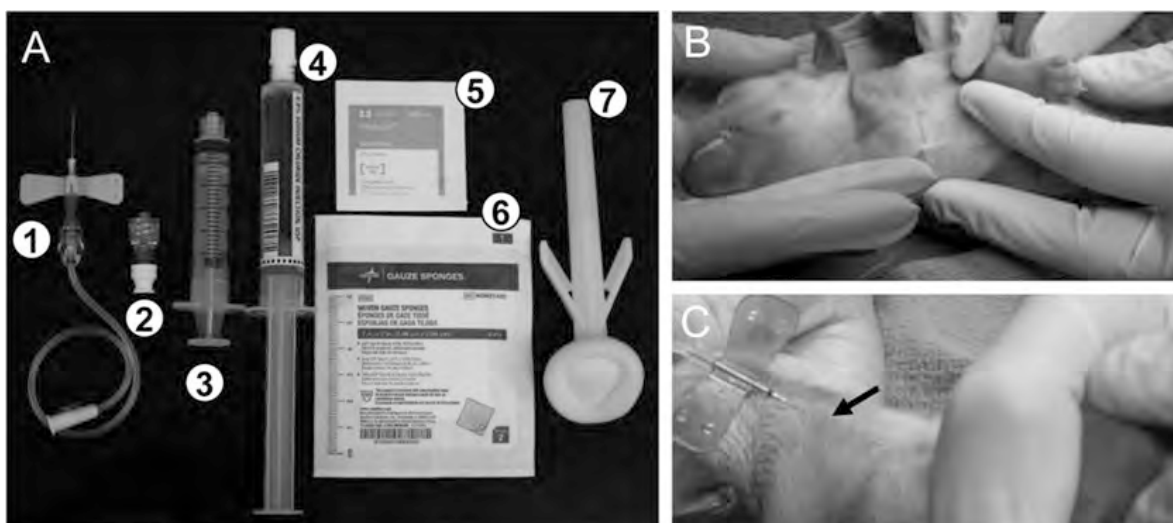


Fig. 2 Systemic injection of neonatal dog puppies via the jugular vein. **(a)** Materials required for the procedure (1). a butterfly needle attached to the infusion line, (2) a Luer lock connector, (3) the 3-mL syringe containing the AAV vector, (4) the prefilled saline syringe, (5), alcohol wipe, (6) gauze sponge, (7) a ChloroPrep one step. **(b)** A photograph showing the position of the puppy for injection. **(c)** A photograph showing the placement of the injection needle in the jugular vein

**2.4 Systemic
Delivery of AAV
to Young/Adult Dogs
(Fig. 3)**

1. Personal protective equipment (PPE) including disposable gowns and/or dedicated scrubs, head cover, shoe covers, surgical mask, and gloves.
2. Digital vet scale (DRE, Louisville, KY, USA).
3. Digital thermometer (ReliOn Inc., Spokane Valley, WA, USA).
4. Stethoscope (3 M Littmann, St. Paul, MN, USA).
5. Handheld pulse oximeter (Masimo Rad-57) (DRE, Louisville, KY, USA).
6. Thermal blanket (100% cotton) (Elite Home Products Inc., Saddle Brook, NJ, USA).
7. Animal work table.
8. Animal hair clipper equipped with a size 50 blade (Oster professional product, Sunbeam products, Inc., Boca Raton, FL, USA).
9. Shielded I.V. Catheter (22G $\times$ 1.00 in. needle, or 20G $\times$ 1.00 in. needle) (Becton Dickinson company, Franklin Lakes, NJ, USA).



Fig. 3 Materials required for the systemic injection of young/adult dog via the cephalic vein (1) Infusion pump, (2) shielded IV catheter, (3) Luer lock, (4) connector tubing, (5) 30 mL syringe, (6) prefilled saline syringe, (7) medical tape, (8) alcohol wipe, (9) gauze sponge, (10) handheld pulse oximeter, (11) ChlorPrep one step, (12) stethoscope, (13) thermal blanket, (14) digital thermometer, (15) animal hair clipper

10. SmartSite Needle free valve (CareFusion, San Diego, CA, USA).
11. Micro-Bore IV Extension set (A 60 in. tube line with 0.3 mL volume capacity, and contains Luer lock connectors as well as a slide clamp) (NeoChild, Oklahoma City, OK, USA).
12. Medical Tape.
13. Vet wrap (3 M, Maplewood, MN, USA).
14. Webcol Alcohol Prep pad (Covidien, Dublin, Republic of Ireland).
15. Syringe with Luer-Lock Tip (30 mL) (Becton Dickinson Company, Franklin Lakes, NJ, USA).
16. BD PosiFlush Pre-filled saline (0.9% Sodium chloride) syringe for IV injection (Becton Dickinson Company, Franklin Lakes, NJ, USA).
17. Infusion pump (KD Scientific Inc., Holliston, MA, USA).
18. Sterile gauge sponges (4 in. × 4 in.) (Covidien, Mansfield, MA, USA).
19. ChloraPrep one step (2% Chlorhexidine gluconate and 70% Isopropyl alcohol) (CareFusion, San Diego, CA, USA).
20. Recombinant AAV vector (*see Note 1*).

3 Methods

3.1 Systemic Delivery of AAV to Neonatal Mice

1. Follow the institutional guidelines for animal care and handling. Obtain the approval from institutional animal care and use committee (ACUC). Wear all PPE before working or handling animals. Use aseptic techniques while preparing the AAV vector for injection and during the injection procedure.
2. Keep the neonates in a pre-warmed cage by keeping a heat pad beneath the cage. Separate the dam from the pups (*see Note 2*).
3. Mix the food dye with stock AAV virus at a 1:100 dilution (*see Note 3*).
4. Fill the syringe with the AAV vector. Get rid of air bubbles (*see Note 4*).
5. Anesthetize the animal using a thermal shock by wrapping the puppy with a piece of gauze and keeping on ice for few seconds (*see Note 5*).
6. Secure the neonate body on its side on the Wee Sight transilluminator by placing a gauze pad on the skin and a tape over the gauze (*see Note 6*) (Fig. 1b).
7. Use the magnifier to visualize the facial vein (Fig. 1c).
8. Slowly insert the needle into the vein and infuse the AAV vector (*see Note 7*) (Fig. 1d). Once all the solution is injected, keep the needle in the vein for additional 15 s (*see Note 8*).

9. After removing the needle, gently pressure the injection site to avoid bleeding.
10. Allow 2–3 min for the neonate to recover, and after it is conscious, place the neonate back in the cage. If necessary the animal can be warmed up by keeping on the investigator's hands or a pre-warmed gauze pad.
11. Monitor the neonates for signs of distress.
12. Return the injected pup to the mother after the pup becomes conscious and restores ambulation.

3.2 Systemic Delivery of AAV to Adult Mice

1. Follow the institutional guidelines for animal care and handling. Obtain the approval from institutional animal care and use committee (IACUC). Wear all PPE before working or handling animals. Use aseptic techniques while preparing the AAV vector for injection and during the injection procedure.
2. Weigh the mouse and calculate the volume of the AAV vector needed to reach a certain viral genome (vg) particles/body weight (*see Note 9*).
3. Position the mouse in the rodent restrainer (Fig. 4a).

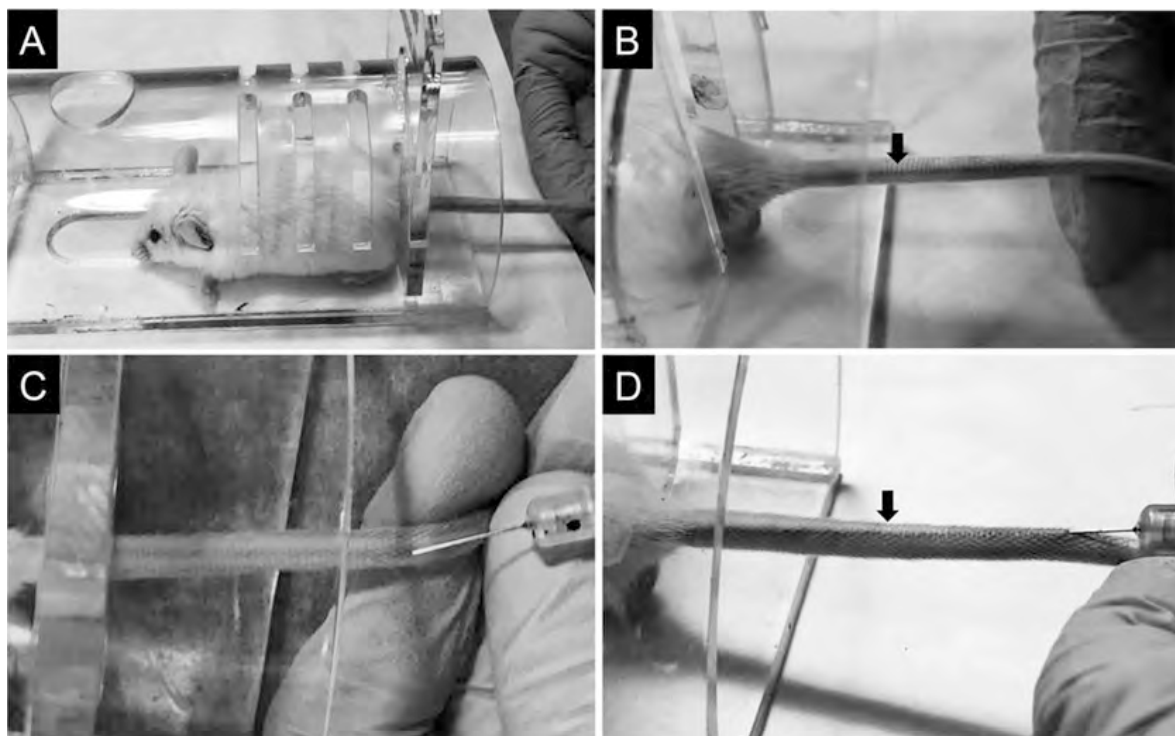


Fig. 4 Systemic injection in adult mice via the tail vein. (a) An adult mouse inside the rodent restrainer. (b) A photograph showing the prominent lateral tail vein (arrow). (c) A photograph showing the placement of the injection needle in the tail vein. (d) A photograph showing the color of the tail becomes dark with a successful injection of the AAV. The color change is due to the food dye in the AAV solution

4. Gently warm the tail using a heating pad at its lowest setting to allow vasodilation.
5. Hold the distal end of the tail with the nondominant hand.
6. Slightly rotate the tail to allow clear visualization of the lateral tail vein (Fig. 4b).
7. Clean the injection site with alcohol pad.
8. Insert the needle to the tail vein by holding the tail and needle positioning parallel to the tail (*see Note 10*) (Fig. 4c, d).
9. Inject the AAV vector at a steady rate of ~1 mL/min.
10. Once the AAV vector is injected, carefully remove the needle and apply pressure to the injection site to avoid bleeding (*see Note 11*).
11. Transfer the mouse to the cage and observe the mouse for at least 4 h.

3.3 Systemic Delivery of AAV to Neonatal Dogs

1. Follow the institutional guidelines for animal care and handling. Obtain the approval from institutional animal care and use committee (ACUC). Wear all PPE before working or handling animals. Use aseptic techniques while preparing the AAV vector for injection and during the injection procedure.
2. Monitor the newborn puppy for the body weight and responsiveness (*see Note 12*). Supplement the puppy with formula as needed (*see Note 13*). Consult with the veterinary doctor immediately for unexpected changes such as sudden drop of the body weight or temperature.
3. Weight the puppy to determine the injection volume. A normal neonatal puppy can tolerate up to 25 μ L liquid/gram body weight (*see Note 14*).
4. Measure the vital signs (heart rate, respiration rate, rectal body temperature, and blood oxygen saturation) (*see Note 15*).
5. Gently shave the neck hair on the side to allow better observation of the jugular vein for injection.
6. Connect the saline prefilled BD PosiFlush syringe with the IV extension line and attach them to the infusion set. All parts are connected through Luer lock fitting. Prefill the IV extension line and the infusion set with saline to remove air from the lines. Lock the sliding clamp on the extension line. Draw the exact volume of AAV vector (calculated based on body weight and desired dose) into the 3 mL syringe and remove any air bubbles from the syringe. Replace the BD PosiFlush syringe with the AAV syringe. Check the extension line and infusion set for any air bubble.
7. Perform injection procedure with the help of three investigators. The first investigator places a preheated thermal blanket

on the animal working table, positions the puppy supine on the blanket, then gently restrains the forelimbs using both hands (Fig. 2b). The second investigator restrains the head and scrubs the ventral side of the neck with the ChloroPrep, then gently inserts the needle in the jugular vein (Fig. 2c). Once the needle enters the vein, blood will appear in the needle hub. The third investigator unlocks the sliding clamp on the extension line and pulls the plunger back to visualize blood in the infusion line. This will confirm that the needle is correctly placed inside the vein. Inject the AAV vector at the rate of 2 mL/min, then flush the infusion line with saline [12].

8. While pressing the entry site of the needle with a finger, retract the butterfly from the jugular vein. Continue to pressure the injection site to avoid bleeding.
9. Remeasure the vital signs.
10. Return the puppy back to the dam and closely monitor the puppy for the following 24 h. Consult with veterinary doctor immediately with any concerns.

3.4 Systemic Delivery of AAV to Young/Adult Dogs

1. Follow the institutional guidelines for animal care and handling. Obtain the approval from institutional animal care and use committee (ACUC). Wear all PPE before working or handling animals. Use aseptic techniques while preparing the AAV vector for injection and during the injection procedure.
2. Weigh the dog to determine the injection volume. Up to 6.2 mL/kg body weight (6.24×10^{14} viral genome particles/kg body weight) can be tolerated in young adult dystrophic dogs [7].
3. Measure the vital signs (heart rate, respiration rate, rectal body temperature, and blood oxygen saturation).
4. Shave the cranial side of the lower forelimb (Fig. 5a) to allow better observation of the cephalic vein (Fig. 5b) during the injection procedure (*see Note 16*).
5. Place a thermal blanket on the animal working table. Place the dog on the table in the sternal recumbency position with the lower forelimb extended. Occlude the cephalic vein at the elbow with your thumb or using a tourniquet by one investigator.
6. Catheterize the cephalic vein by another investigator. Scrub the cranial aspect of the lower forelimb with ChloroPrep. Pull the lower forelimb skin distally to stabilize the vein. Slowly insert the IV catheter needle (bevel up) through the skin and into the vein (Fig. 5c). Blood will appear in the catheter when the needle punctures the vein. Gently slide the catheter into the vein while retracting the needle out. At this time, blood will

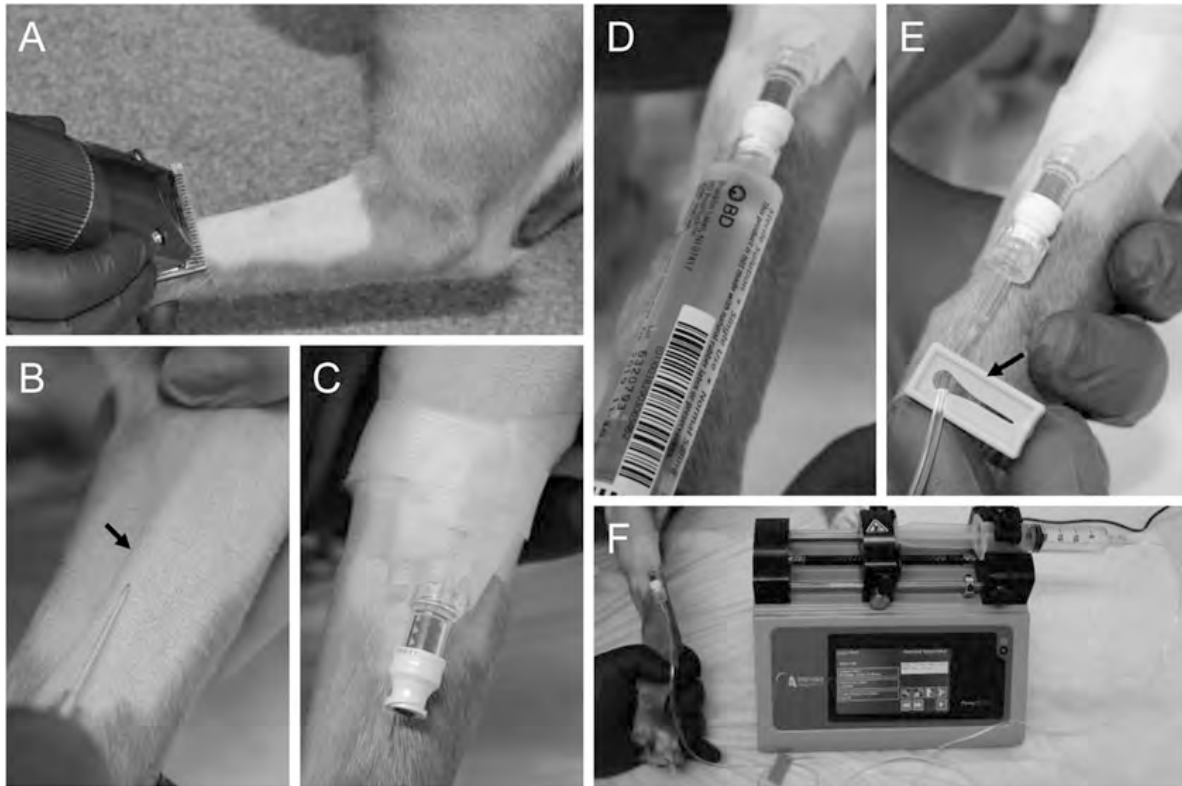


Fig. 5 Systemic injection of young/adult dog via the cephalic vein. (a) Shaving of the cranial aspect of the forelimb. (b) A photograph showing the placement of the needle in the cephalic vein. Arrow, the cephalic vein. (c) A photograph showing the catheterization of the cephalic vein. (d) A photograph showing the AAV containing syringe during the injection. (e) A photograph showing the catheter inserted with the extension line. Arrow, the sliding clamp. (f) A photograph showing injection of AAV using the infusion pump

flow out from the catheter. Quickly occlude the vein at the catheter tip and attach the smart site needle free valve to the catheter. Secure the catheter with tape and wrap it with vet wrap. Flush the catheter with saline to ensure there is no leak.

7. Connect the saline prefilled BD PosiFlush syringe with the IV extension line using the Luer lock fitting (Fig. 5d). Prefill the IV extension line with saline to remove air. Lock the sliding clamp on the extension line (Fig. 5e). Draw the exact volume of AAV vector into the 30 mL syringe. Remove any air bubbles from the syringe. Replace the BD PosiFlush syringe with the AAV syringe. Check the extension line for any air bubble. Secure the syringe to the infusion pump and set the infusion rate to 2 mL/min (Fig. 5f).
8. Attach the IV extension line to the valve and unlock the sliding clamp. Start the infusion and monitor the dog's vital signs closely. Once the infusion is done, flush infusion line with saline and disconnect the extension line from the catheter. While pressing the entry site of the needle with your finger, retract the catheter from the cephalic vein. Continue to pressure the puncture site to avoid bleeding.

9. Continue measuring the vital signs for 5, 10, 30, and 60-min post injection and examine the dog for any adverse reaction.
10. Return the dog to its housing and continue monitoring the dog for 72 h. Consult with the veterinary doctor immediately with any concerns.

