Dystrophin-deficient *mdx* mice display a reduced life span and are susceptible to spontaneous rhabdomyosarcoma

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ABSTRACT **Duchenne muscular dystrophy (DMD) is the most common, lethal genetic disorder of children. A number of animal models of muscular dystrophy exist, but the most effective model for characterizing the structural and functional properties of dystrophin and therapeutic interventions has been the *mdx* mouse. Despite the ~20 years of investigations of the *mdx* mouse, the impact of the disease on the life span of *mdx* mice and the cause of death remain unresolved. Consequently, a life span study of the *mdx* mouse was designed that included cohorts of male and female *mdx* and wild-type C57BL/10 mice housed under specific pathogen-free conditions with deaths restricted to natural causes and with examination of the carcasses for pathology. Compared with wild-type mice, both *mdx* male and female mice had reduced life spans and displayed a progressively dystrophic muscle histopathology. Surprisingly, old *mdx* mice were prone to develop muscle tumors that resembled the human form of alveolar rhabdomyosarcoma, a cancer associated with poor prognosis. Rhabdomyosarcomas have not been observed previously in nontransgenic mice. The results substantiate the *mdx* mouse as an important model system for studies of the pathogenesis of and potential remedies for DMD.—Chamberlain, J. S., Metzger, J., Reyes, M., Townsend, D., Faulkner, J. A. Dystrophin-deficient *mdx* mice display a reduced life span and are susceptible to spontaneous rhabdomyosarcoma. *FASEB J.* 21, 2195–2204 (2007)**

Key Words: muscular dystrophy · aging

As described by many investigators of Duchenne muscular dystrophy (DMD) over the years, DMD is the most common and lethal genetic disease experienced by humans (1). Meryon (2) was the first to describe the symptoms of DMD, followed shortly thereafter by Duchenne's report of 13 cases of "paralysie musculaire pseudohypertrophique," a description based on the transient hypertrophy of the calf muscles of boys in the early stages of the disease that was followed by a progressive and rapid deterioration of the muscles (3). DMD is a recessive, X-linked disorder caused by mutations in the gene that encodes the 427 kDa cytoskeletal protein dystrophin (4). The gene is expressed in skeletal, cardiac, and smooth muscle (5, 6). In muscle, dystrophin plays a critical role by connecting F-actin in the subsarcolemmal cytoskeleton to the dystrophin-glycoprotein (DGC) complex that spans the sarcolemma and attaches to laminin-2 (merosin) in the extracellular matrix (7). Despite identification of the precise anatomical connections of the DGC, a clarification of the actual function of the complex has not been established definitively. The most long-standing hypothesis as to the underlying cause of the progression of the disease (1) and still popular currently (7–13) is that muscular dystrophy causes a structural and/or functional defect in the plasma membrane of skeletal muscle fibers. The hypothesis has evolved slightly to include stabilization of the membrane particularly during contractions (7, 10, 11).

Boys with DMD are difficult to diagnose during the first few years of life, but early symptoms are of a pseudo-hypertrophy, particularly of the calf muscles, followed by an early onset of muscle wasting that appears at ~ 4 years of age (1). The pseudo-hypertrophy results from damage to muscle fibers with subsequent necrosis of fibers, but some regeneration of fibers through activation of satellite cells. The process of degeneration and regeneration produces a larger, but much weaker muscle, hence the term "pseudo-hypertrophy." The muscle wasting follows as the degenerative process overwhelms the capability of the muscle for regeneration. A loss of ambulation occurs between the ages of 8 and 11, years, with subsequent confinement to a wheelchair. Boys with DMD generally die during their late teens to early 20s due to either respiratory or cardiac failure (1, 14). Thus, DMD is a progressive and fatal disease of muscle degeneration

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that occurs with an approximate frequency of 1 in 3500 live male births worldwide (14). As early as the 1950s, a gradual reduction in the incidence of DMD was reported, likely due to genetic counseling, then the introduction of carrier detection in the mid-1960s, and subsequently antenatal gender determination (15).

A great deal of what is known about dystrophin structure-function and the pathogenesis of DMD has come from studies of a variety of dystrophin-deficient animals (8, 16–18), but by far the most prolific model has been the *mdx* mouse, first described in 1984 (19). The dystrophic-like symptoms in the mouse arose from a spontaneous, nonsense mutation in exon 23 of the dystrophin gene (18). For *mdx* mice, limb skeletal muscles display degeneration and regeneration with the associated pseudo- or compensatory hypertrophy (20) also observed in the early stages of DMD in boys (1). The difference is that, for boys, the condition transitions rapidly into muscle atrophy as the degenerative processes outdistance the regenerative processes (1, 14). In contrast, most of the limb muscles of the *mdx* mouse maintain hypertophy throughout much of their life span, but with a considerable loss in specific force and normalized power (20). Compared with the muscle wasting and degeneration observed for boys with DMD, the diaphragm muscle of *mdx* mice most closely approximates the age-related decline in the mass, maximum specific force and maximum normalized power (21–24). McCully and Faulkner (25) demonstrated that contraction-induced injury occurred only with lengthening contractions, and a number of investigators have demonstrated that, under well-controlled conditions, the skeletal muscles of *mdx* mice show a marked susceptibility to lengthening contraction-mediated force decrements (26–32). Progressive histopathology and contractile dysfunction are also evident in cardiac muscles of the *mdx* mouse (33–36). Acute heart failure is also evident in the *mdx* mouse on cardiac stress testing (35).

