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ORIGINAL ARTICLE

Antisense pre-treatment increases gene therapy efficacy in dystrophic muscles

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Abstract

In preclinical models for Duchenne muscular dystrophy, dystrophin restoration during adeno-associated virus (AAV)-U7-mediated exon-skipping therapy was shown to decrease drastically after six months in treated muscles. This decline in efficacy is strongly correlated with the loss of the therapeutic AAV genomes, probably due to alterations of the dystrophic myofiber membranes. To improve the membrane integrity of the dystrophic myofibers at the time of AAV-U7 injection, *mdx* muscles were pre-treated with a single dose of the peptide-phosphorodiamidate morpholino (PPMO) antisense oligonucleotides that induced temporary dystrophin expression at the sarcolemma. The PPMO pre-treatment allowed efficient maintenance of AAV genomes in *mdx* muscles and enhanced the AAV-U7 therapy effect with a ten-fold increase of the protein level after 6 months. PPMO pre-treatment was also beneficial to AAV-mediated gene therapy with transfer of micro-dystrophin cDNA into muscles. Therefore, avoiding vector genome loss after AAV injection by PPMO pre-treatment would allow efficient long-term restoration of dystrophin and the use of lower and thus safer vector doses for Duchenne patients.

Introduction

The dystrophinopathies are pathologies caused by anomalies in the *DMD* gene that encodes the sub-sarcolemmal protein dystrophin. This protein is absent or drastically diminished in Duchenne muscular dystrophy (DMD) while it is present but qualitatively and/or quantitatively altered in the Becker muscular dystrophy (BMD). The dystrophin structure (central rod-domain made of 24 spectrin-like repeats) tolerates large internal deletions (1), which led to the development of two main therapeutic strategies: gene therapy with transfer of micro-dystrophin cDNAs in muscles, and targeted exon skipping. Both approaches have shown encouraging results using adeno-associated viral (AAV)

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vectors, which allow efficient gene transfer into muscles. AAV-mediated delivery of micro-dystrophins into dystrophin-deficient mice has shown remarkable efficiency (2–4) leading to the initiation of an early-phase clinical trial (5).

Exon skipping converts an out-of-frame mutation into an in-frame mutation leading to an internally deleted but partially functional dystrophin. This therapeutic approach has demonstrated some success using antisense oligonucleotides (AONs), but recent studies showed limited clinical benefit (6–9). The novel generation of AON chemistries, in particular tricyclo-DNA (tcDNA) (10) and peptide-phosphorodiamidate morpholino oligonucleotide (PPMO) (11,12), display unprecedented degrees of dystrophin restoration in skeletal muscles, but also restore dystrophin expression in the heart and, to a lesser extent, in the brain for the tcDNAs. AONs have the enormous advantage of not being immunogenic but require regular administration to maintain therapeutic benefit.

The antisense sequences can be expressed in skeletal or cardiac muscles via a small nuclear RNA such as U7snRNA or U1snRNA (13–15). These therapeutic molecules are vectorized in AAV particles, which ensure a permanent production of the antisense in dystrophin-deficient murine models (14–16), as well as in the dystrophin-deficient dog GRMD (17–19). In all dystrophic models, a one-shot treatment of AAV-U7snRNA (AAV-U7) was sufficient to attain substantial levels of restored dystrophin, which is associated with a significant improvement of the muscle force (14–16,18,19).

Despite the high efficiency of AAV-U7 strategy, we recently showed that dystrophin levels decreased significantly already after 6 months in various skeletal muscles in the GRMD dog (18) and between 3 and 12 months in the severely dystrophic dystrophin/utrophin knockout (dKO) mouse (20). This decline in dystrophin was strongly correlated with vector genome loss, most likely due to alterations of the dystrophic myofiber membranes. In the context of an AAV-U7 clinical trial for DMD, AAV genome fate in dystrophic muscles is of major importance since the vector capsid immunogenicity currently limits repeated treatment (21). We recently investigated the vector genome fate in the muscles of the moderately dystrophic *mdx* mouse and showed that non-therapeutic vector genomes were lost quickly after the injection and that this loss was diminished when high doses of vector genomes restored dystrophin at the sarcolemma (20). Hence, two phases of AAV genome loss occur in dystrophic muscles: first, an initial and rapid loss arising after the AAV injection and second, a slow and tardive loss observed in the severely dystrophic models, the dKO mouse and the GRMD dog.

The goal of the present study was to avoid vector genome loss by pre-conditioning the dystrophic muscles for AAV injections. First, we demonstrated that therapeutic AAV genomes were lost from *mdx* muscles only during the first phase just after the AAV-U7 injection and not during the following months. Moreover, induction of transient high dystrophin expression at the sarcolemma of myofibers with peptide-phosphorodiamidate morpholino (PPMO) AONs allowed efficient preservation of AAV genomes in *mdx* muscles. Importantly, the efficacy of AAV-U7-mediated exon skipping as well as AAV-mediated micro-dystrophin gene therapy was markedly improved. Therefore, avoiding vector genome loss after AAV injection by AON pre-treatment would allow efficient long-term restoration of dystrophin in the muscles of DMD patients.

