Dose-Escalation Study of Systemically Delivered rAAVrh74.MHCK7.micro-dystrophin in the *mdx* Mouse Model of Duchenne Muscular Dystrophy

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BACKGROUND

- Duchenne muscular dystrophy (DMD) is the most common severe childhood form of muscular dystrophy[1]
- More than 2000 mutations of the DMD gene account for progressive loss of muscle, ambulation, and ultimately respiratory and cardiac function[1,2]
- DMD therapies targeting the root cause of disease, while applying to a variety of mutations, are needed
- We designed an adeno-associated virus (AAVrh74) vector containing a codon-optimized human micro-dystrophin transgene driven by a muscle-cardiac specific promoter, MHCK7 (rAAVrh74.MHCK7.micro-dystrophin) to achieve targeted skeletal and cardiac muscle expression of a shortened functional dystrophin protein

OBJECTIVE

- To assess the safety and efficacy of systemically delivered rAAVrh74.MHCK7.micro-dystrophin in dystrophin null (*mdx*) mice

METHODS

Animals

- All procedures were conducted in accordance with approval by The Research Institute at the Nationwide Children's Hospital Institutional Animal Care and Use Committee (protocol AR08-00009; AR06-00054)
- C57BL/6 and C57BL/10ScSn-Dmd*mdx*/J mice were maintained under standardized conditions on a 12:12 hour light:dark cycle, with food and water provided ad libitum

Micro-dystrophin gene construct and AAV vector production

- For all gene transfer studies, the human micro-dystrophin cassette contained the (R4–R23/Δ71–78) domains, as previously described[3]
- rAAVrh74.MHCK7.micro-dystrophin was packaged into AAV serotype rh74 capsid using the standard triple transfection protocol, as previously described[4,5]
- Constructs were diluted in Lactated Ringer's solution and was delivered intravenously (IV), as previously described, at doses noted in figure legends

Biological endpoints

- Micro-dystrophin expression was evaluated by immunofluorescence and western blot, as previously described[6]
- For histological evaluation, 12-µm thick cryosections of muscle were processed using:
  - Hematoxylin & eosin staining for morphometric analysis[4]
  - Picrosirius red staining (Polysciences Inc., Mount Arlington, NJ, Catalog #24901) for collagen quantification
  - Immunofluorescence to detect alpha sarcomeric actin expression and localization for assessment of ring fibers
  - Images were captured using a Zeiss (Germany) AxioCam MRC5 camera. All images were processed using National Institutes of Health's ImageJ software or Zeiss Axiovision LE4 software
- Serum analysis
  - Liver enzymes and blood glucose were assessed to evaluate toxicity
  - Creatine kinase was measured using the Creatine Kinase SL Assay (Sekisui Diagnostics, Charlottetown, PE, Canada; Catalog #326-10)
- Taqman quantitative PCR was performed to quantify the number of vector genome copies, as previously described[6-8]

Functional endpoints

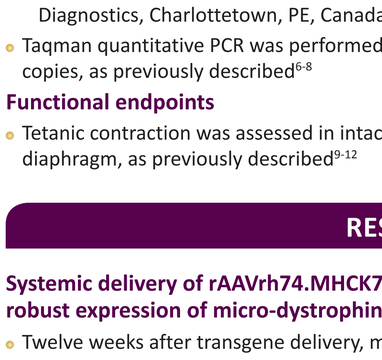
- Tetanic contraction was assessed in intact mice in the tibialis anterior and *ex vivo* in the diaphragm, as previously described[9-12]

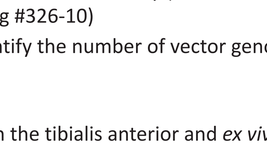
RESULTS

Systemic delivery of rAAVrh74.MHCK7.micro-dystrophin in *mdx* mice promotes robust expression of micro-dystrophin at the sarcolemma membrane

