cardiac muscle even after repeated injections into *mdx* mice of either 20′ MePS or PMO AON[3,4,12] even with doses of PMO that induce high levels of dystrophin in skeletal muscles. However, direct injection of AON or adeno-associated virus (AAV)-mediated AON delivery induced effective dystrophin expression in cardiac muscles, suggesting that efficiency of delivery rather than of exon-skipping is the critical factor in this organ.[13]

One way of enhancing intracellular delivery is to employ cell-penetrating peptides or polymers to provide active transport of AON into muscle fibers. Earlier studies showed that conjugation to an arginine-rich peptide significantly improved PMO-mediated antiviral activity[14] as well as delivery of PMO for dystrophin exon skipping in cell cultures and on intramuscular injection into muscles.[15] More recently, Jearawiriyapaisarn *et al.*[16] used a transgenic mouse that expresses enhanced green fluorescent protein as a positive readout for the efficiency of exon exclusion to evaluate the potency, functional biodistribution, and toxicity of PMOs conjugated to various arginine-rich cell-penetrating peptides containing 6-aminohexanoic acid (X) and/or β-alanine. The greatest restoration of enhanced green fluorescent protein expression in both skeletal and cardiac muscles was observed with PMO tagged with a peptide of (RXRRBR)2XB (PPMO). When applied to the dystrophic *mdx* mice model of DMD, a single i.v. injection of 30 mg/kg of PPMO restored dystrophin in all skeletal muscles to almost normal levels[17] that were maintained by regular biweekly administration over 12 weeks and accompanied by improvement in muscle strength and pathology, with significant lowering of serum creatine kinase levels. Most importantly, i.v. injections of PPMO elicited near-normal levels of dystrophin in cardiac muscle (Figure 2) and prevented dobutamine-induced cardiac failure. Efficient exon skipping was also achieved in smooth muscles in other organs such as the esophagus. Treatment with the PPMO did not cause detectable toxicity. Recently, this PPMO has been shown to considerably ameliorate the severe pathology in the dystrophin–utrophin double null mouse.[18] Together, these findings illustrate the theoretical feasibility of using PPMO to rescue dystrophin expression in both skeletal and cardiac muscles of DMD patients.

However, use of peptides to enhance delivery raises the possibility of an immune response that may prevent repeated administration or cause rejection of targeted tissues or both, especially because DMD patients would require regular life-long administration. Although no immune response was observed in the above study[17] or in previous reports with similar peptides in animal models,[14,19] immunogenicity varies considerably between species, arguing for longer-term studies in a range of animal models. But final verification can come only from clinical trials. It is, therefore, important to develop nonpeptide alternatives to enhance delivery of oligomers. The known sequence and structure of the peptide used in the PPMO provides a basis for modeling such nonpeptide polymers as delivery vehicles with similar or improved function. With this in mind, Wu *et al.* exploited a nonlinear, nonpeptidic dendrimer as a transporter for delivery of PMO. This consists of eight guanidinium head groups bonded to a trifunctional triazine as a core scaffold, which is then conjugated to PMO targeting exon 23 (ref. 6) (termed Vivo-PMO).[20] The study demonstrated that the Vivo-PMO targeting mouse dystrophin exon 23 (Vivo-PMOE23)

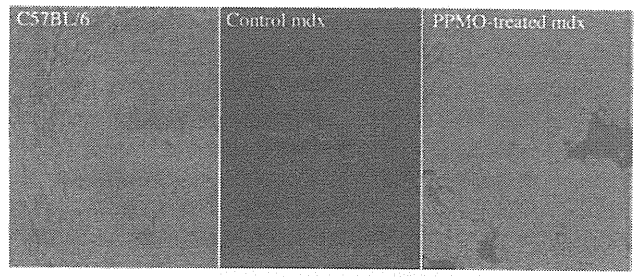

Figure 2 Restoration of dystrophin in cardiac muscles of mdx mice after six intravenous injections (at biweekly intervals) of 30 mg/kg of the PPMOE23 targeting mouse dystrophin exon 23. Muscles were examined 2 weeks after the last injection. Left panel, muscles from heart of normal *C57BL/6*. Middle panel, muscle from heart of control *mdx* mouse. Right panel, PPMO-treated *mdx*. Dystrophin was detected by immunochemistry with the polyclonal rabbit antidystrophin antibody, P7, and visualized with Alexa 594 tagged goat-anti-rabbit Igs. Blue nuclear staining with DAPI.

is highly effective for exon skipping and dystrophin induction in *mdx* mice. A single i.v. injection of 6 mg/kg Vivo-PMOE23 generated dystrophin expression in skeletal muscles at levels equivalent to the injection of 300 mg/kg unmodified PMOE23. Repeated injections of 6 mg/kg Vivo-PMOE23 achieved ~50% and 10% wild-type levels of dystrophin expression in body-wide skeletal muscles and in cardiac muscle respectively, without eliciting a detectable immune response. Vivo-PMOs showed no signs of toxicity at the effective dosage regime that reduced the serum levels of creatine kinase significantly.[20] These results thus offer prospects for the development of new nonpeptide delivery moieties with improved function and low toxicity.

MULTIEXON SKIPPING IN DYSTROPHIC DOGS

Although antisense-mediated exon skipping clinical trials currently conducted in United Kingdom and Netherlands targeting exon 51 show promising results,[8,21] such single exon skipping covers only a proportion of DMD patients. Even if antisense oligos against most exons in the *DMD* gene become available, approximately half of DMD patients will require multiexon skipping by targeting of more than one exon, depending, not on the size but on the type of mutation (*e.g.*, deletion, duplication, point mutation, etc.) and the "phase" of the mutated exon and its neighboring exons. For example, to treat a patient with deletion of exon 7, one needs to target at least two exons (*e.g.*, both exon 6 and exon 8) to put the mutation back in frame (Figure 1). In fact, canine X-linked muscular dystrophy harbors such mutation[22] (*i.e.*, a splice site mutation in intron 6 that excludes exon 7 from the mRNA transcript (Figure 3)) and is therefore, a good model for testing the efficacy and efficiency of double-exon skipping.[23] The dystrophic dog has several further advantages over the *mdx* mouse. First, it provides the prospect of more detailed analyses of clinical condition, such as clinical grading, magnetic resonance imaging, three-dimensional-echocardiography, and electrocardiogram.[23] Second, the canine X-linked muscular dystrophy model, is closer in clinical phenotype than the *mdx* mouse model to human DMD. Indeed, it shows, if anything, a more severe progression than DMD; this, in combination with its similarity in body weight, makes it especially useful for physiological and

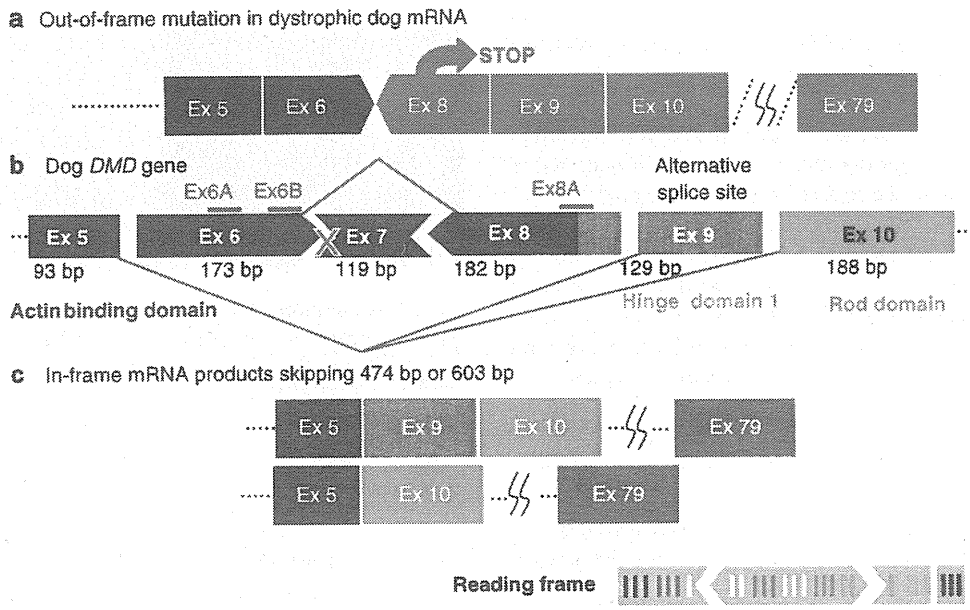

Figure 3 Diagram to illustrate the exon-skipping strategy to restore open reading frame at the mutation site in the CXMD dystrophic dog. A point mutation in the acceptor splice site in intron 6 preceeding exon 7 (X) leads to exclusion of exon 7 from the transcript and loss of open reading frame when exon 6 is spliced to exon 8. To restore open reading frame, requires the loss of at least two further exons: 6 and 8. In the event, exon 9 is also excluded from the transcript but, because it contains a whole number of codon triplets, this does not disrupt the translation of the resultant mRNA. CXMD, canine X-linked muscular dystrophy.

toxicological studies.[23] Finally, there may be some advantage in the fact that many target sites for exon skipping show identity of DNA (mRNA) sequences between dog and human. Drug regulation authorities such as US Food and Drug Administration are inclined to regard antisense oligonucleotides (AOs) of different sequences as different drugs; thus, to target the range of mutations encountered in DMD patients, many AO sequences will need to be designed, tested, and approved. Between man and mouse targeting homologous sites has little predictive value, perhaps due to minor sequence differences.[24] Dogs and humans however, share considerable sequence identity; for most exons in the *DMD* gene one can design a single 20–25mer antisense sequence that is applicable to both and comparison of targeting efficiencies between these species should be explored further.

Overall, the dog experiments provide a promising message for DMD patients. McClorey and colleagues transfected cultured myotubes from dystrophic dogs *in vitro* with a cocktail of antisense oligos targeting exons 6 and 8, noting restoration of reading frame in mRNA.[25] Recently, we sought to test efficacy and toxicity of i.v. PMO induced exon skipping *in vivo* in the DMD dog model.[26] We identified a cocktail that, by either intramuscular injection or systemic i.v. delivery, resulted in extensive dystrophin expression to therapeutic levels. Weekly or biweekly systemic i.v. injections, over the course of 5–22 weeks, with a three-morpholino cocktail (120–200 mg/kg in total of three oligos/injection) targeting exon 6 and exon 8, induced therapeutic levels of dystrophin expression throughout the body, to an average of 26% of normal levels. Expression of dystrophin was associated with significant functional and clinical stabilization, being accompanied by reduced inflammation as observed histologically and by magnetic resonance imaging, improved or stabilized clinical symptoms and timed running tests. Histology and blood tests indicated no evidence of toxicity. Dystrophin expression was also detected in cardiac muscles by immunohistochemistry but, as in the *mdx* mouse,[3,4] less than in skeletal muscles and concentrated in small patches. Recently, we have found that an i.v. injection of peptide-conjugated morpholinos (PPMOs) at 12 mg/kg elicited increased dystrophin expression in the canine heart, as detected by western blotting (Yokota *et al.*, data not shown).

An unexpected observation in the dog study was that, in tissue culture, either of the two antisense oligonucleotide components of the cocktail directed against exon 6 were able, alone, to efficiently induce the desired exon 5–10 splicing in the absence of the sequence against exon 8. By contrast, they did not do this *in vivo*. In addition, excision of exon 8 by the exon 6-specific sequences alone occurred only in the context of the mutant exon 7 splice site (*i.e.*, it did not occur in wild-type dog cells). Similarly, AO administration to human cells produced some disparities in skipping between patients carrying small mutations in the *DMD* gene and wild-type cells.[27] The differences between patterns of skipping *in vivo* versus *in vitro* and between wild-type versus mutant genotypes indicate that the pattern of exon skipping is greatly influenced by variables other than the local presence of target sequence. Thus, it is prudent to consider testing of selected target sequences in multiple systems before committing to a specific sequence for subsequent clinic trials.

