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ABSTRACT

Duchenne muscular dystrophy (DMD) is a fatal progressive muscle wasting disease caused by the defects in the dystrophin gene. The huge cDNA size of dystrophin, 14kb, limits the use of many kinds of virus-based vectors except helper-dependent adenovirus vector (HDAdv) which has a larger cloning capacity of up to 37kb enough to carry the full-length dystorphin cDNA. Some therapeutic studies with HDAdv have been performed in mdx mice that are deficient in dystrophin but display extremely mild clinical phenotype. It is conceivable that a more reliable therapeutic predication should have been achieved with disease models that recapitulate the clinical picture seen in DMD patients. Utrophin/dystrophin double knockout mice, dko mice, exhibit the dystrophic changes in their muscles besides clinical phenotype similar to DMD patients. In this study, we evaluated the therapeutic effect of the full-length dystrophin gene transfer mediated by HDAdv to dko mice. We constructed HDAdv carrying the myc-tagged murine full-length dystrophin (HDAdv-mFLmyc-dys) and injected it to each neonatal dko mouse with a total of 40μl viral vector into the following muscle groups: lower limbs(10μl/limb), upper limbs(5μl/limb) and trunks(5μl/side). Eight weeks after, the histological and physical examinations were performed. We achieved full-length dystrophin expression and prevention of the dystrophic pathology in injected dko mice. Furthermore, motor performance in injected dko mice could be improved and their lifespan became longer. Therapeutic gene transfer with HDAdv to the proximal muscles may ameliorate DMD patients.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is an X-linked recessive disorder that occurs in ~1 in 3500 live male births [1]. DMD patients are affected by severe, progressive muscle wasting and weakness and then become confined to wheelchairs in their early teens and die in their early twenties due to respiratory or cardiac failure. Despite the identification of the defect in the dystrophin gene that is responsible for DMD [2], no therapy for DMD has been successful to date.

Mdx mice, which are deficient in dystrophin but display extremely mild clinical phenotypes, have been used most frequently as DMD model mice. But it is conceivable that a more reliable therapeutic predication should have been achieved with disease models that recapitulate the clinical picture seen in DMD patients. Utrophin/dystrophin double knockout mice (dko mice) were generated to investigate the interrelationship between utrophin and dystrophin in muscles [3][4][5][6]. Dko mice show slack posture and reduced weight, lack of mobility, abnormal breathing patterns, and abnormal field behavior by 4 to 6 weeks of age, followed by progressive muscle weakness with marked kyphosis, waddling gait with joint contractures, and death by 20 weeks [3]. Therefore, dko mice may recapitulate the clinical phenotype seen in DMD, and a reliable therapeutic prediction may be achieved by using these mice as animal models.

The virus-mediated gene delivery of the dystrophin gene could be one of the effective therapeutic approaches for DMD. The helper-dependent adenovirus vector (HDAdv) has a large cloning capacity, up to 37 kb DNA, which is sufficient to package the full-length dystrophin cDNA, 14kb in length [7][8][9][10][11]. We, and others, reported the successful transduction of the full-length dystrophin gene into *mdx* mice using the HDAdv [12][13][14].

Some reports have shown that micro- or truncated versions of dystrophin recovered the dystrophic phenotype in *mdx* mice [15][16][17][18][19], however, the results of a study using dystrophic dogs that received mesoangioblast stem cells suggest that the replacement of microdystrophin produces a modest functional rescue, while alternative full-length dystrophin might be more effective [20]. We speculate that because of dystrophin' s huge size and complex structure, all of its functions have not been disclosed. Thus, we believe that more desirable approaches to replace the full-length dystrophin cDNA are needed.

