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Targeted Disruption of Exon 52 in the Mouse Dystrophin Gene Induced Muscle Degeneration Similar to That Observed in Duchenne Muscular Dystrophy

Eiichi Araki,* Kenji Nakamura,† Kazuki Nakao,† Shuhei Kameya,‡ Osamu Kobayashi,§ Ikuya Nonaka,§ Takuro Kobayashi,* and Motoya Katsuki†

**Department of Neurology, Neurological Institute, Faculty of Medicine, Kyushu University, Fukuoka 812, Japan; †Department of DNA Biology and Embryo Engineering, Institute of Medical Science, University of Tokyo, Tokyo 108, Japan; and ‡Department of Molecular Genetics and §Department of Ultrastructural Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Tokyo 187, Japan*

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Duchenne muscular dystrophy (DMD) is a degenerative disorder of the skeletal muscle in human and is caused by mutations in the dystrophin gene. The *mdx* mouse is a spontaneous mutant and an animal model for DMD. It has a point mutation in exon 23 of the dystrophin gene that eliminates the expression of dystrophin. However, this mutation does not disrupt the expression of four other shorter isoforms that are also expressed from the dystrophin gene through differential promoter usage. We generated another mutant mouse by gene targeting. Exon 52 of the dystrophin gene was disrupted, because the deletion of this exon is known to result in the DMD phenotype in human. In this mouse (*mdx52*), Dp140 and Dp260, shorter dystrophin isoforms, were absent in addition to dystrophin. The skeletal muscles were hypertrophic and the histology exhibited variations in the fiber size with a necrotic and regenerating process. This mouse is thus considered to represent another model for DMD.

Duchenne muscular dystrophy (DMD) is a lethal X-linked degenerative disorder of the muscle. It is caused by mutations within the dystrophin gene, resulting in a lack of dystrophin (1). Dystrophin, 427kD protein, consists of four distinct domains: an N-terminal actin binding domain, a large spectrin-like rod domain, a cysteine-rich domain, and a unique carboxy-terminal domain (2). It is expressed primarily in the muscle and the brain (3). In muscle, it is located under the sarcolemma and is supposed to link the intracellular cytoskeleton and sarcolemmal proteins (4-7). The absence of dystrophin destabilizes the membrane and leads to muscle cell necrosis (8,9). However, the precise mechanism for this process remains unclear. Besides dystrophin, several shorter isoforms are also generated from the dystrophin gene through differential promoter usage (10-13). The mutations within the dystrophin gene affect the expression of these shorter isoforms in some cases (14,15). The deficiency of these isoforms may thus modify the DMD phenotypes.

The *mdx* mouse is a spontaneous mutant and an animal model for DMD. It contains a point mutation in exon 23 at the 5′ end of the dystrophin gene that eliminates the expression of dystrophin (16). The muscle pathology of this mouse includes active fiber necrosis, cellular infiltration, a wide range of fiber size and numerous centrally nucleated regenerating fibers. However, in contrast to DMD, fibrosis and fat tissue replacement are not prominent and no muscle fiber loss is observed, i.e. the regenerating process well compensates fiber necrosis. Moreover, the *mdx* mouse does not show any overt signs of muscle weakness, which is common in DMD patients (17-19). The $mdx^{2cv\text{-}5cv}$ are other mutant mice generated by chemical mutagenesis using N-ethylnitrosourea (20). The mdx^{3cv} has a mutation at the 3′ end of the dystrophin gene, thus resulting in the deficiency of shorter dystrophin isoforms (21). It shows an abnormal bleeding phenotype with a decreased neonatal survival that is different from the *mdx* mouse.

The mutations of these mice are generated haphazardly and they do not exactly correspond to DMD. To induce the same mutation as that seen in one DMD patient, we used the gene targeting method. The exon 52 of the dystrophin gene was disrupted, because the deletion of this exon results in the DMD phenotype in human. In this mouse, shorter isoforms were absent in addition to dystrophin as well. The skeletal muscle showed macroscopic hypertrophy and the histology displayed necrosis and regeneration of muscles. It was therefore considered to represent another model mouse for DMD.

