Characterization of Dystrophin Deficient Rats: A New Model for Duchenne Muscular Dystrophy

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Abstract

A few animal models of Duchenne muscular dystrophy (DMD) are available, large ones such as pigs or dogs being expensive and difficult to handle. Mdx (X-linked muscular dystrophy) mice only partially mimic the human disease, with limited chronic muscular lesions and muscle weakness. Their small size also imposes limitations on analyses. A rat model could represent a useful alternative since rats are small animals but 10 times bigger than mice and could better reflect the lesions and functional abnormalities observed in DMD patients. Two lines of Dmd mutated-rats (Dmdmdx) were generated using TALENs targeting exon 23. Muscles of animals of both lines showed undetectable levels of dystrophin by western blot and less than 5% of dystrophin positive fibers by immunohistochemistry. At 3 months, limb and diaphragm muscles from Dmdmdx rats displayed severe necrosis and regeneration. At 7 months, these muscles also showed severe fibrosis and some adipose tissue infiltration. Dmdmdx rats showed significant reduction in muscle strength and a decrease in spontaneous motor activity. Furthermore, heart morphology was indicative of dilated cardiomyopathy associated histologically with necrotic and fibrotic changes. Echocardiography showed significant concentric remodeling and alteration of diastolic function. In conclusion, Dmdmdx rats represent a new faithful small animal model of DMD.

Introduction

DMD is a severe X-linked muscular dystrophy due to mutations of the DMD gene. It affects all voluntary muscles as well as the heart and breathing muscles in later stages. Despite recent promising new treatments the average life expectancy is severely reduced in DMD patients and a better understanding of the disease and faster evaluation of new treatments are needed [1].

Both large and small animal species deficient for dystrophin have been described and have been extremely useful for pre-clinical studies of DMD. Although they display more features of the human clinical phenotype than mdx mice, large dystrophin-deficient animals such as dogs [2] and pigs [3,4], suffer from individual variability and are costly and time consuming. Mdx mice [5,6] have the advantage of low maintenance costs. Sufficient numbers of animals can also be easily characterized to reach high statistical power. On the other hand, mdx mice exhibit only minor clinical dysfunction [6] and their small size imposes limitations in the analysis of several aspects of the disease. Although each animal model has its own limitations, they have all been essential for the development of treatment strategies that target dystrophin absence, disease progression or muscle regeneration [7]. Nevertheless, new animal models are needed to help pre-clinical research on DMD.

We hypothesized that the rat could represent a useful model of DMD. One of its advantages of over mice is that its behavior is much better characterized. Rats have finer and more accurate
motor coordination than mice and exhibit a richer behavioral display, including more complex social traits [8]. Rats have a convenient size since they are 10 times larger than mouse but are still a small laboratory animal model and allow studies with high statistical power. Until recently, the rat model lacked genetic engineering tools for introducing targeted genetic mutations. But in the last years, we and others have used in rats sequence-specific nucleases, such as meganucleases, zinc-finger nucleases, TALENs, and CRISPRs/Cas9, to efficiently generate precise gene mutations [9–13].

To generate dystrophin-deficient rats, we generated TALENs for Dmd that were microinjected in rat zygotes allowing generation of two Dmd<sup>mdx</sup> rat lines. The muscles of both lines displayed undetectable levels of dystrophin as evaluated by western blot analysis and less than 5% of dystrophin positive fibers by immunohistochemistry. At 3 months of age, forelimb, hindlimb and diaphragm muscles showed severe fiber necrosis and a strong regeneration activity. At 7 months of age, regeneration activity was decreased and muscle showed abundant peri- and endomysial fibrosis with some adipose tissue infiltration. Muscle strength and spontaneous activity were decreased and fatigue was a prominent finding of muscle function analysis. Cardiac muscle was also affected with necrosis and fibrosis and showed signs of progressive dilated cardiomyopathy. Echocardiography showed significant concentric remodeling and alteration of diastolic function. These lesions in skeletal muscle and heart closely mimic those observed in DMD patients. These results indicate that Dmd<sup>mdx</sup> rats represent a new invaluable small animal model for pre-clinical research on DMD.

**Materials and Methods**

**Animals**

This study was approved by the Ethics Committee on Animal Experimentation of the Pays de la Loire Region, France, in accordance with the guidelines from the French National Research Council for the Care and Use of Laboratory Animals (Permit Numbers: CEEA-PIL-2011-45 and CEEA-PIL-01579.01). All efforts were made to minimize suffering. Sprague-Dawley (SD/CD) rats were obtained from Charles River (L’Arbresle, France). The rats were housed in a controlled environment (temperature 21±1°C, 12-h light/dark cycle). Before blood collection, animals were anesthetized with a mixture of ketamine (100 mg/kg, Imalgene, Merial, Lyon, France) and xylazine (10 mg/kg, Rompun, Bayer, Leverkusen, Germany). Rats were then sacrificed by intravenous administration of sodium pentobarbital (300 mg, Dolethal, Vetoquinol UK Ltd, Buckingham, UK). Just after sacrifice, the body weight (g) and the body length (cm) of each rat were determined to define the body mass index calculated as body weight/(body length)<sup>2</sup> (g/cm<sup>2</sup>).

