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Quantitative Antisense Screening and Optimization for Exon 51 Skipping in Duchenne Muscular Dystrophy

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Duchenne muscular dystrophy (DMD), the most common lethal genetic disorder, is caused by mutations in the *dystrophin* (*DMD*) gene. Exon skipping is a therapeutic approach that uses antisense oligonucleotides (AOs) to modulate splicing and restore the reading frame, leading to truncated, yet functional protein expression. In 2016, the US Food and Drug Administration (FDA) conditionally approved the first phosphorodiamidate morpholino oligomer (morpholino)-based AO drug, eteplirsen, developed for *DMD* exon 51 skipping. Eteplirsen remains controversial with insufficient evidence of its therapeutic effect in patients. We recently developed an in silico tool to design antisense morpholino sequences for exon skipping. Here, we designed morpholino AOs targeting *DMD* exon 51 using the in silico tool and quantitatively evaluated the effects in immortalized DMD muscle cells in vitro. To our surprise, most of the newly designed morpholinos induced exon 51 skipping more efficiently compared with the eteplirsen sequence. The efficacy of exon 51 skipping and rescue of dystrophin protein expression were increased by up to more than 12-fold and 7-fold, respectively, compared with the eteplirsen sequence. Significant in vivo efficacy of the most effective morpholino, determined in vitro, was confirmed in mice carrying the human *DMD* gene. These findings underscore the importance of AO sequence optimization for exon skipping.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is one of the most prevalent lethal genetic disorders in boys worldwide, with an incidence of approximately 1 in 3,600–9,337 live male births.[1] DMD is caused by the absence of dystrophin protein due to mutations in the *dystrophin* (*DMD*) gene.[2] Currently one of the most promising therapeutic avenues is exon skipping using antisense oligonucleotides (AOs).[3] Exon skipping can restore the reading frame by removing the mutant exon and/or its flanking exon(s) from the *DMD* pre-mRNA,[4–13] enabling the production of truncated but partly functional dystrophin protein as seen in the milder counterpart disorder, Becker muscular dystrophy (BMD).[14–16] A majority of DMD patients harbor deletion mutations, and 20% of these are amenable to exon 51 skipping.[17]

In September 2016, the US Food and Drug Administration (FDA) conditionally approved the first DMD antisense drug, eteplirsen (Exondys 51), which was developed to exclude exon 51 from mutant *DMD*.[18] Eteplirsen is a phosphorodiamidate morpholino oligomer (morpholino or PMO), an antisense chemistry that has been well established in terms of its safety.[19–21] The FDA's approval of eteplirsen remains controversial because its decision was made with very weak evidence supporting the effectiveness of the drug, both in terms of restoring dystrophin protein to therapeutically beneficial levels and improving clinical outcomes.[22–28] The FDA has previously rejected another drug candidate for *DMD* exon 51 skipping: the 2′-*O*-methyl-phosphorothioate-based AO drisapersen. Although therapeutics must ensure the highest possible benefit for the lowest amount of risk, no significant improvements in muscle function were demonstrated upon treatment with drisapersen, and its use led to concerns over safety.[29] Exon skipping therapy currently faces a major challenge in that its observed efficacy in patients has been very low despite the fact that significant therapeutic effects have been demonstrated in many animal studies.

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It is obvious that exon skipping efficiency is largely dependent on the AO target sequence; however, there has been little debate on the notion that the sequences targeted by eteplirsen and drisapersen might not be the optimal choices for exon skipping therapy.[30] Several groups have undertaken large-scale AO screening efforts to determine effective AO sequences both computationally and empirically. However, the exon skipping effectiveness of designed AOs has not been evaluated both quantitatively and statistically.[4,7,10,13,31] Although restoring dystrophin protein expression is necessary to improve dystrophic muscle function, the ability of AOs to rescue dystrophin protein expression has not been reported with sufficient methods of quantification in previous AO screening studies. They highly relied on RT-PCR from primary DMD muscle cells. It is remarkable that the AO sequences of eteplirsen and drisapersen were determined within this context.[4,7] Thus, there remains the possibility that the effectiveness of exon 51 skipping therapy could be improved by selecting more optimal AO sequences. This also further underlines the need for rigorous AO screening using a more reliable and direct biological measure, such as rescued dystrophin protein in DMD, for validating an AO to be tested in a clinical trial.

Here, we quantitatively evaluate the effectiveness of morpholino-based AOs for exon 51 skipping using a systematic screening method involving in silico, in vitro, and in vivo tests. Combinational screening using computational analysis to predict exon skipping efficiency of designed AO sequences[32] and in vitro tests of morpholino AOs in immortalized DMD patient-derived muscle cell lines reveals that the beginning of the *DMD* exon 51 sequence is a very promising target region for inducing exon skipping; this is notably different from the internal region targeted by eteplirsen and drisapersen. We also demonstrate that optimizing AO sequences enables efficiency in exon 51 skipping and in rescuing dystrophin protein to increase by up to more than 12-fold and 7-fold, respectively, compared with eteplirsen sequence. Significant in vivo exon 51 skipping of the most effective AO identified through in vitro screening was confirmed using transgenic mice harboring the human *DMD* gene. The present findings underscore the importance of selecting optimal AO sequences through thorough AO screening for success in clinical trials.

RESULTS

In Silico Screening of AOs for Exon 51 Skipping

We designed a total of 413 AOs: 204 and 209 AOs with 30-mer and 25-mer lengths, respectively, which cover all possible target sites in *DMD* exon 51. Our exon skipping efficiency algorithm[32] predicted that the highest efficiency for exon 51 skipping was 80.5% for 30-mer AOs and 41.2% for 25-mer AOs in the initial 5′ site of exon 51 (Table S1). In silico screening indicated a very low exon skipping efficiency for the 30-base region targeted by eteplirsen (23.7%), which was ranked 92nd in all 413 AO candidates tested. The same calculation could not be done for 20-mer drisapersen because our predictive model was based on 25- and 30-mer-long sequences; in any case, the drisapersen target site is completely encompassed by that of the 30-mer eteplirsen.

Characterization of Immortalized Clonal Healthy and DMD Skeletal Muscle Cell Lines

Significant issues in preclinical testing with primary DMD muscle cells include low purity of muscle cells and insufficient amounts of mutant *dystrophin* mRNA, which present problems when trying to test AO efficacy. To overcome these hurdles, we generated immortalized clonal skeletal muscle cells from three healthy subjects and two DMD patients with exon 52 and exon 48–50 deletion mutations (ID KM571 and 6594, respectively).[33] All immortalized skeletal muscle cell lines tested expressed easily detectable *dystrophin* mRNA from day 3 after induction of differentiation (Figure S1A). To avoid overestimation of dystrophin protein levels induced by AOs in DMD cells, we selected a cell line (ID 8220) with the highest level of dystrophin protein among three immortalized healthy skeletal muscle cell lines as determined by western blotting to serve as a positive control (Figures S1B and S1C). Dystrophin protein expression in the 8220 cell line was also confirmed by immunocytochemistry (Figure S1D).