Considering that more than 80% of DMD mutations are eligible for the personalized medicine involving the skipping of a single or of multiple exons (22), this combined therapy approach could in theory benefit up to 80% of DMD patients.

Results

Therapeutic AAV genomes are lost from *mdx* muscles after the AAV-U7 injection

We first investigated the kinetics of maintenance of therapeutic vector genomes in *mdx* muscles. The *mdx* mouse is a moderately dystrophic mouse model for DMD that carries a nonsense mutation in exon 23 of the *Dmd* gene (23). We used an AAV1 vector encoding an U7snRNA, AAV1-U7ex23, to induce efficient exon 23 skipping and therefore dystrophin rescue in *mdx* muscles (14). This vector (3E + 10 vector genomes (vg)) was injected into three-month-old *mdx* and wild-type (wt) *Tibialis anterior* (TA) muscles and the number of vector genomes was quantified by quantitative PCR (qPCR) 3, 6, 24 and 39 weeks post-injection. Three weeks post-injection, ten-fold more vector genomes were quantified in wt muscles than in *mdx* muscles confirming our previous demonstration (20) that the vector genomes are drastically lost from the dystrophic muscles during the first three weeks after the AAV injection (Fig. 1A) . Interestingly, the levels of the remaining vector genomes were stable between 3 and 39 weeks post-injection in both wt and *mdx* muscles demonstrating that the genome loss occurred mainly in *mdx* muscles before three weeks and not during the following months.

In *mdx* muscles, this dose of AAV1-U7ex23 allowed a low exon skipping (around 3% of total dystrophin transcripts) at 3 weeks after the injection reaching a plateau (around 45%) by six weeks until the end of the experiment (39 weeks) (Fig. 1B). The kinetics of dystrophin synthesis were followed in these *mdx* muscles by western blotting: 3% of normal dystrophin was expressed at 3 weeks, 30% at 6 weeks and 55% at 24 and 39 weeks (Fig. 1C). In wt muscles, exon skipping level was more than two-fold higher than in *mdx* muscles three weeks post-injection as previously shown (20) and we observed here that a similar discrepancy in exon skipping efficiency between the two muscles was maintained over 9 months (Fig. 1B). Therefore, the initial loss of vector genomes occurring before three weeks led to a reduced exon skipping efficacy in *mdx* muscles compared to wt muscles and a subsequent restrained dystrophin expression level.

AAV genomes are efficiently maintained in Pip6a-PMO rescued *mdx* muscles

We then assessed the impact of the presence of dystrophin at the sarcolemma on vector genome maintenance in dystrophic muscles. For this, we established a two-step protocol, first dystrophin expression was induced temporarily in *mdx* TA myofibers by a single injection of Pip6a-PMO AON, a PPMO that is particularly efficient for mdx exon skipping (11). Then, 1E + 11 vg of a non-therapeutic AAV carrying non-specific sequence (AAV1-U7scr) that was previously shown to be drastically lost after three weeks (20), was injected into the same muscles two weeks after the PPMO injections (Fig. 2A) when dystrophin rescue was already optimal with a mean of 72% of dystrophin-positive myofibers (Supplementary Material, data S1).

As expected, following PPMO pre-treatment and three weeks after AAV1-U7scr injection, immunofluorescence analysis revealed a strong dystrophin restoration with appropriate sarcolemmal location in *mdx* injected muscles (Fig. 2B), between 56 to 98% of normal dystrophin levels when quantified by western