**Design and production of TALE nucleases**

TALE nucleases targeting Dmd exon 23 were designed to recognize the following sites on Rat genome Assembly (Rnor_5.0 - GCA_000001895.3/chrX: 51,878,333-52,510,293):

\[
\text{TCTGCAAAAGCTTTGGAAGCAGACAAAATGGCTTCAACTATCGTGAATTGCGA.}
\]

TALE nucleases were produced, as previously described, by unit assembly method adapted from Huang et al. 2011 [14–16]. For each TALE nuclease subunit, the fragment containing the 16 RVD segment was obtained from single unit vectors: A (NI), T (NG), G (NN) and C (HD), derived from plasmids kindly provided by the laboratory of Dr B. Zhang (Peking University, China). The assembled TALE RVD (DMD-L: TCTGCAAAAGCTTTGGAA; DMD-R: TGGCATTTCAGATTTGGA ) were subcloned in the pVax vector containing the 17th half RVD, the A152/+63 N- and C-terminal truncation points as described by Miller et al. 2011 [17] and contained the wt Fok I catalytic domains or FokI heterodimer-forcing mutations (ELD or KKR) [18] that were constructed by site-directed mutagenesis starting from Addgene plasmids 21872 et 21873 kindly made available by the Joung lab. Assembled TALE nucleases DNAs were cloned into the pVAX vector for mRNA production and in vitro cell transfection.

**In vitro assay of TALE nucleases**

Each subunit of TALE nuclease (0.75 and 1.5 μg each) was nucleaseected into 4.10⁵ C6 cells (Sigma) in 20 μL of V solution using AMAXA FF137 program. Cells were grown into 12 well dishes and collected 48 h after nucleasection. Genomic DNA was extracted with E.Z.N.A. Tissue DNA Kit (OMEGA Biotek). The genomic region encompassing the TALE nuclease target sites was amplified with the primers listed below, typically using 50 ng of genomic DNA, and phusion polymerase (New England Biolabs) following manufacturer instructions. Unpurified PCR product (10 μL, out of 25 μL of PCR reaction) was supplemented with 10 μL of NEBuffer 2 (2X), melted, and annealed (5 min at 95°C, 95°C to 25°C at -0.5°C/30 sec, and 15 min at 4°C) to form heteroduplex DNA. The annealed DNA was treated with 1.5 units of T7 endonuclease I (New England BioLabs) for 20 min at 37°C and run on a 2.4% agarose gel.

**Off-target mutation analysis**

Potential off-target sites were identified by bioinformatics using the PROGNOS program [19] with parameters set for heterodimer-forcing TALEN subunits and looking for pairs of potential TALEN binding sequences with up to 6 mismatches to each cognate target sequences of the TALEN pair separated by spacer of 10 to 30 bp. Candidate off-target sites that were identified had at least a total of 8 mismatches to the TALEN target sequence in the DMD gene. We chose to further look for mutations at the 2

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**Figure 1. Targeted deletion in exon 23 of the rat Dmd gene via TALE nucleases.** DNA cleavage takes place in the spacer sequence located between TALEN DNA binding sequences indicated in blue. Dmd<sup>mdx</sup> rats carries a deletion of 11 nucleotides in exon 23 of the Dmd gene.

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In vitro transcription of TALE nucleases mRNA

As previously described, TALE nuclease plasmids were in vitro transcribed to mRNA and polyadenylated using the mMessage mMachine T7 Ultra kit (Ambion, Austin, TX) following the manufacturer protocol and purified using the MegaClear Kit (Ambion, Austin, TX), quantitated with a NanoDrop-1000 (Thermo Scientific) and stored at −80°C until use [12]. mRNAs encoding each monomer of TALE nucleases for each target sequence were mixed in TE 5/0.1 5 mM Tris-Cl pH 7.5, 0.1 mM EDTA in RNase DNase free water) and stored at −80°C until use. mRNAs were diluted to the working concentration and kept on ice during one day micro-injection procedures and then discarded.

### Microinjection of rat one-cell embryos

Prepubescent females (4–5 weeks old) were super-ovulated with pregnant mare serum gonadotropin (30 IU; Intervet, France) and followed 48 hours later with human chorionic gonadotropin (20 IU; Intervet, France) before breeding. Zygotes were collected for subsequent microinjection using a previously published procedure [12,20]. Briefly, a mixture of TALE nucleases mRNA was microinjected into the cytoplasm of fertilized one-cell stage embryos. Microinjected embryos were maintained under 5% CO2 at 37°C until reimplantation. Surviving embryos were then implanted immediately in the oviduct of pseudo-pregnant females (0.5 dpc) and allowed to develop until term.

### Analysis of Dmd mutation events

DNA from embryos or neonates was extracted from tail biopsy following treatment with Proteinase K as previously described [12]. To analyze mutations, we first used PCR followed by the T7 endonuclease I assay (as described above in the [12]). Briefly, a mixture of TALE nucleases mRNA was microinjected into the cytoplasm of fertilized one-cell stage embryos. Microinjected embryos were maintained under 5% CO2 at 37°C until reimplantation. Surviving embryos were then implanted immediately in the oviduct of pseudo-pregnant females (0.5 dpc) and allowed to develop until term.

### Dystrophin messenger analysis

Total RNA was extracted from muscles with TRIzol reagent (Invitrogen) and 500 ng of this RNA was reverse transcribed using random primers (Invitrogen) and M-MLV reverse transcriptase (Invitrogen). Detection of the Dystrophin mRNA was done by 25 cycles of PCR1 followed by 30 cycles of PCR2 using 1 µl of ADNc or 1 µl of PCR1, 2.5 U of GoTaq DNA polymerase (Promega), 1.5 mM of MgCl2 and 0.2 µM of each following primers (PCR1:
ex20ratDyst-F TCA GAC AAG CCT CAG AAC AA and
ex26ratDyst-R AGTTTTATCCAAACCAGCCT annealing
50°C; PCR2:ex22ratDyst-F AAT GCG CTA TCA AGA GAC
AA and ex24ratDyst-R TCT GCA CTG TTT GAG CTG TT
annealing 55°C). Final PCR products were migrated on an
1.5% agarose gel and revealed with ethidium bromide staining (Fig.
S6).