SIGNIFICANCE OF MULTIEXON SKIPPING

Theoretically, multiple exon skipping could restore open reading frame in >80% both of deletion and nonsense mutations in the *DMD* gene.[28–31] Moreover, since some in-frame deletions are

associated with milder phenotypes than others, selective skipping of more exons than are required for simple restoration of reading frame offers the prospect of selecting options that optimize the functionality of the resultant dystrophin protein. Thus, it has been proposed that a cocktail of AOs targeting exons 45–55, a deletion associated with a high percentage of asymptomatic or mild BMD clinical phenotypes[32] would potentially be applicable to 63% of patients with dystrophin deletions. Currently, techniques for skipping 11 exons simultaneously are not available but might be achieved in future by improved efficacy of AO chemistry or more efficient delivery methods.

AAV U7 GENERATION OF ANTISENSE OLIGONUCLEOTIDES

Perhaps the most efficient way to achieve long lasting exon skipping, without recurrent infusions of antisense oligonucleotides, would be to generate the antisense agent within the target cells. Current studies have used gene vectors expressing modified U7 or U1 small-nuclear RNAs as antisense shuttles (AS-snRNAs).[33–35] Because these expression cassettes are very small (AS-U7 is about 400 nucleotides) there is sufficient room within gene vectors to combine several copies of different AS-snRNAs designed to target multiple exons within a gene or even different genes simultaneously.

Although a number of viral vectors could be used for the delivery of such AS-snRNA chimeras in tissue culture as well as *in vivo*, AAV have come to the fore, offering the advantage of stable long-term expression. Current AAV8-, AAV1- and AAV6-capsids effect efficient and widespread transduction of muscles in mice after tail vein administration,[36] with promising new serotypes pending.[37,38] Systemic delivery of AAV vectors harboring AS-U1 in the *mdx* mouse resulted in effective body-wide dissemination of the therapeutic construct and significant improvement of muscle function suggestive of overall maintenance of muscle mass and strength.[39] Similar results have been obtained with the AS-U7 system:[35] sustained dystrophin rescue to near wild-type levels and restoration of normal levels of muscle resistance to mechanical stress. In addition, no immune response has been reported, against the rescued dystrophin, due perhaps to fact that the rescued truncated dystrophin is represented in the repertoire of pre-existing revertant fibers, which naturally occur in dystrophic mice. However, while the long-term stability of corrected fibers was clearly demonstrated in the *mdx* mouse,[13,40] the AAV(AS-snRNA) approach still faces problems arising from immune sensitization against AAV, that would prevent the application of repeated treatment unless an effective regime of immunomodulation can be developed.[36]

For most myopathic disorders, to be of practical clinical therapeutic value, a genetic therapy would, ideally, provide treatment of the whole skeletal and cardiac musculature. As has been demonstrated by initial experimental trials in murine models, this cannot be achieved by intramuscular injections; only a systemic injection can approach this objective. Such a systemic delivery procedure is not without risk and entails long and expensive development, in particular to overcome the immune problems.[41,42] First, production of the large quantity of vector required to treat even a single patient is a daunting task that is the objective of a number of methods for large scale AAV production currently being developed.[43] Second, practicability of the AAV(AS-snRNA) technology requires development of a safe and effective protocol for systemic administration. This needs to be tested in a large animal, such as the canine X-linked muscular dystrophy dog, to permit evaluation of the dose range and the protocols of administration of the vectors required to achieve therapeutic effectiveness while remaining safe. In order to anticipate, on a rational basis, the adaptation of such a protocol to trials in man, it is important to conduct such studies in conditions that mimic clinical practice as closely as possible.

ONGOING THERAPEUTIC TRIALS USING ANTISENSE OLIGONUCLEOTIDES

Two European consortia are involved in clinical trials using two different antisense oligonucleotide chemistries. One group is based in Holland, closely associated with the Leiden University Medical School (Prof Gert van Ommen and Dr Jan Verschuuren) and works in close collaboration with the company Prosensa, which also sponsored these studies. The second group is based in United Kingdom, where a consortium of four Universities (MDEX consortium) is led by F.M., and works in close collaboration with AVI Biopharma, which is sponsoring the present study.

Both groups are targeting exon 51, although using two different primary sequences, and different backbones. The Dutch study utilizes a 21-mer 2′OMePS,[7] whereas the MDEX Consortium is employing a 30 PMO.[44] Both groups elected to study patients with deletions who would benefit from exon 51 skipping (50, 52, 52–63, 45–50, 48–50, and 49–50), both because cumulatively these account for 13% of all DMD deletions,[7,28] and more especially because the resulting protein has been clearly demonstrated to be extremely functional, as suggested by several multigenerational families deleted for the same domains with no symptoms whatsoever.[45–47]

The Dutch consortium have completed and published in 2007 the result of a proof of concept study in which four DMD boys have received a single injection of the 2′OMePS into the tibialis anterior. This was well tolerated and accompanied by specific skipping of exon 51 as well as detection of sarcolemmal dystrophin in 64–97% of myofibers of the biopsied muscle; the amount of dystrophin ranged from 3 to 12% of that found in the normal control muscle and with intensities in individual fibers ranging from 17 to 35%.[8]

The MDEX consortium performed a similar study using the PMO AO, but with a different design: a dose escalation study in seven DMD boys, who received either 0.09 or 0.9 mg in one of the two extensor digitorum brevis muscles, whereas the contralateral muscle received saline. The results, recently published,[21] demonstrated clearly detectable dystrophin expression in 44–79% of myofibers, with intensity of dystrophin staining averaging 17% greater than the levels in the contralateral muscle and, in the most positive fibers, up to 42% of that in healthy muscle fibers.[21]

Both studies have been followed by repeated systemic administration studies. The Dutch consortium recently completed a study in which four group of DMD boys received escalating doses of the 2′OMePS antisense to skip exon 51, subcutaneously, at doses of 0.5, 2.0, 4.0, and 6.0 mg/kg, weekly for 5 weeks. All 12 children (3/group) had a muscle biopsy at the beginning and the end of the study. While the results of this study have not yet been published, Dr Goemans reported at the World Muscle Society meeting in

2009 (Geneva)[48] that the study drug was well tolerated and that a dose–response in exon skipping and dystrophin production was observed. All boys who received the 2'OMePS AO have been enrolled in an extension study that is currently underway.

Encouraging results have also been announced by the analysis of the first four cohorts of the boys recruited into the MDEX systemic study using the PMO. In this study, seven groups of DMD boys received escalating doses of PMO (0.5, 1.0, 2.0, 4.0, 10, and 20 mg/kg) for a period of 12 weeks. All patients had a pretreatment and post-treatment muscle biopsy. At the time of writing only the first four cohorts have completed the study, and the preliminary analysis indicates that in the three patients in the 2.0 and 4.0 mg/kg cohorts there was accurate skipping of exon 51. In one of the patients at the 2.0 mg/kg dose, the appearance of skipped mRNA was accompanied by a several fold increase in expression of dystrophin protein in the post-treatment samples using both western blotting and immunofluorescent analysis (fivefold on western blot and approximately sevenfold on immunocytochemistry). While the results of the patients recruited into the last two cohorts will not be available until the 2nd quarter of 2010, both these results, and those from the Dutch consortium are very encouraging. Two pivotal multicentric phase III studies are currently being planned, one by Prosensa/GSK, using the 2'OMePS AO, and one by AVI Biopharma, using the PMO AO, and are both likely to start in 2010. The design will be a randomized placebo controlled study which is likely to last for ~1 year. Additional studies are also being planned by Prosensa (a multicentre phase I/II study targeting exon 44 with a 2'OMePS, whereas target optimization for exon 43, 45, 46, and 52 are being pursued, possibly followed by further clinical studies in 2011–2012). In addition AVI Biopharma has initiated a preclinical program which is anticipated to lead to an IND/IMPD filing in early 2010 for it's lead peptide-conjugated PMO (PPMO) to skip exon 50 and thus into a clinical study which is currently being planned.

Prospects

As attested by the above accounts, the potential for use of exon skipping as a therapeutic strategy for DMD has developed from a plausible notion in the mid-1990's[1,2] to the point where early clinical trials show that it holds realistic prospects of providing genuine therapeutic benefit. There remain, however, substantial barriers: some scientific, some regulatory, with occasional interaction between the two.

The major scientific issues concern the choice of sequence for any given exon and the enhancement of delivery and effectiveness of that sequence to the majority, ideally all, of the muscle fibers in the body.

Although, effective sequences that promote skipping of a number of exons have been identified, we have no reliable method for determining whether any given sequence is optimal. A thorough screen for optimal sequences alone and in combination requires the ease of use of a tissue culture system and although a broad correspondence has shown between the *in vitro* and *in vivo* activities of different chemistries and adjuncts[49,50] it is evident from the canine studies[26] that myogenic cultures cannot be relied upon to inform us accurately as to the *in vivo* activity of various sequences. A recent study of equivalent sequences that target human and mouse exons confirms the view that the efficacy of targeting is highly context dependent[24] and that we should be wary of generalizing the applicability of specific results from one test system.

As to delivery, most work in the *mdx* mouse favors PMO over 2'OMePS backbone chemistry, but neither shows great promise for entering cardiac muscle in useful amounts and even in skeletal muscle, effectiveness is patchy. We are therefore in need of developments such as the addition of cell-penetrating moieties which, in turn, will entail extensive animal studies to establish dosage regimes that provide efficacy with minimal toxicity.

For the AAV(AS-snRNA) approach, the ideal would be a single body-wide delivery to 100% of cardiac and skeletal muscle cells, with the reasonable expectation that this would need to be repeated rarely, perhaps never if we are lucky. At present, such efficient delivery does not seem to be possible with a single infusion and the potential immune complications; generation of neutralizing antibodies and of cell-mediated response to residual viral antigens mandates a thorough appraisal of multiple delivery protocols.

For regulatory bodies, antisense induced exon skipping represents an extreme example of agents that are highly targeted to the individual patient, and poses a potentially educative challenge to the appropriateness of standard procedures. The combination of a need for at least one different oligonucleotide sequence for each target exon and the large number of different exons, together with the small numbers of patients who might benefit from skipping of some specific exons, raises considerable obstacles to the conduct of standard safety and efficacy regimes. The problem is further compounded by the fact that sequence-specific side effects are likely to be species-specific and therefore not reliably assessable on animal models. A requirement for a full toxicological workup of each individual sequence would be a major disincentive for manufacturers to extend their interests beyond a small number of the more widely applicable target exons or even to seek to optimize sequences for the commoner exon targets. Moreover, many target exons would be relevant to too few patients to permit conduct of any form of conventionally designed trial. Thus, imposition of the normal regulatory processes would constitute a major impediment to the application of exon-skipping therapy across the range of patients who might benefit from it. A positive exploration of these issues would act as a trailblazer to the benefit of the progress of personalized medicine in general.

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Review

Gene therapy for muscle disease

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ABSTRACT

The molecular mechanisms of Duchenne muscular dystrophy (DMD) have been extensively investigated since the discovery of the dystrophin gene in 1986. Nonetheless, there is currently no effective treatment for DMD. Recent reports, however, indicate that adenoassociated viral (AAV) vector-mediated transfer of a functional dystrophin cDNA into the affected muscle is a promising strategy. In addition, antisense-mediated exon skipping technology has been emerging as another promising approach to restore dystrophin expression in DMD muscle. Ongoing clinical trials show restoration of dystrophin in DMD patients without serious side effects. Here, we summarize the recent progress in gene therapy, with an emphasis on exon skipping for DMD.

Contents

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Introduction

Muscular dystrophies are heterogeneous genetic disorders, characterized by progressive degeneration and weakness of the skeletal and cardiac muscles. DMD is severe and the most common type of muscular dystrophy; worldwide, approximately one in every 3500 boys born is afflicted with DMD.

The *DMD* gene is the largest known gene in humans, comprising over 79 exons, with a coding sequence of 11 kb and spans no less than 2.3 Mb of genomic DNA. DMD is caused by deletion (65%), duplication (15%), or nonsense and other small mutations (20%) in the *DMD* gene, all of which disrupt the open reading frame [1].