The structural and functional properties of the skeletal muscles of *mdx* mice have undergone extensive investigation over the past 30 years (21, 37–44) and a wide variety of genetic interventions have utilized the *mdx* mouse model (reviewed in ref. 8, 45). In particular, dystrophin replacement in muscles of *mdx* mice, either *via* germ-line insertion in transgenic animals or after delivery to adults using viral vectors, has demonstrated the possibility of a therapy for DMD using gene therapy (22, 46–48). Despite the diversity of the structural and functional studies and sophistication of the genetic interventions, the inter-relationships between muscle structure and function with age throughout the life span of the *mdx* mouse have only recently been appreciated (20, 23, 49), and very few genetic studies have focused on the effect of the age of the mice (50). Furthermore, although interesting, none of these studies have resolved the issue as to whether the absence of dystrophin and the impaired structure and function of the diaphragm and the heart affect the life span of the *mdx* mouse.

A previous study attempted to establish, under conventional housing conditions, the consequences of dystrophin deficiency on the survival of *mdx* mice (42, 51), but by 18 months *mdx* mice had difficulty grooming and obtaining food and water unaided, and the last *mdx* mouse died at 24 months. These observations were inconsistent with our studies that examined groups of six *mdx* mice for up to 28 months of age (20). To resolve the issue, for the first time a true life span study of the *mdx* mouse was designed under specific pathogen-free housing conditions for cohorts of male and female *mdx* and C57Bl/10 wild-type mice, with deaths restricted to natural causes. The hypothesis was tested that, compared with wild-type mice, the absence of dystrophin in the striated muscles of *mdx* mice shortens the life span. The results validate the previous observations of the status of the *mdx* mouse as a highly effective model system in which to pursue the underlying pathological mechanisms of DMD disease, causes of the shortened life span, and potential therapeutics for remediation of DMD.

MATERIALS AND METHODS

Animals

Breeding colonies for this study were initially purchased from The Jackson Laboratory (Bar Harbor, ME, USA). The breeding pairs consisted of wild-type mice (C57BL/10ScSn/J) and *mdx* mice (C57BL/10ScSn-*Dmd*^{mdx}/J) and were housed in a specific pathogen-free barrier facility at the University of Michigan. Litters of mice were weaned at 3 wk of age and separated into cages of male or female mice. To monitor life spans, 83 wild-type mice (38 male and 45 female) and 94 *mdx* mice (47 male and 47 female) were monitored daily after weaning. Dates were recorded for all mice that died spontaneously during the study. In other cases, mice that showed signs of distress such as visible tumors, listlessness, or that stopped eating and were deemed close to death, were sacrificed and the veterinary staff performed a necropsy on the carcass. Veterinary care for the mice was provided by the University of Michigan Unit for Laboratory Animal Medicine (ULAM) faculty members and veterinary residents. All protocols were performed in accordance with the guidelines of the University of Michigan's Committee on the Use and Care of Animals and the USPHS *Guide for the Care and Use of Laboratory Animals* (DHHS Pub. No. 85–23 (NIH), Revised 1985, Office of Science and Health reports, Bethesda, MD, USA). The mice were housed up to four mice per cage in cages with microisolator lids and 1/8 Bed-O-Cobs bedding (The Andersons, Maumee, OH, USA). Equal 12 h light/dark cycles were maintained with the changeover at 8 AM and 8 PM. The mice were fed *ad libitum* with Purina Laboratory Rodent Diet 5001 (PMI Nutrition International, St. Louis, MO, USA).

Life span analysis/necropsy

The status of the *mdx* and wild-type mice entered in the "Life span Study" were monitored by ULAM caretakers at least twice a day. Mice that appeared at risk were monitored even more frequently. The vast majority of the mice in each of the four groups, male and female and *mdx* and wild-type, died of natural causes and were found dead in their cages. Ten mice were diagnosed with life-threatening tumors or conditions that the pathologist deemed warranted euthanasia. Cage

cards were maintained for each mouse in each cage, with the date of birth registered; at the time of death, the date of the death was cited and the age in months and days was calculated. When the cause of death was known, the cause was recorded on the cage card. Carcasses were examined by a veterinarian pathologist, and tumors or abnormal growths were dissected from the carcass and prepared for the appropriate analyses.

Muscle histology

For analysis of muscle morphology, the heart, tibialis anterior, extensor digitorum longus, quadriceps, soleus, diaphragm, tongue, and gastrocnemius muscles were excised rapidly after sacrifice, coated in Optimal Cutting Temperature (O.C.T; Sakura Finetek, Torrance, CA, USA) and frozen in liquid nitrogen-cooled isopentane. Serial cryosections of 5 μm thickness that had equilibrated to room temperature were stained with hematoxylin and eosin-phloxine (H&E). Coverslips were positioned on the slides and attached using a drop of Permount (Fischer Scientific, Hampton, NY, USA) and dried at room temperature overnight before viewing under a light microscope. Brightfield photographs were taken using a Nikon E1000 microscope (Melville, NY, USA) and a SPOT-2 camera system (Diagnostic Instruments, Sterling Heights, MI, USA).

