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Probing the pathogenesis of Duchenne muscular dystrophy using mouse models

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Abstract

Investigations using mouse models have provided seminal insights into the pathogenesis of Duchenne muscular dystrophy and the development of novel therapeutics. Several important methods have been considered standard-in-the-field for analyses of skeletal muscle weakness, strength, endurance, and histopathology, as well as responses to therapeutics such as glucocorticoids, disease modifying drugs which are part of the current standard of care for patients with this disease. Here we describe optimized genetic, genomic, and physiologic assays to probe dystrophic pathobiology in the *mdx* mouse and related strains.

Keywords

mdx; Duchenne; muscular dystrophy; exercise; glucocorticoids; skeletal muscle

1. Introduction

Duchenne muscular dystrophy (DMD) is an X-linked, progressive, muscle wasting disease arising from the inheritance of a nonsense mutation encoding the gene Dystrophin [1]. No cure currently exists for DMD, highlighting an urgent need for novel therapeutic strategies. In broad terms, therapeutic approaches are focused on: (a) restoration of dystrophin protein expression using pharmacological [2] and gene-therapeutic/editing approaches [3] or (b) developing disease modifying drugs that modulate aberrant cellular signaling/function in dystrophic muscle. Current standard of care for patients with DMD is the glucocorticoid class of steroid drugs, which have been proven to delay disease progression, but have myriad undesirable side effects associated with long term therapy [4]. Studying DMD in relevant model organisms is a critical step in improving our understanding of the molecular pathogenesis of DMD. By leveraging a combination of genetic, genomic, and chemical approaches, this line of investigation can inform novel therapeutic strategies aimed at abolishing or mitigating DMD progression. Importantly, the use of accepted and

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standardized translational and pre-clinical physiologic methods in order to rigorously test new hypotheses on the effects of innovative therapies is paramount to the successful advancement of treating DMD [5].

One of the most widely used models to investigate the pathogenesis of DMD is the *mdx* mouse, the equivalent rodent analogue to the DMD mutation in humans [6]. The *mdx* mutant allele is a spontaneously occurring nonsense point mutation within exon 23 of the mouse dystrophin gene (located on the mouse X chromosome), leading to a complete absence of Dystrophin protein [7]. While *mdx* mice largely recapitulate many of the fundamental molecular abnormalities seen in human DMD, this mouse model has a milder phenotype compared to humans. The severity of disease in male *mdx* mice peaks at approximately 6–8 weeks of age, with significant recovery and slower rates of disease progression thereafter. Much of this milder phenotype in mice has been attributed to the ability for robust and sustained skeletal muscle regenerative capacity in certain inbred mouse strains [8]. Despite the milder phenotype that is seen in these mice compared to DMD patients [9], they remain an important tool for proof-of-concept studies that seek to elucidate disease mechanisms and therapeutic strategies, particularly given the relative ease of genetic manipulation in mice, as we have used in our work [10].

There are several other well accepted small and large mammalian models of DMD [7] that elicit more severe and progressive phenotypes such as the Dystrophin/Utrophin double-knockout mouse [11,12], *mdx* mouse strains harboring telomerase deficiency [8] and the dystrophic golden retriever [13,14]. While proof-of-concept studies are often performed in *mdx* mice, it is often necessary to validate disease mechanisms or efficacy of novel therapeutic strategies in these next-phase models that more closely recapitulate the severity and tempo of human disease. Furthermore, recent studies suggest that large animal models such as the golden retriever can provide a platform for *de novo* discovery in the field, as exemplified by the recent discovery of Notch signaling as a disease modifier in a golden retriever model of DMD [15]. In addition, dystrophinopathic zebrafish models have been developed as a tool for high throughput genetic and chemical screens [16].

This article will focus on rigorous methods related to proof-of-concept experiments in *mdx* mice, with an emphasis on compound mutant generation, drug administration, phenotyping and transcriptomic analyses.