The purpose of this study is to examine whether gene transfer by HDAdv to the limited muscles works as a gene therapy for muscular dystrophy. In this study we hypothesized that we would be able to estimate the clinical efficacy of gene transfer to limited muscle, when we used the serious symptomatic model mice. Micro-dystrophin was successfully transferred to the mouse whole-body by adeno-associated virus vector (AAV) *via* blood

vessels [15]. But in the present study, in order to avoid the vector infecting to the organs that gene transduction is unnecessary, we transferred the full-length dystrophin cDNA into multiple skeletal muscles of the extremities and trunks of dko mice, and analyzed the therapeutic efficacy of this therapy by histological and physical observations.

Our results suggest the first time that by using dko mice even multiple intramuscular administrations of HDAdv carrying full-length dystrophin could restore not only the histological abnormalities but also other various symptoms: skeletal deformity, small body weight, and poor motor performance. We concluded on the basis of these results that the gene transfer by the direct intramuscular injection of HDAdv could be an effective treatment for muscular dystrophy.

RESULTS

The structure of HDAdv-myc-mFLdys and full-length dystrophin expression.

We constructed HDAdv-myc-mFLdys as shown in Figure 1. To identify dystrophin proteins delivered from HDAdv, myc-tag was inserted into the dystrophin cDNA between 6886-nt and 6887-nt in frame. According to Western blot analysis of the protein extracted from the HDAdv-infected COS7 cells, the expressed protein could be accurately detected by mouse-monoclonal anti-c-myc antibody (clone9E10) and rabbit-polyclonal anti-dystrophin antibody (dystrophin H-300). The expression of full-length dystrophin in the injected muscles was also verified by Western blotting by using dystrophin H-300. The expressed protein migrated to the position of the endogenous dystrophin extracted from wild-type muscle, whose molecular weight was 427 kDa (Supporting Information).

Dystrophin transduction could vastly improve the dystrophic pathology in vector-injected muscles of dko mice.

In this study, we injected HDAdv-mFLmyc-dys into following muscles; triceps branchii, quadriceps femoris, tibialis anterior (TA) and paraspinal muscles, bilaterally at the age of seven days and we examined muscle pathology at eight weeks after injection. Because the proximal skeletal muscles are more severely affected in DMD, we evaluated the efficacy within these muscles.

When we evaluated the pathological or physical changes, we selected TA muscle representatively, because it was the simplest to be identified and removed. As shown in Figure 2a, dystrophin was clearly observable in injected dko TA muscles and localized beneath the sarcolemma similar to wild-type muscle. Immunohistochemical staining for myc-tag undoubtedly indicated that the detected dystrophin was derived from the

transferred gene. Also, we found the delivered dystrophin in other injected muscles (data not shown). We found focal and patchy dystrophin expression in injected dko muscles. Quantitative analysis of the percentage of dystrophin-positive area in injected dko muscle averaged 25.3 ± 6.7 % (n=3), even 8 weeks after injection. The dystrophin positive fibers were mostly observed in injected muscles, and slightly observed in their adjacent muscles. We examined the late fate of transgenic dystrophin with *mdx* mouse which live longer than dko mice. We found transgenic dystrophin in injected *mdx* muscles 13 months after the injection (data not shown).

Next we asked whether dystrophin expression could restore the expression of β-dystroglycan (β-DG), α-sarcoglycan (α-SG), dystrophin associated proteins (DAPs), and neuronal nitric oxide (nNOS). Interestingly, the immunohistochemical stainings for each antigen demonstrated that both β-DG and α-SG were also present at the sarcolemma of the delivered dystrophin positive fibers in the injected dko muscles. More significantly, nNOS expression was also restored to the sarcolemma in the injected dko muscle, similar to wild-type muscle.

Then we counted the percentage of fibers with central nuclei, which reflects the abnormal cycles of fiber degeneration and regeneration. In delivered dystrophin positive fibers of the injected dko group, we found a marked reduction in the number of centrally nucleated myofibers compared with that of the uninjected dko group ($P < 0.03$) (Figure 2b-i).

