Functional and Histological Improvements Comparing 4 Micro-dystrophin Constructs in the mdx Mouse Model of DMD

Rachael A. Potter[1,3], Danielle A. Griffin[1,3], Kristin N. Heller[1], Jerry R. Mendell[1,2], and Louise R. Rodino-Klapac[1,2,3]

[1]Center for Gene Therapy, The Research Institute at Nationwide Children's Hospital, Columbus, Ohio, USA, [2]Department of Pediatrics and Neurology, The Ohio State University, Columbus, Ohio, USA, [3]Sarepta Therapeutics, Inc., Cambridge, Massachusetts, USA

BACKGROUND

- Duchenne muscular dystrophy (DMD) is caused by a mutation in the dystrophin gene, resulting in production of a nonfunctional dystrophin protein and leading to a progressive loss of muscle strength, ambulation, and ultimately respiratory and cardiac failure.[1,2]
- Several AAV-based gene transfer therapies are currently under development, all designed with the goal of restoring production of functional dystrophin protein (NCT03375164, NCT03368742, NCT03362502).
  - –The dystrophin gene is too large to be packaged into an AAV capsid and therefore must be modified in a way that maintains the functional properties of the full-length gene.
  - –Any modification of full-length dystrophin must maintain specific spectrin repeats and hinge regions within the dystrophin protein that are essential for maintaining its function.
- With this in mind, we developed rAAVrh74.MHCK7.micro-dystrophin, which contains a shortened version of the dystrophin gene (micro-dystrophin) under control of the MHCK7 promoter to ensure skeletal and cardiac muscle expression.
- To maximize efficacy, we included essential regions in our transgene sequence (ΔR4-23/ΔC) that would closely resemble native dystrophin structure and ensure proper functioning.

OBJECTIVE

- The objective of this study was to demonstrate and compare the efficacy of 4 unique rAAVrh74 vector constructs following intramuscular delivery in dystrophin null (MDX) mouse model of DMD.

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.
- C57BL/6 and C57BL/10ScSn-Dmdmdx/J mice were maintained under standardized conditions on a 12:12 hour light:dark cycle, with food and water provided ad libitum.

rAAVrh74 vector constructs

- We designed 4 constructs of rAAVrh74 vector (**Figure 1**).
- Constructs were delivered intramuscularly in the left tibialis anterior (LTA) muscle. The contralateral right tibialis anterior muscle (RTA) was untreated and served as the comparator.

Functional endpoints

- Tetanic contraction was assessed in intact mice in the tibialis anterior, as previously described.[3-6]

Biological endpoints

- Micro-dystrophin expression was evaluated by immunofluorescence, as previously described.[7]
- Histological evaluations were performed using 12-µm thick cryosections of muscle, as previously described.[8]