The *DMD* gene encodes dystrophin, which is located beneath the sarcolemma, assembles the dystrophin–glycoprotein complex at the sarcolemma, and links the internal cytoplasmic actin filament network and extracellular matrix, providing physical strength to muscle fibers [2]. At present, there is no effective therapy to stop the lethal progression of the disease, but several therapeutic approaches hold great potential. Here we focus on gene therapy for DMD and summarize AO-mediated exon skipping technology as a most promising therapy.

Adenoassociated virus -mediated gene therapy

Updates on rAAVs

The adenoassociated virus (AAV) is a tiny single-stranded, nonpathogenic, nonreplicative DNA virus belonging to the Parvovirus family. So far, more than 12 serotypes have been identified in primates [3]. Recombinant AAV (rAAV) is a powerful tool to deliver therapeutic genes to skeletal muscle [4–6]. Even in immunologically competent mice, the expression of the exogenous gene was shown to continue for years without evoking immune responses.

Importantly, rAAV has several serotypes that show tropisms to skeletal muscle. rAAV1 and rAAV2 are commonly used for direct delivery to skeletal muscle and mainly used in local treatment. rAAV-6 [7] plus the more recently developed rAAV-8 [8,9], and rAAV-9 [10–12] are powerful in systemic delivery of the therapeutic genes via the circulation to the musculature body-wide, including the diaphragm and heart.

Limited packaging size of rAAV

rAAV has a limitation in the length of the transgene it can accommodate (less than 5.0 kb). Full-length dystrophin, which is nearly 11 kb, cannot be incorporated into an AAV vector. To overcome this limitation, truncated but functional microdystrophins with a large deletion in the central rod domain have been constructed because studies of the genotype–phenotype relationships in DMD and Becker muscular dystrophy (BMD), a milder form of muscular dystrophy with near-normal life expectancy, have suggested that the rod domain has limited function and is largely dispensable [4]. Several types of microdystrophin were administrated to *mdx* mice locally [13] or systemically [7,14–16] and ameliorated pathology and improved muscle function. To expand the packaging capacity of the AAV vector, trans-splicing (ts) of two vectors and recombination of two overlapping (ov) rAAV vectors have been tested (reviewed in Trollet et al. [4]). A hybrid dual-vector system, which combines the features of the ts and ov vectors into a single system, has been reported to work well in skeletal muscle [17].

Immunity against rAAV in dog models

Based on the improvement of pathology and muscle function due to successful AAV-mediated gene transfer into dystrophic mice, preclinical studies using dystrophic dogs [18,19] and nonhuman primates [20,21] were performed. In dogs, considerable cellular immune response was often observed [18,19,22], and transient immune suppression was needed [23]. However, there is no clear explanation of why rAAVs evoke much stronger immune responses in dogs than mice.

Clinical trials

Immunity to AAVs is also a big concern in rAAV-mediated gene therapy for DMD. First, natural AAV infection is quite common in human populations, and preexisting antibodies could block AAV vector-mediated therapy. Second, after the first injection of rAAV vectors, the second injection is known to be much less effective due to a neutralizing antibody. Indeed, clinical trials using AAV vectors suggest that immune response to the vector and/or transgene product is the most important limitation of the rAAV-mediated gene therapy. To diminish a host immune response against the transgene product, utilization of a muscle-specific promoter active in both skeletal and cardiac muscles [24,25] is desirable. Codon optimization has also been demonstrated to be effective to reduce the virus titer [26]. A phase I/II clinical trial of intramuscular delivery of microdystrophin by AAV2.5-CMV-Mini-Dystrophin was initiated in 2006 (PI: JR Mendell; Trial ID: US-679; clinicaltrials.gov identifier: NCT00428935). More information can be obtained at http://www.wiley.co.uk/genetherapy/clinical/, http://www.clinicaltrials.gov, or http://www.mda.org.

Lentiviral vector-mediated gene transfer into muscle stem cells

Lentiviral vectors have a relatively large transgene carrying capacity (7.5–9 kb), integrate into the genomes of both dividing and nondividing cells, and achieve long-term transgene expression in a wide variety of tissues including skeletal muscle. Previously, lentiviral vectors have been used to introduce a mini-dystrophin gene into mouse skeletal muscle [27]. Because the expression levels of mini-dystrophin were low after direct injection of lentiviral

vectors into diseased muscle, this system seemed to be useful in modifying genetically autologous cells *ex vivo* rather than in direct injection *in vivo*. In fact, lentiviral vectors expressing mini-dystrophin transduced mouse satellite cells efficiently, and the transduced cells regenerated muscle fibers after transplantation [28]. Quenneville et al. [29] showed that lentiviral vectors are useful in transducing monkey muscle stem cells. The lentiviral vector has been recently used to modify muscle stem cells to deliver an antisense sequence linked to a modified U7 [30] or U1 [31] small nuclear RNA for restoration of the reading frame.

Antisense oligonucleotide (AO)-mediated exon skipping for *DMD* gene

Skipping of targeted exons

DMD is caused by mutations in the *DMD* gene that disrupt the open reading frame. BMD is also caused by mutations in the *DMD* gene, but in the case of BMD, the open reading frame is maintained. If we can skip (splice out) targeted exons by modification of splicing patterns and restore the reading frame, a shorter dystrophin protein can be restored in the DMD muscle, converting the DMD phenotype to a BMD phenotype. To this end, a number of antisense oligonucleotides (AOs) have been designed and tested *in vitro* [32–34] and *in vivo* [35–37]. Fig. 1 illustrates the skipping of exon 51 using one AO. Whether the resultant shortened dystrophin is functional or not depends largely on the function of the deleted part. In general, truncation of the rod domain is thought to be relatively harmless.

Single exon 51 skipping is expected to be suitable for approximately 13% of DMD patients. Multiple exon skipping is estimated to be applicable to more than 80% of DMD patients. Theoretically, the AO-mediated exon skipping strategy cannot treat patients with mutations in the promoter region, deletion of the first or last (79th) exon, deletion of the domain bound by dystroglycan: exons 62–69 [38] or large deletions (>35 exons) [39]. However, these mutations are rare, and the majority of patients have a mutation in the hotspot located between exons 43 and 55.

Design of AOs

AOs are designed to hybridize specific sequences, such as exon–intron boundaries, and exon splicing enhancer (ESE) sequences in transcripts. AOs interfere sterically with the splicing machinery [40,41]. There are several software programs, such as ESEfinder (http://rulai.cshl.edu/tools/ESE), to design antisense oligonucleotides, but extensive empirical analysis is still required for each exon.

AO chemistry, delivery in vivo, and toxicity

Among the AOs tested so far, AOs having a 2′-*O*-methyl phosphorothioate backbone (2′-*O*-MeAO) and phosphorodiamidate morpholino oligomers (PMOs) (Fig. 2) are commonly used in animal models and in clinical trials [42,43]. 2′-*O*-MeAOs have a chemically modified RNA structure (Fig. 2). The modifications increase the half-life and distribution to tissues. 2′-O-MeAOs have been well tolerated in clinical trials. PMOs have a morpholino backbone, are uncharged, are not recognized by cellular proteins, and, therefore, are rapidly cleared from plasma and excreted in urine. Very high doses of PMOs are reported to be well tolerated by animal models. This would be partly because PMOs hardly evoke innate immune responses.

In vivo delivery of AOs

One limitation of PMO-mediated exon-skipping therapy is that PMOs do not easily enter cardiac muscle. Recently, to improve the uptake of PMOs by cardiocytes, peptide-tagged PMOs (PPMOs) [44] and Octa-guanidine PMOs [45] were developed. These modified

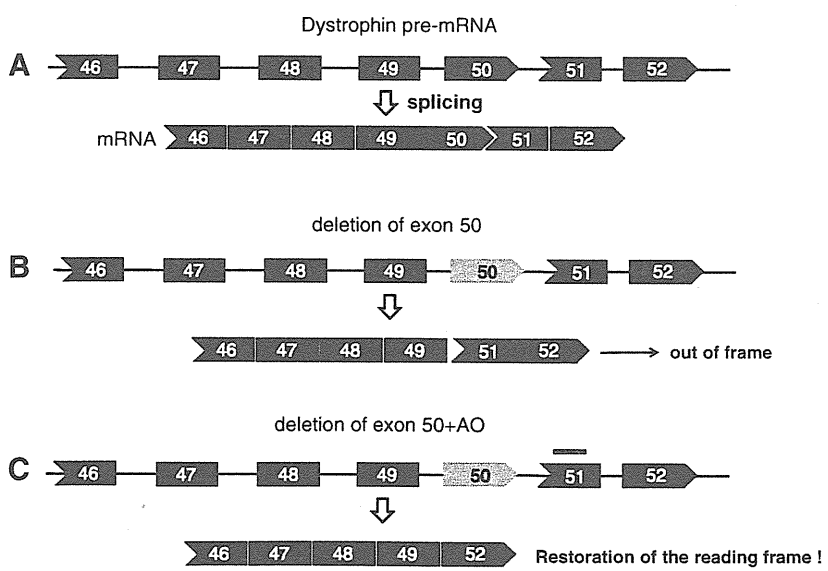

Fig. 1 – Exon skipping therapy for DMD patients with deletion of exon 51. (A) Normal dystrophin transcript and mRNA. (B) Deletion of exon 50 disrupts the open reading frame, leading to a premature stop codon, unstable mRNA, and a truncated protein. (C) Targeted skipping of exon 51 using AO restores the reading frame and produces a shorter but functional dystrophin that lacks exons 50 and 51. Blue bar indicates AO targeting the sequence in exon 51.

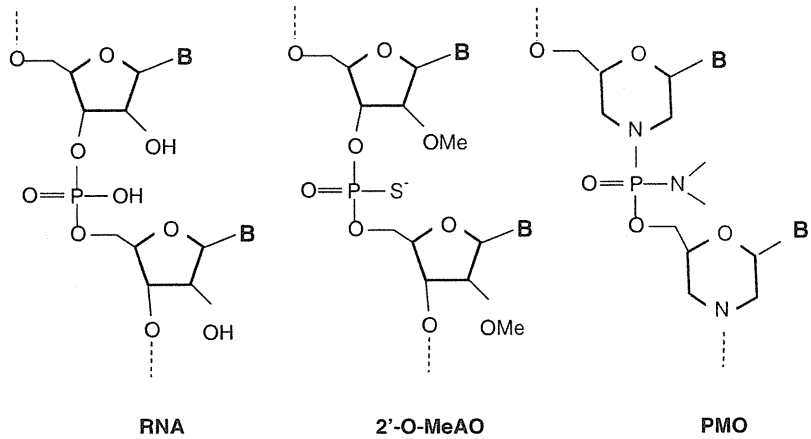

Fig. 2 – Structure of RNA, 2′-*O*-MeAO, and PMO. B: bases (adenine, cytosine, guanine, and thymine).

morpholinos are reported to be more effective than native PMOs in inducing exon skipping in cardiac muscle after intravascular injection. But there are potential concerns that PPMOs might elicit an immune response or have toxicity compared with PMOs due to the protein moiety.

Skipping multiple exons

If fully approved, AVI-4658 and PRO051, both of which target dystrophin exon 51, will be able to treat 13% of DMD patients. To treat more patients, elimination of two or more exons from the final mRNA is required. Theoretically, multiexon skipping using a cocktail of AOs can restore the reading frame of the *DMD* gene in more than 83% of the all DMD patients. Double-exon skipping using AOs has been shown to be feasible in patient-derived cells [46], mouse models, and dystrophic dogs [37]. On the other hand, the efficiency of multiexon skipping is much lower than expected [47]. This is presumably because partial exon skipping results in out-of-frame transcripts. It will be some time before multiple-exon skipping is applied to DMD patients.

