ORIGINAL ARTICLE

Therapeutic impact of systemic AAV-mediated RNA interference in a mouse model of myotonic dystrophy

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Abstract

RNA interference (RNAi) offers a promising therapeutic approach for dominant genetic disorders that involve gain-of-function mechanisms. One candidate disease for RNAi therapy application is myotonic dystrophy type 1 (DM1), which results from toxicity of a mutant mRNA. DM1 is caused by expansion of a CTG repeat in the 3′ UTR of the DMPK gene. The expression of DMPK mRNA containing an expanded CUG repeat (CUGexp) leads to defects in RNA biogenesis and turnover. We designed miRNA-based RNAi hairpins to target the CUGexp mRNA in the human α-skeletal muscle actin long-repeat (HSA LR) mouse model of DM1. RNAi expression cassettes were delivered to HSA LR mice using recombinant adeno-associated viral (rAAV) vectors injected intravenously as a route to systemic gene therapy. Vector delivery significantly reduced disease pathology in muscles of the HSA LR mice, including a reduction in the CUGexp mRNA, a reduction in myotonic discharges, a shift toward adult pre-mRNA splicing patterns, reduced myofiber hypertrophy and a decrease in myonuclear foci containing the CUGexp mRNA. Significant reversal of hallmarks of DM1 in the rAAV RNAi-treated HSA LR mice indicate that defects characteristic of DM1 can be mitigated with a systemic RNAi approach targeting the nuclei of terminally differentiated myofibers. Efficient rAAV-mediated delivery of RNAi has the potential to provide a long-term therapy for DM1 and other dominant muscular dystrophies.

Introduction

A direct approach for treating autosomal dominant gain-of-function genetic disease is inhibition or downregulation of expression of the dominant-acting gene product. For myotonic dystrophy (DM), the molecular effector is a mutant mRNA carrying an expressed DNA repeat, either a CTG repeat from the dystrophy myotonia-protein kinase gene (DMPK; type 1 [DM1]) or an expanded CCTG repeat expressed from intron 1 of the CCHC-type zinc finger nucleic acid binding protein gene (CNBP; type 2 [DM2]) (1–4). The expanded repeat regions do not result in protein mutation because they are located in the 3′ UTR and intronic pre-mRNA, respectively, but lead to nuclear transcript accumulation that affects the localization and activities of RNA-binding proteins, including the muscleblind-like proteins (MBNLs) and the CELF protein CUGBP1 (5–9). MBNL proteins regulate alternative splicing of a subset of mRNAs, and their CUGexp binding and high level of sequestration in DM nuclear foci contributes to the wide-ranging manifestations of disease, such as delayed muscle relaxation (myotonia), insulin insensitivity, muscle weakness and cardiac arrhythmias (10–12) [reviewed in (13,14)]. CELF proteins have been directly linked to DM muscle phenotypes through missplicing of the insulin receptor and the muscle-specific chloride channel mRNAs (15). Additionally, upregulation of CUGBP1 in mice reproduces some features of DM, including muscle wasting, and knockout mice show improved muscle histopathology (16,17).

The major aspects of the DM1 phenotype are potentially reversible by targeting the nuclear CUGexp DMPK mRNA. Recent therapeutic strategies targeting a similar CUGexp mRNA have
shown success in the HSA1-8 transgenic mouse model of DM1, which expresses CTGexp from within the 3′ UTR of the α-skeletal muscle actin gene, HSA or ACTA1 (18). The HSA1-8 mouse exhibits nuclear foci, myotonia and many of the splicing defects that are characteristic of the human disease. Strong expression from the HSA promoter results in 2–8-fold higher levels of CUGexp mRNA in HSA1-8 mice than in muscles of individuals with DM1 (19). Consistent with the DM phenotype, the HSA1-8 mouse also displays robust myotonia and pronounced pre-mRNA splicing changes, although muscle wasting and weakness are less severe (20). The explanation for this discrepancy is unknown, but may relate to differences in developmental expression (later onset in mice) and size of the repeat tract (220 repeats versus >3000 in DM1 patients) (21). In terms of developing a whole-body treatment for DM1, the deficiency of the HSA1-8 mouse model is the lack of a multisystem phenotypic representation of the human condition due to muscle-specific expression of the CUGexp mRNA. Therefore, the HSA1-8 mouse, as a muscle-specific disease model, provides a platform for developing strategies targeting a widespread and abundant tissue, but does not allow for evaluation of therapeutic efficacy for disease complications in motor neurons and other organs. Several groups have used antisense oligonucleotide (ASO) strategies in the HSA1-8 mouse for mitigation of phenotypic effects in muscle as a proof of principle (19-22,24). These studies served to validate CUGexp mRNA targeting as a route to therapeutic intervention.