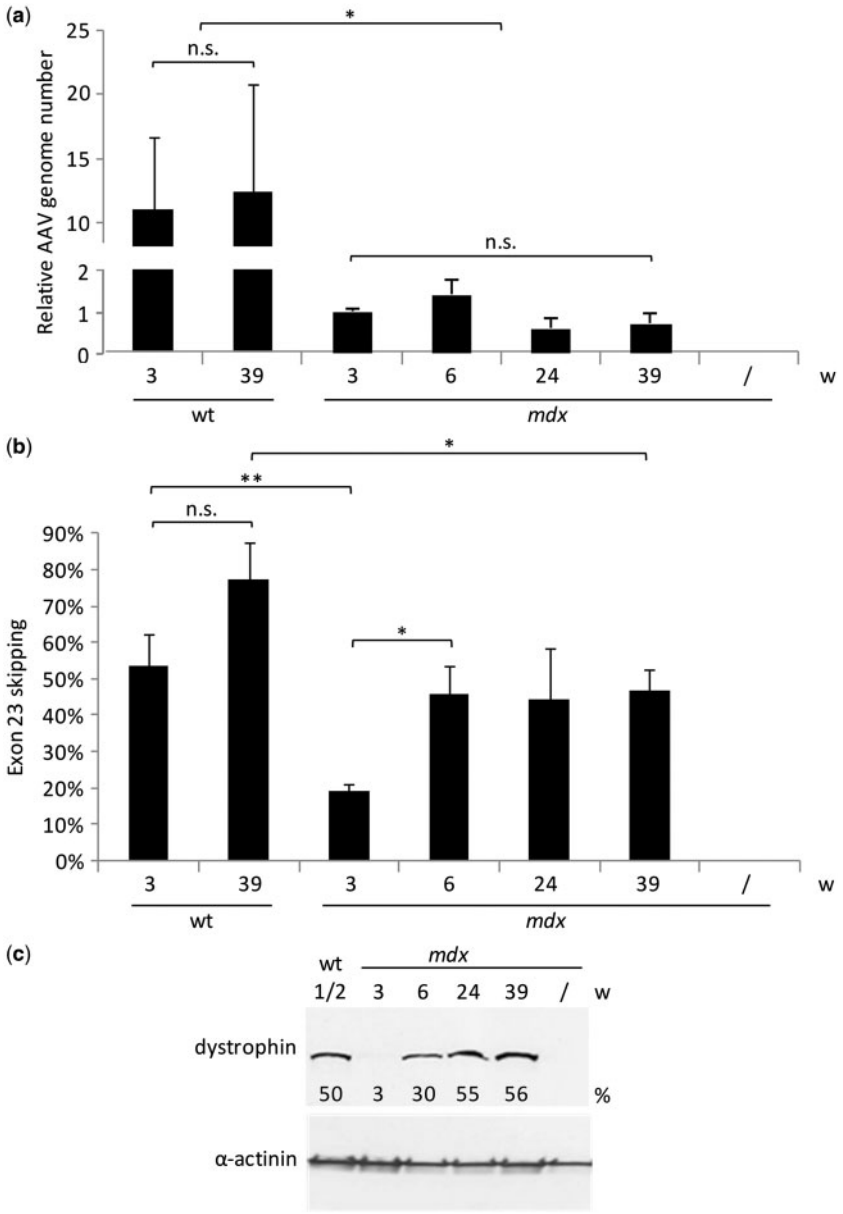

Figure 1. Kinetics of therapeutic vector genome maintenance in *mdx* muscles. *Tibialis anterior* (TA) muscles of wild-type (wt) and *mdx* mice were injected with 3E + 10 vector genomes (vg) of AAV1-U7ex23 and the mice were sacrificed 3, 6, 24 and 39 weeks later (3, 6, 24 or 39w). (**A**) Quantification of AAV genomes by absolute Taqman qPCR in injected *mdx* TAs. AAV genome content is expressed as the AAV genome number relative to the value obtained for the *mdx* muscles injected with AAV1-U7ex23 at three weeks post-injection. (**B**) Quantification of exon 23 skipping performed by relative TaqMan qPCR and expressed as a percentage of total dystrophin transcripts. (**C**) Dystrophin restoration was evaluated by western blotting with NCL-DYS1 monoclonal antibodies (upper panel) on whole protein extracts from the treated muscles (lower panel: α-actinin). The result of one representative TA is shown per condition. Dystrophin restoration was quantified by ImageJ software and expressed as the percentage of dystrophin expression in wt muscle. The data presented in (A) and (B) represent the mean values of three or four TAs per group ± SEM. One of two representative experiments is shown. n.s., non-significant, $^{*}P \leq 0.05$, $^{**}P \leq 0.001$, Student's *t*-test.

blotting (Fig. 2C), illustrating the high PPMO efficiency for dystrophin restoration. The vector genome content was 6 times lower in non-PPMO-treated *mdx* muscles than in wt muscles, as already shown (20). In contrast, the PPMO-treated *mdx* group had significantly increased numbers of vector genomes, with levels exceeding that of wt muscles, although not to significance (Fig. 2D). Therefore, a significant dystrophin expression induced by PPMO pre-treatment at the time of AAV1-U7scr injection protects against the rapid loss of AAV1-U7scr genomes in *mdx* muscles comparable to what was observed in wt muscles.

Pip6a-PMO pre-treatment allows important dystrophin rescue at low dose of therapeutic AAV-U7ex23

To evaluate the benefit of an AON pre-treatment on the dystrophin rescue mediated by the therapeutic AAV1-U7ex23, Pip6a-PMO AONs were injected into *mdx* TAs two weeks before injection of a low dose of the vector (1E + 10 vg) (Fig. 3A), which alone allowed only a weak dystrophin rescue. The benefit of AAV1-U7ex23 injection was analysed six months later when dystrophin rescue induced by the single PPMO injection was nearly abolished (Supplementary Material, data S1a). Levels of

