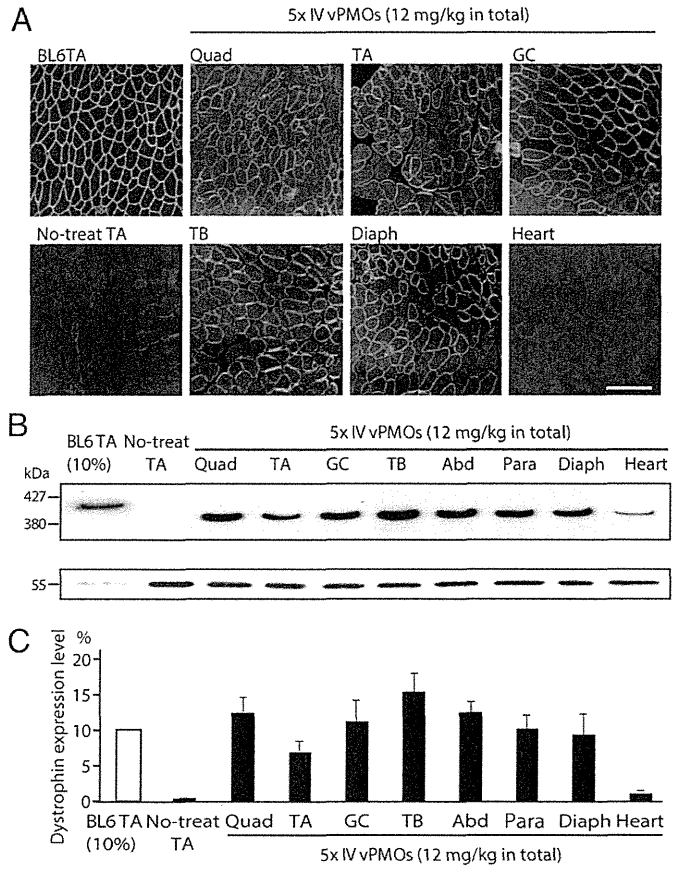

Fig. 3. Systemic i.v. injections of the mixture-ESE2 in *mdx52* mice restore dystrophin expression in body-wide skeletal muscles. (*A*) Immunohistochemical staining of dystrophin exon 57 in quadriceps (Quad), TA, gastrocnemius (GC), triceps brachii (TB), diaphragm (Diaph), and heart muscles in *mdx52* mice after five consecutive biweekly systemic injections of 12 mg/kg of the mixture-ESE2. Representative data are shown. BL6TA, TA muscle from wild-type C57/BL6; no-treat TA, untreated TA muscle from *mdx52* mice. (Scale bar, 100 μm.) (*B*) Western blotting analysis with mouse monoclonal antibody DYS2 after the repeated vPMOs systemic injections into *mdx52* mice. Representative data are shown. vPMO-injected muscles show 380-kDa quasidystrophin bands (*Upper*) and α-tubulin (*Lower*) in Quad, TA, GC, TB, abdominal (Abd), paraspinal (Para), Diaph, and heart muscles of treated *mdx52* mice. BL6TA (10% wt/wt), TA muscle from a 10% (wt/wt) extract of wild-type C57/BL6 mice. (*C*) Semiquantitative analysis of dystrophin expression after AO injection. Data (n = 4) are presented as mean ± SD $*P < 0.05$; $**P < 0.01$.

Exon 45–55 Skipped Quasidystrophin Ameliorates Skeletal Muscle Function. To examine the function of the exon 45–55 skipped quasidystrophin, we performed a battery of physiological and blood tests after five biweekly i.v. injections with the mixture-ESE2. Serum CK levels were significantly reduced in the treated mice, suggesting the protection of muscle fibers against degeneration (Fig. S6*A*). In addition, significant improvement in maximum forelimb grip force and improvement tendency in treadmill endurance and time latency to fall in rotarod test were observed in treated *mdx52* mice compared with untreated *mdx52* mice (Fig. S6 *B–D*).