Cellular immune response were not increased by vector-injection

To estimate the cellular immune response triggered by the HDAdv-mFLmyc-dys, the injected muscles were stained for CD4 and CD8, eight weeks after injection. The number of CD4-positive cells in wild-type group was 1.0 ± 1.2 (n=10), the injected dko group was 4.1 ± 2.2 (n=15), and the uninjected dko group was 13.0 ± 4.0 (n=15). That of CD8-positive cells in wild-type group was 1.3 ± 1.8 (n=15), the injected dko group was 5.7 ± 2.7 (n=15), and the uninjected dko group was 7.5 ± 3.2 (n=15). We found more immune cells in the injected dko group than in wild-type group (CD4; P=0.0004, CD8; P=0.0002). However, in the injected dko group, we detected much fewer CD4-positive cells (P<0.0001) and CD8-positie cells (P=0.1548) than in the uninjected dko group (Figure 2b-ii).

Vector-injected dko mice increased body weight and lived longer.

We compared the development and the lifespan among the following three groups; injected dko mice; uninjected dko mice; wild-type mice.

The uninjected dko mice showed a small body size, severe kyphosis, muscle atrophy and joint contractures, similar to symptoms observed in DMD patients. Age-matched the

injected dko mice showed obvious improvements in these deformations (Figure 3a). Mice in figure 3a were 9 weeks old. The body weight of the injected dko mouse was 21.8 g while that of the uninjected dko mouse was 13.8 g. Because of extremely milder kyphosis and muscle atrophy in the injected dko mouse, it seemed to be much bigger than the uninjected dko mouse.

We weighed weekly the mice in the three groups (Figure 3b). The body weight curve of the injected dko group differed significantly from that of the uninjected dko group at approximately three weeks following injection. The body mass of the injected dko mice was large compared with that of the uninjected dko mice, but not comparable to the mass of wild-type mice.

Kaplan-Meier analysis (Figure 3c) exhibited the difference in the survival rate with a statistical significance between the injected dko mice and the uninjected dko mice ($P<$ 0.05), which indicated that the injected dko mice lived longer than the uninjected dko mice.

Vector-injected mice improved in motor performance.

We assessed the motor performance among the 3 groups by foot print analysis and locomotor activity at the age of nine weeks. The representative results of foot print analysis are shown in Figure 3d. The averages of the length of steps of the injected dko mice, the uninjected dko mice and wild-type mice were 37.2 ± 3.1 mm (n=6), 27.3 ± 6.5 mm (n=7) and 52.5 ± 6.7 mm (n=10), respectively. The injected dko mice walked with a significantly longer stride than that of the uninjected dko mice ($P<$ 0.01). The injected dko mice also showed an improvement in features of gait and standing, while the uninjected dko mice could not lift their own body weight, dragged their pelvis when walking, and showed a reluctance to walk (Supporting video).

We analyzed locomotor activity by using automated infrared sensors, which could detect the infrared rays radiated from a mouse isolated in a cage (Figure 3e). The number obtained from this assay demonstrated how many times mice moved. The averages of counts during 24-hour periods of the injected dko mice, the uninjected dko mice and wild-type mice were 9643 ± 5948 (n=6), 3859 ± 3189 (n= 6) and 18563 ± 7840 (n= 10), respectively. Collectively, these observations indicate that the injected dko mice significantly improved their motor activity over that of the uninjected dko mice ($P<$ 0.05).

To verify if treatment with HDAdv-mFLmyc-dys could improve the force generating of dko muscle, we measured the muscle force of TA when mice were 9 weeks old. Maximal tetanic force of the injected dko muscles was 0.394 ± 0.115 N/cm^2 (n=4), and that of the uninjected dko muscles was 0.278 ± 0.20 N/cm^2 (n=3).We found the maximal tetanic force of the injected dko muscles tended to be higher than that of the uninjected dko

muscles (*P*=0.2888).

DISCUSSION

The present treatments for DMD are palliatives for the various clinical symptoms, but can prolong the lifespan of DMD patients. Gene therapy, in contrast to the makeshift therapy conducted now, is essential treatment to compensate for primary defects in a gene. If we can prove the efficacy of full-length dystrophin cDNA transfer to the several muscles in the model mouse which resembles DMD patient displaying clinical symptoms, we can expect the efficacy of gene transfer by the direct vector injection to several muscles of a DMD patient.