In Vitro Screening of Exon 51 Skipping AOs

Based on the in silico screening results, we selected eight 30-mer AOs, including both high-ranking and low-ranking sequences, spaced at least four bases apart from each other for in vitro screening (Table 1). In the present study, all tested AOs, including eteplirsen and drisapersen sequences, were synthesized using the morpholino chemistry that has been demonstrated to be well tolerated in patients enrolled in clinical trials.[21] Here, we termed morpholinos having the same sequences as eteplirsen and drisapersen (produced by Gene Tools) as "analog eteplirsen" and "analog drisapersen." In RT-PCR, five of our morpholino AOs (Ac0, Ac5, Ac26, Ac30, and Ac48) at 10 μM showed significantly higher skipping efficiency compared with analog eteplirsen and drisapersen in immortalized DMD skeletal muscle cells harboring an exon 52 deletion (Figure 1A). Of the tested AOs, Ac0 in particular had the highest skipping efficiency, reaching up to 72%, which was 4 and 25 times more efficient than analogs of eteplirsen and drisapersen, respectively. In western blotting, Ac0 also induced the highest levels of dystrophin protein, reaching up to 16% of levels in the healthy control cell line, followed by Ac48 at 13% (Figure 1B).

Time-Course Analysis with Ac0, Ac48, and Analog AOs of Eteplirsen and Drisapersen

The persistent effects of Ac0, Ac48, and analogs of eteplirsen and drisapersen at 5 μM were examined in KM571 cells, which have an exon 52 deletion. The superiority of Ac0 to Ac48, with respect to exon skipping efficiency and dystrophin protein rescue, was observed at days 2 and 11 post-transfection compared with analog AOs of eteplirsen and drisapersen (Figure 2).

Dose-Dependent Effects of Ac0, Ac48, and Analog Eteplirsen and Drisapersen

RT-PCR showed that Ac0 at the highest concentration of 10 μM induced up to 74% and 64% exon 51 skipping in DMD KM571 (exon 52 deletion) and 6594 cells (exon 48–50 deletion), respectively, which were significantly higher than Ac48, analogs of eteplirsen and

Table 1. AO Sequences Used in In Vitro and In Vivo Tests, and the Ranking of Predicted Exon Skipping Efficiency by the AO Predictive Tool

Name	Oligo Sequence (5′ to 3′)[a]	Length (mer)	Distance from Ac	Predicted Skip (%)	Ranking
hEx51_Ac9	CCACAGGTTGTGTCACCAGAGTAACAGTCT	30	9	80.5	1
hEx51_Ac0	GTGTCACCAGAGTAACAGTCTGAGTAGGAG	30	0	80.1	2
hEx51_Ac5	AGGTTGTGTCACCAGAGTAACAGTCTGAGT	30	5	73.0	4
hEx51_Ac26	GGCAGTTTCCTTAGTAACCACAGGTTGTGT	30	26	66.3	12
hEx51_Ac30	AGATGGCAGTTTCCTTAGTAACCACAGGTT	30	30	55.5	25
Eteplirsen	CTCCAACATCAAGGAAGATGGCATTTCTAG	30	65	23.7	67
hEx51_Ac48	ATGGCATTTCTAGTTTGGAGATGGCAGTTT	30	48	10.6	128
hEx51_Ac141	TTATAACTTGATCAAGCAGAGAAAGCCAGT	30	141	1.8	142
hEx51_Ac207	atacCTTCTGCTTGATGATCATCTCGTTGA	30	207	NA	NA
Drisapersen	TCAAGGAAGATGGCATTTCT	20	67	NA	NA
hEx51_Ac0-29-mer	TGTCACCAGAGTAACAGTCTGAGTAGGAG	29	0	NA	NA
hEx51_Ac0-28-mer	GTCACCAGAGTAACAGTCTGAGTAGGAG	28	0	NA	NA
hEx51_Ac0-27-mer	TCACCAGAGTAACAGTCTGAGTAGGAG	27	0	NA	NA
hEx51_Ac0-26-mer	CACCAGAGTAACAGTCTGAGTAGGAG	26	0	NA	NA
hEx51_Ac0-25-mer	ACCAGAGTAACAGTCTGAGTAGGAG	25	0	33.3	10[b]

Ac, acceptor splice site.
[a]Uncapitalized nucleotides indicate intronic sequence.
[b]The ranking in 25-mer AOs.

drisapersen (Figures 3A and 3C). At the lowest concentration (1 μM), Ac0 showed 12 and 10 times higher exon skipping efficiency compared with analog eteplirsen in KM571 and 6594 cells, respectively. Interestingly, 1 μM Ac0 induced higher levels of exon 51 skipping than 10 μM analog eteplirsen (24% efficiency versus 15% in KM571 and 24% efficiency versus 21% in 6594, respectively). Quantitative western blotting revealed that 10 μM Ac0 rescued dystrophin protein expression in DMD cell lines at up to 21% of healthy cell line levels (Figures 3B and 3D). At 1 μM, the relative ratio of Ac0 to analog eteplirsen represented 7.1 and 3.3 times higher efficiency in producing dystrophin protein in KM571 and 6594 cell lines, respectively. Ac0 at 1 μM enabled the production of rescued dystrophin protein at higher or comparable levels than analog eteplirsen at 10 μM (10% versus 6% in KM571 and 11% versus 10% in 6594, respectively), confirming that Ac0 is more than 10-fold effective at producing dystrophin protein compared with analog eteplirsen concentration-wise. Analog drisapersen did not work effectively for both exon skipping and dystrophin production in the DMD muscle cell lines. The exon skipping response to Ac0 and Ac48 occurred in a dose-dependent manner that was greater than analog eteplirsen and analog drisapersen (Figure 3E). The dose-responsiveness of Ac0 with respect to dystrophin protein production was also higher than the analog ones in both DMD cell lines (Figure 3E).

Immunocytochemical Assessment of Dystrophin Protein Rescue

Immunocytochemistry revealed that Ac0 and Ac48 at 10 μM yielded more dystrophin-positive myotubes and displayed stronger signal intensity in DMD skeletal muscle cell lines harboring exon 52 and exon 48–50 deletion mutations compared with analog eteplirsen (Figure 4).

Length Optimization of Ac0 Morpholino

In silico and in vitro screening revealed that the initial 5′ region of exon 51 is an important region for influencing exon 51 skipping. To optimize the sequence length of Ac0 targeting this region, we compared the skipping efficiencies of Ac0 morpholinos of different lengths (25- to 30-mer), in which nucleotides at the 5′ site were systematically removed (Table 1). In vitro testing in immortalized DMD muscle cells treated with 1 μM of these AOs showed that 25- to 30-mer Ac0 morpholinos produced efficient exon skipping (>20%) (Figure 5), an effect that was not observed with Ac48, analog eteplirsen, and analog drisapersen at the same dose (Figure 3). The statistically significant effectiveness of 30-mer Ac0 was confirmed at 1 and 3 μM doses compared with the shorter Ac0 morpholinos in both cell lines (IDs KM 571 and 6594).

