mdx^{5cv} Mice Manifest More Severe Muscle Dysfunction and Diaphragm Force Deficits than Do *mdx* Mice

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Duchenne muscular dystrophy (DMD) is characterized by progressive skeletal muscle dysfunction leading to premature death by the third decade of life. The *mdx* mouse, the most widely used animal model of DMD, has been extremely useful to study disease mechanisms and to screen new therapeutics. However, unlike patients with DMD, *mdx* mice have a very mild motor function deficit, posing significant limitations for its use as a platform to assess the impact of treatments on motor function. It has been suggested that an *mdx* variant, the *mdx*^{5cv} mouse, might be more severely affected. Here, we compared the motor activity, histopathology, and individual muscle force measurements of *mdx* and *mdx*^{5cv} mice. Our study revealed that *mdx*^{5cv} mice showed more severe exercise-induced fatigue, Rotarod performance deficits, and gait anomalies than *mdx* mice and that these deficits began at a younger age. Muscle force studies showed more severe strength deficits in the diaphragm of *mdx*^{5cv} mice compared to *mdx* mice, but similar force generation in the extensor digitorum longus. Muscle histology was similar between the two strains. Differences in genetic background (genetic modifiers) probably account for these functional differences between *mdx* strains. Overall, our findings indicate that the *mdx* and *mdx*^{5cv} mouse models of

DMD are not interchangeable and identify the *mdx*^{5cv} mouse as a valuable platform for preclinical studies that require assessment of muscle function in live animals. (Am J Pathol 2011, 179:2464–2474; DOI: 10.1016/j.ajpath.2011.07.009)

Duchenne muscular dystrophy (DMD), the most prevalent muscular dystrophy, is an X-linked recessive disorder affecting 1 in 3500 male births. DMD is typically diagnosed around 3 years of age with a rapid progression of muscle weakness that results in wheelchair dependence by 12 years of age and death by the third decade of life.¹ Molecularly, DMD stems from mutations in the dystrophin gene that typically result in a lack of dystrophin protein expression in skeletal and cardiac muscles.^{2,3} Loss of dystrophin compromises the muscle fiber membrane, leading to cycles of muscle fiber degeneration and regeneration, chronic inflammation, and accumulation of fibrotic tissue.^{4–6} Currently, there is no definitive treatment for DMD, but several gene-, cell-, and drug-based therapeutic approaches are being evaluated.^{7–12} Assessing and comparing the efficacy of these treatments require an easily available, well-characterized animal model with measurable and reproducible motor deficits, histologic pathology, and physiological alterations of muscle function.

The *mdx* mouse is the most widely studied animal model of DMD and has been extensively used in preclinical studies over the past 20 years. It arose from a spontaneous nonsense mutation in exon 23 of the dystrophin gene in an inbred C57BL/10 background.¹³ The *mdx*

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muscles lack dystrophin expression and show histopathological features similar to DMD.^{13–16} However, contrary to DMD patients, *mdx* mice have a milder phenotype, with minimal fibrosis in all muscles with the exception of the diaphragm,¹⁷ a near normal life span,¹⁸ and only minimal motor deficits.^{13,19–23} In particular, treatment effect on overall motor function has been difficult to assess, posing a challenge for preclinical studies.^{24,25}

As a result, *mdx* variants were generated with the hope of obtaining a more severe phenotype.^{26–28} However, these alternative DMD mouse models had histopathological features and mild functional impairment similar to the *mdx* mouse. Among these, *mdx*^{5cv} mice harbor a mutation affecting exon 10 that selectively disrupts expression of full-length dystrophin such as in *mdx* mice.²⁹ However, *mdx*^{5cv} mice have recently been preferred to *mdx* mice because of their very low level of dystrophin-expressing revertant fibers,³⁰ allowing a more accurate quantification of the efficiency of gene and cell therapy in restoring dystrophin expression. In addition, *mdx*^{5cv} mice are on the C57BL/6 background that allows rapid transfer of the dystrophin mutation onto transgenic and knock-out mice to dissect molecular aspects of disease.

Although *mdx* and *mdx*^{5cv} mice have been generally regarded as similar, a recent study found significant differences in gene expression profiles in all muscle groups examined, and a qualitative histologic examination suggested a more severe pathology in *mdx*^{5cv} mice.³¹ We therefore sought to determine whether *mdx*^{5cv} mice have more severe motor function deficits that render them distinct from *mdx* mice and better suited to study the effect of treatments on motor function and endurance.

Materials and Methods

Experimental Animals

All experimental mice were bred in-house and kept under similar conditions. Original C57BL/6J and C57BL/10SnJ breeders were obtained from The Jackson Laboratory (Bar Harbor, ME). C57BL/10ScSn-*Dmd*^{mdx} (referred to as *mdx*) and BL6Ros.Cg-*Dmd*^{mdx5cv} (referred to as *mdx*^{5cv}) breeders were kind gifts from Dr. Arthur Burghes (Ohio State University, Columbus, OH) and Dr. Louis Kunkel (Harvard Medical School, Boston, MA), respectively. Breeders were fed a high-fat diet (Teklad Global 19% protein rodent diet, no. 2019; Harlan, Indianapolis, IN), whereas weaned mice were switched to a low-fat diet (Teklad Global 18% protein rodent diet, no. 2018; Harlan). Animal breeding and experimental procedures followed approved protocols by the Institutional Animal Care and Use Committee. Unless otherwise stated, male mice were used.