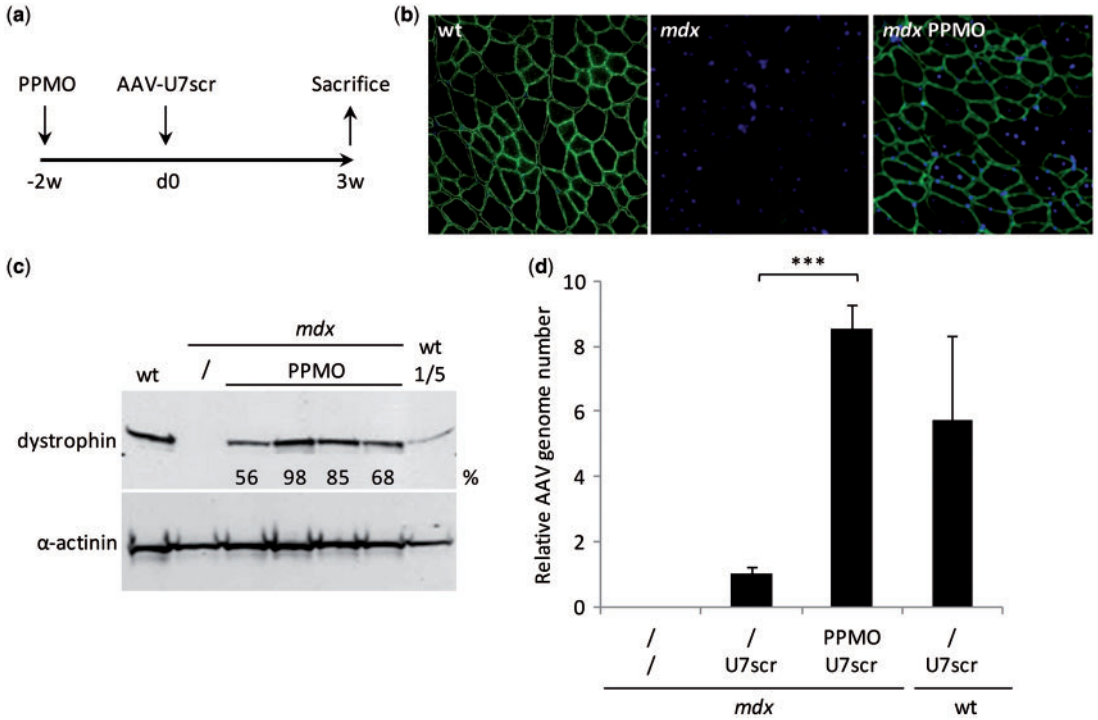

Figure 2. Effect of dystrophin restoration by Pip6a-PMO pre-treatment on vector genome maintenance. (**A**) TAs from *mdx* and wt mice were injected with 1 nmole of Pip6a-PMO two weeks (-2w) before the injection of 1E + 11 vg of the non-therapeutic AAV1-U7scr vector (day 0, d0). Control *mdx* and wt TAs were injected with AAV1-U7scr vector alone. Four TAs were injected per group. The mice were sacrificed 3 weeks later (3w). (**B**) Dystrophin rescue monitored by immunostaining with the NCL-DYS2 monoclonal antibody on transverse sections of TA muscles. One representative immunostained section is shown per condition. (**C**) Dystrophin restoration evaluated by western blotting with NCL-DYS1 monoclonal antibodies (upper panel) on whole protein extracts from the PPMO-treated muscles (lower panel: α-actinin). Dystrophin restoration was quantified by ImageJ software and expressed as the percentage of dystrophin expression in wt muscle. (**D**) Quantification of AAV genomes by absolute Taqman qPCR. AAV genome content is expressed as the AAV genome number relative to the value obtained for the non PPMO-treated *mdx* muscles. The data represent the mean values of four muscles per group ± SEM. n. s.: non-significant, $^{***}P < 0.001$, Student's *t*-test. One of two representative experiments is shown.

exon 23 skipping analysed by nested RT-PCR (Fig. 3B) and quantified by qPCR (Fig. 3C) in *mdx* TAs treated with AAV1-U7ex23 or PPMO alone were low as expected, respectively 9 and 6% of skipped transcripts, leading to the synthesis of rescued dystrophin around 2% of the normal level (Fig. 3E). Conversely, TAs treated sequentially with PPMOs then with AAV1-U7ex23 showed 54% of skipped transcripts (Fig. 3C) and a dystrophin expression at 20% of its normal level (Fig. 3E). Moreover, the vector genome number was 8-fold higher in the combined PPMO/AAV1-U7ex23 treated muscles than in AAV1-U7ex23 only injected muscles (Fig. 3D). These data demonstrate that the PPMO pre-treatment induced maintenance of the therapeutic U7ex23 genomes in *mdx* muscles six months after the AAV-U7ex23 injections and remarkably resulted in a 10-fold increase of the rescued dystrophin amount.

Pip6a-PMO pre-treatment significantly increases the efficacy of AAV1 mediated micro-dystrophin gene therapy

To evaluate the efficacy of an AON pre-treatment on AAV-micro-dystrophin gene therapy, we injected Pip6a-PMO AONs into *mdx* TAs two weeks before the injection of AAV1-MD1 vector (1E + 10 vg) expressing a murine micro-dystrophin (24) (Fig. 4A). Four weeks later, a strong dystrophin restoration was observed in PPMO-treated *mdx* TAs induced by the PPMO pre-treatment (Fig. 4C). The AAV genome copy number and micro-dystrophin expression were 3-fold higher in the PPMO/AAV1-MD1 treated muscles than in AAV1-MD1 only treated muscles (Fig. 4B and C), illustrating the PPMO pre-treatment benefit on AAV-micro-dystrophin gene therapy. This experiment establishes the proof of concept that the AON pre-treatment is capable of enhancing all AAV-based gene therapies for DMD.

