Wild-Type Mouse Models to Screen Antisense Oligonucleotides for Exon-Skipping Efficacy in Duchenne Muscular Dystrophy

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Abstract

A readily available animal model is essential for rapidly identifying effective treatments for Duchenne muscular dystrophy (DMD), a devastating neuromuscular disorder caused by the lack of dystrophin protein, which results from frame-disrupting mutations in the DMD gene. Currently, the mdx mouse is the most commonly used model for antisense oligonucleotide (AO)-mediated exon skipping pre-clinical studies, with a mild phenotype. However, the accessibility of mdx mouse colonies particularly in developing countries can constrain research. Therefore in this study we explore the feasibility of using wild-type mice as models to establish exon-skipping efficiency of various DMD AO chemistries and their conjugates. Four different strains of wild-type mice and six different AO chemistries were investigated intramuscularly and the results indicated that the same exon-skipping efficiency was achieved for all tested AOs as that from mdx mice. Notably, levels of exon-skipping obtained in C57BL6 and C3H and mdx mice were most closely matched, followed by ICR and BALB/C mice. Systemic validation revealed that wild-type mice are less responsive to AO-mediated exon skipping than mdx mice. Our study provides evidence for the first time that wild-type mice can be appropriate models for assessing DMD AO exon-skipping efficiency with similar sensitivity to that of mdx mice and this finding can further accelerate the development of effective DMD AOs.

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Supporting Information files.

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Introduction

Duchenne muscular dystrophy (DMD) is an X-linked, lethal neuromuscular disorder caused by frame-disrupting mutations in the DMD gene, which ultimately result in the lack of functional dystrophin protein [1]. Currently there is no treatment available in clinic except for the use of steroid [2–4]. Numerous approaches for treating DMD are being investigated with antisense oligonucleotide (AO)-mediated exon-skipping therapies showing great promise from pre-clinical studies and phase II clinical trials [5–9]. However recent failure with Drisapersen from GSK and Prosensa phase III clinical trials highlights the importance of identifying safer and more effective AOs.

Animal models play a critical role in identifying effective treatments for DMD. Currently available DMD animal models include dystrophin-deficient mice (mdx) [10–13], dystrophin/utrophin double knock-out mice (DKO) [14], dystrophic dog [15,16] and transgenic dystrophic pig [17], transgenic humanized hDMD mice [18]. Although recently different variants of mdx mice containing different mutations e.g. mdx2cv, mdx3cv, mdx4cv, mdx5cv and exon 52 deficient mdx52 have emerged and been extensively used for different purposes [19–21]. Among them, mdx has been the most commonly used animal model for pre-clinical studies, particularly for DMD AO drug screen, which greatly contributes to ongoing clinical trials [10,13,22,23]. However, the accessibility and maintenance of mdx mouse colonies are costly, particularly in developing countries, as mdx mouse colonies are not widely distributed worldwide and also require special diets and higher-standard breeding condition compared to healthy wild-type mice, which can be a burden for researchers with limited resources. In addition, although in vitro models substantially facilitated the optimization of AO sequences for DMD, in vitro models are not completely predictive of in vivo efficacy for some chemistry e.g. PMO (phosphorodiamidate morpholino oligomer) and PNA (peptide nucleic acid), which are notoriously difficult to transfect in vitro, but showing high activity in vivo [24–28]. Thus, a readily available and more cost-effective alternative in vivo model which allows researchers to identify uptake in different genetic background before optimization will further speed up the development of DMD AOs.

Therefore, we wished to investigate whether wild-type mice can be used to evaluate in vivo uptake and exon-skipping efficiency of new exon-skipping therapeutics before proceeding to optimization of a therapeutic regime in mdx mice or appropriate animal models.
Importantly, this would lower the financial barrier of innovation in the DMD field and lead to new therapeutic molecules for DMD and other genetic diseases [29,30]. Here we demonstrated that DMD AO exon-skipping efficacy in wild-type mice is comparable to that of mdx mice via local intramuscular evaluation of six different AO chemistries, though systemic validation in C57BL6 and C3H with PMO revealed less effective exon skipping observed in wild-type mice than that of mdx mice.