We demonstrated previously that RNAi silencing of a cytoplasmic mRNA via systemic delivery of a rAAV vector was effective at reducing histological, biochemical, immunological and functional disease features in skeletal muscles of an FRG1 over-expressing transgenic mouse model of facioscapulohumeral muscular dystrophy (34). In a separate study of the FRG1 mouse, intramuscular injection of rAAV-delivered RNA hairpins prevented FRG1 phenotype development in the TA muscles (35). Although RNAi is believed to function mainly in the cytoplasm, studies of nuclear expressed shRNAs in cells in vitro have shown that RNAi can reduce levels of nuclear 7SK and U6 snRNAs (36). In addition, studies demonstrated that shRNAs could reduce the level of nuclear-retained, mutant DMPK mRNA in DM1 cells in vitro (37). We therefore explored whether a nuclear localized CUGexp mRNA could be reduced using rAAV-mediated systemic delivery of therapeutic RNAi. To test this possibility, we applied systemic RNAi delivery in the HSA1-8 mouse model of DM using the muscle-tropic rAAV serotype 6 (rAAV6) (38). We chose the HSA1-8 mouse model because it was the most disease representative model that was well characterized and available for study, anticipating that the DMPK mRNA is accessible because of successful viral-mediated RNAi silencing in DM1 patient cells (37).

In this study, we show that intravenous injection of rAAV-HSA-RNAi in HSA1-8 mice mitigates the pathological effects of CUGexp mRNA expression, including myotonia, myofiber size variability, misregulated splicing and the focal accumulation of CUGexp mRNA and Mbnl1. Bodywide delivery of artificial miRNA expression cassettes to muscle is well tolerated in HSA1-8 mice at doses sufficient to modulate the disease phenotype. These results demonstrate that a nuclear localized pathological mRNA can be targeted with systemic RNAi delivery, raising the possibility of using rAAV-mediated RNAi delivery for long-term treatment for DM.

**Results**

**Evaluation of candidate sequences for HSA1-8 transgene silencing**

To develop vectors capable of targeting the HSA1-8 mRNA, we initially screened for target sequences specific for human α-skeletal muscle actin mRNA (ACTA1) in the HSA1-8 transgenic mice. Sixteen target sequences within the HSA mRNA were chosen based on established design rules, and excluded regions that were homologous with the mouse Acta1 mRNA sequence (39-42). For in vitro testing of the HSA shRNAs, the HSA gene cDNA sequence was cloned downstream of firefly luciferase (Luc; Fig. 1A). Candidate HSA shRNAs were expressed from the mouse U6 (mU6) promoter and compared for knockdown efficiency in a co-transfection assay in COS-1 cells. Controls for knockdown efficiency were plasmids lacking an shRNA sequence or expressing a β-galactosidase (β-gal) shRNA. HSA mRNA reduction ranged from 55 to 88% of control levels. From the 16 shRNA target sequences, two were chosen for testing in vivo based on their differing locations either in the coding sequence or in the 3′ UTR where miRNAs typically bind for translational repression. Based on mRNA target regions known to be effective for gene knockdown generally and on in vitro activity criteria, HSA4 (targeting exon 5) and HSA10 (targeting exon 7 in the 3′ UTR) were selected for further testing.

We initially assessed HSA4 and HSA10 guide siRNA expression levels processed from the shRNAs in vitro, using northern blot analysis (Supplementary Material, Fig. S1). rAAV plasmids with the HSA4 and HSA10 hairpin expression cassettes were transfected into HEK293 cells, and total RNA was isolated 48 h later. We compared northern blot results from transfection of rAAV plasmids containing an shRNA, a 5′ extended shRNA and a microRNA (miR) carrying the same HSA target sequences. Radioactively labeled ASO probes identified guide strand siRNAs produced from all three types of hairpins. RNAi hairpins expressed from miRNA templates are typically found at much lower levels than shRNAs, and therefore, the siRNAs produced from the miR hairpins are difficult to detect even with sensitive radioactive probes (29). Because the miRNA-based shuttles generally have proven safe and effective at these expression levels as measured in vivo, we chose the mir-30-based RNAi hairpins for systemic RNAi therapeutic testing (43). The rAAV6-HSA-RNAi vectors developed for in vivo testing also carried the reporter gene human placental alkaline phosphatase (hPLAP) for identification of transduced myofibers (Fig. 1B).

**Systemic administration of AAV6-HSA-RNAi vectors**

The RNAi vectors were tested for the ability to silence the CUGexp repeat-containing HSA mRNA in HSA1-8 mice following systemic
Histological changes in HSA<sup>LR</sup> mice are mild, making it difficult to quantitate the effects on myopathy in hematoxylin and eosin (H&E)-stained cryosections. However, we found that wheat germ agglutinin fluorescent staining of the myofiber sarcolemma clearly delimited myofibers in rAAV6-HSA10-treated HSA<sup>LR</sup> and control mice to facilitate measurement of cross-sectional areas (CSAs; Fig. 2B). Hindlimb muscles in HSA<sup>LR</sup> mice are known to exhibit unusual variability of myofiber size, including a population of abnormally large fibers that may reflect activity-dependent hypertrophy resulting from myotonia (18). Frequency distribution analysis showed a reduction in the larger HSA<sup>LR</sup> myofibers following rAAV-HSA10 administration shifting the size distribution more in line with the WT FVB strain. Therefore, treatment of HSA<sup>LR</sup> mice with rAAV6-HSA10 had an effect on myofiber morphology of the HSA<sup>LR</sup> mice. In contrast, the rAAV6-HSA4 vector administration did not lead to changes in myofiber morphology compared with the untreated HSA<sup>LR</sup> mice in H&E-stained cryosections and were not evaluated further with CSA measurements.

