

a QuantiTect Reverse Transcription kit (Qiagen) according to the manufacturer's instructions. The cDNA product (1  $\mu$ L) was then used as the template for PCR in a 20- $\mu$ L reaction with 0.10  $\mu$ L of Ex Taq Hot Start Version (Takara). The reaction mixture composed of 10 $\times$  PCR buffer (Roche), 10 mM of each dNTP (Qiagen), and 10  $\mu$ M 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  $^{\circ}$ C for 4 min, then 94  $^{\circ}$ C for 0.5 min, 60  $^{\circ}$ C for 0.5 min, 72  $^{\circ}$ C for 1.2 min for 35 cycles, and at 72  $^{\circ}$ 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- $\mu$ m cryosections were cut at 100- $\mu$ m 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- $\alpha$ -sarcoglycan monoclonal mouse antibody (Novocastra Laboratories), anti- $\beta$ -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  $\mu$ g of protein from the TA muscle of a WT mouse as a positive control, 20  $\mu$ g of protein from the TA muscle of untreated *mdx52* as a negative control, and 20  $\mu$ g 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<sup>2</sup> for 1 h. The membrane was then incubated with the C-terminal monoclonal antibody DYS2 (Novocastra Laboratories), anti- $\alpha$ -sarcoglycan monoclonal mouse antibody (Novocastra Laboratories), anti- $\beta$ -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- $\alpha$ -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  $\chi^2$  test. All data are reported as mean values  $\pm$  SD or  $\pm$  SEM. The level of significance was set at  $P < 0.05$ .

**ACKNOWLEDGMENTS.** This work was supported by Intramural Research Grant (22-5) for Neurological and Psychiatric Disorders of National Center of Neurology and Psychiatry (NCNP); Health and Labour Sciences Research Grants for Translation Research (H21-Translational Research-011); Health and Labour Sciences Research Grants for Translation Research (H21-Clinical Research-015); Comprehensive Research on Disability Health and Welfare (H23-Neuromuscular Disease-005) from the Ministry of Health, Labour, and Welfare of Japan; Foundation to Eradicate Duchenne; US Department of Defense (W81XVH-09-1-0599); the National Institutes of Health (1P50AR060836, 5T32AR056993, U54HD071601, R24HD050846, and K26OD011171); Muscular Dystrophy Association; University of Alberta; The Friends of Garrett Cumming Research; HM Toupin Neurological Science Research; and Muscular Dystrophy Canada.