- Twelve weeks after transgene delivery, micro-dystrophin protein expression was demonstrated using immunofluorescence in both the left- and right-side skeletal muscles including: tibialis anterior (TA), quadriceps (QUAD), gastrocnemius (GAS), psoas major (PSOAS), and triceps (TRI) (**Figure 1**)
  - Mean (±SEM) expression of micro-dystrophin in treated mice was 46.7±8.08%, 66.8±6.18%, and 78.3±4.7% for 2.0x10^{12} vg (low) dose, 6.0x10^{12} vg (intermediate) dose, and 1.2x10^{13} vg (high) dose, respectively, across all tissues (**Figure 1**)
- An intermediate dose of rAAVrh74.MHCK7.micro-dystrophin restored expression of neuronal nitric oxide synthase in skeletal muscles (**Figure 1**)

Figure 1. Robust micro-dystrophin expression at the sarcolemma membrane of muscle tissues after systemic delivery of rAAVrh74.MHCK7.micro-dystrophin





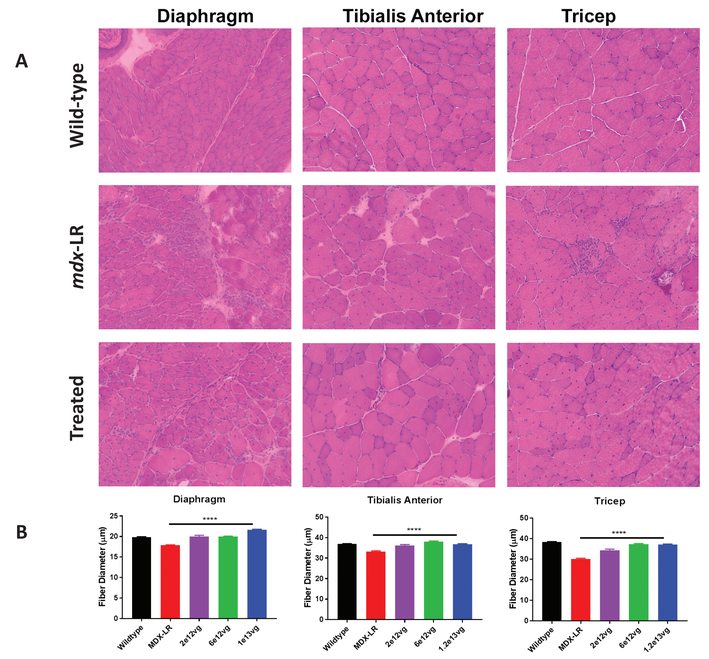
Mice received rAAVrh74.MHCK7.micro-dystrophin IV at 8x10^{13} vg/kg, 2x10^{14} vg/kg, or 6x10^{14} vg/kg, corresponding to 2.0x10^{12} vg (low), 6.0x10^{12} vg (intermediate), and 1.2x10^{13} vg (high) total dose, respectively. (A) Immunofluorescent staining for micro-dystrophin using an N-terminal dystrophin antibody in the heart, diaphragm, and tibialis anterior demonstrates robust expression in rAAVrh74.MHCK7.micro-dystrophin-treated animals 12 weeks post-injection (6.0x10^{12} vg) compared to Lactated Ringer's (vehicle)-injected *mdx* control mice (*mdx*-LR, second row panel). Beta-sarcoglycan staining in the vector-treated cohorts represents normalization of the dystrophin-associated protein complex. 20x images are shown. (B) Immunofluorescent quantification demonstrates percent fiber expression of dystrophin. Immunofluorescence intensity in both intermediate-dose (6x10^{12} vg) and high-dose cohorts (1.2x10^{13} vg) are above 50% in all tissues. Data are reported as means ± SEM of n = 5 per treatment group. (C) Neuronal nitric oxide synthase expression is demonstrated in *mdx* mice treated with rAAVrh74.MHCK7.micro-dystrophin. Abbreviations: BSG, beta-sarcoglycan; DIA, diaphragm; GAS, gastrocnemius; GLUT, gluteal; HRT,cardiac; nNOS, neuronal nitric oxide synthase; PSO, psoas major; QD, quadriceps; TA, tibialis anterior; TRI, triceps.