Effect of Ac0, Ac48, and Analog Eteplirsen and Drisapersen on Primary DMD Patient-Derived Skeletal Muscle Cells

We also tested the AOs in primary DMD skeletal muscle cells with exon 45–50 (ID 4546) or exon 49–50 deletion mutations (ID 4555) to validate whether the superior efficacy of 30-mer Ac0 is consistent for other muscle cell types and deletion mutation patterns. RT-PCR showed that Ac0 achieved significantly higher exon skipping efficiency in both primary DMD muscle cells compared with Ac48, analog eteplirsen, or analog drisapersen (Figures 6A–6D): up to five and seven times higher efficiency were observed compared with analog eteplirsen and drisapersen, respectively. A significant efficiency of Ac0-mediated exon 51 skipping was also confirmed in primary healthy skeletal muscle cells (Figures 6E and 6F). Interestingly, with increasing exon 51 skipping efficiency, spontaneous exon 52 skipping, which does not disrupt the reading frame, was

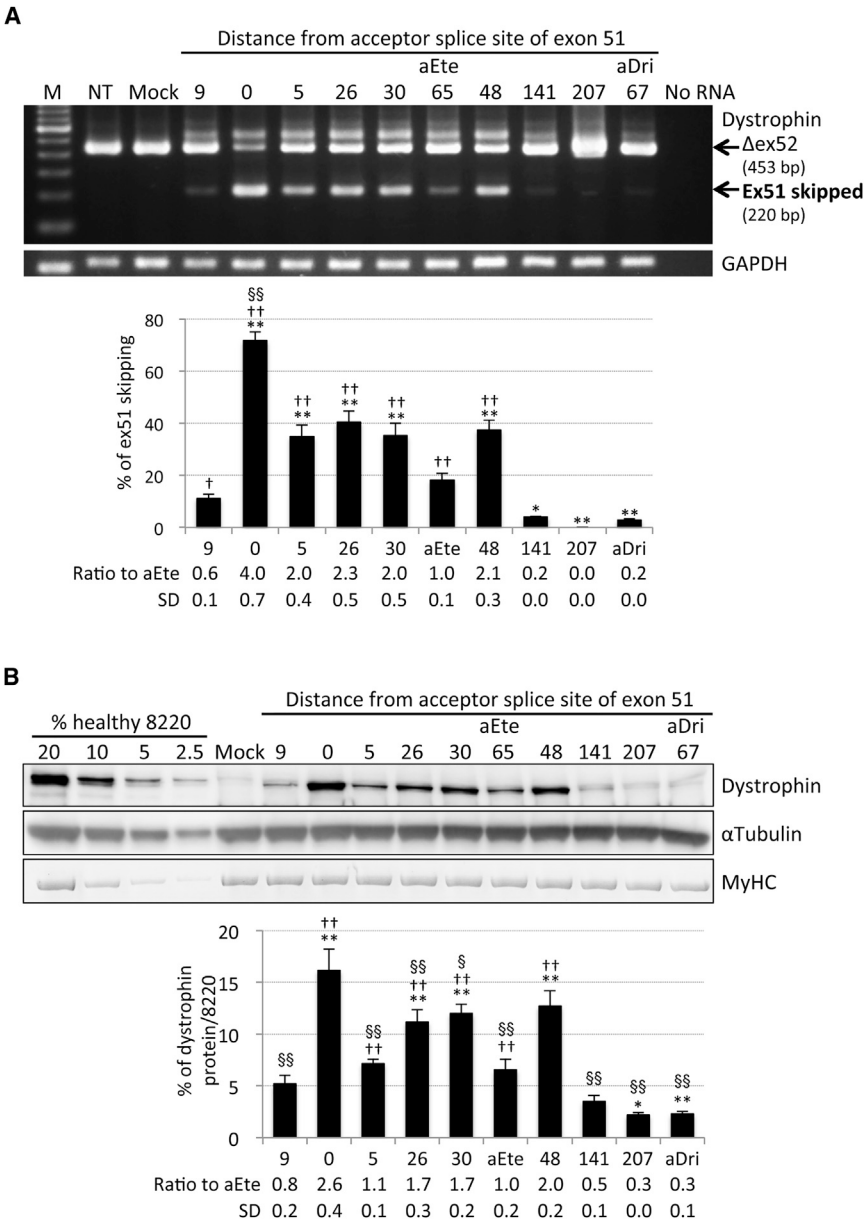

Figure 1. In Vitro Screening of Designed AOs and Analog AOs of Eteplirsen, aEte, and Drisapersen, aDri, at 10 μM, in Immortalized Clonal Exon 52-Deleted DMD-KM571 Skeletal Muscle Cells

Differentiated myotubes were harvested at day 5 following transfection (see also Figure S5). (A) Efficiency of exon 51 skipping as measured by one-step RT-PCR. Representative images are shown. Blank, no RNA template; M, 100 bp marker. (B) Efficiency in inducing truncated dystrophin protein as measured by quantitative western blotting with the anti-dystrophin C-terminal antibody. Rescued dystrophin protein levels are calculated using calibration curves with healthy 8220 cells. Data represent mean ± SD from three to four independent experiments. $^{**}p < 0.01$ versus aEte; $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ versus aDri; $^{\S\S}p < 0.01$ versus all of the AOs in (A) and versus Ac0 in (B).

observed in primary healthy and DMD muscle cells and an immortalized DMD muscle cell line with exon 48–50 deletion (ID 6594) (Figure S2).

Efficacy of Murine Ac0, Ac48, Eteplirsen, and Drisapersen in *mdx52* Mice

To test the suitability of an in vivo animal model as an alternative tool to screen AO drug candidates for patients, we performed intramuscular injections of mouse versions of human Ac0, Ac48, eteplirsen, and drisapersen sequences into the tibialis anterior (TA) muscles of *Dmd* exon 52-deleted *mdx52* mice (Figure S3A). Unlike the results of the in vitro screening in human cell models, no significant differences in exon 51 skipping efficiency or dystrophin protein rescue were observed between mouse versions of Ac0, Ac48, and eteplirsen (Figures S3B and S3C). In immunohistochemistry, all mouse analog AOs showed extensive expression of dystrophin-positive myofibers, with the signal intensity consistently increasing with administrated dose (5 and 20 μg) (Figure S3D). These results indicate that effective regions for exon skipping in the human *DMD* gene are different from those in animals.

In Vivo Efficacy of Ac0 Morpholino and Analog Eteplirsen in *hDMD/Dmd* Null Mice

A major hurdle in the development of exon skipping therapy is that human-specific AOs cannot always be tested in an appropriate animal model. This limits the evaluation of in vivo effects of AOs designed for patients. Here, we developed a new mouse model that has the full-length human *DMD* gene but lacks the entire mouse *Dmd* gene (*hDMD/Dmd* null) to test the in vivo efficacy of human AOs. This mouse model was employed to avoid cross-reaction between human sequences and mouse sequences (note that conventional *mdx* mice still have the mouse *dystrophin* mRNA, which can cross-react with human-targeting AOs) and was obtained by cross-breeding *hDMD* mice[34] and *Dmd* null mice.[35] Ac0, Ac48, analog eteplirsen, or analog drisapersen was injected into the TA muscles of these mice, and the effectiveness of in vivo exon 51 skipping was analyzed 2 weeks after the injection. The result showed significantly greater exon skipping efficiency in mice treated with Ac0 compared with analog eteplirsen (Figure 7). Faintly visible exon 51-skipped bands were found in Ac48-treated mice, with an average exon skipping efficiency of 1.11% (±0.46% SE). On the other hand, no quantifiable exon 51-skipped bands were observed in mice treated with analog drisapersen (Figure S4).

