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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. Surprisingly, the body weight of the treated mice showed a statistically significant reduction. Unexpected weight loss cautions potential side effects of CRISPR therapy. In the current funding period, we discovered the underlying mechanism that is responsible for the low CRISPR efficiency in our original study. We also repeated the study in a larger cohort of female mdx mice to determine whether weight loss is a real safety concern. Our new study showed widespread dystrophin restoration in both skeletal muscle and the heart, significant improvement in cardiac hemodynamic function and a lack of body weight reduction. Our results support further development of systemic CRISPR therapy for Duchenne muscular dystrophy.					
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					
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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.

Poor dystrophin restoration in long-term systemic AAV CRISPR therapy is due to selective depletion of the gRNA AAV vector.

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 better understand poor dystrophin restoration, we directly quantified the edited dystrophin transcripts in skeletal muscle and heart. **Figure 1** illustrates the method we used to quantify edited and unedited dystrophin transcripts as well as the relative percentage of each.

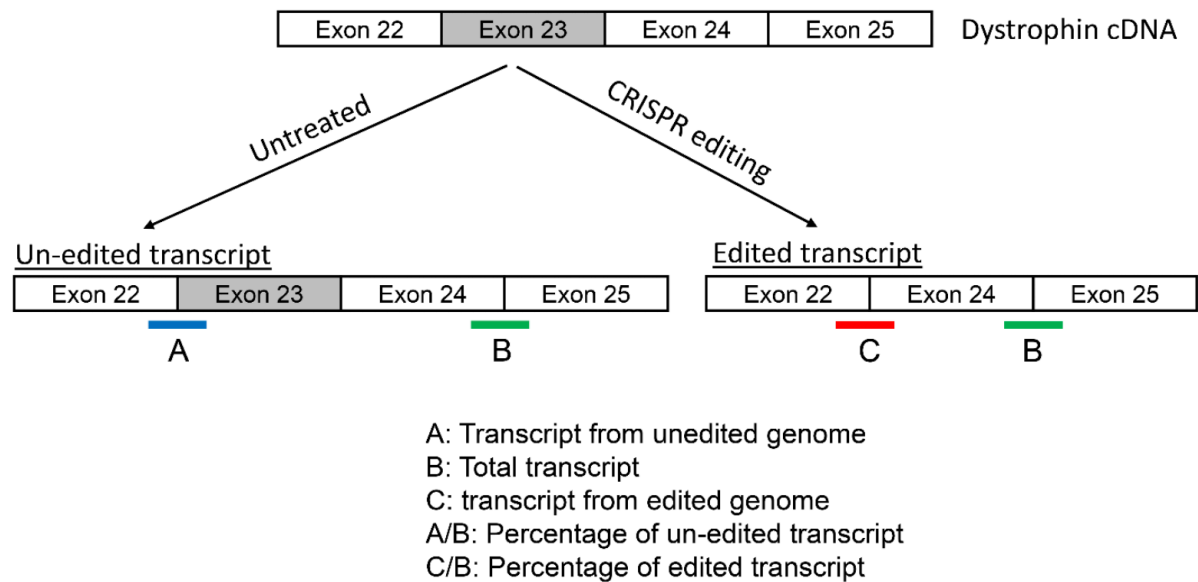
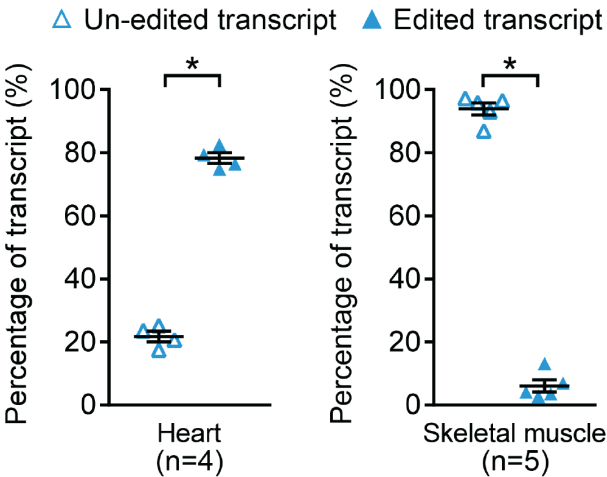


Figure 1. Schematic outline of the strategy used to quantify dystrophin transcript. The mdx mouse has a nonsense mutation in the exon 23 of the dystrophin gene. In our CRISPR therapy, the mutated exon 23 and its surrounding intronic sequences are removed from the mutated gene. The resulting transcript does not contain exon 23. To detect unedited and edited transcripts, PCR experiments were designed to amplify the junctions of exons 22-23 (*product A* in the figure) and exons 22-24 (*product C* in the figure), respectively. The exons 24-25 junction (*product B* in the figure) was used as the internal control for all dystrophin transcripts (both edited and unedited). In untreated mdx mice, we expect *product B*= *product A*. CRISPR treated mdx mice have both edited and unedited transcripts. We expect *product B*= *product A* + *product C*. The percentage of the edited dystrophin transcript was calculated using the formula: (*product C*)/(*product B*). The percentage of the unedited dystrophin transcript is calculated using the formula: (*product A*)/(*product B*). Exon 23 is shaded to indicate mutation.

Figure 2 shows the results from our digital droplet PCR (ddPCR) quantification. In the last progress report, we showed there was essentially undetectable dystrophin expression in skeletal muscle by immunostaining and western blot. Consistent with this finding, we barely detected any edited transcripts in skeletal muscle (**Figure 2**). In the last progress report, we showed dystrophin restoration in the heart. Consistent with this finding, we detected a high percentage of edited transcripts in the heart (**Figure 2**).

Figure 2. Quantification of dystrophin transcripts by ddPCR.



To further understand the poor editing outcome, we examined Cas9 protein expression, the Cas9 transcript level and the Cas9 vector genome copy number (Figures 3 and 4).

Figure 3. Heart and skeletal muscle had similar levels of Cas9 protein. Cas9 expression was determined by quantitative western blot. We found that there was no difference between the heart and skeletal muscle in Cas9 protein expression.

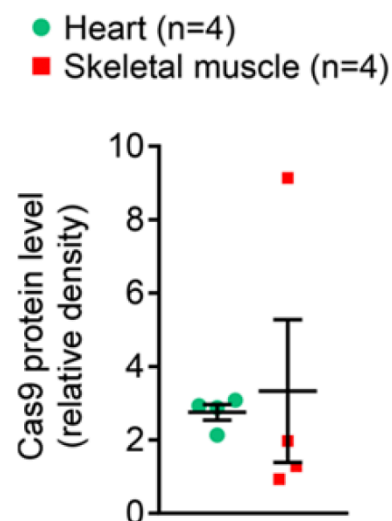
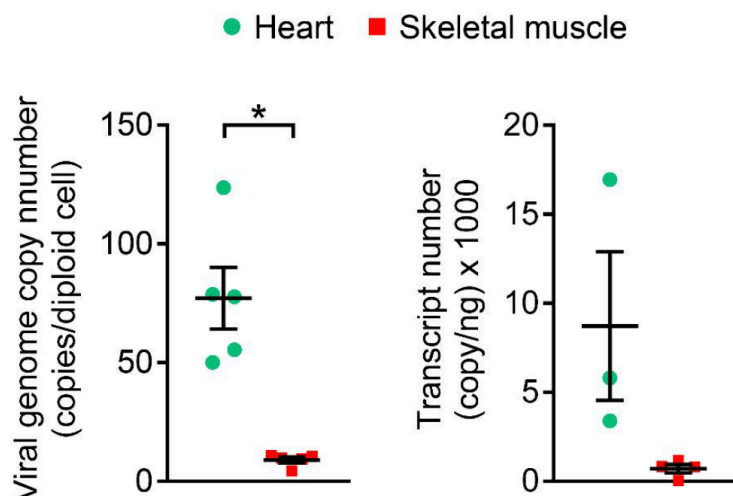


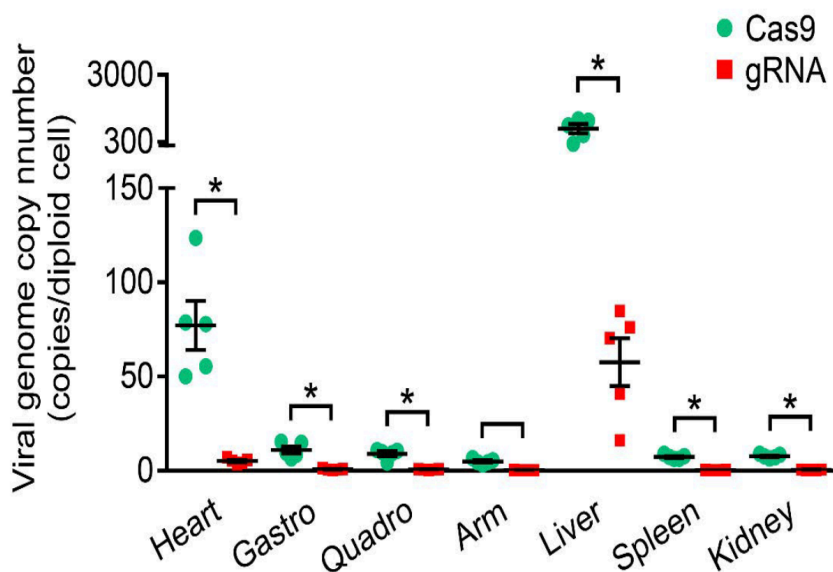
Figure 4. Quantification of the Cas9 AAV genome copy number and Cas9 transcript at 18 months in our original study. The Cas9 AAV genome copy number was determined by TaqMan PCR. The Cas9 cDNA copy number was determined by digital droplet PCR. Asterisk, statistically significant ($p < 0.05$).



Despite the fact that skeletal muscle had significantly lower quantity of the Cas9 AAV vector genome copy number, there was no difference in the transcript level and protein level between skeletal muscle and the heart. Hence, the difference in dystrophin gene editing between skeletal muscle and heart is not due to the difference in Cas9 expression.

Next we compared the Cas9 and gRNA AAV vector genome copy number and their relative ratio (Figures 5 and 6).

Figure 5. The Cas9 and gRNA AAV genome copy number quantification. TaqMan PCR was performed on DNA extracted from the heart, gastrocnemius, quadriceps, arm muscle, liver, spleen and kidney. In all the tissues examined, the genome copy number of the Cas9 vector was significantly higher than that of the gRNA vector. Asterisk, statistically significant ($p < 0.05$). Most importantly, while the gRNA vector was detectable at a low level in the heart, it was essentially “zero” in skeletal muscle. This explains why there was no editing in skeletal muscle.



In summary, we found that there was no editing on the mutated dystrophin gene in skeletal muscle. Disproportional loss of the gRNA AAV vector genome (to the undetectable level in skeletal muscle) resulted in the lack of gene editing. Consequently, no dystrophin expression was detected in skeletal muscle in treated mice.

While some gene editing was detected in the heart, the efficiency was low, very likely due to the same reason, i.e. Disproportional loss of the gRNA AAV vector genome.

Collectively, our detailed molecular analysis revealed the selective loss of the gRNA AAV vector genome is a hurdle for long-term systemic CRISPR therapy. Strategies that can increase gRNA expression or preserve the gRNA AAV vector genome may help overcome this hurdle.

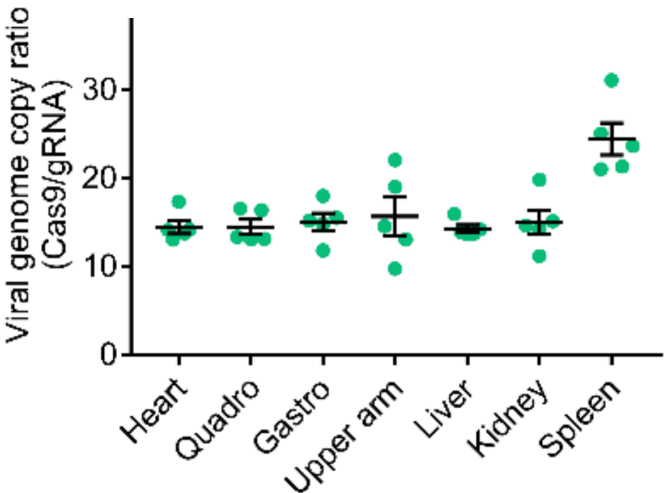


Figure 6. The Cas9 to gRNA vector genome copy number ratio. The Cas9 to gRNA vector genome copy number ratios were calculated for the heart, quadriceps, gastrocnemius, upper arm muscle, liver, kidney and spleen. It reached ≥ 15 , suggesting that the gRNA vector genome was selectively depleted following long-term systemic AAV CRISPR therapy in mdx mice.

Vector dose optimization significantly improved dystrophin expression following long-term systemic AAV CRISPR therapy in mdx mice.

A highly appealing approach to overcome the gRNA AAV vector genome depletion is to simply increase the dose of the gRNA vector at the time of CRISPR therapy. To this end, we performed a new study in which we intentionally used 10-fold more gRNA AAV than what was used in our previous study. Because cardiomyopathy is the primary concern for late-stage DMD patients, we decided to focus on the heart in the new study. We have previously shown that female mdx mice display more severe cardiomyopathy than male mdx mice (Bostick B, Yue Y, Duan D. *Gender influences cardiac function in the mdx model of Duchenne cardiomyopathy*. Muscle & Nerve 42(4):600-603, 2010.). Hence, we opted to conduct the new study in female mdx mice.

Table 1 (below) shows the age, body weight and AAV vector dose in our previous study and the new study. In our previous study, the gRNA AAV vector does is $3.63\text{E}+12$ vg/mouse ($1.61\text{E}+14$ vg/kg). In the new study, the gRNA AAV vector does is $3.00\text{E}+12$ vg/mouse ($1.82\text{E}+14$ vg/kg), hence, an approximately 10-fold increase. 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. Heart function was examined by invasive cardiac catheter assay at 18 months of the age. Tissues were harvested following the heart function assay and examined for dystrophin expression, dystrophin gene editing and off-target editing.

	n	Sex	Age at injection (m)	Body weight at injection (g)	AAV vector (vg/mouse)		AAV vector (vg/kg)	
					Cas9	gRNA	Cas9	gRNA
Study (old)	5	male	1.54 ± 0.02	22.6 ± 0.43	$7.20\text{E}+12$	$3.63\text{E}+12$	$3.19\text{E}+14$	$1.61\text{E}+14$
Study (new)	8	female	1.48 ± 0.05	16.1 ± 0.68	$1.00\text{E}+13$	$3.00\text{E}+13$	$6.07\text{E}+14$	$1.82\text{E}+15$

Figure 7 shows dystrophin expression examined by high power immunostaining and quantification.

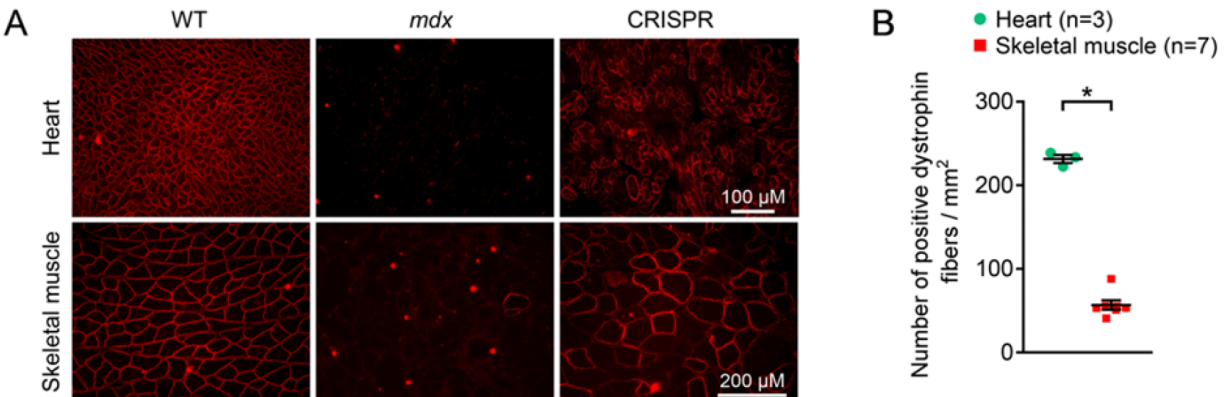


Figure 7. Examination of dystrophin expression by immunofluorescence staining. Systemic AAV-9 CRISPR therapy was performed in 6-week-old female *mdx* mice at the dose of 1×10^{13} vg /mouse and 3×10^{13} vg/mouse for the Cas9 and gRNA vectors, respectively. Mice were evaluated at 18 months of age. **A**, Representative dystrophin immunostaining photomicrographs from wild type (WT), *mdx* and CRISPR-treated *mdx* mice. **B**, Quantification of dystrophin positive myofibers.

Figure 8 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 9 shows dystrophin expression examined by western blot in both heart and skeletal muscle.

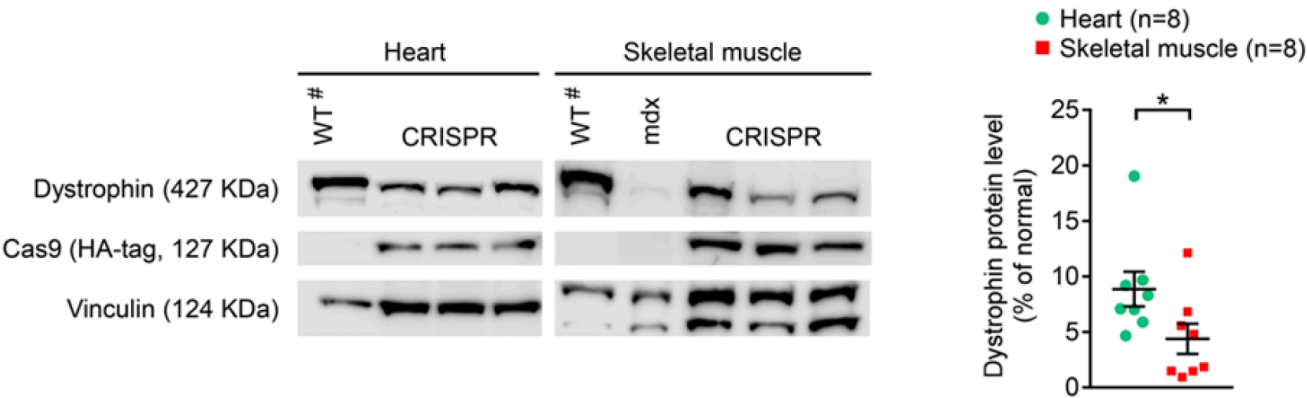


Figure 9. Examination of dystrophin expression by western blot. Systemic AAV-9 CRISPR therapy was performed in 6-week-old female *mdx* mice at the dose of 1×10^{13} vg /mouse and 3×10^{13} vg/mouse for the Cas9 and gRNA vectors, respectively. Mice were evaluated at 18 months of age. **Left**, Representative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

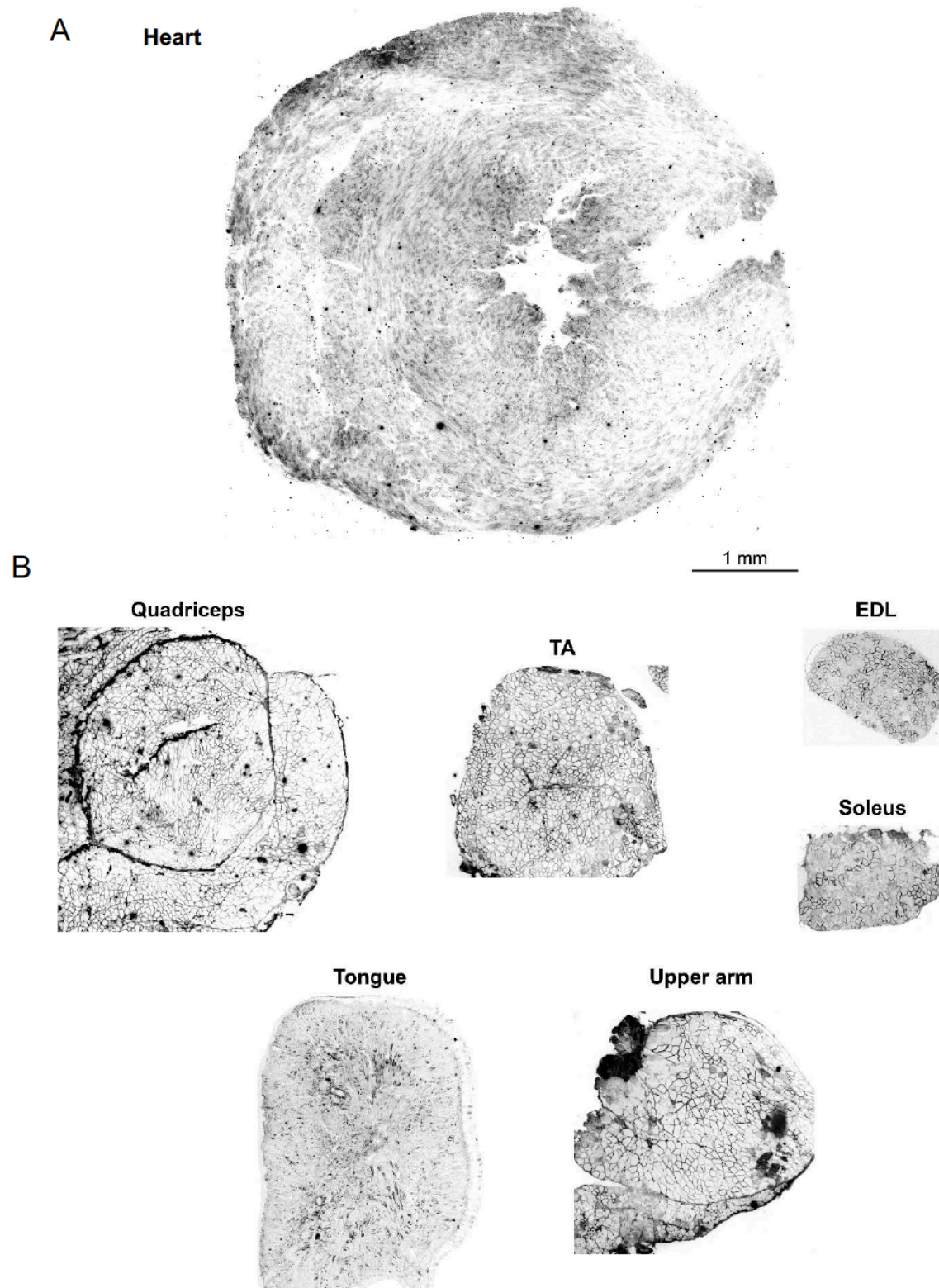


Figure 8. Representative dystrophin immunofluorescence staining in the new study. A, Representative full-view dystrophin staining images of the heart. **B,** Representative low-power dystrophin staining images from the quadriceps (Quadro), upper arm muscle, extensor digitorum longus (EDL), tibialis anterior (TA), soleus and tongue.

Figure 10 shows infra-red western blot comparison of dystrophin expression in the heart from the old study and the new study.

