RNA Interference Improves Myopathic Phenotypes in Mice Over-expressing FSHD Region Gene 1 (*FRG1*)

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Muscular dystrophies, and other diseases of muscle, arise from recessive and dominant gene mutations. Gene replacement strategies may be beneficial for the former, while gene silencing approaches may provide treatment for the latter. In the last two decades, muscle-directed gene therapies were primarily focused on treating recessive disorders. This disparity at least partly arose because feasible mechanisms to silence dominant disease genes lagged behind gene replacement strategies. With the discovery of RNA interference (RNAi) and its subsequent development as a promising new gene silencing tool, the landscape has changed. In this study, our objective was to demonstrate proof-of-principle for RNAi therapy of a dominant myopathy *in vivo*. We tested the potential of adeno-associated viral (AAV)-delivered therapeutic microRNAs, targeting the human Facioscapulohumeral muscular dystrophy (FSHD) region gene 1 (*FRG1*), to correct myopathic features in mice expressing toxic levels of human FRG1 (FRG1^{-high} mice). We found that *FRG1* gene silencing improved muscle mass, strength, and histopathological abnormalities associated with muscular dystrophy in FRG1^{-high} mice, thereby demonstrating therapeutic promise for treatment of dominantly inherited myopathies using RNAi. This approach potentially applies to as many as 29 different gene mutations responsible for myopathies inherited as dominant disorders.

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INTRODUCTION

The concept of muscle gene therapy arose soon after dystrophin mutations were identified as the underlying cause of Duchenne muscular dystrophy (DMD) in 1987.[1] Because DMD was a recessive disorder caused by the lack of normal dystrophin in muscle, several research groups began developing dystrophin gene replacement strategies as potential treatments for DMD.[2] For many years, this was the sole focus of the nascent muscle gene therapy field, but as mutations in other myopathy-related genes were subsequently identified, the field expanded beyond DMD to include other muscle disorders.[3] These disease gene identification studies, combined with important advancements in adeno-associated viral (AAV) vector development and delivery over the last two decades, contributed to several successful preclinical gene therapy trials in animal models of various myopathies.[2,4–13] Importantly, one recently translated study showed the first promising Phase I clinical trial data for gene therapy of limb girdle muscular dystrophy in humans.[14] Thus, steady progress in a relatively short period of time supports that gene therapy may someday be an effective method for treating inherited disorders of muscle.

Nevertheless, most of the current progress in the field has been primarily directed toward developing therapies for recessive disorders, while approaches to treat dominant myopathies were largely unexplored by comparison.[2,4–11,13] This disparity in research focus is significant, as two of the three most common muscular dystrophies are dominant (facioscapulohumeral muscular dystrophy, FSHD; myotonic dystrophy, DM), and more than half of all currently known myopathy-related disease genes are linked to dominant disorders.[15] One reason the muscle gene therapy field principally focused on recessive myopathies relates to the technical feasibility of the strategies necessary to treat each class of disorders. Specifically, recessive disorders require gene replacement, while dominant diseases would potentially benefit from disease gene silencing.[15] Historically, feasible molecular tools existed to accomplish the former, but not the latter. This disparity could change however, with the recent emergence of RNA interference (RNAi) as a promising therapeutic approach to silence dominant disease genes.[16] The initial work in this area mostly focused on treating neurodegenerative disease,[16] but we hypothesized that RNAi could also be an effective mechanism to silence genes associated with dominant myopathies, which has not been previously illustrated.[15] The goal of this study was to demonstrate proof-of-principle that RNAi-based gene therapy could correct muscle abnormalities in a mouse model of dominant myopathy. To do this, we used the FRG1^{-high} transgenic mouse line, which develops myopathy caused by muscle-specific over-expression of

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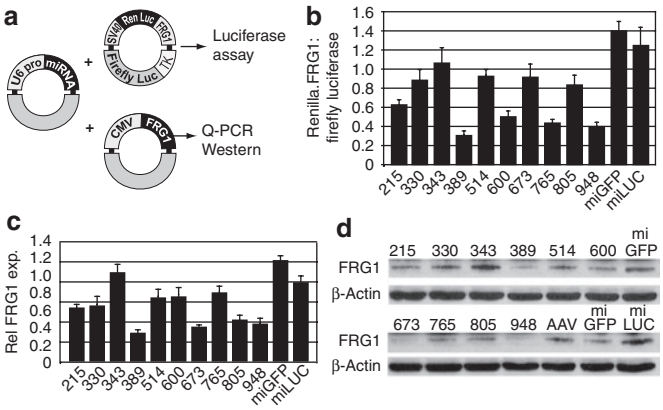