We observed the expression of dystrophin in muscles of dko mice injected with HDAdv-myc-mFLdys. The average of transduction efficiency reached 25% for one-time injection in dko muscles 8 weeks after injection. The previous studies have reported that mosaic expression of full-length dystrophin at 30% level in *mdx* mice could achieve partial improvements in histopathology [21], while mosaic expression of truncated dystrophin at 20% level in *mdx* mice could not achieve morphological improvements [16]. Our results, that even 25% of full-length dystrophin expression in dko mice can improve physiologically as well as histopathologically, suggest full-length dystrophin would more efficiently prevent severe dystrophic pathology and functional deficiency. Moreover, we found dystrophin expression not only in the injected muscles but also their adjacent muscles. Weak barrier between muscles of neonatal dko mice and the pressure of injection may allow HDAdv-mFLmyc-dys to penetrate beyond muscles. This fact will help to explain the general phenotypic improvements by intramuscular administration of HDAdv-mFLmyc-dys.

The previous study reported that the intramuscular deliver of the high-capacity adenoviral vector encoding full-length murine dystrophin resulted in stable expression of recombinant dystrophin for 5 months in dystrophin-deficient mice treated as neonates [22]. We confirmed HDAdv-mFLmyc-dys had kept expressing for 13 months long with one-time injection in *mdx* mouse. If we accomplish higher efficiency and longer period of full-length dystrophin expression with HDAdv, we could make much better results.

In addition, the data of DAPs and nNOS suggests that the delivered dystrophin restored the expression of DAPs and nNOS. In DMD patients and mdx mice, nNOS is absent from the sarcolemma [23], and nNOS-related defects may contribute to the pathophysiology of dystrophinopathy [24][25][26]. A previous study examining the effects of truncated microdystrophin mediated with rAAV6 showed that microdystrophin was not sufficient to restore sarcolemmal nNOS expression in tranduced myofibers [27].

Our results suggest that the recovery of nNOS with full-length dystrophin may reflect other functions of dystrophin.

The centrally nucleated myofibers seen in DMD indicate an active regenerating process, while normal muscle fibers are uniform in size and have peripheral nuclei. Therefore, we assessed the improvement in pathology by the number of centrally nucleated fibers. For treated dko mice, the assessment was performed in dystrophin positive fibers to estimate the therapeutic efficacy. Treated dko mice showed a significant decrease in the number of centrally nucleated fibers compared to those of untreated dko mice. This observation suggests that the transferred dystrophin prevents dystrophic changes, normalizes the myofibers, and remedies the primary causes of DMD.

The expression of delivered dystrophin was immunohistologically detected in 9 week-old treated dko muscles, which suggests that intramuscular administration can lead to dystrophin expression in approximately 8 weeks. Previously, we reported the ability to repeatedly inject HDAdv carrying the full-length dystrophin cDNA [12], and that repeated injections may improve the efficiency of subsequent dystrophin transfer. Repeated injections at appropriate intervals are thought to allow the delivered dystrophin to be expressed for a longer-term.

The immunological response to viral antigens and therapeutic protein expressed by the delivered gene would prevent effective treatment. We examined the cellular immune response triggered by the HDAdv-mFLmyc-dys with the number of CD4-positive and CD8-positive cells [12]. We cannot deny the immunological effect by HDAdv-mFLmyc-dys in injected dko group considering with the data of wild-type and injected dko group, but we found much fewer number of the immune cells in the injected dko muscles than in the uninjected dko muscles. Our results suggest that the vector-injection would slightly induce the cellular immune response in transduced muscles but would not aggravate the T cell activation in dystrophic muscles [28].