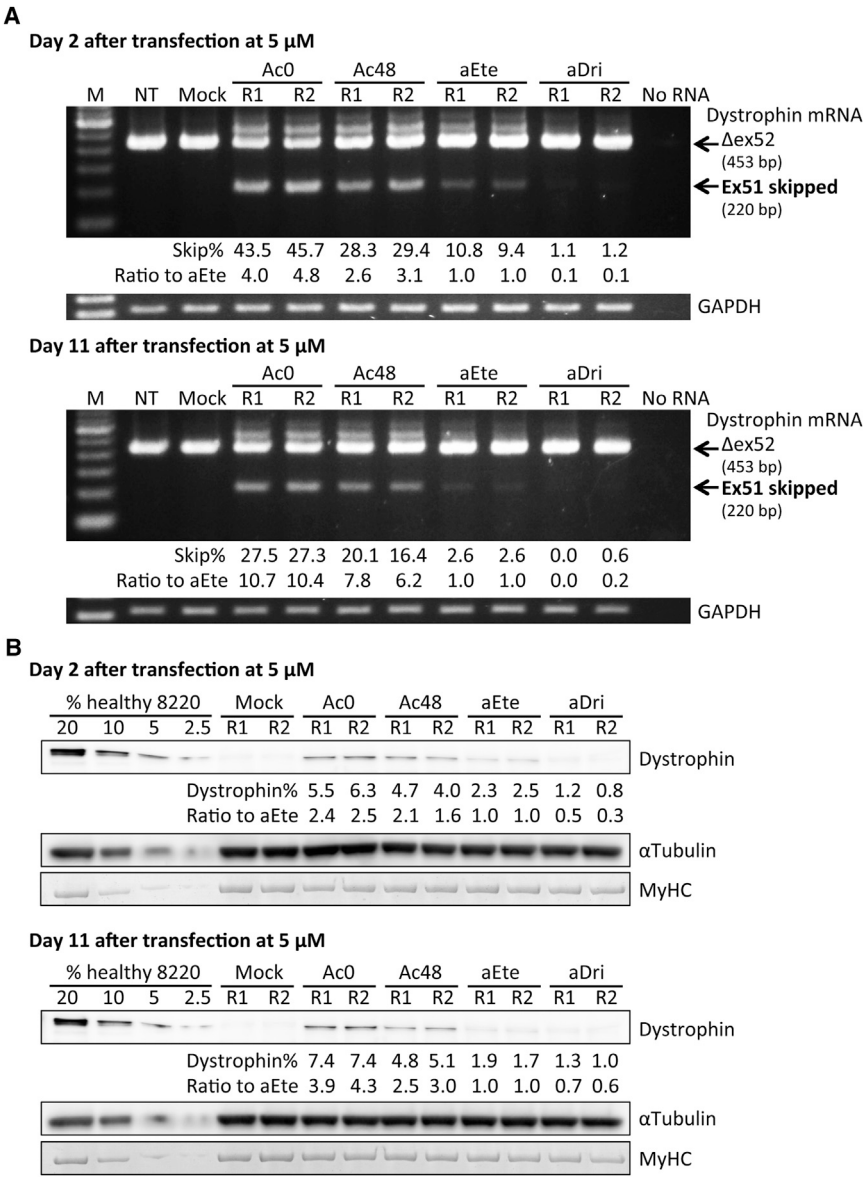

Figure 2. Time-Course Analysis of Dystrophin Exon 51 Skipping and Protein in an Exon 52-Deleted DMD-KM571 Cell Line Transfected with Ac0, Ac48, and Analog AOs of Eteplirsen and Drisapersen at 5 μM

Samples were collected at days 2 and 11 post-transfection (see also Figure S5). (A) RT-PCR analysis of exon 51 skipping. Blank, no RNA templates; M, 100 bp marker; R, replicate number. (B) Quantification of induced dystrophin protein as measured by western blotting with the anti-dystrophin C-terminal antibody. Representative replicates from three independent experiments are shown.

DISCUSSION

Currently, a major challenge for antisense-mediated exon skipping in DMD is that there has been little demonstrated therapeutic efficacy of exon 51 skipping with drisapersen or eteplirsen.[18,29] Whereas drisapersen was rejected, the FDA approved eteplirsen. However, at the same time, the FDA review team also stated that functional improvement with the drug was not proven.[18] Controversy over the approval process was centered on whether the amount of dystrophin protein produced by the drug is sufficient to be reasonably likely to translate into a clinical benefit. The rescued dystrophin levels induced by eteplirsen were reported to be in the range of up to 0.28% of healthy muscle as detected by western blotting, which is far less than the 10% of therapeutically beneficial levels estimated from BMD patients and preclinical trials.[18] These cases compel us to return to a basic concept of exon skipping: that the target sequence of a given AO primarily determines the therapeutic effect of the strategy.

Here, through evaluation of AOs designed using computational and experimental approaches, we revealed that the efficacy of exon 51 skipping largely depends on the given AO sequences and/or its target positions within the *DMD* gene. We have previously developed machine-learning algorithms to predict the exon skipping efficiency of designed AOs.[32] Although, in theory, the number of AOs for a target exon reaches hundreds of candidates, in practice, all of them will not be empirically tested. Although the evaluation of AO sequences and/or the nature of a target exon is the first screening step for identifying an AO for clinical use, no quantitative method to predict exon skipping efficacy has been available to give a robust rationale for AO sequences to proceed to in vitro screening. Because of this, many effective AO sequences may have been overlooked in previous screening efforts. Our AO predictive software tool quantitatively predicted that the initial 5′ site of exon 51 was a potentially important region for inducing exon 51 skipping, whereas the internal region targeted by eteplirsen or drisapersen was predicted to have lower efficiency. Through in silico screening, 91 of 413 AOs, which encompass the entire exon 51 with 30- or 25-mer in length, were revealed to be potentially more effective at exon 51 skipping than the 30-mer eteplirsen sequence. In contrast, the second best AO sequence (Ac48), as determined by in vitro screening, was computationally predicted to have a lower exon skipping efficiency than the eteplirsen sequence. As such, although the present prediction software cannot completely substitute for in vitro screening, the use of a precise computational assessment as the first screening step remains useful, because it can greatly help prevent the oversight of potentially effective AO sequences. The accuracy of in silico AO prediction could still be further improved by