Systemic administration of rAAV6-HSA10 to HSA<sup>LR</sup> mice reduces the level of CUG<sup>exp</sup> HSA transcript and coincides with expression of the HSA10 guide siRNA

To evaluate the level of silencing of the CUG<sup>exp</sup> HSA<sup>LR</sup> transcript, we performed quantitative PCR using total RNA from the TA muscles of the rAAV-HSA10-treated mice. RT-qPCR results showed a reduction in CUG<sup>exp</sup> HSA transcript to as low as 5% of the levels in untreated HSA<sup>LR</sup> mice at the 8 week post-injection time point (Fig. 3A). HSA<sup>LR</sup> transcript reduction ranged from 60 to 95% and is consistent with the level of CUG<sup>exp</sup> HSA<sup>LR</sup> mRNA reduction, previously shown to be necessary for decreasing myotonic discharges and reversing splicing defects (19,24).

To verify the production and to estimate the number of artificial siRNAs made from the miRNA-based hairpins in rAAV6-HSA10-treated muscle tissue, we performed small RNA qPCR. Total RNA was analyzed by small RNA RT-qPCR using primers homologous to the HSA<sup>LR</sup> miRNA scaffold and reporter gene in the AAV transduction in tibialis anterior (TA), quadriceps and gastrocnemius muscles as is typically seen for rAAV gene delivery (38). Quantitation of serum levels of the liver enzymes alanine transaminase (ALT) and aspartate aminotransferase (AST) at 3–4 weeks post-injection showed no elevation, a time when oversaturation of the RNAi pathway can result in liver damage (28,44). ALT and AST levels remained in the normal range at 8 and 10–12 weeks post-treatment (Supplementary Material, Fig. S2; normal range: ALT, 26–56; AST, 66–132). To assess muscle tissue damage, serum levels of creatine kinase (CK) were also measured and found to be normal (normal range: CK 100–1500).

Treatment of the HSA<sup>LR</sup> mice with rAAV6-HSA10 reduces myotonic discharges in muscle

Reduced levels of the Mbnl1 splicing factor in the HSA<sup>LR</sup> mice lead to misregulated alternative splicing of the muscle-specific chloride channel, Clcn1, resulting in loss of the chloride channel activity (12,46). The loss of chloride conductance causes repetitive action potentials, or myotonia, that are detected by electromyographic (EMG) techniques. HSA<sup>LR</sup> mice treated with rAAV6-HSA10 displayed a reduction in myotonia grade in the TA muscle with variation between individual mice tested at 8 weeks post-injection (Fig. 4A). Myotonia continued to lessen with analysis at the 12 week time point (Fig. 4B) and was consistently decreased in the quadriceps muscle (Fig. 4C). No change was detected in untreated HSA<sup>LR</sup> mice (point at right in all panels Fig. 4), and only minor variations were observed in the rAAV6-HSA4-treated animals (Fig. 4D).
Splicing regulation is improved in the rAAV6-HSA10-treated HSA LR mice

Splicing defects in HSA LR and Mbnl1 knockout mice are highly concordant, and recapitulate many, but not all changes in human DM1 (11). To assess RNAi effects on splicing regulation, we examined three exons that are regulated by Mbnl1 and strongly affected in DM1. Alternative splicing of Atp2α1 exon 22 switches from near complete inclusion in normal muscle to strong skipping in DM1 and HSA LR muscles. The TA muscles from the rAAV6-HSA10-treated HSA LR mice displayed on average 75 and 85% inclusion of exon 22 at 8 and 12 weeks post injection, respectively (Fig. 5A; P < 0.005). Cln1 mRNA splicing was largely corrected, as indicated by a reduction in inclusion of exon 7a from 20 to 8%, a level approaching WT levels (Fig. 5B and C; Supplementary Material, Fig. S3B; P < 0.0001 for untreated versus treated; ns for treated versus WT 8 weeks post injection). Consistent with previous observations that HSA LR transgene expression is approximately 2-fold higher in quadriceps than in TA muscles (19), we observed less reversion of splicing defects in Atp2α1 mRNA in the quadriceps muscle, as well as the gastrocnemius muscle (Supplementary Material, Figs S3A and S3C). A third mRNA, from the M line titin gene, m-Ttn, was also examined for reversion of splicing and displayed up to a 60% shift to the adult mRNA isoform (Supplementary Material, Fig. S3C). To control for non-specific or vector related effects on the disease phenotype, we injected 4 × 10^{12} vg of a rAAV6 containing either the miRNA sequence for targeting bacterial lacZ RNA (βgal) or only the hPLAP gene without an RNAi hairpin sequence (47). There was not a significant difference in the percent exclusion of exon 22 of the Atp2α1 mRNA in the HSA LR TA muscles using either control vector compared with untreated mice (Fig. 5D). In addition, splicing changes were not significant with the rAAV6-HSA4 vector, possibly from lower activity of the HSA4 hairpin, because expression of the HSA4 miR was comparable to HSA10 miR in vitro (Supplementary Material, Figs S1 and S3D). The lack of Atp2α1 mRNA splicing changes with rAAV6-HSA4 treatment was consistent with the inability of this vector to reduce myotonia in these mice.