Treated dko mice showed obvious differences in body size and weight and both extremely milder kyphosis and muscle atrophy compared with that observed in untreated dko mice. The increased body weight in treated dko may reflect the increased muscle mass which was supposed from the histological restoration. In addition, the relatively larger body size and the improvements of kyphosis and joint contractures may allow the treated dko mice to access their food or water more easily. The lifespan of the treated dko mice was longer than that of untreated dko mice, possibly due to the improvements in eating and drinking behavior. The causes of death in DMD patients are most likely respiratory or cardiac failure and the same factors may contribute to the death of dko mice [16][17]. Although we have not transferred the dystrophin gene to muscles that

affect respiratory or cardiac function directly, the longer lifespan of treated dko mice is remarkable.

The results of our foot print analyses showed that treated dko mice had significantly longer stride lengths than that of untreated dko mice ($P < 0.01$), and showed improvement in their walking gait. The physical restorations in treated dko mice, such as increased body weight and the improvements on kyphosis and joint contractures, may also contribute to the improvements in gait and the length of steps. The locomotor activity in treated dko mice also increased over that in untreated dko mice ($P < 0.05$). Together, these results suggest that motor performance can be improved by local therapy in multiple muscles.

The maximal tetanic force of treated dko TA tended to become stronger than that of untreated dko TA. Because multiple injections may well bring the same efficacy to each injected muscle, the improvement of force generation by the several treated muscles would result in the improvement of motor performance. We speculate that the higher transduction efficiency, the greater muscle force and the better improvements of motor performance.

We successfully transferred the full-length dystrophin cDNA by local injection into multiple muscles and confirmed the efficacy of this therapy by histological and physical observations. Our results suggest that multiple intramuscular administrations of HDAdv carrying full-length dystrophin cDNA can reduce symptoms and compensate for lost functions in DMD patients. Many questions remain to be considered about the efficacy of gene transfer, the term of expression, immunological response, and the transduction to diaphragm and cardiac muscles which affect the prognosis of DMD patients. We hope various approaches to establish effective therapies for DMD patients will continue and that the results of this study will be helpful.

MATERIAL AND METHODS

Cells cultures

Cell lines used in this study are COS7 (monkey kidney cells, American Type culture Collection, Manassas, VA, USA), HEK293 (human embryonic kidney cells) and Cre-293 (HEK293 stably expressing the Cre recombinase) [7]. All cells were grown in DMEM (Dulbecco's modified Eagle's medium, Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS, USA), 50U/ml penicillin and 50U/ml streptomycin (SIGMA, St. Louis, MO, USA). The cultures were incubated at 37° C in a humidified atmosphere containing 5% CO_2.

Preparation of helper virus

We have reported detailed information concerning the helper virus, AdAsw [7]. Helper virus was constructed using the COS/TPC method [10]. First, a cosmid pAdex-ASw-lox-4.6λ was generated by ligating the 4.6-kb *Eco*RV fragment of λ-phage (nt 2087 to nt 6683) into *Swa*I-digested pAdex-ASw-lox containing two parallel *lox*P sites encompassing the adenovirus packaging signal φ. Next, 0.5 μg of the *EcoT22I*-digested E3-positive wild-type adenovirus genome covalently linked to the terminal protein and 14.5 mg of pAdex-ASw-lox-4.6λ [7] were mixed. HEK293 cells were transfected with the mixed DNA by the calcium phosphate method. Recombinant viruses were purified and propagated according to standard procedures [29].