Figure 1 ***In vitro*** **screen to identify lead miFRG1 sequences.** (**a**) Plasmids used for testing *FRG1* gene silencing in HEK293 cells. (Left) Each *FRG1*-targeted miRNA (miFRG1) was cloned downstream of the mouse U6 promoter (U6 pro). U6.miFRG1 expression plasmids were cotransfected with *FRG1* target plasmids expressing *Renilla* luciferase (RenLuc)-*FRG1* fusion transcripts (right, top) or the human *FRG1* open reading frame (right, bottom). In the Luciferase-*FRG1* expression plasmid, human *FRG1* was placed downstream of the *Renilla* luciferase stop codon, thereby serving as a 3′ UTR. This plasmid also contained a separate Firefly luciferase reporter, which was useful as a transfection control. SV40, SV40 promoter; TK, herpes simplex virus (HSV) thymidine kinase promoter; CMV, cytomegalovirus promoter. (**b**) Luciferase assay screen. *FRG1* gene silencing was initially determined by measuring the ratio of *Renilla*:Firefly luciferase activity from cotransfected cell lysates. Numbers on X-axis indicate miFRG1 sequences; numbers correspond to position on the *FRG1* cDNA. MiGFP and miLUC control miRNAs do not target *FRG1*. (**c**) Relative FRG1 mRNA and (**d**) protein expression in HEK293 cells cotransfected with CMV.FRG1 and indicated miFRG1 expression plasmids. (**c**) *FRG1* levels were determined by Taqman assay and normalized to human β-actin expression. Data represent SEM from two independent experiments performed in triplicate. Western blot in (**d**) shows representative data from three independent experiments. The U6.miFRG1.948 sequence consistently knocked down human *FRG1* levels in all three assays. It was subsequently used in all *in vivo* experiments. eGFP, enhanced green fluorescent protein; MiGFP, microRNAs targeting eGFP.

the human FSHD region gene 1 (*FRG1*).[17] As the gene name suggests, FRG1^{-high} mice were initially developed as a putative model of autosomal dominant FSHD, but the pathogenic mechanisms underlying this disorder are not altogether resolved; indeed, recent data support a model in which *DUX4* over expression is a primary pathogenic insult underlying FSHD.[17–23] Thus, both *FRG1* and *DUX4* may be candidate targets for RNAi therapy, but there are no published animal models stably expressing the latter. We therefore focused on *FRG1* in this study, and tested the potential of AAV-delivered, *FRG1*-targeted microRNAs to correct myopathy in FRG1^{-high} mice. Our results demonstrate the therapeutic promise of RNAi therapy for FSHD candidate genes specifically, and dominant myopathies in general.

RESULTS

Myopathy in FRG1^{-high} mice results from muscle-specific overexpression of the human *FRG1* coding region.[17] We therefore hypothesized that *FRG1* knockdown using RNAi would improve myopathic phenotypes. To do this, we designed ten different U6 promoter-driven artificial microRNAs targeting sequences in the human *FRG1* coding region (**Supplementary Figure S1**). We then identified our lead *FRG1*-targeted microRNA (miFRG1) using *in vitro* screening assays. First, we cotransfected U6.miFRG1 constructs with a plasmid expressing a *Renilla* luciferase-FRG1 fusion transcript and a separate firefly luciferase transfection control (**Figure 1a**). We then measured miFRG1-mediated gene silencing indirectly by determining the ratio of *Renilla* to firefly luciferase activity from transfected cell lysates, 2 days later (**Figure 1b**). To confirm these gene silencing data against a normal *FRG1* open reading frame, we cotransfected individual U6.miFRG1 plasmids with a CMV.FRG1 expression vector into HEK293 cells and measured *FRG1* transcript and protein levels by real-time PCR and western blot, respectively (**Figure 1c–d**). We identified two different miFRG1 sequences that consistently silenced *FRG1* using all three assays, and we chose one of these (miFRG1.948; heretofore referred to as miFRG1) for *in vivo* studies because it catalyzed slightly better silencing at the protein level (**Figure 1d**).