No Detectable Toxicity After Repeated Delivery of AOs into *mdx52* Mice. To further monitor any potential toxicities in the major organs induced by treatment with AOs, we compared a series of standard serum markers as indicators of liver and kidney dysfunction in WT, untreated, and treated *mdx52* mice after five biweekly 12 mg/kg injections with the mixture-ESE2. Serum aspartate amino transferase was reduced in the treated mice (Fig. S7). No significant differences were detected between untreated and treated *mdx52* mice groups in the levels of aspartate transaminase, alanine aminotransferase, total bilirubin, alkaline phosphatase, blood urea nitrogen, creatinine, sodium ion, chloride ion, and potassium ion (Fig. S7). These data confirm that this AO combination was nontoxic in vivo.

Discussion

In this paper, we demonstrated that rescue of dystrophin expression by skipping exons 45–55 is feasible both in *H2K-mdx52* myotubes in vitro and in dystrophic *mdx52* mice in vivo. As far as

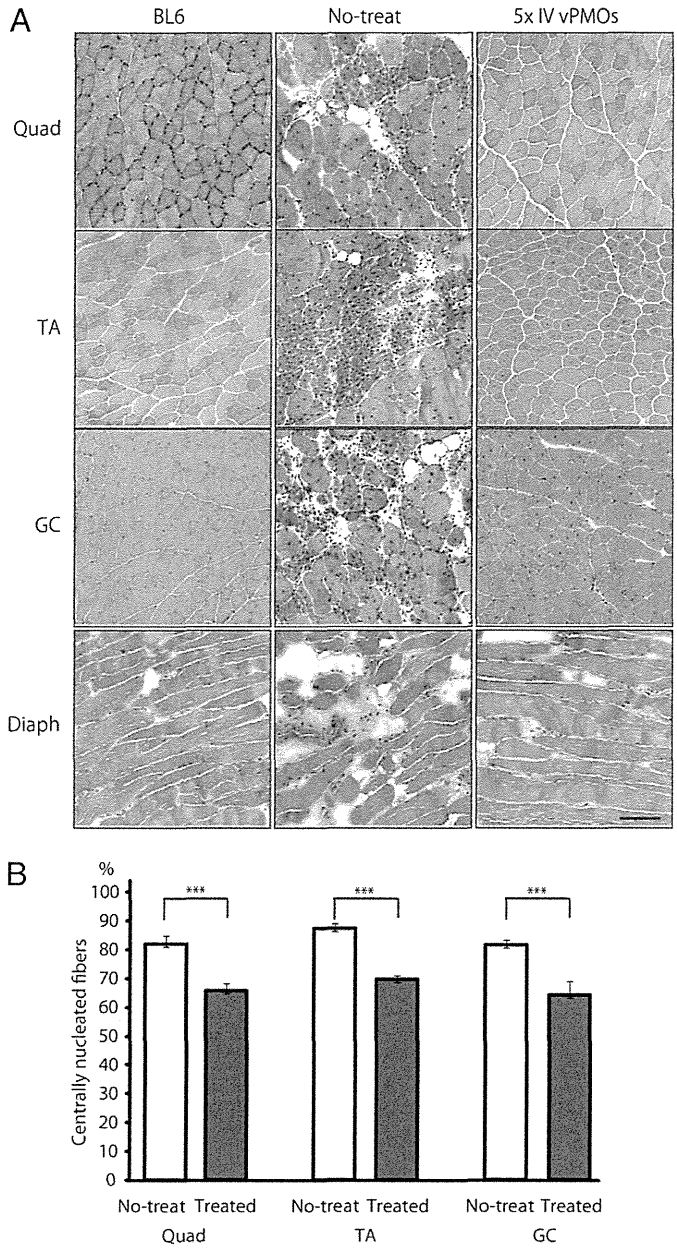

Fig. 4. Exon 45–55 skipped quasidystrophin ameliorates skeletal muscle pathology in *mdx52* mice. (*A*) H&E staining in quadriceps (Quad), TA, gastrocnemius (GC), and diaphragm (Diaph) muscles of WT (BL6), untreated (no-treat), and treated *mdx52* mice (five times i.v. vPMOs). Representative data are shown. (*B*) Measurement of centrally nucleated fibers (CNFs) after systemic exon 45–55 skipping (treated) and nontreated *mdx52* muscles (no-treat) in Quad, TA, and GC muscles. Data (n = 4) are presented as mean ± SD $***P < 0.001$.