Western blot analysis
Total proteins from muscle were extracted using 400 µL of
RIPA extraction buffer containing protease inhibitors (Roche) and
ground with TissueLyser II (Qiagen). 50 µg of protein extracts
were loaded on a 3–8% Tris-Acetate Precast polyacrylamide gel of
NuPAGE Large Protein blotting kit (Invitrogen). After Red
Ponceau staining, membranes were incubated with two different
mouse anti-Dystrophin antibodies: NCL-DYS2 (1:100, Novocastra),
MANEX 1011C (1:100, MDA Monoclonal Antibody
Resource) and with an anti-GAPDH antibody (1:10000,
Imgenex). Detection was performed using a secondary anti-mouse
IgG HRP-conjugated antibody (1:2000, Dako) or secondary anti-
mouse IgG HRP-conjugated antibody (1:2000, Dako).
Immunoblots were revealed with ECL Western blotting substrate (Pierce)
and exposed to ECL-Hyperfilm (Amersham).

Histopathological evaluation
After gross examination, *tibialis cranialis*, *extensor digitorum
longus*, *biceps femoris* muscles of the hindlimb, *soleus* and *biceps
brachii* muscles of the forelimb, diaphragm and heart were
sampled and divided in two parts. Furthermore, *tibialis cranialis*,
*extensor digitorum longus*, *soleus* and heart were weighted. The
first part was frozen and 8-µm-thick serial sections were cut for
immunohistology and histoenzymology assays, the second part was
fixed in 10% neutral buffered formalin, embedded in paraffin wax,
and 5-µm-thick sections were cut and routinely stained with
hematoxylin eosin saffron for histopathological evaluation. Addi-
tional sections were stained with Picrosirius red stain for collagen,
Gomori’s trichrome and Alizarin red staining for calcium. All
lesions were reported by a skilled pathologist certified by the
European College of Veterinary Pathology.

Immunohistochemistry and histoenzymology assays
Immunohistochemical analysis involved the use of immunoper-
oxidase techniques on frozen sections. MANDYS110 clone 3H10,
provided by the Wolfson Centre for Inherited Neuromuscular
Disease, NCL-DYSB and NCL-DYS2 (Novocastra Laboratories,
Newcastle on Tyne, UK) mouse monoclonal antibodies were used
for dystrophin protein detection (1:50 for each one). Other
primary antibodies used were IVD5;A9 (1:50, Developmental Studies Hybridoma Bank, Iowa City, IA), NCL-g-Sarc (1:10, Novocastra Laboratories) and NCL-DRP2 (1:60, Novocastra Laboratories) for alpha-sarcoglycan, gamma-sarcoglycan, and utrophin detection respectively, NCL-MHCd (1:20, Novocastra Laboratories) for developmental myosin heavy chain isoform, anti-complement 5b-9 (1:250, Calbiochem, Strasbourg, France) and anti-CD3 (1:100, Dako, Glostrup, Denmark) for lymphocytes. Briefly, transverse cryosections were incubated in PBS with 5% normal goat serum (Dako) for 1 hour at room temperature. They were then incubated with primary antibody in 5% PBS overnight at 4°C and with biotinylated secondary antibodies (E433, 1:250, Calbiochem, Strasbourg, France) and anti-CD3 (1:100, Dako, Glostrup, Denmark) for lymphocytes.

Histopathology

Morphometric analysis was done using a digital camera (Nikon DFX 1200; Nikon Instruments, Badhoevedorp, the Netherlands) combined with image-analysis software (NIH; Nikon). To determine the proportion of dystrophin-positive fibers in the *biceps femoris* muscle of 3 and 7 month-old *Dmdmdx* rats (5 individuals in each group), a total of 1,000 fibers were counted in sections immunolabelled with Mabdy110 antibody and the percentage of fibers expressing dystrophin was determined. To determine the percentage of MyoHCDev fibers, at least 1,000 fibers were numbered on randomly selected microscopic fields. Fiber size was documented measuring minimal Ferret diameter on at least 250 fibers (292±61 per sample), on randomly selected microscopic fields in picrosirius red-stained sections. Fibrosis was determined as the ratio of areas rich in collagen using specific picrosirius staining on the total muscle area in an overall cross section. Repeatability was tested by the same operator measuring five times the same
sample. The intra-assay variations coefficients were always lower than 5%.

Serum creatine phosphokinase levels

Blood was collected just before sacrifice under anesthesia. Creatine phosphokinase (CK) activity was determined at the Central Laboratory of the National Veterinary School (E.N.V) at Nantes using a commercial kit (LaRoche Diagnostic). Results were expressed as CK levels [23].

Skeletal muscle function

The in vivo tests were performed in the same sequence for each rat, with equivalent time of rests between the tests [23].

Grip test. Rats were placed with their forepaws or four paws on a grid and were gently pulled backward until they released their grip [24]. A grip meter (Bio-GT3, BIOSEB, France), attached to a force transducer, measured the peak force generated. Five tests were performed in sequence with a short latency between each test, and the reduction in strength between the first and the last determination was taken as an index of fatigue [24]. Results are expressed in grams (g) and normalized to the body weight (g/kg).