RESULTS continued

Systemic delivery of rAAVrh74.MHCK7.micro-dystrophin: histopathology and muscle function

- Quantification of histological parameters demonstrated a significant reduction in central nucleation in all skeletal muscles analyzed in a dose-dependent manner, with no significant decreases between the muscles of the intermediate dose versus the high dose (**Figure 2**)

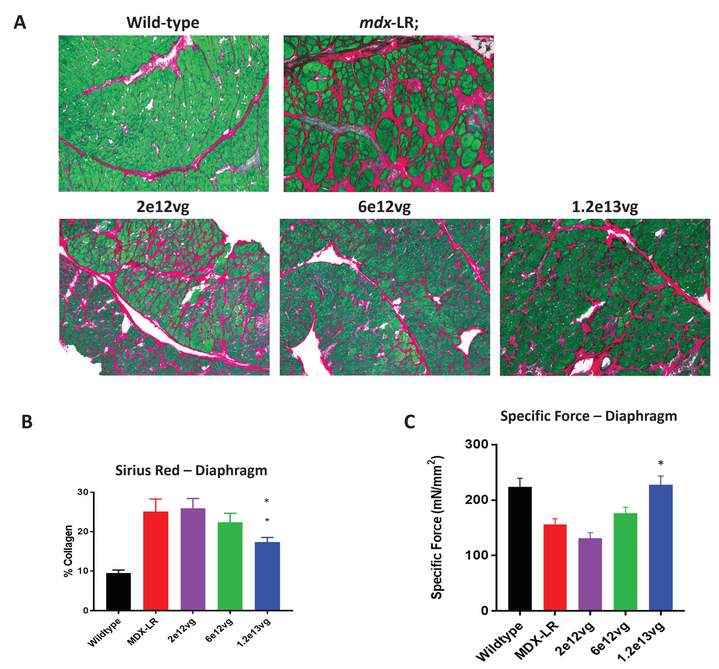
Figure 2. Systemic treatment with rAAVrh74.MHCK7.micro-dystrophin improves muscle pathology in *mdx* mice



Muscle tissue sections were stained using hematoxylin & eosin (H&E) and morphology was analyzed by measuring fiber diameter. (A) Representative images of diaphragm, tibialis anterior, and tricep muscle from wild-type, *mdx*-LR and vector-dosed mice (rAAVrh74.MHCK7.micro-dystrophin). 20x images are shown. (B) Quantification of average fiber size demonstrates a normalization of fiber size to that of wild-type mice across all tissue in *mdx* mice treated with various doses of rAAVrh74.MHCK7.micro-dystrophin (total dose of 2.0x10^{12} vg, 6.0x10^{12} vg, or 1.2x10^{13} vg). Data represent n = 5 per treatment group. Data were analyzed by One-Way ANOVA followed by Tukey's post hoc analysis. ****=p<0.0001 vs vehicle-treated (Lactated Ringer's) *mdx*-LR controls.

- Gene transfer with rAAVrh74.MHCK7.micro-dystrophin significantly decreased collagen deposition in the diaphragm, which corresponded to a significant increase in force output of the diaphragm to levels comparable to that observed in wild-type mice (**Figure 3A-B**)

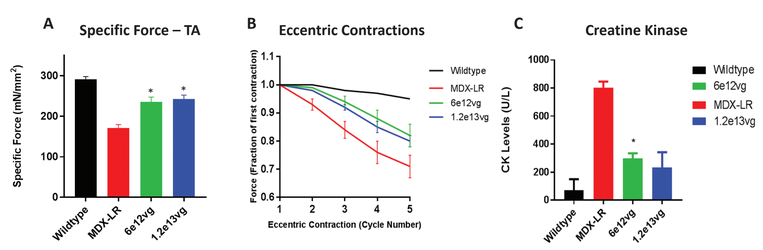
Figure 3. Reduction of fibrosis after systemic treatment with rAAVrh74.MHCK7.micro-dystrophin is associated with improvement in diaphragm muscle function