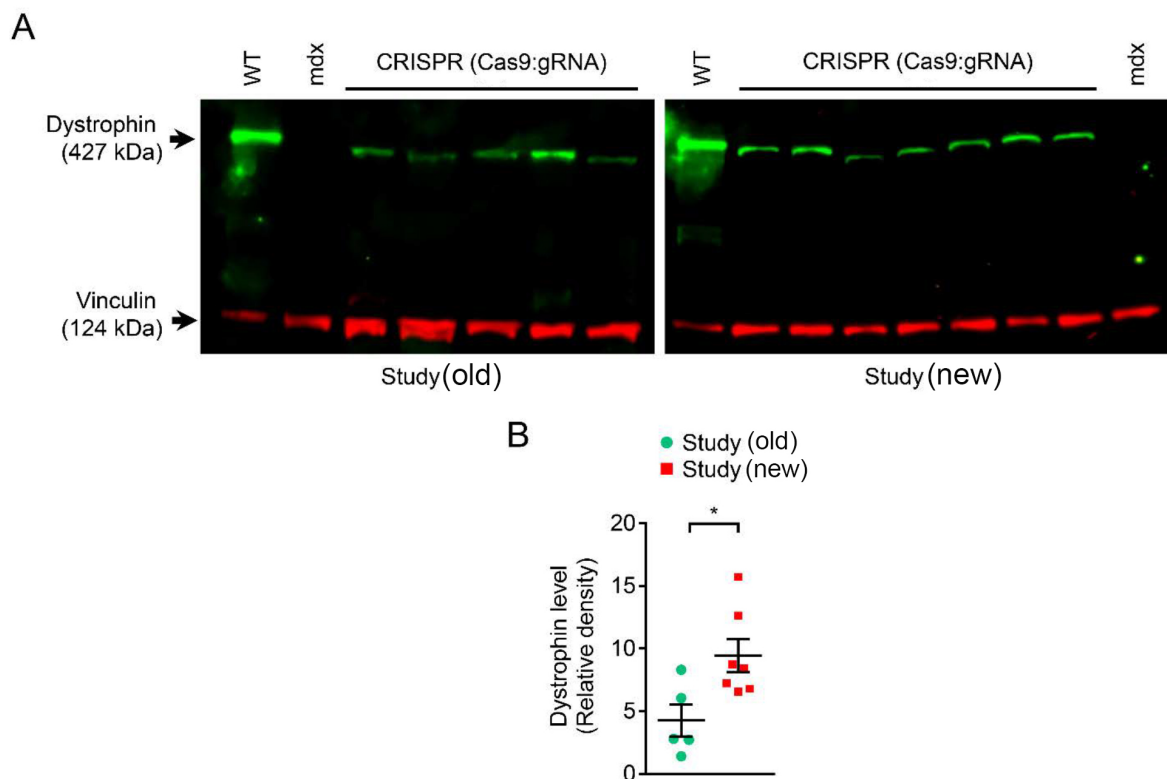


Figure 10. Quantification of dystrophin restoration in the heart by two-color western blot. To compare dystrophin expression between the old study and the new study, we performed LICOR two-color infrared fluorescence western blot using heart lysates collected at 18 months of age from the old study and the new study. **A**, Western blot images. Heart lysates from BL10 and mdx mice were included as positive and negative controls, respectively. The loading of the BL10 lane was one-fourth of the other lanes. N/A, no AAV injection. **B**, Western blot quantification results. Asterisk, statistically significant ($p < 0.05$).

Next, we quantified the amount of total dystrophin transcripts, edited and unedited dystrophin transcripts. **Figure 11** (next page) shows results. In our previous study, we only detected gene editing in the heart but not in skeletal muscle (**Figure 2**). Now, we detected edited transcripts in both heart and skeletal muscle. Notable, now 50% of the dystrophin transcripts were edited in skeletal muscle (**Figure 11, left panel**). 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 both heart ($p < 0.05$) and skeletal muscle (a trend was observed but did not reach statistical significance).

Dystrophin transcript quantification results (**Figure 11**) are consistent with dystrophin protein expression results (**Figures 7 to 10**). Together, these results suggest that increasing the quantity of the gRNA AAV vector at the time of dosing has successfully overcome the poor editing seen in our previous study.

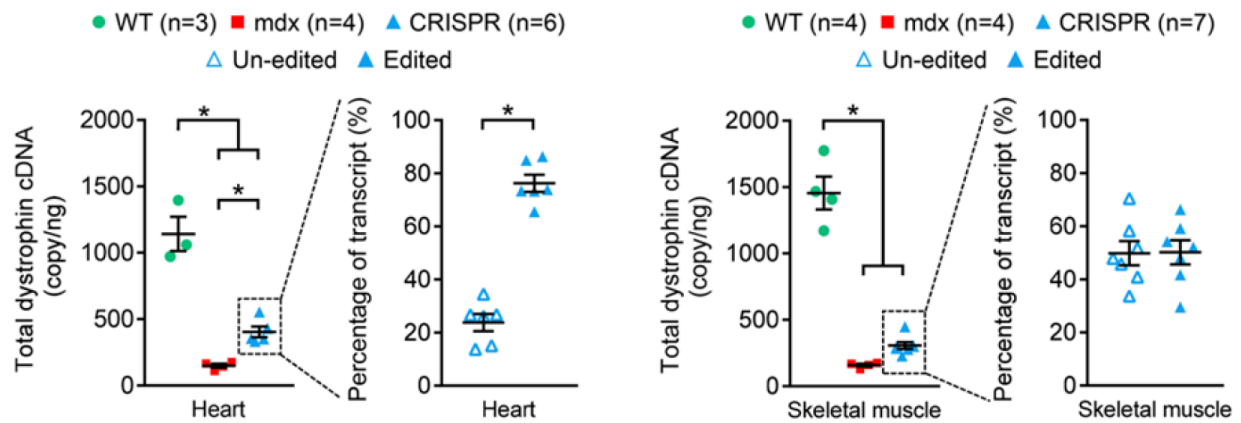


Figure 11. Quantification of dystrophin restoration in the heart by two-color western blot. To compare dystrophin expression between the old study and the new study, we performed LICOR two-color infrared fluorescence western blot using heart lysates collected at 18 months of age from the old study and the new study. **A**, Western blot images. Heart lysates from BL10 and mdx mice were included as positive and negative controls, respectively. The loading of the BL10 lane was one-fourth of the other lanes. N/A, no AAV injection. **B**, Western blot quantification results. Asterisk, statistically significant ($p<0.05$).

Vector dose optimization improved the balance of Cas9 and gRNA vector genome copy number in long-term systemic AAV CRISPR therapy in mdx mice.

In light of these encouraging results, we next examined Cas9 expression and AAV vector genome copy number. **Figure 12 shows** Cas9 expression at the protein and transcript level, Cas9 AAV vector genome copy number quantification in skeletal muscle and the heart.

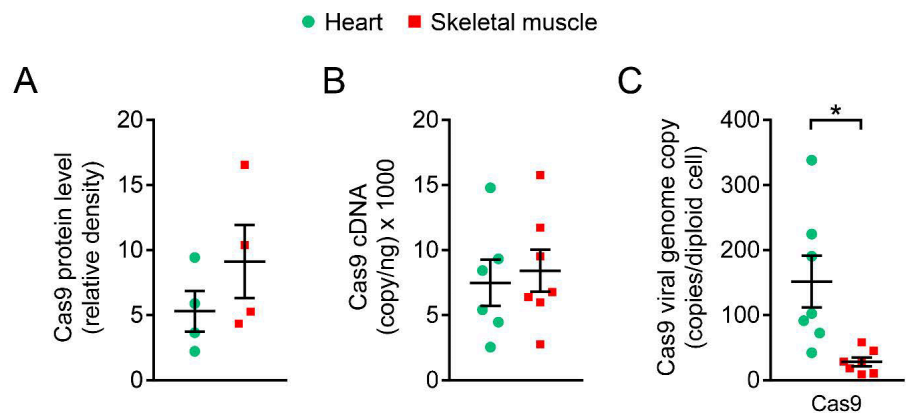


Figure 12. Evaluation of Cas9 protein, Cas9 transcript and Cas9 AAV genome copy number in the new study. The Cas9 protein was examined by quantitative western blot. The Cas9 cDNA copy number was determined by digital droplet PCR. The Cas9 AAV genome copy number was determined by TaqMan PCR. Asterisk, statistically significant ($p<0.05$).

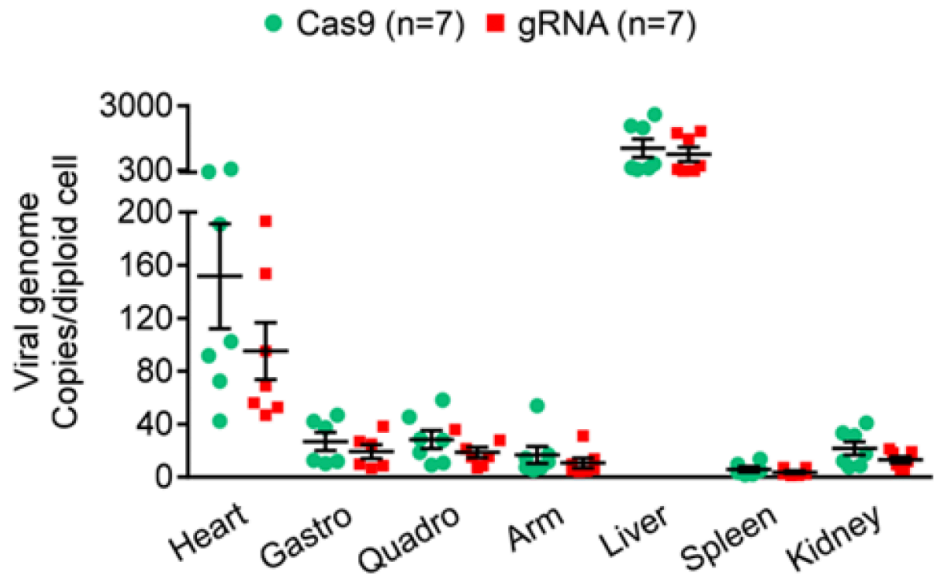
Consistent with the results of our initial study, we found a significantly higher amount of the Cas9 AAV vector genome copy number in the heart than that in skeletal muscle (**Figures 3 and 4**). Similarly, there was no difference in Cas9 expression between the heart and skeletal muscle. This

suggests that altering the gRNA AAV vector dosage has nominal impact on the stability and expression of the Cas9 AAV vector.

Next, we compared the Cas9 and the gRNA AAV vector genome copy number side by side. In contrast to what we saw in our initial study (**Figure 5**), we now see fairly comparable levels of the Cas9 AAV vector genome and the gRNA AAV vector genome in the harvested tissue (**Figure 13**).

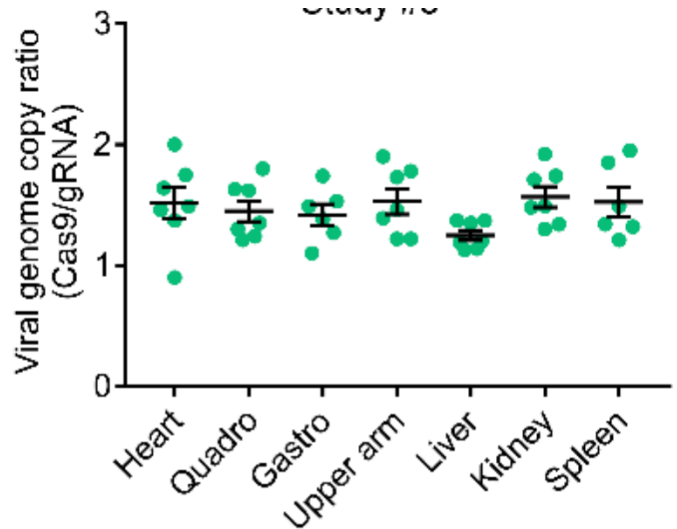
Figure 13. The Cas9 and gRNA AAV genome copy number quantification.

TaqMan PCR was performed on DNA extracted from indicated tissues. In all of them, the genome copy number of the Cas9 vector was similar to that of the gRNA vector..



Finally, we determined the relative ratio of the Cas9 and gRNA AAV vector genome (**Figure 14**). In sharp contrast to what we saw in our initial study (**Figure 6**). The ratio is now ~1.5.

Figure 14. 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.



In summary, we have successfully overcome the gRNA AAV vector depletion hurdle.

Vector optimization enabled significant improvement in heart/skeletal muscle histology and cardiac function without increasing off-target editing

The ultimate goal of the AAV CRISPR therapy is to ameliorate histopathology and improve function. In light of the improved dystrophin expression in both skeletal muscle and the heart, we examined histology and function. For functional study, we have to choose between skeletal muscle function and cardiac function since both assays are terminal. Only one type of the assay can be conducted in each individual mouse. In light of the importance of cardiomyopathy, we opted to focus

on cardiac function assay in these treated mice. However, we examined muscle fibrosis in both the heart and skeletal muscle. **Figure 15** shows skeletal muscle histology and fibrosis. **Figure 16** shows heart histology and fibrosis. In both heart and skeletal muscle, we detected reduction in fibrosis.

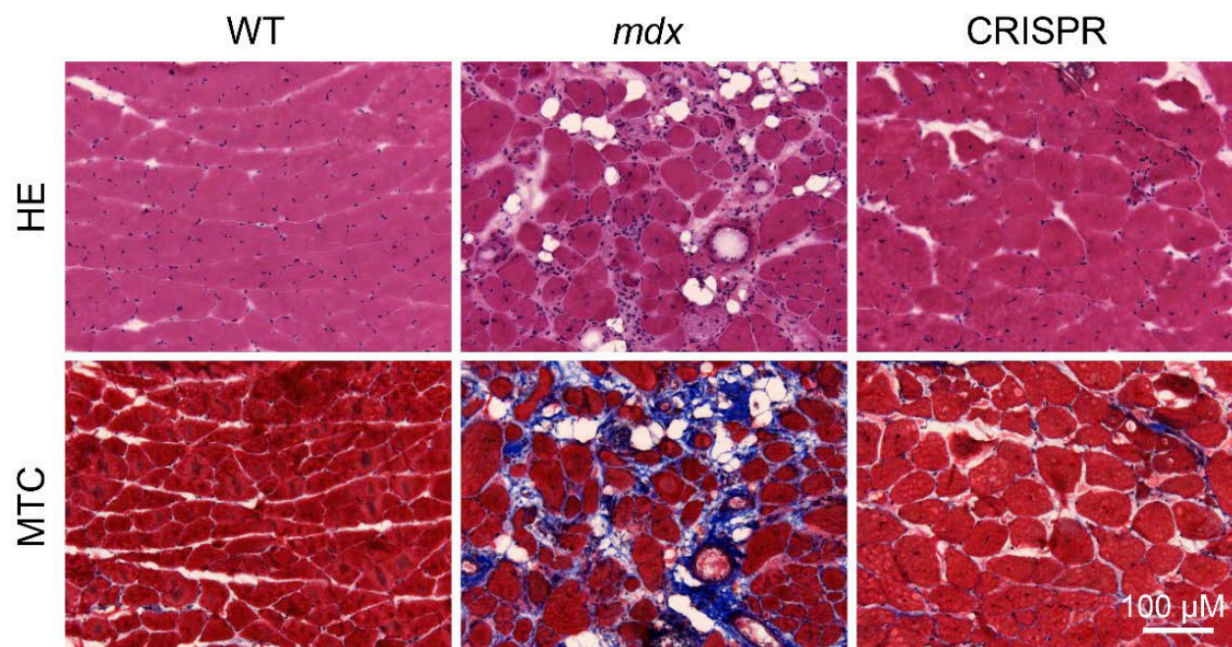


Figure 15. Evaluation of skeletal muscle histology and fibrosis in the new study. Representative high-power images of HE and MTC staining of the gastrocnemius from normal, untreated mdx and CRISPR treated mdx mice.

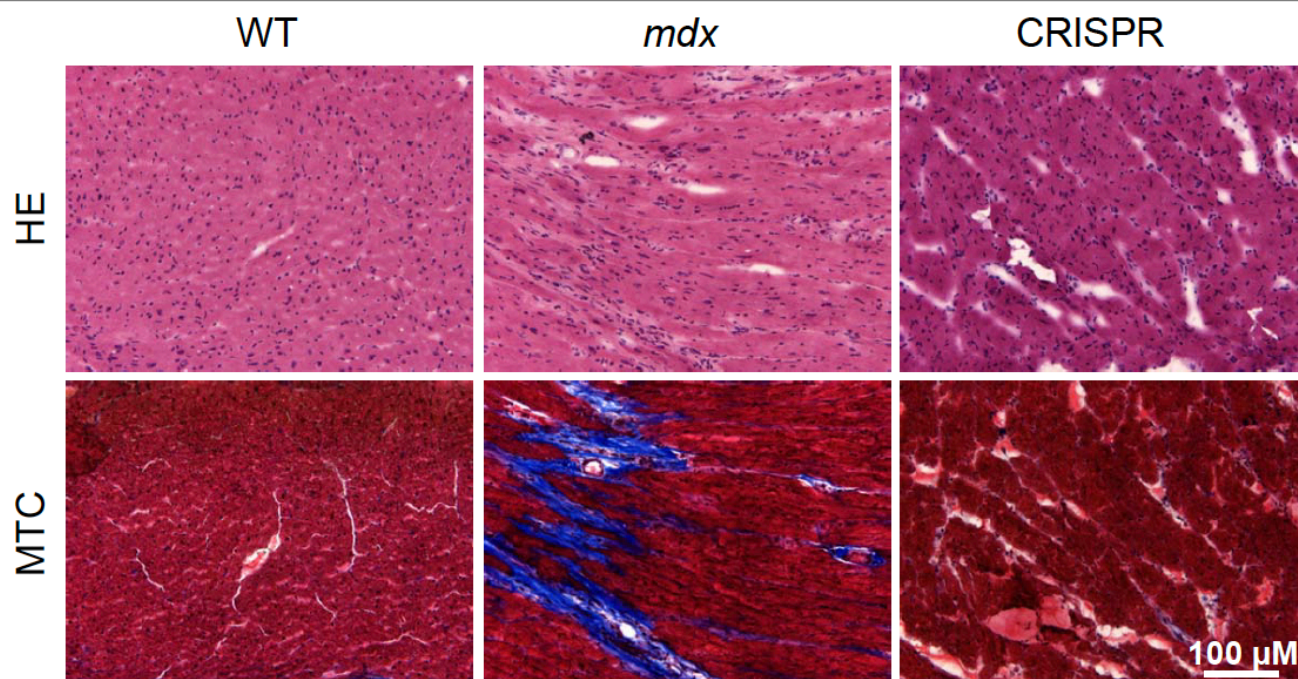


Figure 16. Evaluation of heart histology and fibrosis in the new study. Representative high-power images of HE and MTC staining of the heart from normal, untreated mdx and CRISPR treated mdx mice.

For functional study, we evaluated ECG and we also studied heart hemodynamics using the cardiac catheter assay. **Table 2** below shows ECG results. Improvement was noted in all parameters.

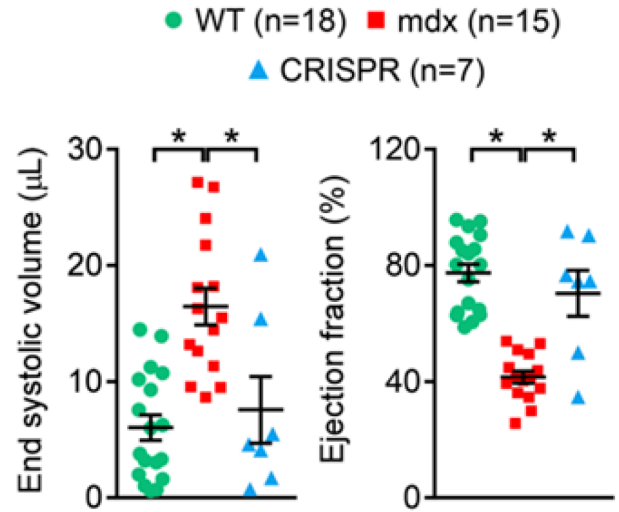
	WT	mdx	CRISPR
n	18	15	8
Age	18.36 ± 0.55	18.87 ± 0.48	18.28 ± 0.04
Heart rate (bpm)	557.74 ± 16.22	615.00 ± 14.78*	586.45 ± 9.05
PR Interval (ms)	40.96 ± 0.80	33.73 ± 0.80*	36.85 ± 1.13
QRS Duration (ms)	8.68 ± 0.23	10.17 ± 0.34*	9.54 ± 0.59
QTc (ms)	18.81 ± 0.57#	25.52 ± 1.14	22.2 ± 1.32
Cardiomyopathy Index	0.66 ± 0.04	1.08 ± 0.05*	0.89 ± 0.06
Q amplitude (μV)	-29.27 ± 5.58	-96.15 ± 18.18*	-47.25 ± 14.55

*, Significantly different from WT

#, Significantly different from mdx and CRISPR

Table 3 (below) and **Figure 17** show hemodynamic results. Statistically significant improvement was found in end-systolic volume and ejection fraction (**Figure 17**). A trend of improvement was seen in all other parameters (**Table 3**).

Figure 17. Quantitative evaluation of the end-systolic volume and ejection fraction by cardiac catheterization. *, Significantly different.



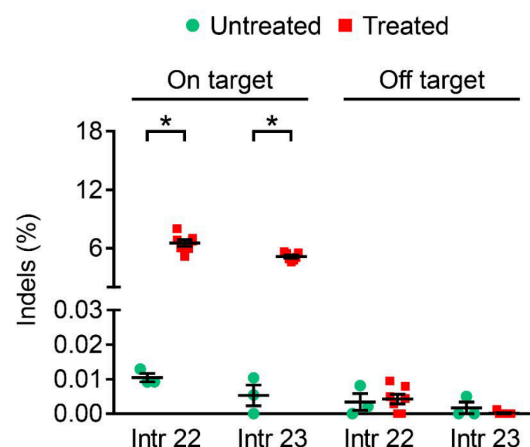
	WT	mdx	CRISPR
n	18	15	7
Age	17.99 ± 0.41	18.82 ± 0.49	18.29 ± 0.04
End diastolic volume (μL)	20.90 ± 1.72	23.27 ± 1.90	19.91 ± 2.90
Max pressure (mmHg)	101.73 ± 1.44 #	86.47 ± 5.00	91.50 ± 2.57
Stroke volume (μL)	16.96 ± 1.12	10.17 ± 0.61*	12.95 ± 1.12
Cardiac output (mL/min)	10218.58 ± 651.91	6002.17 ± 327.11*	7462.68 ± 725.59
dP/dt max (KmmHg /sec)	13571.17 ± 541.32	10402.07 ± 958.94*	11047.29 ± 1036.77
dP/dt min (KmmHg /sec)	-11863.11 ± 498.94	-9414.93 ± 821.74*	-11363.86 ± 894.92
Tau (ms)	7.48 ± 0.55	8.42 ± 0.36	6.87 ± 0.47

*, Significantly different from WT

#, Significantly different from mdx and CRISPR

Off-target cutting is a major safety concern for CRISPR therapy. To this end, 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 18**).

Figure 18. 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 ($p < 0.05$).



Long-term systemic AAV CRISPR therapy in mdx did not result in body weight reduction.

An important finding of our original study is a significant reduction of the body weight in CRISPR-treated mdx mice compared to untreated mice. This may represent a potential safety concern. However, we did not see apparent abnormality in histology of major internal organs. We also did not notice any abnormality in the behavior, food/water intake and activity of CRISPR treated mice. Further, we did not detect off-target editing by deep sequencing.

An alternative explanation to our finding is that body weight reduction we saw may represent a coincidence due to the small sample size ($n=5$) or the particular batch of the mice used in the study. To determine whether this is the case, we examined the bodyweight in the treated and untreated mice in our new study. **Table 4** (below) shows the results. As expected, the bodyweight of aged mdx mice was significantly lower than that of wild type control mice. Importantly, we found no difference in the body weight between untreated mdx mice and CRISPR-treated mdx mice ($n=8$) (**Table 4**). Hence, the original observation of bodyweight reduction was not repeated in the new study. Based on the new finding, we conclude that the long-term CRISPR therapy does not result in body weight loss. Together with the finding that there was no off-target editing (**Figure 18**), we conclude that long-term systemic CRISPR therapy is safe in mdx mice.