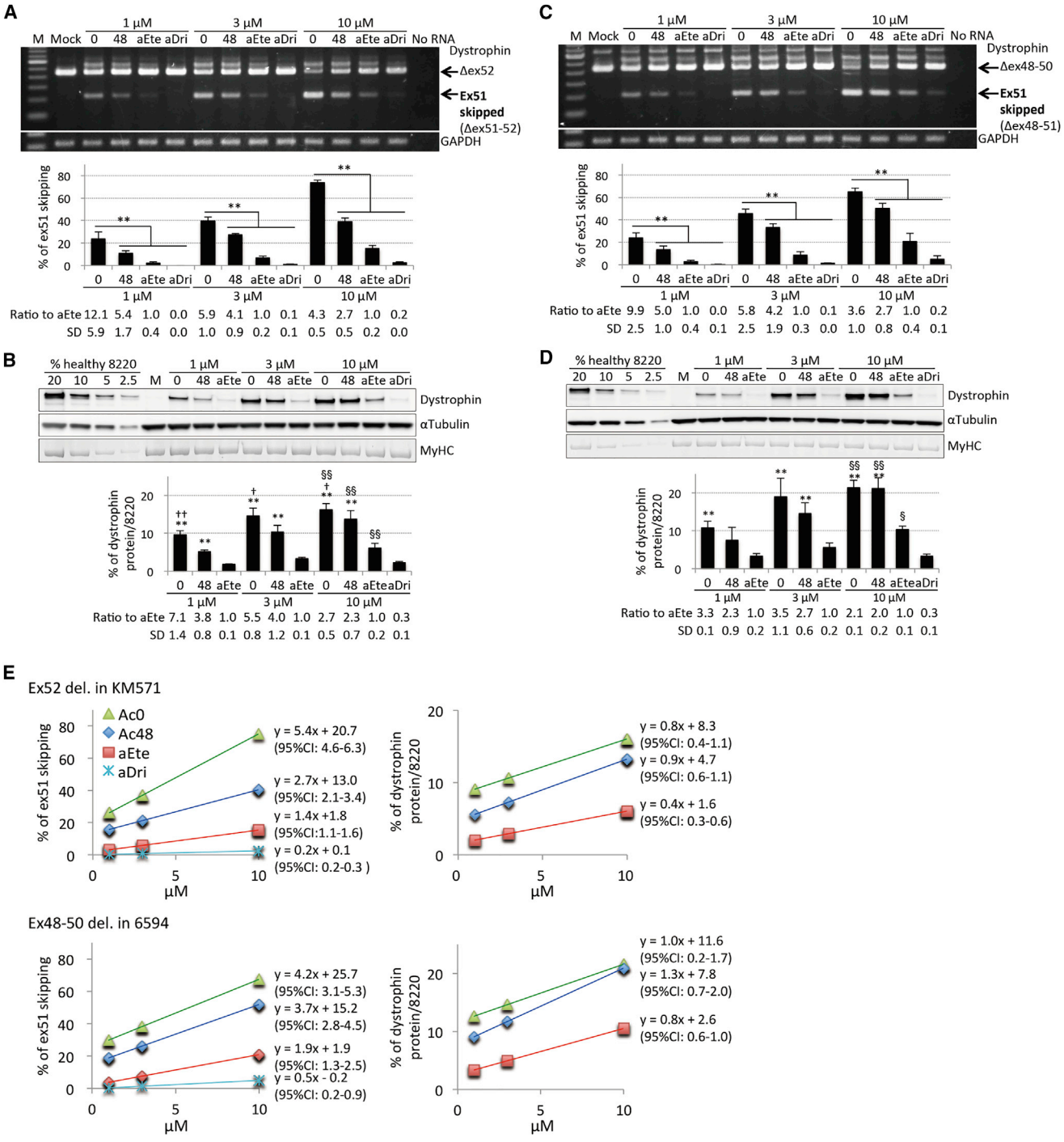

Figure 3. Dose-Dependent Effects of Ac0, Ac48, and Analogs of Eteplirsen and Drisapersen in Immortalized DMD Skeletal Muscle Cells as Measured by One-Step RT-PCR and Quantitative Western Blotting

DMD skeletal muscle cells were transfected with AOs at 1, 3, and 10 µM and harvested at day 5 post-transfection (see also Figure S5). (A and B) Exon 51 skipping efficiency (A) and expression levels of rescued dystrophin protein (B) in DMD muscle cells with an exon 52 deletion mutation (ID KM 571). (C and D) Efficacy of skipping exon 51 (C) and rescuing dystrophin protein expression (D) in DMD muscle cells harboring exon 48–50 deletion mutation (ID 6594). Data represent mean ± SD from three to seven independent experiments in the KM571 cell line and from three to four independent experiments in the 6594 cell line. (E) Dose-responsiveness to the AOs

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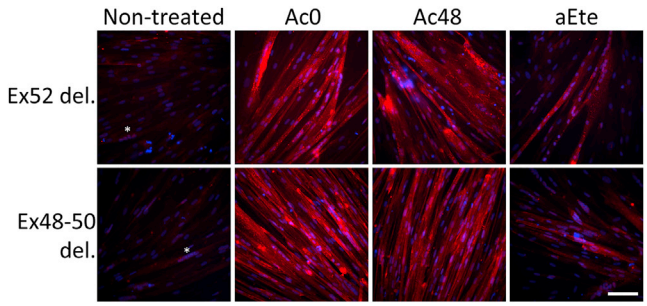

Figure 4. Immunocytochemistry in Immortalized DMD-Patient-Derived Skeletal Muscle Cells

Cells with exon 52 (ID KM571) and exon 48–50 deletion mutations (ID 6594) were used. Cells at day 5 post-transfection with 10 μM Ac0, Ac48, and analog eteplirsen (aEte) were stained with anti-dystrophin C-terminal antibody (see also Figure S5). Red signals indicate dystrophin-positive myotubes. Blue signals indicate nuclei counter-stained with DAPI. Asterisks (*) indicate representative false-positive myotubes due to their contraction or detachment from the culture plate. Representative images are shown from three independent experiments. Scale bar, 100 μm.

integrating additional factors influencing exon skipping into the algorithm, such as chemical properties of AOs (e.g., cell membrane permeability and turnover in target cells) and pre-mRNA conformational landscapes/accessibility of designed AOs to target RNA sites altered by *DMD* mutations.

It is clear that the efficacy of exon skipping as a therapy for DMD correlates with the expression levels of rescued dystrophin protein, a key determinant of whether muscle function will be improved by treatment. The present finding highlights the need for a way to assess the ability of AOs to restore dystrophin protein production in vitro. Because the effectiveness of human-specific AOs cannot currently be examined in humanized animal models harboring applicable *DMD* mutations, in vitro screening using DMD-patient-derived muscle cells is of great importance, representing a final stage at present for assessing the ability of AOs to produce dystrophin protein from *DMD* mutant variants. Primary DMD muscle cells are the most widely used cell type in AO screening efforts, but they do not always express sufficient levels of mutant *DMD* mRNA for properly examining exon skipping efficiency. This has been evidenced by the fact that in previous screening methods, exon skipping efficiency has been assessed with extra PCR cycles, using a nested PCR approach that likely overestimates levels of skipped transcripts.[4–11,13] Accordingly, quantitative screening of exon 51 skipping AOs based on their ability to rescue dystrophin protein has never been reported in primary DMD cells.[4,7,10,13,31] In this study, we demonstrated the feasibility of AO screening via quantification of dystrophin protein induced by exon 51 skipping using immortalized DMD patient cell lines. Through a combination of in silico and in vitro screening, morpholino-based AOs showed significantly greater effectiveness at facilitating exon 51 skipping and rescuing dystrophin protein expression than the eteplirsen sequence. The most effective AO, Ac0, displayed ~12-fold greater exon skipping efficiency and ~7-fold greater rescued dystrophin protein expression compared with analog eteplirsen at a transfection dose of 1 μM. In addition, we demonstrated for the first time that the observed exon skipping efficiency for an AO correlates well with the amount of dystrophin protein it was able to restore; i.e., AOs that showed the best exon skipping efficiencies also consistently best rescued dystrophin expression. Overall, these findings indicate that in vitro screening with an appropriate DMD cell model (such as immortalized DMD cells) expressing sufficient *DMD* mRNA for the evaluation of exon skipping effect can help reveal the therapeutic potential of designed AOs for progression into clinical trials.