Materials and Methods

Antisense oligonucleotide

Six different AO chemistries were evaluated in this study, PMO was purchased from GeneTools (Oregon, USA), and peptide-conjugated PMOs were synthesized with >90% purity by Sarepta Therapeutics (formerly AVI Biopharma Inc, Oregon, USA). PNA and peptide-PNA conjugates were synthesized with high-performance liquid chromatography purification to >90% purity by Panagene (Daejeon, Korea). Details of tested AOs were shown in Table 1. Different AO lengths and sequence positions with respect to the murine DMD exon 23 boundary region were shown in Table 1. These six strains of wild-type mice including C57BL6, C3H, BALB/C and ICR, and mdx mice were used in all experiments (3 mice each group). The experiments were carried out in the animal unit, Tianjin Medical University (Tianjin, China) according to procedures authorized and specifically approved by the institutional ethical committee (Permit Number: SYXK 2009–0001). For intramuscular studies, TA (tibialis anterior) muscles of animals were injected with various AOs, mice were killed by cervical dislocation at desired time points i.e. 48 hr, 2-week and 4-week after injection, and muscles tissues were snap-frozen in dry ice-cooled isopentane and stored at −80°C.

RNA extraction and nested RT-PCR analysis

Total RNA was extracted from tested muscle tissues with Trizol reagent as per manufacturer’s protocol (Invitrogen, UK) and 400 ng of RNA template was used for 20 μl RT–PCR with OneStep RT–PCR kit (Qiagen, UK). The primer sequences for the initial RT–PCR were Exon20Fo: 5'-CAGAATTCTGC-3' and Exon21Ro: 5'-TTCTTCAGCTTG-3'. The cycle conditions were 70°C for 3 minutes, 94°C for 15 s, 60°C for 30 s, and 72°C for 2 minutes for 25 cycles. The products were examined by electrophoresis on a 2% agarose gel. The quantification of full-length and skipped RT-PCR bands was based on densitometry of gel images and analyzed using ImageJ software (http://rsbweb.nih.gov/ij/).

cDNA synthesis

cDNA synthesis was carried out using Applied Biosystems High capacity cDNA reverse transcription kit. Reactions contained 2 μg RNA, 1× RT Buffer, 4 mM dNTP mix, RT random primers and MultiScribe reverse transcriptase in a final volume of 20 μl. The reaction was performed in a Techne TC-412 thermocycler: 25°C for 10 min, 37°C for 120 min, and 95°C for 5 min.

Quantitative PCR

Real-time PCR were performed using an Applied Biosystems StepOnePlus qPCR System with SYBR Green dye. Reactions were carried out in 20 μl reactions containing 100 ng cDNA template, 2× master mix (2× reaction buffer, 0.025 U/μl Taq polymerase, 5 mM MgCl2 and 200 μM dNTP) and 250 nM primers. Cycling was initiated at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min. Fluorescence was measured at the end of the elongation phase in each cycle. The homogeneity of amplicons was verified by melt curve. Primers used to measure total DMD transcripts were designed within exons 20 and 21: (exon 20 forward: 5’ CCACTGCTGAATTTTGACT-3’ and exon 21 reverse: 5’ CACCCCGTATCTGTTGAGAT-3’). Exon23-containing transcripts were amplified by exon 23 forward (5’ ATT-GAG-GGG-GAC-3’) and reverse primer spanning exon 24 (5’ AATCCAT-3’). Identical PCR conditions were used to evaluate Exon23 skipping at RNA level was evaluated 48 hr after injection of AOs into TA muscles.

Table 1. Antisense oligonucleotide nomenclature.

| Name | Sequence |
|------|----------|
| PMO | 5’-GCGCAATCTGCCGCTTACTGAAT-3’ |
| PNA | 5’-GCGCAATCTGCCGCTTACTGAAT-3’ |
| 2’OmePS | 5’-GCGCAATCTGCCGCTTACTGAAT-3’ |
| 8-MSP-PMO | 5’-RXXRXXRXXRXXRXXRXX-3’ |
| MOE25(PS) | 5’-GCGCAATCTGCCGCTTACTGAAT-3’ |
| MSP | ASLNIA |

MSP, muscle-specific peptide and B - L-alanine [34], doi:10.1371/journal.pone.0111079.0001