We next cloned U6.miFRG1 or an U6.miGFP control microRNA into our AAV.CMV.hrGFP proviral vector.[24] The miGFP control microRNA, which targets sequences in the enhanced green fluorescent protein (*eGFP*) gene, does not direct knockdown of *FRG1* or our hrGFP reporter. We then made AAV6 viral vectors expressing hrGFP alone (AAV.hrGFP), or hrGFP with miFRG1 or microRNAs targeting eGFP (miGFP) (AAV.miFRG1 or AAV.miGFP, respectively; **Figure 2a**), and injected 5×10^{10} DNAse resistant particles of each vector into the lower limbs of newborn FRG1^{-high} or wild-type male littermates. For all wild-type mouse injections, and FRG1^{-high} mice injected with AAV.miFRG1 vectors, this delivery approach produced robust and widespread hrGFP expression in most major muscles of the lower limbs, including the adductors (add), gastrocnemius (gas), tibialis anterior (TA), and gluteus maximus (glut), up to 14 weeks post injection (**Figure 2b–c**). The quadriceps muscle was inconsistently hrGFP positive, and showed the least amount of transduction of all major lower limb muscles (**Figure 2c**). In contrast, we did not observe abundant hrGFP expression in AAV.hrGFP or AAV.miGFP-injected FRG1^{-high} mice 14 weeks after injection, although it was present at 3 weeks (**Supplementary Figure S3**).

We next examined muscle size in AAV.miFRG1- and control-treated mice, since muscle size deficits are the most obvious gross abnormality in FRG1^{-high} animals. We found that AAV.miFRG1-treated lower limb muscles were visually larger than AAV.miGFP-injected controls, and isolated add, gas, and glut muscles from the former weighed significantly more than those from animals that received AAV.miGFP or AAV.hrGFP vectors (approximately twofold average increase; **Figure 2d–e**). Moreover, AAV.miFRG1-injected FRG1^{-high} add, gas, and glut muscles were indistinguishable in size from wild-type controls (**Figure 2d–e**). We observed similar trends in TA muscle sizes of all treated and control groups, but none of these changes were statistically significant. In contrast, AAV.miFRG1 treatment did not restore FRG1^{-high} quad muscles to wild-type sizes. This insignificant ~1.3-fold mean quadriceps size correction was likely due to low average transduction, since quad weights trended higher in individual FRG1^{-high} mice with the most AAV.miFRG1-derived hrGFP expression (**Figure 2d–e** and **Supplementary Figure S2**).

We next correlated these gross muscle improvements with *FRG1* mRNA and protein knockdown using real-time PCR,