Muscle tissue sections were stained using picrosirius red stain to assess collagen content. (A) Representative images of diaphragm tissue sections show a decrease in amount of tissue stained red, indicative of reduction in collagen content, a marker of fibrosis, in *mdx* mice treated with rAAVrh74.MHCK7.micro-dystrophin compared to *mdx*-LR control mice. 20x images are shown. (B) Dose-dependent reduction in collagen accumulation (%) in the diaphragm. Quantification of the percent of collagen in the diaphragm is decreased to levels that are observed in wild-type in *mdx* mice treated at both intermediate (6.0x10^{12} vg) and high (1.2x10^{13} vg) doses. Data represent n = 5 per treatment group. Data were analyzed by One-Way ANOVA followed by Tukey's post hoc analysis. *=p<0.05 vs *mdx*-LR (Lactated Ringer's) control mice. (C) Dose-dependent restoration of diaphragm muscle function in *mdx* mice treated with rAAVrh74.MHCK7.micro-dystrophin. Following 12 weeks of treatment, diaphragm muscle strips were harvested to measure specific force (normalized to cross sectional area). Treatment restored force to wild-type levels.

- Twelve weeks post-treatment, specific force increased in the diaphragm (**Figure 3C**) and tibialis anterior muscle (**Figure 4**), with intermediate and high doses eliciting force outputs at wild-type levels

Figure 4. Functional benefits to skeletal muscle in intermediate- and high-dose cohorts



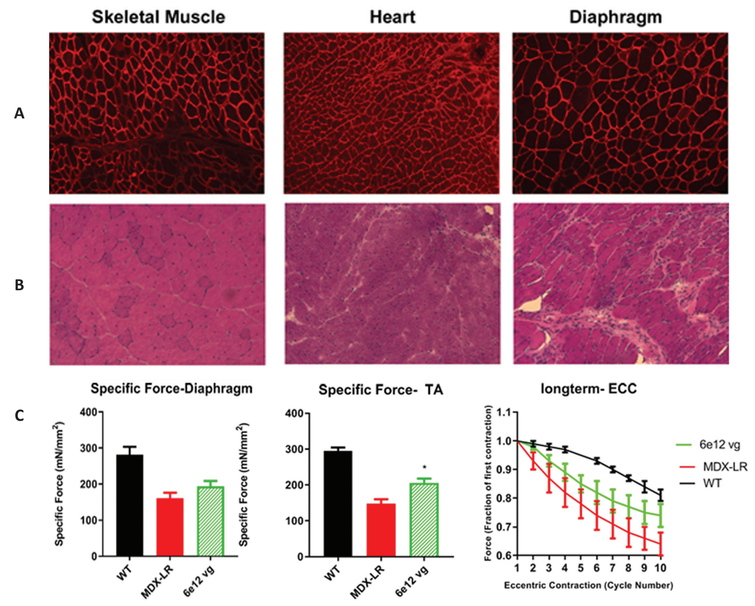
mdx mice received Lactated Ringer's solution (*mdx*-LR) (controls) or rAAVrh74.MHCK7.micro-dystrophin IV at 2x10^{14} vg/kg or 6x10^{14} vg/kg, corresponding to an intermediate (6.0x10^{12} vg) or high (1.2x10^{13} vg) total dose, respectively. (A) Following 12 weeks of treatment, left and right tibialis anterior muscles were harvested to measure specific force (normalized to tibialis anterior weight). Treatment restored force to wild-type levels (*p=<0.01). (B) Treatment rescued tibialis anterior muscles from fatigue after a rigorous protocol of eccentric contractions. (C) Creatine kinase levels in the serum decreased with intermediate-dose (6.0x10^{12} vg) and high-dose (1.2x10^{13} vg) treatment. Data represent n = 5 per treatment group. Data were analyzed by One-Way ANOVA followed by Tukey's post hoc analysis. *=p<0.05 vs *mdx*-LR control mice.