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MATERIALS AND METHODS

Construction of the Targeting Vector

The mouse dystrophin gene was isolated from a 129/SvJ genomic library (Stratagene) using exon 52 of the mouse dystrophin gene as a probe. To construct the targeting vector, an 8.5kb *Eco*RV genomic fragment was cloned into a pBluescript (Stratagene). Then the 2.5 kb *Hinc*II fragment containing exon 52 was replaced with the 1.2 kb neomycine gene derived from pMC1*neo*polyA (Stratagene), thus resulting in the deletion of the exon 52. A 1.9 kb herpes simplex virus thymidine kinase gene fragment was attached to the 3′ end of the dystrophin-neomycin fragment for negative selection.

Gene Targeting and the Generation of Mutant Mice

The CCE embryonic stem (ES) cell line (a kind gift of Dr. E. Robertson, Harvard University) was cultured essentially as previously described (22). 3-10 $\times$ 10^7 ES cells were transfected with 50μg of the linearized targeting vector DNA by electroporation at a setting of 500 μF capacitance, 270 V/1.8 mm (BTX Inc, ECM 6000). G418 (250 μg/ml; Sigma) and the pyrimidine analog GANC (5μM; a gift of Nihon Syntex) were added to the medium for selection 24-48 hr after the transfection. ES cell clones with a targeted disruption of the dystrophin gene were identified by Southern blot analysis of *Bam*HI-digested genomic DNA. The 5′ and 3′ flanking regions and the neomycin gene were used as hybridization probes. Southern blottings were analyzed using a Fujix Bioimage Analyzer BAS2000. Chimeric mice were generated from C57BL/6J blastocysts injected with dystrophin disrupted ES cells essentially as described (23). Female mice heterozygous for the mutation (X^{mdx52}/X) were obtained by mating male chimeric mice to C57BL/6J females. They were then further intercrossed to obtain hemizygous mutant male mice (X^{mdx52}/Y). The genotypes of the mice were determined by Southern blot analysis of the DNA prepared from the segments of the tails.

Western Blot Analyses

Total cellular protein was extracted from fresh tissue specimens as described (24). The protein concentrations were determined by the Coomasie Plus calorimetric assay (Pierce). A 50μg of total protein from the brain, skeletal muscle and sciatic nerve and a 40μg from the retina were electrophoresed on 6% SDS polyacrylamide gels and blotted on an immobilon-P membrane (Milipore). The primary antibodies were used at the following dilutions: a polyclonal anti-dystrophin antibody, P34a (antigen amino acid number: 3495-3544) (25), 1:2000; monoclonal anti-dystrophin antibodies, Dys-1, Dys-2 (Novocastra) and MAB1694 (Chemicon), 1:100. The membranes were incubated with each of these antibodies and peroxidase-labeled anti-IgG was used as the second antibody. The development was performed according to the protocol of the ECL detection kit (Amersham).

Histology and Immunohistochemistry

The organs of the mutant mice and wild mice were excised postmortem and then were rapidly frozen in liquid nitrogen cooled isopentane. 10μm Frozen sections were prepared and stained with haematoxylin and eosin. For immunostaining, the sections were incubated with a polyclonal antibody for dystrophin, 60kD, at a dilution of 1:500 and then were stained using fluorescein isothiocyanate (FITC)-labeled anti-rabbit IgG as described previously (26).