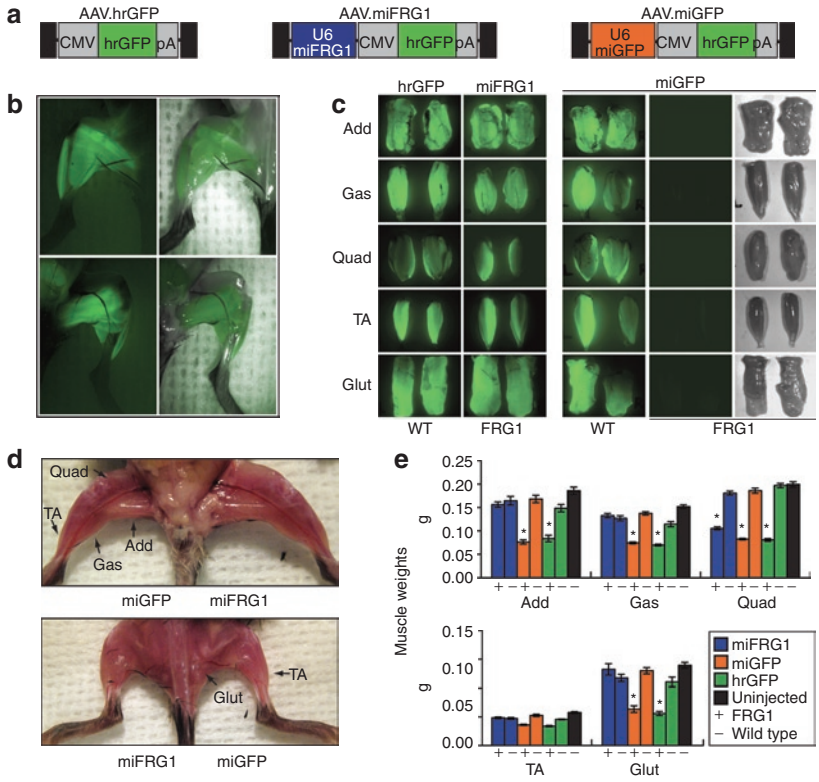

Figure 2 ***FRG1*** **gene silencing improves muscle mass in FRG1^{-high} mice.** (**a**) Adeno-associated viral (AAV) vectors used for *in vivo* studies. All contain a CMV promoter-driven humanized *Renilla* GFP (hrGFP) cassette with an SV40 polyA (pA) signal. The AAV.miFRG1 and AAV.miGFP vectors also contain upstream U6.miRNA expression sequences. The miGFP sequences target eGFP, and do not impact levels of hrGFP, which is a different gene. Flanking black rectangles indicate AAV inverted terminal repeats (ITRs). (**b**) GFP epifluorescence shows near saturation of lower limb musculature in adult mice injected intramuscularly as newborns with 5×10^{10} DNAse resistant particles (DRP) of AAV6. (**c**) In all groups except FRG1-high mice injected with the AAV.miGFP virus, this delivery method produced high transduction in adductors (add), gastrocnemius (gas), tibialis anterior (TA), and gluteus (glut) muscles, shown by hrGFP epifluorescence in isolated muscles. Transduction was lower and less consistent in the quadriceps (quad). In contrast, 14 weeks after AAV.miGFP injections, hrGFP epifluorescence was not evident in lower limbs of FRG1^{-high} mice. (**d**,**e**) AAV.miFRG1 significantly improved muscle mass in FRG1^{-high} mice compared to uninjected or control-injected muscles. In (**d**) vectors were unilaterally injected as indicated. Dorsal (bottom) and ventral (top) views from the same representative animal are shown. In (**e**) data represent the mean weights ± s.e.m. of male FRG1^{-high} (+) or wild-type (–) muscles injected with indicated vectors ($n = 14$ muscles per group). *Indicates significant difference from wild-type counterpart (ANOVA with Bonferroni post-test, $P < 0.05$). CMV, cytomegalovirus promoter; GFP, green fluorescent protein; miGFP, microRNAs targeting eGFP.

western blot, and immunofluorescence staining. We measured a statistically significant ~55% reduction of over expressed human *FRG1* mRNA in FRG1^{-high} muscles treated with AAV.miFRG1 compared to AAV.miGFP controls (**Figure 3a**). AAV.miFRG1 also reduced endogenous mouse *FRG1* mRNA by ~50%, despite having one mismatch with this transcript (**Supplementary Figure S1**). Expectedly, total *FRG1* mRNA knockdown corresponded to a marked decrease in FRG1 protein levels by western blot (**Figure 3b**), but since RNAi rarely produces complete knockdown of abundant targets like *FRG1* in FRG1^{-high} mice, we were surprised to note that human FRG1 was nearly or completely undetectable in moderately- to highly-transduced myofibers (**Figure 3c**). This knockdown in AAV.miFRG1-transduced FRG1^{-high} muscles was associated with wild-type histology. Specifically, all wild-type groups and transduced muscles from AAV.miFRG1-treated FRG1^{-high} mice lacked the fibrosis, fat deposition, and myofiber degeneration and regeneration (indicated by increases in myofibers with smaller diameters and/or centrally-located nuclei) seen in untreated or control-treated FRG1^{-high} animals (**Figure 4**).

Finally, we determined whether *FRG1* knockdown in FRG1^{-high} mice improved overall hind limb muscle function. To do this, we measured grip strength weekly in AAV.miGFP-, AAV.miFRG1-, or AAV.hrGFP-injected FRG1^{-high} and wild-type male mice for 5 weeks, and compared these values to uninjected wild-type littermates. We found that AAV.miFRG1-treated FRG1^{-high} animals were significantly stronger (~1.7-fold average increase) than age-matched AAV.miGFP- or AAV.hrGFP-injected controls (**Figure 5** and **Supplementary Figure S4**). In contrast, hind limb grip strength from AAV.miFRG1-treated FRG1^{-high} and wild-type mice, and all injected wild-type animals, were not significantly different from uninjected wild-type controls (**Figure 5** and **Supplementary Figure S4**). We therefore concluded that RNAi-mediated knockdown of *FRG1* improved myopathy in individual myofibers, isolated muscles, and whole limbs from FRG1^{-high} mice.

DISCUSSION

Gene therapy refers to a therapeutic approach for disease that uses nucleic acids instead of drugs (http://www.asgct.org/about_gene_therapy/defined.php). For many years, this definition was almost