Results

C57BL6 mice are good models for assessing DMD AO exon skipping efficacy

To evaluate the feasibility of C57BL6 as an in vivo test system for AO-mediated exon skipping, we chose three well-studied and representative AO chemistries including PMO (phosphorodiamidate morpholino oligomer), PNA (peptide nucleic acid) and 2’ Ome PS (2’O-methyl RNA with phosphorothioate backbone) in our study [25,26,28,31–33]. Two microgram of PMO, 5 μg of PNA or 5 μg of 2’Ome PS was injected into TA muscles of adult C57BL6 mice and exon 25 skipping at RNA level was evaluated 48 hr after injection of AOs into TA muscles.
injection. RT-PCR results revealed that effective exon skipping can be achieved in C57BL6 with PMO and PNA, approximately 53.1% and 25.4%, respectively (Fig. 1A and B). Compared to PMO and PNA, much less efficient exon skipping was detected with 2'Ome PS, with only about 7.7% (Fig. 1B). Subsequent sequencing confirmed exon 23 exclusion in murine DMD gene (Fig. 1D). When compared with mdx mice (Fig. 1B), comparable level of exon skipping was achieved in C57BL6 for all tested AOs with the same order of efficiency, in which PMO showed the highest activity, followed by PNA and 2'Ome PS. Subsequent quantitative PCR results corroborated with the RT-PCR data and demonstrated the same pattern of exon skipping efficiency for PMO, PNA and 2'Ome PS in mdx mice (Fig. 1C). Consistently, further evaluations of PMO and PNA derivatives e.g. B-MSP-PMO (B refers to B peptide and MSP represents muscle-specific peptide), PNA-MSP and MOE PS (2'O-Methoxyethyl with phosphorothioate backbone), which were evaluated in mdx mice previously [25,27,34,35], in C57BL6 revealed that effective exon skipping can be induced in C57BL6 at 48 hr after local intramuscular injection, with B-MSP-PMO being better than PNA-MSP and MOE PS (Figs. S1A and B), suggesting that C57BL6 is a viable animal model for DMD AO screen.

Similar duration of exon skipping effects can be established between C57BL6 and mdx mice

Given effective exon skipping observed in C57BL6 with PMO, PNA and 2'Ome PS, we wished to examine whether the similar duration of exon skipping effects can be induced in C57BL6 to that of mdx mice as reported earlier [26,27]. The same amount of PMO (2 μg) and PNA, 2'Ome PS (5 μg) was injected into TA muscles of adult C57BL6 mice, respectively, and tissues were harvested at different time-points i.e. 2- and 4-week post-injection. Subsequent RT-PCR revealed almost complete exon 23 exclusion was obtained in TA muscles treated with PMO at 2-week which decreased at 4-week time-point, suggesting the peak time for measuring PMO exon skipping is 2-week post-injection. For PNA, consistent with our previous observation in mdx mice [25], PNA was higher at the 4-week time-point in C57BL6, with about 41.3% exon 23 skipping (Fig. 2A and B). In contrast, there is no significant difference in exon skipping efficiency detected for 2'Ome PS between 2- and 4-week time-points in C57BL6, with 2-week time-point (9.5%) showing a marginal increase compared to 4-week time-point (5.8%), corroborating with the earlier report [26]. Consistent with the data from 48 hr time-point, 2'Ome PS shows much less activity than those of PMO and PNA. In addition, no inflammation was observed in treated TA muscles at any time-point tested as examined by Hematoxylin & Eosin staining (Fig. S2). Overall, similar duration of exon skipping effects

Figure 1. Effective exon skipping in C57BL6 and mdx mice by local intramuscular injection of different AOs. (A) RT-PCR for detecting exon skipping at the RNA level with treated TA muscles 48 hr after intramuscular injection of 2 μg PMO, 5 μg PNA and 5 μg 2'Ome PS in C57BL6 and mdx mice. The numbered Δexon23 is for exon 23 skipping. –ve stands for RT-PCR blank control and the same below unless otherwise specified. (B) Quantitative analysis of exon skipping induced by different AOs in C57BL6 and mdx mice (**P<0.001; *P<0.05, n = 3). (C) Quantitative PCR validation for detecting exon skipping at the RNA level with treated TA muscles 48 hr after intramuscular injection in mdx mice. (D) Sequence analysis of the exon 23 skipped band in the RT-PCR products. doi:10.1371/journal.pone.0111079.g001
was established in C57BL6 to that of mdx mice, further supporting C57BL6 is a good model system for DMD AO screen. Other wild-type strains are viable model systems for screening DMD AO.