Body and Individual Muscle Weights

Body weights were measured once a week from multiple litters. For individual muscle weights, the quadriceps, gastrocnemius, tibialis anterior, and triceps muscles from left and right limbs as well as the diaphragm were care-

fully dissected. Muscles were cleaned from fat and tendons before weighing. Weights of the left and right muscles for each individual mouse were averaged and then normalized to the tibia length (mg/cm).

Tibia Length

The tibia, knee, and ankle joints from the left hind limb were cleaned from tissue. Hind limbs were photographed, and tibia length from the knee to the ankle joint was measured with ImageJ software version 1.39u (NIH, Bethesda, MD).

Rotarod Assay

Ten to 15 mice per group (C57BL/6, C57BL/10, *mdx* and *mdx*^{5cv}) were tested on a Rotarod apparatus (Columbus Instruments, Columbus, OH) once a week in the morning, following a previously described protocol.³² Briefly, mice were placed on the stationary rod that was gradually brought to a constant speed of 20 rpm. At this point, the latency to fall was recorded with a maximum time on the rod of 5 minutes (300 seconds). Results from three trials (with a 1-minute rest between trials) per mouse per session were recorded and averaged. Mice were acclimated 2 weeks before first data collection.

Exercise-Induced Fatigue Assay

A previously described protocol³³ was followed with the following modifications. Activity measurements were taken on a Photobeam Activity System (San Diego Instruments, San Diego, CA) under dim light in the early morning before and after a single bout of treadmill exercise. Activity was recorded for 1 hour at 5-minutes interval. The DMD_M.2.1.002 TREAT-NMD standard operating procedure (SOP; Experimental Protocols for DMD Animal Models, <http://www.treat-nmd.eu/research/preclinical/dmd-sops>, last accessed June 30, 2011) was followed, and the first 5 minutes of recorded activity were discarded from analysis. Treadmill (Columbus Instruments) settings were 15° decline, 10 m/min speed, with a 10-minute run. For each individual mouse, post-exercise values were normalized to pre-exercise levels as follows. Ambulation, rearing, and distance traveled are expressed as the percentage of change in post-exercise levels compared with pre-exercise levels: [(Activity after exercise/Activity before exercise) × 100] – 100. Pre-exercise rest time values were subtracted from post-exercise values.

Grip Test

The M.2.2_001TREAT-NMD SOP was followed. Five grip strength measurements for forelimbs and hind limbs were taken for 5 to 7 mice per group on a strength meter (Columbus Instruments) and averaged. Force measurements (g) were normalized to body weight (g).

Footprint Test

The footprint test was performed as previously described.³⁴ Briefly, hind paws and forepaws of mice were painted blue and red, respectively. Mice were allowed to walk along a 50-cm long and 10-cm wide corridor lined with paper to record their footprints. Three parameters were measured (see Supplemental Figure S1 at <http://ajp.amjpathol.org>) on tracings where a consistent straight gait pattern was achieved: hind paw base width (distance between left and right hind paw footprints), separation (distance between the center of the front and hind footprints of the same side), and stride length. For each mouse, measurements from two to three sets of footprints per trial were averaged. Six to 10 male mice per group were analyzed at 8 weeks, 12 weeks, and 1 year of age.

Evans Blue Dye Injections and Quantifications

Evans Blue dye (MP Biomedicals, Inc., Irvine, CA) was administered intraperitoneally (5 μ L/g of body weight). For visualization on tissue sections, Evans Blue dye was administered 16 to 24 hours before sacrifice.³⁵ For spectrophotometric quantifications, mice were injected 40 hours before sacrifice, and tissues were analyzed as previously described.³⁶ Optical density values at 260 nm (SpectraMax M2; Molecular Devices, Sunnyvale, CA) were normalized to the individual muscle weight (mg).

Immunostaining for CD45 and Collagen III

Immunostaining of CD45 and collagen III were done as previously described³⁷ on 8- μ m frozen sections with the following primary antibodies: rat anti-mouse CD45 (1:200; AbD Serotec, Raleigh, NC) and collagen III (1:20; Southern Biotech, Birmingham, AL). For CD45 staining, antibody binding was visualized with DAB substrate (Vector Laboratories, Burlington, ON, Canada) and viewed under a light microscope. For collagen III staining, antibody signal was visualized with a fluorescein-conjugated secondary antibody and viewed under a fluorescence microscope.

Quantification of Inflammation and Collagen Deposition from Tissue Sections

Quantification was done as previously described.³⁸ The percentage of areas immunoreactive for CD45 and collagen III were quantified on five sections from each specimen separated by at least 50 μ m. Five nonoverlapping areas of each section were digitally captured with the use of an Olympus microscope (Olympus, Center Valley, PA) equipped with a high-resolution digital camera. The immunoreactive area was determined for each marker after manual outlining with the use of ImageJ software (NIH) and was expressed as a percentage of the visualized section area. The average from five sections was calculated for statistical comparisons (mean $\pm$ SD).