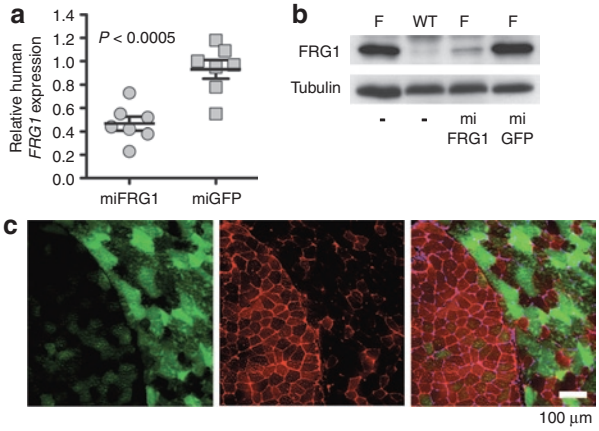

Figure 3 ***In vivo*** **knockdown of** ***FRG1*** **in FRG1^{-high} mice.** (**a**) Taqman assay showed that adeno-associated viral (AAV).miFRG1 vectors reduced *FRG1* mRNA expression by a statistically significant average of 55% in FRG1^{-high} muscles, compared with AAV.miGFP-injected controls (n = 7; ANOVA). FRG1 expression was determined by Taqman assay (human *FRG1* primer/probe) normalized to mouse GAPDH expression. (**b**) Representative western blot confirmed FRG1 protein knockdown *in vivo*. F, FRG1^{-high} muscles; (–), uninjected. Tubulin served as a loading control. (**c**) Immunofluorescence stain of AAV.miFRG1-injected FRG1^{-high} muscle cryosections. Green shows transduced myofibers (humanized *Renilla* GFP (hrGFP) epifluorescence) and red stain shows FRG1 expression by immunostaining with DMA-AP-1 FRG1 primary antibody and Alexa-594 labeled secondary antibody. Importantly, FRG1 immunofluorescence inversely correlated with AAV.miFRG1 transduction, thereby demonstrating the efficacy of our FRG1 knockdown strategy. GFP, green fluorescent protein; miGFP, microRNAs targeting eGFP.

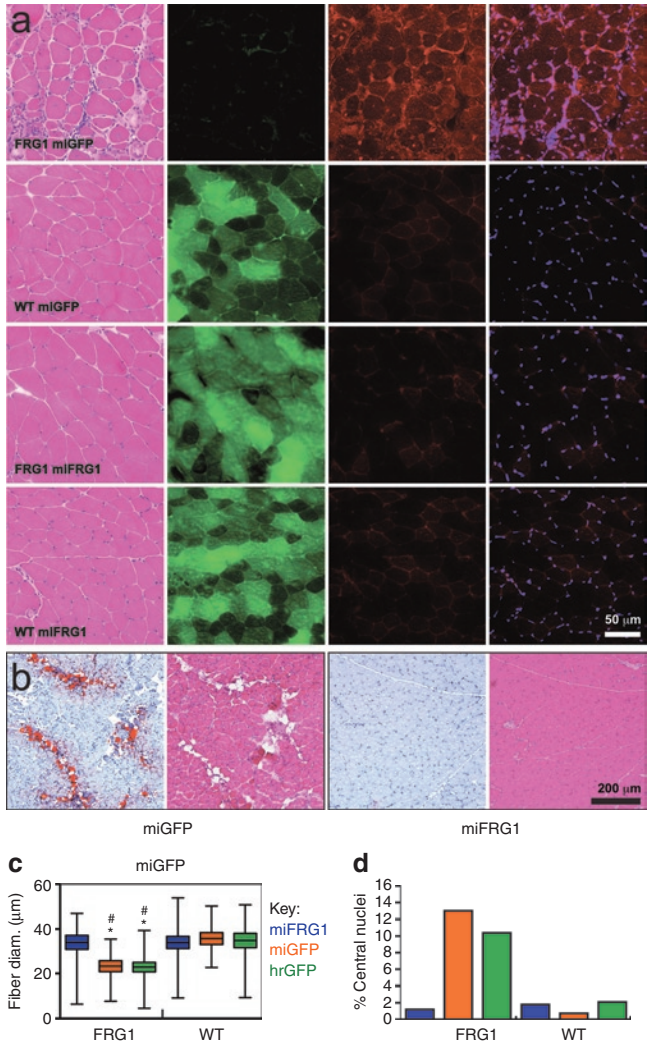

Figure 4 ***FRG1*** **gene silencing improved myopathic histology in FRG1^{-high} mice.** FRG1^{-high} mice show several histological indicators of myopathy, including fibrosis, fat deposition, myofiber size variability, and myofibers with centrally located nuclei. (**a**) Control adeno-associated viral (AAV).miGFP vectors had no impact on any of these histolopathological phenotypes. In contrast, AAV.miFRG1-transduced FRG1^{-high} muscles were indistinguishable from all wild-type groups. Muscle cryosections were stained with (left to right): hematoxylin and eosin (H&E), humanized *Renilla* GFP (hrGFP) (epifluorescence), FRG1 antibodies followed by red-labeled secondaries, and DAPI. (**b**) Oil Red O stain (with corresponding H&E-stained serial sections) shows fat-infiltrated lesions in AAV.miGFP- but not AAV.miFRG1-transduced FRG1^{-high} muscles. (**c**,**d**) *FRG1* gene silencing normalized myofiber size defects and the number of centrally nucleated myofibers in FRG1^{-high} mice. In **c**, *indicates significant differences between the comparable wild-type group, P < 0.05. #Indicates significant differences from FRG1^{-high} mice injected with miFRG1, P < 0.05. Statistics were determined using one way ANOVA with Kruskal–Wallis post-test. GFP, green fluorescent protein; miGFP, microRNAs targeting eGFP.