	WT	mdx	CRISPR
n	20	20	8
Age (m)	18.2 ± 0.44	18.74 ± 0.45	18.53 ± 0.07
BW (g)	30.2 ± 0.91 †	22.36 ± 0.39	22.43 ± 0.88

†, Significantly different from mdx and CRISPR

Additional accomplishments that have benefited from this grant.

Accomplishment benefit from this grant 1. In this study, we have proposed to use optical imaging technology to evaluate heart remodeling. During this funding period, we published one

research article on the development of an automatic approach to quantify heart damage in dystrophic mouse heart using the optical polarization tractography technology (Wang et al 2018).

Accomplishment benefit from this grant 2. During this funding period, we published a review article on using gene editing to treat neuromuscular diseases (Nelson and Gersbach, 2017).

Accomplishment benefit from this grant 3. Genomic changes induced by CRISPR editing has been a hot topic in the field of AAV CRISPR-mediated gene therapy. We studied the genomic editing outcome in mdx mice at one year after systemic delivery in neonatal mdx mice. Using unbiased unidirectional genomic DNA sequencing, we found indel formation, deletion, inversion and AAV genome integration at the target site. We presented our preliminary results in American Society of Gene and Cell Therapy 21st annual meeting (see Conference presentation #1).

Accomplishment benefit from this grant 4. An important question that has been asked on DMD CRISPR therapy is whether it can target satellite cells, the so called muscle stem cells. We evaluated this question in using the whole muscle graft model. Our results suggest that AAV-9 CRISPR delivery to mdx mice can indeed target myogenic stem cells. However, the efficiency is very low. We presented our preliminary results in American Society of Gene and Cell Therapy 21st annual meeting (see Conference presentation #2). We will wrap up the study in the next funding period and submit it for publication in a peer-reviewed journal.

Accomplishment benefit from this grant 5. Based on the results from years 1 and 2, we filed a patent application on systemic AAV CRISPR therapy. Please see invention section for detail.

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. In this funding period, we have identified disproportional depletion of the gRNA vector genome as the culprit causing poor dystrophin restoration in long-term systemic CRISPR therapy for DMD in mdx mice. However, the underlying mechanisms are unclear. For example, it is unclear whether gRNA AAV genome depletion is due to inflammatory dystrophic microenvironment in mdx muscle. To this end, we will perform intramuscular injection in mdx mice to see if we can observe gRNA AAV vector depletion inside mdx muscle.
2. In this funding period, we have developed a novel strategy to overcome the issue of poor CRISPR editing efficiency observed in our year 1 study. Using this strategy, we demonstrated improved gene editing efficiency in female mdx mice. We also showed improvement of cardiac function. In the next funding period, we will perform the corresponding study in male mdx mice to see if the improved CRISPR editing we saw in this funding period can be reproduced. In the male mdx study, we will focus on evaluating skeletal muscle function because we have previously shown male mdx mice have more severe muscle disease in skeletal muscle (Hakim CH, Duan D. *Gender differences in*

contractile and passive properties of mdx extensor digitorum longus muscle. Muscle & Nerve 45(2):250-256, 2012).

3. As stated in “***Accomplishment benefit from this grant 2***” (see section 3), we have also taken the opportunity and studied AAV CRISPR editing of muscle stem cells. We will wrap up the study and submit it for publication in a peer-reviewed journal.

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) Wang Y, Ravanfar M, Zhang K, **Duan D**, Yao G. *Automatic quantification of microscopic heart damage in a mouse model of Duchenne muscular dystrophy using optical polarization tractography.* **Journal of Biophotonics** 11(4):e201700284, **2018**.
- 2) Nelson CE, Robinson-Hamm JN, Gersbach CA. *Genome engineering: a new approach to gene therapy for neuromuscular disorders.* **Nature Review Neurology** 13:647-661, **2017**

6.3. Conference presentations

- 1) Christopher E. Nelson, Matthew Gemberling, Matthew L. Oliver, Chady H. Hakim. Ruth M. Castellanos Rivera, Aravind Asokan, Dongsheng Duan, Charles A. Gersbach. **Long-term Evaluation of Genome Editing Outcomes for Duchenne Muscular Dystrophy.** 2018 American Society of Gene and Cell Therapy 21st annual meeting. Chicago, IL, USA. May 16-19, 2018.
- 2) Michael E. Nance, Ruicheng Shi, Chady H. Hakim, Nalinda Wasala, Yongping Yue, Xiufang Pan, Tracy Zhang, Carolyn A. Robinson, Michael Kyba, N. Nora Yang, Shi-jie Chen, Kathryn R. Wagner, Charles A. Gersbach and Dongsheng Duan. **AAV-9 CRISPR mediated satellite cell editing restored dystrophin expression after complete degeneration in a xenograft model.** 2018 American Society of Gene and Cell Therapy 21st annual meeting. Chicago, IL, USA. May 16-19, 2018. **(outstanding poster award)**

- 3) **Duan D.** AAV therapy for Duchenne muscular dystrophy by gene replacement and gene editing. September 11, 2017 **McGill University**. Montreal, Canada. (Oral Presentation, no abstract available)
- 4) **Duan D.** Gene replacement and gene repair therapy for Duchenne muscular dystrophy. October 25, 2017 **University of British Columbia**. Vancouver, British Columbia, Canada. (Oral Presentation, no abstract available)
- 5) **Duan D.** CRISPR therapy for DMD in mdx mice. June 26 2018 Parent Project Muscular Dystrophy 2018 Annual Conference. Scottsdale, AZ. USA (Oral Presentation, no abstract available)
- 6) **Duan D.** CRISPR editing therapy for Duchenne muscular dystrophy. Aug 19, 2018 One Health Innovations-- One Health Applications of CRISPR/cas9 symposium. Kansas City, MO.

7. Participants/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

Contribution to project: Dr. Duan has overseen all aspects of the 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: 2.64 calendar months

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

Funding Support: Levo Therapeutics, Grant, Allen Foundation, R21NS103007, Sarepta Therapeutics

Name: Matthew Gemberling

Project Role: Postdoc

Researcher Identifier: N/A-Not Key

Nearest person month worked: 2.28 calendar months

Contribution to Project: Analysis of tissue DNA and RNA

Funding Support: R33DA041878, Levo Grant

Name: Christopher Nelson

Project Role: Postdoc

Researcher Identifier: N/A-Not Key

Nearest person month worked: 3.00 calendar months

Contribution to Project: Analysis of tissue DNA and RNA

Funding Support: Sarepta Therapeutics, as of 5/15/18 received NIH K99EB023979

Other Support – Progress Report YR 2
Duan, Dongsheng, PI

Previous Active Grants That Have Closed

A translational pathway towards a clinical trial using the second-generation AAV micro-dystrophin vector
10% effort, (5% effort during ncte), PI

Department of Defense (MD130014)

Wendy A. Baker, Grants Officer; USA Medical Research ACQ Activity; 820 Chandler Street; Fort Detrick, MD 21702

09/01/2014-12/31/2017 (extension)

The goal of this grant will explore an AAV-8 human micro-dystrophin gene vector in adult dystrophic dogs.

The specific aims are (1) to validate therapeutic efficacy of the human version of the $\Delta R2-15/\Delta R18-19/\Delta R20-23/\Delta C$ microgene AAV-8 vector in adult dystrophic dogs by local injection and (2) to explore bodywide AAV-8 gene therapy in young adult dystrophic dogs using the human version of the $\Delta R2-15/\Delta R18-19/\Delta R20-23/\Delta C$ microgene vector.

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

Evaluation of the human version second-generation AAV micro-dystrophin vector in adult dystrophic dogs 5% effort, PI

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

Rick Moss; Managing Director; 195 Dufferin Avenue; Suite #605; London, ON N6A 1K7 CANADA

7/1/14-12/31/2017 (extension)

Goal: We propose to generate the human version microgene vector and confirm its function in adult DMD dogs.

The specific aims are: (1) to engineer a codon-optimized second-generation human dystrophin microgene in a customer-optimized expression cassette and package it in an AAV-8 vector; (2) to validate the efficacy of the human version vector in dystrophin deficient mdx mice by systemic gene transfer; (3) to validate the efficacy of the human version vector in adult dystrophic dogs by local gene transfer; (4) to explore systemic gene therapy in young adult dystrophic dogs.

(This is a supplementary grant to the DOD grant awarded September 2014 that has been approved for extension to 12/31/2017).

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

R16/17-independent nNOS anchoring mechanism

10% effort, PI

NIH/NIAMS (R21 AR067985-01A1)

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

04/01/2016-03/31/2018

The goal is to identify the dog nNOS-binding domain and develop relevant gene delivery vectors.

The specific aims are (1) to identify the canine specific nNOS-binding domain in dog dystrophin and (2) to develop the nNOS-binding canine dystrophin adeno-associated virus (AAV) vector.

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

Fine-needle microscopic tractography for in vivo high-resolution imaging of muscle damage

2% effort, Co-PI (PI: Gang Yao)

University of Missouri, Interdisciplinary Pilot Studies in Translational Science and Biomedical Innovations

Debbie Taylor, MA204 Medical Sciences Building, University of Missouri

07/01/2017 to 06/30/2018

The goal and aim of this project is to develop a new microscopic imaging method for minimal invasive imaging of muscle damage.

(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

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

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

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

5% effort, PI

Solid Biosciences

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

06/01/2016-05/31/2019

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

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

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

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

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 NGA 4V3; CANADA

08/01/2017-07/31/2020

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)

New Active Grants

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

0% effort, PI

Ryan's Quest

David Schultz

PO Box 2544, Hamilton, NJ 08690

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

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

0% effort, PI

Ryan's Quest

David Schultz

PO Box 2544, Hamilton, NJ 08690

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

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

(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

10/01/2017-09/30/2019

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)

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

04/01/2018-03/31/2019

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

04/01/2018-03/31/2019

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

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)

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

12/07/2017-07/31/2018

(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)

Other Support – Progress Report YR 2
Gersbach, Charles A., Co-I

Previous Active Grants That Have Closed

Title: Engineering Morphogenetic Factors for Enhanced Genetic Reprogramming

Time commitments: 2 academic/0.25 summer months (18.7% calendar)

Supporting Agency: National Institutes of Health, 1DP2-OD008586 (Gersbach, PI)

Point of Contact:

Program Official (PO)

Basavappa, Ravi

301-435-7204

basavapr@mail.nih.gov

Performance Period: 9/30/11-6/30/16

Level of Funding:

Goals/Aims: To use protein engineering of transcription factors to increase the efficacy of genetic reprogramming of mammalian cells for regenerative medicine.

Overlap: None

Title: Photoregulated Gene Expression for Spatiotemporal Control of Morphogenesis

Time Commitments: 0

Supporting Agency: NSF Career Award, CBET-1151035 (Gersbach, PI)

Point of Contact:

Friedrich Srienc

Program Director

703.292.7029

fsrienc@nsf.gov

Performance Period: 4/1/12-3/31/17

Level of Funding:

Goals/Aims: To use light-inducible gene expression to spatially control the development of muscle and bone tissue in vitro.

Overlap: None

Title: Circulatory System and Integrated Muscle Tissue for Drug and Tissue Toxicity

Time Commitments: 0.5 academic months (or 4.1% calendar)

Supporting Agency: National Institutes of Health, 4UH3-TR000505-03 (Truskey, PI)

Point of Contact:

Long Nguyen

Grants Management Specialist

NIH-NCATS

nguyen1@mail.nih.gov

Performance Period: 7/24/12-6/30/17

Level of Funding:

Goals/Aims: To develop a circulatory system that consists of a high-pressure arterial system interfaced with various tissue microcirculatory test beds. A computer controlled pump will produce flow that mimics arterial flow.

Overlap: None

Title: Genetic Correction of Duchenne Muscular Dystrophy by Engineered Nucleases

Time Commitments: 0.25 academic/0.25 summer months (or 4.1% calendar)

Supporting Agency: Muscular Dystrophy Association, (Gersbach, PI)

Point of Contact:

Karen Smith
Grants Manager
520-529-2000
ksmith@mdausa.org

Performance Period: 8/1/13-7/31/16

Level of Funding:

Goals/Aims: To evaluate gene correction by artificial zinc finger nucleases and TALENs in cultured cells as a therapeutic approach to muscular dystrophy.

Overlap: None

Title: Genome Editing of Stem Cells for Analysis of Osteoarthritis Causal Variants

Time Commitments: 0.96 academic months (or 8% calendar)

Supporting Agency: NIH, 1R21AR065956 (Guilak and Gersbach, co-PI)

Point of Contact:

Program Official(PO)
Tyree, Bernadette
301-594-5032
tyreeb@mail.nih.gov

Performance Period: 4/1/14-3/31/16

Level of Funding:

Goals/Aims: To examine how genetic variation is linked to osteoarthritis by generating cartilage tissue from stem cells that have been modified to contain the gene variant of interest.

Overlap: None

Title: Targeted Gene Editing of Skeletal Muscle Progenitor Cells

Time Commitments: 0

Supporting Agency: Duke Coulter Translational Partnership, (Gersbach and Smith, Co-PIs)

Point of Contact:

Kristi D. Viles
Project Leader
919-668-8154
k.viles@duke.edu

Performance Period: 9/1/15-8/31/16

Level of Funding:

Goals/Aims: To evaluate gene delivery to muscle stem cells with widely-used vectors based on adeno-associated virus (AAV) and use molecular evolution strategies to develop novel AAV vectors with improved transduction of muscle stem cells.

Overlap: None

Title: Cell and Gene Modification Core

Time Commitments: 0.46 academic months (or 3.8% calendar)

Supporting Agency: NIH, 1P30AR066527(Li, PI)

Point of Contact:

Program Official(PO)

TSENG, HUNG H
tsengh@mail.nih.gov

Performance Period:

Goals/Aims: The main goal of the Cell and Gene Modification Core is to make available state-of-the-art gene and cellular modification techniques to the Duke SDRG membership.

Overlap: None

Title: Scaffold-Mediated Gene Delivery for Engineering of Osteochondral Tissues

Time Commitments: 0.74 academic/0.22 summer months (or 8% calendar)

Supporting Agency: NIH, 1R21AR067467 (Gersbach and Guilak, co-PIs)

Point of Contact:

Program Official(PO)

Wang, Fei

301-594-5055

wangf@mail.nih.gov

Performance Period: 7/1/15-6/30/17

Level of Funding:

Goals/Aims: To functionalize scaffolds with viral gene carriers in a spatially controlled manner for enhancing healing of osteochondral defects.

Title: Multiplex CRISPR/Cas9-Based Genome Engineering for the Genetic Correction of Duchenne Muscular Dystrophy

Time Commitments: 0.50 summer effort (or 4.1% calendar)

Supporting Agency: W81XWH-15-1-0469, USAMRMC MD140071 (Gersbach)

Point of Contact:

Marielena McGuire, PhD

Program Manager

301-619-8969

Marlena.v.mcguire.civ@mail.mil

Performance Period: 9/1/15-8/31/17

Level of Funding:

This proposal will focus on the correction of a mouse model of DMD (Duchenne Muscular Dystrophy with custom-engineered nucleases that treat this disease.

Title: Engineering Targeted Epigenetic Modifiers for Precise Control of Gene Regulation

Time Commitments: 1.28 academic/0.22 summer effort (or 12.5% calendar)

Supporting Agency: NIH, 1R01DA036865 (Gersbach and Crawford, co-PI)

Point of Contact:

Program Official(PO)

Satterlee, John S

satterleej@mail.nih.gov

410-360-4734

Performance Period: 9/15/13-5/31/18

Level of Funding:

Goals/Aims: In this proposal we will develop tools for precisely modifying genome structure that will catalyze innovative advances in gene and cell therapy, drug development, and basic science.

Overlap: None

Title: Human EGFRvIII-specific BiTE for the treatment of Glioblastoma

Time Commitments: 0.9 academic months/0.3 summer months (or 10% calendar); Effort ended on project

Supporting Agency: NIH, 1U01NS090284 (Sampson)

Point of Contact:

Jane W. Fountain

Program Officer

Phone: 301-496-1431

Email: fountai@ninds.nih.gov

Performance Period: 7/1/15-6/30/20

Level of Funding:

Goals/Aims: To perform Investigational New Drug (IND) application required toxicity, stability and pharmacokinetic analysis of human EGFRvIII-specific BiTE for the treatment of glioblastoma.

Overlap: None

Title: Engineering Novel Genome Engineering Systems

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

Supporting Agency: Locus Biosciences (Gersbach, PI)

Point of Contact:

Dave Ousterout, PhD

6845 Piershill Lane

Cary, NC 27519

Performance Period: 7/1/16-6/30/18

Level of Funding:

The purpose of this sponsored research agreement is to develop novel technologies for genome editing of mammalian cells.

Overlap—None

Title: Defining the Mechanisms of Gene Regulation and Drug Resistance in Cancer

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

Supporting Agency: Thorek Memorial Foundation (Gersbach)

Point of Contact:

Timothy Maeder, Executive Director

850 West Irving Park Road

Chicago, IL 60613

tmaeder@thorekfoundation.org

Performance Period: 2/1/17-1/31/18

Level of Funding:

In this project, we will use our new technology to complete the first genome-wide screen of endogenous regulatory contributions to cancer drug. We will use imatinib resistance in K562 cells as a model system. Imatinib is a tyrosine kinase inhibitor and first line treatment for chronic myelogenous leukemia

Overlap—None

Title: A Platform Technology for High-Thoroughput Screening of Gene Regulatory Elements

Time Commitments: 0.38 academic month (or 3.1% calendar)

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

Point of Contact:

John Oxaal, CEO

300 West Duke Street
Durham, NC 27701

Performance Period: 11/15/16-11/14/17

Level of Funding:

Goals/Aims: The goal of this project is to develop a platform technology to assign function to putative regulatory elements in high-throughput to identify the gene regulatory mechanisms responsible for cell phenotype and disease states.

Overlap: None

Current Research Support

Title: Decoding and Reprogramming the Corticosteroid Transcriptional Regulatory Network

Time Commitments: 1.44 academic/0.6 summer months (or 17% calendar)*

Supporting Agency: NIH, 1U01-HG007900-01 (Reddy)

Point of Contact:

Program Official (PO)

PAZIN, MICHAEL J

pazinm@mail.nih.gov

Performance Period: 7/1/14-11/30/17

Level of Funding:

Goals/Aims: To comprehensively map the genomic effects of treatment with common anti-inflammatory drugs.

Overlap: None

Title: Investigating Autophagy in GSD-1a

Time Commitments: 0.3 summer months (or 2.5% calendar)

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:

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: 0.6 academic/0 summer months (or 5% calendar)

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:

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: 0.57 academic/0.57 summer months (or 9.5% calendar)

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:

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 W81XWH-15-1-0469 funded work is supporting the proof-of-principle studies used in the R01AR069085 proposal as preliminary data.

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:

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: Epigenome Editing Technologies to Control Diverse Biological Functions

Time Commitments: .45 academic and 0.8 summer months (or 10.4% calendar)

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:

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: 0.57 academic 0.18 summer months (or 6.2% calendar)

Supporting Agency: 1UG3-TR002142-01- 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:

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 (or 2.1% calendar)

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

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: 9/1/16-8/31/19

Level of Funding:

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 W81XWH-15-1-0469 funded work is supporting the proof-of-principle studies used in the R01AR069085 proposal as preliminary data.

New Active Grants

Title: Regulatory Mechanisms of CD4+ T Cell Differentiation

Time Commitments: 0.9 academic 0.3 summer (10% calendar)

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:

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: 0.60 academic (5% calendar)

Supporting Agency: NIH 1R21NS103007-01A1 (Gersbach)

Point of Contact:

Robert D. Riddle, Program Official

301-402-3315

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

Level of Funding:

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: 0.55 academic (4.5% calendar)

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:

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: 0.50 academic (4% calendar)

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:

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

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

Time Commitments: 1.20 academic (10% calendar) (Dr. Gersbach's effort will not start until 9/1/18)

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

Other Support – Progress Report YR 2

Yao, Gang, Co-I

Previous/active grants that have closed

Title: Fine-needle microscopic tractography for in vivo high-resolution imaging of muscle damage

Level of Effort: 5% effort, PI

Funding Agency: University of Missouri, Interdisciplinary Pilot Studies in Translational Science and Biomedical Innovations

Debbie Taylor, MA204 Medical Sciences Building, University of Missouri

Performance Period: 07/01/2017 to 06/30/2018

Level of Funding:

Project Goals and Specific Aims: The goal of this project is to develop a new microscopic imaging method for minimal invasive imaging of muscle damage.

Overlap/complement with the current proposal: None

Current Research Support

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 06/30/2018 (no-cost extension to 06/30/19)

Level of Funding:

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

Overlap/complement with the current proposal: None

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

Level of Effort: 4% effort, Co-I

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: 06/01/2016-05/31/2019

Level of Funding:

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

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:

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

Overlap/complement with the current proposal: None

New Active Grants

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:

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

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

Funding Agency: Solid Biosciences

Project Goals and Specific Aims: The goal of this project is to determine the maximum gene therapy dosage in dog models of DMD.

Performance Period: 04/01/2018-03/31/2019

Level of Funding:

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

Overlap/complement with the current proposal: None

Organizations that have 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.

FULL ARTICLE

Automatic quantification of microscopic heart damage in a mouse model of Duchenne muscular dystrophy using optical polarization tractography

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¹Department of Bioengineering, University of Missouri, Columbia, Missouri

²Department of Molecular Microbiology & Immunology, University of Missouri, Columbia, Missouri

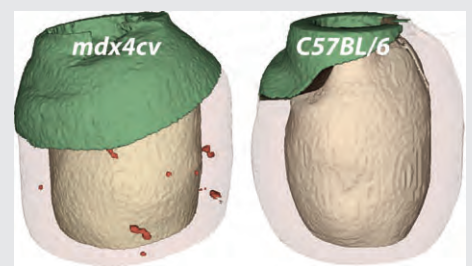
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Funding information

U.S. Department of Defense (US), Grant/Award number: MD150133; University of Missouri System Research Board

Quantification of microscopic myocardium damage in a diseased heart is important in studying disease progression and evaluating treatment outcome. However, it is challenging to use traditional histology and existing medical imaging modalities to quantify all microscopic damages in a small animal heart. Here, a method was developed for fast visualization and quantification of focal tissue damage in the mouse heart based on the fiber alignment index of the local myofiber organization obtained in optical polarization tractography (OPT). This method was tested in freshly excised hearts of the *mdx4cv* mouse, a commonly used mouse model for studying Duchenne cardiomyopathy. The hearts of age-matched C57BL/6 mice were also imaged as the normal controls. The results revealed a significant amount of damage in the *mdx4cv* hearts. Histology comparisons confirmed the damage identified by OPT. This fast and automatic method may greatly enhance preclinical studies in murine models of heart diseases.



KEYWORDS

fiber orientation, heart, optical coherence tomography, polarization, tractography

1 | INTRODUCTION

The mechanical force required for efficient blood pumping is produced by the highly coordinated contraction of cardiac muscles. The myocardial fibers form an intricate cross-helical architecture that plays a key role in optimizing the overall force generation [1–3]. Changes in the myocardial fiber organization have been linked to many cardiac diseases such as hypertrophic and dilated cardiomyopathy [4–6]. As a part of the “remodeling” process, structural changes in the heart often begin as a compensatory mechanism [7] to adapt for the degraded cardiac functions that arise due to disease-induced tissue damage. Many lesions start as focal structural alterations at the cellular level that

may appear as myocyte disarray, necrosis, and fibrosis [8]. These small focal lesions eventually lead to changes in global heart morphology. It is important to evaluate heart structural changes at both the microscopic and global levels to thoroughly understand the cardiac remodeling process during disease progression.