RESULTS

Generation of the Exon 52 Disrupted Mice

We cloned the mouse dystrophin gene containing exon 52 to construct the targeting vector. The part of exon 52 was replaced with the neomycin resistance gene. The targeting vector contained 6.2kb of homologous sequences from the dystrophin gene and the herpes simplex virus thymidine kinase gene at the 3′ end of the homologous region (Fig.1A). After the targeting vector was electroporated into ES cells, we isolated a total of 64 ES cell clones which were selected by both G418 and GANC resistance. As shown in Figure 1A, a 14-kb *Bam*HI fragment covers the exon 52 of the dystrophin gene. After the introduction of the targeting vector, the targeted allele contains another *Bam*HI site derived from the *pMC1neo*polyA. Therefore, Southern hybridization with the 5′ or 3′ probe gives rise to a 7.6kb or 4.7kb fragment from the targeted allele, respectively (Fig.1A). With these probes, one clone showed the pattern expected from the homologous recombination. The Southern blot analysis with a neomycin probe, an internal probe, showed that there was no random integration of the targeting sequence. We generated chimeras by injecting this clone into the blastocysts of the C57BL/6J mouse. Chimeric males were then crossed with the C57BL/6J females. The germline transmission of the disrupted dystrophin gene was confirmed by Southern blot analysis (Figure 1B). Heterozygous female mice (X^{mdx52}/X) were further crossed with C57BL/6J males to finally obtain the exon 52 disrupted male mice (X^{mdx52}/Y). They were born at a ratio of 5/24 according to the laws of Mendelian transmission. The mutant mice did not show either skeletal muscle weakness or behavioral changes on our observations up to the age of 18 months old.

Western Blot Analyses

The Dys-2 or MAB1694 antibody, which recognizes the final 17 amino acids of dystrophin, detected 427kD, 260 kD, 140kD, 116kD, and 71kD bands in the tissue specimens of the wild mouse as previously reported (Figure 2) (11,12,21,25). The 427kD band, which represents dystrophin, was not present in the mutant mouse. The Dys-1 antibody recognizes the domain situated to the N-terminus from the exon 52 encoded region. It also did not detect any band in mutant mouse, thus indicating that there was no truncated N-terminal fragment of dystrophin. The 71kD non-muscle isoform and 116kD peripheral nerve isoform were present in the mutant mouse. The expression of the 140kD isoform was disrupted in the brain, which was also confirmed with P34a which detects 140kD isoform (25). The 260kD retinal isoform was not detected (Fig.2).

Histological Analyses

We examined the tissue specimens of 4 and 4.5 month old mutant mice, respectively. Macroscopically, all dissected muscles were whitish and hypertrophic, especially, tibialis anterior and extensor digitorum longus muscles which were about one and