4 Notes

1. Many different methods have been developed for the production and purification of recombinant AAV vectors (reviewed in refs. [19](#), [20](#)). For the protocols described in this chapter, AAV vectors were generated using the transient plasmid transfection method [[17](#)]. AAV vector titer was determined using quantitative polymerase chain reaction. AAV vector was dialyzed in HEPES buffer (Sodium chloride 8.7 g, HEPES 4.75 g, 10 N Sodium Hydroxide 1.5 mL, Distilled water up to 1 L, pH adjusted to 7.8). AAV-6 and -8 are the first AAV serotypes shown capable of systemic delivery [[5](#), [21](#)]. Since then, a number of AAV serotypes and engineered AAV capsids were found to have the unique systemic delivery property. Some of these AAV serotypes also demonstrated specific tissue tropism following systemic delivery. For example, intravascular injection of AAV-9 results in preferential transduction of the heart in mice [[8](#)]. For the protocols described in this chapter, we have used AAV-1, -6, -8, -9 and their tyrosine mutants [[22](#)].
2. Neonatal facial vein injections need to be carried out between 24 and 48 h after birth. During this time window, the facial vein is clearly visible.
3. The food dye is added to allow easy visualization and confirmation of injection.
4. A newborn mouse can tolerate up to 100 μ L of volume solution.
5. Do not leave the neonate on ice for longer than 1 min to avoid complications related to hypothermia.
6. The newborn mouse should be positioned with its neck turned gently for clear visualization of the facial vein and the head secured by fingers without interfering respiration.
7. Accumulation of solution at the head or neck area (but not in other parts of the body) suggests injection failure. A successful injection should result in the diffusion of the dye throughout the body. The entire puppy will turn the color of the dye.
8. This will allow sufficient time for the AAV solution to flow through the PE-10 tubing and it will also prevent any back flow of the solution.

9. The total AAV vector volume (mL) needed to reach a certain viral dosage (vg/body weight) is calculated using the following formula: Injection volume (mL) = [dosage (vg/body weight) $\times$ body weight]/viral titer (vg/mL).
10. Injection should be done approximately 1 in. distal to the midpoint of the tail. Starting at the far end of the tail to allow second or third injection attempt if the first attempt fails.
11. If the injection creates a bleb, the injection at that site should be terminated. In the case of an unsuccessful injection, up to three additional injections can be attempted.
12. The body weight should be monitored every 2–4 h. Bowel movement and/or urination may cause transient body weight fluctuation. Puppies that fail to gain weight should not be used for AAV injection.
13. The Just Born puppy formula produces best results for nutrition supplementation in our hands.
14. We have performed neonatal AAV injection in puppies as early as 24 h after the birth. Successful systemic AAV injection can also be achieved in puppies up to 1 week of the age.
15. Throughout the injection procedure, make sure the puppy is wrapped in a thermal blanket and kept warm. If needed, thermal blanket can be pre-warmed to 37 °C.
16. Besides the cephalic vein, other veins can also be used such as the jugular vein and saphenous vein. For injection through these veins, shave the related area. For example, for saphenous vein injection, we shave the lateral side of the distal tibia above the tarsus joint.

Acknowledgments

The research on systemic AAV delivery in the Duan lab was supported by the National Institutes of Health (NS-90634, AR-70517 and AR-69085), Department of Defense (MD150133), Jesse's Journey-The Foundation for Gene and Cell Therapy, Hope for Javier, Jackson Freel DMD Research Fund and Solid Biosciences Inc. The authors thank Keqing Zhang for the assistance in figure preparation.

Disclosure: D.D. is a member of the scientific advisory board, an equity holder of Solid Biosciences, LLC. D.D. is an inventor on a patent licensed to Solid Biosciences, LLC.

References

1. Russell S, Bennett J, Wellman JA et al (2017) Efficacy and safety of voretigene neparvovec (AAV2-hRPE65v2) in patients with RPE65-mediated inherited retinal dystrophy: a randomised, controlled, open-label, phase 3 trial. *Lancet* 390(10097):849–860. [https://doi.org/10.1016/S0140-6736\(17\)31868-8](https://doi.org/10.1016/S0140-6736(17)31868-8)
2. FDA News Release (2017) FDA approves novel gene therapy to treat patients with a rare form of inherited vision loss. <https://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm589467.htm>. Accessed 19 Dec 2017
3. Duan D (2016) Systemic delivery of adeno-associated viral vectors. *Curr Opin Virol* 21:16–25. <https://doi.org/10.1016/j.coviro.2016.07.006>
4. Mendell JR, Al-Zaidy S, Shell R et al (2017) Single-dose gene-replacement therapy for spinal muscular atrophy. *N Engl J Med* 377(18):1713–1722. <https://doi.org/10.1056/NEJMoal706198>
5. Gregorevic P, Blankinship MJ, Allen JM et al (2004) Systemic delivery of genes to striated muscles using adeno-associated viral vectors. *Nat Med* 10(8):828–834
6. Yue Y, Ghosh A, Long C et al (2008) A single intravenous injection of adeno-associated virus serotype-9 leads to whole body skeletal muscle transduction in dogs. *Mol Ther* 16(12):1944–1952. <https://doi.org/10.1038/mt.2008.207>
7. Yue Y, Pan X, Hakim CH et al (2015) Safe and bodywide muscle transduction in young adult Duchenne muscular dystrophy dogs with adeno-associated virus. *Hum Mol Genet* 24(20):5880–5890
8. Bostick B, Ghosh A, Yue Y et al (2007) Systemic AAV-9 transduction in mice is influenced by animal age but not by the route of administration. *Gene Ther* 14(22):1605–1609
9. Ghosh A, Yue Y, Long C et al (2007) Efficient whole-body transduction with trans-splicing adeno-associated viral vectors. *Mol Ther* 15(4):750–755. <https://doi.org/10.1038/sj.mt.6300081>
10. Bostick B, Yue Y, Lai Y et al (2008) Adeno-associated virus serotype-9 microdystrophin gene therapy ameliorates electrocardiographic abnormalities in mdx mice. *Hum Gene Ther* 19(8):851–856. <https://doi.org/10.1089/hum.2008.058>
11. Ghosh A, Yue Y, Shin J-H et al (2009) Systemic trans-splicing AAV delivery efficiently transduces the heart of adult mdx mouse, a model for Duchenne muscular dystrophy. *Hum Gene Ther* 20(11):1319–1328
12. Yue Y, Shin JH, Duan D (2011) Whole body skeletal muscle transduction in neonatal dogs with AAV-9. *Methods Mol Biol* 709:313–329. https://doi.org/10.1007/978-1-61737-982-6_21
13. Bostick B, Shin JH, Yue Y et al (2012) AAV micro-dystrophin gene therapy alleviates stress-induced cardiac death but not myocardial fibrosis in >21-m-old mdx mice, an end-stage model of Duchenne muscular dystrophy cardiomyopathy. *J Mol Cell Cardiol* 53(2):217–222. S0022-2828(12)00179-4 [pii]. <https://doi.org/10.1016/j.yjmcc.2012.05.002>
14. Pan X, Yue Y, Zhang K et al (2013) Long-term robust myocardial transduction of the dog heart from a peripheral vein by adeno-associated virus serotype-8. *Hum Gene Ther* 24(6):584–594. <https://doi.org/10.1089/hum.2013.044>
15. Hakim CH, Yue Y, Shin JH et al (2014) Systemic gene transfer reveals distinctive muscle transduction profile of tyrosine mutant AAV-1, -6, and -9 in neonatal dogs. *Mol Ther Methods Clin Dev* 1:14002. <https://doi.org/10.1038/mtm.2014.2>
16. Zhang Y, Yue Y, Li L et al (2013) Dual AAV therapy ameliorates exercise-induced muscle injury and functional ischemia in murine models of Duchenne muscular dystrophy. *Hum Mol Genet* 22(18):3720–3729. <https://doi.org/10.1093/hmg/ddt224>
17. Pan X, Yue Y, Zhang K et al (2015) AAV-8 is more efficient than AAV-9 in transducing neonatal dog heart. *Hum Gene Ther Methods* 26(4):54–61
18. Hakim CH, Pan X, Kodippili K et al (2016) Intravenous delivery of a novel microdystrophin vector prevented muscle deterioration in young adult canine Duchenne muscular dystrophy dogs. *Mol Ther* 24(Suppl 1):S198–S199
19. Kotin RM, Snyder RO (2017) Manufacturing clinical grade recombinant adeno-associated virus using invertebrate cell lines. *Hum Gene Ther* 28(4):350–360. <https://doi.org/10.1089/hum.2017.042>
20. Clement N, Grieger JC (2016) Manufacturing of recombinant adeno-associated viral vectors for clinical trials. *Mol Ther Methods Clin Dev* 3:16002. <https://doi.org/10.1038/mtm.2016.2>

21. Wang Z, Zhu T, Qiao C et al (2005) Adeno-associated virus serotype 8 efficiently delivers genes to muscle and heart. *Nat Biotechnol* 23 (3):321–328
22. Zhong L, Li B, Mah CS et al (2008) Next generation of adeno-associated virus 2 vectors: point mutations in tyrosines lead to high-efficiency transduction at lower doses. *Proc Natl Acad Sci U S A* 105(22):7827–7832. <https://doi.org/10.1073/pnas.0802866105>

Chapter 17

Considerations on Preclinical Neuromuscular Disease Gene Therapy Studies



Dongsheng Duan

Abstract Numerous neuromuscular and non-neuromuscular diseases are amenable to gene therapy. Rigorously designed and carefully conducted preclinical studies are essential to translate these muscle gene therapies to human patients. Many general guidelines have been published in recent years on how to enhance reproducibility and improve predictive value of preclinical studies. These are excellent guidelines to follow in preclinical gene therapy studies. However, they are not tailored specifically for muscle gene therapy. In this chapter, I discuss considerations in the design of a preclinical neuromuscular disease gene therapy study based on our experience in the preclinical development of adeno-associated virus (AAV) microdystrophin gene therapy. I also discuss adapting the design of phase III clinical trials to animal studies to improve their reproducibility. This chapter is not intended to be all-inclusive and to cover all possible scenarios. Due to the complexity of the candidate diseases that can be treated by muscle gene therapy, it is critical to consider disease-specific issues in the design of each preclinical muscle gene therapy study.

Keywords Preclinical study · Gene therapy · Muscle disease · Experimental design · Adeno-associated virus · Animal model · Dystrophin · Muscular dystrophy

D. Duan (✉)

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

Department of Neurology, School of Medicine, University of Missouri,
Columbia, MO, USA

Department of Biomedical Sciences, College of Veterinary Medicine,
University of Missouri, Columbia, MO, USA

Department of Bioengineering, University of Missouri, Columbia, MO, USA
e-mail: duand@missouri.edu

17.1 Introduction

Muscle gene therapy refers to therapies that are based on delivering genetic materials (nucleic acids) to muscle to treat diseases. While the majority of muscle gene therapy aims at treating neuromuscular diseases (such as muscular dystrophy), investigators have also explored treating non-muscle diseases with muscle gene therapy. In the latter case, the muscle is used as a platform to produce and secrete a therapeutic protein such as factor IX, antitrypsin, or lipoprotein lipase [1–4]. In this chapter, I limit the discussion to preclinical gene therapy studies for treating neuromuscular diseases. Currently, clinical muscle gene therapy has only been conducted in a few neuromuscular diseases. Among these, Duchenne muscular dystrophy (DMD) gene therapy, specifically systemic adeno-associated virus (AAV)-mediated micro-dystrophin gene therapy, has attracted significant attention in recent years [5]. For this reason, I focus on experiences gained and lessons learned in the preclinical development of AAV micro-dystrophin gene therapy to illustrate bench-to-bedside translation of muscle gene therapy. Specifically, I discuss issues related to animal models, gene delivery vectors, and outcome measurements. I also highlight some common misconceptions and important precautions in statistical analysis and data interpretation. Finally, I discuss the preclinical randomized controlled trial (pRCT), a new strategy to conduct late-stage animal studies using best practices learned from human trials.

17.2 Overview of Preclinical Muscle Gene Therapy

Broadly speaking, preclinical studies can be divided into early explorative studies and late investigational new drug (IND)-enabling studies. Early explorative studies establish the proof of principle. Late IND-enabling studies define the risk-benefit profile of the therapy to support regulatory approval of a human trial. In the context of neuromuscular disease gene therapy, early explorative studies have four major goals. The first goal is to identify the therapeutic gene. This can be the disease-causing gene (a gene causing disease when mutated, e.g., the DMD gene, the gene that encodes dystrophin), a homologue of the disease gene (e.g., the utrophin gene), or a disease-modifying gene (e.g., the follistatin gene) [6–8]. This gene can also be an endogenous gene (e.g., the alpha-sarcoglycan gene) or an engineered synthetic gene (e.g., the micro-dystrophin gene) [9, 10]. In some neuromuscular diseases, the disease-causing mutation occurs in the untranslated region (UTR). For example, type I myotonic dystrophy is due to CTG repeat expansion in the 3'-UTR of the DMPK gene. For these diseases, the goal should include the identification of disease-causing mutations.

The second goal of early explorative studies is to determine the optimal gene therapy strategy. The most commonly used gene therapy strategies are gene replacement, gene knockdown, gene repair, and disease-modifying gene therapy. Gene replacement therapy is used to treat a recessive disease by providing a functional

copy of the mutated gene (e.g., treating autosomal recessive limb-girdle muscular dystrophy 2D by expressing the alpha-sarcoglycan gene) [9]. Gene knockdown therapy is used to treat dominant muscle diseases (e.g., treating autosomal dominant limb-girdle muscular dystrophy 1D with RNA interference-mediated silencing of the mutated myotilin gene) [11]. Gene repair therapy aims to remove or correct the mutation. The recently developed clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) editing has yielded promising results in removing the dystrophin gene mutation in animal models of DMD [12, 13]. It should be pointed out that gene repair therapy is not limited to DNA level modification. RNA-level modifications such as RNA trans-splicing and exon skipping are powerful gene therapy tools to bypass the DNA mutation and produce a functional protein [14, 15]. Disease-modifying genes are genes that do not cause the disease themselves but rather they modify disease severity [16]. Disease-modifying gene therapy hence does not target the mutated disease gene directly. Instead, it aims at genes that regulate downstream pathogenic events such as degeneration/regeneration, muscle atrophy, necrosis, and fibrosis. Cytosolic calcium overloading is a common feature in muscular dystrophies. Supraphysiological levels of calcium kill muscle cells by activating proteases and phospholipases. Overexpression of the calcium pump, the sarco/endoplasmic reticulum calcium ATPase (SERCA) gene, or silencing of the SERCA inhibitor sarcolipin has been shown to mitigate muscular dystrophy in mice [17–19].

The third goal of early explorative studies is to select the gene transfer vector and the delivery route. A number of non-viral and viral vectors have been explored for muscle gene therapy. After several decades of research, AAV now stands out as the leading vector for muscle gene therapy (see Chaps. 10 and 11 for additional discussion on AAV) [20]. The delivery route of muscle gene therapy has evolved from early direct intramuscular injection to regional perfusion and now to whole-body systemic delivery. The muscle is one of the most widely distributed tissues in the body. Many muscle diseases not only affect the skeletal muscle but also compromise the heart. Hence, body-wide systemic delivery via intravenous injection is the preferred delivery route for many neuromuscular disorders [21, 22]. A small number of muscle diseases only affect a selective set of muscles (e.g., oculopharyngeal muscular dystrophy). In these cases, direct muscle injection will meet the therapeutic need [23].

The fourth and perhaps the most important goal of early explorative studies is to demonstrate therapeutic efficacy in a relevant animal model. Ideally, this should include demonstration of the intended molecular and biochemical changes, amelioration of histopathology, improvement of physiological functions, and/or the survival rate or lifespan following administration of the candidate gene therapy vector. Early explorative studies are often conducted in academic laboratories. These studies establish the proof of principle for treating a particular neuromuscular disease with a tailored gene therapy approach.

IND-enabling studies are prompted by the positive findings from early explorative studies. The aim is to generate adequate data to support the safety and the scientific basis for an early-phase clinical trial (see Chap. 39 for additional discussion on IND-enabling study). These studies are usually designed with significant

input from the regulatory agency such as the Pre-IND Consultation Program at the Center for Biologics Evaluation and Research (CBER) and the Food and Drug Administration (FDA) (<https://www.fda.gov/Drugs/DevelopmentApprovalProcess/HowDrugsareDevelopedandApproved/ApprovalApplications/InvestigationalNewDrugINDApplication/Overview/default.htm>) [24]. Some IND-enabling studies are conducted in accordance with good laboratory practice (GLP). IND-enabling studies are expected to generate efficacy, pharmacology, and toxicology data. The pharmacology includes pharmacodynamics (PD) and pharmacokinetics (PK). PD refers to the effects a drug has on the body, or simply, drug effects. PK describes how a drug moves through the body, including absorption, distribution, metabolism, and excretion. In the context of gene therapy, these often include but are not limited to dose finding, vector biodistribution in target and nontarget tissues, vector replication, vector shedding, transgene expression in target and nontarget tissues, immune response (T-cell and B-cell responses to the viral capsid and/or transgene product and the innate immune response), blood chemistry, histopathology, insertional mutagenesis, germline transmission, and, in certain cases, long-term evaluation [25]. For gene therapy studies, IND-enabling studies also include characterization and validation of the method used for vector production and purification [25].

17.3 Preclinical Development of AAV Micro-Dystrophin Gene Therapy for DMD

17.3.1 Dystrophin and DMD

DMD is caused by frameshift or frame-aborting mutations in the dystrophin gene. These mutations eliminate production of the dystrophin protein, a subsarcolemmal cytoskeletal protein that stabilizes the muscle cell membrane and protects muscle from contraction-induced injury. In the absence of dystrophin, muscle undergoes degeneration and necrosis and eventually is replaced by fibrofatty tissue. DMD is an X-linked recessive disease. It occurs at a frequency of 1 in 5000 newborn boys [26]. Without medical intervention, patients die prematurely in their 20s to 30s from respiratory and/or cardiac muscle failure. Currently, there is no cure for DMD.

17.3.2 Animal Model

The dystrophin gene was discovered in 1987 [7]. Right around the time of dystrophin gene cloning, two naturally existing dystrophin-deficient animal models were identified, including the mdx mouse and the golden retriever muscular dystrophy (GRMD) dog [27, 28]. Subsequently, a large collection of additional DMD models were discovered in nature or developed using chemical induction or genetic engineering. Currently, ~100 DMD models exist in a broad range of

species including invertebrates (worms and flies), zebrafish, mice, rats, cats, rabbits, dogs, pigs, and nonhuman primates [29, 30]. For mouse and dog models, there also exists a great variety of strains/breeds that differ either in the genetic background or in the mutation type (see Chap. 3 for additional discussion on the DMD model). In addition to the existing models, new DMD models are continuously being generated [31–33]. The existence of so many options allows for a maximal coverage of the entire spectrum of the human disease and makes it possible to meet different experimental needs. However, this also makes it challenging to decide on the best model to use.

In the development of AAV micro-dystrophin gene therapy, several parameters have been considered in model selection. The most important one is the study purpose or the study question. The chosen animal model should allow investigators to answer the question being asked in the study. Selection is further narrowed by the cost, availability, genetic background, age, sex, and existing knowledge on the model (e.g., do we know the natural history of the model? Are there robust outcome measurement assays to study the model? Has the model being utilized previously in testing other experimental therapies?). It should be pointed out that DMD is not the only neuromuscular disease that has multiple animal models. This is also the case for many other neuromuscular diseases such as myotonic dystrophy, facioscapulohumeral muscular dystrophy, and spinal muscular atrophy. The considerations discussed here may also help model selection in designing preclinical gene therapy studies for other neuromuscular diseases.

At the early exploratory stage of AAV micro-dystrophin gene therapy, the primary question is whether the highly abbreviated micro-dystrophin gene can treat muscle disease [10]. In this context, young adult mdx mice become the most appropriate model because (a) the model is readily available, easy to handle and breed, and low-cost (hence, no problem for designing experiments with a meaningful sample size); (b) an array of outcome measurements have been developed to study biochemical, histological, and physiological changes following experimental intervention; (c) mdx mice have been extensively used in transgenic studies to demonstrate therapeutic benefits of the full-length dystrophin gene and various truncated dystrophin genes; (d) mdx mice have been widely used in cell therapy studies and adenoviral vector-based gene therapy studies; (e) mdx mice have also been used to test various drug therapies; (f) the natural history data is available for mdx mice; and (g) there exists a background-matched normal control, the C57Bl/10 mouse. Indeed, all early AAV micro-dystrophin gene therapy studies were performed in mdx mice [34–41].