We previously described that the accessibility of an AO to a target site could be a factor influencing exon skipping efficiency because of altered RNA conformation due to a mutation.[32] Throughout the in vitro tests using immortalized or primary DMD muscle cells, the statistically significant effectiveness of Ac0 morpholino was consistently observed in four mutation patterns that have also been tested in eteplirsen or drisapersen clinical trials.[29,36] However, the exon skipping efficiency values of Ac0 given at a dose of 10 μM to DMD muscle cells spanning the mentioned four mutation patterns ranged widely from around 15% to 74%. The variation may relate to the notion that *DMD* mutation type can alter AO target site accessibility, as we have suggested previously.[32] Also, it is possible that immortalization through an as yet unknown mechanism may have influenced AO exon skipping efficiency, especially because obtained efficiency values were found to be similar within cells that were immortalized (exon 52 deletion, exon 48–50 deletion) or cells that were of primary nature (exon 45–50 deletion, exon 49–50 deletion, normal *DMD*). Nevertheless, this finding stresses the need for AOs to be screened against a variety of *DMD* mutation patterns to get a more reliable idea of how the AO will realistically perform in patient populations. Additionally, the assessment of therapeutic outcomes from exon 51 skipping needs to be carefully considered because of differences in the functionality and stability of truncated proteins, arising from structural differences owing to variously skipped exons, as observed in BMD phenotypes with different in-frame mutations.[37,38]

As revealed in the present study using *mdx52* mice, effective AO sequences and/or target positions to skip a certain exon are different between species, which had not previously been demonstrated. This difference may be because of species specificity in how the splicing machinery works or in the *dystrophin* gene sequence itself. An *hDMD* mouse model with the normal human *DMD* gene may be a solution for assessing in vivo exon skipping efficacy of human-specific

analyzed by regression model. Statistical validity of regression equations in skipping and producing dystrophin protein was $p < 0.008$ and $p < 0.014$, respectively. Plots indicate values of exon skipping or dystrophin protein levels predicted in the regression analysis. The regression slope and 95% confidence interval (CI) are shown in individual AOs. *$p < 0.05$, **$p < 0.01$ versus analog eteplirsen (aEte); $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ versus Ac48; $^{\S}p < 0.05$, $^{\S\S}p < 0.01$ versus analog drisapersen (aDri) in the same concentration.

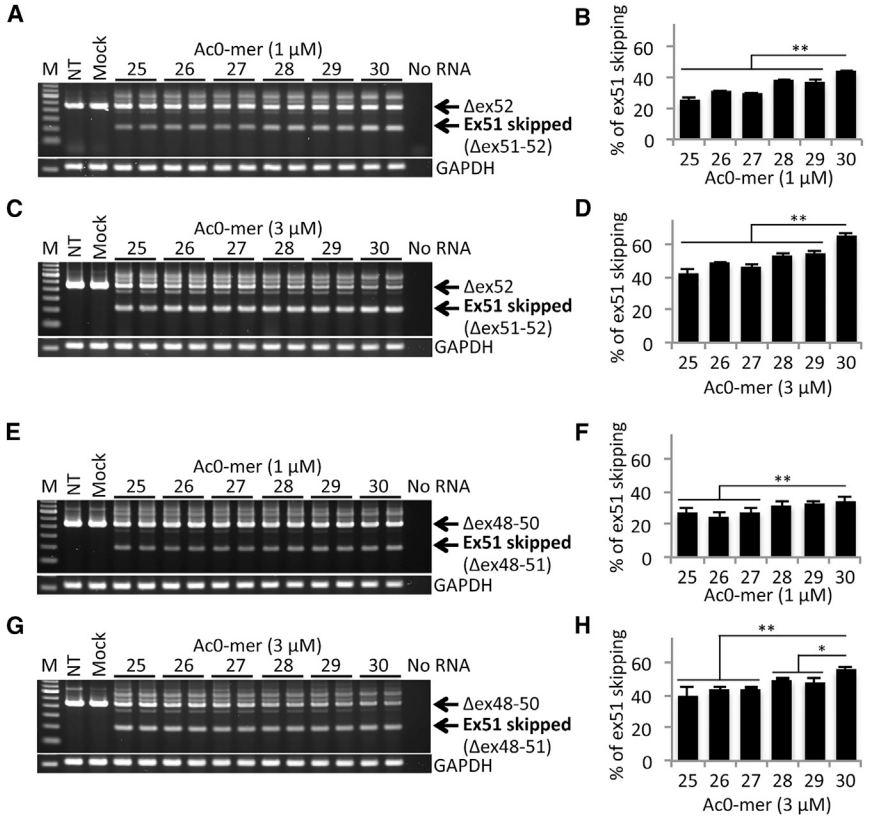

Figure 5. Length Optimization of Ac0 Morpholino

Immortalized DMD muscle cells were transfected with Ac0 morpholinos composed of 25-, 26-, 27-, 28-, 29-, and 30-mer and harvested at day 5 post-transfection (see also Figure S5). Representative images (A and C) and quantification (B and D) of exon 51 skipping induced by Ac0 morpholinos at 1 μM (A and B) and 3 μM (C and D) in DMD muscle cells with an exon 52 deletion (ID KM 571) are shown as represented by RT-PCR. Also shown are representative images (E and G) and quantification (F and H) of the same parameter at 1 μM (E and F) and 3 μM (G and H) in immortalized DMD muscle cells with a deletion of exons 48–50 (ID 6594). Data represent mean ± SD from three independent experiments.

AOs. Because *hDMD* mice retain the normal mouse *Dmd* gene, which could interrupt the precise evaluation of efficacy with a human AO, the *hDMD/Dmd null* mouse, as developed here, could be a more appropriate model. Using this model, we confirmed the significantly greater in vivo exon 51 skipping efficiency of Ac0 than analog eteplirsen. On the other hand, we observed that the difference in exon skipping efficiency between Ac0 and analog eteplirsen was not as high in vivo as seen in vitro. We believe this is due to AO uptake differences between the two models. First, these *hDMD/Dmd null* mice have healthy muscle; this limits the uptake of our AOs because prevailing theories suggest that PMO-based AO uptake is increased in the presence of active muscle regeneration or of "leaky" muscle membranes due to DMD-related muscle damage.[39] Second, AOs were delivered in vitro with the use of a transfection reagent in our experiments, which facilitated uptake as opposed to the case in vivo. Given this, there are thus expected differences in AO efficacy results obtained from in vitro and in vivo studies. Although in vivo studies in these *hDMD/Dmd null* mice better simulate the physiological dynamics of AOs, and are hence possibly more predictive of how such AOs will perform in patients, in vitro studies, on the other hand, are advantageous for easily screening a multitude of AOs in a short amount of time and across different DMD mutation patterns. An ideal screening process for AOs in DMD treatment should, therefore, consist of both in vitro and in vivo testing to capture the full potential of an AO as a candidate for human clinical trials.

It is of note that a major limitation of using this healthy mouse model expressing normal human dystrophin protein is that studies concerning treatment effects on dystrophin rescue or disease progression cannot be conducted. Exon skipping AOs will disrupt the reading frame of the normal human *DMD* gene in this model, rather than fixing it as in patient cases. This exon-skipped human *DMD* transcript is expected to be unstable and prone to degradation, which is more difficult to measure accurately; this can be another reason why Ac0 exhibited a substantial decrease in performance in this model (compared with in vitro results). Our ability to produce *DMD* mutations of interest in *hDMD/Dmd null* mice could surmount these limitations and make feasible the assessment of dystrophin protein rescue by human-specific AOs, further advancing the clinical utility of this model. Together with the development of such humanized animals, access to a precise in silico screening system and a variety of immortalized DMD muscle cell lines with different mutations will enhance our capacity to find AO drug candidates targeting other *DMD* exons.

In conclusion, the present study demonstrates that the development of more effective antisense morpholino drugs for exon 51 skipping is possible, further supporting the feasibility of dystrophin protein rescue via exon skipping in DMD patients. Our study validated that a key component of success in exon skipping therapy is selecting the right AO sequence. We also described a model for systematic AO screening using the quantification of both potential exon skipping efficiency and rescued dystrophin protein levels for the selection of an optimal AO sequence to be tested in a clinical trial. Using this screening method, we identified an AO sequence, Ac0, that was significantly more effective at rescuing dystrophin protein expression when compared with clinically investigated AO sequences. Finally, we note that the screening process of an AO drug candidate needs to be more recognized by regulators, patients, advocates, and scientists so that their decision and expectation regarding the candidate drug can be made in the same direction.