Ongoing clinical trials of exon skipping

Clinical trials using intramuscular administration of 51 AOs, PRO051 (2′-O-Me AO), and AVI-4658 (PMO) have been performed in Europe by Prosensa and AVI BioPharma respectively. PRO051 and AVI-4658 were both designed to induce exon 51 skipping in the *DMD* gene and, therefore, can treat DMD patients with deletions such as 45–50, 47–50, 48–50, 49–50, 50, or 52. AVI BioPharma reported the initial data of systemic treatment with AVI-4658 (a phase 1b/2 clinical study) in the United Kingdom, which resulted in the successful restoration of dystrophin in the 2-mg/kg dose cohort (http://www.avibio.com/). AVI-4658 is well tolerated and so far has caused no serious side effects in treated patients. A phase 1/2 dose-ranging safety study using PRO051 was performed on 12 patients at two European clinical centers. The study demonstrated that PRO051 was also well tolerated up to 6 mg/kg and that novel dystrophin expression was detected in the patients in response to injections above 0.5 mg/kg [48] (also refer to http://prosensa.eu/technology-and-products/Pipeline/PRO-051.php or http://www.parentproject.org.au/html/s02_article/article_view.asp?art_id=679&nav_catid=214&nav_top_id=78).

However, the consequences of long-term administration of both AOs should be carefully examined because AOs have a transient effect and must be readministered to sustain the effect.

Conclusions

Development of gene therapy for DMD has long been a challenge, but recent strategies, such as AAV-8 or AAV-9-mediated systemic delivery of microdystrophin and exon skipping, hold great potential. AO-induced exon skipping is a mutation-specific approach. Both the mutation and splicing patterns of dystrophin mRNA must be examined individually, and the AO sequences used would differ from patient to patient. One concern is that the efficacy and safety of each variation must be tested on the same backbone, requiring more time to get approval from the regulatory authorities.

Although AO-mediated exon skipping has shown promising results, the authors predict that a combination of exon skipping and other therapeutic approaches, such as viral vector-mediated gene transfer, stem cell-based therapy, or additional strategies of enhancing muscle regeneration, will become the standard approach for future DMD therapy.

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In-frame Dystrophin Following Exon 51-Skipping Improves Muscle Pathology and Function in the Exon 52–Deficient *mdx* Mouse

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A promising therapeutic approach for Duchenne muscular dystrophy (DMD) is exon skipping using antisense oligonucleotides (AOs). In-frame deletions of the hinge 3 region of the dystrophin protein, which is encoded by exons 50 and 51, are predicted to cause a variety of phenotypes. Here, we performed functional analyses of muscle in the exon 52–deleted *mdx* (*mdx*52) mouse, to predict the function of in-frame dystrophin following exon 51-skipping, which leads to a protein lacking most of hinge 3. A series of AOs based on phosphorodiamidate morpholino oligomers was screened by intramuscular injection into *mdx*52 mice. The highest splicing efficiency was generated by a two-oligonucleotide cocktail targeting both the 5′ and 3′ splice sites of exon 51. After a dose-escalation study, we systemically delivered this cocktail into *mdx*52 mice seven times at weekly intervals. This induced 20–30% of wild-type (WT) dystrophin expression levels in all muscles, and was accompanied by amelioration of the dystrophic pathology and improvement of skeletal muscle function. Because the structure of the restored in-frame dystrophin resembles human dystrophin following exon 51-skipping, our results are encouraging for the ongoing clinical trials for DMD. Moreover, the therapeutic dose required can provide a suggestion of the theoretical equivalent dose for humans.

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INTRODUCTION

Duchenne muscular dystrophy (DMD) is a severe muscle disorder characterized by mutations in the *DMD* gene that mainly disrupt the reading frame leading to the absence of functional protein.[1] A related allelic disorder, Becker muscular dystrophy (BMD), which shows a much milder phenotype, typically results from shortened but in-frame transcripts of the *DMD* gene that allow expression of limited amounts of an internally truncated but partially functional protein.[2] Antisense oligonucleotide (AO)-mediated exon-skipping therapy for DMD, which is a splice modification of out-of-frame dystrophin transcripts, has been demonstrated to exclude specific exons, thereby correcting the translational reading frame, resulting in the production of "Becker-like," shortened but partially functional protein.[3–7] As a result of exon skipping, DMD could be converted to the milder BMD.[4]

The principle underlying exon-skipping therapy for DMD has been demonstrated in cultured mouse or human cells *in vitro*.[7–13] In addition, *in vivo* studies in murine or canine animal models have provided preclinical evidence for the therapeutic potential of AO-mediated exon-skipping strategies for DMD.[11,14–19] However, the number of patients who have the same mutation as the mice or dogs is estimated to be quite low.[20,21] On the other hand, a hot spot for deletion mutations between exons 45 and 55 accounts for >60% of DMD patients with deletion mutations.[20,22] In particular, exon skipping that targets exon 51 is theoretically applicable to the highest percentage (13%) of DMD patients with an out-of-frame deletion mutation.[20,23–25] Recently, efficient in-frame dystrophin expression following an exon 51-skipping approach has been successfully demonstrated in human subjects using local intramuscular AO injection.[23,24]

The functionality of the dystrophin protein produced by exon 51-skipping has been inferred by the identification of patients harboring the corresponding in-frame deletions (*e.g.*, in BMD patients).[6,25] In-frame deletions near hinge 3 (refs. 12,26,27), which is encoded by exons 50 and 51, are predicted to lead to BMD; however, the severity of this disease can vary considerably.[25,28–32] Consequently, it is desirable to use an animal model to investigate the molecular functionality of in-frame dystrophin lacking hinge 3 following exon 51-skipping. In the exon 52–deficient *mdx* mouse (*mdx*52), exon 52 of the *Dmd* gene has been deleted by gene-targeting, resulting in the production of a premature termination codon in exon 53 (refs. 7,33). This mouse lacks dystrophin and shows dystrophic features as well as muscle hypertrophy.[33] It would be meaningful in predicting whether exon 51-skipping led to an accumulation of BMD-like dystrophin that was able to

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correct the dystrophic histology and muscle function in the *mdx*52 mouse.[11,23,27,34]

Here, we showed the efficient restoration of dystrophin function: the reading frame was restored by exon 51-skipping, and we observed considerable amelioration of the skeletal muscle pathology and function in *mdx*52 mice. We describe the first successful effort at systemic rescue of in-frame dystrophin lacking hinge 3 and recovery of muscle function by AO-mediated exon 51-skipping in a mouse model.

RESULTS

Two AOs targeting the 5′ and 3′ splice sites achieved efficient exon 51-skipping in *mdx*52 mice

Skipping exon 51 of the murine *Dmd* gene in the *mdx*52 mouse corrects the open-reading frame, resulting in the production of truncated dystrophin that lacks two-thirds of the hinge 3 region and resembles human dystrophin following exon 51-skipping (**Figure 1a**).[11,27,34] We first identified effective AO sequences by intramuscular injection into the tibialis anterior (TA) muscles of *mdx*52 mice.[14] To optimize the screening dose in the TA muscle, Murine B30 (mB30) AO was injected into 8-week-old *mdx*52 mice at doses of 1–10 μg.[15] The mB30 AO designed to skip murine exon 51 was based on human B30 (ref. 11), which targets human exon 51 (**Figure 2a**). Mice were euthanized 2 weeks after the injection; the TA muscles were isolated and analyzed by reverse transcription (RT)-PCR and immunohistochemistry. Using RT-PCR with primers flanking exons 50 and 53, the cDNA band equivalent to the mRNA missing exons 51 and 52 was detected. We found that mB30 restored dystrophin expression in a highly dose-dependent manner (**Supplementary Figure S1**). Then, we designed 13 AO sequences targeting either exonic sequences or exon/intron junctions of murine dystrophin exon 51 (refs. 9,11,35,36). The sequences and compositions of these AOs are described in **Table 1** and **Figure 2a**. We then directly injected one or two of the 14 AOs into the TA muscle of *mdx*52 mice. Two weeks after the injection, we analyzed RNA fractions by RT-PCR and cryosections by immunohistochemistry and western blotting. Among the AOs examined, 51D, mB30, and 51I were shown by RT-PCR to be capable of inducing exon 51-skipping at a level approaching 50% (**Figure 2b,c**). 51D and 51I were designed to target the 5′ splice site and an exonic site of exon 51, respectively. It has been reported that a combination of two AOs directed at appropriate motifs in target exons induces more efficient exon skipping than that induced by a single injection.[34,37] We therefore injected combinations of two AOs into the TA muscles of *mdx*52 mice, and found that a combination of two AOs, 51A plus 51D, showed ~75% skipping efficiency, the highest among the combinations that we examined by RT-PCR (**Figure 2d,e**). 51A is targeted to the 3′ splice site of exon 51. We then showed by immunohistochemistry that the combination of 51A plus 51D rendered 50–70% of the fibers dystrophin-positive in cross-sections (**Figure 2f**), and produced ~50% dystrophin expression on western blots compared with the normal control (**Figure 2g**). Taking these results together, we concluded that the co-injection of two AOs, 51A plus 51D, was the optimal combination to skip exon 51 of the murine *Dmd* gene.

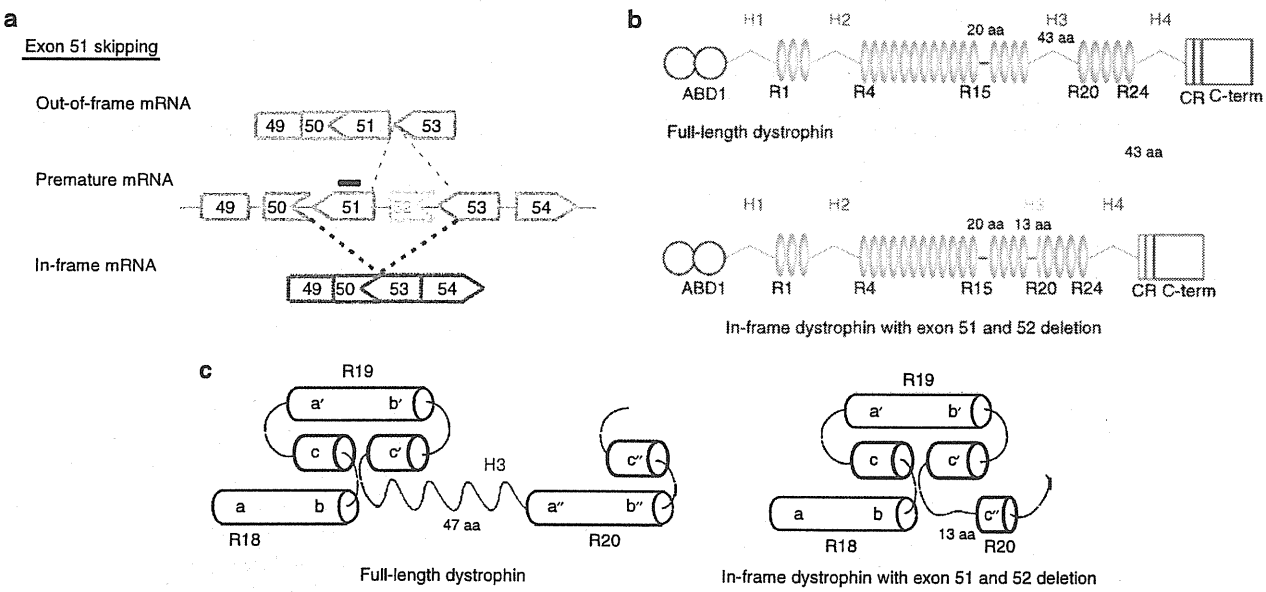

Figure 1 Strategy for exon 51-skipping in *mdx52* mouse. (**a**) Exon 51-skipping by appropriate phosphorodiamidate morpholino oligomers, indicated by a black line, can restore the reading frame of dystrophin in the *mdx*52 mouse, which lacks exon 52 in the mRNA of the murine *Dmd* gene, leading to out-of-frame products. (**b**) The molecular structure of in-frame dystrophin lacking hinge 3 induced by exon 51-skipping is shown below the full-length dystrophin. The protein contains the actin-binding domain 1 (ABD1) at the N-terminus, the central rod domain containing 24 spectrin-like repeats (R1–24), four hinge domains (H1–4), a 20-amino acid insertion between spectrin-like repeats 15 and 16 (segment 5), the cysteine-rich domain (CR), and the C-terminal domain (C-term). The hinge 3 is encoded by exons 50 and 51; therefore, most of this region is lost after exon 51-skipping in the *mdx*52 mouse. (**c**) Predicted nested repeat model with one long helix, one short helix, and overlap between the "a" helix of the following repeat with the "b" and "c" helices of the preceding repeat, forming the triple helix. The predicted structure of full-length dystrophin (upper) and in-frame dystrophin with exon 51 and 52 deletion (lower).