Quantification of microscopic myofiber disruption is required to assess the level and extent of the heart damage [9]. Histological evaluation is conventionally used to study myocardial damage. Although an automatic image analysis method has been developed [9], histological processing is still labor intensive and only practical when evaluating a limited number of locations. Because microscopic heart damages are known to be highly location-dependent [8], a

method that can quantify microscopic tissue damage in the entire heart would be valuable. Magnetic resonance imaging (MRI)-based diffusion tensor imaging has achieved great success in visualization of the global myocardial fiber architecture in the heart [10]. Diffusion MRI has also been applied in imaging myocardial fiber disarray in human [5] and animal models of heart diseases [11–13]. However, the spatial resolution in MRI is limited to sub millimeters, which makes it very challenging to reveal microscopic structural changes in the myocardium. A sufficient spatial resolution is especially important in imaging the hearts of small animals such as mice, which are the most commonly used species to study heart diseases [14, 15].

Optical coherence tomography (OCT) is a nondestructive optical imaging technique that can provide depth-resolved tissue images at high speed. High-resolution OCT has been applied successfully to image heart tissue [16] and visualize myofiber disarray in infarcted mouse hearts [17]. As a functional extension of OCT, polarization-sensitive OCT (PSOCT) [18] has been developed to image the optical polarization properties in tissue. Because cardiac muscles show strong intrinsic optical birefringence, PSOCT provides additional useful contrast in imaging heart tissues [19]. Recently, optical polarization tractography (OPT) was developed based on the Jones matrix implementation of PSOCT [20, 21]. Owing to its capability of calculating depth-resolved local fiber orientation, OPT has shown potential in visualization of fiber organization in multiple fibrous tissues including skeletal muscle, heart, artery and cartilage [22–26].

In this study, we developed an image processing method that can visualize and quantify microscopic heart damages using the fiber orientation images obtained in OPT. This method was applied to image the heart of the *mdx4cv* mouse, a widely used animal model for Duchenne muscular dystrophy (DMD). Duchenne cardiomyopathy often occurs in late-stage DMD patients and is the leading cause of mortality in DMD patients [27]. Because a point mutation in exon 53 of the dystrophin gene abolishes dystrophin expression, *mdx4cv* mice develop a progressive cardiomyopathy that shares many histological and functional features as in the human disease [28, 29]. We recently demonstrated the global structural remodeling that occurs in the heart of *mdx4cv* mice [26]. In this study, we explored the possibility of using OPT to evaluate microscopic heart damage in this model. We also verified the damage locations identified in OPT using standard histology.

2 | METHODS

2.1 | Samples and imaging procedure

Six 7-month-old *mdx4cv* mice (referred to as the “*mdx4cv*” group) and 6 age-matched normal C57BL/6 mice (referred to as the “BL6” group) were used in this study. All animal

experiments were approved by the Institutional Animal Care and Use committee and were in accordance with National Institutes of Health (NIH) guidelines. All experimental mice were generated in-house in a barrier facility using breeders purchased from the Jackson Laboratory (Bar Harbor, Maine). All mice were maintained in a specific-pathogen free animal care facility on a 12-hour light (25 lx):12-hour dark cycle with access to food and water ad libitum.

The hearts were excised, rinsed and imaged immediately after euthanasia. A single-camera spectral domain Jones matrix-based PSOCT system was used to image the hearts as described in detail previously [30, 31]. Briefly, an electro-optic modulator (EO-AM-NR-C1, Thorlabs Inc., Newton, New Jersey, USA) was used to create alternating right- and left-circularly polarized incident light at 50 kHz for Jones matrix imaging. For each incident polarization, the horizontal and vertical polarization components of the backscattered signal were acquired using a customized spectrometer equipped with a 1024-pixel linear scan CCD (AVIIVA SM2, e2v, Bievres, France). The image resolution was measured as 12.4 μm in the lateral direction and 5.9 μm in the depth direction.

Because the light cannot image through the entire heart, a rotational scanning procedure was applied to image from the epicardium surface of the entire heart as described in our previous studies [23, 26]. The excised heart was first mounted on a 20-gauge needle platform that passed through the heart apex and center of the base. The platform was then attached to a rotational stage with the mounting needle aligned along the rotational axis. During imaging, the incident light was scanned repetitively along the long axis of the heart (the B-scan) while the stage was rotated continuously over 360° at a speed of 1.25°/s. Three thousand six hundred B-scans were acquired at a speed of 12.5 B-scans/s to complete an entire heart scanning.

After optical imaging, the hearts were immediately frozen in liquid nitrogen-cooled 2-methylbutane in Tissue-Plus® optimal cutting temperature compound (Scigen Scientific, Gardena, California) for histological processing. Histological sections of 10 μm thickness were sectioned from the tissue block and stained with hematoxylin and eosin (HE). Dystrophin was detected using a monoclonal antibody (MANDYS8, Sigma, Saint Louis Missouri) and a second antibody (Alexa fluor 594 F(ab)2 fragment of goat antimouse IgG(H + L), Invitrogen, Rockford, Illinois). Histological images were acquired using a Nikon Eclipse E800 microscope equipped with a Leica DFC 7000 T camera.

2.2 | Image processing

The raw PSOCT dataset had a size of 1024 $\times$ 2000 $\times$ 3600 pixels along the A-, B- and C-scan directions and covered an imaging area of 5.2 $\times$ 8.0 mm $\times$ 360° (A $\times$ B $\times$ C). The C-scan dimension was represented in degrees because the actual scanning range at the heart surface depended on the heart diameter and its surface geometric shape. The

pixel-wise Jones matrix of the tissue sample was constructed from the image data acquired at 2 different incident polarization as described previously [21, 31]. The depth resolved fiber orientation (θ) was then calculated from the Jones matrix images using a recursive algorithm starting from the heart surface [32, 33]. The measured fiber orientation had a range of $[-90^\circ, 90^\circ]$ with the zero degree being aligned with the rotational direction of the stage (C-scan).

To examine the possible difference in intensity signal between the BL6 and *mdx4cv* hearts, the depth-resolved OCT intensity was fitted using an exponential decay function $\exp(\mu z)$ to obtain the signal attenuation coefficient μ (mm^{-1}) over the imaging depth (z) between 0.1 and 0.7 mm at each imaging location. Microscopic tissue damage leads to disarrayed or randomized fiber orientation [22]. To identify regions with disarrayed fiber orientation, the “fiber alignment index” (FAI) was computed as [24, 34]:

$$\text{FAI} = \sqrt{\langle \cos 2\theta \rangle^2 + \langle \sin 2\theta \rangle^2}, \quad (1)$$

where θ indicates the local fiber orientation obtained in OPT, and the symbol “ $\langle \rangle$ ” denotes the averaged value within a moving evaluation window. The FAI calculation was applied to the en face images of fiber orientation that were constructed by extracting pixels at each equal-transmural depth from the heart surface. To expedite the computation, the en face image was down-sampled by a ratio of 4 to 500×900 pixels with a corresponding pixel size of $16 \mu\text{m} \times 0.4^\circ$ in the B- and C-scan directions, respectively. A 7×7 moving evaluation window ($112 \mu\text{m} \times 2.8^\circ$ in B-scan $\times$ C-scan) was used within each en face plane. This FAI calculation relied on the same circular statistics as the “circular standard deviation” [9] that was previously applied to calculate fiber disarray in histology samples. However, FAI was computationally more efficient to calculate. FAI may have a value between 0 and 1 with a value of 1 indicating a homogeneous distribution of fiber orientation and a value of 0 suggesting a random distribution.

Because entropy is a standard method used to characterize randomness, the entropy of the fiber orientation image was also calculated for comparison. The fiber orientation images were converted to 8-bit gray scale images and the entropy was calculated as:

$$\text{Entropy} = - \sum_{i=1}^{256} P_i \cdot \log_2(P_i), \quad (2)$$

where P_i represents the histogram count at the i th gray scale level of the local fiber orientation image within the same moving evaluation window as used in the FAI calculation. A homogenous image would have a low entropy, whereas a high-entropy value suggests a more randomized orientation distribution.

The heart tissue has a sophisticated myocardial fiber organization. Organized fiber transitions exist in many

regions, especially at the junction between the 2 ventricles [35] and at the vicinity of major vessels [26]. Such transitional areas also have large angular variations that may result in a lower FAI or higher entropy value. To prevent these transitional areas from being identified as tissue damage, edge detection was applied to the fiber orientation images to identify such transitional regions. The fiber orientation images were first smoothed using a 5×5 pixel median filter followed with a 5×5 pixel mean filter to remove small fluctuations. The Sobel edge detector (3×3 pixels) was then applied with a segmentation threshold of 0.1. Finally, a binary dilation operation (3×3 pixels) was applied on all detected edge pixels to obtain a binary map of fiber transition. Such edge pixels were excluded from the final damage map. In addition, signal saturation occasionally happened during the rotational imaging procedure due to tissue surface glaring. Because saturated signals caused incorrect orientation calculation, regions with saturated signals were also excluded from the image analysis.

The aforementioned image processing procedure was applied to the entire depth-resolved en face images of fiber orientation obtained in OPT. To construct the 3D image of the heart damage, the heart surface was first identified using an intensity threshold-based segmentation method [36]. Next, all the image data were polar-transformed based on the corresponding rotation position in the C-scan as described in our previous studies [23, 26]. Once all damage locations were segmented, the overall damage ratio was quantified as the ratio of the total number of pixels of damaged area and total number of pixels of the entire heart image. The Kolmogorov-Smirnov test was applied to verify the normal distribution of the results. Student's t test or the nonparametric Mann-Whitney test was applied to compare the group differences between the BL6 and *mdx4cv* hearts. All image processing was implemented using Matlab. The 3D visualization was created using the 3DSlicer software (www.slicer.org).

3 | RESULTS AND DISCUSSIONS

Figure 1 shows representative dystrophin immunofluorescence staining of a BL6 heart and an *mdx4cv* heart as described in Section 2.1. In the high-resolution ($\times 20$) images, the existence of dystrophin at the sarcolemma is clearly visible for the BL6 heart; however, no dystrophin was observed in the *mdx4cv* heart. Because dystrophin plays a key role in maintaining the structure integrity in cardiac muscle, its absence makes myocardial fibers susceptible to damage [27]. Figure 1 also shows example cross-sectional images of intensity and fiber orientation obtained from the same hearts. The fiber orientation images revealed structural variations that were not discernable in the intensity results. In particular, the fiber orientation results were noticeably noisier in the *mdx4cv* heart.

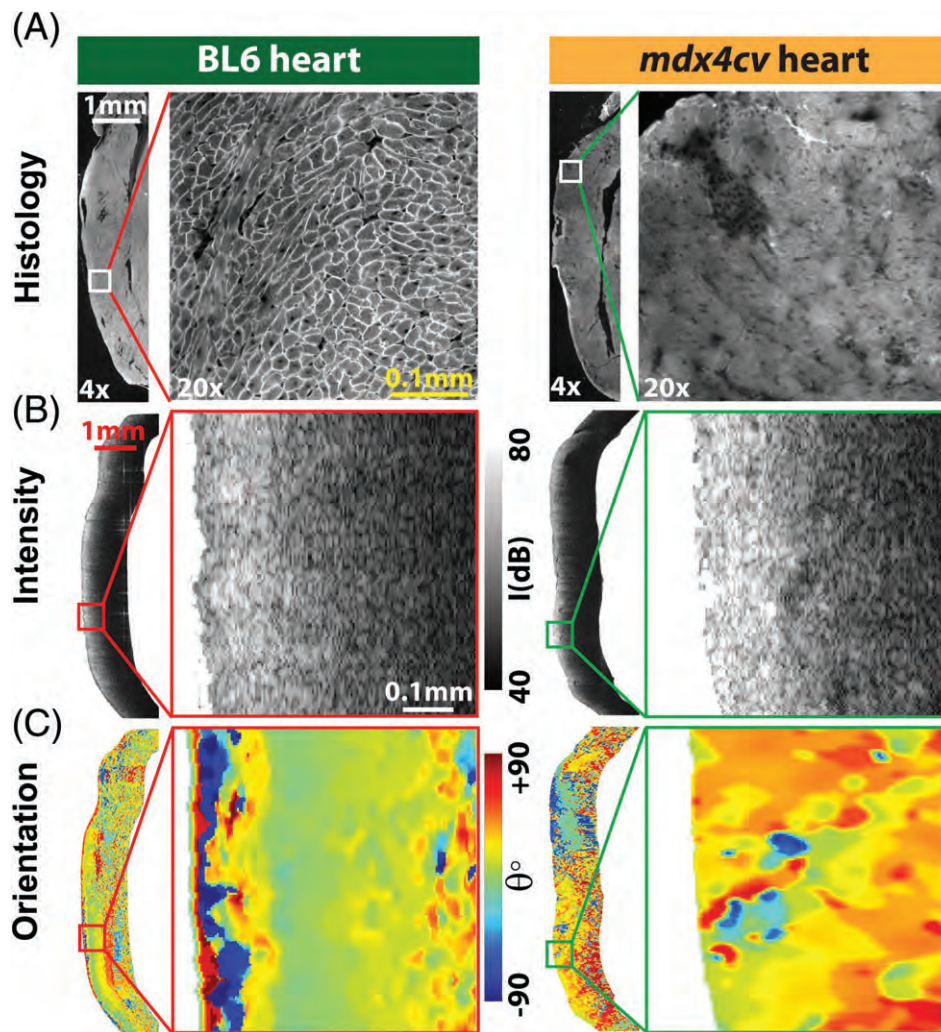


FIGURE 1 (A) Histological images of the dystrophin distribution in heart tissue using immunofluorescence staining as described in Section 2.1. The images of the global histology section were acquired using a $\times 4$ objective and the zoom-in images were acquired using a $\times 20$ objective. Also shown are cross-sectional images of (B) OCT intensity and (C) OPT fiber orientation from the same 2 hearts

Figure 2 shows the example of the proposed image processing in 3 representative samples with (a) homogeneous, (b) random and (c) transitional fiber distributions. The corresponding histograms of the quantitative image parameters were also shown by the side of each image. In the “homogeneous” sample, the fibers were oriented within a narrow range between -60° and -30° , whereas the “random” sample appeared to be noisy and had a wide orientation distribution between -90° and $+90^\circ$. The “transitional” sample showed a pattern where the fibers merged from both the left and the right at a junction located at the center. The corresponding fiber distribution showed 2 groups: one between -10° and $+10^\circ$ and the other between $+50^\circ$ and $+80^\circ$.

The signal intensity varied in the intensity images; but no clear pattern was observed in the regions with clearly random fibers. Similarly, the variation of the fitted attenuation coefficients did not seem to be damage-related. The attenuation coefficients in the center of the “homogeneous” sample appeared to be similar to those in the “random” sample. Each of the 2 diagonal half-regions in the “transitional” sample appeared to have homogeneous fiber orientations. However, they showed quite different attenuation coefficients that were also different from those in the

“homogeneous” sample. Overall, the attenuation coefficients in the 6 BL6 hearts were $-5.42 \pm 0.38 \text{ mm}^{-1}$, which was not significantly different from the $-5.21 \pm 0.37 \text{ mm}^{-1}$ value obtained in the 6 *mdx4cv* hearts ($P = .35$, Student’s *t* test).

Both the entropy and FAI differentiated the “homogeneous” sample from the “random” sample. The entropy values were 2.5 to 4.5 in the “homogeneous” sample, but greater (4.5–5.5) in the “random” sample. The FAI was greater than 0.9 in the “homogeneous” sample, but mostly less than 0.9 in the “random” sample. With a broader range of values, entropy appeared to resolve more details in the “homogeneous” sample. On the other hand, the FAI was able to resolve more details in the “random” sample. The color-coded FAI image revealed subregions with different degrees of randomness, which were not clear in the entropy image. For example, the central region of the “random” sample was significantly more random as shown in the FAI result, but not in the entropy result.

As expected, the entropy had a broad distribution (2.5–5.5) in the “transitional” sample. The corresponding histogram distribution seemed to be the combination of that obtained in the “homogeneous” and the “random” sample,

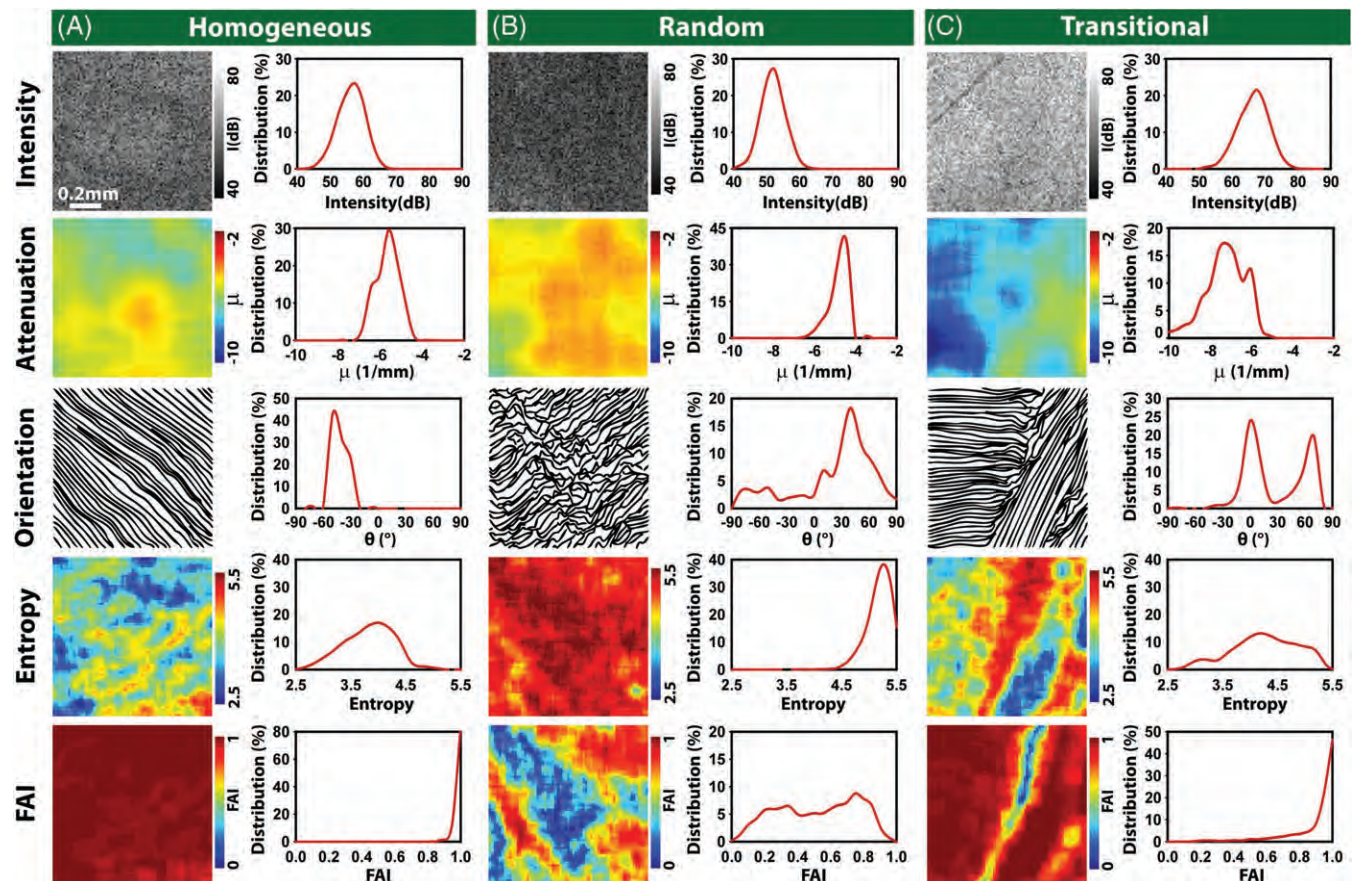


FIGURE 2 Examples of image processing in 3 representative cases of (A) homogeneous, (B) random and (C) transitional orientation distributions. For each case, the gray scale intensity images, pseudo-color images of intensity attenuation coefficient (μ), tractography, pseudo-color images of the computed entropy and FAI were shown. The corresponding histograms of each image were also shown

with the high-entropy portion coming from the transitional area. The same observation was made in the FAI results. However, the part of the random contribution was significantly less in FAI (low FAI) than entropy (high entropy). The pseudo-color FAI image only showed a narrow strip on the transitional boundary with a significantly low FAI, while the entropy was higher within a much larger region surrounding the transitional boundary. These observations confirmed that FAI had better performance in identifying regions with a greater variation in fiber orientation, and thus was better suited for the purpose of this study. It is also worth mentioning that the fiber orientation data did not generally follow the circular normal distribution (or the Von Mises distribution) in tissues with damage or transitional fiber orientation as it did in some other tissues [37].

Based on the results shown in Figure 2, an empirical cut-off threshold of $FAI_{TH} = 0.36$ was used to segment the sample into damaged ($FAI < 0.36$) and nondamaged ($FAI \geq 0.36$) regions. The FAI_{TH} images in Figure 3 showed the damaged areas in light gray and nondamaged background in black. The “homogeneous” sample showed no damaged areas (Figure 3A). The central diagonal and lower-left regions of the “random” sample were identified as damaged areas in the FAI_{TH} result (Figure 3B). The edge-detected image (ED_0) correctly identified some

boundaries inside the damaged areas. The truly damaged regions remained after removing the identified edges ($FAI_{TH} \notin ED_0$). In the “transitional” sample (Figure 3C), the transitional boundary was incorrectly identified as the damaged area in the FAI_{TH} result. However, this region with high FAI values coincided with the identified edges in the ED_0 image. Very few pixels were left after removing all the edge pixels from the FAI_{TH} result. To clean up these minor leftover pixels, an erosion-dilation operation (4-pixel in size) was applied to the final $FAI_{TH} \notin ED_0$ image in subsequent processing.

Figure 4 shows the comparison of applying the aforementioned processing to a BL6 heart and an *mdx4cv* heart. The images shown in (A) to (E) were example equal-transmural en face images extracted at $200 \mu m$ from the heart surface. The B-scans were along the vertical direction in these images, whereas the C-scans (360° rotation) were along the horizontal direction. While the ventricular areas showed organized fiber orientation, the atrial and aortal tissues showed very noisy fiber orientation data (Figure 4B). Such a difference was used to manually lineate the boundaries between the atria and ventricles. Several additional anatomical features in the intensity images (Figure 4A) were used to identify the left ventricle (LV) and right ventricle (RV) including the left atrium (LA), left cardiac vein (LCV)

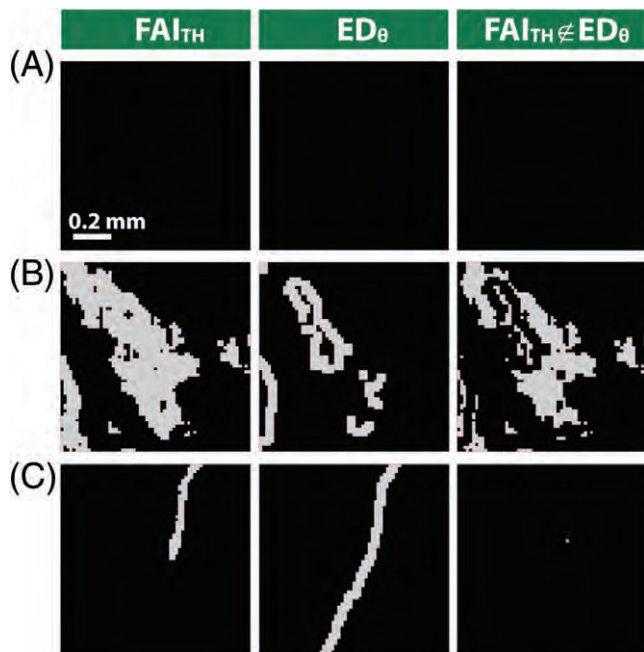


FIGURE 3 Examples of damage segmentation in 3 representative samples with (A) homogeneous, (B) random and (C) transitional orientation distributions as shown in Figure 2. FAI_{TH} represents the binarized image using a threshold of $FAI = 0.36$. ED_0 represents the edge-detected image of the raw fiber orientation image using a Sobel edge detector with a threshold of 0.1. The $FAI_{TH} \neq ED_0$ shows the segmented tissue damage after excluding all pixels identified in the ED_0 images

and right cardiac vein (RCV) as described previously [26, 38]. The boxes in the intensity images marked the region-of-interest (ROI, $2.4 \text{ mm} \times 70^\circ$ in B-scan $\times$ C-scan) of the LV and RV used for subsequent quantitative calculations. The ROI-LV was located below the left atrium and the ROI-RV was centered on the RCV. As shown in Figure 4B, significant fiber orientation transitions existed at the boundaries of the RV. These distinct features were used to delineate the RV as marked using the dashed lines in Figure 4C,E.