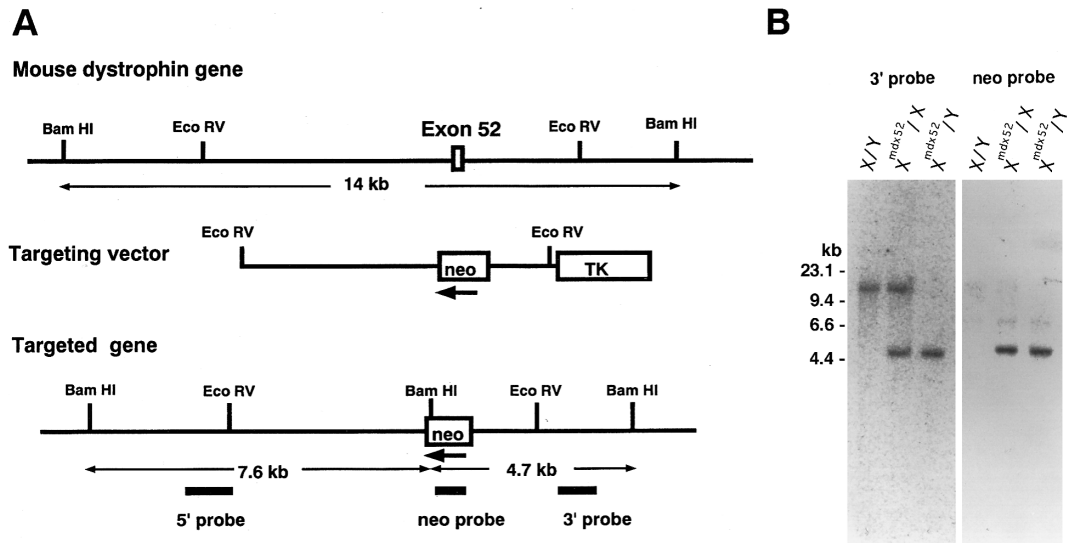

FIG. 1. (**A**) The targeted disruption of the dystrophin gene. The homologous recombination resulted in the replacement of the exon 52 of the dystrophin gene with the neomycin resistance gene (*neo*). The herpes simplex virus thymidine kinase gene (*TK*) was attached to the 3′ end of the targeting vector for negative selection. The probes used for Southern blot analysis are shown. (**B**) Southern blot analysis of genomic DNAs from the tails of wild-type male (X/Y), heterozygous mutant female (X^{mdx52}/X) and hemizygous mutant male (X^{mdx52}/Y) mice. *Bam*HI-digested DNAs were hybridized with the probes.

a half time as large as age matched wild mouse muscles (Figure 3A). There were striking morphologic changes in the diaphragm and all limb muscles on light microscopy. Among them, the diaphragm was most severely affected with an increase in connective tissue (Figure 3B). The soleus (red) and extensor digitorum longus and tibialis anterior (white) muscles were almost equally affected; there was also marked variation in the fiber diameter with scattered to grouped necrotic fibers with phagocytosis (Figure 3C). More than 90% of the muscle fibers had centrally placed nuclei. Dystrophin was completely absent along the muscle surface membrane except for a few "revertant" fibers in mutant mice on immunostaining (Figure 3E). These microscopic findings were similar to those of *mdx* mice. No pathological changes were observed in the heart, brain or stomach, even though dystrophin was negative in these tissues.

DISCUSSION

The dystrophin gene is a complex and extremely large gene, covering more than 2.5 megabases and containing 79 exons. Besides dystrophin, other shorter isoforms are also expressed in various tissues through the

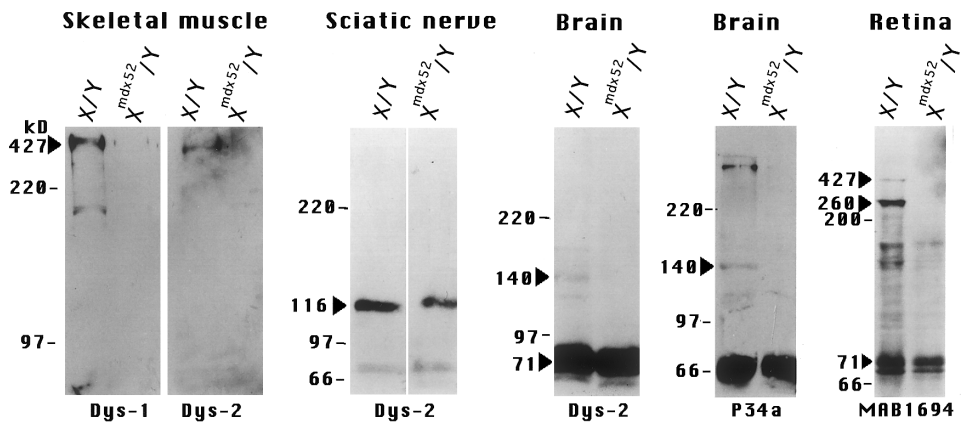

FIG. 2. Western blot analysis of mouse tissue specimens with anti-dystrophin antibodies, Dys-1, Dys-2, P34a, and MAB1694. In hemizygous mutant male (X^{mdx52}/Y), 427kD, 260kD and140kD isoforms of dystrophin are absent but 116kD and 71kD isoforms are present. Molecular weights are indicated on the left.

