A Deoxyribonucleic Acid Decoy Trapping DUX4 for the Treatment of Facioscapulohumeral Muscular Dystrophy

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Facioscapulohumeral dystrophy (FSHD) is characterized by a loss of repressive epigenetic marks leading to the aberrant expression of the DUX4 transcription factor. In muscle, DUX4 acts as a poison protein though the induction of multiple downstream genes. So far, there is no therapeutic solution for FSHD. Because DUX4 is a transcription factor, we developed an original therapeutic approach, based on a DNA decoy trapping the DUX4 protein, preventing its binding to genomic DNA and thereby blocking the aberrant activation of DUX4’s transcriptional network. In vitro, transfection of a DUX4 decoy into FSHD myotubes reduced the expression of the DUX4 network genes. In vivo, both double-stand DNA DUX4 decoys and adeno-associated viruses (AAVs) carrying DUX4 binding sites reduced transcriptional activation of genes downstream of DUX4 in a DUX4-expressing mouse model. Our study demonstrates, both in vitro and in vivo, the feasibility of the decoy strategy and opens new avenues of research.

INTRODUCTION

Facioscapulohumeral dystrophy (FSHD, OMIM 158900)1 is one of the most common muscular dystrophies. FSHD onset usually in teenage or early adult years and the disease progresses slowly (for review see Wagner2) The major symptoms are a rapid loss of selected muscles, including the muscles of the face, the shoulder, and the upper arm. So far there is no curative or preventive treatment. The genetic basis of the disease has been recently elucidated: FSHD is characterized by a loss of repressive epigenetic marks within the D4Z4 array located on the subtelomeric part of chromosome 4, leading to chromatin relaxation and, when associated with a permissive chromosome 4, to the expression of the normally silenced DUX4 protein3 whose open reading frame (ORF) is present in each D4Z4 repeat.4,5 DUX4 is a transcription factor resulting in a poison protein through induction of downstream genes.1,6 DUX4 expression is extremely low but it has been robustly found in adult and fetal FSHD muscle cells and biopsies.3,2–4 DUX4 might be the major trigger of FSHD onset/progression by disrupting several cellular pathways and inducing cell death in different models (for review see DeSimone et al.15). Several laboratories, including ours, have developed therapeutic strategies based on DUX4 silencing mediated by RNA interference, antisense oligonucleotides (AONs), artificial miRNAs, the short-spliced form of DUX4 (DUX4 s), or drugs.11–19

In this study, we describe a new strategy based on a DUX4 decoy. DUX4 is a transcription factor, and DUX4 binding sites have been previously identified.5,20 They include the minimum sequences of the DUX4 binding motif in non-repetitive elements and MaLR-associated sites, which are TAATCCAAATCA and TAAYBYAATCA, respectively (according to International Union of Pure and Applied Chemistry [IUPAC] nomenclature). We sought to develop a DNA decoy-based therapy strategy by trapping the endogenous DUX4, thereby preventing its binding to genomic DNA and thereby blocking transcriptional activation of genes downstream of DUX4.

RESULTS

DUX4 Decoys Reduce Transcriptional Activation of Genes Downstream of DUX4 In Vitro

The 5’-TAATCCAAATCA-3’ DUX4 binding motif, previously described to bind DUX4 in an electromobility shift assay (EMSA),6 was used to design six double-strand oligonucleotide DNA decoys. They differ in their structures (linear or circular), chemical modifications (presence or not of phosphorothioate links and/or hexaethylene glycol linkage [18-HEG]), or lengths (Figure 1A). Their capacity to inhibit DUX4 action was explored after transfection into FSHD myotubes, and the expression levels of three well-characterized DUX4 downstream genes were analyzed. All of the constructs were able to diminish ZSCAN4 and TRIM43 expression (9%–61% mRNA left).

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compared to a random decoy (Figure 1B). D3 appeared to be the best decoy but its transfection was associated with a high toxicity, with cells detaching within the days following transfection. For MBD3L2, decoys D6–D11 led to a decrease (from 36% ± 9% to 59% ± 5% mRNA left) with a statistical significance from 0.08 to 0.1.

Using a univariate test of significance, followed by a Newman-Keuls post hoc test, we analyzed the different properties of the decoys. We did not see differences between the different decoys even if D7 and D11, which carry phosphorothioate links but no hexaethylene glycol linkage, seemed to give the best results.