we know, this is a unique report of a successful rescue of dystrophin using this technique to skip a large block of 10 exons. Currently, trials of exon 51 skipping using systemic delivery of PMO and 2′O-methyl phosphorothioate antisense oligonucleotides (2′O-MePS) AOs are underway (8, 9). Although exon 51 is the single target exon in the *DMD* gene whose skipping would restore ORF to the largest proportion of DMD mutations, it would still be applicable to only some 10% of DMD patients (or 17% of DMD patients with deletion mutations) (Fig. S1*B*) (17). The goal of treating a broader range of DMD patients with a single treatment has fostered interest in skipping multiple exons. In particular block skipping of exons 45–55 commends itself as a most promising approach (13, 18). Exon 45–55 skipping is known to have two major advantages. First, it would be applicable to ~63% of DMD patients with dystrophin deletion mutations (13). Second, the majority of individuals with a deletion of exons 45–55 of the *DMD* gene fall into the category of BMD patients with exceptionally mild, sometimes almost asymptomatic skeletal muscle involvement (12, 13). Nakamura et al. reported two Japanese patients with exon 45–55 deletions (28 and 42 y old) showing no symptoms except for high blood CK level (12). Similarly, Ferreiro et al. reported a 69-y-old individual with exon 45–55 deletion showing only high CK level without showing any symptoms (19). Such observations raise the hope that antisense-mediated exon 45–55 skipping will have the potential not only to convert DMD patients to a mild BMD clinical phenotype but also to ameliorate the condition of some of the more severe BMD patients with mutations within the exon 45–55 hotspot region.

It is not known why deletion of exons 45–55 leads to very mild phenotypes. The Leiden database used in this study consists of reports from many different countries and sites, raising the possibility of inconsistencies arising from incorrect mapping of some patients or differences in the criteria of diagnosis for Duchenne, Becker, and intermediate cases. However, we have no reasons to suspect any systematic bias with this large dataset. The resulting products of exon 45–55 skipping lead to a truncation at the middle of two rod spectrin repeats (rod repeats 17 and 22) as we have previously pointed out (Fig. 1*A*) (11). Interestingly, the number of rod repeats in this quasidystrophin between the remaining adjacent hinge domains H2 and H4 (16 spectrin repeats) is exactly the same as that between H2 and H3 in full-length dystrophin, which might indicate a requirement of such a spacing for protein function or stability (Fig. 1*A*).

Our results indicate that the truncated dystrophin induced by skipping exons 45–55 restored all of the dystrophin-associated proteins except nNOS. It was reported that nNOS is anchored at the subsarcolemma through its binding to the rod domain of dystrophin at the 16 and 17 rod repeats encoded by exons 42–45 and by interaction with α1-syntrophin (20, 21). The truncated dystrophin induced by skipping exons 45–55 would lack half of the 17 rod repeat, thus disturbing the site-responsible anchoring of nNOS and possibly rendering its subsarcolemmal localization unstable. Although nNOS was not restored after the mixture oligo injections, it is encouraging to note that mild BMD patients with deletions of exons 45–55 also lack nNOS at the subsarcolemma (22).

A previous in vitro study by van Vliet et al. showed that the exon 45–55 skipping frequencies with 2′O-MePS AOs were minimal and comparable to those observed in untreated myocytes of DMD patients with exon 45–47 deletions (16). Here, we redesigned the mixture of vPMOs using online software called Human Splicing Finder and ESEfinder to detect ESEs (23, 24), but paying attention to the avoidance of formation of self- or heteroduplex of the AOs, which, we reasoned, could diminish the efficacy of multiple exon skipping. With this aim, we used OligoAnalyzer 3.1 (25) to design the mixture in which most of combinations of ΔG forces of different AOs were above −5 kcal/mole.