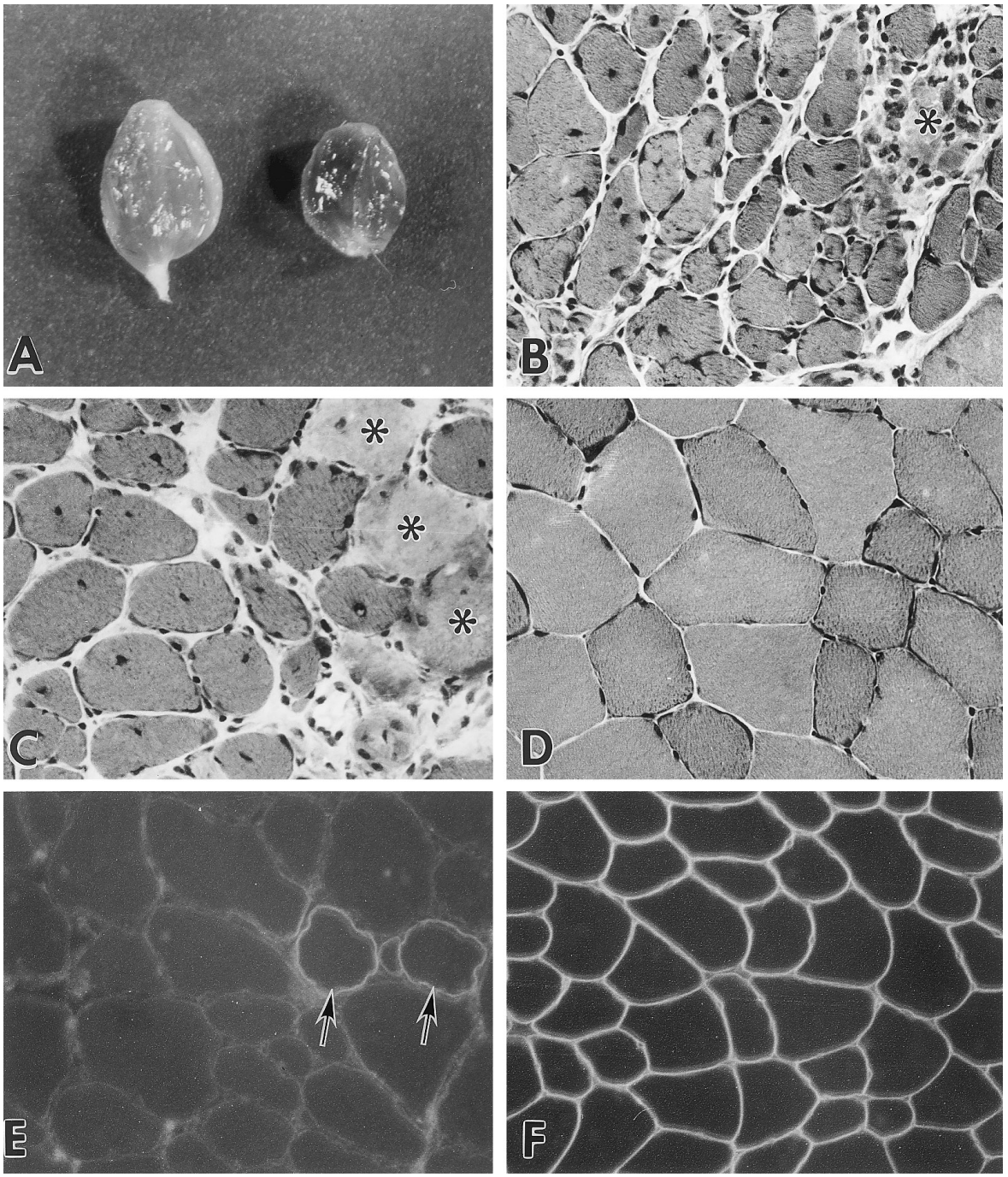

FIG. 3. The skeletal muscle from a hemizygous mutant male (X^{mdx52}/Y) at 4.5 months of age. (**A**) Dissected tibialis anterior muscles. The mutant mouse muscle (left) is paler and hypertrophic than that of age matched wild type mouse muscle (right). (**B**) The diaphragm which seemed most affected showing a marked variation in fiber size, necrotic fibers (asterisk), many regenerating fibers with centrally placed nuclei and interstitial fibrosis. (**C**) In the tibialis anterior muscle from a mutant mouse, a variation in fiber size with aggregated necrotic fibers (asterisks) is also observed. Almost all of the fibers have centrally placed nuclei. Interstitial fibrosis is minimal. (**D**) Control tibialis anterior muscle. (**E**) The dystrophin is negative except for a few revertant fibers (arrows) in all mutant mouse muscles. (**F**) Control muscle with positive dystrophin reactivity along the muscle surface membrane. B–D, heamatoxylin and eosin; E and F, fluorescent immunostaining with a polyclonal anti-dystrophin antibody (60kD). B–F, ×280.