Double-Strand DNA DUX4 Decoys Reduce Transcriptional Activation of Genes Downstream of DUX4 In Vivo

As a next step toward translation, in vivo testing of potential therapeutic decoys was realized. A DUX4 expression plasmid (pCS2) was
intramuscularly electrotransferred into the *tibialis anterior* (TA) of C57BL/6 mice. As electrotransfer efficiency might vary between mice, inducing a variation in DUX4 levels independently of the presence of decoys, a statistical correlation between DUX4 expression and the DUX4 target gene *Tm7sf4* was first established \( \left( r^2 = 0.89 \right) \) (Figure 2A). Decoys were intramuscularly electrotransferred in the presence of the pCS2 plasmid. In order to avoid any bias relative to electrotransfer efficiency and because the decoy mechanism of action is to trap DUX4 without impacting DUX4 mRNA expression, the decoy efficacies were analyzed by calculating the expression level of *Tm7sf4* relative to DUX4. We observed a 40%-53% reduction in the presence of the decoys (with the exception of D6) compared to a random sequence decoy (Figure 2B). This demonstrates that most of the decoys, once transfected into muscle fibers, are stable enough to trap DUX4, diminish its binding to genomic DNA sequences, and consequently inhibit the activation of genes downstream of DUX4. Subcellular distribution of the decoy as well as its presence at the time of sacrifice have been documented through electrotransfer of a rhodamine-labeled D7 decoy (D7-Rho) with an identical efficacy to the native D7 decoy (Figures 2B and 2C). The D7-Rho localized to the cytoplasm of muscle fibers as expected (Figure 2C).
Decoy Stability
We next investigated decoy stability under conditions mimicking systemic delivery. The molecular stability of the DNA decoys was thus examined in the presence of nucleases. In differentiation medium, all (with the exception of D3) decoys showed high molecular weights (Figure 3A), which might correspond to concatemerized forms and which disappear in the presence of nucleases (Figures 3B and 3C). By exonuclease III, decoys D6 and D9 are totally degraded (Figure 3B). Because exonuclease III catalyzes the stepwise removal of mononucleotides starting from a 3’-OH at nicks and blunt ends in double-strand DNA, the presence of the hexaethylene glycol may have affected the ligation efficiency, leading to the formation of nicks (Figure 3B). In the presence of nuclease S1, which preferentially degrades single-strand DNA, D7, D8, and D11 presented lower molecular weights, thus suggesting that the TTTT single-strand loop triggers the degradation (Figure 3C). Finally, all of the decoys were degraded after 8-h treatment in fetal bovine serum (FBS) (Figure 3D), but the presence of the hexaethylene glycol led to a stronger resistance to nucleases (D6 and D9, Figure 3D).

AAVs Carrying DUX4 Binding Sites Reduce Transcriptional Activation of Genes Downstream of DUX4 In Vivo
We thus decided to create an adeno-associated virus (AAV) vector carrying two DUX4 decoy sequences (AAV-decoy). TA muscles of C57BL/6 mice were transduced with either AAV-decoy or an AAV-control (AAV-GFP) 14 days before the pCS2 electrottransfer, and the expression levels of several murine genes downstream of

Figure 3. Molecular Stability of the DNA Decoys in the Presence of Nucleases
(A–D) Decoys were incubated in the presence of differentiation medium (A), exonuclease III (B), nuclease S1 (C), or FBS for 0, 1, 2, 4, 8, 10, or 18 h (D) and loaded on a 15% acrylamide gel. L, 50-bp ladder. D3–D11 indicate the different decoys. Lengths of the double strands: D3, 82 bp; D6, 34 bp; D7, D8, and D9, 40 bp; D11, 28 bp.
AAV-decoy vector to inhibit DUX4-mediated activation of unchanged (p = 0.14). This result demonstrated the ability of the approach. The decoy does not require any promoter to be effective.

PAX3/7 binding sites are very close, and it was previously demonstrated that Pax3 and Pax7 compete with DUX4 after overexpression or activation of a specific disease. FSHD is particularly suited disease for this approach because DUX4 is produced at a very low level, which facilitates its neutralization in quantitative terms.