Tumor immunohistochemistry

Tumors were excised from the underlying skeletal muscle mass and frozen cryosections were prepared as described in the previous section. Sections were incubated with antibodies against MyoD, Pax7, desmin, and myogenin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunohistochemistry was performed using the Vectastain ABC kit (Vector Laboratories, Burlingame CA, USA). Briefly, tissue was fixed with cold methanol for 2 min. Slides were incubated with blocking solution (1% Tween-20, 3% BSA, 2% gelatin in PBS) for 30 min, followed by primary antibody [MyoD, myogenin, and Pax7 (1:400), desmin (1:100)] for 30 min, then biotin-labeled secondary antibody (Vector Laboratories, Burlingame CA, USA) for another 30 min, streptavidin-horseradish peroxidase (Vector Laboratories, Burlingame CA, USA) for 15 min, and AEC (3-amino-9-ethylcarbazole) (Sigma, St. Louis, MO, USA) substrate for 10 min, which produces an insoluble end product that is red in color. Slides were washed three times for 5 min with PBS and 1% BSA between incubations. Photography was as described in the previous section.

RESULTS

Longevity of *mdx* and wild-type mice

Compared with the longevity of wild-type mice, the longevity of the *mdx* mice was reduced substantially (**Fig. 1*A***). The first male and female *mdx* mice began to die at 14 months of age whereas the wild-type mice did not begin to die until 18 months (males) and 20 months (females). The longest lived of the *mdx* mice died at 28 months, whereas the longest lived wild-type mouse survived until 36 months. For average life span, female wild-type mice lived 27.0 months and male wild-type mice lived 26.5 months (Fig. 1*B*). In contrast, the average life span for female *mdx* mice was 22.5 months and for male *mdx* mice 21.5 months (Fig. 1*B*). These numbers represent a 17% reduction in life span for female *mdx* mice and a 19% reduction for male *mdx* mice.

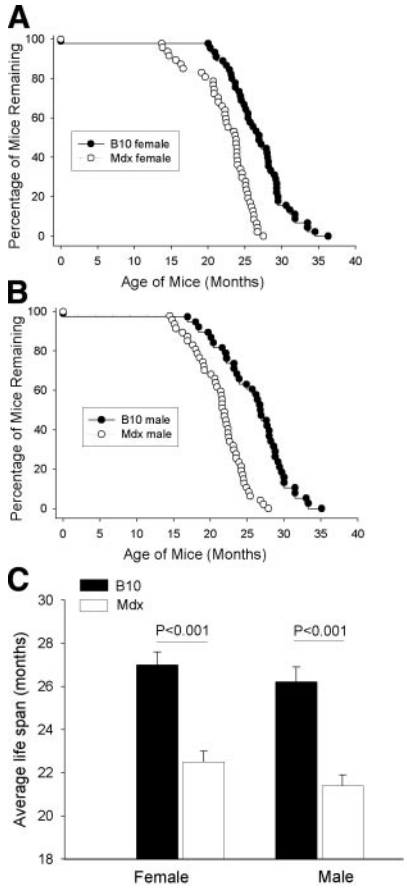

Figure 1. Life span analysis for wild-type and *mdx* male and female mice. Graphs showing the age at death for the female (*A*) and male (*B*) mice. Circles denote the age at which each animal died. *C)* Histogram showing the average age at death of male and female wild-type (C57BL/10) and *mdx* mice. The average life span between wild-type and *mdx* males and between wild-type and *mdx* females was highly significant, as shown.

Morphological analysis of muscles

Because significant histopathological abnormalities were observed in both the diaphragm and heart muscles of the old *mdx* mice, a reasonable assumption is that their reduced life span resulted primarily from respiratory and/or cardiac failure, as has been observed in boys with DMD (14, 52). The obvious exceptions were the mice that developed tumors (see below). The diaphragm muscle has been described as the most severely affected muscle in the *mdx* mouse (23, 24). In young *mdx* mice, the diaphragm muscles display relatively little inflammation compared with that evidenced in diaphragm muscles of 26 months of age *mdx* mice, and none was observed in wild-type diaphragms at 24 months (**Fig. 2**). For both old, 26 months of age, male and female *mdx* mice, the diaphragm muscle displayed