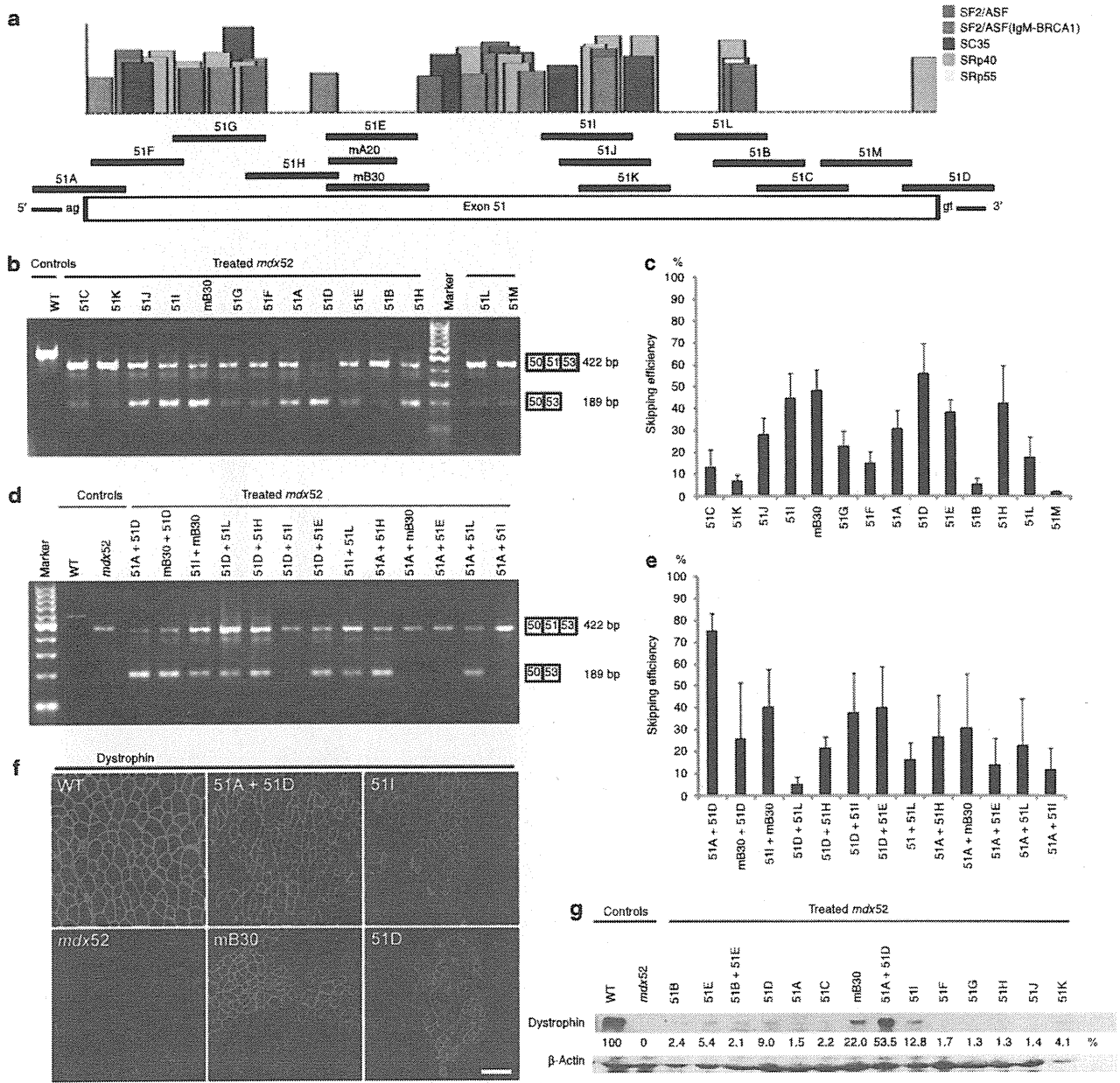

Figure 2 Local intramuscular injection into *mdx52* mice. The restoration of dystrophin in the tibialis anterior (TA) muscles was examined 2 weeks after the injection of 10 µg of one or a combination of two antisense oligonucleotides (AOs). (**a**) Fourteen different AOs designed to skip exon 51 of the murine *Dmd* gene. Each AO targets either an exonic splicing enhancer (ESE) or the 5′ or 3′ splice site, indicated by black lines. The certainties of ESE sites according to ESEfinder 3.0 are indicated by colored boxes. Candidates for splicing enhancer-binding proteins are shown (red, SF2/ASF; purple, SF2/ASF (IgM-BRCA1); blue, SC35; green, SRp40; yellow, SRp55). A murine B30 AO (mB30) corresponding to human B30 was designed.[13] (**b**) Effectiveness of the 14 different AOs for exon 51-skipping detected by RT-PCR. Representative data are shown. Skipped products (50–53) are compared with unskipped products (50–51–53). WT, wild-type mouse. (**c**) Quantitative analysis by RT-PCR of exon 51-skipping by 14 different AOs. The percentages of in-frame transcripts in each lane of **b** are shown. The data ($n = 3$) are presented as mean ± SEM. (**d**) Effectiveness of 13 different combinations of two AOs targeting exon 51 of the murine *Dmd* gene. Representative data are shown. Skipped products (50–53) are compared with unskipped products (50–51–53). *mdx*52, untreated *mdx*52 mouse. (**e**) Quantitative analysis of exon 51-skipping by 13 different combinations of two AOs. The percentages of in-frame transcripts in each lane of **d** are shown. The data ($n = 3$) are presented as mean ± SEM. (**f**) Immunohistochemical staining of dystrophin in TA muscle of WT, untreated and treated *mdx*52 mice. The results for AOs 51A plus 51D, 51I, mB30 and 51D are indicated. Dystrophin was detected with a rabbit polyclonal antibody P7. Bar = 100 µm. (**g**) Western blotting to detect expression of dystrophin in WT, untreated and treated *mdx*52 mice. Representative results for 10 single AOs and three combinations of two AOs. A quantitative analysis (see Materials and Methods) normalized to the expression of β-actin (upper panel), and western blotting to detect β-actin expression (lower panel) are shown. Dystrophin was detected with the Dys2 monoclonal antibody. Note that additional bands between the unskipped and skipped products are visible in some analyses. This is due to heteroduplex formation and has been described previously.[13] bp, base pair.

Table 1 Length, annealing coordinates, sequences of all AOs targeting mouse exon 51

AO	Length (bp)	Annealing coordinates	Sequences
51A	25	−18+7	CTGGCAGCTAGTGTTTTTGAAAGAA
51B	25	+171+195	TCACCCACCATCACTCTCTGTGATT
51C	25	+184+208	ATGTCTTCCAGATCACCCACCATCA
51D	25	+10–15	TTGTTTTATCCATACCTTCTGTTTG
51E	25	+66+90	ACAGCAAAGAAGATGGCATTTCTAG
51F	25	+3+27	TCACTAGAGTAACAGTCTGACTGGC
51G	25	+24+48	CCTTAGTAACCACAGATTGTGTCAC
51H	25	+47+71	TTCTAGTTTGGAGATGACAGTTTCC
51I	25	+125+149	CAGCCAGTCTGTAAGTTCTGTCCAA
51J	25	+130+154	AGAGACAGCCAGTCTGTAAGTTCTG
51K	25	+135+159	CAAGCAGAGACAGCCAGTCTGTAAG
51L	25	+162+186	TCACTCTCTGTGATTTTATAACTCG
mA20	20	+68+87	GCAAAGAAGATGGCATTTCT
mB30	30	+66+95	CTCCAACAGCAAAGAAGATGGCATT TCTAG

AOs restored body-wide dystrophin expression in a highly dose-dependent manner in *mdx52* mice

To examine the effect of systemic delivery, we intravenously injected a single dose of 51A plus 51D into 8-week-old *mdx*52 mice, at 80 (ref. 15), 160 or 320 mg/kg. Mice were euthanized 2 weeks after the injection; the muscles were isolated and analyzed by RT-PCR and the cryosections by immunohistochemistry. We found that the AOs restored body-wide dystrophin expression in a highly dose-dependent manner, with the 320 mg/kg dose showing ~45% skipping efficiency by RT-PCR (**Figure 3a,b**) and 45% dystrophin-positive fibers by immunohistochemistry (**Figure 3c**) in the gastrocnemius (GC) muscle.

Repeated systemic delivery of AOs induced highly efficient in-frame dystrophin in skeletal muscles body-wide

Next, we intravenously injected 320 mg/kg/dose of 51A plus 51D into 8-week-old *mdx*52 mice, seven times at weekly intervals. Two weeks after the final injection, whole-body skeletal muscles and the heart were examined. By RT-PCR, we identified cDNA bands corresponding to exon 51 having been skipped in nearly all skeletal muscles of treated mice (**Figure 4a**). The levels of skipping efficiency were variable: ~67% in the quadriceps (QC), 64% in the GC, 63% in the abdominal, 54% in the paraspinal, 43% in the triceps, 29% in the TA, 24% in the deltoid, 21% in the intercostal, 18% in the diaphragm, and 3% in the heart muscles (**Figure 4b**). Dystrophin expression was also evaluated by quantitative western blotting (**Figure 4c**). The expression levels in the QC, GC, and triceps muscles were the highest at 30–40% of normal levels. Those in the TA, intercostal, paraspinal, and diaphragm muscles showed modest expression at 10–20% of normal levels, whereas the dystrophin expression level in the heart was only 1% of normal levels (**Figure 4d**). We detected 60–80% dystrophin-positive fibers in all skeletal muscles by immunohistochemistry, most prominently in the QC, GC, and paraspinal muscles (**Figure 4e**). Furthermore,

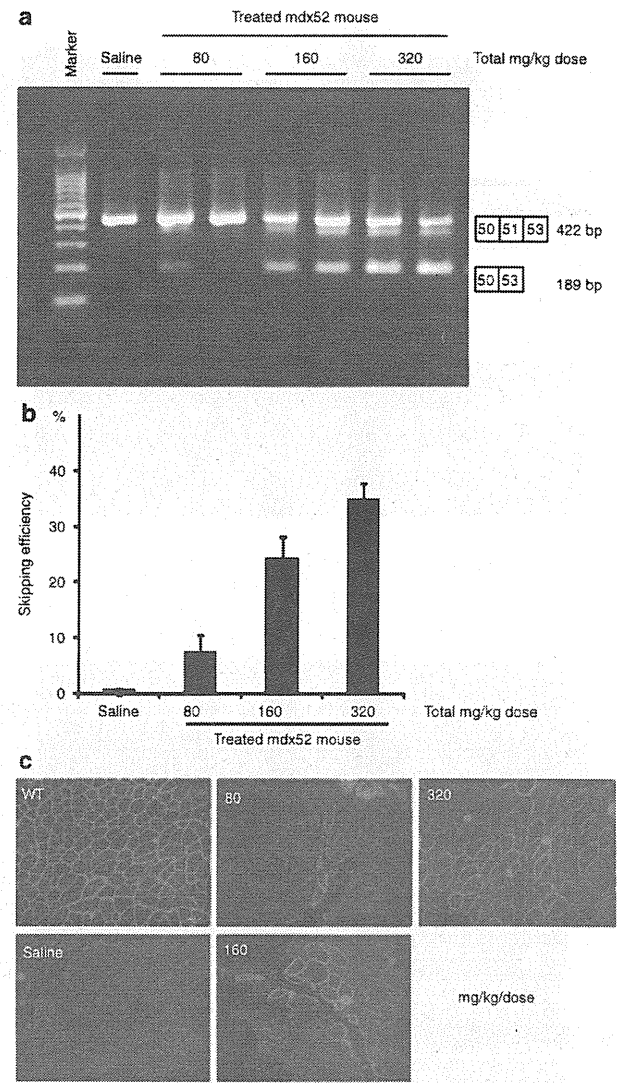

Figure 3 Dose-escalation study of systemic delivery of antisense oligonucleotides (AOs) to *mdx52* mice. Restoration of dystrophin in the gastrocnemius muscle 2 weeks after single intravenous co-injections of 80, 160, or 320 mg/kg/dose of AO. Intravenous saline injection into *Mdx*52 mice was used as a control. (**a**) Detection of exon 51-skipped dystrophin mRNA by RT-PCR. Representative data are shown. Skipped products (50–53) are compared with unskipped products (50–51–53). The additional bands between the unskipped and skipped products is due to heteroduplex formation. (**b**) Quantitative analysis of exon 51-skipping by AO. The percentages of in-frame transcripts are shown. The data ($n = 3$) are presented as mean ± SEM. (**c**) Immunohistochemical staining of dystrophin in the quadriceps muscles of a treated *mdx*52 mouse. Dystrophin was detected with rabbit polyclonal antibody P7. Bar = 100 μm. bp, base pair.

most of the nonpositive fibers in our study showed weak dystrophin signals.