Construction of HDAdv

The murine full-length dystrophin cDNA fragment was extracted from plasmid pCCL-DMD [30] and ligated into pCAGGS [31]. This plasmid was named pCAG-mFLdys [12]. pPN13, which has the inverted terminal repeats (ITR), a packaging signal φ, and part of the *Emx-2* gene [32] as a 13kb-stuffer, with pBluescript as a backbone, was ligated the dystrophin expressing cassette extracted from pCAG-mFLdys. We inserted a myc-tag oligonucleotide, derived from the myc protein and consisting of 10 amino acids (EQKLISEEDL) [33], into the dystrophin cDNA at a unique *SwaI* site. By undertaking these steps, we constructed pPN-myc-mFLdys. pPN-myc-mFLdys was digested at the *NotI* site, and transfected to Cre-293 cells by the calcium phosphate precipitation. Transfected Cre-293 cells were infected with the helper virus AdASw at a multiplicity of infection (m.o.i.) of 3, then propagated repeatedly. The virus was purified by CsCl density gradient and the particle titer of the purified HDAdv (particles/ml) was measured by the optical density at 260nm [34]. Finally, we obtained a titer of 5.8×10^{12} virus particles/ml HDAdv-myc-mFLdys. Helper virus contamination was less than 1%. The expression of myc-tagged dystrophin was confirmed by Western blotting analysis. Protein samples were extracted from the infected COS7 cells and the injected skeletal muscle by using cell lysis buffer. Equal amounts of protein were fractionated by SDS-PAGE. Following transfer onto a nitrocellulose membrane (Amersham Bioscience, Piscataway, NJ, USA), blots were probed with an antibody against dystrophin, rabbit polyclonal anti-dystrophin antibody, dystrophinH-300 (1:100; Santa Cruz Biotechnology, Santa Cruz CA, USA), and myc-tag, mouse monoclonal anti-c-myc antibody (mouse IgG1 isotype), clone9E10, (1:50; SIGMA, USA) followed by the relevant horseradish peroxidase-conjugated immunoglobulin. Blots were developed by using ECL reagent (Amersham Bioscience).

Animal models

All animal experiments in this study were approved by the Kumamoto University Committee on Animal Research.

We used C57BL/10 mice as a normal control. The dko mice used in this study were originally generated by Anne E. Deconinck, *et al.* [3]. Experimental dko mice were obtained by crossing heterozygous mice in utrophin locus on a *mdx* background. Genotype was determined by PCR analysis with DNA from tail biopsies. PCR analysis used three primers described by Anne E. Deconinck, *et al* [3] and reactions were carried out on genomic DNA for 30 cycles under the following conditions: 94°C, 30s; 53°C, 30s; 72°C, 25s. Mice were genotyped by six days of age.

The injection protocol

The dko mice were injected with HDAdv-myc-mFLdys into following muscles; triceps branchii, quadriceps femoris, tibialis anterior (TA) and paraspinal muscles, bilaterally, using a 30-gauge half needle on a Hamilton syringe at the age of seven days. Each muscle was injected same volume of the HDAdv-mFLmyc-dys, 5 μl, and a total of 40 μl per mouse. Prior to the injections, the mice were anesthetized by placing them on ice. Eight weeks after the injections, we performed an assessment of motor performance and a histological analysis.

Histological examination and immunostaining

After killing the mice by cervical dislocation, we removed injected muscles and froze them in isopentane pre-cooled with liquid nitrogen. Frozen muscles were sectioned at 10 μm in thickness and stained with H&E or immunofluorescence. The primary antibody for dystrophin was dystrophinH-300 (1:100; Santa Cruz Biotechnology), for the myc-tag was clone9E10 (1:50; SIGMA), for β-DG was mouse monoclonal anti-β-DG (1:50; NCL-b-DG, Novocastra, Newcastle upon Tyne, UK), for α-SG was monoclonal anti-α-SG (Adhalin) (1:100; NCL-a-SARC), and for nNOS was a polyclonal anti-nNOS C-terminus antibody, NOS1 (R-20) (1:50; Santa Cruz Biotechnology). The secondary antibody for dystrophin and nNOS was Alexa546-labeled goat anti-rabbit IgG (H+L) (1:100; Molecular Probes, Eugene, OR, USA), and for the myc-tag, β-DG, and α-SG, was Alexa488-labeled goat anti-mouse IgG (H+L) (1:1000; Molecular Probes).

The efficiency of transduction was evaluated by the proportion of dystrophin-positive area to the entire cross section of removed lower limb muscles, eight weeks after injection. The dystrophin-positive areas were determined with dystrophin H-300.