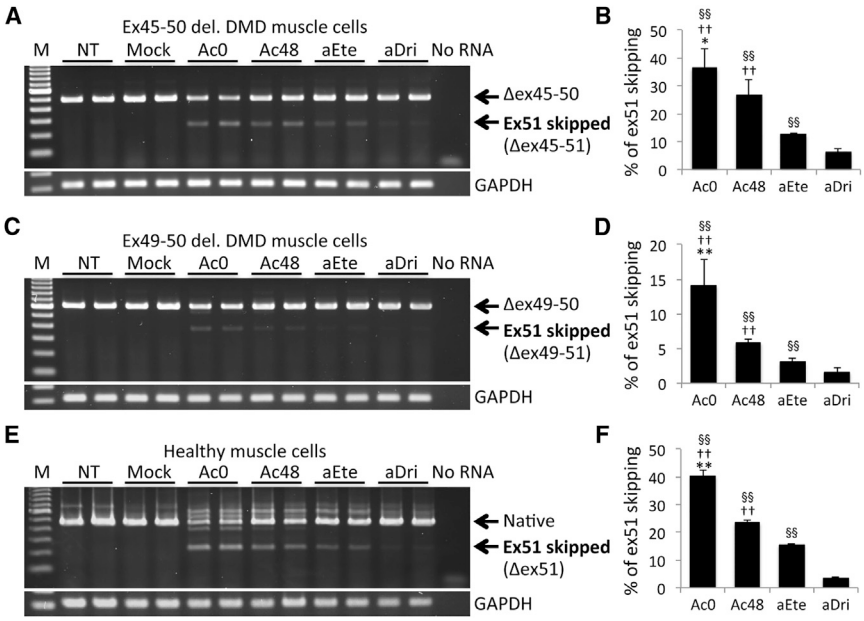

Figure 6. Exon 51 Skipping Efficiency Induced by Ac0, Ac48, Analog AOs of Eteplirsen, aEte, and Drisapersen, aDri, in Primary DMD and Healthy Skeletal Muscle Cells

Differentiated myotubes were transfected with Ac0, Ac48, and analog eteplirsen and drisapersen at 10 μM and then harvested 3 days later (see also Figure S5). Representative one-step RT-PCR images (A, C, and E) and quantification (B, D, and F) of exon 51-skipping efficiency are shown in primary DMD cells with a deletion of exons 45–50 (ID 4546) (A and B), exons 49–50 (ID 4555) (C and D), and in primary healthy muscle cells (E and F). Data represent mean ± SD from at least triplicate wells in each condition. M, 100 bp marker. *$p < 0.05$, **$p < 0.01$ versus Ac48; $^{††}p < 0.01$ versus aEte; $^{§§}p < 0.01$ versus aDri.

MATERIALS AND METHODS

Design and In Silico Screening of AOs

413 30-mer and 25-mer AOs targeting exon 51 were designed and analyzed using the AO predictive algorithm we recently developed (Table S1).[32] Based on predicted exon skipping efficiencies, eight AOs spaced at least four bases apart were selected for in vitro screening (Table 1). Target sequence specificities of selected AOs, eteplirsen, and drisapersen were analyzed using The University of California, Santa Cruz Genome Browser (http://genome.ucsc.edu/index.html), confirming that the AO sequences theoretically do not bind any non-target RNA sequences with 100% identity.

Antisense Morpholinos

All antisense sequences, including analog AOs of eteplirsen[7,40] and drisapersen,[4,41] were synthesized with the morpholino chemistry by Gene Tools.

Cells

Human-derived skeletal muscle cell lines were obtained with the help of Dr. Francesco Muntoni of the MRC Centre for Neuromuscular Diseases Biobank (NHS Research Ethics Committee reference 06/Q0406/33, HTA license number 12198) in the context of Myobank, affiliated with Eurobiobank (European certification). Immortalized human skeletal muscle cells derived from three healthy subjects (IDs 8220, CHQ, and KM155) and two DMD patients harboring deletion mutations of exon 52 (ID KM571) and exons 48–50 (ID 6594) in the *DMD* gene, respectively, were generated by transduction with human telomerase-expressing and cyclin-dependent kinase 4-expressing vectors in the Institute of Myology human cell immortalization platform, as previously described.[33] The three immortalized healthy muscle cell lines were characterized, and the clonal line 8220, which showed the highest dystrophin expression, was selected as a positive control to prevent overestimation of rescued dystrophin expression in immortalized DMD cells. Primary skeletal muscle cells derived from DMD patients with deletion mutations of exons 45–50 (ID 4546) and exons 49–50 (ID 4555) and a healthy subject were prepared by the BioBank of Skeletal Muscle, Nerve Tissue, DNA, and cell lines.

AO Transfection

To mimic as closely as possible the in vivo effects of AO-mediated exon skipping therapy, we used mature, differentiated myotubes expressing sufficient levels of *DMD* mRNA for in vitro screening. Cells were cultured in proliferation conditions with growth medium (GM): DMEM/F12 with skeletal muscle supplement mix (Promocell), 20% fetal bovine serum (Life Technologies), and antibiotics (50 U penicillin and 50 μg/ml streptomycin; Life Technologies). Immortalized and primary DMD skeletal muscle cells were seeded at $1.7 \times 10^4/cm^2$ and $2.2 \times 10^4/cm^2$, respectively, in collagen type I-coated 12- or 24-well culture plates. Two days after seeding, at approximately 80%–90% confluence, GM was replaced with differentiation medium (DM): DMEM/F12 with 2% horse serum (GE Healthcare), 1× insulin-transferrin-sodium selenite (ITS) solution (Sigma), and antibiotics. After 3 days in DM, cells were transfected with AO at 1, 3, 5, or 10 μM containing 6 μM Endo-porter transfection reagent (Gene Tools) (concentrated AOs at 1 mM were incubated at 65°C for 10 min just before diluting with DM). Two days following AO transfection, AO-containing DM was replaced with regular DM. Cells were harvested at days 2, 5, or 11 after AO transfection (days 5, 8, or 14 following differentiation). A schematic of the culture plan is shown in Figure S5.