ACTIMETER. The motor behavior was examined with an open field actimeter [25]. For this analysis, rats were individually placed in an automated photocell activity chamber (Leticia model LE 8811, Bioseb, France) which consists of a plexiglass chamber (45 cm × 45 cm × 50 cm) surrounded by two rows of infrared photobeams. The first row of sensors was raised at a height of 3 cm for measuring horizontal activity and the second row placed above the animal for vertical activity. The spontaneous motor activity was measured for 5 min using a movement analysis system (Bioseb, France), which dissociates resting and ambulatory time (s), distance traveled (cm), stereotyped, and rearing movements.

Echocardiography

Two-dimensional (2-D) echocardiography was performed on rats using a MyLab70 Family - Ultrasound Systems – Esaote with a CAI29 transducer [26]. In order to look for possible structural remodelling, the left ventricular end-diastolic diameter and the
free wall end-diastolic thickness were measured during the diastole from long and short-axis images obtained by M-mode echocardiography. Furthermore, the systolic function was assessed by the ejection fraction, while transmitral flow measurements of ventricular filling velocity were obtained using pulsed Doppler, with an apical four-chamber orientation. Thus, Doppler-derived mitral deceleration time, the early diastolic (E), the late diastolic (A) and the ratio E/A were obtained to assess diastolic dysfunction associated to an evaluation of the isovolumetric relaxation time. To avoid bias in the analysis, experiments were done in a blind fashion.

Statistics

Differences between means were analyzed by unpaired Student’s t-test. When the Student test was not applicable a Mann Whitney test was used. All analyses were performed using SigmaStat 3.1 software (Logi Labo, Paris, France) and Xlstat (Addinsoft, Paris, France). The data are presented as mean ± S.E.M. with the significance level set at \( p < 0.05 \).

Results

Generation of Dmd mutated rats

We generated a pair of TALENs targeting exon 23 of the rat Dmd gene in order to generate rats with Dmd mutations comparable to that of the mdx mouse [Fig. S1] [11,14]. The TALE DNA-binding domains were fused either to the wild-type FokI nuclease domain that can form homodimers or to mutated forms (so called ELD or KKR mutants for each monomer) that can only form heterodimers and thus will have less potential off-target effects. Plasmids encoding wild-type or ELD/KKR TALENs were transfected into rat C6 cells at different doses and were found to induce mutations in dose dependent fashion as evidenced by the PCR-T7 endonuclease I assay [Fig. S1]. Even if the wild-type pair was more effective at inducing mutations, we chose to use the heterodimeric ELD/KKR TALEN pair for the sake of improved specificity.

The mRNAs of each ELD/KKR TALEN monomer were microinjected into the cytoplasm of 387 rat zygotes, 320 were viable and 294 were transferred into pseudopregnant females, as previously described [Table S1] [11,12]. This resulted in the 88

Figure 5. Dmdmdx rats are characterized by muscle weakness and by a decrease in a spontaneous activity. Forelimb grip test and locomotor activity of Dmdmdx rats were analyzed at the age of 3 months. Compared to wild-type littermate controls (WT), Dmdmdx rats were characterized by muscle weakness and showed a lower strength in absolute values (A) and when normalized to body weight (B). Dmdmdx rats showed fatigue with a decreased force grip across the five pulls, and significant values are obtained for the two last trials that were reduced to \( \sim 70\% \) when compared to Trial 1. At 3 months, when compared to WT, Dmdmdx rats were less active and the number of movements (C), the total distance travelled (C), the time of activity (D) and the number of rearing (D) were significantly lower. Values are means ± SEM; *\( p < 0.05 \) vs WT; \$\( p < 0.05 \) vs Trial 1. doi:10.1371/journal.pone.0110371.g005
newborns and among them 11 showed mutations (3.74% of the transferred zygotes and 12.5% of newborns). These results are similar to our previous ones injecting other engineered sequence-specific nucleases and thus Dmd TALENs did not trigger embryo toxicity and allowed to induce Dmd gene mutations as expected [9–12].

The Dmd mutations observed in newborn rats were deletions ranging from 1 to 24 bp and insertions of a few nucleotides.
Two of the newborns presented mutations that did not disrupt the open reading frame and were not further analyzed. In the other newborns, the mutations disrupted the open reading frame and resulted in premature stop codons. Some animals with Dmd mutations detected in tail DNA did not transmit the mutation to their offspring, most likely because it was not present in the germ line due to late activity of TALENs after injection, while several of them transmitted the mutations to the offspring in a Mendelian manner: two of these rat lines were further analyzed (lines 61 and 71). Both lines displayed the same muscle anatomo-pathological and functional characteristics and therefore only line 61, hereafter called Dmdmdx+ is presented in this report. Dmdmdx+ mutation is a deletion of 11 bp in exon 23 leading to a coding frame shift and premature stop codon 81 bp after the mutation (Fig. 1 and Table S2).

Female offspring of mutant founders that carried the mutation did not show premature death and were indistinguishable from wild-type littermate controls (Fig. S2 A). X-linked DMD mainly affects boys and we have therefore only characterized male Dmdmdx+ rats in this study. When compared to wild-type littermate controls of the same ages, Dmdmdx+ rats are smaller and less heavy (Table 1 and Fig. S2 B). As illustrated by the increase in weight with age, the growth of Dmdmdx+ rats is significantly altered as soon as 4 weeks of development (Fig. S2 C).

Founder 61 and its offspring were analyzed for potential off target mutations at other loci. The closest homologous sequences to the ones recognized by Dmd TALENs showed eight mismatches. We chose to further look for mutations at the 2 candidate sites located on the X-chromosome because they could potentially cosegregate with the Dmd mutations and affect the mutant rat phenotype (Table S2). They were analyzed no mutations could be detected by T7 endonuclease I assays (data not shown). Altogether, these results indicate that rat lines with a specific disruption of the Dmd coding frame by premature stop codons were successfully generated.