Discussion

We previously showed that therapeutic AAV genomes are rapidly lost from dystrophic muscles during AAV-U7-mediated exon-skipping therapy (20). However, a strong dystrophin rescue induced by high dose of AAV-U7 in *mdx* muscles prevents this vector genome loss. Here, we showed that from the same low dose of AAV-U7 injected in *mdx* and wild-type muscles, ten-fold more vector genomes were maintained in wt muscles than in *mdx* muscles three weeks after the injections and their levels were stable between this time point until the end of the experiment, nine months later in both wt and *mdx* muscles. Therefore, the genome loss occurred principally in *mdx* muscles before three weeks, when dystrophin is not yet present at the sarcolemma, and not during the following months. Hence, in the moderately dystrophic *mdx* muscles, we could observe the initial and rapid AAV genome loss arising after the AAV injection and not the slow and tardive loss previously observed in the severely dystrophic models, dKO mice and GRMD dogs, where a massive vector genome loss is observable between 3 and 12 months (18,20). In a second AAV-U7 study performed in GRMD dogs (19), this vector genome loss was not observed

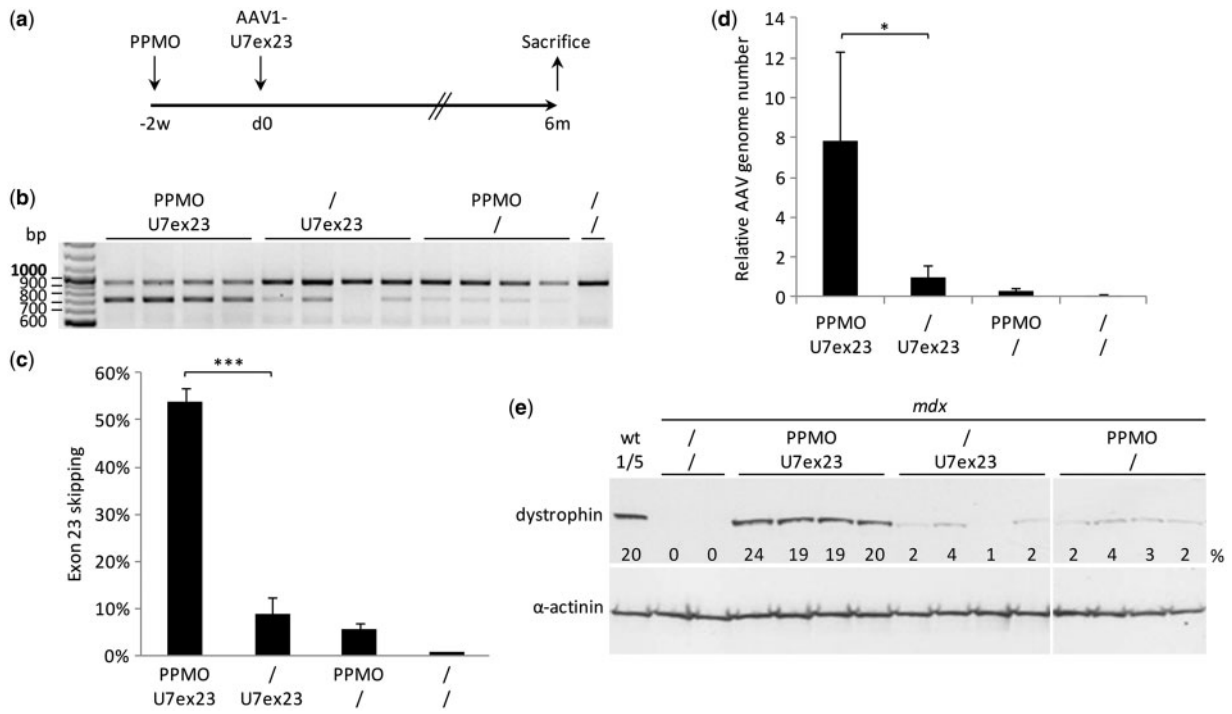

Figure 3. Effect of Pip6a-PMO pre-treatment on dystrophin rescue by low dose of AAV1-U7ex23. (**A**) *Mdx* TAs were injected with 1 nmole of Pip6a-PMO two weeks (-2w) before the injection of 1E + 10 vg of therapeutic AAV1-U7ex23 vector (day 0, d0). Control *mdx* TAs were injected with PPMO or AAV1-U7ex23 vector alone. Four TAs were injected per group. The mice were sacrificed 6 months later (6m). (**B**) Level of exon 23 skipping estimated by nested RT-PCR. The 901 bp PCR product corresponds to full-length dystrophin transcripts whereas the 688 bp product corresponds to transcripts lacking exon 23. (**C**) Quantification of exon 23 skipping performed by relative TaqMan qPCR and expressed as a percentage of total dystrophin transcripts. (**D**) Quantification of AAV genomes by absolute Taqman qPCR. AAV genome content is expressed as the AAV genome number relative to the value obtained for the non PPMO-treated *mdx* muscles. The data presented in (C) and (D) represent the mean values of the four TAs per group ± SEM. * $P < 0.05$, *** $P < 0.001$, Student's *t*-test. (**E**) Dystrophin restoration evaluated by western blotting with NCL-DYS1 monoclonal antibodies (upper panel) on whole protein extracts from the treated muscles (lower panel: α-actinin). Dystrophin restoration was quantified by ImageJ software and expressed as the percentage of dystrophin expression in wt muscle.

certainly because the dogs were followed for 3.5 months and sacrificed before the onset of the vector genome loss observed in Vulin *et al.* (18).