CONCLUSIONS

- Systemic delivery of rAAVrh74.MHCK7.micro-dystrophin resulted in robust expression of a functional shortened dystrophin protein in cardiac and skeletal muscles throughout the body in an *mdx* mouse model of DMD
- Marked improvements in histopathological hallmarks of disease that were observed in rAAVrh74.MHCK7.micro-dystrophin treated *mdx* mice coincided with significant improvements in functional outcomes and provides evidence to support the ability of restored micro-dystrophin expression to prevent neuromuscular degeneration
- Lack of toxicities observed from necropsy and serum chemistry panel in *mdx* mice treated with rAAVrh74.MHCK7.micro-dystrophin suggests that administration of a high vector dose may be tolerated
- Findings from this preclinical study provide proof of principle for safety and efficacy of systemic delivery of rAAVrh74.MHCK7.micro-dystrophin at high vector titers, supporting initiation of a Phase I/II safety study in boys with DMD

- Six months post-injection, micro-dystrophin transgene expression was demonstrated in 6 skeletal muscles (TA, GAS, QUAD, TRI, PSOAS, and GLUT), both left and right, in addition to the diaphragm and heart of all treated mice
  - Specifically, cardiac expression equaled ≥95% positive fibers, as shown with immunofluorescent staining of micro-dystrophin in all treated mice (**Figure 5**)

Figure 5. Long-term durability after a single systemic administration of rAAV.MHCK7.micro-dystrophin in *mdx* mice

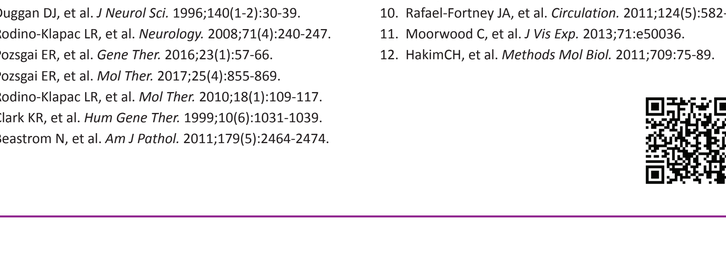


Long-term effects of rAAVrh74.MHCK7.micro-dystrophin in *mdx* mice treated with an intermediate dose. (A) Representative images of heart, diaphragm, and tibialis anterior following immunofluorescent staining for micro-dystrophin using an N terminal dystrophin antibody demonstrates robust expression in rAAV.MHCK7.micro-dystrophin-treated animals 6 months (24 weeks) post-injection. 20x images are shown. (B) Hematoxylin & eosin staining demonstrates normalization of muscle environment in skeletal muscle, heart, and diaphragm tissue in dosed cohorts. 20x images are shown. (C) Functional improvements are demonstrated by the observed increase in specific force in the diaphragm compared to the control cohort (*mdx*-LR), increase in specific force in the tibialis anterior muscle, and improvements in eccentric contractions in the tibialis anterior muscle. Data represent mean ± SEM of n = 5 per treatment group. Data were analyzed by One-Way ANOVA followed by Tukey's post hoc analysis. *=p<0.05 vs *mdx*-LR (Lactated Ringer's) controls mice.

Biodistribution and safety of systemic delivery of rAAVrh74.MHCK7.micro-dystrophin in *mdx* mice

- Vector genome copies were detected at varying levels in all collected tissues. As expected, the highest levels were seen in skeletal muscle and the heart, as well as clearance organs (liver). The lowest levels were detected in gonad, lung, and kidney in all dose levels
- No adverse effects due to treatment with rAAVrh74.MHCK7.micro-dystrophin were reported, either by clinical or organ-specific laboratory assessments or by necropsy
- No other abnormal muscle pathologies were observed in mice treated with rAAVrh74.MHCK7.micro-dystrophin, including ring fiber formation (**Figure 6**), indicating restoration of a functional shortened protein at the sarcolemmal membrane of muscle fibers

Figure 6. Absence of ring fibers in *mdx* mice treated with rAAVrh74.MHCK7.micro-dystrophin construct containing Hinge 2



Using an alpha sarcomeric actin antibody to evaluate the presence or instance of ringed fibers, there were no instances of ringed fibers in wild-type mice, *mdx*-LR untreated mice, or rAAVrh74.MHCK7.microdystrophin-treated mice (construct contains Hinge 2). Images are representative of the gastrocnemius and taken at 20X.

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