Despite positive results from young adult mdx mice, there are caveats with using this model. For example, these mice do not manifest the dystrophic clinical phenotype and do not model classic dilated cardiomyopathy of DMD. To determine whether AAV micro-dystrophin therapy can ameliorate disease in a symptomatic model, investigators turned to more severe mouse models such as aged mdx mice, dystrophin/utrophin double knockout mice, dystrophin/MyoD double knockout mice, and DBA/2J-mdx mice [39, 42–46]. Cardiac function of young mdx mice is well preserved. These mice also lack heart pathology. Interestingly, the heart of the aged female mdx mouse displays dilated cardiomyopathy similar

to that seen in DMD patients [47]. Aged female mdx mice are thus used to determine whether AAV micro-dystrophin therapy can mitigate Duchenne cardiomyopathy [48, 49].

Following the proof-of-principle studies in mice, the next challenge is whether AAV micro-dystrophin therapy can be scaled up to treat affected boys. This question is addressed in the canine DMD model [50]. The body weight of a boy is ~700- to 800-fold higher than that of a mouse but only ~2- to 4-fold higher than that of a dog. Importantly, the clinical course and manifestations of dystrophin-deficient dogs are similar to those of DMD patients [29]. AAV micro-dystrophin gene therapy in the dog model bridges the gap between mice and human patients [50–55]. Together, efficacy and pharmacokinetic and dose-finding studies in different animal models pave the way to the regulatory approval of three early-phase systemic AAV micro-dystrophin gene therapy trials in DMD patients [10].

It is worth pointing out that preclinical studies are not only limited to the disease model. They are also performed in normal animals. For example, FDA requires toxicology data from at least two species (one rodent and one non-rodent). In the case of systemic AAV micro-dystrophin gene therapy, normal nonhuman primates were used as the non-rodent species for toxicology and biodistribution study [56].

The discussion above mainly focused on animal models used in the development of systemic AAV micro-dystrophin gene therapy. However, these models may not be ideal for testing other DMD gene therapy approaches. The most common mutations in DMD patients are deletion mutations between exons 43 and 55 [57]. However, the majority of early DMD models carry point mutations outside this hotspot region. For example, mdx mice carry a nonsense point mutation in exon 23, and GRMD dogs carry a point mutation in intron 6 that disrupts splicing [27, 28]. For exon skipping and CRISPR/Cas9 gene-editing therapy, models that share mutations similar to those of DMD patients would be more useful for translational purposes. To meet these needs, several new models carrying a deletion mutation in this region were generated recently [30–33]. To study the human dystrophin gene in the mouse genome, a transgenic hDMD mouse, which carries the full-length human dystrophin gene, was generated on the background of the mdx mouse [58, 59]. Subsequently, a deletion mutation was introduced in the mutation hotspot region (exons 43–55) in the human dystrophin gene [31, 33]. There is a high likelihood that gene repair reagents developed in these models can be directly used in human studies because of the sequence identity.

Some DMD gene therapy-related questions can also be addressed without applying gene therapy to diseased animals. For example, one of the fundamental questions in dystrophin gene replacement therapy is the level of expression. Will low-level expression offer any benefit? Will high-level expression cause toxicity? To study whether low-level dystrophin expression can ameliorate DMD, we used the chemically induced mdx3cv mice which express 4–5% dystrophin in all muscle cells [60–62]. The Aartsma-Rus lab crossed mdx mice with Xist^{Δh} mice. Due to random X-chromosome inactivation, the resulting mdx-Xist^{Δhs} mice showed a wide range of low-level dystrophin expression [63–65]. These studies echo well with clinical findings and suggest that marginal level dystrophin expression is still ben-

eficial [66, 67]. The toxicity of supraphysiological dystrophin overexpression was studied in transgenic mdx mice [68, 69]. These studies suggest that 50-fold overexpression is not toxic. However, ≥ 100 -fold overexpression is associated with cardiac toxicity.

The age, gender, and genetic background are known factors that greatly affect study outcome [70–75]. Mdx display an acute necrotic phase between the age of 2 and 6 weeks. This phase does not exist in human patients [29]. It is thus advisable to avoid performing gene therapy studies in this age range. It has been demonstrated that mouse sex may greatly impact skeletal muscle function, cardiomyopathy manifestation, and inflammation [76–79]. We also noticed that sex profoundly influences disease severity in mdx mice. Male mdx mice show more severe skeletal muscle disease, while female mice display more advanced cardiomyopathy [47, 80]. It is worthwhile to point out that animal sex may not only modify disease manifestations but may also change transduction profile of the AAV vector [81, 82]. Hence, depending on the study question, animal sex should be carefully considered in experimental design. One often made mistake is mixing male and female animals in the same experimental group. This is especially problematic when the ratios of male and female animals are not equal between the gene therapy group and control groups (untreated controls and normal controls). Such a design can greatly confound data interpretation.

There are ample publications documenting the influence of the genetic background on animal behaviors, activities, organ/tissue functions, and disease phenotype [70, 71, 83–88]. Of particular interests to muscle gene therapy are the publications that demonstrate the impact of the genetic background on muscle and heart function [75, 89–93]. In the context of mdx mice, it has been shown that the genetic background profoundly influences the frequency and expansion of revertant fibers, the rarely occurring dystrophin positive fibers seen in dystrophin-deficient muscle [94]. Tachycardia is a characteristic feature in DMD patients. Interestingly, we found that this phenotype is preserved in C57Bl/10 background mdx mice but not in FVB background mdx mice [95, 96].

Collectively, these findings underscore the importance of model selection as a critical aspect in the design of preclinical muscle gene therapy studies. As a rule of thumb, one should keep in mind that no model is perfect because humans are not animals. The other point that can never be overemphasized is the nature history study. Such studies should begin as soon as the model becomes available and continue through the course of preclinical development and after the completion of a preclinical gene therapy study.

17.3.3 Gene Delivery Vector

Gene therapy vectors can be broadly classified as viral and non-viral vectors. Viral vectors can be further divided into DNA viral vectors and RNA viral vectors [97]. All these vectors have been tested in human patients for different gene therapy needs [98]. A number of factors are considered when determining which vector is

the best for a particular application. These include, but are not limited to, the nature of the disease (recessive or dominant, affected organ(s) and tissues(s), disease gene and gene product, etc.), biological features of the vector (carrying capacity, tropism, replication competence, persistence, immunogenicity, genotoxicity, etc.), and vector manufacture (production, purification, storage, etc.).

DMD gene therapy faces several unique challenges. First, the dystrophin gene is one of the largest genes in the genome, and the dystrophin coding sequence exceeds 11 kb. Second, the disease affects both skeletal and cardiac muscle. Skeletal muscle accounts for ~40% body weight and spreads throughout the body. The heart is located in the chest cavity, and it beats continuously. Third, the dystrophic muscle is highly inflamed. Fourth, DMD is a chronic disease, and an effective therapy requires lifelong expression of a therapeutic dystrophin gene. To meet these needs, an ideal vector for DMD gene therapy should have a large packaging capacity, have the ability to efficiently transduce all body striated muscle, result in persistent transduction, and be non- or minimally immunogenic.

Since the discovery of the dystrophin gene in 1987, many different vector systems have been tested for DMD gene therapy. The non-viral plasmid vector did not meet the needs because it only led to poor and transient transduction in the muscle [99, 100]. The retroviral vector did not work because it could not transduce postmitotic muscle tissue [101]. The adenoviral vector failed because of its immune toxicity [102]. AAV seems to meet most of the needs of DMD gene therapy. AAV can lead to high-level, persistent, whole-body muscle transduction without inducing a strong cellular immune response. Further, AAV is now considered a medicine [103]. Several AAV-based gene therapy drugs have received regulatory approval for commercial use, such as Luxturna for treating an inherited childhood blindness disease called Leber congenital amaurosis and Glybera (also called Alipogene tiparvovec) for treating lipoprotein lipase deficiency. The only shortcoming of the AAV vector is its less than 5 kb packaging capacity. Fortunately, this problem is solved with the development of the micro-dystrophin gene (≤ 4 kb).

The wild-type AAV virus is composed of an approximately 4.7 kb single-stranded DNA genome and a ~25 nm icosahedral capsid. An intact AAV virus genome has two inverted terminal repeats (ITRs) at the 5' and 3' end. In the middle are the expression cassettes for making AAV replication proteins, capsid proteins, and the assembly-activating protein. In a recombinant AAV vector, viral gene expression cassettes are replaced by a transgene (reporter gene or therapeutic gene) expression cassette. The ITR serves as the AAV replication origin and packaging signal. The ITR is the only viral element present in an AAV vector.

Two decisions are made in AAV vector selection, one for the vector genome and the other for the viral capsid. For the vector genome, we need to decide on the genome configuration and expression cassette. Two genome configurations exist including single-stranded AAV (ssAAV) and self-complementary AAV (scAAV) [104]. The ssAAV has the exactly same single-stranded genome configuration as that of wild-type AAV. In the scAAV vector, the terminal resolution site from one ITR is deleted [105]. This allows packaging of a double-stranded vector genome with two wild-type ITRs at the ends and the mutated ITR in the middle. This con-

figuration bypasses single-strand to double-strand conversion, a rate-limiting step in AAV transduction, and yields rapid-onset and higher-level transduction in the muscle [104, 106, 107]. The trade-off of the scAAV vector is the reduction of the packaging capacity from 5 kb to 2.5 kb. A number of therapeutic genes have been packaged in scAAV such as the factor IX gene for treating hemophilia B and the survival motor neuron gene for treating spinal muscular atrophy [108, 109]. The size of a functional micro-dystrophin gene is in the range of 3.8–4 kb [10]. This exceeds the 2.5 kb packaging capacity of scAAV. Hence, only ssAAV is used for micro-dystrophin gene therapy.

A basic expression cassette is composed of a promoter, a transgene, and a polyadenylation (pA) signal. Sometimes, additional regulatory elements (such as the microRNA target site) are included to fine-tune transgene expression (see Chap. 8 for additional discussion on expression cassette design). At the early exploratory stage, investigators often use a ubiquitous promoter such as the cytomegalovirus (CMV) promoter. However, tissue-specific promoters (if available) should be used for large animal studies and IND-enabling studies. In the case of AAV micro-dystrophin gene therapy, a variety of muscle-specific promoters have been used including abbreviated promoters derived from the muscle creatine kinase gene (e.g., CK6, CK8, MHCK7, and minimized MCK) and synthetic promoters (e.g., Spc5-12) [10, 110, 111]. The transgene refers to the cDNA rather than the intron-containing full-length gene. Several strategies have been used to improve expression. These include codon optimization of the cDNA sequence [40], inclusion of a small intron between the promoter and the transgene [112], and utilization of a gene variant that shows an increased activity (e.g., the Padua variant of the factor IX gene for hemophilia B gene therapy) [113]. To minimize the immunogenicity of the transgene, one may also consider removing CpG motifs by introducing silent mutations [114, 115]. A unique issue for AAV micro-dystrophin gene therapy is the configuration of the microgene. In the microgene, ~70% of the dystrophin coding sequence is removed. More than 30 different microgene configurations have been published [10]. The difference in the constitution of the microgene may greatly impact its biological activity. For example, inclusion of dystrophin spectrin-like repeats 16 and 17 in the microgene allows restoration of neuronal nitric oxide synthase to the sarcolemma [39, 116]. Polyadenylation is critical for nuclear export of the RNA transcript and the stability/translation of mRNA. A variety of pA signals are available [117]. SV40 virus late pA (SV40 pA) and bovine growth hormone pA (bGH pA) are commonly used strong pA signals [118]. In the case of the AAV micro-dystrophin vector, a 49 bp synthetic pA is used due to the packaging limit of the AAV vector [119].

Another important decision to make in AAV vector selection is the viral capsid. Capsid not only determines tissue tropism but also influences intracellular trafficking and processing of the viral particle. The triangulation number *T* reflects the size and complexity of the capsid. Although the *T*-number for AAV is merely 1, the AAV capsid still comes with many different “flavors.” Twelve AAV serotypes and hundreds of AAV variants have been published [120]. Newer AAV capsids are continuously being generated.

Among existing AAV serotypes, AAV1 and AAV6 (especially tyrosine-modified AAV6) are the best for direct intramuscular injection [121]. AAV6, AAV8, and AAV9 are the top serotypes for intravascular delivery [21]. For the purpose of systemic muscle gene therapy, a capsid with enhanced muscle transduction and reduced non-muscle transduction would be very appealing. Hence, there have been continuous efforts from many academic laboratories as well as industry to further improve the existing serotypes. Using AAV8 and/or AAV9 as the benchmark, several new muscle tropic AAV capsids were developed in the last few years. These new capsids were engineered either by rational design or by forced evolution [122]. They include AAV2i8, AAVB1, AAV587MTP, AAVM41, AAV9.45, AAV9.61, AAVMyo, and AAVNP22 and AAVNP66 [123–129]. Except for AAVNP22 and AAVNP66 [128], most of these newly developed muscle tropic AAVs are developed and tested in mice. It should be mentioned that AAV tropism may exhibit significant interspecies difference. For example, AAV8 is the best for the mouse liver, but AAV3B is the best for human liver cells [130, 131]. AAV9 is considered as a cardiotropic capsid in murine studies, but AAV8 outperforms AAV9 in the neonatal dog heart [132–134]. Because the same AAV serotype may exhibit distinctive behavior in different species, additional studies are needed to determine whether these newly developed muscle tropic AAV variants can outperform existing serotypes (AAV8 and AAV9) in large animals and human patients.

At the time when the first micro-dystrophin gene was engineered [135], the best studied AAV was AAV serotype 2 (AAV2). Methods have also been developed for the production and purification of AAV2 vectors. Hence, AAV2 was used in early micro-dystrophin studies [34–36]. The discovery of the systemic transduction property of several newer AAV serotypes (AAV6, AAV8, and AAV9) opens the door to whole-body muscle therapy [136–138]. These serotypes (especially AAV8 and AAV9) are now not only the most popular AAV capsids in preclinical muscle gene therapy studies but are also the chosen serotypes in several ongoing human trials. For example, AAV9 was used in type 1 spinal muscular atrophy patients and DMD patients [10, 22], AAV8 was used in X-linked myotubular myopathy patients and hemophilia B patients [139, 140], and AAVrh74 (a serotype similar to AAV8) was used in DMD patients [10, 141].

Additional issues to consider in AAV capsid selection are the pre-existing neutralizing antibody (NAb) titer, AAV purity, AAV production methods, and AAV titer determination. The preinjection NAb titer is usually not measured in murine studies (unless the study goal is to test readministration). However, it is essential to measure the preinjection NAb titer in large animal studies because of the high prevalence of NAb in these animals and the high cost of the large animal study [142–145]. It has been shown that even a relatively low NAb titer may compromise AAV transduction [146–148]. AAV purity has been shown to significantly influence its transduction efficiency in a serotype- and tissue-independent manner [149]. For this reason, the stock AAV vector should be checked for purity, at least, by silver stain for capsid purity and by quantifying the endotoxin level for contamination before dosing large animals [150]. Numerous methods have been developed to produce and purify AAV [151–153]. Research grade AAV is usually made using the transient transfection

method in adherent 293 cells. For systemic delivery in human patients, scalable methods (such as baculovirus production system, herpes simplex virus production system, and transient transfection in suspension 293 cells) are often used to generate clinical grade AAV [154–156]. Research grade AAV can be used in most preclinical studies. However, clinical grade AAV should be used for IND-enabling toxicity study. The AAV titer is usually determined by quantitative PCR (qPCR) and expressed as viral genome particles per milliliter (vg particles/ml or vg/ml). Despite a seemingly simple technique, significant inter- and intra-laboratory variations have been reported [157–160]. Some technical issues in the design of the qPCR protocol may have contributed to this inconsistency. For example, using ITR-specific qPCR may lead to the overestimation of the titer [158]. The conventional method that is used for titrating ssAAV may underestimate the scAAV titer [161]. Hence, optimization and validation of the AAV titration protocol should be an integral component of a preclinical muscle gene therapy study [162].

17.3.4 Outcome Measurements

In addition to the correct animal model, vector and delivery route, rigorous outcome measurements, and data analysis are also essential to get meaningful results and correct conclusions (either positive or negative). For some outcome measurements, there are established methods/assays widely used in the field. In this case, it is essential to strictly follow published protocols or use established standard operation procedures (SOPs). For protocols described only in the method section of research articles, it is always helpful to contact the authors and get the working protocol. The working protocol often contains more details that are critical for conducting the assay. Sometimes, a detailed protocol may have already been published in a different format in method journals such as *Methods in Molecular Biology* (Springer protocols; published by the Humana Press) [163–165]; *Journal of Visualized Experiments* (JoVE; published by MyJove Corporation) [166–168]; *Current Protocols* series such as *Current Protocols in Molecular Biology*, *Current Protocols in Mouse Biology*, and *Current Protocols in Immunology* (published by John Wiley & Sons Inc); and *Nature Protocols* (published by Nature Publishing Group) [169–172]. A number of SOPs have been established for studying neuromuscular diseases. These SOPs are freely accessible at the Treat-NMD Neuromuscular Network website (<http://www.treat-nmd.eu/research/preclinical/overview/>) and Parent Project Muscular Dystrophy website (http://join.parentprojectmd.org/site/PageServer?pagename=Advance_researchers_sops) [173–176].

While many functional assays exist for studying neuromuscular diseases in mice, few are available for studying large animals. To fill the gap, we and others have begun to develop protocols for evaluating muscle function in canines over the last few years. These include dog gait analysis [177–180], noninvasive dog activity quantification [177, 181], electrical impedance myography [182], in situ single muscle force measurement [183], and, most recently, a physiological assay to study

functional ischemia in dog muscle in vivo [184]. Several points should be considered in the development of a new assay. These include selectivity (the new method is specific for the intended purpose), sensitivity (the lowest value the new method can accurately measure), accuracy (the value obtained from the new method should reflect the actual value in the sample), and reproducibility (the new method can yield the same results on replicates). It should be pointed out that the development, optimization, and validation of a new protocol should not be considered as a trivial undertaking. It often requires multidisciplinary collaboration among investigators who have expertise in different fields including, but not limited to, neuromuscular disease, muscle physiology, veterinary medicine, computer science, bioengineering, and statistics.

Failure to validate/authenticate experimental reagents contributes to irreproducibility in biomedical research. Authentication of key biological and chemical reagents has now attracted a lot of attention from major funding agencies (such as NIH) and publishers [185–189]. Online guidelines and checklists are readily available on NIH (<https://grants.nih.gov/grants/guide/notice-files/NOT-OD-17-068.html>), journal, academic society, and institution websites and will not be discussed in depth in this chapter. However, I'd like to emphasize the importance of antibody validation. Antibodies are among the most commonly used reagents in research. Unfortunately, many commercially available and self-generated antibodies are poorly validated and/or characterized. This has become a major concern in the research community because data generated with these antibodies vary greatly and conclusions drawn are often misleading due to frequent false-positive and false-negative results.

In the case of DMD, we recently evaluated 65 epitope-specific dystrophin monoclonal antibodies in murine and canine muscles by immunostaining and Western blot [190]. These antibodies were originally developed for diagnostic use in human patients [191, 192]. Their research use in the mouse and dog DMD models has never been validated. Interestingly, we found some antibodies work well for immunostaining/Western blot in murine/canine muscle (e.g., Mandys 1), some antibodies work well only for immunostaining in murine/canine muscle (e.g., Mandys 105), some antibodies work well only for Western blot in murine/canine muscle (e.g., Mandra 13), some antibodies work well for immunostaining/Western blot in murine muscle only (e.g., Mandnex 45B), and some antibodies work well for immunostaining/Western blot in canine muscle only (e.g., Mannex 4850D) (Fig. 17.1). There are also antibodies that do not work for immunostaining/Western blot in murine/canine muscle (e.g., Mandhinge 2C) (Fig. 17.1).