Since effective exon skipping was achieved in C57BL6 with comparable level and similar duration of effects to that of mdx mice, we extended our studies to other common laboratory strains including ICR, C3H and BALB/C. To examine the possibility and applicability of these mice as alternatives to mdx mice for DMD AO screen, the same AOs including PMO, PNA and 2’Ome PS were injected into TA muscles of these three different strains of mice, respectively, under the same dosing regimen as did with C57BL6. Treated TA muscles were harvested 48 hr after injection and assayed with RT-PCR. RT-PCR results indicated that significant exon skipping was induced for these three wild-type mice with all tested AOs (Fig. 3). Notably, the same order of exon skipping efficiency was achieved in these wild-type mice as those from C57BL6 and mdx mice, with PMO showing the highest activity, followed by PNA and 2’Ome PS in ICR, C3H and BALB/C mice, respectively (Fig. 3B). The difference was not significant compared to mdx mice. Of note, similar pattern was observed for PNA and 2’Ome PS, with comparable exon skipping achieved between C3H and mdx mice, followed by C57BL6 and ICR and the least exon skipping detected in BALB/C mice, though the overall level for these two chemistries is lower than that of PMO.

Furthermore we cross-compared the exon skipping efficiency between four wild-type and mdx mice based on the same AO chemistry. In regards to PMO, which demonstrated the highest level of exon skipping compared to PNA and 2’Ome PS in any tested animal model, similar exon skipping efficiency was achieved between C3H and mdx mice with the mean value of 63.7% and 60%, respectively (Fig. 3). Second to C3H, significant exon skipping was detected in C57BL6 and ICR, approximately 53.1% and 58%, respectively (Fig. 3G). Although slightly lower level of exon skipping was detected in BALB/C mice, the overall level for these two chemistries is lower than that of PMO.

Intravenous delivery of PMO in C57BL6 and C3H, at a low dose of 25 mg/kg for 3 weekly injections, revealed that detectable level of exon skipping was observed in quadriceps, abdominal and TA from treated C57BL6 and C3H, though the overall level is lower than counterparts from treated mdx mice under identical dosing regimen (Fig. S3). These data indicate that wild-type mice are responsive to AO-mediated exon skipping systemically, though to a less extent than that of mdx mice.
Taken together, our study demonstrated that wild-type mice can be used as alternative animal models for DMD AO screen and a means to ascertain variation in efficacy of AOs in mice with different genetic backgrounds, which can inform about the potential variation in therapeutic efficacy that might be present in the patient population.

**Discussion**

AO-mediated exon skipping therapeutics offers hope for DMD patients based on the outcome from local and systemic trials in human subjects [7–9,23,36,37]. However, to further accelerate the development of effective treatments for DMD, a reliable and cost-
effective animal model is a critical component among others. In this report, we tested the feasibility and applicability of wild-type mice, which are easily accessible and require low maintenance cost, as in vivo models to evaluate DMD AO efficacy and distribution prior to studies in relevant disease models. This study implies that in vivo efficacy of AOs, which may not correlate to efficacy in cell culture, can also be used in wild-type mice for other exons in DMD in which specific models do not exist and even for other genes and for AOs of other functions.

Compared to the in vitro system, in vivo model presents with properly-assembled musculature including the presence of connective tissue and fibroblasts et al. More pertinently, transfection reagents are frequently required to deliver the AOs in cell culture while these could substantially mask the authentic property of tested AOs. Thus, in vitro efficacy does not reflect in vivo efficacy. This notion is supported by previous studies, in which 2’Ome PS showed the greatest exon skipping activity in vitro compared to PMO and PNA but the least effective one in vivo locally and systemically [24,27,38]. In the current study, we reaffirmed findings from previous studies in mdx mice that demonstrated better in vivo exon skipping efficacy of PMO and PNA compared to negatively charged RNA analog -2’Ome PS, which can be attributed to their higher serum stability and sequence-specificity [27,31]. Peptide modifications, such as cell-penetrating peptides (CPPs) and targeting motifs are also more accurately assessed in vivo, irrespective of wild-type or mdx mice [34,39,40]. Our findings further emphasize the importance of in vivo screen for DMD AOs, particularly for neutral PMO and PNA, which are notoriously difficult to transfect in vitro. Moreover, in vivo screen in wild-type mice can be used as a rapid drug screening platform as 48 hr post-injection is sufficient to differentiate the potency of different AOs.