RESULTS

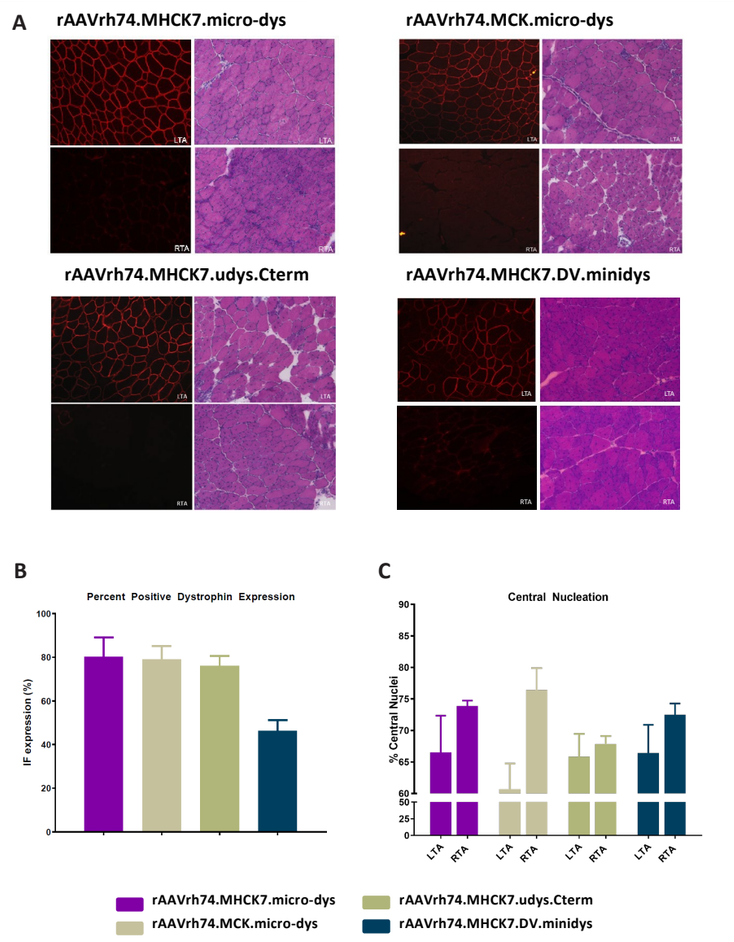
Dystrophin expression

- Four weeks after transgene delivery, micro-dystrophin protein expression was robustly increased with the rAAVrh74.MHCK7.micro-dys construct compared to the other constructs (**Figure 2A** and **B**).
- Percent positive dystrophin expression in mice treated with the rAAVrh74.MHCK7.micro-dys was 80% compared to 78% for the rAAVrh74.MCK.micro-dys, ~75% for the rAAVrh74.MHCK7.udys.Cterm construct, and ~50% for the rAAVrh74.MHCK7.DV.minidys (**Figure 2B**). Additionally, rAAVrh74.MHCK7.micro-dys was assessed using sarcolemma intensity of dystrophin and was significantly more intense at the membrane compared to the three other constructs.

Histopathology

The muscle environment in the rAAVrh74.MHCK7.micro-dys construct was normalized, which is demonstrated by reductions in centralized nucleation (**Figure 2A** and **C**).

Figure 2. Dystrophin expression levels and improvement in muscle morphology depends on promoter and transgene



Mice received vector constructs intramuscularly in the left tibialis anterior (LTA). The untreated right tibialis anterior (RTA) is the contralateral (untreated) limb. **(A)** Representative images of immunofluorescent staining for micro-dystrophin 4 weeks post-injection. 20x images are shown. **(B)** Quantification of dystrophin fiber expression assessed by immunofluorescence. Quantification of intensity of dystrophin expression at the sarcolemma and protein levels are significantly increased in the MHCK7.micro-dys construct compared to the other three constructs (data not shown) **(C)** Improvement in dystrophic pathology (i.e., reduction in central nucleation) was observed in treated TA (LTA) after treatment with the constructs with the micro-dys transgene.

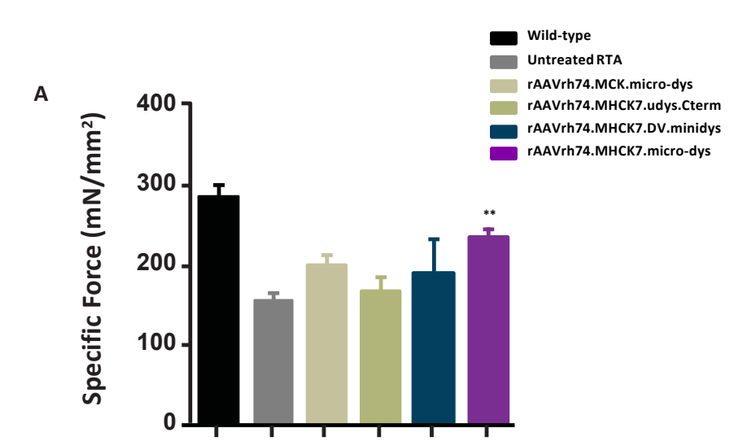
CONCLUSIONS

- The results of this study highlight the importance in the design of the transgene sequence in promoting maximal dystrophin expression.
- Furthermore, these data demonstrate that the microdystrophin transgene sequence (ΔR4-23/ΔC) driven by the MHCK7 promoter produced the most advantageous outcome measures in the mdx mouse among these constructs. Inclusion of the nNOS domain or nNOS sarcolemma localization in the DV.minidys or MHCK7.udys.Cterm constructs did not improve functional outcomes. With no functional benefit and the need for two vectors at high dose, the DV.minidys construct was not chosen as a clinical candidate.
- The findings in this preclinical study provided proof-of-principle for safety and efficacy for systemic delivery of rAAVrh74.MHCK7.micro-dystrophin in a dose-escalation study in the mdx mouse model for DMD.

Muscle function

- Four weeks after transgene delivery, specific force output increased in the tibialis anterior (TA) muscle for the rAAVrh74.MHCK7.micro-dys construct compared to the other constructs, and there was no difference from wild-type levels (**Figure 3A** and **B**).
- Functional benefit was 15% higher in the rAAVrh74.MHCK7.micro-dys construct compared to the rAAVrh74.MCK.micro-dys construct.
- No functional improvements above the Untreated TA control was observed for the rAAVrh74.MHCK7.udys.Cterm and rAAVrh74.MHCK7.DV.minidys constructs.