Various strategies have been suggested on how to validate an antibody [193–197]. Several working groups and consortium have developed online resources that allow users to search for validation information of an antibody. These online antibody databases include, but are not limited to, Antibodypedia (<https://www.antibodypedia.com/>) [198, 199], Human Protein Atlas (<https://www.proteinatlas.org/>) [200], CiteAb (<https://www.citeab.com/>) [201], AbMiner (<https://discover.nci.nih.gov/abminer/>) [202], EuroMAbNet (<https://www.euromabnet.com/guidelines/example1.php>) [203], Biocompare (<https://www.biocompare.com/Antibodies/>)

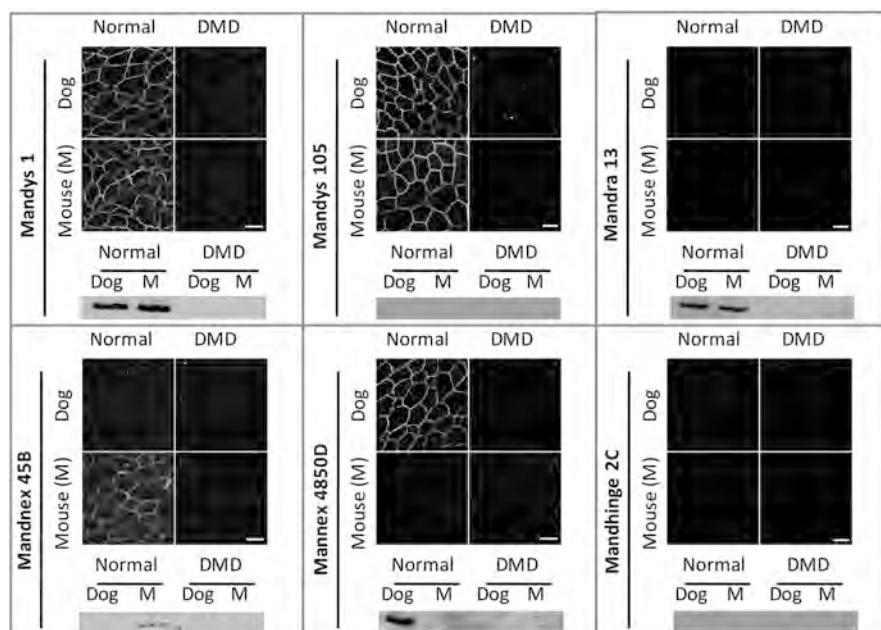


Fig. 17.1 Validation of dystrophin monoclonal antibodies in the muscle of normal and affected mice and dogs by immunostaining and Western blot. Representative photomicrographs illustrate different behaviors of different antibodies. Some (e.g., Mandys 1) can recognize dystrophin in both murine and canine muscle irrespective of detection methods. Some (e.g., Mandys 105, Mandra 13, Mandnex 45B, and Mannex 4850D) recognize dystrophin in a species- and method-dependent manner. In extreme cases (e.g., Mandhinge 2C), an antibody may completely fail to recognize dystrophin in mouse and dog muscle

[204], SelectScience (<https://www.selectscience.net/antibodies/product-directory/>) [204], and BenchSci (<https://www.benchsci.com/>).

17.3.5 Statistics

Statistical considerations for preclinical muscle gene therapy studies are not different from those used for other studies. Several recent review articles have thoroughly elaborated these considerations [205, 206]. Here I only outline some important points.

Statistical methods should be introduced at the study planning stage rather than just for data analysis. Prior to animal experiments, the sample size should be determined using the power analysis. Methods for sample size calculation can be found in publications cited here [207–211]. A number of free online programs are also available such as G*Power (https://download.cnet.com/G-Power/3000-2054_4-10647044.html) and Sample Size Calculator (<http://powerandsamplesize.com/>) [212].

Randomization is another important strategy to improve the stringency and reproducibility of preclinical studies [213, 214]. Depending on the study design, randomization can be achieved with a variety of methods such as simple randomization, block randomization, stratified randomization, and covariate adaptive randomization [215]. Similar to the power analysis used for sample size calculation, several online programs such as Randomization (<http://www.randomization.com/>) and Research Randomizer (<https://www.randomizer.org/>) are available for randomization.

The *t*-test and analysis of variance (ANOVA) are commonly used statistic methods to compare the mean value between two groups and among multiple groups, respectively [216, 217]. It should be noted that these methods are only applicable to parametric data. When the sample size is small, data may not always follow normal distribution. Most animal studies have a sample size of ≤ 10 in each experiment group. Therefore it is important to check if the data is parametric before applying the *t*-test or ANOVA. Sometimes skewed data can be converted to parametric by logarithm transformation. If data are not parametric or if there are outliers, nonparametric tests should be used. The Wilcoxon rank-sum test (also called the Mann–Whitney test) and the Wilcoxon signed-rank test are nonparametric tests for two non-paired and paired group comparison, respectively. The Kruskal–Wallis test is the nonparametric test for multiple group comparison [218]. Very often, multiple outcomes are measured in the same study, or a single outcome is measured repeatedly in a longitudinal study. In the former, the Bonferroni adjustment should be used. In the latter, mixed models should be considered. In these cases, it is always advisable to consult with a statistician.

The *p* value is perhaps the most commonly used but also the most controversial and misused statistical concept [219–223]. In statistical analysis, one usually concerns two types of errors, false positive (type I error) and false negative (type II error). The *p* value quantifies the probability of making a type I error, i.e., the chance of rejecting a null hypothesis that is actually true. The smaller the *p* value, the smaller the chance of the results being false positive. The *p* value does not reflect clinical significance. Interpretation on clinical implications of a statistically significant finding requires input from clinicians and experts in the related field. For example, in hemophilia B gene therapy, $\geq 5\%$ factor IX activity is required for a clinically meaningful improvement. A candidate gene therapy may result in a statistically significant increase of 0.5% activity (e.g., from 1% activity to 1.5% activity). Although this is statistically significant, we cannot consider this gene therapy successful because the improvement is not therapeutically relevant. The *p* value should always be considered with the type II error or power (1-type II error). A *p* value of larger than 0.05 does not necessarily suggest a therapy is not effective. The 0.05 threshold of the *p* value is only based on a somewhat arbitrary convention. A *p* value of >0.05 can also be caused by a small sample size or a lack of statistical power. The power reflects the probability of making (or avoiding) a type II error. The higher the power, the lesser the likelihood of the result being a false negative.

17.3.6 *Data Interpretation*

Misinterpretation and over-interpretation of animal study results contribute to the poor translation of preclinical research findings to clinical benefits [224–226]. Below I illustrate some commonly encountered issues in preclinical muscle gene therapy studies.

The creatine kinase (CK) level is a commonly used blood biomarker for muscle injury. The enzyme is released from muscle to the circulation when muscle is injured. The reduction in the CK level is often considered as an indication of muscle protection by the candidate therapy. However, the CK level can be skewed by a number of factors such as the method used in blood collection, body condition and age of the animal, and the genetic background of the animal [227–230]. For example, the CK level can be artificially raised because of dehydration or inappropriate handling of the animal at the time of blood collection [231]. It should also be noted that the CK level will go down with age in dystrophic subjects even in the absence of any therapy (likely due to the replacement of muscle by fibrofatty tissue) [227].

The context of the experimental setting (such as the strain, age, and sex of the animal or detection method) should be carefully considered in data interpretation. For example, optical imaging of cathepsin activity in live mice is an excellent tool to sensitively monitor muscle inflammation in a noninvasive way [232]. The value in mdx limb muscle is two- to threefold higher than that of aged-matched BL10 mice at 7 weeks. However, the difference between two strains becomes negligible at 52 weeks although mdx mice still have muscle inflammation [233]. Conclusions drawn without the consideration of the mouse age will be misleading in this example. In dystrophin restoration gene therapy, an increase in the percentage of the dystrophin level may have different meanings depending on the detection method (Western blot or immunostaining). Western blot reflects total dystrophin in the whole muscle lysate, while immunostaining reflects dystrophin expression in individual myofibers. A moderate increase detected by Western blot could suggest a uniform low-level increase in all myofibers or a high-level increase in a subset of myofibers (mosaic expression).

Muscle histology is essential for the evaluation of the therapeutic effect. However, there could be significant individual variations among strain-, age-, and sex-matched animals (Fig. 17.2a). Further, since muscle damage is not uniform in mdx mice, there are often huge differences in different regions of the same section from the same muscle (Fig. 17.2a). Correct interpretation can only be obtained from a thorough evaluation of the entire muscle section from all experimental animals.

Degeneration and regeneration are characteristic features in muscular dystrophy. This is frequently evaluated by quantifying the percentage of centrally localized nuclei (% CN). A reduction in the % CN after gene therapy is often considered as an indication of improved muscle protection. In other words, the candidate gene therapy is effective in treating muscle disease. However, this may not always hold true because the % CN will also decrease if the candidate therapy negatively impacts muscle regeneration (a detrimental effect).

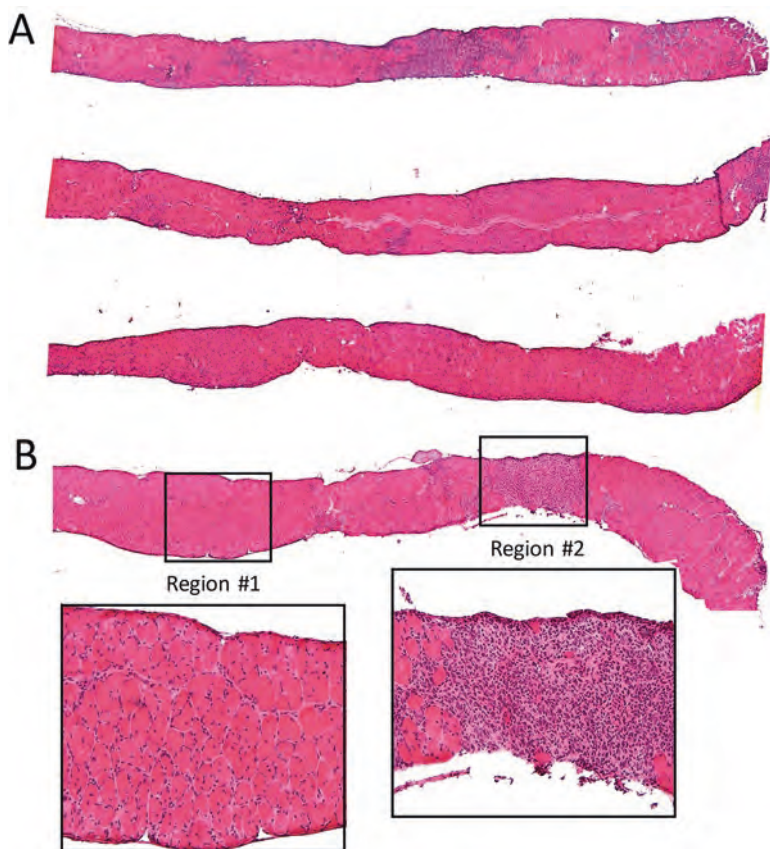


Fig. 17.2 Heterogeneity of the pathology in dystrophic muscle. (a) Diaphragm HE staining from three age- and sex-matched untreated young adult mdx mice. (b) HE staining revealing dramatic differences of muscle pathology in different regions of the same muscle section in the diaphragm of a young adult mdx mouse

A commonly held misconception by inexperienced researchers is that “muscle histology should always correlate with muscle function (and vice versa).” This might be true in some occasions, but there are many exceptions. For example, removing collagen VI from γ -sarcoglycan-deficient mice (a model for limb-girdle muscular dystrophy 2C) attenuates muscle pathology, but muscle force is not improved [234]. Marginal level dystrophin expression improves skeletal muscle function, heart function, and survival in mouse DMD models, but it does not improve muscle histology [60–62].

17.4 Phase III: A New Concept in Preclinical Study

Clinical studies are divided into different phases. Phase I evaluates the safety. Phase II establishes the efficacy. Phase III confirms the safety and efficacy in a large group of patients through a multicenter randomized controlled trial (RCT). Phase IV is post-marketing surveillance. The FDA makes the decision on whether a candidate drug should be approved or not based on the results from the first three phases of the trial (since a go no-go decision has to be made after each phase, the final FDA decision is usually based on the results of the phase III trial). To improve translation from animal studies to human trials, a similar paradigm was proposed recently to divide preclinical studies into four phases [235, 236]. Preclinical phase I is the discovery phase. At this phase, the focus is to elucidate disease mechanism(s) and to identify therapeutic targets (drug targets). Preclinical phase II refers to initial efficacy studies performed by independent (often individual) academic laboratories. Traditionally, a preclinical exploratory study will stop here and move to the stage of the IND-enabling study. It is believed that this approach has contributed to the reproducibility crisis in translational research. To address this issue, a new concept called “preclinical phase III study” is introduced. When positive results are obtained in a preclinical phase II study, the study moves to a large-scale preclinical phase III study instead of an IND-enabling study. In preclinical phase III, multicenter preclinical randomized controlled trials (pRCT) are conducted to confirm and validate the efficacy data obtained from preclinical phase II [235–238]. Preclinical phase IV is initiated based on positive findings from preclinical phase III studies. In preclinical phase IV, IND-enabling PK, PD, and toxicology studies are carried out by academia, industry, or academia/industry collaboration to pave the way for IND application.

Numerous clinical trials that were based on the efficacy data of animal studies have failed to yield a therapeutic drug [224–226]. Among many factors that have caused this translational crisis is poor reproducibility. In fact, it was suggested that more than 70% of published studies cannot be reproduced [239–242]. It is believed that the high failure rate of replication studies is due to the limited sample size (lack of power), lack of blinding and/or randomization, inadequate data report, and flaws in statistical analysis. Two approaches have been used to address this issue, systemic review/meta-analysis and pRCT [237, 243, 244]. In systemic review/meta-analysis, individual published studies are reviewed, and data from each study are pooled together and reanalyzed as a whole [244–248]. Systemic review/meta-analysis provides a less biased opinion on a candidate therapy (or drug). However, its conclusion is limited by the lack of a predefined study protocol, lack of inclusion/exclusion criteria, heterogeneity in materials and methods, inconsistency in data collection and reporting, and difference in study end points [249, 250]. This is further aggravated by publication bias in favor of positive data.

The recently introduced pRCT approach is modeled after the RCT, the gold standard in clinical trials. The pRCT utilizes the same rigorous design and standardized protocols as the RCT. The pRCT utilizes a centralized management structure. It has a study steering committee, a coordinating center, a data monitoring committee, an adjudication committee, a statistical center, and a training center. The study is designed by the statistical center in collaboration with participating laboratories. All participating laboratories use identical animal models, (strain/genetic background, age, sex, animal supplier, and husbandry), reagents (chemical and biological), standardized methodologies, key equipment, and animal protocols. All participating researchers receive centralized training on procedures (data generation, blood/tissue collection and transportation). All participating researchers agree to every aspect of the study design such as blinding, randomization, placebo control, concealment of allocation, and sharing of the original data. Data generated in all participating laboratories are sent to the statistical center for data analysis. Inclusion and exclusion criteria for animals and data points are determined prior to the study. All participating researchers agree on publication (even if results are negative) [236, 237]. The pRCT addresses a number of issues that have contributed to the reproducibility crisis such as the small sample size, inconsistent data handling, publication pressure and bias, and deficiency in methodology reporting. However, the pRCT also faces significant challenges including coordination, standardization, and financial cost. The first pRCT was published recently [251]. This study establishes feasibility of the pRCT. Despite the pRCT being considered a major advance in the field of translational research, it is yet unknown whether and how much the pRCT will improve validity of animal studies and prevent unnecessary (or even harmful) clinical trials in the future. A recent publication argues that excessive standardization in animal studies may undermine translation from bench to bedside due to ignorance of inherent biological variations in animals and humans [252].

Preclinical muscle gene therapy is at the dawn of a rapid growing phase. Incorporating the concept and practice of the pRCT may give us a better chance of success when translating from animal studies to human trials.

17.5 Conclusion

Irreproducibility in preclinical research has placed a significant socioeconomic burden on therapeutic drug development. It is estimated that ~\$28 billion is spent every year in the United State of America on preclinical studies that cannot be reproduced [253]. Concerns on the reliability of preclinical studies have sparked intense discussions in the biomedical research field. To address this critical issue, many excellent general guidelines have been published in recent years on how to rigorously design a preclinical study to improve reproducibility and enhance predictive value for clinical trials [188, 254–258]. These papers have thoroughly discussed fundamental principles, commonly encountered problems, and solutions regarding sample size determination, randomization, blinding, data handling, statistical analysis,

reporting, biological variables, standard operating protocols in outcome measurement, and validation/authentication of experimental reagents [205, 259–266]. These guidelines should always be strictly followed in a preclinical muscle gene therapy study.

According to the gene table of neuromuscular disorders (www.muscle.genetable.fr/), nearly 500 disease genes have been identified for ~900 neuromuscular diseases [267]. It is conceivable that many of these diseases are eligible for muscle gene therapy. The considerations discussed in this chapter may serve as a starting point to shape the design of a preclinical gene therapy study for treating these neuromuscular diseases. However, it should be pointed out that every preclinical muscle gene therapy study has its unique features. There is no one-size-fits-all. Disease-specific issues should always be considered in the design and implementation of a preclinical muscle gene therapy study. There are numerous examples where costly clinical trials have failed despite promising efficacy data in animal models. In light of this, perhaps it is more important to carefully design and rigorously conduct preclinical studies to exclude ineffective candidate gene therapy drugs from going to more expensive and potentially detrimental human trials.

Acknowledgment Muscle gene therapy research in the Duan lab is currently supported by the National Institutes of Health (NS-90634, AR-70517, AR-69085), the Department of Defense (MD150133), Jesse's Journey: The Foundation for Gene and Cell Therapy, Hope for Javier, Jackson Freel DMD Research Fund, Parent Project Muscular Dystrophy, and Solid Biosciences. The author thanks the Duan lab members for helpful discussion. The author thanks Drs. Jianguo (Tony) Sun and Gang (Gary) Yao for their helpful advices on the statistics section (Sect. 17.3.5). The author thanks Emily Million and John D'Alessandro for the help with proofreading the manuscript.

Disclosure The author is a member of the scientific advisory board for Solid Biosciences and an equity holder of Solid Biosciences. The Duan lab has received research support from Solid Biosciences.