Hydroxyproline Assay

Hydroxyproline concentration of muscle samples from 12-week-old and 1-year-old *mdx* and *mdx*^{5cv} mice (5 mice per group) was analyzed as previously described.³⁹

Force Measurements in Diaphragm and EDL Muscles

The extensor digitorum longus (EDL) and diaphragm muscle strips were isolated immediately after euthanasia while the heart was still beating and bathed in oxygenated Krebs-Henseleit solution [95% O₂/5% CO₂ (pH 7.4), 118 mmol/L NaCl, 25 mmol/L NaHCO₃, 5 mmol/L KCl, 1 mmol/L KH₂PO₄, 2 mmol/L CaCl₂, 1 mmol/L MgCl₂, 5 mmol/L glucose] in a circulating bath. Diaphragm contractile strength measurements were done as previously described.^{40–42} This protocol deviated from the M.1.2_002 TREAT-NMD SOP mainly in that strength was assessed at 37°C. This temperature was chosen because it is physiologically more relevant to the *in vivo* function of diaphragm muscle, and reliable measurements can be performed as shown by us^{40,41} and others.^{43–45} Briefly, small linear strips were dissected from the diaphragm and connected to a linear micromanipulator and a fixed hook. Electrical stimulation was achieved with parallel platinum-iridium electrodes. First, twitch contractions were used, and the muscle was stretched until twitches produced maximal force. Thereafter, a paired-pulse protocol was used, and various frequencies of pulse intervals were used, with 10 minutes of rest between each stimulation. At 150 Hz, a smooth tetanus was obtained with the use of this protocol. After a final rest period, fatigue was assessed by sending a 100-Hz (500 milliseconds) impulse every second for 1 minute. After force measurements were taken, tissues were only lightly blotted before weighing to allow use of the same muscle for histologic analysis. This resulted in a 30% to 40% overestimation of muscle weight. As a result, when normalized isometric force measurements (mN/mm²) per unit of cross-sectional area is based on total weight (using muscle density of 1.06 g/cm³), these values are generally 30% to 40% lower than when calculated after complete tissue blotting. Because we did record all 3 muscle dimensions for diaphragm strips (using a stereo dissection microscope at $\times 40$), specific force was calculated by dividing total active force by the central cross-sectional area.

EDL force measurements were taken at 30°C as described previously.^{41,46,47} Briefly, one end of the EDL muscle was tied to a force transducer and the other to a high-speed linear servo-controlled motor. At a resting tension of 1 $\times$ g, stimulation was delivered by two parallel platinum-iridium electrodes. Muscles were adjusted to optimum length, defined as the length for maximal twitch when subjected to an isometric tetanus of 150 Hz, for 500 milliseconds. After a 10-minute rest period, muscles were subjected to an eccentric contraction protocol that consisted of a series of 10 isometric 700-millisecond tetani, at

2-minute intervals, with a 5% lengthening of the muscles (0.5 fiber length per second for duration of 200 milliseconds) when maximal force has developed at 500 milliseconds. When the tetanus ended (at $t = 700$ milliseconds), the muscle was brought back to initial length (at the same speed as the stretch), allowing for full relaxation to the initial length. For EDL muscle, specific force was calculated with the recorded weights and is thus ~30% to 40% lower because of the mild blotting used to preserve histology. All groups were treated identically.

AAV8.MCK.microdystrophin Vector Production and Administration to Mice

High-titer recombinant AAV8 vectors were produced by a modified cross-packaging approach.⁴⁸ The murine microdystrophin construct R4-R23/ Δ 71-72⁴⁶ was placed under the control of a truncated MCK (muscle creatine kinase) promoter/enhancer to drive muscle-specific gene expression (AAV8.MCK.microdystrophin).⁴⁹ Five neonatal *mdx*^{5cv} mice received an intraperitoneal injection of 3.7×10^{11} AAV8.MCK.microdystrophin vector genomes in a total volume of 100 μ L.

Dystrophin Immunostaining

Diaphragm, quadriceps, gastrocnemius, tibialis anterior, and triceps muscles were embedded in 7% gum tragacanth and flash frozen in liquid nitrogen. Unfixed 12- μ m cryosections were processed for immunohistochemistry as previously described⁴⁶ with the use of the anti-dystrophin Manex1a and Manex1011b antibodies (Developmental Studies Hybridoma Bank, Iowa City, IA; 1:50) that also recognize microdystrophin. Fluorescence images were captured under a $\times 20$ objective with a Hamamatsu ORCA-ER digital camera mounted on an Olympus BX61 microscope. Montage images (Slidebook 4.2; Intelligent Imaging Innovations, Denver, CO) of the entire tissue section were used to manually quantify the number of total and microdystrophin-positive myofibers. Values were used to calculate the percentage of microdystrophin-positive fibers.