As shown in Figure 4D, the majority of the BL6 heart had high-FAI values, indicating a well-organized myofiber structure. The atrial and aortal tissues were removed from the subsequent FAI calculation because no clear fiber orientation was obtained from these tissues. The regions close to the major cardiac veins and at the junctions between the LV and RV appeared to have lower FAI values. These transitional regions were successfully identified using edge detection and excluded from the damage calculation. In general, the *mdx4cv* heart showed lower FAI values than the BL6 heart. In addition, the FAI values appeared to be lower in the RV of the *mdx4cv* heart than the LV, suggesting more damaged tissues in the RV. Using the same threshold of $FAI_{TH} = 0.36$, Figure 4E showed no damage in the BL6 heart; on the other hand, several “damaged” regions were identified in the *mdx4cv* heart. The majority of the damages were located in the RV. Figure 4F shows the 3D visualization of the overall tissue damage in the 2 hearts. These

damaged regions were computed from the epicardium until 0.5 mm in depth. The atrial and aortal tissues were shown in solid color. No clear damage was observed in the BL6 heart, whereas some scattered tissue damages were discernable in the *mdx4cv* heart.

Table 1 shows the calculated “damage ratio” in 6 BL6 hearts and 6 *mdx4cv* hearts. All the hearts were processed using the same procedures and parameters as described above. The damage ratio of the whole heart was calculated as the ratio of the number of damaged pixels and the total pixels in the entire heart. The results of the LV and RV were counted within the corresponding evaluation ROIs as shown in Figure 4A. All the results were obtained from the heart surface until 0.5 mm in depth. Overall, independent *t* test confirmed that the *mdx4cv* hearts had significantly greater damage ($t = 10.009$, $P = .000$) in the whole heart ($0.597\% \pm 0.145\%$) than the BL6 hearts ($0.004\% \pm 0.003\%$). No significant damage was identified in the LV and RV of the BL6 hearts. The Kolmogorov-Smirnov test indicated that the damage ratios in both the LV and RV of the *mdx4cv* hearts were significantly non-normal. The nonparametric Mann-Whitney test indicated that the damage ratio in the RV of the *mdx4cv* hearts were significant ($z = -3.077$, $P = .002$) in comparison with the results in the BL6 RV; while the damage ratios in the *mdx4cv* LVs did not reach statistical significance ($z = -2.286$, $P = .065$). Within the *mdx4cv* group, the Wilcoxon signed-rank test indicated that the damage was significantly higher in the RV than in the LV ($z = -1.99$, $P < .05$).

A detailed histological comparison was used to verify identified muscle damage in OPT (Figure 5). Four consecutive sections were acquired over a distance of $240 \mu\text{m}$ with a separation of $80 \mu\text{m}$. As shown in Figure 5, a small portion of the left atrium started to appear in the last histology section (Section 4), but was absent in the previous 3 sections. Therefore, the approximate locations of the histology sections were slightly below the left atrium. To register the OPT results with the histology sections, an initial section plane (Figure 4F) was first placed at this approximate location in the 3D OPT dataset. The 3D OPT data were then gradually rotated, tilted and translated. Meanwhile, the resulting damage images projected in the 2D section plane were examined and compared with the corresponding histology images. The optimal image registration was determined when the locations of tissue damage were best matched between the histology and OPT in all 4 successive sectioning planes.

Figure 5 shows the section-by-section comparison between OPT and histology. OPT identified 2 consistent damage areas at all 4 sectioning positions. Both areas were located in the RV with one area (location-1 in Sections 1-4) close to the heart surface and the other (location-2 in Sections 1-4) located at 250 to $300 \mu\text{m}$ beneath the epicardium. The histology images were also carefully examined to identify any pathological features. Although the histological sections experienced significant deformation during processing,

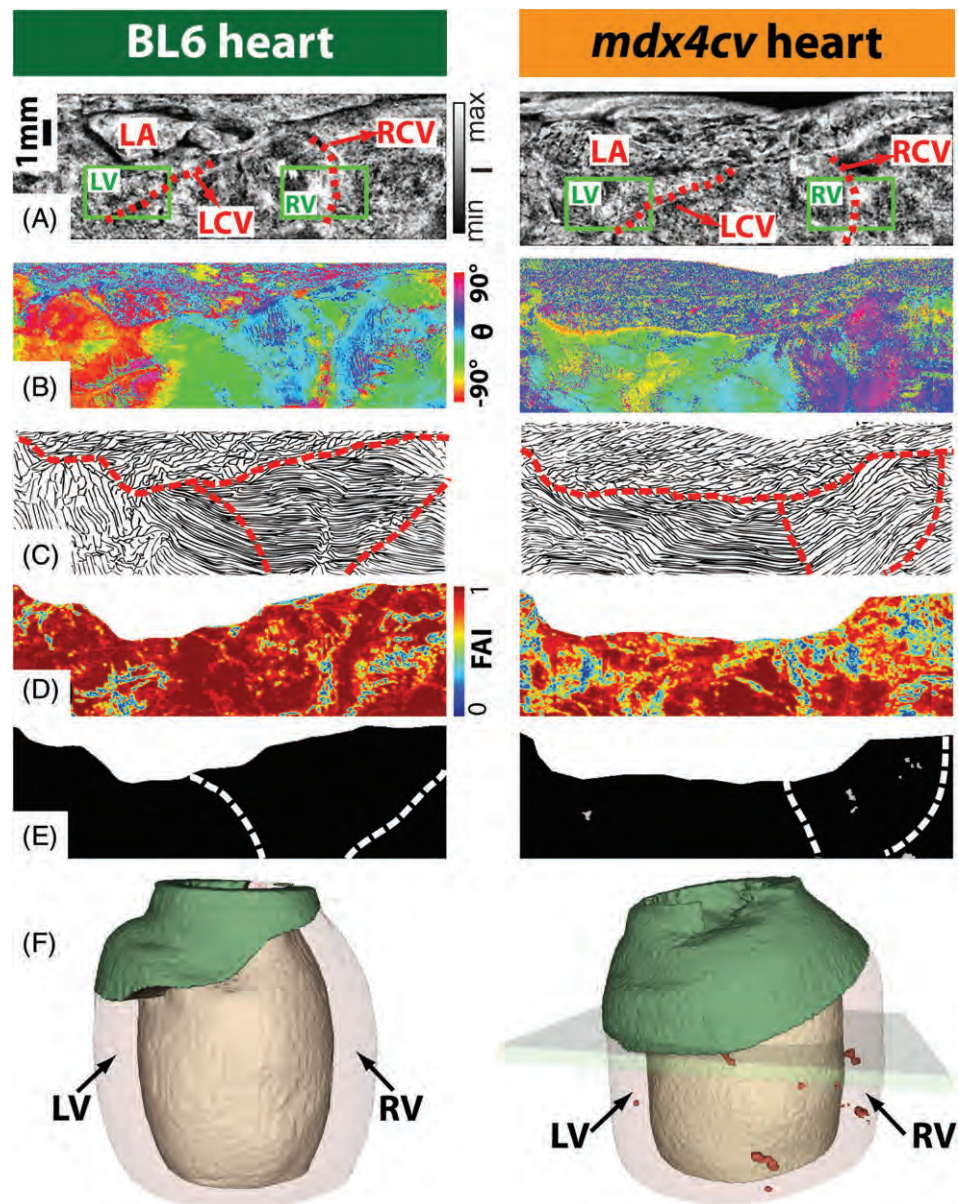


FIGURE 4 Examples of applying the image processing procedure to identify heart damage in a BL6 heart and an *mdx4cv* heart. Images shown are (A) intensity, (B) fiber orientation in pseudo-color, (C) tractography, (D) FAI, (E) segmented tissue damage (light gray) and (F) visualization of the tissue damage in the 3D heart. All images shown in (A) to (E) are *en face* results extracted at 200 μm from the heart surface. The dashed lines in (A) mark the locations of LCV and RCV. The boxes in (A) indicate the ROIs in the LV and RV for follow-up quantitative assessment. The dashed lines in (C) and (E) mark the boundaries of the RVs. The atrial and aorta tissues were removed from the fiber alignment and damage calculation in (D) and (E). The horizontal plane in the 3D image of the *mdx4cv* heart (F) marks the location of histology sections for validation

2 pathological areas (locations 1 and 2) appeared consistently at similar locations in all 4 sections. Both areas showed increased number of nuclei due to inflammatory cell infiltration and the existence of fibrosis. The damage location 1 was barely visible in Sections 1 and 2, but became more prominent in Sections 3 and 4, which was similar in both OPT and histology. In the last sectioning position, OPT identified another damaged area (location 3 in Section 4) at the junction of LV and RV. Histology

also revealed inflammation and fibrosis at a similar region. However, a close examination of the histology revealed an additional damaged area (location-3) in Section 2, which was not identified in OPT.

The finding of more damage in the RV was consistent with previous reports [39, 40]. Such dystrophic pathology has been linked to pulmonary arterial hypertension [41], which can be attributed to diaphragm degeneration [39]. Tissue damage-induced fiber disarray significantly impaired the integrity of the myocardial fiber organization. In fact, large variations occurred when analyzing the global myofiber structure in the RV of the *mdx4cv* heart [26]. In addition, both the histology and OPT results showed that the microscopic heart tissue damages were focal and had inhomogeneous distribution across the heart. It would be challenging to use conventional histology to fully evaluate and quantify such small and inhomogeneous damages unless multiple large sections were analyzed.

TABLE 1 Comparison of percentage of tissue damage in the BL6 and *mdx4cv* hearts

Mouse	ROI-LV (%)	ROI-RV (%)*	Whole (%)*
BL6	0.000 $\pm$ 0.000	0.000 $\pm$ 0.000	0.004 $\pm$ 0.003
<i>mdx4cv</i>	0.306 $\pm$ 0.550	1.843 $\pm$ 2.131	0.597 $\pm$ 0.145

The data shown were mean $\pm$ SD obtained from 6 BL6 hearts to 6 *mdx4cv* hearts.

* $P < .001$.

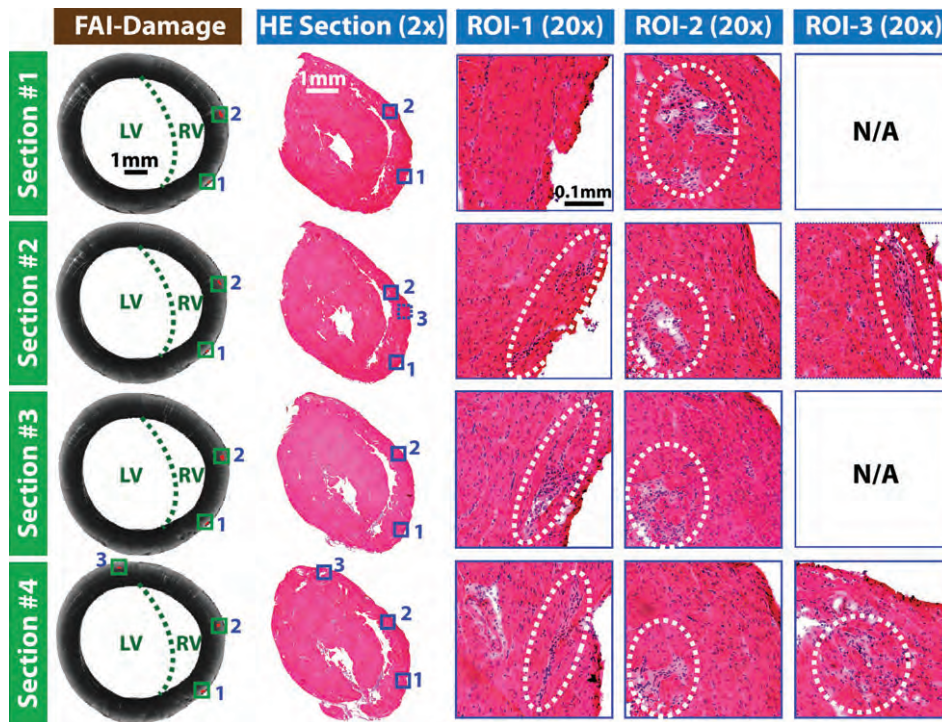


FIGURE 5 A comparison of the regions of muscle damages identified in OPT with histology (HE stain). Four successive histology sections were obtained with an 80 μm distance between 2 adjacent sections. Four OPT intensity images equally positioned over the same 240 μm distance were shown and overlaid with the damaged regions marked in boxes. The global histology images were acquired using a $\times 2$ objective. The histology images of the small ROIs were acquired using a $\times 20$ objective

Sufficient spatial resolution is also important to detect such a small damage.

An empirical fiber alignment threshold was used in this study, which needs to be validated by imaging more samples. Although the histological comparison confirmed most of the pathological features, some features (location 3 in Section 2) was not detected in the OPT results. Tissue deformation in histological processing may have contributed to the observed discrepancies. It was noted that the missed feature appeared to be limited to Section 2, suggesting a relatively smaller volume than the other features. It was likely that smaller pathological features were overlooked due to the large morphological operations used during image processing. A better imaging resolution with optimized imaging processing parameters may help to improve the detection sensitivity. In addition, Table 1 shows that OPT still detected some very small amount (0.004%) damages in the whole BL6 hearts. A close examination indicated that the majority of those identified damages in the BL6 hearts were located at the proximity of the atria, aorta, or the transitional regions. Therefore, it is likely that some minor false positive “damage” signals were not eliminated during image processing. Finally, the calculation of fiber alignment was susceptible to the image noise that became more significant at deeper depths. Although a longer imaging wavelength may be applied to improve the light penetration depth, the effective imaging depth in OPT is likely to be limited to 1 to 2 mm in most tissues as in any OCT based imaging methods. For deeper imaging in large samples, endoscopic implementations such as a needle probe [42] may be necessary.

4 | CONCLUSION

Here, we developed an image processing method that can automatically segment and quantify microscopic tissue damages in the heart. The proposed method identified tissue damage-related fiber disarray by analyzing the fiber alignment of the orientation images obtained in OPT. This method was applied to quantify microscopic tissue damages in the heart of *mdx4cv* mice, a common mouse model of DMD. The results revealed significant tissue damages in the *mdx4cv* heart that were not found in the age-matched C57BL/6 heart. The tissue damages identified in OPT were histologically confirmed. More histological validation is warranted to further investigate the sensitivity and specificity of this new technology. In comparison to conventional histology, OPT has the advantage of being fast and nondestructive. It can quickly visualize and quantify the extent of the tissue damage in the whole heart, which can be used to guide in-depth histological and biochemical analyses. In comparison to MRI-based imaging technology, OPT provides a more economical solution for routine laboratory application. OPT has much better resolution and thus can detect small tissue damage. Although its imaging depth is limited to 1-mm, it is well positioned for imaging the hearts of small animals such as mice.

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Genome engineering: a new approach to gene therapy for neuromuscular disorders

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Abstract | For many neuromuscular disorders, including Duchenne muscular dystrophy, spinal muscular atrophy and myotonic dystrophy, the genetic causes are well known. Gene therapy holds promise for the treatment of these monogenic neuromuscular diseases, and many such therapies have made substantial strides toward clinical translation. Recently, genome engineering tools, including targeted gene editing and gene regulation, have become available to correct the underlying genetic mutations that cause these diseases. In particular, meganucleases, zinc finger nucleases, TALENs, and the CRISPR–Cas9 system have been harnessed to make targeted and specific modifications to the genome. However, for most gene therapy applications, including genome engineering, gene delivery remains the primary hurdle to clinical translation. In preclinical models, genome engineering tools have been delivered via gene-modified cells or by non-viral or viral vectors to correct a diverse array of genetic diseases. In light of the positive results of these studies, genome engineering therapies are being enthusiastically explored for several genetic neuromuscular disorders. This Review summarizes the genome engineering strategies that are currently under preclinical evaluation for the treatment of degenerative neuromuscular disorders, with a focus on the molecular tools that show the greatest potential for clinical translation of these therapies.

Neuromuscular disorders impair motor function, and are often debilitating and prematurely fatal. The genetic causes of many of these conditions have been identified, and owing to advances from the genomic revolution in understanding gene structure and function, gene therapy strategies can now be designed to replace the mutated genes. Until recently, gene therapy relied on exogenous gene replacement. However, the advent of genome engineering technologies, such as gene editing, has enabled correction of the underlying mutations. Gene editing creates permanent genetic modifications in the native chromosomal context under the control of the gene's *cis*-acting regulatory elements, and might be preferable to exogenous gene delivery for many disease indications.

Genome engineering tools, including meganucleases¹, zinc finger nucleases (ZFNs)^{2,3}, transcription activator-like effector nucleases (TALENs)⁴, and the type II CRISPR system⁵, can be directed to make sequence-specific modifications in preselected genomic locations. Genome engineering tools are now being developed for clinical translation, and are entering clinical trials for various conditions⁶, including HIV, haemophilia and

sickle cell disease^{6–10}. In addition, substantial preclinical progress has been made in the adaptation of genome engineering for neuromuscular disorders.

This Review highlights recent advances in genome engineering technologies for the correction of neuromuscular conditions, with a focus on diseases for which published reports of these approaches are available, including muscular dystrophies, spinal muscular atrophy (SMA) and nucleotide repeat disorders. We discuss the challenges and prospects for clinical translation, and consider future directions for genome editing.

Genome engineering tools

As genome engineering tools can be targeted to any DNA sequence, researchers have begun to explore these tools for the potential treatment of genetic diseases. Current therapies that aim to correct the genetic deficiency in neuromuscular disorders include gene replacement, exon skipping and nonsense readthrough compounds, all of which have entered clinical trials^{11–17}. These technologies have been applied to various neuromuscular disorders, including Duchenne muscular dystrophy (DMD), SMA and myotonic dystrophy^{18–27}. A morpholino exon-skipping

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Key points

- Genetic neuromuscular conditions are debilitating and often prematurely fatal, and few standard treatment options are available
- Genome engineering provides a viable method of correcting the underlying genetic mutations in neuromuscular diseases
- A recent discovery, the highly adaptable CRISPR–Cas9 system, is already finding widespread use for cell therapy and *in vivo* gene editing
- Ongoing challenges include efficient gene delivery, identification and reduction of off-target interactions, and immunogenicity of genome engineering tools, delivery vectors and other neoantigens

Exon skipping

A technique in which antisense oligonucleotides are introduced that bind motifs within an mRNA, promoting alternative splicing and excluding the targeted exon from the final transcript.

Nonsense readthrough compounds

Small molecules that suppress stop codon recognition and allow ribosomes to translate an mRNA, despite nonsense mutations.

Morpholino

A non-ionic synthetic nucleic acid with DNA bases covalently attached to morpholine rings with a phosphorodiamidate backbone.

Homology-directed repair (HDR). A precise method of DNA repair that uses the sister chromosome or a provided DNA template to restore the damaged genome or introduce new DNA sequences.

Safe-harbour locus

A genomic region where targeted integrations can be performed without disrupting endogenous gene activity or negatively affecting cell function.

Non-homologous end joining

(NHEJ). A DNA repair pathway that religates double-strand breaks, with the possibility of producing small insertions or deletions that might lead to gene knockout.

Directed evolution

A process that uses multiple rounds of mutagenesis and user-defined selection to alter the function of a protein or nucleic acid.

compound, eteplirsen, has received FDA approval for the treatment of DMD. Recently, molecular tools have become available that correct the underlying chromosomal abnormality, and might be preferable to the existing gene therapy approaches.

Editing the genome. The ability to create site-specific double-strand breaks (DSBs) (FIG. 1a) with targeted nucleases allows the introduction of various sequence modifications to, theoretically, any gene. Homologous recombination mediated by oligonucleotides was the first method that allowed site-specific genetic modifications^{28,29}, but at relatively low frequencies. Site-specific DSBs enhance recombination by several orders of magnitude³⁰. In the presence of a donor template, homology-directed repair (HDR) can be used to insert designed sequences and repair mutated genes³¹ (FIG. 1b). For example, DSBs in a so-called safe-harbour locus, such as ROSA26 (REFS 32, 33) or AAVS1 (REFS 34, 35), facilitates targeted integration of replacement genes that are provided with homology arms corresponding to the safe-harbour sequence.

Without a donor template, DSBs can resolve through non-homologous end joining (NHEJ), traditionally considered to be an error-prone pathway that often produces insertions or deletions (indels) in the repaired cut site (FIG. 1c). Indel formation in the coding region can be used to knock out a gene in order to block a particular pathway or to reverse toxic gain-of-function mutations. Through the simultaneous generation of two DSBs, larger regions of genomic DNA can be deleted^{36,37} — an approach that can be used to correct the reading frame of a gene or remove nonsense mutations in non-essential regions of a gene.

Site-specific nucleases. Characterization of the I-SceI meganuclease³⁸ marked the first discovery of endonucleases with sufficient target site specificity to address unique sites in eukaryotic genomes (FIG. 2a). Meganucleases are a family of enzymes that target relatively large (14–40 bp) DNA sequences³⁹. The DNA recognition and endonuclease activities reside within the same protein motif, making re-engineering of DNA specificity a challenge. However, the site specificity of these proteins can be altered via computational design, site-directed mutagenesis, and directed evolution^{40–43}.

The DNA-binding motif of the Cys2–His2 zinc finger domain was engineered as a sequence-specific DNA-targeting tool with 3–4 bp recognition by each

domain^{3,44,45}. Through the development of arrays of zinc finger domains, DNA specificity was extended to up to 18 bp of DNA, with almost every triplet available for recognition^{3,46} (FIG. 2b). Fusions of zinc fingers to other protein domains created programmable nucleases (zinc finger nucleases, or ZFNs)⁴⁷, transcription factors⁴⁸ and recombinases^{49,50}. An important consideration for ZFN design is that the DNA recognition and nuclease activity are encoded by discrete units, unlike in meganucleases. Challenges in protein engineering of new zinc finger arrays include design of the linkage between each zinc finger, and the difficulty of targeting any desired sequence, due in part to crosstalk between adjacent fingers, which creates unpredictable context dependency.

Organisms from the plant pathogen genus *Xanthomonas* encode a class of DNA recognition proteins known as transcription activator-like effectors (TALEs)^{51,52} (FIG. 2c). The basic subunit, a repeat variable diresidue, recognizes a single nucleotide^{53,54}, and can be assembled in arrays to target specific DNA sequences^{55–57}. The earlier development of ZFNs prepared investigators for rapid adaptation of TALEs as nucleases (TALENs)⁵⁸, transcription factors⁵⁹, recombinases⁶⁰ and other fusions. Compared with ZFNs, TALEs are easier to design to target new sequences: no re-engineering of linkages between repeats is necessary, as there seem to be significantly fewer interactions between domains. However, TALENs present a different challenge of cloning and maintaining highly repetitive TALE arrays. New molecular cloning strategies, including Golden Gate assembly, have addressed some of these challenges⁶¹.