We examined the expression of components included in the *dystrophin*–glycoprotein complex in the QC by immunohistochemistry. The expression of α-sarcoglycan correlated well with that of dystrophin (**Figure 4f**). We also observed the recovery of β-dystroglycan and α1-syntrophin expression at

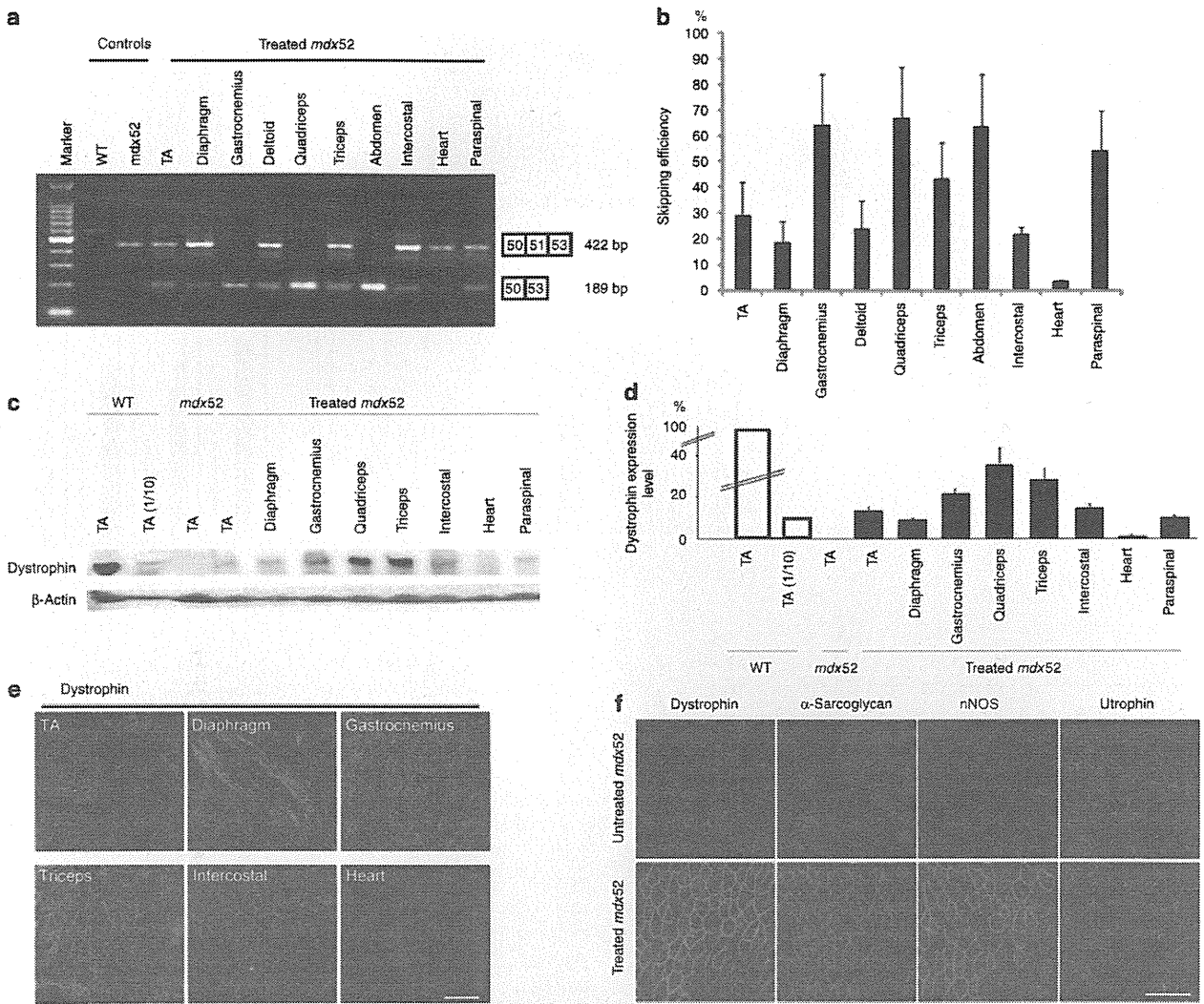

Figure 4 Repeated systemic delivery of antisense oligonucleotides (AOs) to *mdx52* mice. The restoration of dystrophin in various muscles after seven weekly intravenous co-injections of 320 mg/kg/dose of AOs was examined. (**a**) Detection of exon 51-skipped dystrophin mRNA by RT-PCR. Representative data are shown. Skipped products (50–53) are compared with unskipped products (50–51–53). The additional bands between the unskipped and skipped products is due to heteroduplex formation. (**b**) Quantitative analysis of exon 51-skipping by AOs. The percentages of in-frame transcripts are shown. The data ($n = 3$) are presented as mean ± SEM. (**c**) Western blotting after AO injections to detect the expression of dystrophin (upper panel) and β-actin (lower panel) in the TA, diaphragm, gastrocnemius, quadriceps, triceps brachii, intercostal, heart, and paraspinal muscles of a treated *mdx*52 mouse. Representative results are shown. Dystrophin was detected with the Dys2 monoclonal antibody. (**d**) Quantitative analysis of dystrophin expression after AO injection. The data ($n = 4$) are presented as mean ± SEM. TA(1/10): 10% of WT samples. (**e**) Immunohistochemical staining of dystrophin in the TA, diaphragm, gastrocnemius, triceps brachii, intercostal, and heart muscles of a treated *mdx*52 mouse. Dystrophin was detected with rabbit polyclonal antibody P7. Bar = 100 μm. (**f**) Immunohistochemical staining of dystrophin, α-sarcoglycan, neuronal nitric oxide synthase (nNOS) and utrophin in the quadriceps muscle of an untreated *mdx*52 mouse (upper panel) and a treated *mdx*52 mouse (lower panel). Bar = 100 μm. *mdx*52, untreated *mdx*52 mouse; TA, tibialis anterior; WT, wild-type mouse.

the sarcolemma (data not shown). On the other hand, utrophin expression was diminished in dystrophin-positive fibers (**Figure 4f**).

In-frame dystrophin largely lacking hinge 3 ameliorated skeletal muscle pathology

The *mdx*52 mice skeletal muscle shows hypertrophy and an increased ratio of centrally nucleated fibers.[17] Two weeks after seven consecutive weekly i.v. injections of the combination of AOs, the wet weight of the extensor digitorum longus muscle tended to be slightly lower in treated mice than in untreated mice (**Figure 5a**). We observed less muscle degeneration and fewer cellular infiltrates in the treated TA muscle compared with the untreated TA muscle (**Figure 5b**). We then evaluated the detailed histological changes in the treated muscles and compared them with the changes in the untreated muscles. The fiber size variation in the treated TA muscle was less than that in the untreated TA muscle (**Figure 5c**). We found a significant decrease in the mean cross-sectional area of muscle fibers in treated mice compared with those in untreated mice (**Figure 5d**). The percentages

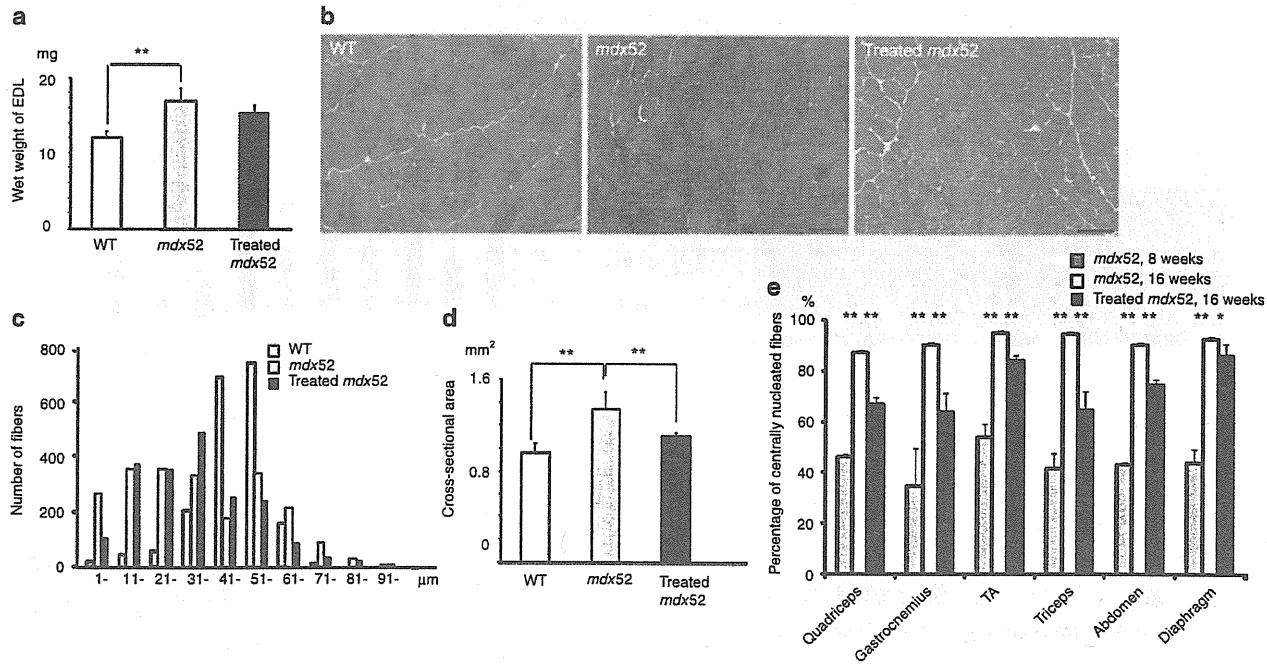

Figure 5 Amelioration of pathology in body-wide muscles of *mdx52* mice after seven weekly intravenous co-injections of 320 mg/kg/dose of antisense oligonucleotides. (**a**) Wet weight of the extensor digitorum longus (EDL) muscles of wild-type (WT), untreated and treated 16-week-old *mdx*52 mice. The data ($n = 4$) are presented as mean ± SEM. $^{**}P < 0.01$. (**b**) Hematoxylin and eosin staining of cryosections in tibialis anterior (TA) muscle of WT, untreated and treated *mdx*52 mice. Bar = 100 μm. (**c**) Histogram of muscle fibers in the TA muscle of WT, untreated and treated 16-week-old *mdx*52 mice. (**d**) Cross-sectional area of EDL muscles of WT, untreated and treated 16-week-old *mdx*52 mice. The data ($n = 4$) are presented as mean ± SEM. $^{**}P < 0.01$. (**e**) The ratio of centrally nucleated fibers in the quadriceps, gastrocnemius, TA, triceps brachii, abdominal, and diaphragm muscles of untreated 8-week-old (dark gray), 16-week-old *mdx*52 mice (light gray), and treated 16-week-old *mdx*52 mice (black). The data ($n = 4$) are presented as mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$. *mdx*52, untreated *mdx*52 mouse.

of centrally nucleated fibers were lower in the triceps, GC, QC, and abdominal muscles than in the diaphragm and TA muscles (**Figure 5e**). These changes reflect the amelioration of muscle fiber hypertrophy and dystrophic changes in the treated *mdx*52 mice.