2. Materials

2.1. Animals, instruments, and equipment

1. Mice harboring the *Dmd*^{*mdx*} allele (referred to as *mdx* mice) on a C57BL/10ScSn background and C57BL/10ScSn control mice from Jackson Laboratory.
2. Modular belt treadmill system with an electric shock stimulus containing multiple lanes and an adjustable incline (Columbus Instruments).
3. Grip strength meter (Columbus Instruments).
4. Passive wire hang apparatus (*see* Note 1).

5. Straight 22-gauge, bulb-tipped mouse gavage needle (Kent Scientific).
6. Tissue-lyzer (Qiagen).
7. GeneChip Mouse 430A 2.0 arrays (Affymetrix).
8. GeneChip Hybridization Oven 645 (Affymetrix).
9. Fluidics Station 450 (Affymetrix).
10. GeneArray Scanner 3000 7G Plus (Affymetrix).

2.2. Reagents

1. 3 mg/mL prednisolone syrup.
2. 1% Evans blue dye (EBD). Prepare EBD for injection by making a 1% (weight/volume) solution in sterile PBS, then pass the EBD solution through a 0.2 μ m filter.
3. Aurum Total RNA Mini Kit (Bio-Rad).
4. Dystrophin Forward primer: ATT TTG AGG CTC TGC AAA GTT C
5. Dystrophin Reverse primer: GCC CCT CAA TCT CTT CAA ATT C
6. WT probe: 5' tag[JOE] TGA AAG AGC AAC AAA ATG GCT TC [BHQ1]3' tag
7. *mdx* probe: 5' tag[6FAM] TGA AAG AGC AAT AAA ATG GCT TC [BHQ1]3' tag
8. Although Iowa Black is recommended by Jackson Laboratories as the 3' tag, we substitute it with Blackhole Quencher 1 (BHQ1) as shown above.
9. PCR reaction components for use in a Roche Lightcycler machine:
 - a. 2X Mastermix (Roche Lightcycler 580 Probes Master) – 7.5 μ L
 - b. Forward primer – 0.9 μ M (0.27 μ L of 50 μ M solution)
 - c. Reverse primer – 0.9 μ M (0.27 μ L of 50 μ M solution)
 - d. WT probe – 0.2 μ M (0.375 μ L of 10 μ M solution)
 - e. *mdx* probe – 0.2 μ M (0.375 μ L of 10 μ M solution)
 - f. Sterile H₂O – 5.21 μ L
 - g. DNA (from tail snip) – 1 μ L of 10 ng/ μ L stock
10. RNA Later stabilizing reagent (Qiagen).
11. Qiazol (Qiagen).
12. Chloroform.

¹The passive wire hang apparatus is custom made by stacking two mouse cages on top of one another on two sides and is then spread out to the width of a metal cage top so that the metal cage top rests on top of and between the two stacks. The home cage of the mouse being used during the experiment is placed below and open for when the mouse falls.

13. iScript Kit (Bio-Rad).
14. WT Expression Kit (Ambion).
15. GeneChip WT Terminal Labeling and Hybridization Kit (Affymetrix).

3. Methods

3.1. Genetic manipulation, breeding strategy of *mdx* mice, and genotyping

The example method of breeding in order to introduce gain or loss of function of an additional gene into the *mdx* background will be described using our gene of interest (*Klf15*) as shown in our prior work [10,17].