This difference between the DMD models in term of vector genome loss is certainly due to differences in the phenotypic severity of the dystrophin deficient animals. The *mdx* mouse, which is historically the primary animal model for DMD, presents histological signs of muscular dystrophy, but has a little muscle weakness and relatively normal lifespan. On the other hand, the dKO mouse suffers from a much more severe and progressive muscle wasting, impaired mobility and premature death, similar to DMD patients (25,26). GRMD dogs are a larger animal model exhibiting also a severe dystrophic phenotype which parallels the human disease with early muscle weakness leading to locomotor, respiratory, digestive and cardiac impairments (27). Although *mdx* mice are mildly affected, they present a peculiar phase of rapid cycles of necrosis and regeneration between three and ten weeks of age. Our experiments were performed on three-month-old *mdx* mice, thus the massive initial loss of vector genomes described in their muscles cannot be attributed to this severe period of necrosis.

To slow down the loss of therapeutic vector genomes from dystrophic muscles and achieve long-term restoration of dystrophin expression, we previously proposed recurrent systemic injections of AONs to prevent the progressive reappearance of a dystrophic phenotype caused by the partial loss of AAV genomes over time (20). Instead of these successive therapies, we demonstrate here the synergistic benefit of the reciprocal treatment combination with first a single AON injection to restore dystrophin at the myofiber sarcolemma and secure membrane integrity followed by a single systemic injection of AAV-U7 vector to induce a strong and long-lasting expression of dystrophin in muscles. A significant dystrophin rescue by PPMO pre-treatment at the time of AAV-U7 injection allows an efficient maintenance of the vector genomes in *mdx* muscles three weeks later. Additionally, this initial maintenance of vector genomes increases dystrophin restoration by AAV-U7, around 8-fold at RNA level and 10-fold at protein level up to six months later. Of course, this does not rule out our previous hypothesis that AON administration subsequent to AAV treatment could supplement AAV-mediated dystrophin restoration and act so as to maintain or even increase dystrophin levels, especially as a life-long treatment will be required in DMD patients. We also showed that an AON pre-treatment confers an increased benefit to AAV-mediated micro-dystrophin cDNA transfer into *mdx* muscles. Indeed, four weeks after injection, micro-dystrophin expression was already 3-fold higher in PPMO-treated *mdx* mouse compared to untreated one.

The PPMO pre-treatment results in substantial dystrophin expression at the time of AAV-U7 injection that likely reduces the membrane abnormalities. Once established, an AAV-U7 mediated high dystrophin expression will be maintained because it will by itself prevent transgene loss (20). The same scenario is conceivable for micro-dystrophin gene therapy. By allowing the maintenance of high vector genome content in the decisive period between AAV injection and AAV-mediated transgene expression in the treated dystrophic muscles, PPMO-mediated dystrophin restoration guarantees a higher therapeutic benefit