One important question regards the potential side effects of this approach. The decoy does not require any promoter to be effective and does not encode a message by itself. However, DUX4 and PAX3/7 binding sites are very close, and it was previously demonstrated that Pax3 and Pax7 compete with DUX4 after overexpression of DUX4 in C2C12 cells, and substitution mutants in which DUX4 homeodomains are replaced by Pax7 homeodomains retain the ability to inhibit differentiation and to induce cytotoxicity after overexpression. However, DUX4 and PAX7 expressions are spatiotemporally different: DUX4 is rather expressed in myofibers than in myoblasts, and PAX7 is expressed in both quiescent and activated satellite cells before being downregulated to allow cell differentiation. Because AAV vectors do not efficiently target muscle satellite cells, an interaction between the decoy and PAX7 is not likely. As it was previously demonstrated that phosphorothioate morpholino oligomer (PMO)-based antisense oligonucleotides can be found in PAX7-expressing satellite cells after intravenous injections, an AAV-based therapeutic approach would be privileged over a nucleotide-based decoy. Moreover, AAV-based therapy has the potential to provide long-lasting, one-time treatment.

Another important point is the location of the decoy within muscle fibers. DUX4 being a transcription factor, it is mainly located within the nucleus, and our study has shown that oligonucleotide-based decoys are mostly located within the cytoplasm. However, we have previously demonstrated that once translated, DUX4 protein is able to diffuse into nearby nuclei within myotubes, thus demonstrating its presence also in the cytoplasm. The oligonucleotide-based decoys may therefore bind the DUX4 protein during its cytoplasmic phase. This might be very important for the efficiency of this therapeutic approach because in contrast to antisense approaches directed to DUX4 silencing, which needs to target all nuclei of a myofiber in order to suppress DUX4 protein synthesis and in consequence needs high intra-fiber levels, a decoy-based approach with a cytoplasmic distribution will intercept the DUX4 protein irrespective of the nuclei that are involved in DUX4 synthesis. However, AAV-based decoys may trap the DUX4 protein directly within the nuclei because AAV genomes are located in the nucleus.

These observations lead to a crucial question: how can the decoys trap the DUX4 protein, assuming that the human genome may contain hundreds of DUX4 binding sites? The sequence of the decoy we chose, which is one of the strongest DUX4 binding sequences according to a recent study, or the fact that AAV genomes remain primarily episomal after transduction may be part of the answer, which still needs to be deciphered. However, several articles have already described double-strand oligonucleotide decoy-based approaches for other transcription factors, among them E2F, STAT3, or nuclear factor kB, to treat a wide range of diseases such as liver fibrosis, acute myeloid leukemia, myocardial infarction, or ovarian cancer, and a trial of a STAT3 decoy oligonucleotide in head and neck cancer has been already performed. These results demonstrate that targeting a transcription factor using a double-strand oligonucleotide is a viable and safe therapeutic approach to treat several diseases where transcription factors are involved in the pathogenesis. FSHD is a particularly suited disease for this approach because DUX4 is produced at a very low level, which facilitates its neutralization in quantitative terms.

During the past two decades, many noncoding oligonucleotide strategies have been developed to modify gene expression through repression or activation of a specific pathway, including small interfering RNAs to silence gene expression, antisense oligonucleotides to reduce protein translation, RNA decoys to compete with natural targets, or ribozymes to cleave mRNAs (for review see Shum and Rossi). In this study, we developed a double-strand deoxyribonucleic acid decoy cis-element to block the binding of DUX4 to its genomic DNA target sequences. One of our decoys (D3) was toxic after transfection in muscle cells. D3 carries phosphorothioate linkages, which improve oligonucleotide stabilization and internalization in most cells in a diffusive manner, a decoy-based approach with a cytoplasmic distribution will intercept the DUX4 protein irrespective of the nuclei that are involved in DUX4 synthesis. However, AAV-based decoys may trap the DUX4 protein directly within the nuclei because AAV genomes are located in the nucleus.

DUX4 were analyzed. A 34% decrease (p = 0.066) of the Tm7sf4/ DUX4 ratio was observed in the presence of AAV-decoy, as well as a 51% (p = 0.02) fold decrease for DuxBl (Figure 4). Wfdc3 remained unchanged (p = 0.14). This result demonstrated the ability of the AAV-decoy vector to inhibit DUX4-mediated activation of Tm7sf4.