Mice

Animal studies were approved by the Animal Care and Use Committee at the University of Alberta, Children's National Medical Center, and National Center of Neurology and Psychiatry (NCNP). Male and female *Dmd* exon 52-deficient *mdx52*[42] and wild-type mice (Jackson Laboratory) with a C57BL/6J background were prepared at age

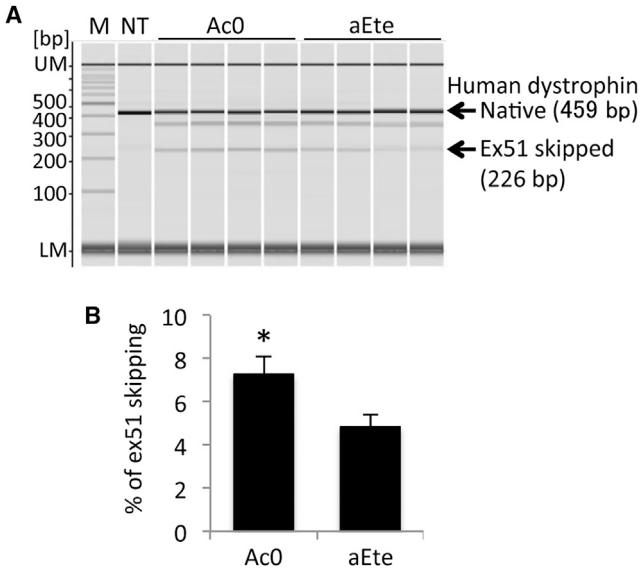

Figure 7. In Vivo Efficacy of 30-mer Ac0 Antisense Morpholino in the *hDMD/Dmd Null* Mouse Model

Exon skipping efficacy was analyzed by RT-PCR with tibialis anterior muscles 2 weeks after the intramuscular injection of Ac0 morpholino or analog eteplirsen (aEte) (50 μg in 30 μL of saline). (A) Densitometry analysis of exon 51 skipping as represented by a microchip-based capillary electrophoresis system. (B) Averaged percentage of exon 51 skipping efficiency (mean ± SE). n = 7 in each group. *p < 0.05. LM, lower marker dye; M, marker; NT, non-treated muscle; UM, upper marker dye. See also Figure S4 for Ac48 and analog drisapersen results.

4–8 weeks. *Dmd* mutation in affected mice was confirmed by genotyping with PCR. A transgenic mouse model harboring the human *DMD* gene and lacking the mouse *Dmd* gene (*hDMD*/*Dmd* null mouse) was generated by cross-breeding male *hDMD* mice (Jackson Laboratory)[34] with female *Dmd null* mice.[35]

Intramuscular Injection

Mouse version morpholinos of Ac0, Ac48, eteplirsen, or drisapersen at 5 or 20 μg in 40 μL of saline were intramuscularly injected into TA muscle under inhalation anesthesia with isoflurane as previously described (Figure S2A).[43] Fifty micrograms of Ac0 morpholino and analog eteplirsen in 30 μL of saline was injected into TA muscles of *hDMD/Dmd null* mice. All muscle samples were harvested 2 weeks after intramuscular injection.

Exon Skipping Analysis by RT-PCR

Total RNA was extracted with TRIzol (Invitrogen) as previously described.[32,43] RT-PCR to detect dystrophin mRNA was performed with the SuperScript III One-Step RT-PCR System (Invitrogen) and 0.2 μM forward and reverse primers (Table S2) for 200 and 320 ng of total RNA in immortalized and primary skeletal muscle cells, respectively. Primers were designed using Primer3Plus software, and their specificity was confirmed in healthy human skeletal muscle cells (line 8220). The RT-PCR conditions were as follows: 50°C for 5 min; 94°C for 2 min; 35 cycles at 94°C for 15 s, 60°C for 30 s, and 68°C for 35 s; and 68°C for 5 min. PCR products were separated on a 1.5% agarose gel and visualized by SYBR Safe DNA Gel Stain (Invitrogen). Using ImageJ software (NIH) or the MCE-202 MultiNA system (Shimadzu), we calculated the efficiency of exon 51 skipping using the following formula: exon 51-skipped transcript intensity/(native + intermediate + exon 51-skipped transcript intensities) × 100 (%). Unknown top bands above the native band, possibly coming from unexpected splicing events, were excluded from quantification of skipping efficiency. The sequences of the PCR products were confirmed with Big Dye Terminator v3.1 (Applied Biosystems). *GAPDH* or *18S ribosomal RNA* was used as an internal control.

Western Blotting

Cells were harvested with RIPA buffer (Thermo Scientific) containing cOmplete, Mini, EDTA-free protease inhibitor cocktail (Roche) and then homogenized by passing through a 21G needle 10 times. The supernatants as loading samples were prepared by centrifugation at 14,000 × *g* for 15 min at 4°C. Protein from muscle tissues was prepared as previously described.[43] Protein concentrations were adjusted using the Bradford assay with supernatants diluted 100 times with distilled water. Proteins in a sample buffer containing 10% SDS, 70 mM Tris-HCl (pH 6.8), 5 mM EDTA, 20% glycerol, 0.004% bromophenol blue, and 5% 2-mercaptoethanol were heated at 70°C for 10 min. Western blotting was then done as previously described.[32,43,44] Twelve and thirty micrograms from cells and tissues, respectively, were used for SDS-PAGE. Blots were incubated with a rabbit polyclonal antibody against dystrophin C-terminal (1:2,500, ab15277; Abcam) in the blocking solution or DYS1 antibody against dystrophin rod domain (1:400; Leica Biosystems) for 1 hr at room temperature. The primary antibody was reacted with horseradish peroxidase (HRP)-conjugated anti-rabbit or mouse immunoglobulin G (IgG) H+L antibody (1:10,000; Bio-Rad). Signals were detected with the ECL select detection reagent (GE Healthcare). Expression levels of dystrophin protein induced by AOs were quantified using calibration curves (R^2 = 0.93–0.99) from dystrophin protein levels of healthy 8220 skeletal muscle cells diluted with protein from non-treated DMD cells or wild-type mice using ImageJ (NIH). α-Tubulin (Abcam) was detected on the same membrane as a loading control. Myosin heavy chain (MyHC) on post-transferred gels was stained with Coomassie Brilliant Blue (Bio-Rad) as a loading control/differentiation marker.

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde for 5 min at room temperature. After washing with PBS containing 0.01% Triton X-100, cells were blocked with 10% goat serum (Life Technologies) in PBS with 0.05% Triton X-100 for 20 min and then incubated with anti-dystrophin C-terminal (ab15277) or rod-domain (DYS1) antibody at 1:50 dilution in blocking solution overnight at 4°C. Dystrophin signals were detected with Alexa 488- or 594-conjugated secondary antibody (1:500). Desmin (1:80; Abcam) and MyHC-fast type (1:30; Leica Biosystems) were detected to confirm myogenic differentiation of cells. Cells were stored in SlowFade Gold Antifade Mountant with DAPI (Invitrogen) at 4°C until analyzed.

Immunohistochemistry

Dystrophin-positive muscle fibers on cryosections from TA muscles of non-treated and treated *mdx52* mice were detected with the ab15277 antibody as previously described.[45] Signal intensity of dystrophin in the treated mice was compared with that in wild-type using neutral density filters (Eclipse TE 2000-U; Nikon).

Statistical Analysis

For determining the significance of efficiencies in exon skipping and dystrophin protein rescue, we prepared datasets from at least three independent experiments in immortalized cells, triplicate wells in primary cells, and three to seven mice. The statistical analysis between AO-treated groups was performed by one-way ANOVA followed by a post hoc Tukey-Kramer multiple comparison test. Simple linear regression analysis was performed for dose-responsiveness to AOs. Statistical significance was set at $p < 0.05$ for all analyses.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.ymthe.2017.07.014.

AUTHOR CONTRIBUTIONS

Conceptualization, T.Y.; Methodology, Y.E., K.M., Y.A., V.M., T.Y.; Software, W.D.; Investigation, Y.E., K.R.Q.L., N.T., B.B., B.M., M.C.V., J.S.N., Y.H., J.L., A.T., Y.A.; Writing, Y.E., K.R.Q.L., R.M., T.Y.; Funding Acquisition, T.Y.; Resources, Y.E., K.M., Y.A., K.N., S.T., V.M., W.D., T.Y.; Supervision and Project Administration, Y.E., R.M., T.Y.

CONFLICTS OF INTEREST

The authors have no conflicts of interest. A patent application on the technology disclosed in the manuscript has been filed.

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