1. Homozygous male *mdx* (genotype: $Dmd^{mdx} Y$) and homozygous female *mdx* mice (genotype: $Dmd^{mdx} Dmd^{mdx}$) can be bred with minimal difficulty to maintain the colony. The experiments are typically conducted with male mice of the *mdx* background as the human disease is X-linked and manifests predominantly in males. Thus when planning experiments, one must take this into consideration in order to generate a sufficient sample size of male mice.
2. To generate *Klf15* null mice harboring the homozygous *mdx* allele ($Klf15^{-/-} / mdx$ mice), see 3–6.
3. Cross $Klf15^{-/-}$ male mice (a systemic knockout; C57BL/6 background) with homozygous *mdx* female mice to generate the F1 generation. The F1 generation will then consist of the genotypes: male $Klf15^{+/-} /$ homozygous for the *mdx* allele (*mdx* allele is X-linked) and female $Klf15^{+/-}$ mice heterozygous for the *mdx* allele.
4. Intercrossing the F1 generation ($Klf15^{+/-} / Dmd^{mdx} Y$ males crossed with $Klf15^{+/-} / Dmd^{mdx} Dmd^{WT}$ females) will subsequently generate both male and female mice that are $Klf15^{+/-}$ and homozygous for the *mdx* allele yielding the F2 generation ($Klf15^{+/-} / Dmd^{mdx} Y$ males and $Klf15^{+/-} / Dmd^{mdx} Dmd^{mdx}$ females).
5. Subsequent intercrossing of littermate males and females of the F2 generation ($Klf15^{+/-} / Dmd^{mdx} Y$ males and $Klf15^{+/-} / Dmd^{mdx} Dmd^{mdx}$ females) will yield male study mice and littermate controls.
6. It is extremely important to strictly use littermate controls in order to control for the mixed background of the parental strains (see Note 2).
7. To generate mice with skeletal muscle-specific transgenic gain of function harboring the homozygous *mdx* allele ($Klf15^{Tg}$), see 8–10. These mice contain a single copy of the *Klf15* transgene under control of the muscle creatine kinase promoter.

²The phenotype may differ with each generation, so it is imperative to keep a record of which generation of interbreeding is being studied. An alternative approach is to start with strains of the same pure genetic background. For example, *Klf15* null mice can be backcrossed to purity on the C57BL/10-ScSn background. Alternatively, *mdx* mice are available already backcrossed to purity on a C57BL/6 background. Again, it is important to note that the disease phenotypes may vary significantly based on genetic background of the mouse strain.

8. Cross male Tg mice (not harboring the *mdx* allele; C57BL/6 background) with females homozygous for the *mdx* allele (C57BL/10-ScSn background).
9. All *male* offspring of this cross will be homozygous for the *mdx* allele and will be either positive (study mice; Tg *mdx*) or negative (non-transgenic littermate controls) for the transgene.
10. Again, it is extremely important to strictly use littermate controls in order to control for the mixed background of the parental strains (*see* Note 3).
11. Genotyping for the *mdx* allele and other point mutation models is best done by using endpoint/primer competition PCR [18]. This is a modified TaqMan protocol that amplifies the region around the mutation with PCR primers and uses 2 fluorophore-labeled 23-mer TaqMan probes that recognizes either the WT dystrophin allele or the mutant *mdx* allele. The magnitude of the fluorophore signal at the end of all the PCR cycles can be used to reliably and efficiently differentiate WT, heterozygotes, and homozygotes at this locus. Our description for *mdx*-specific PCR conditions can be found in the **Reagents** section and a similar protocol can also be found on the Jackson Laboratories website (jaxmice.jax.org/strain/001801.html).

3.2. Administration of prednisolone to *mdx* mice by oral gavage

1. Oral gavage to *mdx* mice can be begun after weaning (at approximately 3 weeks of age). It is advised that extreme precaution be taken if initiating treatment at such an early time point due to significant weakness and muscle fiber degeneration that manifests at this age [9,19]. Consider using a small amount of volume for gavage (e.g. 50 μ L).
2. We use a low and intermittent dosing regimen to elicit the ergogenic effects of prednisolone at 5 mg/kg oral gavage given 3 times weekly, a dose similar to that in prior clinical studies in DMD patients and in *mdx* mice [4,20,21]. The stock prednisolone syrup is diluted into mouse drinking water and is made fresh prior to each day of dosing.
3. It is advisable to administer the smallest volume of gavaged material that is technically feasible and accurate. For young mice (e.g. age 3–5 weeks) use a gavage volume of 50–100 μ L. For older mice, use a maximal gavage volume of 200 μ L.
4. It is important to weigh the mice in the beginning of each week as dosing volumes will change as they continue to grow.