Western Blot Analysis

Five, 50- μ m diaphragm tissue sections were homogenized in ice-cold protein lysis buffer [125 mmol/L Tris-HCl, 4% SDS, 4M urea, 5% 2-Mercaptoethanol, protease inhibitor cocktail (Roche, Indianapolis, IN)] with the use of a pellet pestle motor (Kimble Chase, Vineland, NJ). Protein lysates were centrifuged at $14,000 \times g$ for 15 minutes at 4°C to remove insoluble material. Protein concentration was determined on a Nanodrop 1000 (Thermo Scientific, Waltham, MA) before adding 4 $\times$ SDS-PAGE reducing sample buffer. Proteins were resolved on a 4% to 12% gradient SDS Bis-Tris gel (Invitrogen, Carlsbad, CA) and transferred to a nitrocellulose membrane that was cut at the 60-kDa molecular weight mark. The top membrane (molecular weight >60 kDa) was incubated with the Manex1011b antibody (1:1000; Developmental Studies

Hybridoma Bank) to detect dystrophin and microdystrophin, whereas the bottom was used for protein loading control with the use of an anti-actin antibody (1:500; clone 20 to 33; Sigma-Aldrich, St. Louis, MO). After incubation with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories Inc., West Grove, PA), signal was detected by chemiluminescence (Thermo Scientific) on X-ray film (Research Products International, Mt. Prospect, IL).

Densitometric Analysis

Densitometric analysis was performed with the ImageJ software (NIH) on scanned X-ray films taken at nonsaturating exposures. Microdystrophin and dystrophin band intensities were normalized to actin for each sample. Percentages of microdystrophin levels were calculated relative to dystrophin in wild-type mice.

Quantification of Diaphragm Myofibers with Centrally Located Nuclei

Two 12- μ m diaphragm sections were stained with hematoxylin and eosin. Bright-field pictures were taken with a 10 $\times$ objective and a color Olympus DP71 camera mounted on a Zeiss Axioskop microscope (Zeiss, Thornwood, NY). For each section, two random nonoverlapping fields were photographed, and the numbers of total myofibers and of myofibers with a centrally located nucleus were counted and used to calculate the following ratio per mouse: total number of myofibers with central nuclei/the total number of myofibers counted.

Statistical Analyses

For comparisons between two groups we used a two-tailed unpaired Student's *t* test (significance set at $P < 0.05$). For multiple group comparisons, a two-way analysis of variance followed by the Bonferroni test for pairwise comparisons was used. For experiments with repeated measures, a repeated-measure analysis of variance was performed to detect significant differences between groups.

Results

*Differences in Body and Muscle Weights between *mdx*^{5cv} and *mdx* Mice*

We first determined whether significant weight or growth differences were present between *mdx* and *mdx*^{5cv} mice because they might affect parameters measured in motor function tests. In agreement with previous reports,^{15,50,51} we found that *mdx* mice had a significantly lower body weight than wild-type mice between 8 and 20 weeks of age (Figure 1A). *mdx*^{5cv} mice also had a significantly lower body weight compared with wild-type controls but also compared with *mdx* mice at most ages tested. This difference could not be attributed to intrinsic strain differ-

Figure 1. Comparison of body and muscle weight among wild-type, *mdx*, and *mdx*^{5cv} mice. **A:** Body weight of wild-type and dystrophic mice between 8 and 20 weeks of age. *mdx*^{5cv} mice have a significantly lower body weight than wild-type mice at all ages studied ($P < 0.05$). Significant differences between *mdx* and *mdx*^{5cv} mice are indicated. * $P < 0.05$, ** $P < 0.01$. Values are means $\pm$ SEs ($n = 5$ to 25 mice per group). **B:** Individual muscle weight normalized to tibia length for 42-week-old male mice. Data are means $\pm$ SDs ($n = 4$). QD, quadriceps; TA, tibialis anterior; GS, gastrocnemius; TC, triceps; DIA, diaphragm. No statistically significant differences were found.

ences because C57BL/6 and C57BL/10 wild-type mice had similar body weights.

Because we observed more abdominal fat accumulation in wild-type mice than in either dystrophic mouse strain, we measured tibia length, an alternative measure of growth that is linked to muscle mass but independent of fat.⁵² Tibia length showed no difference between wild-type and dystrophic mice or between *mdx* and *mdx*^{5cv} mice (see Supplemental Figure S2, A and B, at <http://ajp.amjpathol.org>). Furthermore, measurements of body length (base of the neck to the base of the tail) taken after sacrifice in 1-year-old mice were similar between all strains (data not shown). These results suggest that weight differences between dystrophic and wild-type mice as well as between *mdx* and *mdx*^{5cv} mice do not reflect differences in growth or overall mouse size but may reflect differences in body fat. To further test for differences between *mdx* and *mdx*^{5cv} mice in muscles involved in motor function, we compared the weight of freshly isolated limb and diaphragm muscles from male dystrophic mice and found no significant differences (Figure 1B). Therefore, *mdx* and *mdx*^{5cv} mice are similar in size and individual muscle weight, allowing for a direct comparison of their motor function.

Differences in Rotarod Performance, Fatigue, and Gait between *mdx*^{5cv} and *mdx* Mice

Next, we evaluated three modalities of muscle function in male mice: Rotarod performance, exercise-induced fatigue, and gait. For all tests performed, we found no significant differences between wild-type C57BL/6 and C57BL/10 mice (Figure 2A; see also Supplemental Figures S3 and S4 at <http://ajp.amjpathol.org>). For simplicity, we are showing C57BL/6 data to highlight the severity of motor deficits in dystrophic strains relative to wild-type performance. Rotarod performance was assayed with a protocol that challenges both motor coordination and endurance.³² The performance of *mdx* mice was not significantly different from either C57BL/10 or C57BL/6 wild-type mice, although it appeared mildly affected after 10 weeks of age (Figure 2A). In stark contrast, *mdx*^{5cv} mice showed a significant motor deficit at all ages compared with both wild-type and *mdx* mice which was particularly pronounced between 6 and 10 weeks. Therefore, the Rotarod assay showed a deficit in motor coordination and possibly endurance that is only present in *mdx*^{5cv} mice.