Although systemic evaluation of PMO in wild-type mice did not turn out as efficient as that from mdx mice under identical dosing regimens, detectable level of exon skipping was observed in C57BL6 and C3H with a low dose of PMO, suggesting wild-type mice, particularly C57BL6 and C3H, are viable animal models for systemic evaluation of DMD AOs. We speculate the difference between wild-type strains and mdx mice can be attributed to the condition of muscle membrane e.g. membrane leakage in mdx mice, which likely impacts on systemic delivery more than local administration by facilitating the vascular escape of AOs. In addition, we chose intravenous administration for our systemic studies since it was reported previously that intravenous injection led to higher level of AO detection in body-wide tissues and increased exon skipping efficiency in muscles compared to intraperitoneal and subcutaneous administration in mdx mice [41]. Furthermore, in our earlier report, we carried out a side-by-side comparison between intravenous and intraperitoneal injections in dystrophin/utrophin double knock-out mice (DKO) and the data demonstrated that intravenous administration is more effective in enhancing AO-mediated exon skipping than intraperitoneal injection [42]. Therefore, the overall low level of exon skipping observed in systemic studies is likely due to the low dose applied and unlikely related to the delivery routes used.

Ultimately, except for the hDMD mouse [18], all mouse models are primarily used to evaluate effects of backbone chemistries, peptide modifications and dosing regimens on delivery efficiency rather than efficacy of specific sequences because of sequence differences between human and murine DMD genes. Functional improvement in DMD mice, while useful in previous studies to demonstrate that restoration of dystrophin expression at a specific level can lead to functional improvement, is not as crucial for the above-mentioned uses for a variety of reasons. First, these mice do not fully recapitulate human pathology, so functional improvements may not be translatable; second, the dystrophin restored by exon skipping in humans and mice have different domains removed so it may have different functional effects; third, sequence-specific differences may have a bigger role to play on exon skipping once the AO is within the cell, thus the inability to test human sequences in mice means the results are not directly translatable. Moreover, the differences in systemic distribution between wild-type strains may provide insights into the variation that will be present in human patients and can inform better designs for AOs. Thus, our study indicates that it is beneficialfinancially and therapeutically to evaluate AO chemistry and modifications in wild-type strains despite the inability to assess functional improvements.

Conclusions

In summary, we demonstrated that wild-type mice are a practicable system for DMD AO screen locally and systemically, with wild-type mice showing less responsive to AO-mediated exon skipping than mdx mice systemically. The application of this model system can potentially open up a new avenue for DMD study and further accelerate the development of DMD AOs for the treatment of DMD patients.

Supporting Information

Figure S1 Evaluation of other AO chemistries in C57BL6 mice intramuscularly. (A) RT-PCR for detecting exon skipping at the RNA level with treated TA muscles 48 hr after intramuscular injection of 2 μg B-MSP-PMO, 5 μg PNA-MSP and 3 μg MOE. The numbered Δexon23 is for exon 23 skipping. (B) Quantitative evaluation of exon skipping induced by different AOs in C57BL6 (**P<0.001 and *P<0.05, n = 3).

Figure S2 Routine hematoxylin and eosin staining for examining muscle morphology. Hematoxylin and eosin staining of TA tissue sections from treated C57BL6 mice with 2 μg PMO, 5 μg PNA and 5 μg 2’Ome PS by local injection at different time-points e.g. 48 hr, 2 and 4 weeks after injection, and C57BL6 normal controls. Scale Bar = 100 μm. No difference was observed between treated and untreated mdx mice.

Figure S3 Systemic evaluation of PMO in wild-type and mdx mice. (A) RT-PCR results for systemic validation in C57BL6, C3H and mdx mice with PMO intravenously, at a low dose of 25mg/kg for 3 weekly injections. The numbered Δexon23 is for exon 23 skipping. (B) Quantitative evaluation of exon skipping in body-wide muscles in treated C57BL6, C3H and mdx mice (⁎P<0.001, n = 3).

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Author Contributions

Conceived and designed the experiments: HY. Performed the experiments: LC GH BG. Analyzed the data: LC GH. Wrote the paper: HY LC.
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