To test whether the phenotypic improvement correlated with vector dose additional HSA LR, mice were examined that had received a lower dose of rAAV6-HSA10. Tail vein injection of 2 × 10^{12} vg resulted in no change in myotonia (data not shown), reduced hPLAP staining and limited correction of Atp2α1 and Cln1 mRNA splicing (Supplementary Material, Fig. S4) (34,47). Splicing assays showed a trend toward reversal to the adult isoform between the 8 and 12 week time points post treatment that reached significance at 25% reduction of exon 7a inclusion for Cln1 mRNA splicing. The lower vector dose resulted in less reduction of the
Figure 3. HSA mRNA reduction in TA muscles and copy number quantitation of the artificial HSA miRNAs expressed in vivo following systemic treatment of HSA10 mice with AAV6-HSA10. (A) RT-qPCR was performed using total RNA isolated from TA muscles harvested 8 weeks post injection of AAV6-HSA10. Relative quantitation, ΔCt, was determined from comparing HSA mRNA levels to non-transgenic WT mice, with normalization to an endogenous control in each mouse (one-way ANOVA statistical methods with Dunnett’s multiple comparison test of all samples to the control HSA10; P < 0.01±SD; n = 7 treated and three WT and three untreated HSA10 mice). (B) Absolute quantitation of levels of HSA10 mRNA were determined with small Taqman qPCR (stem-loop PCR) and compared with small Taqman qPCR from an RT that was spiked with known quantities of synthetic HSA10 miRNA. Gapdh expression was used as an endogenous control to relate quantitation to genomic DNA copy number. All qPCRs were performed in triplicate; n = 7 treated and 3 untreated HSA10 mice.

neonatal mRNA isoforms compared with higher rAAV6-HSA10 dose (Fig. 5), suggesting a dose dependency of the effect. The mitigation of the skeletal muscle disease in the HSA10 mice at the 2-fold lower dose is supported by evidence of a threshold effect near the level of the 2 × 10^12 vg dose for rAAV6 muscle transduction with systemic delivery in mice (48).

Nuclear foci reduction and Mbnl1 nuclear redistribution in rAAV6 HSA10-treated HSA10 mice

RNA FISH detection of CUGexp HSA transcripts identified numerous bright nuclear foci in the TA of the HSA10 mice (Fig. 6A). A significant decrease in the number of foci was observed in the rAAV6-HSA10-treated mouse muscles (n = 7) at 8 week post injection compared with untreated HSA10 mice (Fig. 6B; P < 0.0001; n = 4 fields at 20 × magnification; n = 3 HSA10). We also observed a significantly more diffuse pattern of Mbnl localization in the nuclei of treated muscle cryosections with immunohistochemical detection of Mbnl1 immunofluorescence compared with the bright, punctate immunofluorescent signals in the untreated HSA10 mice (Fig. 6C). The transition to a more diffuse nuclear pattern of staining shows a normalization of Mbnl1 localization that is more similar to its localization in WT mice.

RNA biomarker levels are downregulated in the rAAV6-HSA10-treated HSA10 mice

To determine whether treatment led to a change in the expression levels of miRNAs associated with disease pathology, we examined the expression of two genes, Atp1a4 and Mstn1, previously shown to be upregulated in the HSA10 mouse (Supplementary Material, Fig. S5) (24). RT-qPCR quantitation of the level of these transcripts, compared with WT FVB mice, showed an approximately 2-fold relative decrease compared with the levels in untreated HSA10 mice at 8 weeks post injection (P < 0.0001).

Discussion

Therapeutic RNAi has shown great promise for treating diseases of the liver, brain and eye, where changes in gene expression can ameliorate disease (49,50). Previous studies from our laboratory and others have demonstrated the potential for success of muscle disease RNAi therapy. We now add to this list the potential for systemic RNAi therapy of muscle disease, regardless of the intracellular localization (nuclear or cytoplasmic) of the target mRNA. Unlike most mRNA targeting studies where the mRNA is cytoplasmic, there is compelling evidence that the majority of the CUGexp RNA is located in the nucleus both in DM1 and the HSA10 mouse model (7,18,19). Early DMPK mRNA silencing studies in vitro using lentivirus delivery of DMPK shRNA expression cassettes were successful in reducing the DMPK CUGexp mRNA and supported development of this approach as a bodywide treatment for DM1. This was the first study suggesting that therapeutic RNAi could work on a nuclear target, and soon after Robb et al. (36) reported RNAi activity could be targeted to the nuclear RNAs 7SL and U6 snRNA. Controversy regarding the possibility of RNAi functioning in the nucleus has been longstanding, and originated from studies showing that intron targeting was ineffective and that RNAi factors had a predominantly cytoplasmic localization (51). However, mounting evidence from studies in mammalian cells now supports the activity of RNAi in the nucleus (52). Careful cell fractionation studies indicate that Ago2 and other RNAi factors are present in the nucleus in multiprotein complexes (53–55). Nuclear Dicer and Ago2 isolated from nuclei are catalytic activity, and nuclear Ago2 associates with endogenous miRNAs. These data refute the early assumption that RNAi functioned only in the cytoplasm. Despite the compelling evidence for RNAi in the nucleus, it is still possible that the pathogenic population of HSA CUGexp mRNA is found in the cytoplasm as a minor population and is targeted by the therapeutic RNAi, whereas the nuclear accumulated HSA CUGexp mRNA is gradually degraded over time by other mechanisms (19).