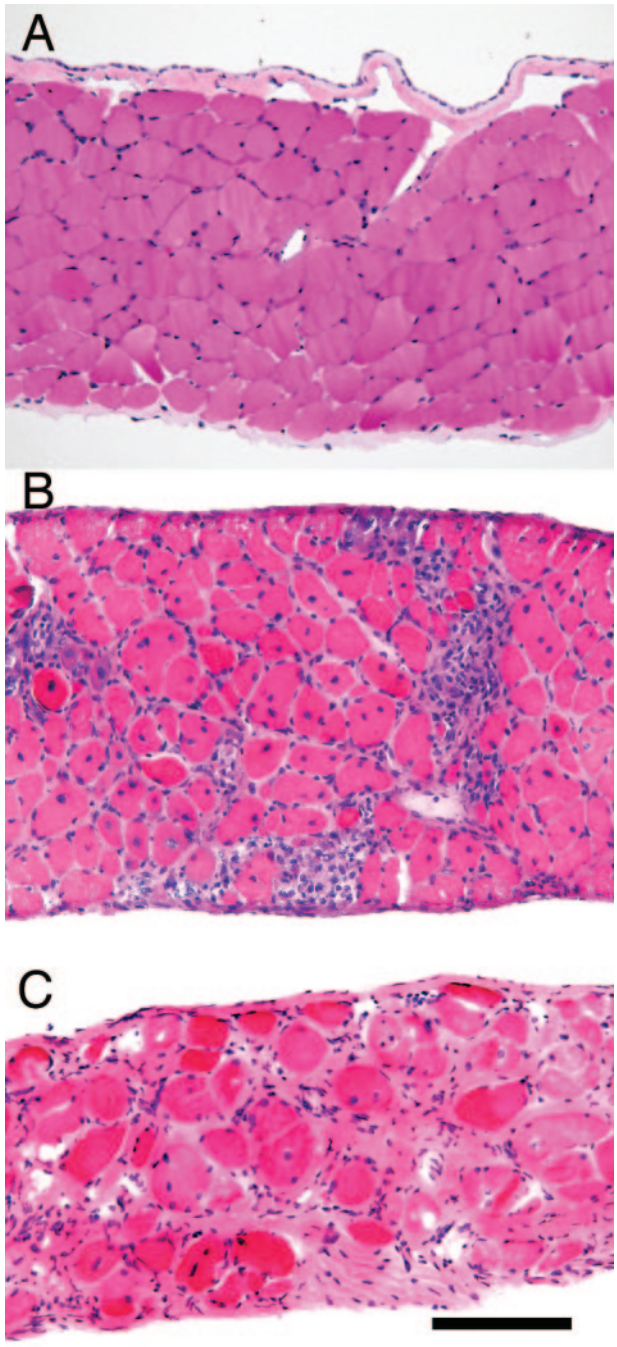

Figure 2. Histology of wild-type and *mdx* mouse diaphragm muscles. Diaphragms were excised, frozen in O.C.T., and 5 μm cryosections were prepared and stained with hematoxylin and eosin. *A)* 24-month-old wild-type mouse diaphragm. *B)* 4-month-old *mdx* mouse diaphragm. *C)* 26-month-old *mdx* mouse diaphragm. Scale bar = 250 μm.

a large degree of fibrotic infiltration and loss of muscle fibers (Fig. 2*C*). Surprisingly, the most severely affected muscle other than the diaphragm was the tongue, a muscle that has not been studied extensively in the *mdx* mouse. The fibrosis and loss of muscle fibers were particularly severe in the central portion of the tongue (**Fig. 3**). The hearts of old *mdx* mice displayed frequent scattered areas of fibrotic lesions and mononuclear cell infiltration (**Fig. 4**). The lesions were characterized by scattered fibrosis, with no suggestion of any regional specificity in the occurrence of the lesions.

As expected from the extensive histochemical and histological studies that have focused on the limb muscles of young *mdx* mice (37, 39, 42, 51, 53, 54), the limb muscles display the characteristic features of dystrophic muscle pathology. For young *mdx,* compared with age-matched wild-type mice, extensive morphological and functional differences have been described in detail (for references see above). Furthermore, the maximum force and power of soleus and extensor digitorum longus (EDL) muscles have been compared for *mdx* and wild-type mice from 6 to 26 months of age (20). In contrast, the dramatic increase in the degree of morphological abnormalities in the skeletal muscles of old compared with young *mdx* mice have not been reported. These abnormalities include increased necrosis, fibrosis, and accumulation of adipocytes. Representative photographs of skeletal muscles from a 24-month-old wild-type mouse are displayed in **Fig. 5*A, B*** and from 4-, 6-, and 26-month-old *mdx* mice in Fig. 5*C–H*. The photographs were of muscles obtained from mice that were sacrificed while in good health and from mice that were not a part of the life span study shown in Fig. 1. As expected, the 24 month wild-type muscles displayed no morphological abnormalities. In contrast, the *mdx* mice displayed typical dystrophic features at each age examined. The dystrophic features included centrally located myofibers, necrotic fibers, small caliber regenerating fibers, moderate amounts of fibrosis, and some fatty infiltration. All skeletal muscles exam-

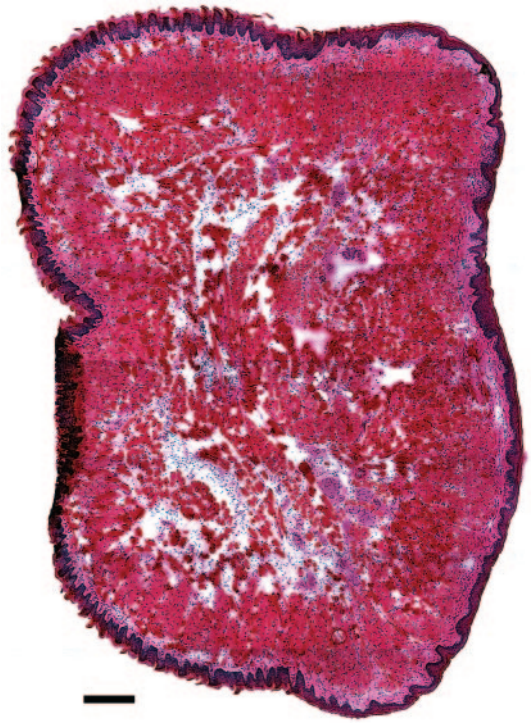

Figure 3. Montage images of the most affected nonrespiratory muscle in old *mdx* mice. 5 μm transverse cryosection of the entire tongue. Scale bar: 10 μm.