References

1. Stoes ES, Nierman MC, Meulenberg JJ, Franssen R, Twisk J, Henny CP, Maas MM, Zwiderman AH, Ross C, Aronica E, High KA, Levi MM, Hayden MR, Kastelein JJ, Kuivenhoven JA (2008) Intramuscular administration of AAV1-lipoprotein lipase S447X lowers triglycerides in lipoprotein lipase-deficient patients. *Arterioscler Thromb Vasc Biol* 28(12):2303–2304. <https://doi.org/10.1161/ATVBAHA.108.175620>
2. Herzog RW, Mount JD, Arruda VR, High KA, Lothrop CD Jr (2001) Muscle-directed gene transfer and transient immune suppression result in sustained partial correction of canine hemophilia B caused by a null mutation. *Mol Ther* 4(3):192–200
3. Manno CS, Chew AJ, Hutchison S, Larson PJ, Herzog RW, Arruda VR, Tai SJ, Ragni MV, Thompson A, Ozelo M, Couto LB, Leonard DG, Johnson FA, McClelland A, Scallan C, Skarsgard E, Flake AW, Kay MA, High KA, Glader B (2003) AAV-mediated factor IX gene transfer to skeletal muscle in patients with severe hemophilia B. *Blood* 101(8):2963–2972

4. Flotte TR, Trapnell BC, Humphries M, Carey B, Calcedo R, Rouhani F, Campbell-Thompson M, Yachnis AT, Sandhaus RA, McElvaney NG, Mueller C, Messina LM, Wilson JM, Brantly M, Knop DR, Ye GJ, Chulay JD (2011) Phase 2 clinical trial of a recombinant Adeno-associated virus vector expressing alpha 1 antitrypsin: interim results. *Hum Gene Ther* 22(10):1239–1247. <https://doi.org/10.1089/hum.2011.053>
5. Duan D (2018) Micro-dystrophin gene therapy goes systemic in Duchenne muscular dystrophy patients. *Hum Gene Ther* 29(7):733–736. <https://doi.org/10.1089/hum.2018.012>
6. Mendell JR, Sahenk Z, Malik V, Gomez AM, Flanigan KM, Lowes LP, NA L, Berry K, Meadows E, Lewis S, Braun L, Shontz K, Rouhana M, Clark KR, Rosales XQ, Al-Zaidy S, Govoni A, Rodino-Klapac LR, Hogan MJ, Kaspar BK (2015) A phase I/IIa follistatin gene therapy trial for Becker muscular dystrophy. *Mol Ther* 23(1):192–201. <https://doi.org/10.1038/mt.2014.200>
7. Kunkel LM (2005) 2004 William Allan award address. Cloning of the DMD gene. *Am J Hum Genet* 76(2):205–214
8. Gilbert R, Nalbantoglu J, Petrof BJ, Ebihara S, Guibinga GH, Tinsley JM, Kamen A, Massie B, Davies KE, Karpati G (1999) Adenovirus-mediated utrophin gene transfer mitigates the dystrophic phenotype of mdx mouse muscles. *Hum Gene Ther* 10(8):1299–1310
9. Mendell JR, Rodino-Klapac LR, Rosales-Quintero X, Kota J, Coley BD, Galloway G, Craenen JM, Lewis S, Malik V, Shilling C, Byrne BJ, Conlon T, Campbell KJ, Bremer WG, Viollet L, Walker CM, Sahenk Z, Clark KR (2009) Limb-girdle muscular dystrophy type 2D gene therapy restores alpha-sarcoglycan and associated proteins. *Ann Neurol* 66(3):290–297. <https://doi.org/10.1002/ana.21732>
10. Duan D (2018) Systemic AAV micro-dystrophin gene therapy for Duchenne muscular dystrophy. *Mol Ther* 26(10):2337–2356. <https://doi.org/10.1016/j.ymthe.2018.07.011>
11. Liu J, Wallace LM, Garwick-Coppens SE, Sloboda DD, Davis CS, Hakim CH, Hauser MA, Brooks SV, Mendell JR, Harper SQ (2014) RNAi-mediated gene silencing of mutant myotilin improves myopathy in LGMD1A mice. *Mol Ther Nucleic Acids* 3:e160. <https://doi.org/10.1038/mtna.2014.13>
12. Zhang Y, Long C, Bassel-Duby R, Olson EN (2018) Myoediting: toward prevention of muscular dystrophy by therapeutic genome editing. *Physiol Rev* 98(3):1205–1240. <https://doi.org/10.1152/physrev.00046.2017>
13. Nelson CE, Robinson-Hamm JN, Gersbach CA (2017) Genome engineering: a new approach to gene therapy for neuromuscular disorders. *Nat Rev Neurol* 13(11):647–661. <https://doi.org/10.1038/nrneurol.2017.126>
14. Berger A, Maire S, Gaillard MC, Sahel JA, Hantraye P, Bemelmans AP (2016) mRNA trans-splicing in gene therapy for genetic diseases. *Wiley Interdiscip Rev RNA* 7(4):487–498. <https://doi.org/10.1002/wrna.1347>
15. Spitali P, Aartsma-Rus A (2012) Splice modulating therapies for human disease. *Cell* 148(6):1085–1088. <https://doi.org/10.1016/j.cell.2012.02.014>. S0092-8674(12)00214-0 [pii]
16. Vo AH, McNally EM (2015) Modifier genes and their effect on Duchenne muscular dystrophy. *Curr Opin Neurol* 28(5):528–534. <https://doi.org/10.1097/WCO.0000000000000240>
17. Goonasekera SA, Lam CK, Millay DP, Sargent MA, Hajjar RJ, Kranias EG, Molkentin JD (2011) Mitigation of muscular dystrophy in mice by SERCA overexpression in skeletal muscle. *J Clin Invest* 121(3):1044–1052. <https://doi.org/10.1172/JCI43844>. 43844 [pii]
18. Voit A, Patel V, Pachon R, Shah V, Bakhutina M, Kohlbrenner E, McArdle JJ, Dell'Italia LJ, Mendell JR, Xie LH, Hajjar RJ, Duan D, Fraidenraich D, Babu GJ (2017) Reducing sarco-lipin expression mitigates Duchenne muscular dystrophy and associated cardiomyopathy in mice. *Nat Commun* 8(1):1068. <https://doi.org/10.1038/s41467-017-01146-7>
19. Shin J-H, Bostick B, Yue Y, Hajjar R, Duan D (2011) SERCA2a gene transfer improves electrocardiographic performance in aged mdx mice. *J Transl Med* 9:132. <https://doi.org/10.1186/1479-5876-9-132>. 1479-5876-9-132 [pii]
20. Wang D, Zhong L, Nahid MA, Gao G (2014) The potential of adeno-associated viral vectors for gene delivery to muscle tissue. *Expert Opin Drug Deliv* 11(3):345–364. <https://doi.org/10.1517/17425247.2014.871258>

21. Duan D (2016) Systemic delivery of adeno-associated viral vectors. *Curr Opin Virol* 21:16–25. <https://doi.org/10.1016/j.coviro.2016.07.006>
22. Mendell JR, Al-Zaidy S, Shell R, Arnold WD, Rodino-Klapac LR, Prior TW, Lowes L, Alfano L, Berry K, Church K, Kissel JT, Nagendran S, L'Italien J, Sproule DM, Wells C, Cardenas JA, Heitzer MD, Kaspar A, Corcoran S, Braun L, Likhite S, Miranda C, Meyer K, Foust KD, Burghes AHM, Kaspar BK (2017) Single-dose gene-replacement therapy for spinal muscular atrophy. *N Engl J Med* 377(18):1713–1722. <https://doi.org/10.1056/NEJMoa1706198>
23. Malerba A, Klein P, Bachtarzi H, Jarmin SA, Cordova G, Ferry A, Strings V, Espinoza MP, Mamchaoui K, Blumen SC, St Guily JL, Mouly V, Graham M, Butler-Browne G, Suhy DA, Trollet C, Dickson G (2017) PABPN1 gene therapy for oculopharyngeal muscular dystrophy. *Nat Commun* 8:14848. <https://doi.org/10.1038/ncomms14848>
24. FDA (2013) Guidance for industry: preclinical assessment of investigational cellular and gene therapy products. <https://www.federalregister.gov/documents/2013/11/25/2013-28173/guidance-for-industry-preclinical-assessment-of-investigational-cellular-and-gene-therapy-products>
25. Lima BS, Videira MA (2018) Toxicology and biodistribution: the clinical value of animal biodistribution studies. *Mol Ther-Meth Clin Dev* 8:183–197. <https://doi.org/10.1016/j.omtm.2018.01.003>
26. Mendell JR, Lloyd-Puryear M (2013) Report of MDA muscle disease symposium on newborn screening for Duchenne muscular dystrophy. *Muscle Nerve* 48(1):21–26. <https://doi.org/10.1002/mus.23810>
27. Sicinski P, Geng Y, Ryder-Cook AS, Barnard EA, Darlison MG, Barnard PJ (1989) The molecular basis of muscular dystrophy in the mdx mouse: a point mutation. *Science* 244(4912):1578–1580
28. Cooper BJ, Winand NJ, Stedman H, Valentine BA, Hoffman EP, Kunkel LM, Scott MO, Fischbeck KH, Kornegay JN, Avery RJ et al (1988) The homologue of the Duchenne locus is defective in X-linked muscular dystrophy of dogs. *Nature* 334(6178):154–156
29. McGreevy JW, Hakim CH, McIntosh MA, Duan D (2015) Animal models of Duchenne muscular dystrophy: from basic mechanisms to gene therapy. *Dis Model Mech* 8(3):195–213. <https://doi.org/10.1242/dmm.018424>
30. Sui T, Lau YS, Liu D, Liu T, Xu L, Gao Y, Lai L, Li Z, Han R (2018) A novel rabbit model of Duchenne muscular dystrophy generated by CRISPR/Cas9. *Dis Model Mech* 11(6):dmm032201. <https://doi.org/10.1242/dmm.032201>
31. Veltrop M, van Vliet L, Hulsker M, Claassens J, Brouwers C, Breukel C, van der Kaa J, Linssen MM, den Dunnen JT, Verbeek S, Aartsma-Rus A, van Putten M (2018) A dystrophic Duchenne mouse model for testing human antisense oligonucleotides. *PLoS One* 13(2):e0193289. <https://doi.org/10.1371/journal.pone.0193289>. ARTN e0193289
32. Amoasii L, Long C, Li H, Mireault AA, Shelton JM, Sanchez-Ortiz E, McAnally JR, Bhattacharyya S, Schmidt F, Grimm D, Hauschka SD, Bassel-Duby R, Olson EN (2017) Single-cut genome editing restores dystrophin expression in a new mouse model of muscular dystrophy. *Sci Transl Med* 9(418):eaan8081. <https://doi.org/10.1126/scitranslmed.aan8081>
33. Young CS, Mokhonova E, Quinonez M, Pyle AD, Spencer MJ (2017) Creation of a novel humanized dystrophic mouse model of Duchenne muscular dystrophy and application of a CRISPR/Cas9 gene editing therapy. *J Neuromuscul Dis* 4(2):139–145. <https://doi.org/10.3233/JND-170218>
34. Wang B, Li J, Xiao X (2000) Adeno-associated virus vector carrying human minidystrophin genes effectively ameliorates muscular dystrophy in mdx mouse model. *Proc Natl Acad Sci U S A* 97(25):13714–13719
35. Harper SQ, Hauser MA, DelloRusso C, Duan D, Crawford RW, Phelps SF, Harper HA, Robinson AS, Engelhardt JF, Brooks SV, Chamberlain JS (2002) Modular flexibility of dystrophin: implications for gene therapy of Duchenne muscular dystrophy. *Nat Med* 8(3):253–261

36. Fabb SA, Wells DJ, Serpente P, Dickson G (2002) Adeno-associated virus vector gene transfer and sarcolemmal expression of a 144 kDa micro-dystrophin effectively restores the dystrophin-associated protein complex and inhibits myofibre degeneration in nude/mdx mice. *Hum Mol Genet* 11(7):733–741
37. Yue Y, Li Z, Harper SQ, Davisson RL, Chamberlain JS, Duan D (2003) Microdystrophin gene therapy of cardiomyopathy restores dystrophin-glycoprotein complex and improves sarcolemma integrity in the mdx mouse heart. *Circulation* 108(13):1626–1632
38. Liu M, Yue Y, Harper SQ, Grange RW, Chamberlain JS, Duan D (2005) Adeno-associated virus-mediated microdystrophin expression protects young mdx muscle from contraction-induced injury. *Mol Ther* 11(2):245–256. <https://doi.org/10.1016/j.ymthe.2004.09.013>. S1525-0016(04)01461-3 [pii]
39. Lai Y, Thomas GD, Yue Y, Yang HT, Li D, Long C, Judge L, Bostick B, Chamberlain JS, Terjung RL, Duan D (2009) Dystrophins carrying spectrin-like repeats 16 and 17 anchor nNOS to the sarcolemma and enhance exercise performance in a mouse model of muscular dystrophy. *J Clin Invest* 119(3):624–635. <https://doi.org/10.1172/JCI36612>
40. Foster H, Sharp PS, Athanopoulos T, Trollet C, Graham IR, Foster K, Wells DJ, Dickson G (2008) Codon and mRNA sequence optimization of microdystrophin transgenes improves expression and physiological outcome in dystrophic mdx mice following AAV2/8 gene transfer. *Mol Ther* 16(11):1825–1832. <https://doi.org/10.1038/mt.2008.186>
41. Townsend D, Blankinship MJ, Allen JM, Gregorevic P, Chamberlain JS, Metzger JM (2007) Systemic administration of micro-dystrophin restores cardiac geometry and prevents dobutamine-induced cardiac pump failure. *Mol Ther* 15(6):1086–1092
42. Gregorevic P, Allen JM, Minami E, Blankinship MJ, Haraguchi M, Meuse L, Finn E, Adams ME, Froehner SC, Murry CE, Chamberlain JS (2006) rAAV6-microdystrophin preserves muscle function and extends lifespan in severely dystrophic mice. *Nat Med* 12(7):787–789
43. Gregorevic P, Blankinship MJ, Allen JM, Chamberlain JS (2008) Systemic microdystrophin gene delivery improves skeletal muscle structure and function in old dystrophic mdx mice. *Mol Ther* 16(4):657–664. <https://doi.org/10.1038/mt.2008.28>
44. Yue Y, Liu M, Duan D (2006) C-terminal truncated microdystrophin recruits dystrobrevin and syntrophin to the dystrophin-associated glycoprotein complex and reduces muscular dystrophy in symptomatic utrophin/dystrophin double knock-out mice. *Mol Ther* 14(1):79–87
45. Wang B, Li J, Fu FH, Xiao X (2009) Systemic human minidystrophin gene transfer improves functions and life span of dystrophin and dystrophin/utrophin-deficient mice. *J Orthop Res* 27(4):421–426. <https://doi.org/10.1002/jor.20781>
46. Hakim CH, Wasala NB, Pan X, Kodippili K, Yue Y, Zhang K, Yao G, Haffner B, Duan SX, Ramos J, Schneider JS, Yang NN, Chamberlain JS, Duan D (2017) A five-repeat micro-dystrophin gene ameliorated dystrophic phenotype in the severe DBA/2J-mdx model of Duchenne muscular dystrophy. *Mol Ther Methods Clin Dev* 6:216–230. <https://doi.org/10.1016/j.omtm.2017.06.006>
47. Bostick B, Yue Y, Duan D (2010) Gender influences cardiac function in the mdx model of Duchenne cardiomyopathy. *Muscle Nerve* 42(4):600–603. <https://doi.org/10.1002/mus.21763>
48. Bostick B, Shin J-H, Yue Y, Duan D (2011) AAV-microdystrophin therapy improves cardiac performance in aged female mdx mice. *Mol Ther* 19(10):1826–1832
49. Bostick B, Shin J-H, Yue Y, Wasala NB, Lai Y, Duan D (2012) AAV micro-dystrophin gene therapy alleviates stress-induced cardiac death but not myocardial fibrosis in >21-m-old mdx mice, an end-stage model of Duchenne muscular dystrophy cardiomyopathy. *J Mol Cell Cardiol* 53(2):217–222. <https://doi.org/10.1016/j.jymcc.2012.05.002>. S0022-2828(12)00179-4 [pii]
50. Duan D (2015) Duchenne muscular dystrophy gene therapy in the canine model. *Hum Gene Ther Clin Dev* 26(1):57–69. <https://doi.org/10.1089/humc.2015.006>
51. Shin J-H, Pan X, Hakim CH, Yang HT, Yue Y, Zhang K, Terjung RL, Duan D (2013) Microdystrophin ameliorates muscular dystrophy in the canine model of Duchenne muscular dystrophy. *Mol Ther* 21(4):750–757. <https://doi.org/10.1038/mt.2012.283>

52. Yue Y, Pan X, Hakim CH, Kodippili K, Zhang K, Shin J-H, Yang HT, McDonald T, Duan D (2015) Safe and bodywide muscle transduction in young adult Duchenne muscular dystrophy dogs with adeno-associated virus. *Hum Mol Genet* 24(20):5880–5890
53. Hakim CH, Kodippili K, Jenkins G, Yang HT, Pan X, Lessa TB, Leach SB, Emter C, Yue Y, Zhang K, Duan XS, Yao G, Schneider JS, Yang NN, Chamberlain JS, Duan D (2017) Single systemic AAV micro-dystrophin therapy ameliorates muscular dystrophy in young adult Duchenne muscular dystrophy dogs for up to two years. *Mol Ther* 25(S1):192–193
54. Hakim CH, Kodippili K, Jenkins G, Yang HT, Pan X, Lessa TB, Leach SB, Emter C, Yue Y, Zhang K, Duan XS, Yao G, Schneider JS, Yang NN, Chamberlain JS, Duan D (2018) AAV micro-dystrophin therapy ameliorates muscular dystrophy in young adult Duchenne muscular dystrophy dogs for up to 30 months following injection. *Mol Ther* 26(S1):5
55. Le Guiner C, Servais L, Montus M, Larcher T, Fraysse B, Moulec S, Allais M, Francois V, Dutilleul M, Malerba A, Koo T, Thibaut JL, Matot B, Devaux M, Le Duff J, Deschamps JY, Barthelemy I, Blot S, Testault I, Wahbi K, Ederhy S, Martin S, Veron P, Georger C, Athanasopoulos T, Masurier C, Mingozzi F, Carlier P, Gjata B, Hogrel JY, Adjali O, Mavilio F, Voit T, Moullier P, Dickson G (2017) Long-term microdystrophin gene therapy is effective in a canine model of Duchenne muscular dystrophy. *Nat Commun* 8:16105. <https://doi.org/10.1038/ncomms16105>
56. Gonzalez JP, Schneider JS, Brown KJ, Golebiowski D, Shanks C, Ricotti V, Laforet G, Quiroz J, Morris CA (2018) Preclinical evaluation of SGT-001 Microdystrophin gene transfer for Duchenne muscular dystrophy. *Mol Ther* 26(S1):390
57. Oudet C, Hanauer A, Clemens P, Caskey T, Mandel JL (1992) Two hot spots of recombination in the DMD gene correlate with the deletion prone regions. *Hum Mol Genet* 1(8):599–603
58. 't Hoen PA, de Meijer EJ, Boer JM, Vossen RH, Turk R, Maatman RG, Davies KE, van Ommen GJ, van Deutekom JC, den Dunnen JT (2008) Generation and characterization of transgenic mice with the full-length human DMD gene. *J Biol Chem* 283(9):5899–5907. <https://doi.org/10.1074/jbc.M709410200>
59. Bremmer-Bout M, Aartsma-Rus A, de Meijer EJ, Kaman WE, Janson AA, Vossen RH, van Ommen GJ, den Dunnen JT, van Deutekom JC (2004) Targeted exon skipping in transgenic hDMD mice: a model for direct preclinical screening of human-specific antisense oligonucleotides. *Mol Ther* 10(2):232–240. <https://doi.org/10.1016/j.ymthe.2004.05.031>
60. Li D, Yue Y, Duan D (2010) Marginal level dystrophin expression improves clinical outcome in a strain of dystrophin/utrophin double knockout mice. *PLoS One* 5(12):e15286. <https://doi.org/10.1371/journal.pone.0015286>
61. Li D, Yue Y, Duan D (2008) Preservation of muscle force in mdx3cv mice correlates with low-level expression of a near full-length dystrophin protein. *Am J Pathol* 172(5):1332–1341. <https://doi.org/10.2353/ajpath.2008.071042>
62. Wasala NB, Yue Y, Vance J, Duan D (2017) Uniform low-level dystrophin expression in the heart partially preserved cardiac function in an aged mouse model of Duchenne cardiomyopathy. *J Mol Cell Cardiol* 102:45–52. <https://doi.org/10.1016/j.yjmcc.2016.11.011>
63. van Putten M, van der Pijl EM, Hulsker M, Verhaart IE, Nadarajah VD, van der Weerd L, Aartsma-Rus A (2014) Low dystrophin levels in heart can delay heart failure in mdx mice. *J Mol Cell Cardiol* 69:17–23. <https://doi.org/10.1016/j.yjmcc.2014.01.009>
64. van Putten M, Hulsker M, Young C, Nadarajah VD, Heemskerk H, van der Weerd L, 't Hoen PA, van Ommen GJ, Aartsma-Rus AM (2013) Low dystrophin levels increase survival and improve muscle pathology and function in dystrophin/utrophin double-knockout mice. *FASEB J* 27(6):2484–2495. <https://doi.org/10.1096/fj.12-224170>
65. van Putten M, Hulsker M, Nadarajah VD, van Heiningen SH, van Huizen E, van Iterson M, Admiraal P, Messemaker T, den Dunnen JT, 't Hoen PA, Aartsma-Rus A (2012) The effects of low levels of dystrophin on mouse muscle function and pathology. *PLoS One* 7(2):e31937. <https://doi.org/10.1371/journal.pone.0031937>
66. Nicholson LV, Johnson MA, Bushby KM, Gardner-Medwin D (1993) Functional significance of dystrophin positive fibres in Duchenne muscular dystrophy. *Arch Dis Child* 68(5):632–636