Based on the early reports of RNAi activity in nuclei, we sought to develop a bodywide delivery system in a relevant disease mouse model that could lead to a clinical RNAi therapy for DM1. The HSA10 mouse model was studied, because no genetically stable mouse model had been developed, based on DMPK expression, that recapitulated as many of the predominant features of DM1 as in the HSA10 mouse model (56). Significant reduction of the HSA10 mRNA was achieved with IV tail vein single injection at 8–12 weeks following treatment. Reduction of the HSA10 mRNA was accompanied by significant reversal of alternative splicing defects in Atp2u1, Clcn1 and m-Ttn miRNAs in TA muscles that
express levels of the expanded repeat more comparable to those found in DM1 muscle. The reduction of HSA_{LR} mRNA was also accompanied by a reduction in myotonic discharges coupled with correction of a proportion of alternative splicing events in Clcn1 pre-mRNA processing. As expected, CUGEXP RNA foci were less prevalent and their reduction was consistent with redistribution of a proportion of myonuclear Mbnl1. Although many of the original histological defects are no longer found in the HSA_{LR} mice, HSA_{LR} transgene expression is accompanied by an increase in myofiber size (of potentially compensatory fibers), an effect that is reversed with rAAV6-HSA10 treatment. This decrease in larger HSA_{LR} myofibers has also been observed with effective ASO treatment (24).

More recently, it was shown that synthetic siRNAs targeting CUG repeats (siGAC) could reduce the CUG_{EXP} RNA when electrooporated into the TA muscle of the HSA_{LR} mice, and was consistent with previous RNAi studies in DM cells in vitro (37,57). Targeting the CUG sequence results in preferential allelic silencing of DMPK CUG_{EXP} mRNA and is an attractive strategy, although there remains the potential for reduction of other unrelated CUG repeat-containing mRNAs as observed for one of eight mRNAs analyzed. Additional studies using RNase H active ASOs targeting the CUG_{EXP} mRNA achieved partial reduction, but also caused muscle damage, again suggesting a generalized repeat track targeting strategy may cause unwanted off-target effects (23,57). This might be mitigated through design of RNAi hairpins that target specific SNPs in the CUG_{EXP} mRNAs. The CTG_{EXP} mutation and SNPs found on the haplotype are likely to be shared predominantly by individuals with DM1, because the disease prevalence in ethnic populations is positively correlated with the frequency of premutation alleles (58,59). However, partial knockdown of both the mutant and non-mutant alleles is predicted to significantly reduce the burden of the CUG_{EXP} mRNA and may not interfere with the function of DMPK, because knockout of the mouse gene leads to only mild phenotypic effects in aged animals (60). Experimental evidence also supports the protective function of CUG_{EXP} mRNA foci, which may allow tolerance of some mutant mRNA without manifestation of disease (61–63).

Since discovery of the DNA repeat expansion basis of DM1 and deciphering the toxic RNA gain-of-function mechanism several approaches for therapy development have been undertaken. These include methods to interfere with protein binding of toxic CUG_{EXP} repeat RNA (64,65), overexpression of MBNL1 (66), drugs that alter signaling (67), and both synthetic and expressed ASOs (19,22,24). Of the synthetic ASOs, the most effective tested in mice are the MOE-gapmers (2′-O-methoxyethyl end-modified ASOs) that are substrates for RNase H cleavage when bound to DMPK mRNA outside of the repeat region (24). Because both RNase H and DMPK CUG_{EXP} mRNA are predominantly nuclear, it may be possible to deliver low levels of the MOE-gapmers for efficacy that was achieved in mice, thereby reducing the chance of adverse treatment reactions. However, most of the ASO and protein drug delivery will require continuous treatment and could produce toxicities with repeated dosing. In contrast, rAAV-mediated delivery of genes has progressed beyond testing in