use of different promoters on its gene. A gene product of the 71 kD (Dp71) protein is regulated by a promoter situated in the intron 62 and is expressed in a wide range of tissues (10). The peripheral nerve isoform, Dp116, is regulated by another promoter located in the intron 55 (11). The Dp140 is expressed in the central

nervous system and its promoter is thought to be in the intron 44 (13). The retinal isoform, Dp 260, is transcribed from a promoter upstream to the exon 30 (12). Therefore, the position of the mutation in the dystrophin gene affects not only the expression of dystrophin but also the expression of these isoforms and thus may cause the difference in phenotypes. To generate a model mouse, we induced the mutation at exon 52, which corresponds to the mutation found in human DMD patient. The exon 52 consists of 118 bases and it encodes a C-terminal part of the spectrin-like rod domain (27). The disruption of the exon 52 in the mouse caused an aberrant splicing between exon 51 and 53 and then produced a frameshift mRNA resulting in the deficiency of dystrophin (data not shown). It also eliminated the expression of the Dp140 and Dp260, but did not affect the expression of the Dp71 and Dp116. Our mutant mouse is therefore unique regarding the expression of dystrophin isoforms. In the *mdx* mouse, the expression of shorter isoforms is preserved because the *mdx* mutation is upstream from the promoters of these isoforms. On the other hand, the mdx^{3cv} mouse has a mutation which disrupts the production of all shorter isoforms (21). The *mdx52* is thus considered to be an important model for studying the normal functions of Dp260 and Dp 140 and their roles in the disease phenotype.

Dp260 is localized on the outer plexiform layer of the retina and its deficiency is supposed to cause abnormal electroretinogram seen in some DMD patients (12). The effect of this deficiency on the retina has also been investigated in *mdx52* (S. Kameya et al, in submission).

The function of the Dp140 in the brain remains unclear. Some DMD patients show a mild cognitive impairment and several studies report that this finding correlates the deletions in the exon 45-52 region (13,28). Such deletions particularly disrupt the expression of the Dp140, because it is transcribed from the promoter that is probably located in the intron 44. Therefore, its deficiency may contribute to the cognitive impairment in DMD patients. In *mdx52*, the deficiency of Dp 140 did not have any definite effect on the phenotype and also did not cause any pathological changes in the brain. The behavior looked normal. Further examinations are necessary, however, to reveal the effect of this deficiency on the brain.

Our mutant mice did not have any apparent muscle weakness by 1 year of age. They showed muscle hypertrophy in their limb muscles similar to that seen in DMD patients. The overall muscle pathology was "dystrophic" showing muscle fiber necrosis, regeneration and variations in fiber size. However, the fibrosis and fat infiltration, commonly seen in DMD muscles, were minimal. In human, the deleted exon 52 induces a typical DMD phenotype of muscle weakness from early infancy. This difference thus suggests the species response to the same mutation of the dystrophin gene. The skeletal muscle pathology of *mdx52* was similar to that of the *mdx* mouse rather than the dy^{+}/dy^{+} mouse (29). The diaphragm of *mdx52* also exhibited severe degeneration as seen in the *mdx* and DMD diaphragm (30). On the other hand, the muscle hypertrophy of the limb muscle seen in *mdx52* is not observed in *mdx* mice. The muscles of *mdx52* may be more sensitive to the stress induced by muscle contraction, which results in compensated muscle hypertrophy. It has recently been found that fibrosis and fatty deposits appear in the limb muscle of old *mdx* mice (31,32). Therefore, a longer follow-up study is necessary to clarify whether or not *mdx52* develop progressive muscle weakness and advanced dystrophic changes with age.

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