The discovery of a bacterial adaptive immune system, the CRISPR–Cas system^{62,63}, has made genome engineering more accessible than ever before⁶⁴. The CRISPR–Cas9 systems uses a DNA-targeting mechanism that is programmed through Watson–Crick RNA–DNA pairing rather than through protein–DNA interactions. A single guide RNA (gRNA) is required to direct the Cas9 nuclease to the desired DNA sequence^{65–68} (FIG. 2d). The only design limitation is the presence of a protospacer adjacent motif (PAM) sequence recognized by Cas9. The PAM sequence varies depending on the species of origin, but the PAM for the most commonly used Cas9, derived from *Streptococcus pyogenes*, is 5′-NGG-3′. Following the path of the earlier genome engineering technologies, Cas9 was rapidly developed as a targeted nuclease, transcriptional activator, transcriptional repressor and epigenome modifier^{5,64}. The use of a single gRNA to target Cas9 to the genome makes the CRISPR–Cas9 system easily adaptable. Genetic targets can now be screened on a scale that was previously impossible, with genome-wide libraries of gRNAs being assembled for functional screens^{69–72}.

Gene regulation. Zinc finger proteins (ZFPs), TALEs and catalytically deactivated Cas9 (dCas9) have also been adapted with effector molecules to enhance or repress transcription and modify epigenetic states⁷³. A tetramer of the herpes simplex virus VP16 activation domain, known as VP64, is one of the most commonly

used transcriptional activator domains, and has been attached to ZFPs⁷⁴, TALEs⁵⁹ and dCas9 (REFS 75–77) in various studies (FIG. 3). To repress transcription, the KRAB heterochromatin-forming domain has been fused to ZFPs⁷⁴, TALEs⁷⁸ and dCas9 (REFS 75, 79) (FIG. 3). Effector molecules that modify epigenetic states have also been investigated, most notably DNA methyltransferases⁸⁰, the TET1 methylcytosine dioxygenase⁸¹, and acetyltransferases⁸² (reviewed in detail elsewhere⁷³).

Rapid generation of animal models of neuromuscular disease. Genome engineering can also be used to produce animal models of neuromuscular disease. Rapid one-step generation of novel mouse and rat models⁸³ or other larger animal models of disease can be accomplished via the CRISPR platform. As reported in 2016, a new pig model of DMD was generated by targeted disruption of dystrophin (*DMD*) exon 29 in porcine zygotes⁸⁴, and a similar approach has been used to create a rhesus monkey model of this disease⁸⁵. Targeted gene knockouts and knock-ins can model loss-of-function and gain-of-function mutations. In addition, regions of animal model genomes can be humanized to enable preclinical evaluation of human-targeted genome engineering therapies.

Delivery methods

Cell therapy. Repopulation of skeletal muscle with myoblasts has been proposed as a potential treatment for severe neuromuscular disorders⁸⁶. Owing to the challenge of obtaining sufficient numbers of human myoblasts, muscle progenitors have been derived from induced pluripotent stem cells (iPSCs) through the use of conditional *PAX7* expression⁸⁷. Engraftment of these cells into damaged muscles has been shown to improve muscle contractility in *mdx* mice, a standard animal model of DMD⁸⁷. Autologous cell therapy is preferred as it removes the immune rejection barrier of allogeneic cell sources, although gene editing might be used to create universal donor cells by eliminating MHC expression⁸⁸. By means of genome engineering, patient-derived cells such as iPSCs can be genetically corrected, then used to repopulate dystrophic muscle and improve the muscle phenotype^{89–91}.

Hurdles to cell therapy include the difficulty of obtaining sufficient number of cells to engraft in all affected tissues, migrate from the injection site, and both repair muscle fibres and reconstitute the self-renewing progenitor cell pool. Moreover, some tissues are not amenable to the invasive procedure. For example, to improve patient outcomes in DMD, improvements in cardiac function are required in addition to repopulation of skeletal muscle.

Viral gene delivery. Systemic administration of gene editing technologies is probably the most comprehensive method for correcting mutations in the skeletal muscle, cardiac muscle and nervous system with a single administration. Viral vectors are currently the most likely candidates for systemic gene delivery or highly efficient delivery to cultured patient cells⁹². Adeno-associated

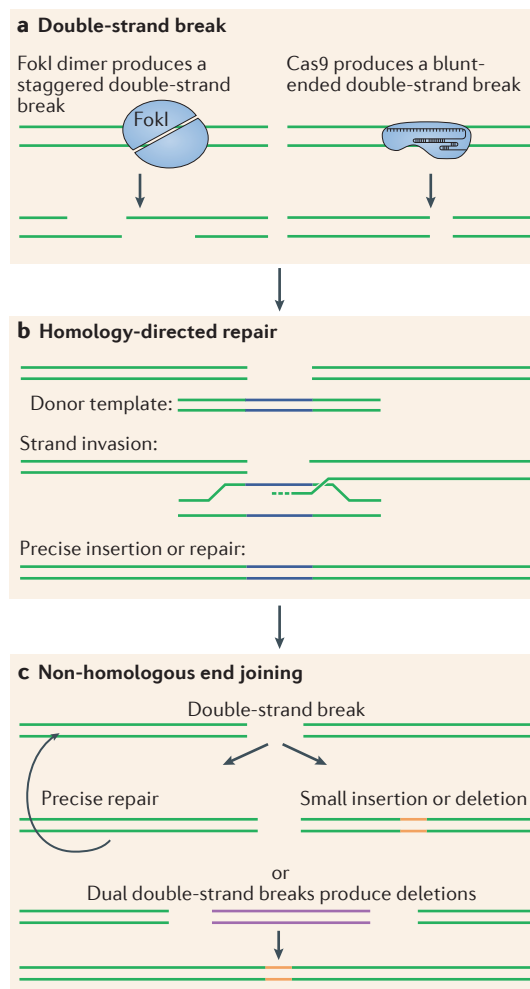


Figure 1 | Strategies for genome editing. **a** | Targeted nucleases generate double-strand breaks within the genome. FokI and meganucleases produce staggered breaks, whereas Cas9 produces blunt-ended breaks. **b** | Following introduction of a donor template with arms homologous to the region of interest, endogenous repair mechanisms can drive homology-directed repair for site-specific gene repair or safe-harbour integration (insert shown in blue). **c** | Through non-homologous end joining in the absence of a donor template, targeted nucleases can generate small insertions or deletions (also known as indels; orange shading) in the target site to disrupt a gene, or dual nucleases can generate targeted deletions of genomic regions (purple) to remove non-essential exons or mutated regions of a gene.

Cys2–His2 zinc finger domain

A finger-like structure of ~30 amino acids that recognizes three or four base pairs. Proteins containing multiple Cys2–His2 zinc finger domains can be used for sequence-specific DNA targeting.

Golden Gate assembly

A cloning method that allows scarless ligation of multiple DNA fragments and is well-suited for cloning repetitive sequences, including TALE arrays.

Protospacer adjacent motif (PAM)

A sequence motif that is recognized by the Cas9 enzyme. Several CRISPR systems have been described with varying PAM requirements.

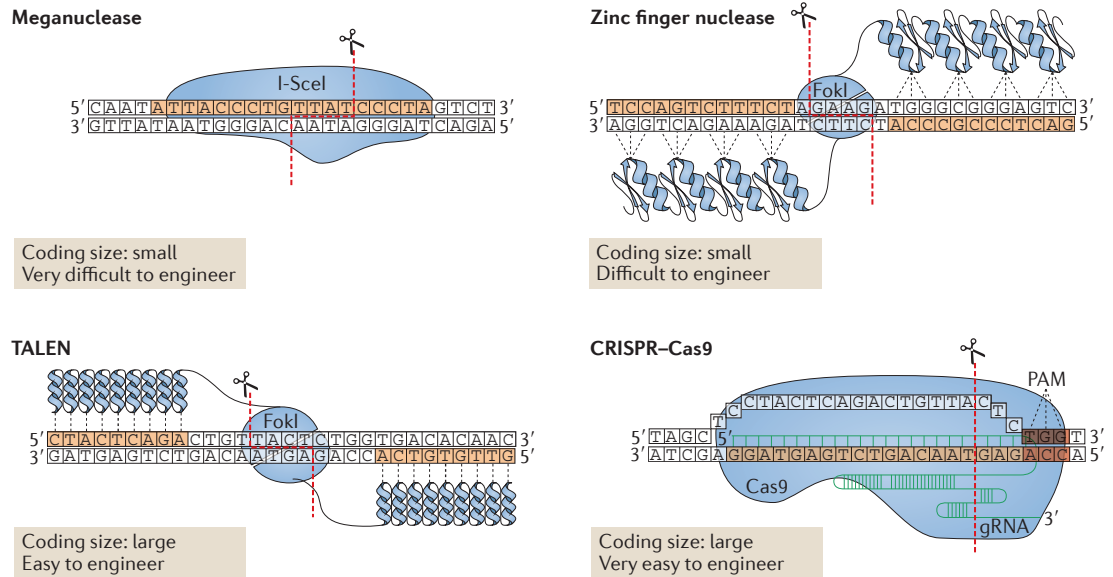


Figure 2 | Genome engineering tools. **a** | The meganuclease family of endonucleases, which includes I-SceI, recognizes 14–40 bp regions. **b** | Zinc finger arrays can be created in which each domain recognizes 3 bp of DNA. To create a nuclease, the FokI catalytic domain is fused to two antiparallel arrays, and double-strand breaks are generated following FokI dimerization at the target site. **c** | Assembled transcription activator-like effector (TALE) arrays recognize 1 bp for each repeat variable diresidue, and are similarly fused to the FokI nuclease. **d** | The CRISPR–Cas9 system uses the bacterial enzyme Cas9 targeted to specific regions of DNA by a designed guide RNA (gRNA). Editing can only occur if the target sequence is followed by a protospacer adjacent motif (PAM).

AAVs are considered to be ideal vectors in part because AAV rarely integrates into the host genome and, instead, exists in an episomal state⁹⁹. However, AAV integration has been detected at homologous loci and sites of DNA damage¹⁰⁰. AAV is also replication-deficient and non-pathogenic. A limitation of AAV vectors for gene editing is that gene packaging is restricted to ~4.7 kb, with the exact size restriction depending on the serotype^{101,102}. This restriction poses a challenge for CRISPR–Cas9-based gene editing, as the *S. pyogenes* Cas9 (SpCas9) is encoded by a ~4.1 kb cDNA, and it requires additional regulatory elements plus the gRNA expression cassette to generate a fully functional gene editing system. Other Cas9 enzymes, such as Cas9 from *Staphylococcus aureus* (SaCas9), are encoded by smaller transgenes, and might be better suited for AAV packaging¹⁰³. Novel AAVs with improved tropisms, pharmacological properties and immune system evasion have been developed using rational design, DNA shuffling and directed evolution^{97,104–109}. AAV vectors have been used to deliver various genome engineering tools, including meganucleases, ZFNs⁸ and CRISPR–Cas9 (REFS 103, 110–113), to several different cell types and tissues.

Another prominent candidate for gene delivery is lentivirus. Safety concerns about random integration of this virus into the host genome and sustained nuclease expression were originally a major setback, but integrase-deficient lentiviral vectors are now available for transient expression^{114,115}. Other safety features that have enabled several clinical successes with lentiviral gene therapies include self-inactivating long terminal repeats that limit aberrant transcriptional modulation of nearby genes¹¹⁵.

Lentivirus is an attractive prospect for the delivery of genome engineering tools for several reasons. First, integrase-deficient lentivirus remains episomal and provides transient expression¹¹⁵, which can be ideal for the expression of nucleases. Second, the larger packaging size relative to AAV allows the delivery and expression of SpCas9 or SpCas9 fusion proteins and multiple gRNAs simultaneously¹¹⁶. Last, lentiviral particles can also deliver protein cargo, such as meganucleases, ZFNs and TALENs^{117,118}. However, owing to the limited viral titres and tissue penetration of lentiviral vectors relative to AAV, this approach is currently not feasible for systemic delivery to large volumes of muscle tissue.

Adenovirus might also prove to be an effective gene delivery vector, as it has a much larger genome than AAV and remains episomal. Adenovirus was demonstrated to be efficient for the delivery of TALENs in human cells¹¹⁹ and, most recently, for the *in vivo* delivery of CRISPR–Cas9 (REF. 120). However, concerns about immunogenicity¹²¹ and adjuvant properties of adenovirus¹²² have limited its use relative to the other gene therapy vehicles.

Non-viral gene and protein delivery. In light of the safety and immunogenicity concerns surrounding viral vectors^{121,123}, non-viral vectors are being developed to deliver nucleic acids, including plasmids, small interfering RNA and genome engineering tools^{124,125}. Common approaches include introduction of an oligonucleotide or plasmid as a direct injection^{126,127}, or facilitated by electroporation, sonoporation, or packaging into polymeric vehicles or nanoparticles¹²⁸. Compared with systemic viral transduction, the generation of efficient non-viral vectors has

Small interfering RNA
Short, double-stranded RNA that mediates sequence-specific silencing of targeted mRNAs.

proven challenging. Nevertheless, non-viral delivery has progressed to clinical trials for delivery of nucleic acids to the liver and tumours, as well as pulmonary delivery for cystic fibrosis¹²⁵. In a study published in 2014, the CRISPR–Cas9 system was delivered to hepatocytes via hydrodynamic tail vein injection of plasmid DNA and oligonucleotides to correct hereditary tyrosinaemia in mice¹²⁹. In skeletal muscle, non-viral delivery approaches have included direct plasmid injection, oligonucleotides for exon skipping, and oligonucleotides for genome editing^{130,131}. Direct delivery of Cas9–gRNA ribonucleoprotein has been demonstrated in primary cells and in local settings *in vivo*, such as the inner ear¹³², although systemic administration of ribonucleoprotein complexes will be considerably more challenging.

Overall, the genome engineering revolution has provided us with a versatile toolbox for the modification or repair of essentially any gene. As discussed in the sections that follow, investigators are now applying these technologies to the study and treatment of neuromuscular disorders (TABLE 1).

Duchenne and Becker muscular dystrophy

The most highly developed application of genome engineering for neuromuscular diseases is in the treatment of DMD^{133,134}, an X-linked disorder that affects 1 in 5,000 live male births and is caused by mutations in the *DMD* gene¹³⁵. Approximately 35% of DMD-associated mutations are spontaneous¹³⁶, which is partially explained by the large size of the *DMD* gene (2.5 Mb). DMD results in early difficulty with mobility, loss of ambulation in the teenage years, and death from cardiac and pulmonary dysfunction, typically in the 20s. Patient outcomes are only slightly improved by long-term corticosteroid therapy^{137,138}. The causative mutations are heterogeneous, and they include large deletions, duplications and nonsense mutations, all of which cause complete loss of functional dystrophin (FIG. 4). Gene therapy holds considerable promise for the treatment of monogenic disorders such as DMD. However, the 14 kb cDNA of dystrophin exceeds the packaging limits of many viral vectors, including AAV.

A related condition, Becker muscular dystrophy (BMD), results from mutations of the *DMD* gene that produce much milder patient phenotypes, which vary widely depending on the nature of the mutation. Mutations associated with a BMD phenotype are typically in-frame internal deletions or duplications. Insights into the BMD genotype and highly variable phenotype have motivated the study of structure–function relationships in the various regions of the dystrophin protein. This analysis has revealed regions of the dystrophin cDNA that can be removed (for example, the rod domain) without significantly decreasing the function of the protein. These findings have motivated the development of internally shortened *DMD* transgenes, termed minidystrophin and microdystrophin, which are compatible with dual or single AAV administration^{139–141}.

Antisense oligonucleotides that are complementary to splice acceptors can cause exons to be skipped during RNA splicing, resulting in an internally shortened RNA. Patients with out-of-frame mutations can have exons

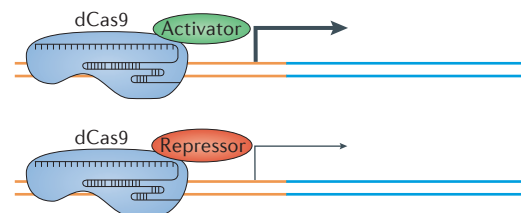


Figure 3 | Gene activation or repression. Activation domains (green), such as VP64, or repression domains (red), such as KRAB, can be fused to programmable DNA-binding proteins — in this case, catalytically deactivated Cas9 (dCas9) — to activate or repress gene expression by targeting promoter regions.

skipped to recover an internally shortened but functional form of dystrophin¹¹. An estimated 83% of mutations in patients with DMD can be addressed through skipping of various exons¹⁴², and skipping of only exon 51 is applicable to around 13% of patients. Oligonucleotides can be delivered intravenously or in a viral vector by expression as small nuclear RNAs¹⁴³. A large body of work has demonstrated successful restoration of dystrophin expression by exon skipping in cultured cells and animal models. In September 2016, an exon-skipping phosphorodiamidate morpholino oligomer that induces skipping of human exon 51 was granted accelerated approval by the FDA¹⁴⁴. Oligonucleotides targeting other exons, as well as additional — potentially more effective — oligonucleotide chemistries, are under development¹⁴⁵. However, there is still substantial room for improvement in addressing the symptoms of DMD, owing to delivery challenges and toxicities of certain oligonucleotide chemistries that ultimately limit the efficiency of dystrophin restoration.

The exon-skipping studies, in addition to the treatment of preclinical models of DMD with minidystrophin and microdystrophin, has motivated attempts to apply genome engineering to this disease. Early efforts used meganucleases to demonstrate that insertions or deletions could change the reading frame of the *DMD* gene, which could potentially be useful for correcting frameshift mutations. A well-characterized meganuclease target site was inserted within a *DMD* gene harbouring a frameshift mutation in the context of a plasmid. Successful recovery of dystrophin expression was observed following co-transfection of the plasmid and a meganuclease expression plasmid *in vitro*, and following electroporation of the same plasmids into muscle tissue *in vivo*¹⁴⁶ (FIG. 4b). Following these results, Popplewell *et al.* engineered a meganuclease targeting intron 44, upstream of the mutational hotspot (exons 45–55), to drive meganuclease-mediated HDR with an integration-deficient lentivirus template containing exons 45–52 (REF. 147) (FIG. 4c).

ZFNs and TALENs have also been adapted for *DMD* gene repair. Our group used two ZFNs targeted to the introns surrounding exon 51 to remove this exon in DMD patient myoblasts¹⁴⁸ (FIG. 4d). A clonal cell line containing the corrected gene was used for transplantation *in vivo*, and produced properly localized human dystrophin expression in the mouse sarcolemma. We

have also developed TALENs for frameshift correction. These TALENs were targeted to exon 51 of the *DMD* gene to restore functional protein expression by introducing indels upstream of the premature stop codon¹⁴⁹ (FIG. 4b). Li *et al.* demonstrated TALEN-based and CRISPR–Cas9-based editing in DMD patient-derived iPSCs by deleting exons, introducing frameshifts, and knocking in exons. Using these methods, they were able to differentiate iPSCs to myogenic cells and detect dystrophin expression. Importantly, they also showed proof of principle for exon 44 knock-in to restore regions of the gene that are critical for protein function⁸⁹ (FIG. 4c).

Our group has demonstrated that CRISPR–Cas9-mediated deletions of exon 51 or the entire mutational hotspot of exons 45–55, which are applicable to approximately half of all patients with DMD¹⁴², can

restore dystrophin expression in patient-derived myoblasts¹⁵⁰ (FIG. 4d). As a proof of principle for cell therapy, human dystrophin was detected *in vivo* after transplantation of these genetically corrected myoblasts¹⁵⁰. A similar approach to deleting exons 45–55 was subsequently demonstrated in iPSCs⁹⁰ — a cell source that could potentially be sufficiently expanded to deliver cells to the large mass of skeletal muscle necessary to treat DMD, if safety and regulatory concerns can be addressed. In another study, adenoviral delivery of Cas9 and gRNAs led to highly efficient, selection-free restoration of dystrophin expression in patient cell populations, indicating potential applications in cell therapy¹⁵¹.

Although deletion of whole exons can restore partial dystrophin function and recapitulate a BMD-like genotype, the structure of the dystrophin protein is still

Table 1 | **Genome engineering approaches for neuromuscular disorders**

Disorder	Gene mutation	Chromosomal location	Prevalence*	Amenable to:	Refs
Duchenne muscular dystrophy (DMD)	Deletion, duplication or nonsense mutation in dystrophin (<i>DMD</i>)	Xp21.2–p21.1	1 in 5,000	• Indel frame restoration • Deletion • Homologous repair • Targeted gene replacement • Activation of compensatory gene	89,90, 111–113, 146–154, 161
Spinal muscular atrophy (SMA)	Deletion or mutation in survival of motor neuron 1 (<i>SMN1</i>)	5q13.2	1–9 in 100,000	• Homologous repair of <i>SMN1</i> • Editing of compensatory <i>SMN2</i>	171–174
Facioscapulohumeral muscular dystrophy (FSHD)	D4Z4 repeats	4q35.2	1 in 20,000	• Double homeobox 4 (<i>DUX4</i>) silencing • Poly(A) removal	189
Trinucleotide repeat disorders					
Myotonic dystrophy (type I)	Trinucleotide repeat in myotonic dystrophy protein kinase (<i>DMPK</i>)	19q13.32	1–5 in 10,000	• Site-specific RNA endonucleases • Trinucleotide repeat shortening	215,216
Friedreich ataxia	Trinucleotide repeat in frataxin (<i>FXN</i>)	9q21.11	1–9 in 100,000	• <i>FXN</i> activation • Trinucleotide repeat shortening	199,200
Oculopharyngeal muscular dystrophy (OPMD)	Trinucleotide repeat	14q11.2	1 in 100,000	• Trinucleotide repeat shortening	206,208
Congenital muscular dystrophies					
Muscle–eye–brain disease	Heterogeneous mutations in genes such as <i>POMT1</i> , <i>POMT2</i> , <i>FKTN</i> , <i>FKRP</i> , <i>LARGE</i> , <i>POMGNT1</i> , <i>ISPD</i> , <i>SEPN1</i> and <i>LMNA</i>	Various	Unknown	• Targeted gene replacement • Removal of inhibitors of muscle function • Activation of compensatory gene	232
Walker–Warburg syndrome	Various, but primarily in <i>POMT1</i> , <i>POMT2</i> , and <i>FKRP</i>	• 9q34.13 • 14q24.3 • 19q13.32	1–9 in 1,000,000	• Targeted gene replacement • Removal of inhibitors of muscle function • Activation of compensatory gene	232
Fukuyama congenital muscular dystrophy	Various in <i>FKTN</i> ; founder mutation is 3 kb insertion of tandemly repeated sequences	9q31.2	1–9 in 1,000,000	• Targeted gene replacement • Region deletion	231
Myopathies					
Dysferlin deficiencies (limb-girdle type 2B)	Various in dysferlin (<i>DYSF</i>)	2p13.2	1–2 in 1,000,000	• Targeted <i>DYSF</i> gene replacement	217–219
Tibial muscular dystrophy	11 bp in titin (<i>TTN</i>)	2q31.2	1–9 in 100,000	• Homologous repair of <i>TTN</i> • Targeted <i>TTN</i> replacement	NA
Limb-girdle muscular dystrophy type 1A	In myotilin (<i>MYOT</i>) exon 2 or 9	5q31.2	<1 in 1,000,000	• Gene knockout • Gene repression	224
Desminopathy	Various in desmin (<i>DES</i>)	2q35	Unknown	• Gene replacement	NA

*Prevalence numbers from Orphanet. *DMPK*, DM1 protein kinase; *FKRP*, fukutin-related protein; *FKTN*, fukutin; *ISPD*, isoprenoid synthase domain containing; *LARGE*, LARGE xylosyl- and glucuronyltransferase; *LMNA*, lamin A/C; NA, not applicable; *POMGNT1*, protein O-linked mannose N-acetylglucosaminyltransferase 1; *POMT*, protein O-mannosyltransferase; *SEPN1*, selenoprotein N.