**DISCUSSION**

During the past two decades, many noncoding oligonucleotide strategies have been developed to modify gene expression through repression or activation of a specific pathway, including small interfering RNAs to silence gene expression, antisense oligonucleotides to reduce protein translation, RNA decoys to compete with natural targets, or ribozymes to cleave mRNAs (for review see Shum and Rossi). In this study, we developed a double-strand deoxyribonucleic acid decoy cis-element to block the binding of DUX4 to its genomic DNA target sequences. One of our decoys (D3) was toxic after transfection in muscle cells. D3 carries phosphorothioate linkages, which improve oligonucleotide stabilization and internalization in most cells in a sequence-independent, but length-dependent, binding to several cellular proteins, including laminin and fibronectin. However, phosphorothioates are also toxic due to their non-specific binding to proteins, and this could explain the D3-mediated toxicity. We established a proof of principle for this therapeutic strategy capable of being delivered at a level significant enough to treat skeletal muscle pathology intramuscularly.

One important question regards the potential side effects of this approach. The decoy does not require any promoter to be effective and does not encode a message by itself. However, DUX4 and PAX3/7 binding sites are very close, and it was previously demonstrated that Pax3 and Pax7 compete with DUX4 after overexpression of DUX4 in C2C12 cells, and substitution mutants in which DUX4 homeodomains are replaced by Pax7 homeodomains retain the ability to inhibit differentiation and to induce cytotoxicity after overexpression. However, DUX4 and PAX7 expressions are spatiotemporally different: DUX4 is rather expressed in myofibers than in myoblasts, and PAX7 is expressed in both quiescent and activated satellite cells before being downregulated to allow cell differentiation. Because AAV vectors do not efficiently target muscle satellite cells, an interaction between the decoy and PAX7 is not likely. As it was previously demonstrated that phosphorothioate morpholino oligomer (PMO)-based antisense oligonucleotides can be found in PAX7-expressing satellite cells after intravenous injections, an AAV-based therapeutic approach would be privileged over a nucleotide-based decoy. Moreover, AAV-based therapy has the potential to provide long-lasting, one-time treatment.

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Further studies are now required to systemically inject the decoy in DUX4-expressing mice. Several hurdles will need to be overcome, including an efficient delivery in the body’s largest organ, an AAV pre-existing immunity, and an immune response triggered by the AAV vectors, but many laboratories are looking for solutions such as an AAV-specific plasmapheresis column or immunoglobulin G (IgG)-cleaving endopeptidases to reduce anti-AAV antibodies.

Overall, our study provides the proof of principle for a new therapeutic approach for FSHD, based on deoxyribonucleic acid decoy, an approach never developed for a neuro-muscular disease before.

MATERIALS AND METHODS

Cell Culture
Primary FSHD1 cells were derived from biopsies as previously described, and the myogenicity was determined by CD56 labeling. The cells were enriched in CD56+ cells using a magnetic-activated cell sorting (MACS) column when the percentage was below 85%. Cells were cultivated in proliferation medium (4 vol of DMEM, 1 vol of 199 medium, 20% FBS, 50 μg/mL gentamycin [Life Technologies, Saint-Aubin, France]) supplemented with 5 μg/mL insulin, 0.2 μg/mL dexamethasone, 0.5 ng/mL basic fibroblast growth factor (bFGF), 5 ng/mL human epidermal growth factor (hEGF), and 25 μg/mL FBS. Differentiation was induced by replacing the proliferation medium by DMEM supplemented with insulin (10 μg/mL).

For D3 decoy, forward and reverse oligonucleotides were annealed at equimolar concentration in a final volume of 50 μL and heated at 95°C for 4 min. For all other decoys, naked oligonucleotides were hybridized. Briefly, 10 μg of decoy was heated at 95°C for 4 min and the temperature was decreased to 20°C with a ramping of 1°C using a Veriti thermal cycler (Applied Biosystems). The ligation was performed overnight with the T4 ligase according to the manufacturer’s protocol (Life Technologies, Saint-Aubin, France). Cells were seeded in six-well plates 72 h before differentiation. Transfections were realized at day 2 of differentiation using Lipofectamine 2000 according to the manufacturer’s protocol (Invitrogen) with a ratio of 1:5 between DNA and RNAiMAX (Life Technologies, Saint-Aubin, France). The collected virus was purified by a Veriti thermal cycler (Applied Biosystems). The AAV-decoy was injected at 2.5 × 1010 viral genomes (vg)/TA.