Analysis of dystrophin expression

Western blot analysis of dystrophin expression in tibialis cranialis and cardiac muscles was performed using two antibodies directed against epitopes located at the C terminus and within exons 10–11. No expression of dystrophin or of a truncated dystrophin could be detected in muscle tissues analyzed from Dmd mutated animals (Fig. 2A).

Immunohistological analysis of biceps femoris and cardiac muscles using mouse monoclonal antibodies directed against three different epitopes (located within exons 11–12, 38–39 and exon 78) revealed rare scattered dystrophin positive fibers in all Dmd mutated animals (Fig. 2B-E for epitope within exons 38–39 and data not shown). Dystrophin positive fibers in biceps femoris of Dmdmdx+ of mutated animal of line 61 after immunolabelling represented 4.9±0.3% (n = 5) of total number of fibers. Such dystrophin positive fibers are frequently observed in animal models of dystrophin deficiency [26]. The exact mechanism involved in the generation of the so-called revertant myofibers remains poorly understood.

The dystrophin-associated protein complex is severely affected in skeletal muscle of DMD patients. We therefore examined the expression of alpha- and gamma-sarcoglycan, two proteins of the dystrophin-associated complex. The expression of alpha-sarcoglycan (Fig. S3 A, B) and gamma (data not shown) was weak and scarce compared to the systematic expression in myofibers of wild-type rats [27]. Altogether, these results indicate that Dmdmdx+ rats are bona fide dystrophin deficient animals.

Histopathological evaluation of muscles

Before histopathological evaluation, skeletal muscle and heart weights were measured in 3 and 7 month-old rats. Fast muscles tibialis anterior and extensor digitorum longus muscles reached significantly lower weights, of around 25% less, in 7 month-old Dmdmdx+ rats compared to wild-type controls (Table 1). In contrast, the slow soleus skeletal muscle was not significantly affected. The loss of skeletal muscle mass that was detected is consistent with the lower body weight of Dmdmdx+ rats (Fig. 2C) and indicates that muscular atrophy place in Dmdmdx+ rats, preferentially in fast muscles. The heart weights were slightly higher at 3 months and significantly lower at 7 months in Dmdmdx+ rats compared to littermates (Table 1). At 3 months, hearts of Dmdmdx+ rats were markedly dilated with left ventricular wall thinning and increased diameter of the ventricular cavity. Ratio between left ventricle wall thickness and ventricular cavity diameter was significantly decreased compared to age-related wild-type littermate controls at 5 and 7 months (Table 1). All

Table 2. Comparison of pathological and functional characteristics between patients and animal models of Duchenne disease.

| Muscle histopathology                        | DMD patient | GRMD dog | DMD pig | HFMD cat | Mdx mouse | Dmdmdx+ rat |
|----------------------------------------------|-------------|----------|---------|----------|-----------|-------------|
| Revertant fibers                             | 1 to 3%     | <1%      | ND      | ND       | 5%        | 5%          |
| Necrotic fibers                              | 0.5 to 3.5% | 2%       | absent  | present  | 5%        | >10%        |
| Regeneration                                 | ND          | 15%      | ND      | present  | 10%       | 10%         |
| Calcification                                | mild        | mild to  | marked  | ND       | severe    | mild        |
| Fibrosis                                     | marked      | present  | present | diaphragm | late & mainly | marked     |
| Lipomatosis                                  | severe      | absent   | absent  | absent   | absent    | mild        |
| Cardiomyopathy                               | marked, major cause of death | mild | absent | present | absent or late | marked |

| Muscle function                              | Strength reduction | Locomotion |
|----------------------------------------------|--------------------|------------|
| DMD patient                                  | marked             | severely impaired |
| GRMD dog                                     | marked             | impaired   |
| DMD pig                                      | ND                 | impaired   |
| HFMD cat                                     | ND                 | normal     |
| Mdx mouse                                    | mild               | impaired   |
| Dmdmdx+ rat                                  | marked             | impaired   |

Table S2. Two of the newborns presented mutations that did not disrupt the open reading frame and were not further analyzed. In the other newborns, the mutations disrupted the open reading frame and resulted in premature stop codons. Some animals with Dmd mutations detected in tail DNA did not transmit the mutation to their offspring, most likely because it was not present in the germ line due to late activity of TALENs after injection, while several of them transmitted the mutations to the offspring in a Mendelian manner: two of these rat lines were further analyzed (lines 61 and 71). Both lines displayed the same muscle anatomo-pathological and functional characteristics and therefore only line 61, hereafter called Dmdmdx+ is presented in this report. Dmdmdx+ mutation is a deletion of 11 bp in exon 23 leading to a coding frame shift and premature stop codon 81 bp after the mutation (Fig. 1 and Table S2).
together these changes in heart morphology were indicative of a progressive dilated cardiomyopathy in Dmd\textsuperscript{mdx} rats.