exclusively synonymous with gene replacement, the strategy typically used to treat recessive diseases. As RNAi and microRNA-based expression systems emerged in recent years, the gene therapy field evolved to include gene silencing as another possible therapeutic approach. Thus, the number of diseases potentially treatable with nucleic acid therapies expanded. We viewed this expansion as an opportunity to begin developing gene therapies for dominant myopathies, which historically has been an underrepresented area of research.

The "dominant myopathies" classification refers to a diverse group of clinically distinct, currently incurable, and potentially devastating muscle disorders caused by mutations in at least 29 different genes.[15] As a group, dominant myopathies are relatively abundant, possibly affecting as many as 1 in 2,400 to 1 in 3,200 individuals.[15,25] We hypothesized that a common RNAi-based therapeutic strategy, with modifications depending on etiology of each disorder, could potentially benefit a large population of patients affected by dominantly inherited muscle disease. We therefore set out to demonstrate proof-of-principle for this strategy *in vivo*. Accomplishing this required a disease animal model that developed obvious myopathic phenotypes arising from expression of a gene linked to a dominant human myopathy. We used the FRG1^{-high} mouse model in this study, which was initially developed to test the hypothesis that *FRG1* over expression was a primary pathogenic insult underlying FSHD.[17,20] Although the progressive myopathy produced in these mice strongly supported this hypothesis, there have been some conflicting data arguing against the involvement of *FRG1* in FSHD, or at least minimizing its role as a primary pathogenic insult.[17–23,26–36] Thus, it is fair to say that *FRG1* is a controversial FSHD candidate gene.[37] Nevertheless, for this study, we were unconcerned with this ongoing debate, because our primary goal was to demonstrate proof-of-principle for RNAi therapy of dominant myopathies in general, and the FRG1^{-high} line was useful as an outstanding model of dominant

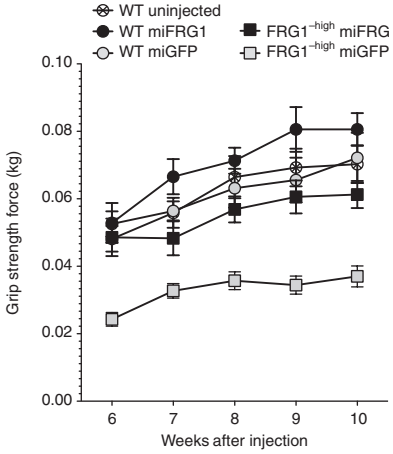

Figure 5 *FRG1* gene silencing improved strength in FRG1^{-high} mice. Hind limb grip strength assay showed that adeno-associated viral (AAV). miFRG1-treated FRG1^{-high} animals were significantly stronger than counterparts injected with AAV.miGFP controls. In contrast, the former group and all injected wild-type controls were not significantly different from uninjected wild-type animals at any time point between 6–10 weeks post injection. Data represent means ± SEM using eight male mice per group (ANOVA with Bonferroni post-test). GFP, green fluorescent protein; miGFP, microRNAs targeting eGFP.

muscle disease.[17,20] We reasoned that its involvement in FSHD, or lack thereof, was irrelevant to the goal of this study. We therefore developed a gene therapy strategy to knockdown pathological levels of human *FRG1* in FRG1^{-high} mouse muscles. Here, we reported that AAV6-delivered artificial microRNAs reduced toxic *FRG1* levels and improved histological and functional muscle abnormalities associated with *FRG1* over expression in mice. Our work therefore supports the therapeutic potential of RNAi therapy for dominant myopathies in general. In addition, it could be applied to FSHD, if additional evidence supporting *FRG1* involvement in the disease emerges; alternatively, our strategy could be modified to target other FSHD candidate genes, such as *DUX4*.[18,19,21–23,38]