![Figure 4. Reduction of myotonic discharges in the rAAV6-HSA10 or rAAV6-HSA4-treated HSA_{LR} mice noted by needle electromyography (EMG). Grades of myotonic discharges for (A) HSA10 treated TA at 8 weeks post injection; (B) HSA10 treated TA at 12 weeks post injection; (C) HSA10 treated quad at 8 weeks post injection and (D) HSA4-treated TA at 8 weeks post injection. Blinded analysis of rAAV6-HSA10 or rAAV6-HSA4-treated HSA_{LR} mice was performed by an experienced practitioner under anesthesia to evaluate myotonic discharges with repositioning of the EMG needle electrode 16 times before arriving at a score on a scale of 0 to 4; 0, no discharge, 1, rare events (less than 50% of needle electrode insertions), 2, occasional events (greater than 50% of needle electrode insertions), 3, frequent events, 4, discharge with most needle electrode insertions. All measurements were repeated two times for individual mice shown. LR, untreated HSA_{LR} mice.](https://academic.oup.com/hmg/article-abstract/24/17/4971/648599)
mice to large animal muscular dystrophy models, the canine model of Duchenne muscular dystrophy (CXMD) and the X-linked myotubular myopathy model (XLMTM) (68–70). rAAV has also shown success with a single application in the brain of non-human primates for Huntington’s disease and has proven safe and effective in a growing number of cases in humans, such as Leber’s congenital amaurosis and lipoprotein lipase deficiency (33,71). Because the HSA LR mouse model does not recapitulate the multisystem aspects of DM1, more work will have to be done to address delivery concerns, especially for disease effects in the CNS and gastrointestinal tract. One option might be to combine other serotypes of rAAV for therapeutic RNAi delivery that show different tissue biodistributions with the predominantly muscle tropic rAAV6 or use another serotype altogether (72,73).

There are over 100 isolated serotypes of rAAV that could be used to deliver RNAi expression cassettes to multiple tissues (32). Our results using rAAV delivery of therapeutic RNAi to reverse DM1 disease features in the HSA LR mouse suggests that further vector development for evaluation in emerging DMPK mouse models will help advance this approach toward clinical testing.

**Methods**

**Plasmids for HSA reporter and RNAi expression cassettes**

Plasmid pLI-ACTA was constructed by inserting a firefly luciferase SmaI/XbaI fragment from pGL3-Basic (Promega Corporation, Madison, WI, USA) into the EcoRV/NheI sites of pRESneo3.
HSA4 5′-ctctgagcagggcatcacc3′ (bold are additions from original shRNA) starting at position 320 of the HSA mRNA and HSA10 5′-ctctgagcagggcatccta3′ starting at position 1269 of the HSA mRNA. These sequences were embedded in the miR30 scaffold (74) with XhoI and SpeI sites added for cloning into the XhoI/XbaI sites of pCR Blunt II TOPO (Invitrogen, Carlsbad, CA, USA) containing an the mU6 promoter and RNA Pol III T termination sequence as described previously (75). An EcoRI fragment containing the miR30-based expression cassette was blunt-ended and ligated into the 5′ Smal I site of the rAAV2 plasmid pARAP4 (kind gift of Dusty Miller, FHRC) containing the reporter gene RSV-hPLAP. The 5′ extended versions of the HSA4, HSA10 and LacZ/bgal shRNA hairpins had an additional 27 nt as described previously (28,57). All RNAi target sequences were chosen based on basic shRNA design rules from the RNAi Consortium of the Broad Institute, Cambridge, MA, USA, based on rules established by Tuschl and co-workers (39–41). MiR30-based hairpin sequences were analyzed for proper folding for Drosha and Dicer recognition with Michael Zuker’s UNAfold program (IDT, Coralville, IA, USA), checked for homology of passenger or guide strands to other known genes, and analyzed for seed sequence homology using the siRNA Seed Locator (Thermo Scientific; Fisher, Pittsburg, PA, USA).

Virus preparation

rAAV6 was prepared and titrated by Southern blot analysis for viral genomes as described (40). Typical titers were 3×10^{12} vg/ml from 30 roller bottles. The RNAi expression cassette sequences were confirmed with the difficult to sequence protocol (RNAi hairpin; Genewiz, Seattle, WA, USA).

Luciferase assays

HSA shRNA plasmids or control plasmids, with either mU6 alone or mU6siβgal, were cotransfected with pLI-ACTA reporter plasmid into COS-1 cells using the FuGENE 6 reagent (Promega, Mountain View, CA, USA) in a 3:1 ratio to DNA, 500 ng total per well of 12-well culture dish. All of the 16 HSA shRNA plasmids were tested in each experiment and repeated in six different experiments performed on different days with luciferase assays performed in triplicate. Cell lysates were prepared 48 h after transfection and assayed on a single well Berthold luminometer using the Luciferase Assay System (Promega). Results from HSA shRNA and reporter transfection assays were normalized to the mU6 or mU6siβgal plasmid.