In-frame dystrophin largely lacking hinge 3 restored skeletal muscle function

To examine the function of the AO-induced dystrophin, we evaluated skeletal muscle function with a battery of tests after seven weekly i.v. AO injections. The protection of muscle fibers against degeneration was supported by a significant reduction in serum creatine kinase levels in the treated mice (**Figure 6a**). Significant improvements in treadmill endurance (**Figure 6b**), maximum forelimb grip force (**Figure 6c**), and specific tetanic force of the extensor digitorum longus muscle (**Figure 6d**) were observed in treated *mdx*52 mice compared with nontreated *mdx*52 mice.

Efficacy of repeated AO injection in *mdx52* mice confirmed by gene expression array

Gene expression array analysis has been widely used to profile gene expression for disease diagnosis and therapy due to its ability to interrogate every transcript in the genome simultaneously. Dystrophic TA muscle has been compared with normal TA muscle in human and *mdx* mice at various stages of the disease.[23,24] To evaluate the gene expression profile of TA muscles following exon 51-skipping, we performed genome-wide gene expression analysis (**Figure 7a**). Gene expression array analysis showed that the gene expression profiles of TA muscles correlated well between the treated and untreated *mdx*52 mice ($r^2 = 0.97$), and there was no unexpected downregulation of housekeeping genes or upregulation of stress-related proteins. We found that dystrophin-associated proteins such as dystrophin, neuronal nitric oxide synthase, and α1-syntrophin were upregulated, α-sarcoglycan and β-dystroglycan levels were unchanged, and utrophin was downregulated. We also found that inflammatory cytokines were downregulated in treated *mdx*52 mice. Quantitative RT-PCR following gene expression array analysis showed that dystrophin and neuronal nitric oxide synthase expression levels were 3.4 and 1.9 times higher than those in the untreated *mdx*52 mice, respectively; however, they were still only 33 and 40% of the normal levels, respectively (**Figure 7b**). Utrophin expression levels were upregulated in the untreated *mdx*52 mice, but downregulated in the treated *mdx*52 mice compared with wild-type (WT) mice (**Figure 7b**). In treated *mdx*52 mice, we observed reduced levels of several C-C class chemokine ligands (Ccls) such as Ccl7, Ccl21b, and Ccl2, which are small cytokines that induce the migration of monocytes and other cell types such as natural killer cells and dendritic cells (**Figure 7c**). This might reflect an improvement of the muscle inflammatory response in the treated *mdx*52 mice.

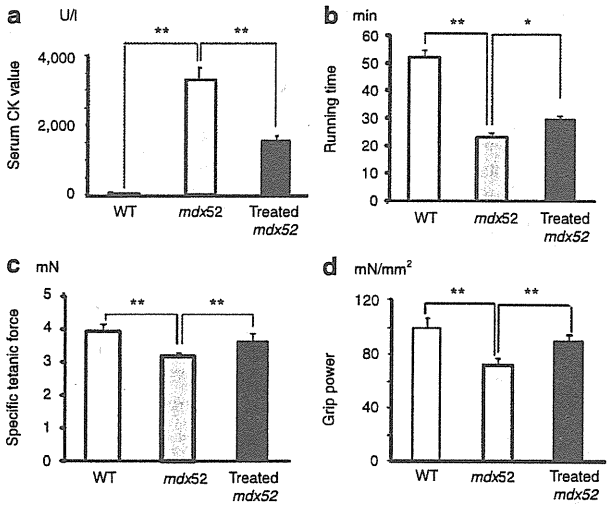

Figure 6 Muscle function in *mdx52* mice after seven weekly intravenous co-injections of 320 mg/kg/dose of antisense oligonucleotides. (**a**) Measurement of serum creatine kinase (CK) levels (IU/l). (**b**) Treadmill performance (min), (**c**) grip power test (mN/g), and (**d**) specific tetanic force of the extensor digitorum longus muscle (mN/mm²). Wild-type (WT), untreated (*mdx*52), and treated 16-week-old *mdx*52 mice were examined. The data ($n = 4$) are presented as mean ± SEM. $^*P < 0.05$; $^{**}P < 0.01$.

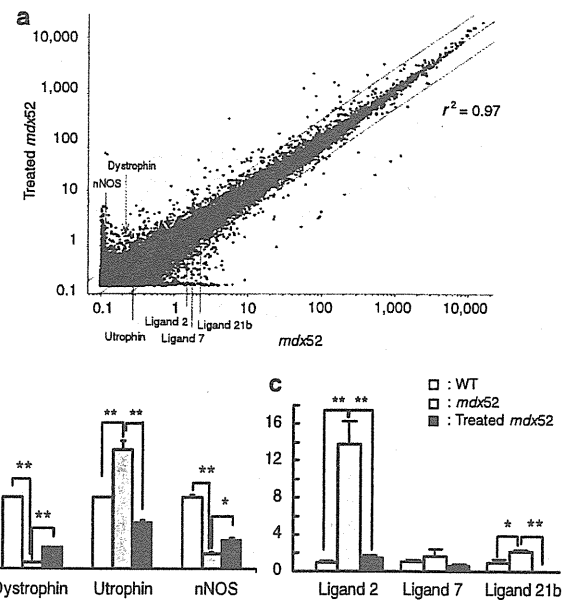

Figure 7 Genome-wide expression analyses by gene expression array using tibialis anterior muscles. Gene expression array analysis after repeated intravenous co-injections of 320 mg/kg/dose of antisense oligonucleotides into *mdx*52 mice. (**a**) A scatter plot of global gene expression analyses by gene expression array in treated and untreated *mdx*52 mice. The upper line shows twofold changes and the lower line shows 0.5-fold changes in gene expression levels between treated and untreated *mdx*52 mice. The positions of dystrophin, utrophin, neuronal nitric oxide synthase (nNOS), and the C-C class chemokine ligands (Ccls) 2, 7, and 21b are indicated. (**b**,**c**) The gene expression levels of dystrophin, utrophin, nNOS (**b**) and CCLs 2, 7, and 21b (**c**) by quantitative PCR, in 16-week-old WT, untreated and treated *mdx*52 mice. The data ($n = 3$) are presented as mean ± SEM. $^*P < 0.05$; $^{**}P < 0.01$. *mdx*52, untreated *mdx*52 mouse; WT, wild-type mouse.

No detectable toxicity after repeated delivery of AOs into *mdx52* mice

No signs of illness and no deaths were noted during the period of AO treatment. To further monitor any potential toxicities in the major organs induced by treatment with AOs, we compared a series of serum markers commonly used as indicators of liver and kidney dysfunction in WT, untreated and treated *mdx*52 mice. No significant differences were detected among the three groups in the levels of creatinine, blood urea nitrogen, aspartate amino transferase, alanine aminotransferase, total bilirubin, alkaline phosphatase, and γ-glutamyl transpeptidase (**Supplementary Figure S2a**). Histological examination of liver and kidney revealed no signs of tissue damage or increased monocyte infiltrations in treated *mdx*52 mice (**Supplementary Figure S2b**). These data confirm that this AO combination was nontoxic in this study.

In vitro exon 51-skipping in DMD 5017 cells with deletion of exons 45–50

We newly designed several AOs based on murine sequences: hAc (51Ac) targeting the 5′ splice site, and hDo1 (51D1) and hDo2 (51D2) targeting the 3′ splice site of human exon 51. The sequences and composition of the AO treatments are described in **Supplementary Table S1**. MyoD-converted fibroblasts (DMD 5017 cells) were examined after 48-hour incubation with a single or two AOs at a final concentration of 1, 5, or 10 μmol/l. Among the AOs examined, hAc plus hDo1, and B30 alone, were shown by RT-PCR to be capable of inducing exon 51-skipping at a level approaching 50 and 30%, respectively (**Supplementary Figure S3**). On the other hand, hAc, hDo1, or hDo2 alone were less effective at inducing exon 51-skipping (**Supplementary Figure S3**). These results suggest that the co-injection of two AOs could induce highly effective exon 51-skipping in DMD cells.

DISCUSSION

This is the first report showing widespread induction of in-frame dystrophin lacking most of hinge 3 following exon 51-skipping, and clear recovery of muscle function to therapeutic levels in a DMD mouse model.

Exon skipping produces several forms of in-frame dystrophin that lack part of the molecular structure depending on the targeted exons.[4–6] The molecular structure of dystrophin is composed of the actin-binding domain 1 at the N-terminus (ABD1), the central rod domain containing 24 spectrin-like repeats (R1–24), four hinge domains, a 20-amino acid insertion between spectrin-like repeats 15 and 16 (segment 5), the cysteine-rich domain, and the C-terminal domain (**Figure 1b**).[27,38] Until now, the in-frame dystrophin formed following exon 23-skipping, which lacks half of the 6th spectrin-like repeat and part of the 7th, ameliorated the muscle pathology and function in *mdx* mice with a point mutation in exon 23 (refs. 15,16). These results were consistent with the fact that in-frame deletions of the central rod domain in humans typically lead to a mild BMD.[6] However, the severity of BMD with in-frame deletions including hinge 3 can vary considerably.[28–32,38]

The hinges are proline-rich, nonrepeat segments that may confer flexibility to dystrophin.[27] Among them, the hinge 3 region is encoded by exons 50 and 51, located between the 19th and 20th spectrin-like repeats, and is prone to deletion mutations.[20,22] Recently, it was reported that hinge 3 is more important than hinge 2 in preventing muscle degeneration and promoting muscle maturation in the microdystrophin$^{\Delta R4-R23}$/*mdx* transgenic mice.[38] The in-frame dystrophin produced following exon-51-skipping is predicted to lack most of the hinge 3 region (**Figure 1b**); hinges 1, 2, 3, and 4 consist of 75, 50, 43, and 72 amino acid residues, respectively.[27,38] The small segment of hinge 3 that remains following exon 51-skipping consists of 13 amino acid residues with two prolines and would not be predicted to function as a hinge (**Figure 1c**). This remaining fragment is very similar to segment 5, which consists of 20-amino acid residues with one proline and is located between the 15th and 16th spectrin-like repeats.[27] Based on the molecular structure of dystrophin, the small segment of the hinge 3 might act as a "turn," which is bound to the helix of the 20th repeat.[27,38] Our results suggest that the hinge 3 region is more essential in the short microdystrophin (167 kDa) than in the almost full-length dystrophin lacking hinge 3 due to exon 51-skipping (420 kDa).[38,39]

In-frame dystrophin expression in skeletal muscle at 20% of normal levels produced moderate/mild BMD phenotypes.[26] Moreover, restoration of 20–30% in-frame dystrophin expression resulted in protection from muscle degeneration and recovery of skeletal muscle function in the transgenic *mdx* mouse.[40] As a first step, we screened optimal AO sequences including mB30 for skipping of exon 51 to induce in-frame dystrophin at up to 20% of normal dystrophin levels. To date, specific AO sequences have been assessed for their efficiency of exon skipping using cell-based experimental systems, with the optimal sequences then used for *in vivo* experiments.[11,41,42] However, the *in vivo* efficacy of phosphorodiamidate morpholino oligomers (PMOs), which are rather difficult to deliver into mammalian cells in culture because of their neutral chemistry,[11,19] could differ from that *in vitro*.[14] Therefore, we used the *mdx*52 mouse to screen AO sequences for exon 51-skipping by intramuscular injection *in vivo*. We also showed that simultaneous delivery of two AO sequences directed against both the 3′ and the 5′ splice sites drove skipping of exon 51 more efficiently than any single or two AO sequences targeting exonic regions in the *mdx*52 mouse. This combination of AOs worked in a synergistic fashion, where the increase in activity was greater than the additive effect of each individual AO.[14,34,37]

We found that the skipping efficiency induced by systemically delivered PMO increased in proportion to the AO dose in the *mdx*52 mouse. A dose-dependent restoration of dystrophin expression in the muscles of *mdx* mice by systemically delivered PMO has also been reported.[43] The therapeutic dose (320 mg/kg/dose) of exon 51-skipping AOs in the *mdx*52 mouse to induce 20–30% of normal dystrophin levels is approximately four times as much as the dose (80 mg/kg/dose) required for exon 23-skipping in the *mdx* mouse. We have to consider the possibility that the difference of the genetic background of mice between *mdx*52 (C57BL/6J) and *mdx* (C57BL/10) mice could influence the properties of exon skipping. Our results suggest that the therapeutic dose of AO required is different depending on which exon is being targeted. Because AO-mediated exon skipping is the first RNA-modulating therapeutic with this mechanism of action, this study using a DMD mouse model could provide a suggestion for human equivalent doses based on body surface area.[44] Recently, press releases from both Prosensa for PRO051 and AVI Biopharma for AVI-4658 have revealed that DMD patients who received 2–6 and 2–20 mg/kg, respectively, induced specific exon 51-skipping and dystrophin expression in a dose-related manner (http://www.prosensa.eu/press-room/press-releases/2009-09-14-PRO051-shows-favourable-results.php; http://investorrelations.avibio.com/phoenix.zhtml?c=64231&p=irol-newsArticle&ID=1433350&highlight=).