67. Waldrop MA, Gumienny F, El Husayni S, Frank DE, Weiss RB, Flanigan KM (2018) Low-level dystrophin expression attenuating the dystrophinopathy phenotype. *Neuromuscul Disord* 28(2):116–121. <https://doi.org/10.1016/j.nmd.2017.11.007>
68. Cox GA, Cole NM, Matsumura K, Phelps SF, Hauschka SD, Campbell KP, Faulkner JA, Chamberlain JS (1993) Overexpression of dystrophin in transgenic mdx mice eliminates dystrophic symptoms without toxicity. *Nature* 364(6439):725–729
69. Yue Y, Wasala NB, Bostick B, Duan D (2016) 100-fold but not 50-fold dystrophin overexpression aggravates electrocardiographic defects in the mdx model of Duchenne muscular dystrophy. *Mol Ther Methods Clin Dev* 3:16045. <https://doi.org/10.1038/mtm.2016.45>
70. Yoshiki A, Moriaki K (2006) Mouse phenome research: implications of genetic background. *ILAR J* 47(2):94–102
71. Linder CC (2001) The influence of genetic background on spontaneous and genetically engineered mouse models of complex diseases. *Lab Anim* 30(5):34–39
72. Zucker I, Beery AK (2010) Males still dominate animal studies. *Nature* 465(7299):690. <https://doi.org/10.1038/465690a>
73. Wald C, Wu C (2010) Biomedical research. Of mice and women: the bias in animal models. *Science* 327(5973):1571–1572. <https://doi.org/10.1126/science.327.5973.1571>
74. Leinwand LA (2003) Sex is a potent modifier of the cardiovascular system. *J Clin Invest* 112(3):302–307
75. Turner MJ, Kleeberger SR, Lightfoot JT (2005) Influence of genetic background on daily running-wheel activity differs with aging. *Physiol Genomics* 22(1):76–85. <https://doi.org/10.1152/physiolgenomics.00243.2004>
76. Glenmark B, Nilsson M, Gao H, Gustafsson JA, Dahlman-Wright K, Westerblad H (2004) Difference in skeletal muscle function in males vs. females: role of estrogen receptor-beta. *Am J Phys Endocrinol Metab* 287(6):E1125–E1131. <https://doi.org/10.1152/ajpendo.00098.2004> [pii]
77. Meyer S, van der Meer P, van Tintelen JP, van den Berg MP (2014) Sex differences in cardiomyopathies. *Eur J Heart Fail* 16(3):238–247. <https://doi.org/10.1002/ehfj.15>
78. Pergola C, Dodt G, Rossi A, Neunhoffer E, Lawrenz B, Northoff H, Samuelsson B, Radmark O, Sautebin L, Werz O (2008) ERK-mediated regulation of leukotriene biosynthesis by androgens: a molecular basis for gender differences in inflammation and asthma. *Proc Natl Acad Sci U S A* 105(50):19881–19886. <https://doi.org/10.1073/pnas.0809120105>
79. Du XJ, Samuel CS, Gao XM, Zhao L, Parry LJ, Tregear GW (2003) Increased myocardial collagen and ventricular diastolic dysfunction in relaxin deficient mice: a gender-specific phenotype. *Cardiovasc Res* 57(2):395–404
80. Hakim CH, Duan D (2012) Gender differences in contractile and passive properties of mdx extensor digitorum longus muscle. *Muscle Nerve* 45(2):250–256. <https://doi.org/10.1002/mus.22275>
81. Dane AP, Cunningham SC, Graf NS, Alexander IE (2009) Sexually dimorphic patterns of episomal rAAV genome persistence in the adult mouse liver and correlation with hepatocellular proliferation. *Mol Ther* 17(9):1548–1554. <https://doi.org/10.1038/mt.2009.139>
82. Voutetakis A, Zheng C, Wang J, Goldsmith CM, Afione S, Chiorini JA, Wenk ML, Vallant M, Irwin RD, Baum BJ (2007) Gender differences in serotype 2 adeno-associated virus biodistribution after administration to rodent salivary glands. *Hum Gene Ther* 18(11):1109–1118. <https://doi.org/10.1089/hum.2007.072>
83. Chandler CH, Chari S, Dworkin I (2013) Does your gene need a background check? How genetic background impacts the analysis of mutations, genes, and evolution. *Trends Genet* 29(6):358–366. <https://doi.org/10.1016/j.tig.2013.01.009>
84. Brayton CF, Treuting PM, Ward JM (2012) Pathobiology of aging mice and GEM: background strains and experimental design. *Vet Pathol* 49(1):85–105. <https://doi.org/10.1177/0300985811430696>
85. Crawley JN, Belknap JK, Collins A, Crabbe JC, Frankel W, Henderson N, Hitzemann RJ, Maxson SC, Miner LL, Silva AJ, Wehner JM, Wynshaw-Boris A, Paylor R (1997) Behavioral

- phenotypes of inbred mouse strains: implications and recommendations for molecular studies. *Psychopharmacology* 132(2):107–124
86. Erickson RP (1996) Mouse models of human genetic disease: which mouse is more like a man? *BioEssays* 18(12):993–998. <https://doi.org/10.1002/bies.950181209>
87. Montagutelli X (2000) Effect of the genetic background on the phenotype of mouse mutations. *J Am Soc Nephrol* 11(11):S101–S105
88. Schauwecker PE (2002) Complications associated with genetic background effects in models of experimental epilepsy. *Prog Brain Res* 135:139–148
89. Lerman I, Harrison BC, Freeman K, Hewett TE, Allen DL, Robbins J, Leinwand LA (2002) Genetic variability in forced and voluntary endurance exercise performance in seven inbred mouse strains. *J Appl Physiol* 92(6):2245–2255. <https://doi.org/10.1152/japplphysiol.01045.2001>
90. Lightfoot JT, Turner MJ, Daves M, Vordermark A, Kleeberger SR (2004) Genetic influence on daily wheel running activity level. *Physiol Genomics* 19(3):270–276. <https://doi.org/10.1152/physiolgenomics.00125.2004>
91. Xing S, Tsaih SW, Yuan R, Svenson KL, Jorgenson LM, So M, Paigen BJ, Korstanje R (2009) Genetic influence on electrocardiogram time intervals and heart rate in aging mice. *Am J Physiol Heart Circ Physiol* 296(6):H1907–H1913. <https://doi.org/10.1152/ajpheart.00681.2008>
92. Kadambi VJ, Ball N, Kranias EG, Walsh RA, Hoit BD (1999) Modulation of force-frequency relation by phospholamban in genetically engineered mice. *Am J Physiol-Heart Circ Physiol* 276(6):H2245–H2250
93. Shusterman V, Usiene I, Harrigal C, Lee JS, Kubota T, Feldman AM, London B (2002) Strain-specific patterns of autonomic nervous system activity and heart failure susceptibility in mice. *Am J Physiol-Heart Circ Physiol* 282(6):H2076–H2083. <https://doi.org/10.1152/ajpheart.00917.2001>
94. Rodrigues M, Echigoya Y, Maruyama R, Lim KRQ, Fukada S, Yokota T (2016) Impaired regenerative capacity and lower revertant fibre expansion in dystrophin-deficient mdx muscles on DBA/2 background. *Sci Rep* 6:38371. <https://doi.org/10.1038/srep38371>. ARTN 38371
95. Bostick B, Yue Y, Lai Y, Long C, Li D, Duan D (2008) Adeno-associated virus serotype-9 microdystrophin gene therapy ameliorates electrocardiographic abnormalities in mdx mice. *Hum Gene Ther* 19(8):851–856. <https://doi.org/10.1089/hum.2008.058>
96. Wasala NB, Lai Y, Shin J-H, Zhao J, Yue Y, Duan D (2016) Genomic removal of a therapeutic mini-dystrophin gene from adult mice elicits a Duchenne muscular dystrophy-like phenotype. *Hum Mol Genet* 25(13):2633–2644. <https://doi.org/10.1093/hmg/ddw123>
97. Nance ME, Duan D (2018) Gene therapy: use of viruses as vectors. Reference module in biomedical sciences: Elsevier. <https://doi.org/10.1016/B978-0-12-801238-3.95711-8>
98. Ginn SL, Amaya AK, Alexander IE, Edelstein M, Abedi MR (2018) Gene therapy clinical trials worldwide to 2017: an update. *J Gene Med* 20(5):e3015. <https://doi.org/10.1002/jgm.3015>. ARTN e3015
99. Duan D (2008) Myodys, a full-length dystrophin plasmid vector for Duchenne and Becker muscular dystrophy gene therapy. *Curr Opin Mol Ther* 10(1):86–94
100. Braun S (2008) Muscular gene transfer using nonviral vectors. *Curr Gene Ther* 8(5):391–405
101. Fassati A, Bresolin N (2000) Retroviral vectors for gene therapy of Duchenne muscular dystrophy. *Neurol Sci* 21(5):S925–S927
102. Karpati G, Gilbert R, Petrof BJ, Nalbantoglu J (1997) Gene therapy research for Duchenne and Becker muscular dystrophies. *Curr Opin Neurol* 10(5):430–435
103. Flotte TR, Gao GP (2017) AAV is now a medicine: we had better get this right. *Hum Gene Ther* 28(4):307–307. <https://doi.org/10.1089/hum.2017.29041.trf>
104. McCarty DM (2008) Self-complementary AAV vectors; advances and applications. *Mol Ther* 16(10):1648–1656. <https://doi.org/10.1038/mt.2008.171>. mt2008171 [pii]

105. McCarty DM, Fu H, Monahan PE, Toulson CE, Naik P, Samulski RJ (2003) Adeno-associated virus terminal repeat (TR) mutant generates self-complementary vectors to overcome the rate-limiting step to transduction in vivo. *Gene Ther* 10(26):2112–2118. <https://doi.org/10.1038/sj.gt.3302134>
106. Ferrari FK, Samulski T, Shenk T, Samulski RJ (1996) Second-strand synthesis is a rate-limiting step for efficient transduction by recombinant adeno-associated virus vectors. *J Virol* 70(5):3227–3234
107. Fisher KJ, Gao GP, Weitzman MD, DeMatteo R, Burda JF, Wilson JM (1996) Transduction with recombinant adeno-associated virus for gene therapy is limited by leading-strand synthesis. *J Virol* 70(1):520–532
108. Benkhalifa-Ziyyat S, Besse A, Roda M, Duque S, Astord S, Carcenac R, Marais T, Barkats M (2013) Intramuscular scAAV9-SMN injection mediates widespread gene delivery to the spinal cord and decreases disease severity in SMA mice. *Mol Ther* 21(2):282–290. <https://doi.org/10.1038/mt.2012.261>
109. Nathwani AC, Gray JT, Ng CY, Zhou J, Spence Y, Waddington SN, Tuddenham EG, Kemball-Cook G, McIntosh J, Boon-Spijker M, Mertens K, Davidoff AM (2006) Self-complementary adeno-associated virus vectors containing a novel liver-specific human factor IX expression cassette enable highly efficient transduction of murine and nonhuman primate liver. *Blood* 107(7):2653–2661. <https://doi.org/10.1182/blood-2005-10-4035>
110. Li X, Eastman EM, Schwartz RJ, Draghia-Akli R (1999) Synthetic muscle promoters: activities exceeding naturally occurring regulatory sequences. *Nat Biotechnol* 17(3):241–245
111. Jaynes JB, Chamberlain JS, Buskin JN, Johnson JE, Hauschka SD (1986) Transcriptional regulation of the muscle creatine kinase gene and regulated expression in transfected mouse myoblasts. *Mol Cell Biol* 6(8):2855–2864
112. Shaul O (2017) How introns enhance gene expression. *Int J Biochem Cell Biol* 91(Pt B):145–155. <https://doi.org/10.1016/j.biocel.2017.06.016>
113. Lozier JN (2012) Gene therapy. Factor IX Padua: them that have, give. *Blood* 120(23):4452–4453. <https://doi.org/10.1182/blood-2012-09-452821>
114. Krieg AM, Yi AK, Matson S, Waldschmidt TJ, Bishop GA, Teasdale R, Koretzky GA, Klinman DM (1995) CpG motifs in bacterial-DNA trigger direct B-cell activation. *Nature* 374(6522):546–549. <https://doi.org/10.1038/374546a0>
115. Faust SM, Bell P, Cutler BJ, Ashley SN, Zhu Y, Rabinowitz JE, Wilson JM (2013) CpG-depleted adeno-associated virus vectors evade immune detection. *J Clin Invest* 123(7):2994–3001. <https://doi.org/10.1172/JCI68205>
116. Lai Y, Zhao J, Yue Y, Duan D (2013) alpha2 and alpha3 helices of dystrophin R16 and R17 frame a microdomain in the alpha1 helix of dystrophin R17 for neuronal NOS binding. *Proc Natl Acad Sci U S A* 110(2):525–530. <https://doi.org/10.1073/pnas.1211431109>. 1211431109 [pii]
117. Proudfoot N, O'Sullivan J (2002) Polyadenylation: a tail of two complexes. *Curr Biol* 12(24):R855–R857
118. Powell SK, Rivera-Soto R, Gray SJ (2015) Viral expression cassette elements to enhance transgene target specificity and expression in gene therapy. *Discov Med* 19(102):49–57
119. Levitt N, Briggs D, Gil A, Proudfoot NJ (1989) Definition of an efficient synthetic poly(A) site. *Genes Dev* 3(7):1019–1025
120. Kotterman MA, Schaffer DV (2014) Engineering adeno-associated viruses for clinical gene therapy. *Nat Rev Genet* 15(7):445–451. <https://doi.org/10.1038/nrg3742>
121. Qiao C, Zhang W, Yuan Z, Shin J-H, Li J, Jayandharan GR, Zhong L, Srivastava A, Xiao X, Duan D (2010) AAV6 capsid tyrosine to phenylalanine mutations improve gene transfer to skeletal muscle. *Hum Gene Ther* 21(10):1343–1348. <https://doi.org/10.1089/hum.2010.003>
122. Nance ME, Duan D (2015) Perspective on adeno-associated virus (AAV) capsid modification for Duchenne muscular dystrophy gene therapy. *Hum Gene Ther* 26(12):786–800
123. Asokan A, Conway JC, Phillips JL, Li C, Hegge J, Sinnott R, Yadav S, DiPrimio N, Nam HJ, Agbandje-McKenna M, McPhee S, Wolff J, Samulski RJ (2010) Reengineering a receptor

- footprint of adeno-associated virus enables selective and systemic gene transfer to muscle. *Nat Biotechnol* 28(1):79–82. <https://doi.org/10.1038/nbt.1599>
124. Pulicherla N, Shen S, Yadav S, Debbink K, Govindasamy L, Agbandje-McKenna M, Asokan A (2011) Engineering liver-detargeted AAV9 vectors for cardiac and musculoskeletal gene transfer. *Mol Ther* 19(6):1070–1078. <https://doi.org/10.1038/mt.2011.22>. mt201122 [pii]
125. Choudhury SR, Fitzpatrick Z, Harris AF, Maitland SA, Ferreira JS, Zhang Y, Ma S, Sharma RB, Gray-Edwards HL, Johnson JA, Johnson AK, Alonso LC, Punzo C, Wagner KR, Maguire CA, Kotin RM, Martin DR, Sena-Esteves M (2016) In vivo selection yields AAV-B1 capsid for central nervous system and muscle gene therapy. *Mol Ther* 24(7):1247–1257. <https://doi.org/10.1038/mt.2016.84>
126. Yu CY, Yuan Z, Cao Z, Wang B, Qiao C, Li J, Xiao X (2009) A muscle-targeting peptide displayed on AAV2 improves muscle tropism on systemic delivery. *Gene Ther* 16(8):953–962. <https://doi.org/10.1038/gt.2009.59>
127. Yang L, Jiang J, Drouin LM, Agbandje-McKenna M, Chen C, Qiao C, Pu D, Hu X, Wang DZ, Li J, Xiao X (2009) A myocardium tropic adeno-associated virus (AAV) evolved by DNA shuffling and in vivo selection. *Proc Natl Acad Sci U S A* 106(10):3946–3951. <https://doi.org/10.1073/pnas.0813207106>
128. Paulk NK, Pekrun K, Charville GW, Maguire-Nguyen K, Wosczyzna MN, Xu J, Zhang Y, Lisowski L, Yoo B, Vilches-Moure JG, Lee GK, Shrager JB, Rando TA, Kay MA (2018) Bioengineered viral platform for intramuscular passive vaccine delivery to human skeletal muscle. *Mol Ther Methods Clin Dev* 10:144–155. <https://doi.org/10.1016/j.omtm.2018.06.001>
129. Weinmann J, Weis S, Sippel J, Lenter M, Lamla T, Grimm D (2018) Massively parallel in vivo characterization of >150 Adeno-Associated Viral (AAV) capsids using DNA/RNA barcoding and next-generation sequencing. *Mol Ther* 26(S1):319–318
130. Wang L, Bell P, Somanathan S, Wang Q, He Z, Yu H, McMenamin D, Goode T, Calcedo R, Wilson JM (2015) Comparative study of liver gene transfer with AAV vectors based on natural and engineered AAV capsids. *Mol Ther* 23(12):1877–1887. <https://doi.org/10.1038/mt.2015.179>
131. Li S, Ling C, Zhong L, Li M, Su Q, He R, Tang Q, Greiner DL, Shultz LD, Brehm MA, Flotte TR, Mueller C, Srivastava A, Gao G (2015) Efficient and targeted transduction of nonhuman primate liver with systemically delivered optimized AAV3B vectors. *Mol Ther* 23(12):1867–1876. <https://doi.org/10.1038/mt.2015.174>
132. Pan X, Yue Y, Zhang K, Hakim CH, Kodippili K, McDonald T, Duan D (2015) AAV-8 is more efficient than AAV-9 in transducing neonatal dog heart. *Hum Gene Ther Methods* 26(4):54–61
133. Pan X, Yue Y, Zhang K, Lostal W, Shin JH, Duan D (2013) Long-term robust myocardial transduction of the dog heart from a peripheral vein by adeno-associated virus serotype-8. *Hum Gene Ther* 24(6):584–594. <https://doi.org/10.1089/hum.2013.044>
134. Yue Y, Ghosh A, Long C, Bostick B, Smith BF, Kornegay JN, Duan D (2008) A single intravenous injection of adeno-associated virus serotype-9 leads to whole-body skeletal muscle transduction in dogs. *Mol Ther* 16(12):1944–1952. <https://doi.org/10.1038/mt.2008.207>
135. Yuasa K, Ishii A, Miyagoe Y, Takeda S (1997) Introduction of rod-deleted dystrophin cDNA, delta DysM3, into mdx skeletal muscle using adenovirus vector. *Nihon Rinsho* 55(12):3148–3153
136. Gregorevic P, Blankinship MJ, Allen JM, Crawford RW, Meuse L, Miller DG, Russell DW, Chamberlain JS (2004) Systemic delivery of genes to striated muscles using adeno-associated viral vectors. *Nat Med* 10(8):828–834
137. Wang Z, Zhu T, Qiao C, Zhou L, Wang B, Zhang J, Chen C, Li J, Xiao X (2005) Adeno-associated virus serotype 8 efficiently delivers genes to muscle and heart. *Nat Biotechnol* 23(3):321–328
138. Bostick B, Ghosh A, Yue Y, Long C, Duan D (2007) Systemic AAV-9 transduction in mice is influenced by animal age but not by the route of administration. *Gene Ther* 14(22):1605–1609