After i.v. injections of our mixture vPMOs into *mdx52* mice, we observed extensive dystrophin-positive fibers and an average of some 8–15% of wild-type levels of dystrophin protein, as determined by Western blotting analysis in a range of skeletal muscles (Fig. 3). The pathology of skeletal muscles was ameliorated; but the skeletal muscle function was only marginally recovered, probably due to the incomplete restitution of quasi-dystrophin (Fig. 4 and Fig. S6).

Whereas conventional PMOs are relatively safe, high-dose administration is required to induce exon skipping systemically (5, 7). Here, we observed high efficacy for systemic rescue with vPMOs that contain a cell-penetrating moiety of octaguanidine dendrimer (Fig. S2*B*). Although the sequences we have used are specific to the mouse, our data validate the principle that carefully designed AOs may be used to realize block skipping of exons 45–55 and, by this means, generate effective amounts of quasidystrophin of near-optimal structure in some 60% of DMD deletion patients. This multiple exon skipping therapy was achieved by use of vPMOs, which produced no evidence of toxicity. The most significant barrier to translating the "mixture approach" to a therapeutic for DMD is the lack of adequate delivery, especially to the heart. Nevertheless, we suggest that a similar approach might pave the way for use of a single-mixture antisense drug that could be applied to treatment of 40–45% of DMD patients.

Materials and Methods

Animals. Exon 52-deficient X chromosome-linked muscular dystrophy mice (*mdx52* mice) were produced by a gene-targeting strategy and are maintained in our facility (26); they have been backcrossed to the C57BL/6J (WT) strain for more than eight generations. Five-week-old male *mdx52* and WT mice were used in this study. The mice were allowed ad libitum access to food and drinking water. The Experimental Animal Care and Use Committee of the National Institute of Neuroscience, National Center of Neurology and Psychiatry (NCNP), Japan, approved all experimental protocols in this study.

Antisense Oligos. AOs for targeted skipping of exons 45–55 in the mouse *Dmd* gene were designed using ESEfinder and Human Splicing Finder software to anneal to the ESEs of each exons (Table S1) (23, 24). All antisense mixtures consist of equal amounts of each antisense oligo. Unmodified morpholinos (PMOs) or octaguanidine dendrimer-conjugated morpholinos (vivo-morpholinos or vPMOs) were used in this study (Gene-Tools) (27, 28).

***H2K-mdx52* Myoblasts.** *H-2Kb*-tsA58 × *mdx52/mdx52* F_1 male mice yielded dystrophin-deficient *H2K-mdx52* myoblasts (29). *H2K-mdx52* myoblasts were grown at 33 °C in medium containing γ-IFN at a concentration of 20 units/mL and 20% (vol/vol) FBS. After the treatment, the cells were grown in differentiation medium containing 5% (vol/vol) horse serum at 37 °C for 1 d.

AO Transfection. Myotubes were differentiated from *H2K-mdx52* cells and were transfected with the vPMO or the PMOs as previously reported (30). In the differentiation medium, the final concentration of the vPMO was a total of 0.1–3 μM and that of the PMO was a total of 10 μM for 10 sequences. After 48 h incubation with the vPMO or the PMO, total RNA was extracted from myotubes using TRIzol (Invitrogen).

AO Injections. Animals were anesthetized by inhalation of sevoflurane (Wako Pure Chemical Industries) for injections. A total of 1.5 μg of vPMOs or 10 μg of PMOs targeting exons 45–55 in a total volume of 36 μL of saline were used for each TA muscle in the *mdx52* mice. Muscle samples were obtained 2 wk after the intramuscular injection.

A total of 12 mg/kg dose of vPMOs in 100 μL of saline was injected into the tail vein of *mdx52* mice, five times at biweekly (every 2 wk) intervals. The mice were examined 2 wk after the final injection. Muscles were obtained immediately, snap frozen in liquid nitrogen-cooled isopentane, and stored at −80 °C for immunohistochemistry and Western blotting. Skeletal muscle tissues were cut and collected in microtubes and snap frozen in liquid nitrogen for reverse transcription PCR (RT-PCR).