Figure 2. Rotarod, exercise-induced fatigue, and gait analysis in dystrophic and wild-type mice. **A:** Rotarod performance. *mdx*^{5cv} mice have a significant deficit in Rotarod performance compared with either wild-type or *mdx* mice (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.005$). No significant difference was found between *mdx* and wild-type mice. Data are mean $\pm$ SE ($n = 5$ to 15 mice). **B–D:** Decrease in rearing events (**B**), ambulation (**C**), and distance traveled (**D**) after exercise compared with pre-exercise levels. Data are mean $\pm$ SD expressed as percentage of change in activity ($n = 5$ to 12 mice). **E:** Increase in rest time after exercise. Data are mean $\pm$ SD ($n = 5$ to 12 mice). Values are normalized to pre-exercise levels. **F–H:** Measurements of hind paw base width (**F**), hind paw to forepaw separation (**G**), and stride length (**H**) in wild-type C57BL/6, *mdx*, and *mdx*^{5cv} mice. Data represent mean $\pm$ SE. **B–H:** Significant differences: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.005$.

To evaluate separately endurance and motor coordination, we tested exercise-induced fatigue and gait, respectively. In preliminary tests, we observed that contrary to *mdx* mice, *mdx*^{5cv} mice were unable to sustain 30 minutes of downhill treadmill running and showed extreme fatigue after 15 minutes. To quantify this difference in exercise-induced fatigue, we compared open field activity of wild-type, *mdx*^{5cv}, and *mdx* mice at 8 weeks, 12 weeks, and 1 year of age before and after a single 10-minute bout of treadmill running. As previously reported,³³ we found no significant differences in baseline levels of ambulation (horizontal activity), rearing events (vertical activity), distance traveled, or resting time between wild-type and dystrophic mice (data not shown). To account for interindividual variability, activity values measured after exercise were normalized to pre-exercise levels for each individual mouse (see *Materials and Methods*). Overall, exercise decreased ambulation, rearing activity, and the distance traveled, and it increased rest time in both wild-type and dystrophic mice, with more pronounced effects in dystrophic mice (Figure 2, B–E). In agreement with previous findings,³³ only rearing events were significantly decreased after exercise in young (8 weeks) *mdx* mice compared with wild-type mice (Figure 2B). By 12 weeks of age, *mdx* mice also showed a significant decrease in ambulation and increased rest time (Figure 2, C and E), whereas the distance traveled was significantly decreased only at 1 year of age (Figure 2D). Thus, *mdx* mice became more sensitive to exercise-induced fatigue with age. By contrast, *mdx*^{5cv} mice showed maximum decreases (>90% drop in activity after exercise) in both ambulation and rearing events, starting as early as 8 weeks of age (Figure 2, B and C). By 12 weeks of age, *mdx*^{5cv} mice showed a significant decrease in distance traveled compared with both wild-type and *mdx* mice. Interestingly, *mdx*^{5cv} mice had a surprisingly uniform response to exercise-induced fatigue, whereas *mdx* mice showed a high level of interindividual variability. The coefficient of variation at 12 weeks was 2.5% for *mdx*^{5cv} mice (*n* = 8) compared with 26.2% for *mdx* mice (*n* = 7) for ambulation, and 1.0% versus 17.1%, respectively, for

rearing events. These results indicate that *mdx*^{5cv} mice are more sensitive than *mdx* mice to exercise-induced fatigue, are affected at an earlier age, and show minimal interindividual variability in this assay.

Motor coordination in dystrophic mice was further evaluated with gait analysis. Visual comparison of gait tracings between wild-type, *mdx*, and *mdx*^{5cv} mice indicated that dystrophic mice, and in particular *mdx*^{5cv} mice, had a shorter stride and that their hind paw did not always land on the same position as the front paw as is normal for mice (see Supplemental Figure S1A at <http://ajp.amjpathol.org>). Gait abnormalities were confirmed by measurements of hind paw base width (Figure 2F), front-hind paw separation (Figure 2G), and stride length (Figure 2H). Both *mdx* and *mdx*^{5cv} mice had a significantly larger hind paw base width than wild-type mice, possibly indicative of muscle weakness. However, measurements of front-hind paw separation and especially stride length showed significant gait abnormalities that were more severe in *mdx*^{5cv} mice than in wild-type and *mdx* mice. This could not be attributed to a smaller body size because tibia length measurements and postmortem examinations showed no differences between strains. Of note, 1-year-old wild-type mice show increased base width and separation, becoming indistinguishable from dystrophic mice. Therefore, the gait test is sensitive to age. Taken together, the above tests all indicate deficits in motor coordination, endurance, and gait that are present earlier, are more severe, and progress more rapidly in *mdx*^{5cv} than in *mdx* mice.