139. Kuntz N, Shieh PB, Smith B, Bonnemann CG, Dowling JJ, Lawlor MW, Muller-Felber W, Noursalehi M, Rico S, Servais L, Prasad S (2018) ASPIRO phase 1/2 gene therapy trial in X-linked myotubular myopathy (XLTM): preliminary safety and efficacy findings. *Mol Ther* 26(S1):4
140. Nathwani AC, Reiss UM, Tuddenham EG, Rosales C, Chowdary P, McIntosh J, Della Peruta M, Lheriteau E, Patel N, Raj D, Riddell A, Pie J, Rangarajan S, Bevan D, Recht M, Shen YM, Halka KG, Basner-Tschakarjan E, Mingozzi F, High KA, Allay J, Kay MA, Ng CY, Zhou J, Cancio M, Morton CL, Gray JT, Srivastava D, Nienhuis AW, Davidoff AM (2014) Long-term safety and efficacy of factor IX gene therapy in hemophilia B. *N Engl J Med* 371(21):1994–2004. <https://doi.org/10.1056/NEJMoa1407309>
141. Mendell JR, Campbell K, Rodino-Klapac L, Sahenk Z, Shilling C, Lewis S, Bowles D, Gray S, Li C, Galloway G, Malik V, Coley B, Clark KR, Li J, Xiao X, Samulski J, McPhee SW, Samulski RJ, Walker CM (2010) Dystrophin immunity in Duchenne's muscular dystrophy. *N Engl J Med* 363(15):1429–1437. <https://doi.org/10.1056/NEJMoa1000228>
142. Mingozzi F (2018) AAV immunogenicity: a matter of sensitivity. *Mol Ther* 26(10):2335–2336. <https://doi.org/10.1016/j.ymthe.2018.09.001>
143. Calcedo R, Franco J, Qin Q, Richardson DW, Mason JB, Boyd S, Wilson JM (2015) Preexisting neutralizing antibodies to adeno-associated virus capsids in large animals other than monkeys may confound in vivo gene therapy studies. *Hum Gene Ther Methods* 26(3):103–105. <https://doi.org/10.1089/hgtb.2015.082>
144. Rapti K, Louis-Jeune V, Kohlbrenner E, Ishikawa K, Ladage D, Zolotukhin S, Hajjar RJ, Weber T (2011) Neutralizing antibodies against AAV serotypes 1, 2, 6, and 9 in sera of commonly used animal models. *Mol Ther* 20(1):73–83. <https://doi.org/10.1038/mt.2011.177>. mt2011177 [pii]
145. Shin J-H, Yue Y, Smith B, Duan D (2012) Humoral immunity to AAV-6, 8, and 9 in normal and dystrophic dogs. *Hum Gene Ther* 23(3):287–294. <https://doi.org/10.1089/hum.2011.125>
146. Scallan CD, Jiang H, Liu T, Patarroyo-White S, Sommer JM, Zhou S, Couto LB, Pierce GF (2006) Human immunoglobulin inhibits liver transduction by AAV vectors at low AAV2 neutralizing titers in SCID mice. *Blood* 107(5):1810–1817. <https://doi.org/10.1182/blood-2005-08-3229>
147. Jiang H, Couto LB, Patarroyo-White S, Liu T, Nagy D, Vargas JA, Zhou S, Scallan CD, Sommer J, Vijay S, Mingozzi F, High KA, Pierce GF (2006) Effects of transient immunosuppression on adeno-associated, virus-mediated, liver-directed gene transfer in rhesus macaques and implications for human gene therapy. *Blood* 108(10):3321–3328. <https://doi.org/10.1182/blood-2006-04-017913>
148. Hurlbut GD, Ziegler RJ, Nietupski JB, Foley JW, Woodworth LA, Meyers E, Bercury SD, Pande NN, Souza DW, Bree MP, Lukason MJ, Marshall J, Cheng SH, Scheule RK (2010) Preexisting immunity and low expression in primates highlight translational challenges for liver-directed AAV8-mediated gene therapy. *Mol Ther* 18(11):1983–1994. <https://doi.org/10.1038/mt.2010.175>. mt2010175 [pii]
149. Ayuso E, Mingozzi F, Montane J, Leon X, Anguela XM, Haurigot V, Edmonson SA, Africa L, Zhou S, High KA, Bosch F, Wright JF (2010) High AAV vector purity results in serotype- and tissue-independent enhancement of transduction efficiency. *Gene Ther* 17(4):503–510. <https://doi.org/10.1038/gt.2009.157>
150. Schnodt M, Buning H (2017) Improving the quality of adeno-associated viral vector preparations: the challenge of product-related impurities. *Hum Gene Ther Methods* 28(3):101–108. <https://doi.org/10.1089/hgtb.2016.188>
151. Kotin RM (2011) Large-scale recombinant adeno-associated virus production. *Hum Mol Genet* 20(R1):R2–R6. <https://doi.org/10.1093/hmg/ddr141>
152. Clement N, Grieger JC (2016) Manufacturing of recombinant adeno-associated viral vectors for clinical trials. *Mol Ther Methods Clin Dev* 3:16002. <https://doi.org/10.1038/mtm.2016.2>
153. Penaud-Budloo M, Francois A, Clement N, Ayuso E (2018) Pharmacology of recombinant adeno-associated virus production. *Mol Ther Methods Clin Dev* 8:166–180. <https://doi.org/10.1016/j.omtm.2018.01.002>

154. Grieger JC, Soltys SM, Samulski RJ (2016) Production of recombinant adeno-associated virus vectors using suspension HEK293 cells and continuous harvest of vector from the culture media for GMP FIX and FLT1 clinical vector. *Mol Ther* 24(2):287–297. <https://doi.org/10.1038/mt.2015.187>
155. Kotin RM, Snyder RO (2017) Manufacturing clinical grade recombinant adeno-associated virus using invertebrate cell lines. *Hum Gene Ther* 28(4):350–360. <https://doi.org/10.1089/hum.2017.042>
156. Clement N, Knop DR, Byrne BJ (2009) Large-scale adeno-associated viral vector production using a herpesvirus-based system enables manufacturing for clinical studies. *Hum Gene Ther* 20(8):796–806. <https://doi.org/10.1089/hum.2009.094>
157. Lock M, Alvira M, Vandenbergh L, Samanta A, Toelen J, Debyser Z, Wilson JM (2010) Rapid, simple, and versatile manufacturing of recombinant adeno-associated viral vectors at scale. *Hum Gene Ther* 21(10):1259–1271. <https://doi.org/10.1089/hum.2010.055>
158. D’Costa S, Blouin V, Broucque F, Penaud-Budloo M, Francois A, Perez IC, Le Bec C, Moullier P, Snyder RO, Ayuso E (2016) Practical utilization of recombinant AAV vector reference standards: focus on vector genomes titration by free ITR qPCR. *Mol Ther Methods Clin Dev* 5:16019. <https://doi.org/10.1038/mtm.2016.19>
159. Ayuso E, Blouin V, Lock M, McGorray S, Leon X, Alvira MR, Auricchio A, Bucher S, Chtarto A, Clark KR, Darmon C, Doria M, Fountain W, Gao GP, Gao K, Giacca M, Kleinschmidt J, Leuchs B, Melas C, Mizukami H, Muller M, Noordman Y, Bockstael O, Ozawa K, Pythoud C, Sumaroka M, Surosky R, Tenenbaum L, van der Linden I, Weins B, Wright JF, Zhang XH, Zentilin L, Bosch F, Snyder RO, Moullier P (2014) Manufacturing and characterization of a recombinant Adeno-associated virus type 8 reference standard material. *Hum Gene Ther* 25(11):977–987. <https://doi.org/10.1089/hum.2014.057>
160. Moullier P, Snyder RO (2008) International efforts for recombinant adeno-associated viral vector reference standards. *Mol Ther* 16(7):1185–1188. <https://doi.org/10.1038/mt.2008.125>
161. Fagone P, Wright JF, Nathwani AC, Nienhuis AW, Davidoff AM, Gray JT (2012) Systemic errors in quantitative polymerase chain reaction titration of self-complementary adeno-associated viral vectors and improved alternative methods. *Hum Gene Ther Methods* 23(1):1–7. <https://doi.org/10.1089/hgtb.2011.104>
162. Werling NJ, Satkunanathan S, Thorpe R, Zhao Y (2015) Systematic comparison and validation of quantitative real-time PCR methods for the quantitation of adeno-associated viral products. *Hum Gene Ther Methods* 26(3):82–92. <https://doi.org/10.1089/hgtb.2015.013>
163. Duan D (2011) Muscle gene therapy: methods and protocols. *Methods in molecular biology*, vol 709. Humana, New York
164. Kyba M (2016) Skeletal muscle regeneration in the mouse: methods and protocols. *Springer Protocols*, vol 1460. Humana Press, New York
165. DiMario JX (2012) Myogenesis: methods and protocols. *Methods in molecular biology*, vol 798. Humana Press/Springer, New York
166. Moorwood C, Liu M, Tian Z, Barton ER (2013) Isometric and eccentric force generation assessment of skeletal muscles isolated from murine models of muscular dystrophies. *J Vis Exp* (71):e50036. <https://doi.org/10.3791/50036>
167. Hakim CH, Wasala NB, Duan D (2013) Evaluation of muscle function of the extensor digitorum longus muscle ex vivo and tibialis anterior muscle in situ in mice. *J Vis Exp* (72):e50183. <https://doi.org/10.3791/50183>
168. Kumar A, Accorsi A, Rhee Y, Girgenrath M (2015) Do’s and don’ts in the preparation of muscle cryosections for histological analysis. *J Vis Exp* (99):e52793. <https://doi.org/10.3791/52793>
169. Grieger JC, Choi VW, Samulski RJ (2006) Production and characterization of adeno-associated viral vectors. *Nat Protoc* 1(3):1412–1428
170. Duricki DA, Soleman S, Moon LD (2016) Analysis of longitudinal data from animals with missing values using SPSS. *Nat Protoc* 11(6):1112–1129. <https://doi.org/10.1038/nprot.2016.048>

171. Bagasra O (2007) Protocols for the in situ PCR-amplification and detection of mRNA and DNA sequences. *Nat Protoc* 2(11):2782–2795. <https://doi.org/10.1038/nprot.2007.395>
172. Jager L, Hausl MA, Rauschhuber C, Wolf NM, Kay MA, Ehrhardt A (2009) A rapid protocol for construction and production of high-capacity adenoviral vectors. *Nat Protoc* 4(4):547–564. <https://doi.org/10.1038/nprot.2009.4>
173. Duan D, Rafael-Fortney JA, Blain A, Kass DA, McNally EM, Metzger JM, Spurney CF, Kinnett K (2016) Standard operating procedures (SOPs) for evaluating the heart in preclinical studies of Duchenne muscular dystrophy. *J Cardiovasc Transl Res* 9(1):85–86. <https://doi.org/10.1007/s12265-015-9669-6>
174. Briguet A, Courdier-Fruh I, Foster M, Meier T, Magyar JP (2004) Histological parameters for the quantitative assessment of muscular dystrophy in the mdx-mouse. *Neuromuscul Disord* 14(10):675–682. <https://doi.org/10.1016/j.nmd.2004.06.008>
175. Nagaraju K, Willmann R, Network T-N, the Wellstone Muscular Dystrophy Cooperative Research Network (2009) Developing standard procedures for murine and canine efficacy studies of DMD therapeutics: report of two expert workshops on “pre-clinical testing for Duchenne dystrophy”: Washington DC, October 27th–28th 2007 and Zurich, June 30th–July 1st 2008. *Neuromuscul Disord* 19(7):502–506. <https://doi.org/10.1016/j.nmd.2009.05.003>
176. van Putten M, Aartsma-Rus A, Grounds MD, Kornegay JN, Mayhew A, Gillingwater TH, Takeda S, Ruegg MA, De Luca A, Nagaraju K, Willmann R (2018) Update on standard operating procedures in preclinical research for DMD and SMA report of TREAT-NMD Alliance workshop, Schiphol airport, 26 April 2015, the Netherlands. *J Neuromuscul Dis* 5(1):29–34. <https://doi.org/10.3233/JND-170288>
177. Shin J-H, Greer B, Hakim CH, Zhou Z, Chung YC, Duan Y, He Z, Duan D (2013) Quantitative phenotyping of Duchenne muscular dystrophy dogs by comprehensive gait analysis and overnight activity monitoring. *PLoS One* 8(3):e59875
178. Jenkins GJ, Hakim CH, Yang NN, Yao G, Duan D (2018) Automatic characterization of stride parameters in canines with a single wearable inertial sensor. *PLoS One* 13(6):e0198893. <https://doi.org/10.1371/journal.pone.0198893>
179. Marsh AP, Eggebeen JD, Kornegay JN, Markert CD, Childers MK (2010) Kinematics of gait in golden retriever muscular dystrophy. *Neuromuscul Disord* 20(1):16–20. <https://doi.org/10.1016/j.nmd.2009.10.007>. S0960-8966(09)00660-9 [pii]
180. Barthelemy I, Barrey E, Thibaud JL, Uriarte A, Voit T, Blot S, Hogrel JY (2009) Gait analysis using accelerometry in dystrophin-deficient dogs. *Neuromuscul Disord* 19(11):788–796. <https://doi.org/10.1016/j.nmd.2009.07.014>. S0960-8966(09)00578-1 [pii]
181. Hakim CH, Peters AA, Feng F, Yao G, Duan D (2015) Night activity reduction is a signature physiological biomarker for Duchenne muscular dystrophy dogs. *J Neuromuscul Dis* 2(4):397–407. <https://doi.org/10.3233/JND-150114>
182. Hakim CH, Mijailovic A, Lessa TB, Coates JR, Shin C, Rutkove SB, Duan D (2017) Non-invasive evaluation of muscle disease in the canine model of Duchenne muscular dystrophy by electrical impedance myography. *PLoS One* 12(3):e0173557. <https://doi.org/10.1371/journal.pone.0173557>
183. Yang HT, Shin J-H, Hakim CH, Pan X, Terjung RL, Duan D (2012) Dystrophin deficiency compromises force production of the extensor carpi ulnaris muscle in the canine model of Duchenne muscular dystrophy. *PLoS One* 7(9):e44438
184. Kodippili K, Hakim CH, Yang HT, Pan X, Yang NN, Laughlin MH, Terjung RL, Duan D (2018) Nitric oxide dependent attenuation of norepinephrine-induced vasoconstriction is impaired in the canine model of Duchenne muscular dystrophy. *J Physiol* 596(21):5199–5216. <https://doi.org/10.1113/JP275672>
185. Capes-Davis A, Neve RM (2016) Authentication: a standard problem or a problem of standards? *PLoS Biol* 14(6):e1002477. <https://doi.org/10.1371/journal.pbio.1002477>
186. Williams M (2018) Reagent validation to facilitate experimental reproducibility. *Curr Protoc Pharmacol* 81(1):e40. <https://doi.org/10.1002/cpph.40>

187. Casadevall A, Ellis LM, Davies EW, McFall-Ngai M, Fang FC (2016) A framework for improving the quality of research in the biological sciences. *MBio* 7(4). <https://doi.org/10.1128/mBio.01256-16>
188. Landis SC, Amara SG, Asadullah K, Austin CP, Blumenstein R, Bradley EW, Crystal RG, Darnell RB, Ferrante RJ, Fillit H, Finkelstein R, Fisher M, Gendelman HE, Golub RM, Goudreau JL, Gross RA, Gubitz AK, Hesterlee SE, Howells DW, Huguenard J, Kelner K, Koroshetz W, Krainc D, Lazic SE, Levine MS, Macleod MR, McCall JM, Moxley RT 3rd, Narasimhan K, Noble LJ, Perrin S, Porter JD, Steward O, Unger E, Utz U, Silberberg SD (2012) A call for transparent reporting to optimize the predictive value of preclinical research. *Nature* 490(7419):187–191. <https://doi.org/10.1038/nature11556>
189. Freedman LP, Inglese J (2014) The increasing urgency for standards in basic biologic research. *Cancer Res* 74(15):4024–4029. <https://doi.org/10.1158/0008-5472.Can-14-0925>
190. Kodippili K, Vince L, Shin JH, Yue Y, Morris GE, McIntosh MA, Duan D (2014) Characterization of 65 epitope-specific dystrophin monoclonal antibodies in canine and murine models of Duchenne muscular dystrophy by immunostaining and western blot. *PLoS One* 9(2):e88280. <https://doi.org/10.1371/journal.pone.0088280>
191. Morris GE, Man NT, Sewry CA (2011) Monitoring Duchenne muscular dystrophy gene therapy with epitope-specific monoclonal antibodies. *Methods Mol Biol* 709:39–61. https://doi.org/10.1007/978-1-61737-982-6_3
192. Nguyen TM, Ginjaar IB, van Ommen GJ, Morris GE (1992) Monoclonal antibodies for dystrophin analysis. Epitope mapping and improved binding to SDS-treated muscle sections. *Biochem J* 288(Pt 2):663–668
193. Uhlen M, Bandrowski A, Carr S, Edwards A, Ellenberg J, Lundberg E, Rimm DL, Rodriguez H, Hiltke T, Snyder M, Yamamoto T (2016) A proposal for validation of antibodies. *Nat Methods* 13(10):823–827. <https://doi.org/10.1038/Nmeth.3995>
194. Bordeaux J, Welsh A, Agarwal S, Killiam E, Baquero M, Hanna J, Anagnostou V, Rimm D (2010) Antibody validation. *BioTechniques* 48(3):197–209. <https://doi.org/10.2144/000113382>
195. Marx V (2013) Finding the right antibody for the job. *Nat Methods* 10(8):703–707. <https://doi.org/10.1038/nmeth.2570>
196. Taussig MJ, Fonseca C, Trimmer JS (2018) Antibody validation: a view from the mountains. *New Biotechnol* 45:1–8. <https://doi.org/10.1016/j.nbt.2018.08.002>
197. Bradbury A, Pluckthun A (2015) Standardize antibodies used in research. *Nature* 518(7537):27–29. <https://doi.org/10.1038/518027a>
198. Alm TL, von Feilitzen K, Uhlen M (2016) Antibodypedia - the Wiki of Antibodies. Poster presented at the 2016 American Society for Cell Biology (ASCB) Annual Meeting, San Francisco, CA, Dec. 3-7, 2016. <https://www.ascb.org/wp-content/uploads/2016/04/2016ASCBMeeting-PosterAbstracts.pdf>. urn:nbn:se:kth:diva-204763
199. Bjorling E, Uhlen M (2008) Antibodypedia, a portal for sharing antibody and antigen validation data. *Mol Cell Proteomics* 7(10):2028–2037. <https://doi.org/10.1074/mcp.M800264-MCP200>
200. Colwill K, Renewable Protein Binder Working Group, Graslund S (2011) A roadmap to generate renewable protein binders to the human proteome. *Nat Methods* 8(7):551–558. <https://doi.org/10.1038/nmeth.1607>
201. Helsby MA, Leader PM, Fenn JR, Gulsen T, Bryant C, Doughton G, Sharpe B, Whitley P, Caunt CJ, James K, Pope AD, Kelly DH, Chalmers AD (2014) CiteAb: a searchable antibody database that ranks antibodies by the number of times they have been cited. *BMC Cell Biol* 15:6. <https://doi.org/10.1186/1471-2121-15-6>
202. Major SM, Nishizuka S, Morita D, Rowland R, Sunshine M, Shankavaram U, Washburn F, Asin D, Kourous-Mehr H, Kane D, Weinstein JN (2006) AbMiner: a bioinformatic resource on available monoclonal antibodies and corresponding gene identifiers for genomic, proteomic, and immunologic studies. *BMC Bioinformatics* 7:192. <https://doi.org/10.1186/1471-2105-7-192>