To estimate the cellular immune response in dystrophin positive area, the serial sections were stained for CD4 and CD8. For CD4 and CD8, the primary antibodies were rat monoclonal anti-mouse CD4 antibody (1:20; clone RM4-5, BD Biosciences), and rat monoclonal anti-mouse CD8 antibody (1:20; clone 53-6.7, BD Biosciences), respectively. For evaluation of cellular immune response in transduced areas, the numbers of CD4-positive and CD8-positive cells were counted in randomized views of 200-power

microscope.

Stained sections were observed using a confocal laser scanning microscope (FLUOVIEW/FV300, Olympus, Tokyo, Japan) and an optical microscope (DP70-WPCXP, Olympus, Tokyo, Japan). The histology was analyzed using computer software, WinROOF (version 5.6, MITANI CORRPORATION, Japan).

Motor performance analysis

Foot print examinations and locomotor activity analyses were performed using 9-week-old mice. For the foot print test, mice were individually placed in a plastic cage (25 cm × 7 cm × 14 cm) to walk voluntarily, and we measured the length from toe to toe. In order to quantify locomotor activity, we used an automated electronic activity counter (NS-AS01, Neuroscience, Tokyo, Japan) [35][36]. Briefly, each mouse was put in clear acrylic cage (24 cm × 17 cm × 12 cm), and their activity was measured for 24 hours using an automated activity counter placed 15cm above the cage. All mice were housed in rooms under controlled temperature (22 ± 2 °C), relative humidity (50 ± 10 %) and 12/12 light/dark cycle. Survival analysis was performed using the Kaplan-Meier analysis and comparisons were made employing the Logrank test.

Muscle force measurements

TA muscles were isolated and removed from the mice at 9 weeks of age. The TA muscles were carefully mounted in a chamber filled with oxygenated Ringer's solution (95% O_2, 5% CO_2) and maintened at 30°C. One tendon of the muscle was attached to a steel hook in the chamber, and the other was tied to the lever arm of a dual-mode servomotor system (Electronic Stimulator, NIHON KOHDEN, Tokyo, Japan) *via* 5-0 surgical silk. Muscles were streched to the length at which single twich showed the highest amplitude (optimal length; Lo), tetanic force was then measured at 20, 50, 100 and 150Hz, 500ms in duration with a rest period of 120s between tetani. The specific force (N/cm^2) was calculated with muscle density, 1.06 g/cm^3 [13] [15] [16] [17] [27].

Statistical studies

Statistical comparisons were performed using two-factor analysis of variance (ANOVA) followed by Mann-Whitney's U test. The *P*-value was set at 0.05

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FIGURE LEGENDS

[Figure1] The structure of HDAdv-myc-mFLdys

Abbreviations are: ITR, inverted terminal repeat of adenovirus; φ, packaging signal; PA, SV40 polyadenylation signal; full-length dystrophin, the murine full-length dystrophin cDNA; CAGP, the CAG promoter; stuffer, a part of the murine *Emx2* gene. Myc-tag was inserted into the dystrophin gene between 6886 nt and 6887 nt.

[Figure 2] Restoration of dystrophin, DAPs and nNOS expressions in the injected dko TA muscles

(a). H&E staining and immunostaining for five kinds of antigen

Top row: wild-type mouse, Middle row: uninjected dko mouse, Bottom row: injected dko mouse.

From left to right: H&E, Dystrophin (dystrophinH-300; 1:100), myc-tag (clone9E10; 1:50), β-dystroglycan (β-DG) (NCL-b-DG; 1:50), α-sarcoglycan (α-SG) (NCL-a-SARC; 1:100) and nNOS (NOS1 (R-20);1:50).

Scale bar = 100 μm.