Histopathological evaluation of tibialis cranialis, extensor digitorum longus, biceps femoris, biceps brachii, soleus and diaphragm skeletal muscles and heart was performed at 3 and 7 month-old. Similar severe lesions were present in all examined striated skeletal muscles and were characterized by excessive fiber size variation with some large rounded hypertrophic fibers and clusters of small centronucleated regenerating fibers (Fig. 3 and Fig. S4). Numerous individual necrotic fibers appeared fragmented or contained invading inflammatory cells (Fig. 3 and Fig. S4), some being CD3-positive lymphocytes (data not shown). No marked endomysial and perimysial fibrosis at 7 months (patchy increase in endomysial connective tissue at 3 months and a clusters of small centronucleated regenerating fibers) was noticed after HES or Alizarin red stainings. ATPase staining revealed a predominance of type 1 fibers with perturbation of the normal random distribution of muscle fiber types, abnormal fiber grouping and presence of some type 2C fibers, described to be observed in case of a regenerating process after fiber necrosis (Fig. S5). These lesions were accompanied by a patchy increase in endomysial connective tissue at 3 months and a marked endomysial and perimysial fibrosis at 7 months (Fig. 3 A–C). The main lesions observed in striated skeletal muscles were quantified by histomorphometry on the biceps femoris muscle. The mean minimal Ferret diameter was 41.1 ± 20.9 μm vs 54.7 ± 16.7 μm and 45.0 ± 22.5 μm vs 54.7 ± 18.5 μm, respectively at 3 and 7 months in Dmd\textsuperscript{mdx} rat vs wild-type age-related controls (n = 5 in each group) showing a significant decrease in individual fiber size in Dmd\textsuperscript{mdx} rats (p < 0.0001) (Fig. 3 G, H). This decreased diameter was illustrated by the modal value that was 30 to 40 μm in 3 and 7 month-old Dmd\textsuperscript{mdx} rats (19.5 ± 1.6% and 19.5 ± 2.6%, respectively) whereas it corresponded to 50 to 60 μm in wild-type littermate controls (19.5 ± 1.6% and 19.7 ± 2.3%, respectively). The smallest fibers (with diameter less than 10 μm) represented 8.0 ± 4.9% and 15.7 ± 7.9% of the total cardiac muscle area in Dmd\textsuperscript{mdx} rats compared to 4.3 ± 3.1% and 3.4 ± 1.4% in wild-type littermate controls at 3 and 7 months, respectively. The increase in the fibrotic area from 3 to 7 months in Dmd\textsuperscript{mdx} rats was significant (p < 0.0001).

Dmd\textsuperscript{mdx} rats have a high level of serum creatine kinase

In DMD patients, muscle damage is characterized by an increase of creatine kinase (CK) activity in the serum. In Dmd\textsuperscript{mdx} rats, CK levels showed a 10-fold augmentation when compared to wild-type littermate controls (CK [U.L\textsuperscript{-1}]). At 3 month-old, CK levels were 1945 ± 620 in Dmd\textsuperscript{mdx} rats (n = 5) and 185 ± 17 in wild-type rats (n = 5) (p < 0.05); At 7 month, these levels were 3965 ± 817 in Dmd\textsuperscript{mdx} rats (n = 5) and 588 ± 397 in wild-type rats (n = 5) (p < 0.05). These results suggest an increased fragility of muscle membrane and confirmed that Dmd\textsuperscript{mdx} rats develop a dystrophic pathology.

Significant muscle weakness in Dmd\textsuperscript{mdx} rats

To examine whether the absence of dystrophin affected muscle function, we performed a grip test on 3 month-old Dmd\textsuperscript{mdx} rats. A significant decrease in forelimb grip strength indicated a generalized alteration in the whole body muscular performance. As illustrated in Fig. 5 (A, B), at the first trial to the grip a 30% weaker force was exerted by Dmd\textsuperscript{mdx} rats compared to wild-type litters. Furthermore, while control rats maintain the same force over five successive pulls, the grip force of Dmd\textsuperscript{mdx} rats decreased significantly and became 70% weaker than that of control rats. Therefore, Dmd\textsuperscript{mdx} rats presented a decreased grip strength test demonstrating muscle weakness, a typical sign observed in DMD patients [24, 29].

Decrease in the spontaneous activity in Dmd\textsuperscript{mdx} rats

Behavioral and locomotor measurements are important parameters that help to define the phenotypes of animal models with neuromuscular disorders such as muscular dystrophies [25]. These assessments were done in an open field plexxiglass chamber equipped with multiple photocell receptors and emitters. At the age of 3 months, Dmd\textsuperscript{mdx} rats were less active than control litters and the number of movements, the total distance travelled and the time of activity were significantly lower respectively 21 ± 7%; 38 ± 7%; 17 ± 5% lower in Dmd\textsuperscript{mdx} rats than in wild-type littermate controls (p < 0.05) (Fig. 5C, D). Furthermore, the horizontal activity as rearing was also significantly impaired and reduced by 55 ± 8% (p < 0.05) in Dmd\textsuperscript{mdx} rats compared to wild-type littermate controls (Fig. 5D). These data demonstrate that motor activity and locomotor behavior are severely affected in Dmd\textsuperscript{mdx} rats.