203. Roncador G, Engel P, Maestre L, Anderson AP, Cordell JL, Cragg MS, Serbec VC, Jones M, Lisnic VJ, Kremer L, Li D, Koch-Nolte F, Pascual N, Rodriguez-Barbosa JI, Torensma R, Turley H, Pulford K, Banham AH (2016) The European antibody network's practical guide to finding and validating suitable antibodies for research. *MAbs* 8(1):27–36. <https://doi.org/10.1080/19420862.2015.1100787>
204. Acharya P, Quinlan A, Neumeister V (2017) The ABCs of finding a good antibody: how to find a good antibody, validate it, and publish meaningful data. *F1000Res* 6:851. <https://doi.org/10.12688/f1000research.11774.1>
205. Aban IB, George B (2015) Statistical considerations for preclinical studies. *Exp Neurol* 270:82–87. <https://doi.org/10.1016/j.expneurol.2015.02.024>
206. Liu C, Cripe TP, Kim MO (2010) Statistical issues in longitudinal data analysis for treatment efficacy studies in the biomedical sciences. *Mol Ther* 18(9):1724–1730. <https://doi.org/10.1038/mt.2010.127>
207. Dell RB, Holleran S, Ramakrishnan R (2002) Sample size determination. *ILAR J* 43(4):207–213
208. Fitts DA (2011) Ethics and animal numbers: informal analyses, uncertain sample sizes, inefficient replications, and type I errors. *J Am Assoc Lab Anim Sci* 50(4):445–453
209. Lenth RV (2001) Some practical guidelines for effective sample size determination. *Am Stat* 55(3):187–193. <https://doi.org/10.1198/000313001317098149>
210. Hardouin JB, Amri S, Feddag ML, Sebillé V (2012) Towards power and sample size calculations for the comparison of two groups of patients with item response theory models. *Stat Med* 31(11–12):1277–1290. <https://doi.org/10.1002/sim.4387>
211. Whitley E, Ball J (2002) Statistics review 4: sample size calculations. *Crit Care* 6(4):335–341
212. Faul F, Erdfelder E, Lang AG, Buchner A (2007) G*Power 3: a flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behav Res Methods* 39(2):175–191
213. Hirst JA, Howick J, Aronson JK, Roberts N, Perera R, Koshialis C, Heneghan C (2014) The need for randomization in animal trials: an overview of systematic reviews. *PLoS One* 9(6):e98856. <https://doi.org/10.1371/journal.pone.0098856>
214. Macleod MR (2014) Design animal studies better. *Nature* 510(7503):35–35. <https://doi.org/10.1038/510035a>
215. Suresh K (2011) An overview of randomization techniques: an unbiased assessment of outcome in clinical research. *J Hum Reprod Sci* 4(1):8–11. <https://doi.org/10.4103/0974-1208.82352>
216. Whitley E, Ball J (2002) Statistics review 5: comparison of means. *Crit Care* 6(5):424–428
217. Bewick V, Cheek L, Ball J (2004) Statistics review 9: one-way analysis of variance. *Crit Care* 8(2):130–136. <https://doi.org/10.1186/cc2836>
218. Whitley E, Bai J (2002) Statistics review 6: nonparametric methods. *Crit Care* 6(6):509–513. <https://doi.org/10.1186/cc1820>
219. Baker M (2016) Statisticians issue warning over misuse of P values. *Nature* 531(7593):151. <https://doi.org/10.1038/nature.2016.19503>
220. Wasserstein RL, Assoc AS (2016) ASA statement on statistical significance and P-values. *Am Stat* 70(2):131–133
221. Wasserstein RL, Lazar NA (2016) The ASA's statement on p-values: context, process, and purpose. *Am Stat* 70(2):129–131. <https://doi.org/10.1080/00031305.2016.1154108>
222. Greenland S, Senn SJ, Rothman KJ, Carlin JB, Poole C, Goodman SN, Altman DG (2016) Statistical tests, P values, confidence intervals, and power: a guide to misinterpretations. *Eur J Epidemiol* 31(4):337–350. <https://doi.org/10.1007/s10654-016-0149-3>
223. Nuzzo R (2014) Scientific method: statistical errors. *Nature* 506(7487):150–152. <https://doi.org/10.1038/506150a>
224. Couzin-Frankel J (2013) When mice mislead. *Science* 342(6161):922–923, 925. <https://doi.org/10.1126/science.342.6161.922>
225. Perrin S (2014) Preclinical research: make mouse studies work. *Nature* 507(7493):423–425. <https://doi.org/10.1038/507423a>

226. van der Worp HB, Howells DW, Sena ES, Porritt MJ, Rewell S, O'Collins V, Macleod MR (2010) Can animal models of disease reliably inform human studies? *PLoS Med* 7(3):e1000245. <https://doi.org/10.1371/journal.pmed.1000245>. ARTN e1000245
227. Hathout Y, Brody E, Clemens PR, Cripe L, DeLisle RK, Furlong P, Gordish-Dressman H, Hache L, Henricson E, Hoffman EP, Kobayashi YM, Lorts A, Mah JK, McDonald C, Mehler B, Nelson S, Nikrad M, Singer B, Steele F, Sterling D, Sweeney HL, Williams S, Gold L (2015) Large-scale serum protein biomarker discovery in Duchenne muscular dystrophy. *Proc Natl Acad Sci U S A* 112(23):7153–7158. <https://doi.org/10.1073/pnas.1507719112>
228. Meltzer HY (1971) Factors affecting serum creatine phosphokinase levels in the general population: the role of race, activity and age. *Clin Chim Acta* 33(1):165–172
229. Gledhill RF, Van der Merwe CA, Greyling M, Van Niekerk MM (1988) Race-gender differences in serum creatine kinase activity: a study among South Africans. *J Neurol Neurosurg Psychiatry* 51(2):301–304
230. Neal RC, Ferdinand KC, Ycas J, Miller E (2009) Relationship of ethnic origin, gender, and age to blood creatine kinase levels. *Am J Med* 122(1):73–78. <https://doi.org/10.1016/j.amjmed.2008.08.033>
231. Parasuraman S, Raveendran R, Kesavan R (2010) Blood sample collection in small laboratory animals. *J Pharmacol Pharmacother* 1(2):87–93. <https://doi.org/10.4103/0976-500X.72350>
232. Baudy AR, Sali A, Jordan S, Kesari A, Johnston HK, Hoffman EP, Nagaraju K (2011) Non-invasive optical imaging of muscle pathology in mdx mice using cathepsin caged near-infrared imaging. *Mol Imaging Biol* 13(3):462–470. <https://doi.org/10.1007/s11307-010-0376-z>
233. Coley WD, Bogdanik L, Vila MC, Yu Q, Van Der Meulen JH, Rayavarapu S, Novak JS, Nearing M, Quinn JL, Saunders A, Dolan C, Andrews W, Lammert C, Austin A, Partridge TA, Cox GA, Lutz C, Nagaraju K (2016) Effect of genetic background on the dystrophic phenotype in mdx mice. *Hum Mol Genet* 25(1):130–145. <https://doi.org/10.1093/hmg/ddv460>
234. de Greef JC, Hamlyn R, Jensen BS, O'Campo Landa R, Levy JR, Kobuke K, Campbell KP (2016) Collagen VI deficiency reduces muscle pathology, but does not improve muscle function, in the gamma-sarcoglycan-null mouse. *Hum Mol Genet* 25(7):1357–1369. <https://doi.org/10.1093/hmg/ddw018>
235. Dirnagl U, Fisher M (2012) International, multicenter randomized preclinical trials in translational stroke research: it's time to act. *J Cereb Blood Flow Metab* 32(6):933–935. <https://doi.org/10.1038/jcbfm.2012.51>
236. Bath PM, Macleod MR, Green AR (2009) Emulating multicentre clinical stroke trials: a new paradigm for studying novel interventions in experimental models of stroke. *Int J Stroke* 4(6):471–479. <https://doi.org/10.1111/j.1747-4949.2009.00386.x>
237. Llovera G, Liesz A (2016) The next step in translational research: lessons learned from the first preclinical randomized controlled trial. *J Neurochem* 139:271–279. <https://doi.org/10.1111/jnc.13516>
238. Amaro S, Llull L (2016) Preclinical randomized controlled multicenter trials in translational stroke research. *Ann Transl Med* 4(Suppl 1):S58. <https://doi.org/10.21037/atm.2016.10.66>
239. Mullard A (2011) Reliability of 'new drug target' claims called into question. *Nat Rev Drug Discov* 10(9):643–644. <https://doi.org/10.1038/nrd3545>. nrd3545 [pii]
240. Baker M (2016) Biotech giant publishes failures to confirm high-profile science. *Nature* 530(7589):141. <https://doi.org/10.1038/nature.2016.19269>
241. Aarts AA, Anderson JE, Anderson CJ, Attridge PR, Attwood A, Axt J, Babel M, Bahnik S, Baranski E, Barnett-Cowan M, Bartmess E, Beer J, Bell R, Bentley H, Beyan L, Binion G, Borsboom D, Bosch A, Bosco FA, Bowman SD, Brandt MJ, Braswell E, Brohmer H, Brown BT, Brown K, Bruning J, Calhoun-Sauls A, Callahan SP, Chagnon E, Chandler J, Chartier CR, Cheung F, Christopherson CD, Cillessen L, Clay R, Cleary H, Cloud MD, Cohn M, Cohoon J, Columbus S, Cordes A, Costantini G, Alvarez LDC, Cremata E, Crusius J, De Coster J, De Gaetano MA, Della Penna N, den Bezemer B, Deserno MK, Devitt O, Dewitte L, Dobolyi DG, Dodson GT, Donnellan MB, Donohue R, Dore RA, Dorrough A, Dreber A, Dugas M, Dunn EW, Easey K, Eboigbe S, Eggleston C, Embley J, Epskamp S,

- Errington TM, Estel V, Farach FJ, Feather J, Fedor A, Fernandez-Castilla B, Fiedler S, Field JG, Fitneva SA, Flagan T, Forest AL, Forsell E, Foster JD, Frank MC, Frazier RS, Fuchs H, Gable P, Galak J, Galliani EM, Gampa A, Garcia S, Gazarian D, Gilbert E, Giner-Sorolla R, Glockner A, Goellner L, Goh JX, Goldberg R, Goodbourn PT, Gordon-McKeon S, Gorges B, Gorges J, Goss J, Graham J, Grange JA, Gray J, Hartgerink C, Hartshorne J, Hasselman F, Hayes T, Heikensten E, Henninger F, Hodsoll J, Holubar T, Hoogendoorn G, Humphries DJ, Hung COY, Immanuel N, Irsik VC, Jahn G, Jakel F, Jekel M, Johannesson M, Johnson LG, Johnson DJ, Johnson KM, Johnston WJ, Jonas K, Joy-Gaba JA, Kappes HB, Kelso K, Kidwell MC, Kim SK, Kirkhart M, Kleinberg B, Knezevic G, Kolorz FM, Kossakowski JJ, Krause RW, Krijnen J, Kuhlmann T, Kunkels YK, Kyc MM, Lai CK, Laique A, Lakens D, Lane KA, Lassetter B, Lazarevic LB, EP LB, Lee KJ, Lee M, Lemm K, Levitan CA, Lewis M, Lin L, Lin S, Lippold M, Loureiro D, Luteijn I, Mackinnon S, Mainard HN, Marigold DC, Martin DP, Martinez T, Masicampo EJ, Maticcotta J, Mathur M, May M, Mechin N, Mehta P, Meixner J, Melinger A, Miller JK, Miller M, Moore K, Moschl M, Motyl M, Muller SM, Munafò M, Neijenhuis KI, Nervi T, Nicolas G, Nilsson G, Nosek BA, Nuijten MB, Olsson C, Osborne C, Ostkamp L, Pavel M, Penton-Voak IS, Perna O, Pernet C, Perugini M, Pipitone RN, Pitts M, Plessow F, Prenoveau JM, Rahal RM, Ratliff KA, Reinhard D, Renkewitz F, Ricker AA, Rigney A, Rivers AM, Roebke M, Rutchick AM, Ryan RS, Sahin O, Saide A, Sandstrom GM, Santos D, Saxe R, Schlegelmilch R, Schmidt K, Scholz S, Seibel L, Selterman DF, Shaki S, Simpson WB, Sinclair HC, Skorinko JLM, Slowik A, Snyder JS, Soderberg C, Sonnleitner C, Spencer N, Spies JR, Steegen S, Stieger S, Strohminger N, Sullivan GB, Talhelm T, Tapia M, te Dorsthorst A, Thomaes M, Thomas SL, Tio P, Traets F, Tsang S, Tuerlinckx F, Turchan P, Valasek M, van 't Veer AE, Van Aert R, van Assen M, van Bork R, van de Ven M, van den Bergh D, van der Hulst M, van Dooren R, van Doorn J, van Renswoude DR, van Rijn H, Vanpaemel W, Echeverria AV, Vazquez M, Velez N, Vermue M, Verschoor M, Vianello M, Voracek M, Vuu G, Wagenmakers EJ, Weerdmeester J, Welsh A, Westgate OC, Wissink J, Wood M, Woods A, Wright E, Wu S, Zeelenberg M, Zuni K, Collaboration ES (2015) Estimating the reproducibility of psychological science. *Science* 349(6251):aac4716. <https://doi.org/10.1126/science.aac4716>. ARTN aac4716
242. Prinz F, Schlange T, Asadullah K (2011) Believe it or not: how much can we rely on published data on potential drug targets? *Nat Rev Drug Discov* 10(9):712. <https://doi.org/10.1038/nrd3439-c1>
 243. Boltze J, Ayata C, Wagner DC, Plesnila N (2014) Preclinical phase III trials in translational stroke research: call for collective design of framework and guidelines. *Stroke* 45(2):357. <https://doi.org/10.1161/STROKEAHA.113.004148>
 244. Macleod MR, O'Collins T, Howells DW, Donnan GA (2004) Pooling of animal experimental data reveals influence of study design and publication bias. *Stroke* 35(5):1203–1208. <https://doi.org/10.1161/01.STR.0000125719.25853.20>
 245. Sena ES, Currie GL, McCann SK, Macleod MR, Howells DW (2014) Systematic reviews and meta-analysis of preclinical studies: why perform them and how to appraise them critically. *J Cereb Blood Flow Metab* 34(5):737–742. <https://doi.org/10.1038/jcbfm.2014.28>
 246. Vesterinen HM, Sena ES, Egan KJ, Hirst TC, Churolov L, Currie GL, Antonic A, Howells DW, Macleod MR (2014) Meta-analysis of data from animal studies: a practical guide. *J Neurosci Methods* 221:92–102. <https://doi.org/10.1016/j.jneumeth.2013.09.010>
 247. Hooijmans CR, Int'Hout J, Ritskes-Hoitinga M, Rovers MM (2014) Meta-analyses of animal studies: an introduction of a valuable instrument to further improve healthcare. *ILAR J* 55(3):418–426. <https://doi.org/10.1093/ilar/ilu042>
 248. Normand SL (1999) Meta-analysis: formulating, evaluating, combining, and reporting. *Stat Med* 18(3):321–359
 249. Esterhuizen TM, Thabane L (2016) Con: meta-analysis: some key limitations and potential solutions. *Nephrol Dial Transplant* 31(6):882–885. <https://doi.org/10.1093/ndt/gfw092>
 250. Zoccali C (2016) Moderator's view: meta-analysis: the best knowledge but not always shining gold. *Nephrol Dial Transplant* 31(6):886–889. <https://doi.org/10.1093/ndt/gfw093>

251. Llovera G, Hofmann K, Roth S, Salas-Perdomo A, Ferrer-Ferrer M, Perego C, Zanier ER, Mamrak U, Rex A, Party H, Agin V, Fauchon C, Orset C, Haelewyn B, De Simoni MG, Dirnagl U, Grittner U, Planas AM, Plesnila N, Vivien D, Liesz A (2015) Results of a preclinical randomized controlled multicenter trial (pRCT): anti-CD49d treatment for acute brain ischemia. *Sci Transl Med* 7(299):299ra121. <https://doi.org/10.1126/scitranslmed.aaa9853>. ARTN 299ra121
252. Voelkl B, Vogt L, Sena ES, Wurbel H (2018) Reproducibility of preclinical animal research improves with heterogeneity of study samples. *PLoS Biol* 16(2):e2003693. <https://doi.org/10.1371/journal.pbio.2003693>
253. Freedman LP, Cockburn IM, Simcoe TS (2015) The economics of reproducibility in preclinical research. *PLoS Biol* 13(6):e1002165. <https://doi.org/10.1371/journal.pbio.1002165>
254. Moher D, Avey M, Antes G, Altman DG (2015) The National Institutes of Health and guidance for reporting preclinical research. *BMC Med* 13:34. <https://doi.org/10.1186/s12916-015-0284-9>
255. Hsieh T, Vaickus MH, Remick DG (2018) Enhancing scientific foundations to ensure reproducibility: a new paradigm. *Am J Pathol* 188(1):6–10. <https://doi.org/10.1016/j.ajpath.2017.08.028>
256. Goodman SN, Fanelli D, Ioannidis JP (2016) What does research reproducibility mean? *Sci Transl Med* 8(341):341ps312. <https://doi.org/10.1126/scitranslmed.aaf5027>
257. Wieschowski S, Chin WWL, Federico C, Sievers S, Kimmelman J, Strech D (2018) Preclinical efficacy studies in investigator brochures: do they enable risk-benefit assessment? *PLoS Biol* 16(4):e2004879. <https://doi.org/10.1371/journal.pbio.2004879>
258. Jarvis MF, Williams M (2016) Irreproducibility in preclinical biomedical research: perceptions, uncertainties, and knowledge gaps. *Trends Pharmacol Sci* 37(4):290–302. <https://doi.org/10.1016/j.tips.2015.12.001>
259. Han S, Olonisakin TF, Pribis JP, Zupetic J, Yoon JH, Holleran KM, Jeong K, Shaikh N, Rubio DM, Lee JS (2017) A checklist is associated with increased quality of reporting preclinical biomedical research: a systematic review. *PLoS One* 12(9):e0183591. <https://doi.org/10.1371/journal.pone.0183591>
260. Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG (2010) Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. *PLoS Biol* 8(6):e1000412. <https://doi.org/10.1371/journal.pbio.1000412>
261. Ramirez FD, Motazedian P, Jung RG, Di Santo P, MacDonald ZD, Moreland R, Simard T, Clancy AA, Russo JJ, Welch VA, Wells GA, Hibbert B (2017) Methodological rigor in preclinical cardiovascular studies: targets to enhance reproducibility and promote research translation. *Circ Res* 120(12):1916–1926. <https://doi.org/10.1161/CIRCRESAHA.117.310628>
262. Gerdes AM (2015) How to improve the overall quality of cardiac morphometric data. *Am J Physiol Heart Circ Physiol* 309(1):H9–H14. <https://doi.org/10.1152/ajpheart.00232.2015>
263. Burgoon LD (2006) The need for standards, not guidelines, in biological data reporting and sharing. *Nat Biotechnol* 24(11):1369–1373. <https://doi.org/10.1038/nbt1106-1369>
264. Deutsch EW, Ball CA, Berman JJ, Bova GS, Brazma A, Bumgarner RE, Campbell D, Causton HC, Christiansen JH, Daian F, Dauga D, Davidson DR, Gimenez G, Goo YA, Grimmond S, Henrich T, Herrmann BG, Johnson MH, Korb M, Mills JC, Oudes AJ, Parkinson HE, Pascal LE, Pollet N, Quackenbush J, Ramialison M, Ringwald M, Salgado D, Sansone SA, Sherlock G, Stoeckert CJ Jr, Swedlow J, Taylor RC, Walashek L, Warford A, Wilkinson DG, Zhou Y, Zon LI, Liu AY, True LD (2008) Minimum information specification for in situ hybridization and immunohistochemistry experiments (MISFISHIE). *Nat Biotechnol* 26(3):305–312. <https://doi.org/10.1038/nbt1391>
265. Taylor CF, Field D, Sansone SA, Aerts J, Apweiler R, Ashburner M, Ball CA, Binz PA, Bogue M, Booth T, Brazma A, Brinkman RR, Michael Clark A, Deutsch EW, Fiehn O, Fostel J, Ghazal P, Gibson F, Gray T, Grimes G, Hancock JM, Hardy NW, Hermjakob H, Julian RK Jr, Kane M, Kettner C, Kinsinger C, Kolker E, Kuiper M, Le Novère N, Leebens-Mack J, Lewis SE, Lord P, Mallon AM, Marthandan N, Masuya H, McNally R, Mehrle A, Morrison

- N, Orchard S, Quackenbush J, Reecy JM, Robertson DG, Rocca-Serra P, Rodriguez H, Rosenfelder H, Santoyo-Lopez J, Scheuermann RH, Schober D, Smith B, Snape J, Stoeckert CJ Jr, Tipton K, Sterk P, Untergasser A, Vandesompele J, Wiemann S (2008) Promoting coherent minimum reporting guidelines for biological and biomedical investigations: the MIBBI project. *Nat Biotechnol* 26(8):889–896. <https://doi.org/10.1038/nbt.1411>
266. Zeiss CJ, Allore HG, Beck AP (2017) Established patterns of animal study design undermine translation of disease-modifying therapies for Parkinson's disease. *PLoS One* 12(2):e0171790. <https://doi.org/10.1371/journal.pone.0171790>. ARTN e0171790
267. Bonne G, Rivier F, Hamroun D (2017) The 2018 version of the gene table of monogenic neuromuscular disorders (nuclear genome). *Neuromuscul Disord* 27(12):1152–1183. <https://doi.org/10.1016/j.nmd.2017.10.005>