- Duchenne (1867) The pathology of paralysis with muscular degeneration (Paralysis Myosclerotique), or paralysis with apparent hypertrophy. *British Medical Journal* 2: 541–542.
- Hoffman EP, Brown RH, Jr., Kunkel LM (1987) Dystrophin: The protein product of the Duchenne muscular dystrophy locus. *Cell* 51:919–928.
- Koenig M, et al. (1989) The molecular basis for Duchenne versus Becker muscular dystrophy: Correlation of severity with type of deletion. *Am J Hum Genet* 45:498–506.
- Yokota T, et al. (2009) A renaissance for antisense oligonucleotide drugs in neurology: Exon skipping breaks new ground. *Arch Neurol* 66:32–38.
- Yokota T, et al. (2009) Efficacy of systemic morpholino exon-skipping in Duchenne dystrophy dogs. *Ann Neurol* 65:667–676.
- Lu QL, et al. (2005) Systemic delivery of antisense oligonucleotide restores dystrophin expression in body-wide skeletal muscles. *Proc Natl Acad Sci USA* 102: 198–203.
- Aoki Y, et al. (2010) In-frame dystrophin following exon 51-skipping improves muscle pathology and function in the exon 52-deficient *mdx* mouse. *Mol Ther* 18:1995–2005.
- Goemans NM, et al. (2011) Systemic administration of PRO051 in Duchenne's muscular dystrophy. *N Engl J Med* 364:1513–1522.
- Cirak S, et al. (2011) Exon skipping and dystrophin restoration in patients with Duchenne muscular dystrophy after systemic phosphorodiamidate morpholino oligomer treatment: An open-label, phase 2, dose-escalation study. *Lancet* 378:595–605.
- Hoffman EP, et al. (2011) Restoring dystrophin expression in duchenne muscular dystrophy muscle progress in exon skipping and stop codon read through. *Am J Pathol* 179:12–22.
- Yokota T, Duddy W, Partridge T (2007) Optimizing exon skipping therapies for DMD. *Acta Myol* 26:179–184.
- Nakamura A, et al. (2008) Follow-up of three patients with a large in-frame deletion of exons 45–55 in the Duchenne muscular dystrophy (DMD) gene. *J Clin Neurosci* 15: 757–763.
- Bérout C, et al. (2007) Multiexon skipping leading to an artificial DMD protein lacking amino acids from exons 45 through 55 could rescue up to 63% of patients with Duchenne muscular dystrophy. *Hum Mutat* 28:196–202.
- Aartsma-Rus A, Janson AA, van Ommen GJ, van Deutekom JC (2007) Antisense-induced exon skipping for duplications in Duchenne muscular dystrophy. *BMC Med Genet* 8:43.
- McCloy G, Moulton HM, Iversen PL, Fletcher S, Wilton SD (2006) Antisense oligonucleotide-induced exon skipping restores dystrophin expression in vitro in a canine model of DMD. *Gene Ther* 13:1373–1381.
- van Vliet L, de Winter CL, van Deutekom JC, van Ommen GJ, Aartsma-Rus A (2008) Assessment of the feasibility of exon 45–55 multiexon skipping for Duchenne muscular dystrophy. *BMC Med Genet* 9:105.
- Aartsma-Rus A, et al. (2009) Theoretic applicability of antisense-mediated exon skipping for Duchenne muscular dystrophy mutations. *Hum Mutat* 30:293–299.
- Nakamura A, Takeda S (2011) Exon-skipping therapy for Duchenne muscular dystrophy. *Lancet* 378:546–547.
- Ferreiro V, et al. (2009) Asymptomatic Becker muscular dystrophy in a family with a multiexon deletion. *Muscle Nerve* 39:239–243.
- Lai Y, et al. (2009) Dystrophins carrying spectrin-like repeats 16 and 17 anchor nNOS to the sarcolemma and enhance exercise performance in a mouse model of muscular dystrophy. *J Clin Invest* 119:624–635.
- Kameya S, et al. (1999) alpha1-syntrophin gene disruption results in the absence of neuronal-type nitric-oxide synthase at the sarcolemma but does not induce muscle degeneration. *J Biol Chem* 274:2193–2200.
- Anthony K, et al. (2011) Dystrophin quantification and clinical correlations in Becker muscular dystrophy: Implications for clinical trials. *Brain* 134:3547–3559.
- Cartegni L, Wang J, Zhu Z, Zhang MQ, Krainer AR (2003) ESEfinder: A web resource to identify exonic splicing enhancers. *Nucleic Acids Res* 31:3568–3571.
- Desmet FO, et al. (2009) Human Splicing Finder: An online bioinformatics tool to predict splicing signals. *Nucleic Acids Res* 37:e67.
- Owczarzy R, et al. (2008) DT SciTools: A suite for analysis and design of nucleic acid oligomers. *Nucleic Acids Res* Jul 36:W163–W169.
- Araki E, et al. (1997) Targeted disruption of exon 52 in the mouse dystrophin gene induced muscle degeneration similar to that observed in Duchenne muscular dystrophy. *Biochem Biophys Res Commun* 238:492–497.
- Summerton J, Weller D (1997) Morpholino antisense oligomers: Design, preparation, and properties. *Antisense Nucleic Acid Drug Dev* 7:187–195.
- Morcós PA, Li Y, Jiang S (2008) Vivo-Morpholinos: A non-peptide transporter delivers Morpholinos into a wide array of mouse tissues. *Biotechniques* 45:613–614, 616, 618 passim.
- Morgan JE, et al. (1994) Myogenic cell lines derived from transgenic mice carrying a thermolabile T antigen: A model system for the derivation of tissue-specific and mutation-specific cell lines. *Dev Biol* 162:486–498.
- Saito T, et al. (2010) Antisense PMO found in dystrophic dog model was effective in cells from exon 7-deleted DMD patient. *PLoS ONE* 5:e12239.