In this study, the dystrophin expression level in treated *mdx*52 mice was restored to roughly 20–30% of normal levels, and creatine kinase levels and skeletal muscle function significantly recovered in the treated mice. These findings show that the in-frame dystrophin lacking most of hinge 3 ameliorates dystrophic histology and functional phenotypes in *mdx*52 mice as well as the dystrophin produced following exon 23-skipping or microdystrophin in *mdx* mice.[15,16] On the other hand, the treadmill endurance of the treated *mdx*52 mice was still considerably inferior to that of WT mice compared with the clear recovery of forelimb grip force and specific tetanic force of the extensor digitorum longus muscle. These findings are similar to the data produced using microdystrophin in the *mdx* mouse.[39] Two possibilities remain to explain the incomplete recovery: insufficient dystrophin expression or defective dystrophin molecular structure due to exon 51-skipping. To examine these possibilities, we are now trying to increase the level of dystrophin expression using a high dose of PMO and using peptide-conjugated PMO in *mdx*52 mice.

The amelioration of the histopathology demonstrated by the reduction of centrally nucleated fibers in treated *mdx*52 mice was marked in the muscles with high levels of dystrophin expression, and the effect was modest in the muscles with low levels of dystrophin, in accordance with a previous report.[15,16] It is noteworthy that high levels of dystrophin expression were seen in severely degenerated muscles, and that low levels of expression were found in less affected muscles. We suggest the possibility that the antigravity muscles, such as the QC, GC, triceps brachii, abdominal, and paraspinal muscles[45] efficiently incorporated PMO into the muscle fibers. We showed that the antigravity muscles were mainly affected in *mdx*52 mice during the period that we examined; therefore, those severely affected muscles had taken up the most AO. Our data support the fragile membrane hypothesis that has been proposed as the background for PMO incorporation into dystrophic muscle.[46,47] This hypothesis also explains the inefficient incorporation of PMO into the diaphragm muscle where dystrophic changes chronically persisted, as previously reported.[15,16]

To screen for changes in gene expression levels influenced by the in-frame dystrophin, we performed gene expression array analysis. The inflammatory chemokines Ccl2, Ccl7, and Ccl21b, which were downregulated after treatment, play important roles in the migration of macrophages, $CD4^+$ and $CD8^+$ T cells to muscle in *mdx* mice.[48] It is also known that depletion of these cells from *mdx* mice decreased the sarcolemmal damage.[49] These data showed that the inflammatory process, which could aggravate the pathology, was prevented in *mdx*52 mice. Measurement of the chemokines might be a beneficial index of the therapeutic effects on *mdx*52 mice.

In conclusion, this report describes the first successful effort at systemic rescue of in-frame dystrophin lacking most of hinge 3 and muscle function by PMO-mediated exon 51-skipping in a mouse model. Because the structure of the in-frame dystrophin lacking most of hinge 3 in mice resembles human dystrophin following exon 51-skipping, our results are extremely encouraging as regards the ongoing systemic clinical trials for DMD. In addition, the therapeutic dose in DMD model mice provides a suggestion of the theoretical equivalent dose in humans.

MATERIALS AND METHODS

Animals. Exon 52–deficient X chromosome–linked muscular dystrophy (*mdx*52) mice were produced by a gene-targeting strategy and maintained at our facility.[33] The mice have been backcrossed to the C57BL/6J WT strain for more than eight generations. Eight-week-old male *mdx*52 and WT mice were used in this study. All experimental protocols in this study were approved by The Experimental Animal Care and Use Committee of the National Institute of Neuroscience, National Center of Neurology and Psychiatry (NCNP), Tokyo, Japan.

Antisense sequences and delivery methods. Thirteen AOs for targeted skipping of exon 51 during dystrophin pre-mRNA splicing in mice were comprehensively designed to anneal to the 5′ splice site (51A), the 3′ splice site (51D), and other intraexonic regions (51B, 51C, 51E, 51F, 51G, 51H, 51I, 51J, 51K, 51L, 51M). The sequences and positions of the AOs are described in **Table 1**. mB30, which corresponds to human B30, was also specifically designed for this study.[11] To design these sequences, we referred to previously published sequences and considered GC content and secondary structure to avoid self- and heterodimerization.[9,11] All sequences were synthesized using a morpholino backbone (Gene Tools, Philomath, OR). Primers for RT-PCR and sequencing analysis were synthesized by Operon Biotechnologies (Tokyo, Japan) and are listed in **Supplementary Table S1.**

Ten micrograms of PMO were injected into each TA muscle of *mdx*52 mice. Muscles were obtained 2 weeks after the intramuscular injection. To examine the optimal therapeutic dose, a total of 80 (ref. 15), 160 or 320 mg/kg/dose of AO was injected into the tail vein of *mdx*52 mice singly. Muscles were isolated 2 weeks after the systemic injection and analyzed by RT-PCR and the cryosections by immunohistochemistry. Following the dose-escalation study, a 320 mg/kg dose of PMO in 200 µl of saline,[50] or 200 µl saline, was injected into the tail vein of *mdx*52 mice or WT mice, seven times at weekly intervals. The mice were examined 2 weeks after the final injection. Muscles were dissected immediately, snap-frozen in liquid nitrogen–cooled isopentane and stored at −80 °C for RT-PCR, immunohistochemistry, western blotting, and gene expression array analysis. Liver and kidney were also frozen in liquid nitrogen and stored at −80 °C for pathological analysis.

RT-PCR and sequencing of cDNA. Total RNA was extracted from cells or frozen tissue sections using TRIzol (Invitrogen, Carlsbad, CA) from treated *mdx*52 mice, and from WT and untreated *mdx*52 mice, which were used as controls, respectively. Two hundred nanograms of total RNA template was used for RT-PCR with a QuantiTect Reverse Transcription kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. The cDNA product (1 µl) was then used as the template for PCR in a 25 µl reaction with 0.125 units of TaqDNA polymerase (Qiagen). The reaction mixture comprised 10× PCR buffer (Roche, Basel, Switzerland), 10 mmol/l of each dNTP (Qiagen), and 10 µmol/l of each primer. The primer sequences were Ex50F 5′-TTTACTTCGGGAGCTGAGGA-3′ and Ex53R 5′-ACCTGTTCGGCTTCTTCCTT-3′ for amplification of cDNA from exons 50–53. The cycling conditions were 95 °C for 4 minutes, then 35 cycles of 94 °C for 1 minute, 60 °C for 1 minute, 72 °C for 1 minute, and finally 72 °C for 7 minutes. The intensity of PCR bands was analyzed by using ImageJ software (http://rsbweb.nih.gov/ij/), and skipping efficiency was calculated by using the following formula [(the intensity of skipped band) / (the intensity of skipped band + the intensity of unskipped band)].[51] After the resulting PCR bands were extracted using a gel extraction kit (Qiagen), direct sequencing of PCR products was performed by the Biomatrix Laboratory (Chiba, Japan).

Immunohistochemistry, and hematoxylin and eosin staining. At least ten 8 µm cryosections were cut from flash-frozen muscles at 100 µm intervals. The serial sections were stained with polyclonal rabbit antibody P7 against the dystrophin rod domain (a gift from Qi-Long Lu, Carolinas Medical Center, Charlotte, NC), anti-α-sarcoglycan monoclonal rabbit antibody (Novocastra Laboratories, Newcastle, UK), anti-β-dystroglycan monoclonal mouse antibody (Novocastra Laboratories), anti-α1-syntrophin monoclonal mouse antibody (Novocastra Laboratories), antineuronal nitric oxide synthase polyclonal rabbit antibody (Zymed, San Francisco, CA), and antiutrophin polyclonal rabbit antibody (UT-2). Alexa-488 or 568 (Molecular Probes, Cambridge, UK) was used as a secondary antibody. 4′,6-diamidino-2-phenylindole containing a mounting agent (Vectashield; Vector Laboratories, Burlingame, CA) was used for nuclear counterstaining. The maximum number of dystrophin-positive fibers in one section was counted, and the TA muscle fiber sizes were evaluated using a BZ-9000 fluorescence microscope (Keyence, Osaka, Japan). Hematoxylin and eosin (H&E) staining was performed using Harris H&E.

Western blotting. Muscle protein from cryosections was extracted with lysis buffer as described previously.[14] Two to twenty micrograms of protein were loaded onto a 5–15% XV Pantera Gel (DRC, Tokyo, Japan). The samples were transferred onto an Immobilon polyvinylidene fluoride membrane (Millipore, Billerica, MA) by semidry blotting at 5 mA/mm² for 1.5 hours. The membrane was incubated with the C-terminal monoclonal antibody Dys2 (Novocastra) at room temperature for 1 hour. The bound primary antibody was detected by horseradish peroxidase–conjugated goat anti-mouse IgG (Cedarlane, Burlington, ON) and SuperSignal chemiluminescent substrate (Pierce, Rockford, IL). Anti-β-actin antibody was used as a loading control. Signal intensity of detected bands of the blots were quantified using ImageJ software and normalized to the loading control.

Serum creatine kinase levels and toxicity tests. Four treated *mdx*52 mice were examined for toxic effects of PMO before injection, 1 week after the third injection, and 2 weeks after the last injection. Blood was taken from the tail artery and centrifuged at 3,000*g* for 10 minutes. The biochemical markers creatine kinase, electrolytes (sodium, potassium, and chloride ions), blood urea nitrogen, total bilirubin, alkaline phosphatase, aspartate transaminase, and alanine transaminase were assayed as described previously.[14] The histology of the liver, lung, and kidney was examined microscopically on cryosections.

Functional testing. The mice were placed on a flat MK-680S treadmill (Muromachi Kikai, Tokyo, Japan) and forced to run at 5 m/minute for 5 minutes. After 5 minutes, the speed was increased by 1 m/minute every minute. The test was stopped when the mouse was exhausted and did not attempt to remount the treadmill, and the time to exhaustion was determined.

The grip strength of the mice was assessed by a grip strength meter (MK-380M; Muromachi Kikai). The mice were held 2 cm from the base of the tail, allowed to grip a woven metal wire with their forelimbs, and pulled gently until they released their grip. Five sequential tests for the exerted force were carried out for each mouse, with 5-second intervals, and the data were averaged.

The extensor digitorum longus muscles were kept in Krebs–Henseleit solution at 25 °C and stimulated with a pair of platinum electrodes using an electronic stimulator (SEN-3301; Nihon Kohden, Tokyo, Japan). A Thermal Arraycorder (WR300; Graphtec, Yokohama, Japan) was used to