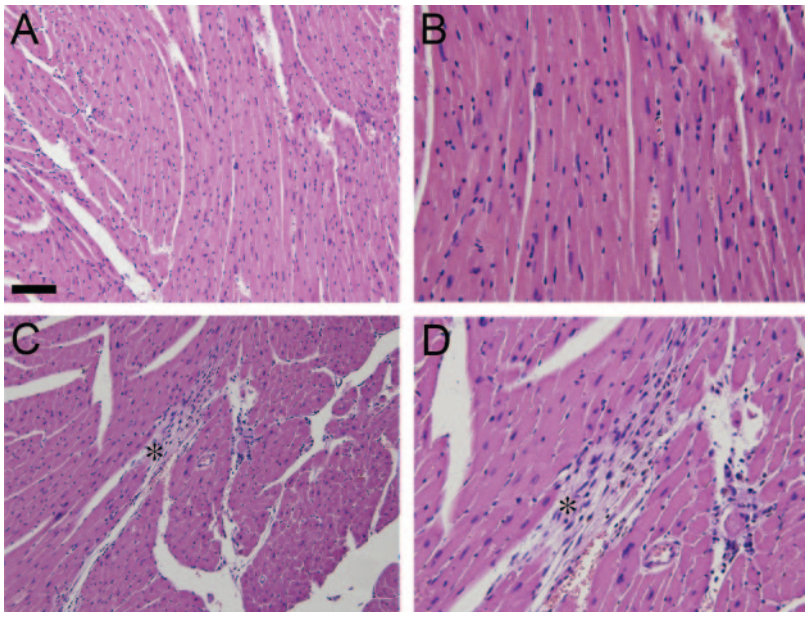

Figure 4. Histology of 20-month-old control and *mdx* mouse hearts. The hearts were excised, frozen in O.C.T., and 5 μm cryosections were prepared and stained with hematoxylin and eosin. *A, B)* 20-month-old hearts from control C57Bl/10 mice; *C, D)* 20-month-old hearts from *mdx* mice. *C, D)* Asterisks denote areas of fibrosis. Scale bar: *A, C)* 100 μm; *B, D)* 50 μm.

ined displayed a moderate to severe degree of dystrophic histopathology by 26 months of age (Fig. 5*C–H*). For example, note the increased necrosis fibrosis and adipocyte accumulation in the 26 months soleus compared with the same muscle group at 4 months (Fig. 5*C, D*). In our experience, the soleus is the most morphologically dystrophic limb muscle in the *mdx* mouse at any age (Figs. 2 and 3 and data not shown).

Necropsy analysis

With the exception of clearly defined tumors (see below), necropsy results were generally uninformative, although one male wild-type mouse developed a suppurative pleuritis and was sacrificed. Analysis of multiple tissues revealed no specific findings in the wild-type mice.

Incidence of tumoregenesis

A surprising outcome of our study was the observation that *mdx* mice were prone to develop sarcomas, particularly rhabdomyosarcomas. Only three of the wild-type mice developed tumors, while tumors were found in seven *mdx* mice. One wild-type and one *mdx* mouse, both females, each developed a lymphosarcoma at ~1.5 years of age. Two additional male wild-type mice developed sarcomas by 30 months of age: one an osteosarcoma, and the other a hemangiosarcoma. In contrast, 6 of the 94 *mdx* mice developed rhabdomyosarcomas, an extremely rare tumor in mice (55). These tumors were found in three male and three female animals. The average age at which the rhabdomyosarcomas were detected was 20 months, with a range between 16.5 and 24 months. No rhabdomyosarcomas were observed in any wild-type mice.

Three tumors were analyzed further to obtain details on the nature of the rhabdomyosarcoma. Low power imaging of one tumor showed normal skeletal muscle connected to a large mass of tightly packed small cells interspersed with larger, often multinucleated cells displaying occasional cross striations (**Fig. 6*A***). A second tumor imaged at higher magnification (Fig. 6*B*) showed randomly arranged eosinophilic cells with considerable variation in cell size and shape, including small, round tumor cells with hyperchromatic nuclei and large, polygonal-shaped tumor cells with abundant eosinophilic cytoplasm, which often contain diagnostic cross striations (inset). The histology of these tumors was suggestive of the alveolar type of rhabdomyosarcoma. To obtain immunological confirmation of the tumor type, cryosections from three tumors were immunostained with markers of skeletal muscle myogenesis. As shown in Fig. 6*C–F*, essentially all tumor cells were positive for myogenin and Pax7 expression while ~50% were positive for MyoD expression. The histology combined with widespread myogenic regulatory gene expression is most compatible with the alveolar form of human rhabdomyosarcoma (55–59).

DISCUSSION

The *mdx* mouse is the most widely studied animal model for Duchenne muscular dystrophy (17). These mice carry a premature stop codon in exon 23 that leads to the absence of detectable dystrophin except in rare, revertant myofibers (18, 60). Most studies of muscle pathology in *mdx* mice have reported that the histopathological abnormalities are milder than in DMD, and numerous reports have stated that the pathology is not progressive except for the diaphragm muscle (21, 24). Invariably, the masses of the limb muscles of young *mdx* mice show a substantial hypertrophy of from 20% to 30% in some studies (21, 38, 41, 42) to as much as 30% to 60% in others (20, 42). As with the pseudo-hypertrophy observed in the calf mus-