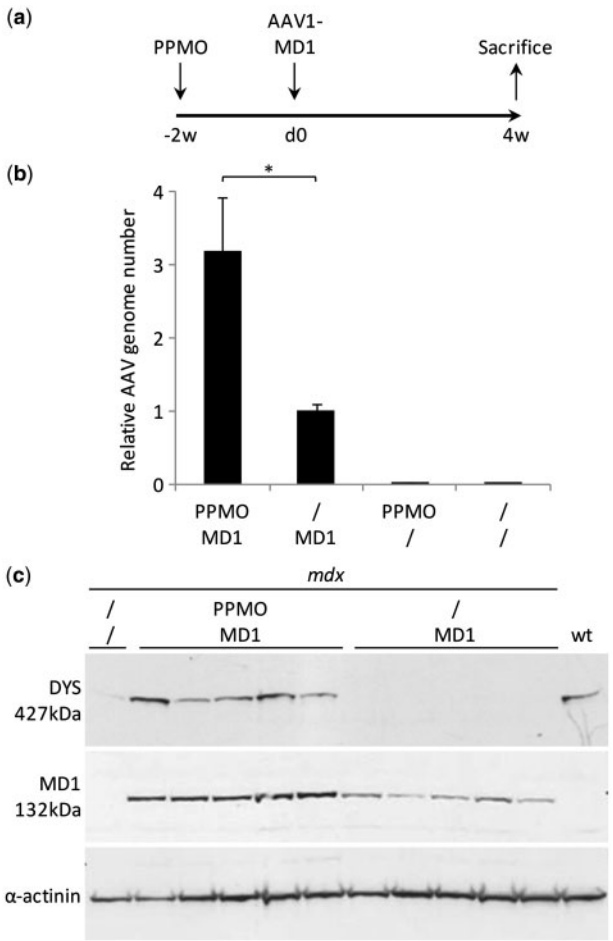

Figure 4. Effect of Pip6a-PMO pre-treatment on AAV1-mediated micro-dystrophin gene therapy. (**A**) *Mdx* TAs were injected with 1 nmole of Pip6a-PMO two weeks (-2w) before injection of 1E + 10 vg of AAV1-MD1 micro-dystrophin expressing vector (day 0, d0). Control *mdx* TAs were injected with PPMO or AAV1-MD1 vector alone. Five TAs were injected per group. The mice were sacrificed 4 weeks later (4w). (**B**) Quantification of AAV genomes by absolute Taqman qPCR. AAV genome content is expressed as the AAV genome number relative to the value obtained for the non PPMO-treated *mdx* muscles. The data represent the mean values of the 5 muscles per group ± SEM. *$P < 0.05$, Student's *t*-test. (**C**) Expression of PPMO-induced dystrophin (DYS, 427kDa) and micro-dystrophin (μDYS, 132kDa) evaluated by western blotting with MANEX1011B monoclonal antibodies (upper panel) on whole protein extracts from the treated muscles (lower panel: α-actinin).

of the AAV based therapy compared to direct AAV injection. Additionally, in condition of poor AAV transduction, especially with systemic delivery some muscles are less transduced than others (28), PPMO pre-treatment could help reaching in these muscles the threshold dose of AAV genomes needed to restore efficiently dystrophin in *mdx* muscles. Nevertheless, the PPMO pre-treatment will not prevent the tardive vector genome loss observed in the severely dystrophic models but will delay significantly its onset by increasing the initial vector genome content in the myofibers.

The fact that the rescue effect by the pre-treatment on AAV genome maintenance was relatively lower for AAV-MD1 (3-fold increase of AAV genome copy number, Fig. 4B) as compared to AAV-U7 mediated treatment (8-fold increase, Fig. 3D) can likely be explained by the different mechanisms of action of the two therapeutic strategies. During AAV-MD1 therapy, the direct transcription and overexpression of the micro-dystrophin cDNA result in rapid dystrophin-mediated membrane sealing and a minor AAV genome loss. In contrast, the U7-mediated exon skipping is dependent on the U7-antisense transcription and its action on intrinsically produced dystrophin mRNA which delays membrane sealing and thereby favours increased AAV genome loss. Therefore, this strong genome loss could be countered more easily by the pre-treatment than the slight loss observed during AAV-MD1 therapy. This assumption is consistent with our observation that, following intramuscular injections with an equivalent low dose of vectors, AAV-MD1 injected muscles presented a strong dystrophin recue with a high percentage of dystrophin-positive fibres (60%) whereas AAV-U7 muscles had little effect at this dose (Supplementary Material, data S2). This strong dystrophin rescue by AAV-MD1 was indeed correlated with three times more vector genomes than in AAV-U7 muscles. Finally, this divergence between AAV-MD1 and AAV-U7 mechanisms of action may explain why in dystrophic dogs robust micro-dystrophin expression was obtained for at least two years (29), whereas U7-mediated benefit declined drastically after six months (18).

Albeit we can not exclude a lower AAV transduction efficiency in dystrophic muscles compared to wild-type tissues, the vector genome loss is certainly due to the fragility of the dystrophic muscle fibres that undergo cycles of necrosis/generation as we observed a similar loss of vector genomes in cardiotoxin-treated normal muscles (20). It was previously proposed that the poor AAV transduction efficiency in *mdx* muscles could be the result of an exacerbated immune response against the transgene product and the AAV capsid that would amplify the cycles of necrosis/generation and leads to the transgene elimination (30).

However, other causes could participate in the process of vector genome loss from the dystrophic muscles. Dystrophic myofibers present abnormally leaky membranes that could passively loose the vector genomes, as exemplified by the creatine kinase (CK) activity greatly elevated in sera of Duchenne patients and preclinical animal models or conversely by Evans blue uptake into dystrophic muscle (31) that has been shown to result from permeabilization of the sarcolemma by the abnormal presence of connexin hemichannels (32). In addition, AAV vectors were also found associated with exosomes termed vexosomes in culture media of AAV producer cells (33). Interestingly, lack of dystrophin at the sarcolemma of *mdx* myofibers was shown to lead to an increased excretion of exosomes that was partially restored by dystrophin rescue (34), suggesting that therapeutic vectors might also be lost through excretion via exosomes from the dystrophic myofibers. However, such secretion mechanisms of AAV vectors remain to be further investigated *in vivo*. Therefore, AON-mediated dystrophin restoration could reduce these membrane abnormalities and thus further preserve the therapeutic vector genomes in the dystrophic myofibers. Recently, oxidative damage has been shown to destabilize transcripts derived from AAV vectors through free radical damage, reducing the transgene expression in dystrophic muscles (35). Interestingly, high expression of micro-dystrophin was able to significantly reduce the proportion of oxidized transcripts in GRMD dog muscles showing a direct correlation between the oxidative stress of the myofibers and the dystrophic phenotype. Hence, lowering the oxidative status by AON pre-treatment might also facilitate a high AAV transgene expression and help reaching the threshold of the therapeutic vector genome number necessary to allow a strong benefit of AAV therapies.