Figure 3. rAAVrh74.MHCK7.micro-dystrophin produces the greatest functional improvement in muscle

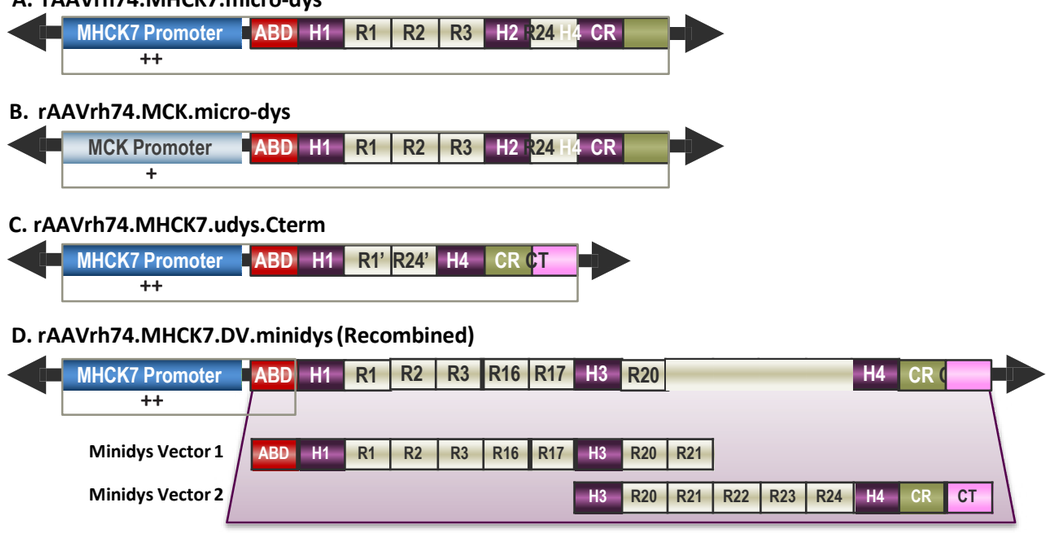


Four weeks after treatment, left tibialis anterior muscles were harvested to measure **(A)** specific force (normalized to tibialis anterior weight). ** indicates statistical differences compared to untreated TA ($p<.05$)

- Eccentric force loss for Dys$^{\Delta R4\text{-}23/\Delta CT}$-*mdx* mice was comparable to that in WT (C57BL/10) mice[1]
- Dys$^{\Delta 2\text{-}15/\Delta 18\text{-}23/\Delta CT}$-*mdx* had an intermediate eccentric force loss[1]
- Data support a role for the dystrophin **R1-R3 membrane-binding domain** in modulating radial force transmission and mechanical vulnerability[1-3]

HSA, human skeletal actin.
*P<0.001 vs C57BL/10.
1. Nelson DM, et al. *Hum Mol Genet*. 2018;27(12):2090-2100.
2. Legardinier S, et al. *Biochim Biophys Acta*. 2008;1784(4):672-682.
3. Zhao J, et al. *Hum Mol Genet*. 2016;25(17):3647-3653.

Figure 1. Construct schematics



* R1-3 has been shown to be essential for maximal protection against eccentric force loss.[9]
** R16/17 contains an nNOS binding domain.
***Hinge domains are important for conferring flexibility,[10] but no differences in restoration of muscle functional capacity have been found between hinge 2 and hinge 3.[11,12] ' indicates partial repeat/hinge.
****Indicates the construct with the addition of the C-terminus localizes nNOS to the sarcolemma.
+MCK promoter directs tissue-specific expression in both skeletal and cardiac muscle.
++MHCK7 promoter drives robust expression selectively in skeletal and cardiac muscle through the addition of α-MHC that enhances expression in cardiac muscle.[13]

ACKNOWLEDGMENTS & DISCLOSURES

No competing financial interests exist for the authors. This study was sponsored by Sarepta Therapeutics, Inc. The authors would like to thank Khampaseuth Thapa for her assistance. Medical writing and editorial support was provided by Health & Wellness Partners, LLC, Upper Saddle River, New Jersey, funded by Sarepta Therapeutics, Inc. This work has been supported by Nationwide Children's Hospital Foundation to L.R.R.K and J.R.M.

REFERENCES

1. Hoffman EP, et al. *Cell*. 1987;51(6):919-928.
2. Koenig M, et al. *Cell*. 1987;50(3):509-517.
3. Pozsgai ER, et al. *Gene Ther*. 2016;23(1):57-66.
4. Koo T, et al. *J Gene Med*. 2011;13:497-506.
5. Liu M, et al. *Mol Ther*. 2005;11:245-256.
6. Schinkel S, et al. *Hum Gene Ther*. 2012;23:566-575.
7. Shin JH, et al. *Gene Ther*. 2011;18:910-919.
8. Rodino-Klapac LR, et al. *J Transl Med*. 2007;5:45.
9. Nelson DM, et al. *Hum Mol Genet*. 2018;27(12):2090-2100.
10. Koenig M, Kunkel LM. *J Biol Chem*. 1990;265(8):4560-4566.
11. Harper SQ, et al. *Nat Med*. 2002;8(3):253-261.
12. Banks GB, et al. *PLoS Genet*. 2010; 6(5):e1000958.
13. Salva MZ, et al. *Mol Ther*. 2007;15(2):320-329.