Finally, we note that over expression of an otherwise normal gene, such as in FSHD and FRG1^{-high} mice, is a unique pathogenesis mechanism for dominant muscle diseases. Indeed, most other dominant myopathies arise from point mutations in one allele of a disease gene, while the other allele remains normal. In some cases, the remaining normal alleles encode essential proteins, and sufficient levels of the wild-type allele may be required to maintain some level of normal muscle function. Thus, any therapeutic benefits of reducing the dominant mutant allele could be counterbalanced if a similar reduction of the remaining wild-type allele causes haploinsufficiency-related myopathy. For example, dominant negative caveolin-3 (*CAV3*) mutations that result in 97% loss of normal *CAV3*, cause severe limb-girdle muscular dystrophy type 1C, but mutations resulting in 16% or 50% normal *CAV3* levels produce only mild hyperCKemia without muscle weakness, or normal phenotypes, respectively.[39–41] Thus, in addition to dominant mutations, *CAV3* loss of function below a certain threshold also contributes to myopathic phenotypes. This was not a concern in our proof-of-principle study here, because dominant myopathy in FRG1^{-high} mice was caused by increased dosage of an otherwise normal gene; we therefore only needed to reduce *FRG1* to sufficiently nontoxic levels. In contrast, for most other dominant myopathies, such as the *CAV3* example above, disease allele-specific RNAi strategies may be required. Importantly, several studies support the feasibility of engineering inhibitory RNA sequences that can distinguish between two alleles differing by a single nucleotide.[42] Each allele-discriminating miRNA must be uniquely designed and empirically validated, since mismatches do not necessarily prevent gene silencing (**Supplementary Figure S1** and ref. 42). Such strategies often require designing additional mismatches in the miRNA with the goal of destabilizing interactions with the normal allele of a disease gene.[42] Thus, our RNAi strategy can be modified for disease allele-specificity, when applicable. Our work therefore supports that RNAi-based gene therapy is a promising candidate strategy for treating dominant myopathies, regardless of the causal genetic mutation. Future studies demonstrating the practicability of allele-specific silencing of dominant myopathy genes will further strengthen this conclusion.

MATERIALS AND METHODS

Cloning of FRG1-targeted microRNAs. Mouse U6 promoter-driven artificial microRNAs targeting human FRG1 (called miFRG1s) were cloned using common molecular techniques as previously described.[43] All microRNAs were based on human mir-30 sequences and structure, but the mature mir-30 portions were replaced by sequences derived from the *FRG1* coding region. Ten different miFRG1s were generated; nomenclature indicates the first position of the miRNA binding site relative to +1 of the *FRG1* coding region. Control U6-driven miGFP and microRNAs targeting firefly luciferase (miLuc) were previously described.[43,44]

Luciferase assay. The luciferase reporter plasmid (**Figure 1a**) was modified from Psicheck2 (Promega, Madison, WI). Human *FRG1* cDNA was cloned downstream of the *Renilla* luciferase stop codon, thereby functioning as a 3′ UTR. A separate TK promoter driven firefly luciferase cassette served as a transfection control. HEK293 cells were cotransfected in triplicate wells (Lipofectamine-2000; Invitrogen, Carlsbad, California) with the luciferase. FRG1 reporter and individual U6.microRNA expression plasmids in a 1:5 molar ratio. *FRG1* gene silencing was determined by measuring Renilla and firefly luciferase activity (Dual Luciferase Reporter Assay System, Promega) 48 hours post-transfection, following manufacturer's instructions. Triplicate data were averaged, and individual experiments performed 3 times; results were reported as the mean ratio of renilla to firefly activity ± SEM.

Real-time PCR and western blot. For *in vitro* work, U6.miFRG1 or control microRNA plasmids were cotransfected with a CMV.FRG1 expression vector into HEK293 cells (5:1 molar ratio). Forty-eight hours later, RNA or protein was extracted (Trizol from Fisher, Waltham, MA and M-PER from Pierce, Rockford, IL respectively). For *in vivo* work, RNA or protein was extracted from muscles injected 11–14 weeks prior, using previously described methods.[23] RNA was quantified by Nanodrop, DNase-treated (DNA-Free, Ambion, TX), and reverse transcribed using random hexamers (Applied Biosystems cDNA Archive Kit; Applied Biosystems, Foster City, CA). Subsequent cDNA samples were then used as template for Taqman Assay using predesigned *FRG1* and human β-*actin* or mouse *GAPDH* control primer/probe sets (Applied Biosystems). Two independent experiments were performed, with each sample run in triplicate. All *in vitro* data were normalized to miLuc-expressing samples. For westerns, protein was quantified by Lowry assay (BioRad, Hercules, CA), 50 µg samples were separated on 15% SDS-PAGE, transferred to PVDF membrane, and incubated with the following antibodies: commercial primary mouse monoclonal antibodies to FRG1 (1:8,000, Abnova, Taipei City, Taiwan); custom polyclonal FRG1 antibodies kindly provided by Dr Peter Jones (DMA-AP-1, 1:500)[45]; mouse monoclonal β-actin antibodies (1:60,000; Sigma, St Louis, MO);