Modification of the heart function in Dmd\textsuperscript{mdx} rats

At 3 months, Dmd\textsuperscript{mdx} rat and wild-type control litters showed the same heart rate values under anesthesia (HR\textsubscript{WT} = 221 ± 13 bpm and HR\textsubscript{Dmd\textsuperscript{mdx}} = 219 ± 12 bpm) (Fig. 6). The echocardiographic investigations revealed structural remodeling with a significant decrease in the LV end-diastolic diameter (EDD\textsubscript{WT} = 7.4 ± 0.4 mm and EDD\textsubscript{Dmd\textsuperscript{mdx}} = 6.3 ± 0.2 mm) associated with thickening of the LV end-diastolic anterior wall in Dmd\textsuperscript{mdx} rats (EDAW\textsubscript{WT} = 1.6 ± 0.1 mm and EDAW\textsubscript{Dmd\textsuperscript{mdx}} = 2.3 ± 0.1 mm). These observations suggest the presence of a concentric remodeling in Dmd\textsuperscript{mdx} rats heart at the age of 3 months. We did not observe any change in systolic function as ejection fraction (EF\textsubscript{WT} = 82.3 ± 3.3% and EF\textsubscript{Dmd\textsuperscript{mdx}} = 83.3 ± 2.6%) and shortening fractions (SF\textsubscript{WT} = 44.3 ± 4.4% and SF\textsubscript{Dmd\textsuperscript{mdx}} = 47.8 ± 2.8%; data not shown). However, diastolic dysfunction markers were observed. We observed
changes in E/A ratio (E/A\text{WT} = 1.7 ± 0.1 and E/A\text{Dmd}\text{mdx} = 1.2 ± 0.1), in Doppler-derived mitral deceleration time (DT\text{WT} = 34.3 ± 1.3 msec and DT\text{Dmd}\text{mdx} = 54.2 ± 1.8 msec) and in isovolumetric relaxation time (IRT\text{WT} = 23.3 ± 0.9 msec and IRT\text{Dmd}\text{mdx} = 34.2 ± 1.3 msec) (Fig. 6). These data demonstrate structural remodeling and altered diastolic function in hearts of Dmd\text{mdx} rats.

Discussion

In this study, we describe the generation and the full characterization of a novel dystrophin deficient rat model (Dmd\text{mdx} rats) with phenotypic properties very close to the human DMD pathology.

DMD is the most common neuromuscular disorder, accounting for approximately 30% of muscular dystrophy patients [30–32]. The responsible gene is located in the short arm of the X chromosome, Xp21, and contains 14 kbp of dystrophin protein coding sequence split in 79 exons. The primary molecular characteristic of DMD patients is the absence of dystrophin protein in skeletal and cardiac muscles. The present results show that dystrophin was undetectable by western blot in skeletal muscles of Dmd\text{mdx} rats. In agreement, immunohistological analysis demonstrated that dystrophin is undetectable in 95% of fibers in skeletal muscles and only some revertant fibers were visible, as in DMD patients and other animal models of DMD [27]. Immunostaining and western blot analyses demonstrated that dystrophin was also undetectable in hearts of Dmd\text{mdx} rats. The absence of dystrophin, as encountered in DMD patients, leads to mosaicism of dystrophin deficient and wild-type cells, instability of the muscle cell membrane and uncontrolled influx of calcium. This initiates a set of downstream pathological processes that ultimately lead to loss of muscle cell proteins, myofiber damage and progressive muscle wasting [33]. Similar to muscle damages in DMD patients, Dmd\text{mdx} rats are characterized by a loss of skeletal muscle mass, muscle fiber necrosis, increased variation in fiber size due to the simultaneous presence of hypertrophic fibers and small centromucleated regenerating fibers, infiltration by inflammatory cells, regenerative activity and interstitial fibrosis in skeletal muscles. All these signs of tissue changes observed in limbs and forelimb skeletal muscles are correlated with a dramatic increase of serum creatine kinase activity in 3 and 7 month-old Dmd\text{mdx} rats. As observed in DMD patients but not in mdx mice [34], fibrosis was severe in all skeletal muscle examined as well as in cardiac muscle of Dmd\text{mdx} rats (Fig. 4). It is well described in dystrophin deficient skeletal myofibers that Ca\textsuperscript{2+} homeostasis alteration is pivotal in the initiation of the fiber damages [35,36]. In skeletal muscle from Dmd\text{mdx} rats at 3 and 7 months, while severe lesions were present no calcification was observed. Thus it will be interesting to further investigate calcium regulation in Dmd\text{mdx} rat skeletal muscles.

Consistent with histomorphological muscle damages, at 3 and 7 months of age, Dmd\text{mdx} rats exhibit muscle dysfunction with decrease in muscle force and reduced locomotor activity. The clinical manifestations of Dmd\text{mdx} rats evaluated at 3 and 7 months of age were progressive with gradual loss of body weight and muscle mass (mainly of fast twitch muscles such as tibialis anterior) as well as in necrosis and degeneration of skeletal muscle fibers including fundamental muscles such as the diaphragm. Additionally, Dmd\text{mdx} rats showed a progressive dilated cardiomyopathy as indicated by morphological analyses, concentric structural remodeling and diastolic dysfunction. Since the majority of DMD patients die from cardiac failure [37] the cardiac dysfunction described in Dmd\text{mdx} rats is an important feature of this new DMD model.

All skeletal and cardiac lesions as well as functional deficits are important features of the rat Dmd\text{mdx} model since they are similar to those of patients. It will be interesting to study the disease onset in this new model as was previously studied in the mdx mouse, in particular to identify the early prenecrotic stages. Importantly, the changes observed between the two time points of this study i.e. 3 and 7 month-old showed a progressive evolution of the pathology in this rat model, in the same manner than those described in DMD patients. Therefore, Dmd\text{mdx} rats will be useful for preclinical assessment of new therapies. In particular, as analyses of dystrophin mRNA in the muscles of Dmd\text{mdx} rats showed that the mutated exon 23 was transcribed (Fig. S6), Dmd\text{mdx} rats could therefore be used for the evaluation of DMD treatment using an exon skipping approaches.

The use of TALENs targeting the rat Dmd gene was efficient at introducing Dmd mutations. Other types of gene-specific nucleases are available, such as ZFNs and more recently CRISPRs/Cas9, that have also been used successfully in rats [9–13,38]. Therefore, the generation of new rat models of neuromuscular disease using artificial sequence-specific nucleases now appears very promising. While this manuscript was in preparation, dystrophin-deficient rats generated with CRISPR/Cas9 targeting exon 2 and exon 16 were reported [38]. The phenotype of the corresponding F0 rats showed characteristics similar to the Dmd\text{mdx} rats in skeletal muscle although progression of the disease was not documented. In the heart, in contrast, even though morphological changes were reported in some 3-month old F0 animals, they were not found statistically significant. The difference between the two models could be due to differences in genetic backgrounds (Wistar-Iamamichi inbred vs Sprague-Dawley outbred strain for Dmd\text{mdx}) or to mosaicism of Dmd mutations in F0 animals studied in the latter report.