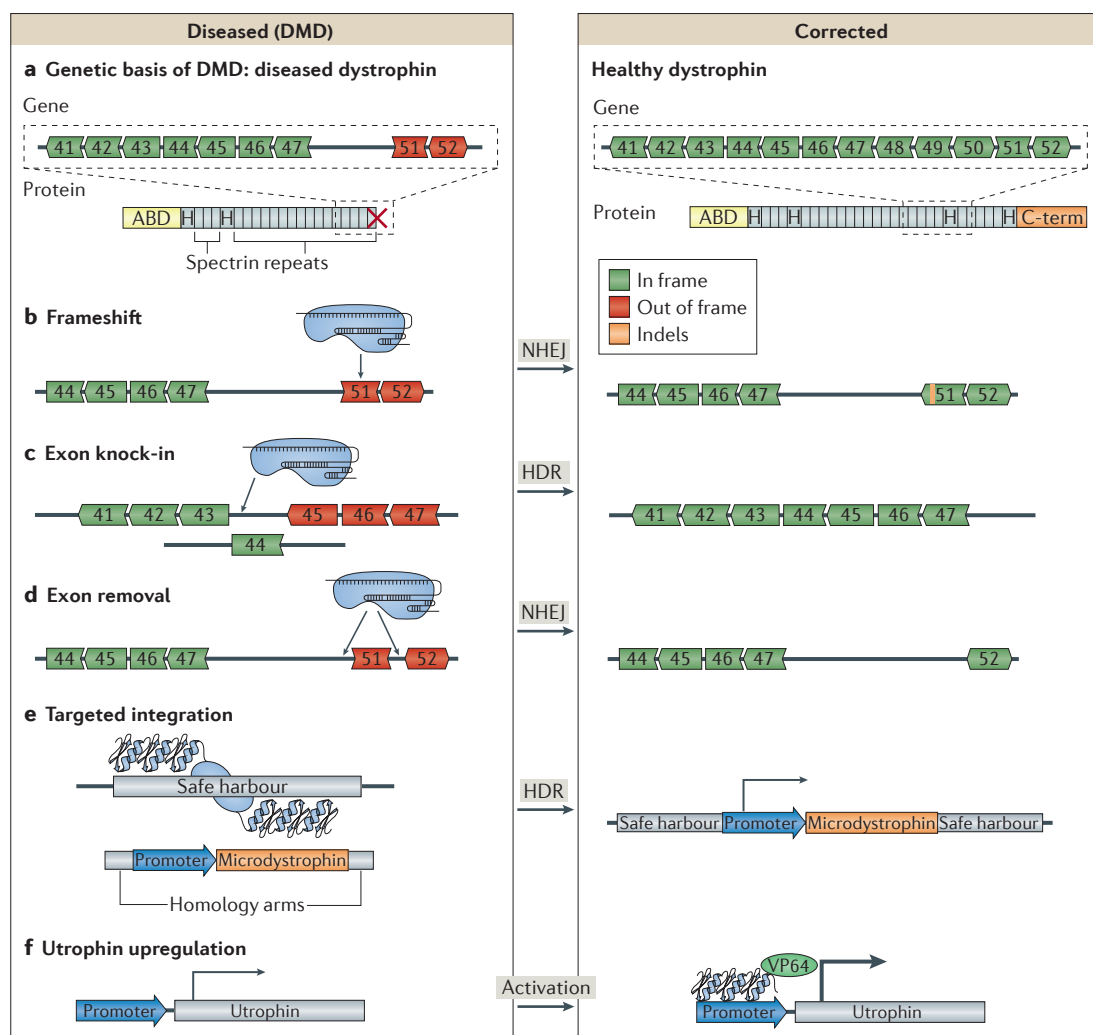


Figure 4 | Genome engineering for Duchenne muscular dystrophy. **a** | Duchenne muscular dystrophy (DMD) is often caused by frameshift mutations resulting from deletion of one or more exons from the *DMD* gene. The figure shows one such mutation, deletion of exons 48–50 ($\Delta 48-50$). **b** | Targeted nucleases have been used to produce indels in frameshifted exons to restore the reading frame. **c** | Nucleases have been used for the targeted integration of missing exons. **d** | Multiplexed targeted nucleases have been used to excise non-essential exons in the human *DMD* gene to recover protein expression. **e** | Zinc finger nucleases have been used to target the integration of microdystrophin into a safe-harbour locus. **f** | Zinc finger protein-based transcriptional activators have been used to upregulate utrophin to compensate for the absence of dystrophin. ABD, actin-binding domain; C-term, carboxyl terminus; HDR, homology-directed repair; NHEJ, non-homologous end joining.

disrupted. However, precisely targeted deletions within *DMD* exons can be used to restore the reading frame while maintaining the native spectrin-repeat structure of the dystrophin rod domain, which is not maintained through exon skipping or deletion¹⁵².

Following these demonstrations of *ex vivo* genome editing to restore dystrophin expression, it was critical to develop a strategy for *in vivo* genome editing that might circumvent the various challenges of cell-based therapy. This endeavour required adaptation of the gene editing tools to develop clinically relevant methods of efficient delivery to skeletal and cardiac tissue. *In vivo* editing of the *DMD* gene was accomplished by adenoviral delivery of Cas9 and gRNAs, providing important proof of principle for *in vivo* dystrophin restoration by

genome editing¹⁵³. However, adenovirus is unlikely to be used to treat DMD in humans, owing to its substantial immunogenicity. Our group and others reported local and systemic *in vivo* genome editing via AAV-mediated delivery of CRISPR–Cas9 in skeletal and cardiac muscle in adult and neonatal *mdx* mice^{111–113}. The AAV vectors capitalize on a generally favourable safety profile and the capacity for systemic delivery to all skeletal and cardiac muscle with muscle-tropic serotypes. These studies demonstrate CRISPR-mediated restoration of dystrophin, improvement in muscle biochemistry, and increased muscle force (FIG. 5). In another recently published study, a 45 kb deletion of exons 52 and 53 in the *mdx*^{dcv} mouse restored dystrophin expression and improved muscle function¹⁵⁴.

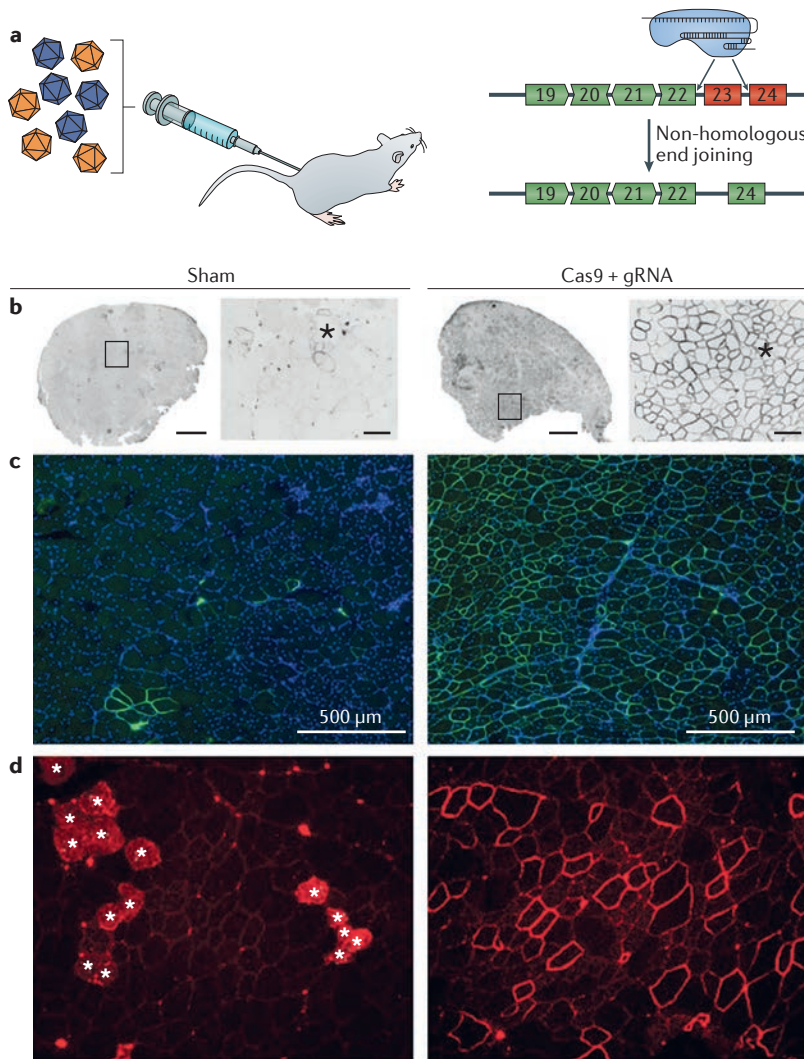


Figure 5 | *In vivo* gene editing in the *mdx* mouse restores dystrophin expression. **a** | Strategy for targeted removal of exon 23 of the *Dmd* gene by CRISPR–Cas9 in the *mdx* mouse, an animal model of Duchenne muscular dystrophy. Dual adeno-associated viral vectors were used to deliver Cas9 and guide RNAs (gRNAs) targeting the intronic regions flanking exon 23. **b** | Tibialis anterior muscle of adult mice treated for 8 weeks (scale bars 600 μm full view, 100 μm inset)¹¹¹. **c** | Tibialis anterior muscle of adult mice 4 weeks post-injection¹¹². **d** | Tibialis anterior muscle of retro-orbitally injected postnatal day 18 mice 8 weeks post-injection (asterisks indicate myonecrosis in unedited *mdx* control mice)¹¹³. Part **b** from 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). Part **c** from Tabebordbar, M. *et al.* *In vivo* gene editing in dystrophic mouse muscle and muscle stem cells. *Science* **351**, 407–411 (2016). Part **d** from Long, C. *et al.* Postnatal genome editing partially restores dystrophin expression in a mouse model of muscular dystrophy. *Science* **351**, 400–403 (2016). Reprinted with permission from AAAS.

The overall robustness of *in vivo* gene editing is supported by the similar outcomes in these three studies, despite differences in Cas9 species, gRNAs, AAV serotypes, AAV doses, and ages of mice at the time of treatment, among other experimental variables¹³⁴. The recent FDA approval of a phase I clinical trial, in which AAV will be used to deliver ZFNs to the liver to provide gene editing therapy for haemophilia¹⁵⁵, is a meaningful step forward for *in vivo* gene editing with AAV vectors.

Deletion of exons to restore dystrophin expression has several advantages relative to other mechanisms of gene repair. First, this approach can address multiple cohorts of patient mutations with a single therapeutic approach and composition. Second, the NHEJ mechanism is more efficient than HDR, which is down-regulated in postmitotic cells such as muscle fibres¹⁵⁴. Last, the technique does not require the addition of a donor DNA repair template. However, not every patient mutation will be amenable to dystrophin restoration by deletion of exons. To make this treatment available to more patients, one approach is to integrate a replacement *DMD* gene into a safe-harbour locus. Recent work has suggested that targeted integration of donor DNA through an NHEJ-based mechanism *in vivo* is more efficient than previously expected¹⁵⁶. Safe-harbour regions of the genome can be targeted for site-specific integration of dystrophin transgenes, such as microdystrophin^{32,33,157–160}. For example, a ZFN-based approach has been used to introduce microdystrophin at the C–C motif chemokine receptor 5 (*CCR5*) locus¹⁶¹. Humans with *CCR5* loss-of-function mutations have no adverse phenotype, so this locus is assumed to be a safe site for integration. The resulting human myoblasts expressed microdystrophin *in vitro* and *in vivo* (FIG. 4e). In addition to safe-harbour sites, transgenes might be targeted to their endogenous promoters, where they would be under the control of natural gene regulation mechanisms. For example, this approach has been used for targeted integration of the factor IX gene to treat haemophilia B⁸.

An alternative approach to enable the treatment of more patients is specific upregulation of compensatory proteins. Utrophin is similar to dystrophin in structure and function but is not expressed at high levels in adult human muscle. Therefore, utrophin upregulation might improve patient outcomes. Small-molecule inducers of utrophin expression have recently entered clinical trials¹⁶². AAV-based delivery of the ZFP ‘Jazz’ (REF. 163) fused to a transcriptional activator has been shown to increase expression of utrophin and ameliorate the dystrophic phenotype *in vivo*^{164,165} (FIG. 4f). With three zinc finger domains, Jazz has only 9 bp of specificity. Improved specificity has been engineered with longer zinc fingers, including ‘Bagly’ and ‘Utroupe’, which have been designed to target 12 bp and 18 bp sequences, respectively^{166,167}.

Spinal muscular atrophy

SMA is a debilitating and fatal disease characterized by substantial muscle wasting and motor neuron death, resulting from a deletion or mutation in the survival of motor neuron 1 (*SMN1*) gene at position 5q13 on chromosome 5 (REF. 24). Gene therapy strategies employing AAV-based *SMN1* gene delivery have improved outcomes in a mouse model of SMA and in clinical trials^{168–170}. An analogous but biologically inactive gene, *SMN2*, has a single base pair alteration at the exon 7 splice site that results in splice silencing and, consequently, loss of proper protein expression²⁴. Conversion of this silencer in *SMN2* to the sequence

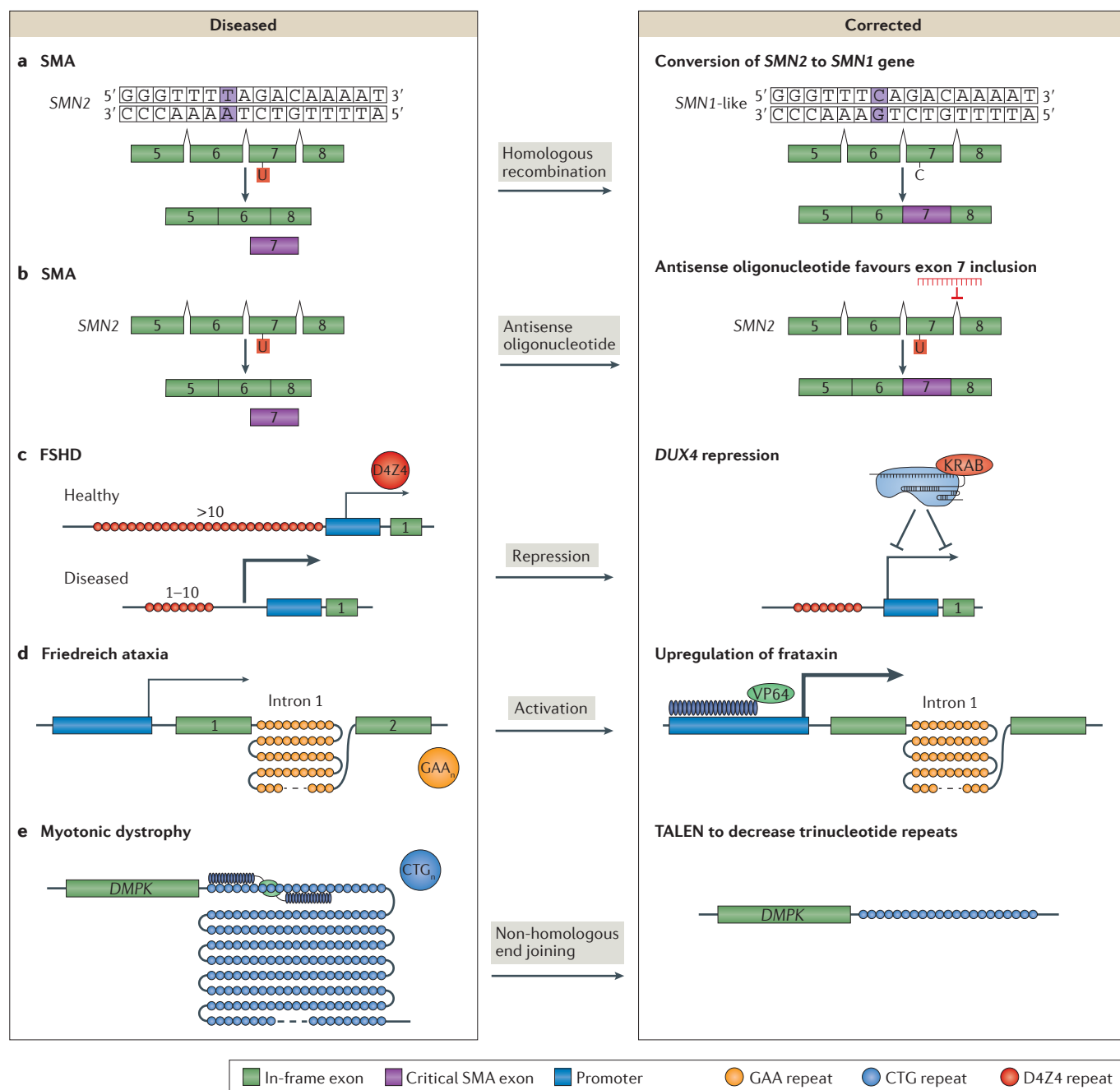


Figure 6 | Genome engineering for other neuromuscular disorders. a | Spinal muscular atrophy (SMA). Oligonucleotide-mediated recombination has been used to convert the survival of motor neuron 2 (SMN2) gene to the absent SMN1 gene. **b** | Also in SMA, antisense oligonucleotides have been used to encourage inclusion of SMN2 exon 7 during splicing. **c** | Facioscapulohumeral muscular dystrophy (FSHD). dCas9–KRAB targeting the double homeobox 4 (DUX4) promoter or exon 1 reduces expression of this gene. **d** | Friedreich ataxia. Targeted gene activation with transcription activator-like effector proteins has been used to increase expression of frataxin in Friedreich ataxia. **e** | Myotonic dystrophy. Transcription activator-like effector nucleases (TALENs) have been adapted for shortening CTG/CAG trinucleotide repeats at the 3' end of the DM1 protein kinase (DMPK) gene.

found in the SMN1 gene has been shown to improve the SMA phenotype. DiMatteo *et al.* successfully converted the SMN2 gene to the SMN1 sequence through homologous recombination by delivering single-stranded oligonucleotides to patient-derived cells¹⁷¹ (FIG. 6a). In another study, corrected patient-derived iPSCs that were

differentiated into motor neurons improved the lifespan and phenotype of an SMA mouse model¹⁷². In other work, antisense oligonucleotides and morpholinos were adapted to alter the splicing of SMN2 at the mRNA level to boost functional SMN protein in the mouse CNS^{173,174} (FIG. 6b). This research establishes gene therapy and gene

editing as a viable approach to SMA. Targeted nucleases could be directed to disrupt the exon 7 splice repressor or, alternatively, *SMN2* could be converted to *SMN1* through HDR.

Facioscapulohumeral muscular dystrophy

Facioscapulohumeral muscular dystrophy (FSHD) is an autosomal dominant disease associated with contraction of the D4Z4 microsatellite tandem repeats on chromosome 4q35 (REFS 175–178), resulting in a toxic gain of function of double homeobox 4 (*DUX4*) expression¹⁷⁸ concomitant with loss of regulatory heterochromatin^{179,180}. Patients with FSHD have 1–10 D4Z4 tandem repeats, which is insufficient for proper heterochromatin formation and repression of *DUX4*, whereas a normal genotype has more than 11 repeats. Monosomy of distal 4q, which leads to haploinsufficiency of *DUX4*, does not cause FSHD¹⁸¹, further supporting a gain-of-function mechanism. Life expectancy is typically not compromised in FSHD, and the patient phenotype is extremely variable, although 95% of patients show symptoms by the second decade of life¹⁸². As many as 20% of affected individuals eventually become wheelchair-dependent.

Currently, no effective treatment is available to slow the progression of muscle weakness and atrophy in FSHD, and the complex genetic and epigenetic causes are incompletely understood. One major challenge is the absence of an accurate animal model of FSHD¹⁸⁰. Several groups have characterized gene-based therapies for FSHD, using patient cells and various animal models that mimic some aspects of the disease^{183–185}. Gene therapy to express compensatory factors¹⁸⁶, or *DUX4* knockdown by morpholinos¹⁸⁴ and RNA interference^{187,188}, have been used with some success in various mouse models. Targeting with nucleases has not been accomplished, probably owing to the presence of a highly homologous region on chromosome 10, which makes specific targeting of the D4Z4 repeats on chromosome 4 difficult. However, targeted repression of *DUX4* was achieved in FSHD myocytes by targeting the promoter or the first exon of *DUX4* with dCas9–KRAB, thereby establishing a potential therapeutic strategy for FSHD¹⁸⁹ (FIG. 6c).

Nucleotide repeat disorders

Around 30 disorders, including myotonic dystrophy, fragile X syndrome, and Huntington disease, are known to originate from nucleotide repeat expansions. These diseases share the mechanism of anticipation, in which subsequent generations gain additional repeats and display increasingly severe phenotypes and early ages of onset. Genome engineering might be a useful approach to shorten the number of nucleotide repeats and reduce the pathological effects¹⁹⁰. Many trinucleotide repeat disorders, including Huntington disease¹⁹¹, are caused by CAG/CTG expansions, and the majority of genome engineering efforts to address repeat expansions have been applied to these targets. Creation of DSBs in regions of CAG/CTG repeats can lead to deletions of repeats¹⁹². However, duplications of repeats were

observed when breaks were produced by ZFNs¹⁹³, suggesting that a preferable approach would be to remove the whole repeat expansion by targeting the regions that flank the repeats. Alternatively, ZFP-based transcriptional repressors can be targeted to CAG repeats to reduce expression of the mutant form of huntingtin protein¹⁹⁴. In a yeast model, a TALEN was adapted for the targeted contraction of CAG or CTG trinucleotide repeats — an approach that is potentially translatable to humans¹⁹⁵.

Friedreich ataxia. Friedreich ataxia affects 2–4 in 100,000 individuals, and results in mobility loss and cardiac dysfunction after the second decade of life¹⁹⁶. Friedreich ataxia is an autosomal recessive neurodegenerative disorder caused by GAA trinucleotide repeat expansions in the first intron of the frataxin (*FXN*) gene on chromosome 9 (REF. 197). Increased numbers of GAA repeats result in protein silencing. AAV-mediated *FXN* delivery was shown to increase frataxin protein levels and improve the phenotype in a conditional knockout mouse¹⁹⁸. A genome engineering approach with a TALE–VP64 targeted to the *FXN* promoter increased frataxin levels in patient fibroblasts and the YG8R mouse model¹⁹⁹ (FIG. 6d). Excision of the expanded GAA repeats has also been accomplished, using ZFNs that target intronic regions flanking the GAA repeats. Deletions of the GAA repeats resulted in increased frataxin levels and normalization of biomarkers in differentiated cells²⁰⁰.

Oculopharyngeal muscular dystrophy. Oculopharyngeal muscular dystrophy (OPMD) presents in the fifth or sixth decade of life with eyelid drooping, difficulty swallowing, and proximal limb weakness^{201,202}. OPMD results from an increase of two or more GCG repeats in the poly(A) binding protein nuclear 1 (*PABPN1*) gene on chromosome 14q11 (REFS 202, 203). *PABPN1* encodes a protein that regulates the length of poly(A) mRNA tails, and the mutant protein forms aggregates in skeletal muscle cell nuclei^{204,205}. OPMD is unique among trinucleotide repeat disorders because only a small repeat expansion is required for pathogenic effects in skeletal muscle²⁰⁵. Clinical progress has been made with protein chaperones and cell therapy. Cabaletta is a trehalose-based treatment that reduces protein aggregates²⁰⁶, and a phase II clinical trial of this agent is ongoing²⁰⁷. In another phase II trial, functional improvement was seen when autologous myoblasts isolated from unaffected muscles were injected into the pharyngeal muscles of 12 patients with OPMD²⁰⁸. The success of this cell-based approach indicates that *ex vivo* gene editing in cells could be useful, but approaches to target the genetic basis of OPMD remain to be explored²⁰⁹.