Animals, infections, blood and tissue collection

All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Washington. All mice used in these studies were of the FVB/N strain (wild-type/genetic background strain for HSA18) or human α-skeletal muscle actin long-repeat (HSA18) homozygous transgenic, line 20b, described previously (18) (gift of C. Thornton). Approximately 15–18 g mice at 4 weeks of age were injected via the tail vein with either 2 or 4×10^{12} vector genomes (vg) of rAAV6-HSA10 or -HSA4 in a total volume of 200 μl. Blood samples (200 μl/mouse) were collected from lightly anesthetized mice as a retro-orbital bleed at stated time points. Serum was sent to Phoenix Central Laboratories (Mukilteo, WA, USA) for muscle CK, liver alanine transaminase (ALT) and liver aspartate aminotransferase (AST) analysis. For CK analysis, serum was diluted 1:2 in water. Animals were euthanized by cervical dislocation 8 or 12 weeks after infection.
after injections, and tissues were collected for analysis. For splicing analysis and real-time PCR, tissues were flash frozen in liquid nitrogen. For histology, histochemistry, immunoﬂuorescence and FISH, tissues were embedded in OCT and frozen in liquid nitrogen-cooled isopentane.

Electromyography
Needle EMG study was performed using a Viking Quest electromyograph (Nicolet, Madison, WI, USA). Mice were anesthetized with 5% isoflurane gas with 100% O2 delivered at 2.5 l/min in an induction chamber, then maintained under anesthesia with a cone facemask connected to a coaxial circuit during the EMG procedure. The quadriceps or TA muscles were sampled in the proximal, middle and distal regions of the muscle belly using a 25-gauge concentric needle electrode after ethanol sterilization of the area. A steel needle electrode (Dantec, Copenhagen, Denmark) was placed subcutaneously as a ground electrode in the muscle about 1 cm proximal to the recording site. EMG analysis of myotonic discharges was performed by a blinded examiner as described previously (76). Myotonic discharges were graded 0, indicating no myotonic discharge; 1, occasional myotonic discharge (less than 50% of needle electrode insertions); 2, myotonic discharge (greater than 50% of needle electrode insertions) and 3, myotonic discharge with nearly every insertion.

Histology and histochemistry
Transverse frozen sections (10 μm) were cut from OCT-embedded tissues. For basic histology analysis, sections were fixed in methanol and stained with H&E/Phloxine using a standard protocol. For analysis of transduction efficiency, sections were stained for hPLAP activity as previously described with slight modifications (34). Briefly, sections were fixed in ice cold 4% PFA/PBS for 10 min, washed with PBS, incubated at 67°C for 90 min in PBS to deactivate endogenous alkaline phosphatases, incubated in AP wash buffer (100 mM Tris–HCl pH 9.5, 100 mM NaCl, 10 mM MgCl2) for 10 min at room temperature, and stained with the BCIP/NBT substrate (Fast BCIP/NBT tablets from Sigma-Aldrich) for 40 min at room temperature. Sections were then washed with PBS, dehydrated and mounted with Permount.

Fluorescence in situ hybridization
Localization of CUGexpanded RNA by fluorescence in situ hybridization (FISH) was carried out as previously described with slight modifications (77). Briefly, 10 mm frozen sections of the TA and quadriceps muscles were fixed in 3% PFA/PBS for 15 min, washed with PBS, permeabilized with 0.5% Triton X-100/PBS for 5 min and blocked in 5% normal goat serum in PBS for 30 min, all at room temperature. Sections were then incubated with the primary antibody (MBNL1 A2764, 1:10 000 in 1% BSA/PBS) overnight at 4°C, washed with PBS, incubated with the secondary antibody (goat anti-rabbit Alexa488, 1:400 in PBS) in the dark for 45 min at room temperature, and stained with Alexa 594-labeled 3′-methyl-CAG RNA probe (Texas red 2-′m-Ttn CTAGCTTTG 3′) and Alexa 488-labeled sense probe (CCACCACAGGACCATGTTATTTC 3′) for a total of 2, 10 μm-thick, cross sections of TA muscle from separate regions of TAs isolated from three different mice for FVB, HSA, or treated mice at 20× magnification on a Leica SP5 confocal microscope. Boundaries were drawn around fibers and enclosed surface areas were determined using the Adobe Photoshop software measure function and pixel-size information from the confocal images. A frequency distribution histogram was generated using GraphPad Prism software. Curves were derived from non-linear regression using a least squares fit in the Gaussian distribution.

Immunoﬂuorescence
Localization of MBNL1 by immunoﬂuorescence was carried out as previously described with minor modiﬁcations (11). Briefly, 10 mm frozen sections of the TA and quadriceps muscles were ﬁxed in 3% PFA/PBS for 15 min, washed with PBS, permeabilized with 0.5% Triton X-100/PBS for 5 min and blocked in 5% normal goat serum in PBS for 30 min, all at room temperature. Sections were then incubated with the primary antibody (MBNL1 A2764, 1:10 000 in 1% BSA/PBS) overnight at 4°C, washed with PBS, incubated with the secondary antibody (goat anti-rabbit Alexa488, 1:400 in PBS) in the dark for 45 min at room temperature, washed with PBS and mounted with DAPI-containing ProLong Gold mounting media (Life Technologies). Images were acquired using a Leica SP5 confocal microscope at the University of Washington Biology Imaging Facility.