Differences in the Contractile Properties of the Diaphragm between *mdx* and *mdx*^{5cv} Mice

We next evaluated whether differences in the severity of motor function deficits between *mdx* and *mdx*^{5cv} mice could stem from differences in limb and/or diaphragm muscle strength. Whole-limb muscle strength was evaluated with the grip test. In agreement with previous studies,^{53,54} both *mdx* and *mdx*^{5cv} mice showed a significant

Figure 3. Limb grip strength and diaphragm force measurements in wild-type, *mdx*, and *mdx*^{5cv} mice. **A:** Grip strength assay of hind limbs and forelimbs of 42-week-old mice. No significant difference was found between *mdx* and *mdx*^{5cv} mice. Grip strength was normalized to body weight. Data are mean $\pm$ SD (*n* = 5 to 6 mice). Significant differences from wild-type mice: **P* < 0.05, ***P* < 0.01. **B:** Tetanic force of diaphragm muscle. *mdx* and *mdx*^{5cv} mice showed significantly lower force generation (smooth tetanus at 150 Hz) than strain-matched wild-type mice. In addition, a significantly lower tetanic force was generated by the diaphragm of *mdx*^{5cv} than *mdx* mice. Significant differences between groups: **P* < 0.05, ****P* < 0.005. **C:** Diaphragm muscle response to the repetitive isometric contraction protocol. The diaphragm of *mdx*^{5cv} mice showed enhanced susceptibility to fatigue compared with wild-type and *mdx* mice (**P* < 0.05). **B** and **C:** Data are mean $\pm$ SE (*n* = 10/group).

Figure 4. Quantification of fibrosis in the quadriceps and diaphragm muscles of wild-type, *mdx*, and *mdx*^{5cv} mice. **A:** Hydroxyproline content in diaphragm muscles of 12-week-old and 1-year-old *mdx* and *mdx*^{5cv} mice. No statistically significant differences were found. **B:** Hydroxyproline content in quadriceps muscles of 12-week-old and 1-year-old *mdx* and *mdx*^{5cv} mice. No statistically significant differences were found. Note difference in scale between panels **A** and **B**. **C:** Representative micrographs of diaphragm muscle sections from 12-week-old and 1-year-old male *mdx* and *mdx*^{5cv} mice stained with an antibody to collagen III. Scale bar = 25 μ m. **D:** Quantification of the percentage of area of the diaphragm section positive for collagen III immunostaining. Values are means $\pm$ SDs from 5 mice per group. No significant differences were found.

deficit in grip strength compared with wild-type mice for both hind limbs and forelimbs (Figure 3A; see also Supplemental Figure S5A at <http://ajp.amjpathol.org>). However, no difference was found between *mdx* and *mdx*^{5cv} mice. Similarly, *ex vivo* measurements of tetanic force and resistance to eccentric contraction-induced damage in the EDL showed no significant differences between *mdx* and *mdx*^{5cv} mice (see Supplemental Figure S5, B and C, at <http://ajp.amjpathol.org>). Therefore, limb muscles of *mdx*^{5cv} mice do not appear to be weaker or more sensitive to eccentric contraction-induced damage than *mdx* mice.

By contrast, the diaphragm of *mdx*^{5cv} mice showed a significantly lower tetanic force (Figure 3B) and enhanced susceptibility to fatigue during 60 repetitive tetani (Figure 3C) compared with *mdx* mice. These differences could not be attributed to baseline differences between the C57BL/6 and C57BL/10 strains (Figure 3, B and C). Diaphragm force and fatigue measurements in both *mdx* and *mdx*^{5cv} mice were significantly lower than in wild-type mice. Therefore, *mdx*^{5cv} mice have a more severe deficit in force generation and resistance to fatigue in the diaphragm and their limb muscle strength is equivalent to *mdx* mice.

Histopathological Parameters in the Diaphragm Are Similar between *mdx* and *mdx*^{5cv} Mice

To determine whether the diaphragm of *mdx*^{5cv} mice had more pronounced histopathology, we quantified and compared connective tissue deposition, immune cell infiltration, and muscle fiber membrane permeability in the diaphragm muscles of *mdx*^{5cv} and *mdx* mice. We further

extended our analysis to limb muscles to complement our muscle strength analysis. We studied mice at 12 weeks when significant differences in motor performance were present between *mdx*^{5cv} and *mdx* mice. We also compared old (45 to 53 weeks of age) *mdx*^{5cv} and *mdx* mice when pathology is most evident.

Collagen deposition, an indicator of fibrosis, was quantified in the diaphragm and quadriceps muscles by measuring hydroxyproline content and by immunohistochemistry. For both *mdx*^{5cv} and *mdx* mice, the diaphragm showed increased collagen deposition between 12 weeks and 1 year of age and higher hydroxyproline content than the quadriceps (Figure 4, A–D). However, no significant differences were found between *mdx* and *mdx*^{5cv} mice in either tissue at either age, suggesting comparable levels of fibrosis. Similarly, quantification of global immune cell infiltration (lymphocytes and macrophages) did not show significant differences between *mdx* and *mdx*^{5cv} mice for either diaphragm or quadriceps muscles (Figure 5, A and B). Finally, quantification of Evans Blue dye incorporation, a measure of myofiber damage,^{35,55–57} also failed to show differences between *mdx* and *mdx*^{5cv} mice in the diaphragm as well as quadriceps, tibialis anterior, gastrocnemius, and triceps muscles at either 12 weeks or 1 year of age (Figure 5, C and D; data not shown). Similar to fibrosis, only the diaphragm showed a significant increase in Evans Blue dye incorporation in older mice (Figure 5C). These results suggest that *mdx* and *mdx*^{5cv} mice are similar for the histopathological parameters we evaluated here. We found no evidence of more severe histopathology in the diaphragm of *mdx*^{5cv} mice that could account for a more severe force deficit compared with *mdx* mice.