or rabbit polyclonal α-tubulin antibodies (1:5,000; Abcam, Cambridge, MA) overnight at 4°C. Following washes, blots were then probed with HRP-coupled goat anti-mouse or goat anti-rabbit secondary antibodies (1:100,000; Jackson ImmunoResearch, West Grove, PA) for 1 hour at room temperature and then developed using Immobilon Western HRP substrate (Millipore, Billerica, MA).

AAV vector delivery to mouse muscle. U6.miGFP and U6.miFRG1.948 were cloned into our AAV.CMV.hrGFP proviral plasmid upstream of CMV.hrGFP. AAV6 particles were generated and titrated as previously described by the Viral Vector Core Facility at The Research Institute at Nationwide Children's Hospital.[23] FRG1^{-high} colonies were maintained by breeding hemizygous FRG1^{-high} mice to C57BL/6 animals. Male FRG1^{-high} and negative littermates were identified by PCR genotyping of genomic DNA from newborn mice (P1 or P2) using primers detecting the HSA.FRG1 transgene (5′-CCAGGGTAAAAAGACCATTGTCG-3′ and 5′-TCGTGCTCAAGGGAACCAAG-3′) and the mouse Y chromosome (*SRY* gene; 5′-GTGTCACAGAGGAGTGGCATTTTAC-3′ and 5′-TTGCTGCTGGTGGTGGTTATGG-3′). Following genotyping, male P1 or P2 mice were injected in the lower limbs with 5×10^{10} DNAse resistant particles per leg with indicated vectors. All mouse procedures were performed following guidelines approved by the Institutional Animal Care and Use Committee (IACUC) at the Research Institute at Nationwide Children's Hospital.

Imaging and histology. *In vivo* AAV transduction was determined by hrGFP epifluorescence using a fluorescent dissecting microscope (MZ16FA, Leica, Wetzlar, Germany) at ×4.63 magnification. Dissected muscles were placed in O.C.T. Compound (Tissue-Tek, Torrance, CA), frozen in liquid nitrogen-cooled isopentane, cut onto slides as 10 µm cryosections, and stained with hematoxylin and eosin (H&E; following standard protocols), Oil Red O/Harris hematoxylin, or DMA-AP-1 FRG1 polyclonal antibodies. Oil Red O stains were performed using a filtered 60% stock solution in dH_2O (stock, 2.5 g Oil red O powder in 500 ml isopropanol). Cryosections were post fixed in 10% formalin for 10 minutes, washed in tap water, and stained in Oil red O working solution for 10 minutes. Slides were then washed in tap water, counter stained in Harris hemotoxylin for 1 minute, rinsed, blued in ammonia water, and washed in tap water. H&E and Oil Red O sections were covered with crystal-mount (Electron Microscopy Sciences, Hatfield, PA), and mounted with Permount (Fisher Scientific). For FRG1 immunohistochemistry, cryosections were post fixed in 4% paraformaldehyde, washed, blocked in 5% milk/phosphate-buffered saline-tween (PBST), incubated overnight at 4°C with DMA-AP-1 FRG1 primary antibody (1:200 in 1% BSA, 20% goat serum, and phosphate-buffered saline), and then with AlexaFluor-594 conjugated goat anti-rabbit secondary antibodies (1:500; 1 hour at RT; Molecular Probes, Carlsbad, CA). Slides were covered in Vectashield plus DAPI. All images were taken from mouse tissue harvested from 11-14 week old male mice, except in **Supplementary Figure S2** (3 week old mice). Muscle cross-sectional fiber diameters and percentage of myofibers with centrally-located nuclei were determined as previously described from five different animals per group (five fields per leg).[23]

Grip strength. Hindlimb grip strength was measured weekly between 6–10 weeks of age as previously described ($n = 8$ male animals per group).[23] Data represent means ± SEM.

SUPPLEMENTARY MATERIAL

Figure S1. miFRG1 sequences.

Figure S2. HrGFP transduction in isolated muscles from male mice used in this study.

Figure S3. AAV.miGFP transduction in 3 week-old FRG1-high mice intramuscularly injected with 5×10^{10} DRP AAV.miGFP at post natal day 1 (P1).

Figure S4. Additional controls for the grip strength assay (**Figure 5**).

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