RT-PCR analysis of alternative splicing
Splicing analysis for Cln1, Atp2a2 and m-Ttn was carried out as previously described (11). Briefly, RNA was isolated from flash frozen tissues using the mirVana miRNA Isolation Kit (Life Technologies). cDNA was synthesized using oligo (dT) plus random hexamer primers and treated with RNase H at 37°C for 35 min. PCR amplification was carried out for 24 cycles using the following primers: CIC-1 forward: 5′ TGAAGGAATACCTCACATCTAAGG 3′, reverse: 5′ CAGCGAAACAAAAGGCCACTGAATG 3′, Atp2a2 forward: 5′ GCCATGCTCCAGTCAAGTTCAGC 3′, R-5′ GGTCACTGTGGTCGCTCCAGAAGC 3′, reverse: 5′ CAGCGAAACAAAAGGCCACTGAATG 3′ and m-Ttn forward: 5′ GCTGAAGTGCTCCACGACAC 3′, reverse: 5′ GATCCGCTTTGGTTGGTGAATG 3′. PCR products were resolved on agarose gels, stained with SYBR Green and scanned with a Molecular Dynamics Storm 860 scanner. Band intensities were quantified by densitometry using GE Healthcare Life Sciences ImageQuant TL 8.1 software.

Small RNA Northern analysis
Total RNA was isolated from rAAV RNAi plasmid-transfected HEK293 cells using the mirVana miRNA Isolation kit (Life Technologies), 25 μg of total RNA per sample was run on Novex 15% TBE-Urea gels with 300 pg and 30 pg of sense or guide equivalent oligonucleotide per RNAi plasmid run concurrently as positive controls. Gels were run in RNase-free 1 × TBE at 100 V for 3–4 h, then stained with ethidium bromide and photographed as a reference for equivalent loading and migration of total RNA.
RNA was electrophobled to 0.45 μm Nytran SPC Membranes (Sigma-Aldrich) in RNase-free 0.5x TBE. Membranes were autocrosslinked in a Stratagene UV Stratalinker 1800. RNA-bound membranes were prehybridized with ULTRAhyb-Oligo Hybridization Buffer (Life Technologies). Ten pmol of each ASO was labeled with 5 μCi/μl of γ-32P ATP (New England Nuclear) by T4 PNK (New England Biolabs) and unincorporated 32P was subsequently removed with G25 spin columns (GE Healthcare). Membranes were incubated with hybridization buffer and the RNA-bound membrane at 32°C. All membranes were washed for 15 min, followed by three washes for 5 min each in 2x SSC/0.5% SDS at 32°C. Membranes were wrapped in one layer of plastic wrap and sandwiched exposed to a phosphor screen and BioMax XAR film (Kodak). Film and phosphor screens were exposed for 5 min each at 32°C. Membranes were washed for 15 min, followed by three washes under standard product protocol conditions. Efficiency of the PDR assays was detected using the Power SYBR Green PCR Master Mix Green method. cDNA was synthesized using the Power SYBR Green PCR Master Mix. cDNA was synthesized as for ACTA1 and qPCR was performed using the ABI 7500. Following primer/probe set was used:

Forward: 5′-CACGAGGCCTGAGCAGGAGA-3′

Reverse: 5′-AGTCCGGTCTCTCTACTAGCC-3′

Relative expression levels of ACTA1 were determined by hybridization probe reactions and normalized to 18s rRNA reference gene (24). cDNA was synthesized using oligo(dT) primers and qPCR was performed under standard reaction conditions on an ABI 7500. Following primer/probe set was used:

Forward: 5′-CACGAGGCCTGAGCAGGAGA-3′

Reverse: 5′-AGTCCGGTCTCTCTACTAGCC-3′

Relative expression levels of ACTA1 were determined using the SYBR Green method. cDNA was synthesized as for ACTA1 and qPCR was performed using the Power SYBR Green PCR Master Mix (Life Technologies, Grand Island, NY, USA). Following primers were used (all designed to span exon junctions): Mstn1-forward: GCCAAGAACCAGGACATCAAGTC, Mstn1-reverse: 5′-CTCTGACTGCGTGTCGATATA-3′; Atp1-forward: 5′-AGTCCGGTCTCTCTACTAGCC-3′, Atp1-reverse: 5′-AGGCACGTGGTGTCTCACCTGAC-3′.

Supplementary Material
Supplementary Material is available at HMG online.

Acknowledgements
We thank James Allen, John Hall and Glen Banks for critical reading of the manuscript and sharing scientific expertise and James Allen and Erik Finn for vector preparation and advice. We are grateful to the JS Chamberlain Lab for critical evaluation of the work and Steven Hauschka for training, guidance and support. Charles Thornton and Thurman Wheeler were generous with their reagents, training and constructive comments and are deeply appreciated. We thank the Gregg Meekins for his EMG expertise and Erin Kirkegaard and Carrie Stoltzman for their technical assistance.

Conflict of Interest statement. There are no conflicts of interest to declare.

Funding
This work was supported by the National Institutes of Health (RO3AR056107, U54HD47175 supplement to J.R.C.) and the Muscular Dystrophy Association (MDA USA; 172830 to J.R.C.).

References