Figure 5. Quantification of inflammation and myofiber membrane damage in wild-type, *mdx*, and *mdx*^{5cv} mice. **A:** Representative micrographs of diaphragm (Dia) and quadriceps (Qua) muscle sections stained with an antibody against the immune cell marker CD45. Scale bar = 25 μ m. **B:** Quantification of the percentage of area of the muscle section positive for CD45 immunostaining. Values are means $\pm$ SDs from 5 mice per group. **C** and **D:** Quantification of the amount of Evans Blue dye incorporation in the diaphragm (**C**) and quadriceps (**D**) muscles of *mdx* and *mdx*^{5cv} mice at 12 weeks and 1 year of age. No significant differences were found between *mdx* and *mdx*^{5cv} mice. Data represent mean $\pm$ SD ($n = 4$).

Figure 6. Microdystrophin expression in the diaphragm of *mdx*^{5cv} mice improves resistance to exercise-induced fatigue. **A** and **B**: Effects of microdystrophin expression in the diaphragm of *mdx*^{5cv} mice on exercise-induced fatigue at 12 weeks of age. AAV8.MCK.microdystrophin *mdx*^{5cv} mice show significant improvements for all activity parameters measured compared with *mdx*^{5cv} mice but do not reach wild-type values. Significant differences among groups shown: **P* < 0.05, ***P* < 0.001, and ****P* < 0.0005. An analysis of variance was used for comparisons. **C**: Western blot analysis of microdystrophin protein expression in the diaphragm of wild-type and AAV8.MCK.microdystrophin *mdx*^{5cv} mice. Actin is shown as a loading control.

Microdystrophin Expression in the Diaphragm Improves Resistance to Exercise-Induced Fatigue in *mdx*^{5cv} Mice

Microdystrophin expression in *mdx* mice has been shown to significantly improve muscle strength and rescue histopathology.^{46,58–61} We sought to determine whether microdystrophin expression in *mdx*^{5cv} mice could lead to measurable improvements in motor function. One-day-old *mdx*^{5cv} pups were treated with AAV8.MCK.microdystrophin following a protocol shown to result in body-wide transduction.⁶² Because sex cannot be established in neonates, female and male wild-type, control *mdx*^{5cv}, and AAV8.MCK.microdystrophin *mdx*^{5cv} mice were evaluated for exercise-induced fatigue and gait at 12 weeks of age and then sacrificed for analysis of microdystrophin expression in limb and diaphragm muscles.

No effect of AAV8.MCK.microdystrophin treatment was detected on any gait parameter measured (stride length, paw separation, hind paw width), even after taking sex differences into account (data not shown). For exercise-induced fatigue, no significant sex differences were found, and data from both sexes were pooled. *mdx*^{5cv} mice treated with AAV8.MCK.microdystrophin showed a significant improvement in exercise-induced fatigue compared with control *mdx*^{5cv} mice for all parameters measured (Figure 6, A and B). As expected, values were below wild-type levels because microdystrophin does not restore neuronal nitric oxide synthase (nNOS) at the

membrane of myofibers,^{33,63} and this protein plays an important role in muscle fatigue.³³

Histologic and Western blot analyses of muscles from AAV8.MCK.microdystrophin-treated *mdx*^{5cv} mice showed that we achieved high transduction of the diaphragm in all injected mice (Figure 6C) and that microdystrophin was correctly localized to the myofiber membrane (see Supplemental Figure S6A at <http://ajp.amjpathol.org>). Furthermore, the percentage of fibers with centrally located nuclei (Table 1), the amount of connective tissue, and the incorporation of Evans Blue dye (see Supplemental Figure S6A at <http://ajp.amjpathol.org>) were visibly reduced. Interestingly, no microdystrophin expression was detected in limb muscles, possibly explaining the lack of improved gait in treated mice. Triceps, gastrocnemius, quadriceps, and tibialis anterior muscles showed large areas of Evans Blue dye-positive fibers (see Supplemental Figure S6B at <http://ajp.amjpathol.org>; data not shown), and most myofibers had centrally located nuclei, similar to control *mdx*^{5cv} mice (data not shown).

Therefore, expression of microdystrophin in the diaphragm significantly improves resistance to exercise-induced fatigue in *mdx*^{5cv} mice independent of nNOS restoration. Because microdystrophin expression was not detected in limb muscles, these results suggest that the diaphragm contributes significantly to the high sensitivity of *mdx*^{5cv} mice to exercise-induced fatigue. They also indicate that this assay is robust and sensitive enough to

Table 1. Comparison of Micro-Dystrophin Expression, Myofiber Regeneration, and Changes in Postexercise Activity in Individual AAV8.MCK.microdystrophin *mdx*^{5cv} Mice

Mouse ID	%μDYS protein	%DYS+fibers	%FCN	Ambulation (%)	Rearing (%)	Distance (%)	Rest time (s)
1	69	51	2.6	−85	−89	−84	1275
2	11	17	14.7	−93	−91	−94	2604
3	20	40	2.2	−82	−83	−73	1189
4	27	30	8.8	−66	−77	−64	694
5	30	30	3.1	−78	−79	−80	1471
<i>mdx</i> ^{5cv} controls	—	—	51 ± 5	−91 ± 2	−97 ± 1	−90 ± 9	2029 ± 707

Ambulation, rearing events, and distance travelled are expressed as percentage of change in activity after exercise. Rest time shown is postexercise value after subtraction of rest time value before exercise. Reference values for control *mdx*^{5cv} mice represent mean ± SD.