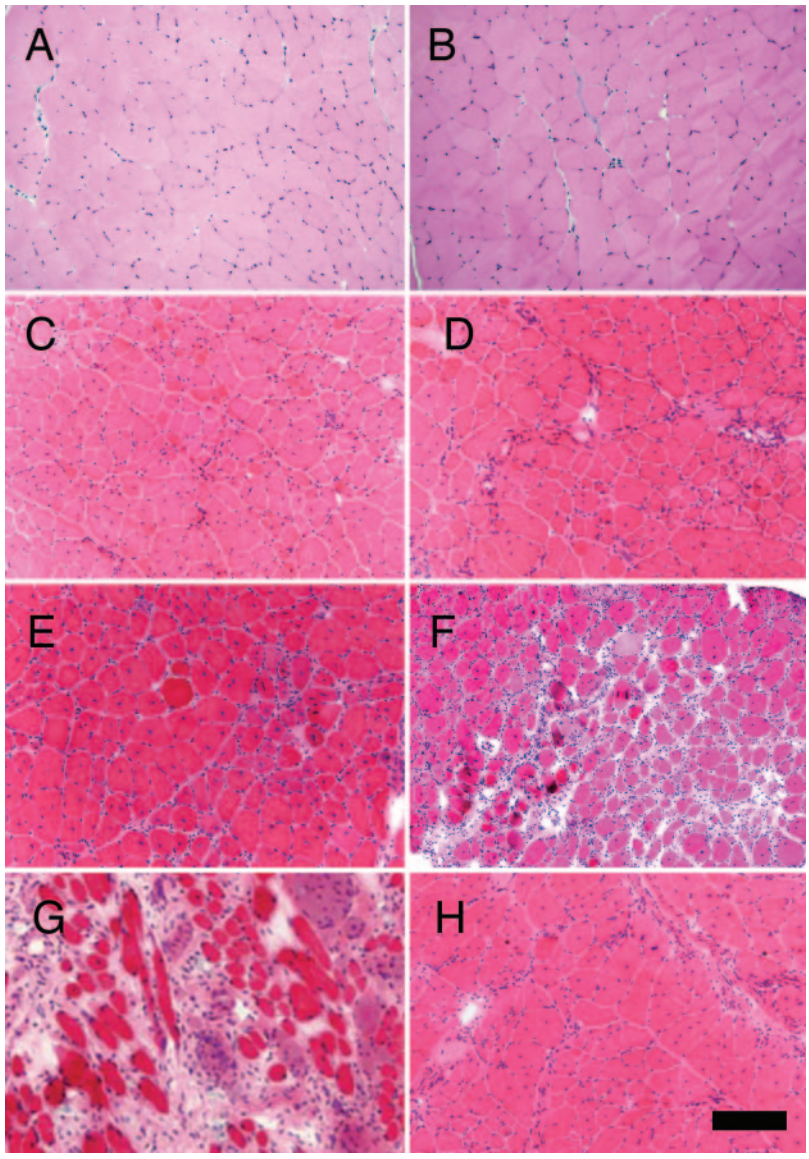

Figure 5. Histology of wild-type and *mdx* mouse limb muscles. The indicated muscles were excised, frozen in O.C.T., and 5 μm cryosections were prepared and stained with hematoxylin and eosin. *A)* 24-month-old wild-type mouse TA; *B)* 24-month-old wild-type mouse quadriceps; *C)* 6-month-old *mdx* mouse TA; *D)* 26-month-old *mdx* mouse TA; *E)* 4-month-old *mdx* mouse soleus; *F)* 26-month-old *mdx* mouse soleus; *G)* 26-month-old *mdx* mouse tongue; *H)* 26-month-old *mdx* mouse quadriceps. Scale bar = 250 μm.

cles of young boys (1), the increased mass of the muscles of the *mdx* mice is attributed to the ongoing cycle of degeneration and regeneration. The damaged fibers with impaired function result in a 25% to 35% loss in maximum specific force (kN/m^2) and normalized power (W/kg), respectively (20).

Life span analyses

The studies of *mdx* mice have focused mainly on comparisons of young age groups of *mdx* and wild-type mice (37, 39). A few have extended the observations of a limited number of conventionally housed *mdx* mice (n=14) throughout their life span (42, 51), but unfortunately these mice were short-lived, with the oldest dying at 24 months. Despite these somewhat random observations, to date there has not been a detailed analysis of the life spans of *mdx* and wild-type mice under controlled conditions that include a specific pathogen-free environment. Our results reveal that muscle histopathology of the old *mdx* animals is more extensive and widespread than what has been observed in young *mdx* mice in all muscle groups analyzed. Furthermore, for female and male *mdx* mice, a 17% and 19% reduction in life span was observed compared with wild-type mice of the same gender. A strikingly high incidence of rhabdomyosarcoma was observed for *mdx* mice, an otherwise extremely rare tumor in mice (55, 58).

The question of interest is whether the observed reduction in life span observed in *mdx* mice is of general significance and thus relevant to the age-dependent progression of the human pathology of muscular dystrophy. A controlled life span study has not been reported in the canine model of DMD, but these dogs are severely affected and many die young (16, 61). Recently, a reduced life span has also been observed in a *Drosophila* model where dystrophin expression was suppressed using RNAi technology (R. Bodmer, personal communication). These observations suggest that interference with dystrophin function in mammals as well as in an insect model affects