Dystrophin was expressed in wild-type mice and injected dko mice beneath the sarcolemma. And β-DG, α-SG and nNOS were expressed in the dystrophin positive fibers of both groups, although myc-tag was expressed only in the injected dko mice. Expressed dystrophin in the injected dko mice was the delivered dystrophin and restored the DAPs and nNOS

(b-i). A reduction in the number of centrally nucleated fibers

Injected dko mice show a significant reduction in the number of centrally nucleated fibers compared with uninjected dko mice (wild-type: 0.51±0.34, uninjected dko: 89.1±4.3, injected dko: 13.7±16.4, uninjected dko *VS* injected dko: $P < 0.05$).

(b-ii). Cellular immune response in muscles

Quantitative data showing the number of CD4-positive and CD8-positive cells in muscles of 9-week-old wild-type, injected dko and uninjected dko mice. There were much fewer immune cells in the injected dko muscles than in the uninjected dko muscles.

[Figure 3] Effects of HDAdv-myc-mFLdys on appearance and motor performance in injected dko mice

(a). Appearance

The injected dko mouse showed obvious differences in size, and extremely milder kyphosis and muscle atrophy compared with the uninjected dko mouse. These mice were

9 weeks old.

(b). Body weight

circle: wild-type mice (2w:6.6±0.3, 4w:12.6±1.0, 6w:19.2±2.1, 8w:20.6±2.9, 10w:22.6±1.6), triangle: injected dko mice (2w:6.3±1.1, 4w:9.4±2.2, 6w:14.1±1.9, 8w:17.0±2.1, 10w:17.6±3.6), square: uninjected dko mice (2w:5.3±1.3, 4w:8.7±2.6, 6w:13.3±2.3, 8w:15.9±3.0, 10w:15.6±2.2). The *P*-values of the body weight between the injected dko and the uninjected dko at each point was 2 weeks-0.1198, 4 weeks-0.2614, 6 weeks-0.6985, 8 weeks-0.425, and 10 weeks-0.2482.

The injected dko mice became heavier than the uninjected dko mice, but not over wild-type mice.

(c). Kaplan-Meier curves

dotted line: wild-type mice (n=10), circle: injected dko mice (n=18), square: uninjected dko mice (n=33)

The injected dko mice significantly lived longer than the uninjected dko mice ($P < 0.05$).

(d). Footprints

The typical pattern of footprint for 3 groups; wild-type mouse(left), uninjected dko mouse (middle) and injected dko mouse (right) revealed the uninjected dko mouse showed wide-based gait and dragged their pelvis when walking, while the injected dko mouse showed improvements in gait .

The length of step was measured the stride from toe to toe.

black: wild-type mice (52.5±6.7), gray: uninjected dko mice (27.3±6.5), white: injected dko mice (37.2±3.1)

The injected dko mice showed a significantly longer stride length than that of the uninjected dko mice ($P < 0.01$).

(e). Locomotor activity

black: wild-type mice (18563 ± 7840), gray: uninjected dko mice (3858 ± 3189), white: injected dko mice (9642 ± 5947)

The injected dko mice significantly improved their motor activity over that of the uninjected dko mice ($P < 0.05$).

(c)-(e) parameters showed significantly difference between the injected dko mice and the uninjected dko mice ($P < 0.05$ for all parameters)

[Supporting Information] The expression of delivered full- length dystrophin in Western blot analysis

Left. protein extracted from COS7 cells (Lane 1) and infected COS7 cells (Lane 2).

Right. protein extracted from skeletal muscles of wild-type mouse(Lane 1), injected dko mouse (Lane 2) and uninjected dko mouse(Lane 3).

dystrophin:dystrophinH-300 (1:100), myc-tag: clone9E10 (1:50)

The expression of full-length dystrophin was confirmed in the injected muscles and the molecular weight of the expressed protein was 427 kDa, the same weight as the dystrophin protein extracted from wild-type muscle.

[Supporting video] Injected dko mouse restored motor performance

Left. The uninjected dko mouse

Right. The injected dko mouse

The injected dko mouse (7 weeks after injection) moved more actively than the uninjected dko mouse.

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