Viral Production
Oligonucleotides for the decoy D3 were cloned into an pG2G backbone, using the restriction enzymes XbaI and HpaI, leading to the AAV-decoy backbone. All of the vectors were produced in human embryonic kidney 293 cells using polyethyleneimine (PEI) (1 mg/mL) by triple transfection at a DNA/PEI ratio of 1:3. The transfected plasmids are pX26 plasmid coding for the adenoviral sequences essential for AAV production, the pREpCap plasmid coding the AAV-1 capsid, and the viral genome carrying the decoy sequences. Three days after transfection, the supernatant was withdrawn and the collected virus was purified by iodixanol gradient and concentrated by Amicon Ultra-15 columns (Merck Millipore, Molsheim, France).

RNA Extraction and RT-PCR
Total RNAs from either cells or murine muscles were extracted using TRIzol according to the manufacturer’s protocol (Life Technologies, Saint-Aubin, France). The reverse transcription, PCR, and qPCR methods were described previously. The primers are described in Table 1. The qPCR Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) standards were followed.

Muscle Injection
All research was conducted according to the French and European regulations and was approved by the French Ministry of Education, Higher Education and Research (agreement no. APAFIS#5555-20160627I3055186 v3). Injections in the TA were performed on 6- to 8-week-old female C57BL/6 mice. After intraperitoneal anesthesia (100 mg/kg ketamine, 10 mg/kg xylazine), the TA was injected with a mix containing 10 μg of decoy and 2 μg of DUX4 plasmid expression pCS2-mkgDUX4, a gift from Stephen Tapscott (Addgene plasmid no. 21156; http://n2t.net/addgene/21156; RRID: Addgene_21156) in 40 μL of PBS-water (1:1). Transcutaneous electrical pulses were applied by two stainless steel external plate electrodes (Tweezertrodes [7 mm], BTX/Harvard Apparatus), and eight square-wave electric pulses of 200 V/cm and 20-ms duration were generated at 500-ms intervals by a Gentronix BTX ECM830 instrument (BTX/Harvard Apparatus). The AAV-decoy was injected at 2.5 × 1010 viral genomes (vg)/TA.

Histological Analysis
Mice were euthanized 4 days following electroporation and muscles were dissected immediately, two-thirds of TA was mounted in OCT and frozen in isopentane cooled in liquid nitrogen, and one-third was directly frozen in liquid nitrogen for RNA extraction. Transverse sections (8 μm) were performed on a cryostat and stained with hematoxylin and eosin (Sigma, Saint- Quentin, France) or laminin (Dako, Courtabœuf, France).

Decoy Synthesis and Stability
The decoys were ordered from Eurogentec. Forward and reverse oligonucleotides for decoys were annealed at an equimolar concentration in a final volume of 50 μL and heated at 95°C for 4 min. The ligation was performed with the T4 ligase according to the manufacturer’s protocol (New England Biolabs, Eevry, France). For decoy stability experiments, 1 μg of pre-annealed decoy was incubated either 30 min at 25°C with 10 U/μL of nuclease S1 (Thermo Scientific, Saint-Aubin, France) or 2 h at 37°C with 160 U/μL of RNase A.
exonuclease III (New England Biolabs, Evry, France), or either during a time course (0, 1, 2, 4, 8, 10, 18, and 24 h) at 37°C with FBS diluted by half. The oligodeoxynucleotides were extracted with phenol and chloroform and examined on a 15% denaturing polyacrylamide gel.

Statistical Analysis
A t test or one-way ANOVA followed by the Newman-Keuls post hoc test were used. Differences were considered statistically different at p < 0.05 (*p < 0.05, **p < 0.01, ***p < 0.001).

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.omtn.2020.10.028.

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AUTHOR CONTRIBUTIONS
J.D. and V.M. conceptualized the study. J.D. and T.V. provided funding. V.M., R.J., A.-C.M., C.H., and J.D. performed and analyzed the experiments. C.H. and J.D. performed statistical analysis. The original draft of the article was written by J.D. Review and editing were performed by V.M. and T.V., and the manuscript was approved by all authors. J.D. supervised the project.

DECLARATION OF INTERESTS
A patent named “Treatment of facioscapulohumeral dystrophy” has been filed and includes J.D., V.M. and T.V. as named inventors. The remaining authors declare no competing interests.

REFERENCES