The genetic heterogeneity and body size of the Golden Retriever Muscular Dystrophy dog model (GRMD) is closer to humans than the mice (Table 2). Therefore, GRMD is an important animal model for DMD. However, one major disadvantage is the large heterogeneity in the clinical phenotypes of GRMD dogs that makes the functional evaluation of potential therapies difficult. The mdx mouse has been important as a DMD model for development of gene therapy approaches. Murine models as mdx, mdx5cv, mdx52 will continue to provide important findings for the basic study of pathogenesis and development of therapies but they do not show significant muscle weakness or cardiac alterations nor any of the skeletal or cardiac muscle lesions developing at late time points in DMD patients, such as fibrosis and muscular atrophy. In contrast, it was shown that mdx/mTR\textsuperscript{KO} mice, combining the mdx mutation with deletion of the gene for the RNA component of telomerase, show skeletal muscle dysfunction and progressive dilated cardiomyopathy as a result of shortened telomers and accelerated depletion of stem cell reservoirs [39,40]. Given the much aggravated skeletal and cardiac muscle phenotype of Dmd\text{mdx} rats compared to mdx mice, it will be interesting to look for any difference in telomere dynamics or stem cell biology between these two closely related species that could be involved.

In summary, our study shows that Dmd\text{mdx} rats represent a promising model to further elucidate mechanisms of DMD and to test novel therapies, in particular those aiming to curtail heart alterations and skeletal muscle disease progression.

Supporting Information

Figure S1 Mutation rates of TALE nucleases targeting exon 23 of the rat Dmd gene. The mutation rates induced by
TALE nuclease were determined using T7 endonuclease assay in C6 cells transfected with indicated amounts of rat Dmd TALE nuclease expression vectors. Homodimeric (WT, 0.75 μg each) and heterodimeric (EDL/KKR; 0.75 μg and 1.5 μg each) TALEN pairs (DMD/EDL) have been evaluated as well as left and right part of the TALEN pair (1.5 μg). The expected sizes of digested fragments are indicated near the gel. The rates of insertion and deletion mutations (indels) detected are indicated below each lane. The TALEN binding sites in exon 23 are indicated in blue.

**Figure S2 Growth of Dmdmdx rats.** Newborns at day 2 (A). While wild-type littermate controls (WT) and Dmdmdx rats were indistinguishable after birth, Dmdmdx rats were noticeably smaller at the age of 16 weeks (B). Body weight values from WT and Dmdmdx rats from 4 to 12 weeks postnatal (C). Values are mean ± SEM; *p<0.05 Dmdmdx vs WT, analyzed by unpaired t test.

**Figure S3 The absence of dystrophin in skeletal muscle fibers is associated with a strong reduction in alpha-sarcoglycan expression in Dmdmdx rats.** Biceps femoris muscle samples were assessed for the expression of proteins of the dystrophin associated protein complex by immunohistochemistry. Compared to the generalized subsarcolemmal expression of alpha-sarcoglycan in wild-type littermate control (WT) rats (A), only some fibers expressed the protein in 7 month-old Dmdmdx rats (B). Bar = 100 μm.

**Figure S4 Muscle changes were present in all striated muscle including skeletal, respiratory and cardiac ones in Dmdmdx rats at 7 month-old.** In extensor digitorum longus (A) and diaphragm (B) muscles, hypercontracted hyalin giant fibers (white arrowhead) and individual necrotic fibers (open arrowhead), sometimes surrounded by some inflammatory cells (white arrow), were associated with foci of small regenerative centro-nucleated fibers (arrow). Some muscle fibers were replaced by fibrotic (*) and fat (black arrowhead) tissue. In cardiac muscle (C), individual fiber necrosis (open arrowhead) elicited some inflammatory cell infiltration (#). Thick bundles of fibrotic tissue surround cardiomyocytes (*). Hemalun eosin saffron staining (A–C). Bar = 100 μm.

**Figure S5 Abnormal fiber type pattern in Dmdmdx rats.** Compared to wild-type littermate control (WT) rats, biceps femoris muscles from 7-month-old Dmdmdx rats were characterized by type 1 predominance and grouping (type 1 fibers in black) and abnormal presence of type 2C (intermediate grey staining, open arrowhead). ATPase staining, pH 4.35. Bar = 100 μm.

**Figure S6 Dystrophin messenger RNA expression in skeletal muscles and hearts of Dmdmdx rats.** Dystrophin mRNA was detected by nested RT-PCR from exon 22 to 24 in tibialis cranialis muscles and in hearts sampled in 7 month-old Dmdmdx rats and wild-type control littersates. No skipping of the mutated exon 23 was detected in muscles from Dmdmdx rats.

**Table S1 Efficacy of generation of Dmd mutants by TALE nuclease microinjection.**

**Table S2 Dmd mutations and potential off target sequences analyzed in founder animals.**

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Yan Cherel died during the course of the study. This manuscript is dedicated to his memory.

**Author Contributions**

Conceived and designed the experiments: YC CG JPC IA CH. Performed the experiments: TL AL LT SR VT VF CLG HG MD LG GT ADC CB JBR YC CG JPC IA CH. Contributed to the writing of the manuscript: IA CH.

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