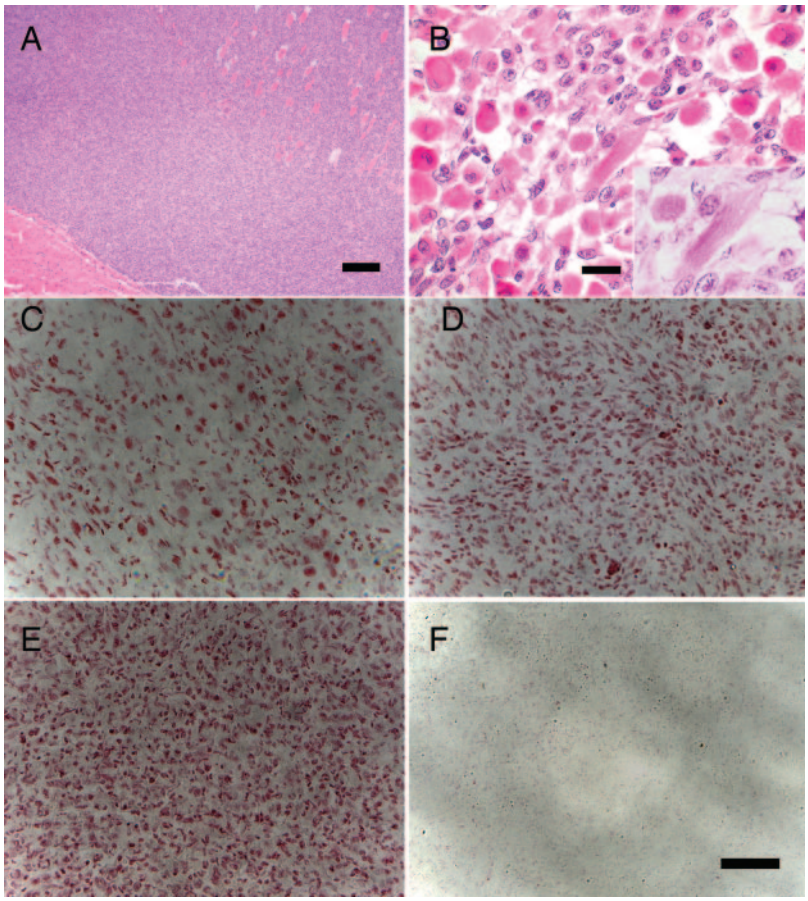

Figure 6. Rhabdomyosarcomas from *mdx* mouse muscles. Tumors were excised, frozen in O.C.T., and 5 μm cryosections were prepared and processed as described below. *A)* Low power image of a tumor stained with hematoxylin and eosin. A section of the underlying skeletal muscle layer is visible in the lower left quadrant. Note the sporadic islands of eosinophilic striated structures. Scale bar = 250 μm. *B)* A second tumor was also sectioned and stained with hematoxylin and eosin and photographed at higher magnification. Note the randomly arranged eosinophilic cells with considerable variation in cell size and shape, including small, round tumor cells with hyperchromatic nuclei containing prominent nucleoli. Also present are large, polygonal-shaped tumor cells with abundant eosinophilic cytoplasm, which often contain diagnostic cross striations (inset). Scale bar = 50 μm; inset is a 2 × magnification. *C–E)* Frozen sections from a third tumor with morphological features similar to those in panel *B* were stained with antibodies against various myogenic regulatory proteins. *C)* Staining for MyoD; *D)* staining for myogenin; *E)* staining for Pax7; *F)* no primary antibody. Approximately 50% of the cells in the tumor were positive for MyoD, while > 90% stained positive for myogenin and Pax7. Scale bar for panels *C–F* is shown in panel *F* and represents 250 μm.

longevity and that this aging phenotype is likely due to a muscle autonomous effect of dystrophin.

Impact of aging on histology

The degree of dystrophic pathology observed in the limb muscles of old *mdx* mice was visibly worse than in 4- and 6-month-old *mdx* mice, but not nearly as extensive as observed in young or old *mdx* diaphragm muscles (21–24, 46). In contrast, the *soleus* and tongue muscles of the older *mdx* mice displayed an extensive dystrophic pathology intermediate in severity between the diaphragm and quadriceps/TA muscles. The extensive histopathology noted in the tongue of the *mdx* mouse has not been reported previously. Muscles of the tongue are organized in a complex pattern consisting of longitudinal, transverse, and vertically oriented myofibers (62). This critical organ is used in various ways during normal suckling, chewing, swallowing, and breathing. In laboratory mice, which lead a sedentary life, the tongue and diaphragm muscles are among the most active and continuously used, which may in part explain the greater degree of dystrophic histopathology relative to other skeletal muscles. While the diaphragm and *soleus* are among the slowest muscles of the mouse (43, 63), the intrinsic tongue muscles are composed almost exclusively of fast-twitch fibers in both rats and mice, suggesting that the degree of dystrophic pathology in *mdx* mice is not a simple reflection of fiber type composition (64–66). The severity of the pathology suggests that the tongue muscle might be useful in the analysis of potential therapeutic interventions.

Contractile properties of skeletal muscles of old *mdx mice*

The diaphragm muscles of 4–6 months and 24 months wild-type mice have specific forces of ~ 225 kN/m^2 (23). In contrast, the diaphragm muscle is the only skeletal muscle of the *mdx* mouse to demonstrate a progressive loss of specific force throughout the life span (20, 23, 49). When the specific force (kN/m^2) is calculated for the total cross-sectional area (CSA) of the diaphragm, with both viable and necrotic tissue included, the specific force is 125 kN/m^2 for a 2-month-old *mdx* mouse, 95 kN/m^2 for a 4- to 6-month-old, and 46 kN/m^2 for a 24-month-old (49). These values are 55%, 42%, and 20% of the values for wild-type mice. Furthermore, at 24 months of age, only 41% of the CSA of the diaphragm muscles of *mdx* mice is composed of viable skeletal muscle fibers (23). Consequently, for 24-month-old *mdx* mice, with a specific force of 116 kN/m^2, even the force development for the viable muscle is still greatly diminished compared with that of age-matched wild-type mice. The present data (Fig. 2*C, D*) are consistent with the magnitude of the histological evidence of deterioration observed in the diaphragm muscle.