RT-PCR and Sequencing of cDNA. Total RNA from the muscles of WT, untreated, or *mdx52* mice were extracted as previously described (7). Two hundred nanograms of RNA template was used for a 20-μL RT-PCR using

a QuantiTect Reverse Transcription kit (Qiagen) according to the manufacturer's instructions. The cDNA product (1 μL) was then used as the template for PCR in a 20-μL reaction with 0.10 μL of Ex Taq Hot Start Version (Takara). The reaction mixture composed of 10× PCR buffer (Roche), 10 mM of each dNTP (Qiagen), and 10 μM of each primer. The primer sequences for the PCR were designed using Primer3 (http://frodo.wi.mit.edu/) and described in Table S2. The cycling conditions were at 95 °C for 4 min, then 94 °C for 0.5 min, 60 °C for 0.5 min, 72 °C for 1.2 min for 35 cycles, and at 72 °C for 7 min. PCR products were separated on a 2% (wt/wt) agarose gel. Bands of the expected size for the transcript were extracted by using a gel extraction kit (Qiagen). Direct sequencing for PCR products was performed at Operon Biotechnologies.

Immunohistochemistry and Histology. At least ten 7-μm cryosections were cut at 100-μm intervals from the quadriceps, the TA, the gastrocnemius, the triceps brachii, the diaphragm, and the heart muscles. The serial sections were stained with antidystrophin antibody such as monoclonal mouse antibody MANEX46B against exon 46, monoclonal mouse antibody MANEX50 against exon 50, and polyclonal rabbit antibody P7 against exon 57 (provided by Qi-Long Lu, Carolinas Medical Center, Charlotte, NC) anti–α-sarcoglycan monoclonal mouse antibody (Novocastra Laboratories), anti–β-dystroglycan monoclonal mouse antibody (Novocastra Laboratories), anti–anti-alpha1-syntrophin polyclonal rabbit antibody (Abcam), and antineuronal nitric oxide synthase polyclonal rabbit antibody (Zymed). Alexa 568 (Invitrogen) was used as a secondary antibody. The maximum number of dystrophin-positive fibers in one section of TA was counted under a BZ-9000 fluorescence microscope (Keyence). H&E staining was performed using Harris H&E.

Western Blotting Analysis. Western blot analysis was performed as previously described (7). Two to 20 μg of protein from the TA muscle of a WT mouse as a positive control, 20 μg of protein from the TA muscle of untreated *mdx52* as a negative control, and 20 μg of protein from the muscles of treated *mdx52* mice were loaded onto a 5–15% (wt/vol) XV Pantera gel (DRC). The samples were transferred onto an Immobilon PVDF membrane (Millipore) by semidry blotting at 5 mA/mm^2 for 1 h. The membrane was then incubated with the C-terminal monoclonal antibody DYS2 (Novocastra Laboratories), anti–α-sarcoglycan monoclonal mouse antibody (Novocastra Laboratories), anti–β-dystroglycan monoclonal mouse antibody (Novocastra Laboratories), anti–anti-alpha1-syntrophin polyclonal rabbit antibody (Abcam), and antineuronal nitric oxide synthase polyclonal rabbit antibody (Zymed) at room temperature for 1 h. Anti–α-tubulin (Abcam) was used as loading controls. The intensity of the bands obtained from the treated *mdx52* muscles was analyzed using ImageJ software (http://rsb.info.nih.gov/ij/) and compared with that from normal WT muscles.

Blood Analysis and Muscle Functional Testing. The blood analysis, grip strength, treadmill, and rotarod tests of the mice were performed as previously described (7).

Statistical Analysis. Statistical differences were assessed by one-way analysis of variance with differences among the groups assessed by a Tukey comparison, or χ^2 test. All data are reported as mean values ± SD or ± SEM. The level of significance was set at $P < 0.05$.

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