%μDYS, percentage of micro-dystrophin protein expression relative to dystrophin in wild-type diaphragm as determined by Western blot analysis (Figure 6C), and values were normalized to actin as a loading control for each lane; % DYS+fibers, percentage of micro-dystrophin positive myofibers per tissue sections; %FCN, percentage of fibers with central nuclei.

detect treatment effects in small numbers of experimental animals.

Discussion

In this study, we report that the *mdx*^{5cv} mouse, a commercially available model of DMD (The Jackson Laboratory stock no. 002379), has more severe functional deficits than the more widely used *mdx* mouse with respect to motor coordination, resistance to exercise-induced fatigue, and gait abnormalities. Furthermore, our study showed that the greater susceptibility of *mdx*^{5cv} mice to exercise-induced fatigue primarily arises from a more severe force-generation deficit and enhanced susceptibility to contraction-induced fatigue of their diaphragm muscle compared with *mdx* mice. This conclusion is based on our *ex vivo* force measurements as well as on the *in vivo* finding that expression of microdystrophin in the diaphragm of *mdx*^{5cv} mice significantly improves their resistance to exercise-induced fatigue. Notably, the beneficial effects of microdystrophin on fatigability in *mdx*^{5cv} mice are probably independent of improved histopathology or nNOS signaling because transgenic expression of microdystrophin in *mdx* mice normalizes histologic parameters but does not protect from exercise-induced fatigue.³³

Overall, our results are consistent with the existence of a genetic modifier of the dystrophic phenotype in *mdx*^{5cv} mice. *mdx*^{5cv} mice were generated by chemical mutagenesis and backcrossed for 11 generations onto the C57BL/6 background (The Jackson Laboratory, personal communication), whereas *mdx* mice arose from a spontaneous mutation on the C57BL/10 strain. The C57BL/10 and C57BL/6 strains share a common ancestor and are generally considered interchangeable. However, genetic differences have been reported^{64,65} as well as some behavioral differences.^{66,67} The effects of this genetic modifier appear silent in the absence of muscular dystrophy because we and others^{33,66,68,69} found no significant differences in motor performance or force measurements between wild-type C57BL/6 and C57BL/10 mice. Furthermore, it appears distinct from recently identified modifiers of the dystrophic phenotype that affect all skeletal muscles and typically result in increased fibrosis.^{28,36,41,70,71} Uniquely, the phenotype of *mdx*^{5cv} mice appears linked to a mechanism that specifically affects the contractile properties of the diaphragm and can be corrected by microdystrophin independent of nNOS restoration. Interestingly, among skeletal muscles, the diaphragm had the largest number of differentially expressed genes between *mdx*^{5cv} and *mdx* mice.³¹ Further studies are needed to elucidate the mechanisms underlying a more severe diaphragm involvement in *mdx*^{5cv} mice because they may be relevant to variability in the DMD phenotype in humans.

The primary goal of this study was to determine whether *mdx*^{5cv} mice have more severe motor function deficits than *mdx* mice, thus providing a mouse model of DMD better suited to study the effect of treatments on motor function and endurance. Currently, preclinical

studies involving *mdx* mice primarily rely on indirect measures (histology and force measurements on a limited subset of muscles) to predict treatment effect on overall motor function and fatigability, which may not be accurate as shown for microdystrophin.³³ Our results indicate that *mdx*^{5cv} mice should be considered as an alternative to *mdx* mice for these studies for several reasons. First, because of the severity of their functional deficits, the performance of *mdx*^{5cv} mice was strikingly inferior to that of wild-type mice, providing a large dynamic range both in terms of motor performance and age, over which treatment effects can be evaluated. This is not the case for the *mdx* mouse, whereby a motor performance deficit is only reliably detectable at older ages or is not detected at all as shown for the Rotarod assay. Second, *mdx*^{5cv} mice show quantifiable and significant deficits at younger ages (typically 6 to 8 weeks) compared with *mdx* mice. Therefore, treatment effects can be assayed much earlier in *mdx*^{5cv} mice and can be followed over longer periods of time. In addition, compounding effects of aging on phenotype can be avoided, especially for some tests such as gait analysis. Third, *mdx*^{5cv} mice tend to show lower interindividual variability in motor activity tests compared with *mdx* mice. This is a significant advantage when planning pilot studies with small numbers of animals.

Therefore, our results show that *mdx*^{5cv} and *mdx* mice are not interchangeable models of DMD. Their differences hint at novel disease mechanisms that may influence disease outcome. Importantly, our findings indicate that the *mdx*^{5cv} mouse model can significantly extend the utility of the mouse in DMD preclinical studies that want to include *in vivo* muscle function as an outcome measure without the compounding effects of exercise-induced muscle damage or old age.

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