A study of structure-function relationships of the EDL and soleus muscles of male *mdx* compared with those for wild-type mice at 6, 17, 24, and 28 months of age (23) showed that throughout the life span of mice, although absolute values for muscle mass ranged from ~40% higher at 5 months to ~20% higher at 28 months, the maximum absolute force and power changed minimally and maximum specific force (kN/m^2) and normalized power (W/kg) were only ~20% lower (23). Unlike the minor age-related changes in the absolute and normalized values for force and power developed by the limb muscles, the capability of the diaphragm muscle to develop force and power undergoes a highly significant, almost linear age-related loss that, by 24 months, of age may affect respiratory function and possibly life span.

Heart function

Advances in cellular and organ-level technologies have allowed new insight into the mechanisms of cardiac dysfunction in mouse models of disease and aging. Using microcarbon fiber technology, the phenotype of single intact cardiac myocytes from *mdx* mice has been described (35). The passive tension extension properties of myocytes from *mdx* and wild-type mice demonstrate that during a stretch of physiologically relevant magnitude, the dystrophin deficiency renders the cell less compliant owing to transient increases in intracellular Ca^{2+}. At a critical threshold, excessive Ca^{2+} overload initiates irreversible hypercontracture and death of the myocyte. At the organ-level *in vivo*, micromanometry catheters demonstrated reduced systolic performance of the heart of the *mdx* mouse and increased acute cardiac failure during cardiac stress testing (35). These deficits in cardiac function likely contribute significantly to the reduced life span of the *mdx* mouse.

Rhabdomyosarcomas in dystrophic mice

The occurrence of spontaneous alveolar rhabdomyosarcomas in dystrophic *mdx* mice is a striking finding since this muscle tumor has not previously been observed to arise spontaneously in mice, and it arises only rarely after significant genetic manipulation of the mouse genome (55, 58, 67, 68). We observed 6 rhabdomyosarcomas in 94 *mdx* mice at an equal frequency in both males and females. Of interest was the observation that no tumors were detected earlier than 16 months of age. Rhabdomyosarcoma does not appear to be unique to *mdx* mice in this longevity study, as one of our groups (Washington) observed numerous additional rhabdomyosarcomas in their *mdx* mouse colonies that were not a part of the longevity study. The incidence of rhabdomyosarcoma in this general colony cannot be determined precisely, since most of the mice were not aged extensively due to the nature of their experimental involvements. Despite these limitations in the experimental design, in a 1 year period we observed 23 tumors associated with skeletal muscles in our *mdx* mouse colony. While most tumors were not analyzed extensively, those that were had morphological characteristics of rhabdomyosarcoma and four out of four examined displayed widespread (>90% of the tumor cells) expression of myogenin (data not shown). The tumors were found with approximately equal frequency in all four limbs. We have never observed a rhabdomyosarcoma in our wild-type mice, nor have any been observed in our transgenic *mdx* mice expressing a functional dystrophin (*e.g.*, ref. 22). We have, however, observed rhabdomyosarcomas in transgenic *mdx* mice expressing nonfunctional dystrophins or other transgenes (*e.g.*, ref. 8).

In humans, rhabdomyosarcoma is a malignant tumor originating from striated muscle. Rhabdomyosarcoma of the head and neck is primarily a disease of the first decade of life, and it is the most common soft tissue sarcoma observed in childhood (69). Investigators have proposed that these tumors are derived from primitive mesenchyme that retained its capacity for skeletal muscle differentiation (70, 71). Recently, the origin of rhabdomyosarcoma has been directly associated with mutations within satellite cells of adult muscle (72), and most cases are associated with the expression of a *Pax3:* or *Pax7:Fkhr* fusion gene that has arisen by chromosomal translocation (73). To our knowledge, there are only two cases reported of rhabdomyosarcoma in DMD patients—one of the alveolar form and another of the embryonal form (74, 75). Rhabdomyosarcomas may be less frequent in DMD patients than in the *mdx* mouse because DMD patients rapidly lose muscle mass and their satellite cell numbers drop significantly as they prematurely acquire proliferative senescence (76). As shown in Figs. 2 and 3, limb muscles of *mdx* mice never lose a major proportion of their myofiber content, suggesting a significantly more robust ability to regenerate myofibers throughout their life span. Also, spontaneously immortalized myoblast lines are easily derived from mouse limb muscles, whereas none have been successfully generated from humans (77).

The only previous example of alveolar rhabdomyosarcomas in mice was observed in transgenic animals that conditionally expressed a *Pax3:Fkhr* fusion gene in differentiating myocytes (55). Why are *mdx* mice unique among all nontransgenic mice in displaying a susceptibility to develop rhabdomyosarcoma? We speculate that the lifelong continuous myofiber degeneration and regeneration that characterize this animal model are associated with continuous and massive activation and proliferation of satellite cells, which greatly increases the chance of developing random and spontaneous mutations. If the appropriate combination of mutations arise or accumulate in a satellite cell, the progeny of the satellite cell might lead to formation of a rhabdomyosarcoma on differentiation into a myocyte, which would then be unable to complete terminal differentiation into myotubes and myofibers. Further analysis of the tumor cells will be required to fully

understand this phenomenon. Despite the limitations, our results suggest that *mdx* mice might be a useful model system not only for DMD, but also to study the origin, progression, and treatment options for alveolar rhabdomyosarcoma. The results also raise the possibility that treatments for DMD that do not slow ongoing myofiber necrosis and regeneration, but which lengthen life span, could lead to an increase in the incidence of rhabdomyosarcoma in older patients. FJ

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