The ability to retain vector genomes clearly depends on the efficiency of the AON pre-treatment. Recent studies showed limited clinical benefit of 2'OMePS and PMO chemistries (6–9)

with a level of restored dystrophin observed in patients far too low to protect skeletal myofibers from necrosis. However, the novel generation of AON chemistries, in particular the tcDNAs (10) and the PPMOs (11,12), display unprecedented level of dystrophin restoration in mice. This new AON generation gives hope to the DMD patients and their family and could be good candidates for a pre-treatment for AAV-based therapies. On the eve of clinical trials using AAV-based therapies for DMD patients, this study underscores the strong impact of combined approaches to improve the benefit of AAV-based therapies allowing the use of lower and thus safer vector doses for a larger level of dystrophin expression in the long term.

Materials and Methods

AAV vector production and animal experiments

A three-plasmid transfection protocol was used with pAAV(U7smOPT-SD23/BP22) (14), pAAV(U7smOPT-scr) (20) and codon optimized pΔR4-R23/ΔCT (MD1) (24) plasmids for generation of single-strand AAV1-U7ex23, AAV1-U7scr and AAV1-MD1 vectors. Vector titers were determined by real-time PCR and expressed as vector genomes per ml (vg/ml). Three-month-old *mdx* mice were injected into the *Tibialis anterior* (TA) muscles with 1 nmole of Pip6a-PMO oligonucleotides (GGCCAAACCTCGGCTTACCTGAAAT) (11). Additionally, 50 µl of AAV1-U7scr, AAV1-U7ex23 or AAV1-MD1 containing 1E + 10, 3E + 10 or 1E + 11 vg were injected into C57BL/6 (wt) or *mdx* TAs. These animal experiments were performed at the Myology Research Center, Paris, France, according to the guidelines and protocols approved by the Institutional Review Board. A minimum of four mice were injected per group for each experiment. At sacrifice, muscles were collected, snap-frozen in liquid nitrogen-cooled isopentane and stored at -80 °C.

Vector genome quantification

Genomic DNA was extracted from mouse muscles using the Puregene Blood kit (Qiagen). Copy number of AAV genomes and genomic DNA were measured on 100ng of genomic DNA by absolute quantitative real-time PCR on a StepOnePlus™ (Applied Biosystems) using the Taqman® Universal Master Mix (Applied Biosystems). Primers (forward: CTCCATCACTAGGGGTTCCTTG and reverse: GTAGATAAGTAGCATGGC) and probe (TAGTTAAT GATTAACCC) were used to specifically amplify the vector genome sequence. As a reference sample, a pAAV plasmid was 10-fold serially diluted (from 10^7 to 10^1 copies). All genomic DNA samples were analysed in duplicates.

RT-PCR analysis

Total RNA was isolated from mouse muscle with NucleoSpin® RNA II (Macherey-Nagel), and reverse transcription (RT) performed on 200ng of RNA by using the Superscript™ II and random primers (Life Technologies). Non-skipped and skipped dystrophin transcripts were detected by nested PCR and quantified as described (16).

Western blot analysis

Protein extracts were obtained from pooled muscle sections treated with 125 mM sucrose, 5 mM Tris-HCl pH 6.4, 6% of XT Tricine Running Buffer (Bio-Rad), 10% SDS, 10% Glycerol, 5% β-mercaptoethanol. The samples were purified with the Pierce Compat-Able™ Protein Assay Preparation Reagent Set (Thermo Scientific) and the total protein concentration was determined with the Pierce BCA Protein Assay Kit (Thermo Scientific). The samples were denatured at 95 °C for 5 minutes and 100 µg of protein were loaded onto Criterion XT Tris-acetate precast gel 3–8% (Bio-Rad). The membrane was probed with primary monoclonal antibodies directed against dystrophin (NCL-DYS1, 1:50, Leica Biosystems; or MANEX1011B, 1:20, kindly gifted by The Muscular Dystrophy Association Monoclonal Antibody Resource (36)) and α-actinin (1:1000, Sigma-Aldrich), followed by incubation with a sheep anti-mouse secondary antibody (horseradish peroxidase conjugated; 1:15000) and Pierce ECL Western Blotting Substrate (Thermo Scientific).

Immunohistochemistry

TA sections of 12 µm were cut and examined for dystrophin expression using the NCL-DYS2 (1:50; Leica Biosystems) or MANEX1B (1:50; kindly gifted by The Muscular Dystrophy Association Monoclonal Antibody Resource (36)) monoclonal antibodies and a goat anti-mouse secondary antibody Alexa 488 (1:1000; Life technologies).

Supplementary Material

Supplementary Material is available at *HMG* online.

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