Myotonic dystrophy. Type I myotonic dystrophy (DM1) is an autosomal dominant disorder that affects 1 in 8,000 people²¹⁰ and causes substantial wasting in skeletal and cardiac muscle. The genetic basis is the expansion of a CTG repeat at the 3' end of the myotonic dystrophy protein kinase (*DMPK*) gene^{211–213}. DM1 manifests when the number of repeats increases from the

normal 5–37 to over 50. Longer repeats ranging into the thousands are associated with younger onset and worse phenotypes, and can be fatal²¹⁰. The mechanism has been identified as a toxic gain of function of (CTG)_n RNA accumulation in the nucleus, leading to sequestration of RNA-binding proteins and widespread aberrant splicing, and/or silencing of the *DMPK* gene²¹⁴. A potential cell therapy that was developed for DM1 utilized iPSCs corrected by TALEN-mediated gene editing²¹⁵ (FIG. 5e). Another approach used site-specific RNA endonucleases to target CUG repeats that accumulated in the nucleus²¹⁶.

Limb-girdle muscular dystrophy

Limb-girdle muscular dystrophies present with weakness of muscles in the shoulder and pelvic girdles. Around 20 subtypes have been described, caused by mutations in several genes including dysferlin (*DYSF*) and α -sarcoglycan (*SGCA*), and with a wide range of phenotypes. Several groups are pursuing gene transfer as a viable option for dysferlin-related myopathies. *DYSF* transgene delivery has been pursued with an AAV-delivered minidysferlin gene²¹⁷. In addition, dual AAV vectors^{218,219} are currently being tested in a phase I clinical trial²²⁰. iPSCs have been reprogrammed with lentiviral constructs carrying *SGCA*; transplantation of these cells into mice restored α -sarcoglycan expression and improved muscle function²²¹. Genome editing to correct *DYSF* and *SGCA* mutations in patient-derived iPSCs has been used to address limb-girdle muscular dystrophy types 2B and 2D, respectively²²². RNA interference has been applied to dominant forms of the disease, suggesting that gene repression or knockouts could be applicable to these disorders^{223,224}.

CMDs and distal myopathies

Around 33 separate congenital muscular dystrophies (CMDs) have been identified, including the dystroglycanopathies Walker–Warburg syndrome, muscle–eye–brain disease and Fukuyama congenital muscular dystrophy. CMDs vary in phenotype, and can result in motor and neurological dysfunction at an early age. Around 20 distal myopathies are also known to exist, including Miyoshi myopathy, tibial distal myopathy and myofibrillar myopathy^{225–229}. Many distal myopathies lead to loss of ambulation, and desminopathy shows rapid progression, often leading to disability and cardiac and respiratory complications. However, most distal myopathies are slow to progress and do not compromise lifespan²²⁹. Owing to the heterogeneity of distal myopathies as a group and even within individual myopathies, genotype–phenotype correlations have proved elusive, so curative and ameliorative treatments have not yet been fully developed²²⁷.

A recent study described an approach to correct mutations in CMD by using NHEJ-mediated deletions to restore mutated splice sites. As a proof of principle, systemically administered AAV carrying CRISPR–Cas9 deleted a small region in the *Lama2* gene in the *dy²¹/dy²¹* mouse model of CMD type 1A²³⁰. The deletion restored proper splicing and protein expression, and improved

muscle function. By designing gRNA pairs for each splice-site mutation, this approach could extend to as many as 40% of *Lama2* mutations.

Another approach to circumvent the problem of heterogeneity in CMD and distal myopathies is to devise therapeutic strategies that compensate for multiple cohorts. Targeting the integration of the missing gene or compensatory genes to safe-harbour regions could be a practical approach, as a single validated and well-characterized nuclease can be used for all patients, and the disease-specific component of the therapeutic would be the transgene insert. Finding transgenes that compensate for large patient cohorts will also be beneficial. For example, AAV-mediated delivery of the *LARGE* gene, which can compensate for defects in the glycosylation of α -dystroglycan, rescues the muscular dystrophy phenotype in several mouse models of dystroglycanopathy^{231,232}. Inhibitors of muscle function, such as myostatin (a negative regulator of muscle mass) or its receptor, can also be removed by gene editing or silenced by gene repression, as has been accomplished with TALENs²³³. However, caution is warranted in any therapeutic strategy that requires knockout or permanent silencing of an endogenous gene involved in normal tissue homeostasis.

Conclusions and future challenges

Genome engineering tools have been widely adopted by the neuromuscular disease field, and recent developments and ongoing work demonstrate the remarkable potential of genome engineering for neuromuscular disorders. However, we face several major challenges in the therapeutic translation of these technologies.

Additional optimization may be required to achieve clinically relevant levels of genome modification. For genome editing in skeletal muscle, for example, gene modification rates as low as 2% have been reported¹¹¹. Low correction rates might still provide significant benefits, such as in DMD, where a small number of corrected nuclei can restore dystrophin expression in a large proportion of muscle fibres, leading to functional improvements. However, higher correction levels might be necessary in diseases caused by gain-of-function mutations. Importantly, the technologies are still very young, and there is substantial room for improvement on the early studies.

Another major and related challenge is to adapt and engineer efficient delivery vehicles for transduction or transfection of cells and tissues associated with neuromuscular disease¹²⁴. Clinical treatment using viral vectors also creates a challenge in terms of scaling production in accordance with Current Good Manufacturing Practice and in a cost-effective manner²³⁴. The development of non-viral vectors for efficient and targeted delivery would be particularly valuable in addressing the concerns of immunogenicity and prolonged nuclease expression associated with viral vectors.

A primary advantage of chromosomal modification by gene editing is the permanence of the correction. Stem and progenitor cells might aid gene correction if muscle progenitors are targeted by gene-correcting

techniques and are allowed to repopulate the diseased muscle. However, non-edited stem and progenitor cells could dilute the impact of gene correction by repopulating the tissue with non-edited cells. Muscle satellite cells are difficult to study, and have shown varying degrees of vector uptake depending on the viral vector used and the age of the animal^{1235–237}. However, evidence suggests that efficient gene editing of satellite cells is possible¹¹². By specifically engineering vehicles to target muscle progenitor cells, we may be able to harness the full potential of *in vivo* genome editing for neuromuscular conditions.

Although genome editing tools allow specific modification in the genome, the potential for off-target interactions persists, and should be screened extensively in advance of clinical development. Off-target nuclease events are dependent on the target sequence and epigenetic state of the target locus. Sensitive and unbiased genome-wide tools, such as Guide-seq, are becoming available for detecting rare off-target nuclease activity²³⁸. Higher-specificity versions of genome engineering tools are also available, including dual Cas9 nickases, which reduce off-target indel formation²³⁹, and engineered Cas9 variants that have reduced or undetectable off-target activity^{240,241}.

The immune response to viral vectors, including the presence of neutralizing antibodies against AAV capsids in humans, has been extensively studied²⁴². New viral vector designs aim to reduce immunogenicity^{242–247}; however, further characterization will be required as human trials progress. In particular, the immune response to genome editing tools such as ZFPs, TALEs, Cas9 and gRNAs will need to be evaluated. The emergence of Cas9-specific antibodies in response to adenovirus-mediated delivery was first reported in 2015 (REF. 120). SpCas9 delivered by AAV also seemed to provoke an immune response in mice²⁴⁸. Responses to the restored or newly expressed disease-related proteins have been noted in a few cases and, thus, immunosuppression or immunotolerance strategies should be carefully considered²⁴⁹.

Despite these challenges, genome engineering, accomplished via cell therapy or systemic delivery of gene editing tools, holds tremendous promise for advancing the science and treatment of neuromuscular disorders. Investigators are actively addressing the current obstacles to clinical advancement, and in the coming years we expect to see substantial preclinical and clinical developments in gene editing technologies in the neuromuscular medicine field.

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Author contributions

All authors contributed equally to the preparation of the manuscript.

Competing interests statement

C.A.G., C.E.N. and J.R.H. are inventors on patent applications related to genome engineering for neuromuscular diseases.

Gene Targeting and Gene Correction

948. Efficient In Vivo Selection of Gene-Targeted Hepatocytes Using Acetaminophen-Induced Liver Toxicity

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Precise gene editing by homologous recombination has low efficiency in vivo. However, selective expansion of rare gene-targeted cells is a promising approach to overcome this problem. We previously described a system for expanding gene-edited hepatocytes by rendering them genetically resistant to CEHPOBA, a compound that causes hepatotoxicity by blocking the tyrosine catabolic pathway. Here we demonstrate selection using a far more readily available and clinically applicable drug, acetaminophen. Acetaminophen-induced liver toxicity results from cytochrome P450 (Cyp)-mediated metabolism in hepatocytes, generating the toxic intermediate NAPQI. We theorized that a deficiency in the enzyme cytochrome P450 reductase (Cypor), the obligate co-factor for most Cyp enzymes would block this process and protect Cypor-deficient hepatocytes. To test this theory, a cohort of mice was administered a CRISPR plasmid targeting the mouse Cypor gene by hydrodynamic tail vein injection. Five mice from this cohort were assigned to the experimental group and were injected twice weekly with high doses of acetaminophen (400 mg/kg). Control mice (n=4) received no acetaminophen.

Results: As expected, acetaminophen injections resulted in significant serum ALT spikes (~1000 IU/L) within 6 hours of administration. However these spikes abated after the tenth bi-weekly dose. After fifteen acetaminophen doses in the experimental group, all mice were sacrificed and liver sections were examined by Cypor immunofluorescence. Mice from the control group had ~1% Cypor-negative hepatocytes whereas experimental mice had regions of Cypor-negative staining comprising 40-51% of the liver area. Sequencing liver genomic DNA showed 35-42% Cypor-localized indels in experimental mice and ~3% in the control group.

Conclusions: These data demonstrate robust clonal expansion of gene-modified hepatocytes using acetaminophen-induced liver toxicity. Acetaminophen-treated mice showed no significant weight loss during treatment and no mortality was observed. Clinical incidents of mild to moderate acetaminophen overdose normally resolve with complete restoration of liver function. With proper safety protocols, genetic protection from acetaminophen toxicity could be used to select gene-modified hepatocytes in humans and achieve clinically relevant levels of transgene expression.

949. Designed Zinc Finger Protein Transcription Factors for Single-Gene Regulation Throughout the Central Nervous System

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The Microtubule Associated Protein Tau (MAPT) is strongly linked to the pathogenesis of several primary tauopathy disorders, which include Alzheimer's Disease, Progressive Supranuclear Palsy, Frontotemporal Dementia, and Corticobasal Degeneration. While genetic and antisense-based approaches to lower intracellular tau levels have proven efficacious and well tolerated in the brain, the development of a single-administration tau-targeted therapy remains a long-standing goal. Advances in Zinc Finger Protein (ZFP) design and recombinant AAV capsid engineering have created new potential for the treatment of tauopathies and other diseases affecting the central nervous system (CNS). We developed ZFP repressors capable of reducing both mouse and human tau levels by up to 98% with no detectable off-target gene regulation in primary and iPS-derived neurons. When focally delivered to the hippocampus, MAPT-targeted ZFPs resulted in >90% tau reduction. Upon intravenous ZFP administration to adult mice, tau levels were reduced by 50-70% across the entire brain. Moreover, ZFP expression and tau reduction were stable out to at least six months, resulted in >80% lowering of CSF-tau, and reduced dystrophic neurites by 50% in APP/PS1 mice with advanced amyloid pathology. Transcriptome-wide specificity assessments detected no off-target activity or secondary effects of tau reduction in the hippocampus or frontal cortex 10 weeks after systemic ZFP administration. The potency, specificity, and stability of the effects that we have observed, coupled with continuing development of gene delivery to the brain and spinal cord, raise the prospect that it will soon be possible to use ZFPs to achieve permanent, CNS-wide regulation of tau or any other chosen gene.

950. Long-Term Evaluation of Genome Editing Outcomes for Duchenne Muscular Dystrophy

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Duchenne muscular dystrophy (DMD) is a debilitating musculoskeletal disease characterized by muscle wasting, loss of ambulation, and premature death by 30 years of age. DMD is caused by deletions,

duplications, and nonsense mutations throughout the DMD gene resulting in the absence of dystrophin protein. Genome editing has emerged as a method to permanently repair the endogenous gene. Multiple groups have used AAV to deliver CRISPR/Cas9 to restore dystrophin expression and improve muscle function in mouse models of DMD. Further development of DMD CRISPR therapy requires careful preclinical evaluation of safety, host response, and the persistence of gene-edited cells. We administered AAV-CRISPR, designed to excise dystrophin exon 23 by simultaneous cutting of two gRNAs, systemically to neonatal and adult mdx mice. Excision of exon 23 will remove the premature stop codon in mdx mice and restore dystrophin expression. To comprehensively characterize gene editing outcomes, we utilized a custom unidirectional sequencing method to quantitatively determine rates of genetic deletion, inversion, AAV integration, indel formation, and chromosomal translocation at the target locus. Illumina Nextera transposase was used to fragment and tag genomic DNA, and then PCR was performed using a locus-specific forward primer and a constant reverse primer specific for the transposon DNA tag. One year after systemic administration into neonatal mice, unbiased unidirectional sequencing of genomic DNA (heart, TA, and diaphragm) showed total on-target editing (8.84%, 1.05%, 2.95%), including indel formation (2.90%, 0.52%, 0.70%), deletions (1.34%, 0.21%, 0.86%), inversions (0.52%, 0.04%, 0.56%), and AAV integrations (4.08%, 0.28%, 0.83%). AAV integrations were consistent with the canonical mechanism of AAV integration into double-strand break sites. No chromosomal translocations were detected in this experiment. To examine longevity of genome editing, we evaluated the same endpoints in mice eight weeks and one year after single-dose administration of AAV-CRISPR. Statistically significant increases in indels, inversions, and deletions, but not AAV integrations, were noted for the one-year time point in the heart, skeletal muscle, and diaphragm. RT-qPCR for edited dystrophin transcript levels shows significant increases in proportions of edited transcript and increases in overall abundance of dystrophin mRNA at one year. Dystrophin protein was detected at both 8 weeks and 1 year by western blot and immunofluorescence histology in multiple skeletal muscles and cardiac muscle. No significant differences were noted in H&E sections of skeletal or cardiac muscle in treated mice compared to age-matched untreated mdx mice. No adverse events were noted in the long-term cohort. This study establishes the feasibility of long-term genome editing in mouse models of disease indicating persistence of gene-edited cells one year after a single administration. In addition, this work shows the presence of alternative on-target genome modifications after delivery of AAV-CRISPR. Additional work will seek to characterize biological implications of these alternative on-target modifications, improve systemic gene deletion efficiency, and characterize humoral and cellular immune response with constitutive and muscle-specific promoters.

951. AAV-Vector Integration into CRISPR-Induced Double-Stranded Breaks

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Introduction: Adeno-associated virus (AAV) vectors are being used as carriers for the components of the CRISPR system or homology repair donors in genome editing applications. Here we characterized homology-independent AAV integration into CRISPR induced DNA breaks *in vitro* and *in vivo* into therapeutically relevant genes. **Methods:** We used AAV to carry CRISPR components (SpCas9, SaCas9 and gRNA against different therapeutic targets) and transduced cells *in vitro* (U2-OS cells and primary mouse neurons). We also injected AAV vectors into hippocampi of adult and inner ears of neonatal mice. We re-analyzed the sequencing data from Bengtsson et al. (2017) to study AAV integration into the *Dmd* gene in mouse muscle. Integration efficiencies were quantified by targeted deep-sequencing and integration-specific qPCR. To allow sequencing of the entire integrated product, we created a minimal AAV construct with a 175bp long payload of the lambda bacteriophage (AAV-λ). Successful packaging of AAV-λ was validated with titration of vector genomes, capsid ELISA, and transmission electron microscopy. TA cloning was used to sequence the integration product after AAV-λ transduction of U2-OS cells co-transfected with guide RNAs and Cas9 encoding plasmids. Finally, AAV was PCR amplified from the genome after *in vitro* transduction and *in vivo* injection to reveal genome-wide random and CRISPR associated AAV integration (AAV-Seq). **Results:** We observed 5-30% integration efficiencies as compared to insertion and deletion (indel) formation in the case of several genes (*Tmc1*, *APP*, *Mecp2*, *Dnmt3*, *FANCF*, *Dmd*, *Tor1A*) *in vitro* and *in vivo* after transduction with AAV. AAV integration was present not only in on-target, but also in validated off-target loci. Targeted deep-sequencing of PCR products revealed that one of the breakpoints in the AAV construct is found in the ITR, more specifically in the hairpin loops. AAV ITR integrants were present in flip and also flop configuration, with the 'a' region in ITR being the most abundant. Surprisingly, there was no difference in integration efficiency using a minimal AAV (175bp) compared to a full-length AAV construct (4.2kb), as assayed by integration-specific qPCR in U2-OS cells (assayed for *Tmc1*, *Tor1A*, *APP*, *VEGF*, *FANCF*) and targeted deep-sequencing. TA cloning after AAV-λ transduction revealed that one ITR is always present in the integrant sequence, while the other end is chewed back from the ITR and variable length sequences (including the full-length sequence) are integrated in CRISPR-induced breaks; with or without indels in the target genomic DNA. Finally, unbiased AAV-Seq identified genome-wide random and

system (CNS), these proteins are located at postsynaptic membrane of GABAergic synapses and contribute to cognitive function. Therefore impairment in CNS due to the lack of dystrophin and DGC have also been described in one third of DMD patients, including autism spectrum disorder, mental retardation and poor academic performance. Dystrophin is expressed in brain tissue, such as cerebellar Purkinje cells, amygdalae and hippocampal neurons in mouse. In dystrophin-deficient *mdx* mouse, which is model mouse with DMD, deficit of dystrophin and DGC in neurons leads to cognitive dysfunction. Microdystrophin therapy with adeno-associated virus (AAV) for *mdx* mouse has been shown to reduce skeletal muscle disease and cardiomyopathy. Meanwhile, it is unclear whether cognitive dysfunction is improved or not. To assess cognitive function and to evaluate microdystrophin therapy in the brain, we designed the quantitative evaluation methods using a combination of behavioural test and recording of spontaneous locomotor activity. We injected 1×10^{13} vector genome per kilogram of AAV9-CMV-microdystrophin via retro orbital vein to 3 days-old male *mdx* mouse. At 8 weeks-old, we performed behavioural test and recording of spontaneous locomotor activity. Western blot analysis confirmed dystrophin expression in brain tissue at 10 weeks after gene transfer. *Mdx* with AAV injection showed significantly reduce freezing time as behavioural test. Our results suggest that microdystrophin therapy improved cognitive dysfunction in *mdx* mouse. This observation methods may be useful in evaluating of *mdx* mouse compared with wild type or AAV injection *mdx* mouse in behavior analysis and cognitive function.

861. AAV-9 CRISPR Mediated Satellite Cell Editing Restored Dystrophin Expression after Complete Degeneration in a Whole Muscle Graft Model

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Abstract. Adeno-associated virus serotype 9 (AAV-9) mediated CRISPR-Cas9 gene editing is a promising approach to restore missing dystrophin for Duchenne muscular dystrophy (DMD) therapy. A permanent therapy likely depends on efficient repairing of satellite cells which are the stem cells responsible for muscle repair/regeneration throughout adult life. It is hotly debated whether AAV-9 CRISPR delivery can edit satellite cells. We addressed this issue using a whole muscle graft model. Specifically, the tibialis anterior (TA) muscle from the donor was grafted to the surgically cleared TA compartment in an immune deficient host mouse. The donor muscle undergoes complete necrosis followed by regeneration from satellite cells. We first determined the relative contribution of the donor and

host satellite cells to the formation of nascent myofibers in the graft. Using cross-transplantation between GFP+ and GFP- donor/host pairs, we found that ~97% regenerated myofibers were derived from the donor. However, traces of host myogenic cells were observed within TA grafts. Next, to investigate satellite cell editing, we treated 6-week-old dystrophin-null *mdx* mice with AAV-9 CRISPR via tail vein injection. 18 months later, we grafted the TA muscle to immune-deficient dystrophin-null host. AAV-9 CRISPR therapy resulted in 22.1 ± 13.0 dystrophin positive myofibers in the *mdx* TA muscle prior to graft. Following grafting, the regenerated muscle contained 4.6 ± 2.7 dystrophin positive fibers. As a control, we also grafted the TA muscle of age-matched *mdx* mice that were not treated by AAV-9 CRISPR. Prior to the grafting, there were 1.4 ± 0.5 dystrophin positive cells. In regenerated grafts, this number was 1.7 ± 1.2 , significantly lower than what was observed in AAV-9 CRISPR treated donors. Subsequent genomic sequencing confirmed the presence of the edited genome only in the grafts derived from AAV-9 CRISPR treated donors. Our data suggest that AAV-9 CRISPR delivery indeed resulted in satellite cell editing. Strategies that can improve the efficiency may have important implications for DMD gene repair therapy (supported by NIH and DOD).

862. Isolated Limb Infusion of rAAVrh74.MCK.GALGT2 in the Rhesus Macaque Allows for Broad Transgene and CT Glycan Expression in Limb Skeletal Muscles

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rAAV vectors require high doses for systemic treatment of cardiac and skeletal muscles, the target tissues in muscular dystrophy. In some instances, isolated limb infusion may allow for lower doses of vector to be used in a manner that reduces overall organ toxicity. Here we report on an isolated limb infusion technique in a non-human primate where hindlimb blood flow is transiently isolated during vector delivery to concentrate vector in targeted leg muscles. A bilateral dose of 2.5×10^{13} vector genomes (vg)/kg/limb of rAAVrh74.MCK.GALGT2 was sufficient to induce GALGT2 expression in 10-60% of skeletal myofibers in all leg muscles. Muscles from a limb receiving isolated limb infusion had, on average, a 12 ± 3 -fold increase in vg compared to the same muscles in an untreated limb after vector was released into the systemic circulation. Variability in AAV biodistribution between different segments of the same muscle, however, was high, suggesting limitations to uniform transduction. These results demonstrate that treatment of muscles throughout the leg with rAAVrh74.MCK.GALGT2 can be accomplished safely using an isolated limb infusion technique where balloon catheters transiently isolate the limb vasculature.

Duan D. CRISPR editing therapy for Duchenne muscular dystrophy. Aug 19, 2018 One Health Innovations-- One Health Applications of CRISPR/cas9 symposium. Kansas City, MO.

CRISPR editing therapy for Duchenne muscular dystrophy

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Duchenne muscular dystrophy (DMD) is the most common lethal muscle disease affecting ~250,000 to 300,000 boys and young men worldwide. DMD is caused by frame-shifting mutations in the dystrophin gene. CRISPR-mediated genome editing holds promise to restore the reading-frame of the mutated dystrophin gene. To explore this innovative therapeutic modality, we generated adeno-associated virus (AAV) vectors that express the Cas9 endonuclease and the guide RNA (gRNA). The gRNA was used to direct Cas9 to the selected targets in the dystrophin gene to directly remove the mutation or to modulate genomic splicing signals to allow production of an in-frame dystrophin transcript. DMD is a chronic disease affecting all muscles in the body. An effective therapy for DMD requires life-long treatment. Targeting satellite cells, the muscle stem cells, will allow regenerated myofibers to express dystrophin. We examined whether satellite cells can be edited by free muscle grafting. Our results suggest that AAV can transduce and edit satellite cells. To study long-term benefits, we treated 6-week-old mdx mice, a DMD model. At 18 months after therapy, we observed bodywide dystrophin restoration. Importantly, CRISPR therapy improved muscle force and heart function (Funded by NIH AR69085 and DOD MD150133).

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