³The offspring of these transgenic crosses should not be used to propagate the colony of study mice as the phenotypes may vary with each successive generation due to mixing of the background strains. For this transgenic breeding strategy, it is better to independently maintain the transgenic colony in a pure C57BL/6 background and likewise maintain the *mdx* colony in the C57BL/10-ScSn background by breeding male and female homozygotes. Each study cohort can be generated from a single cross as described in section 3a above. An alternative approach is to start with strains of the same pure genetic background. For example, transgenic mice can be backcrossed to purity on the C57BL/10-ScSn background. Alternatively, *mdx* mice are available already backcrossed to purity on a C57BL/6 background. Again, it is important to note that the disease phenotypes may vary significantly based on genetic background of the mouse strain.

5. Estimate the length of gavage needle that will pass through the mouth and esophagus to reach the stomach. This can be accomplished by holding the needle next to the mouth and estimating the length to the diaphragm. When ready to gavage the mouse, scruff the mouse behind the ears and shoulders so that it is restrained enough so as not to obstruct passage of the needle. It is important that the neck of the mouse be straightened to ensure ease of passage from the mouth to the esophagus. This is accomplished via gentle dorsiflexion of the gavage needle as it enters the esophagus. Gently insert the ball part of the gavage needle into the mouth and slide it down into the esophagus with extreme ease. No resistance should be felt whatsoever during insertion into the esophagus. Mice will normally lick the shaft of the needle and swallow as the needle is passed through the esophagus and into the stomach. If resistance is felt, do not push the needle forward, retract fully and re-insert carefully. Once the needle is at the level of the stomach (which can be estimated as described above), slowly inject the syringe.

3.3. Treadmill exercise of *mdx* mice

1. When preparing the treadmill for use, the electric shock stimulus should be set at 1 Hz/20% output.
2. In our hands, *mdx* male mice are strong enough to endure rigorous treadmill exercise at 9 weeks of age in order to achieve reproducible results. *mdx* mice have tremendous difficulty performing treadmill exercise at 5–8 weeks of age, even at very low workloads. Therefore, the data are highly variable and difficult to interpret when performed at these ages. Since *mdx* mice have significant muscle weakness, it is advisable not to run them on a horizontal plane, rather, they should be run at a downhill angle of 20°.
3. *mdx* mice need to be slowly acclimated to the treadmill with ease before starting the experiment. We have found the following acclimating protocol most effective for *mdx* mice (see 4 and 5).
4. Acclimate mice for 3 consecutive days prior to the experimental day (see Note 4).
5. Treadmill should be set up at a downhill angle of 20° with the electrical shock turned on and use the following interval protocol: 3 m/min × 5 min and increasing to 5 m/min × 2 min, 7 m/min × 2 min, and 8 m/min × 1 min (total 10 minutes).
6. Assessing time to exhaustion on experimental day: With the treadmill at a downhill angle of 20° use the following interval protocol: 3 m/min × 5 min, 5 m/min × 2 min, 7 m/min × 2 min, 8 m/min × 1 min, 9 m/min × 2 min, 10 m/min × 10 min, 12 m/min × 20 min, 13 m/min × 1 min, 14 m/min × 30 min, and 16 m/min continuously until exhaustion is reached (see Note 5). Exhaustion is

⁴It is extremely important that during the acclimating period, and even more so during the experiment, that the mice be watched closely to prevent injury and to ensure they are running properly on the apparatus.

defined as the time point when mice are unable to avoid repetitive electrical shocks for 5 continuous seconds [22]. Time is then stopped and the electrical shock stimulus for that lane is turned off. The distance run by each mouse can be calculated accordingly.

3.4. Passive wire hang of *mdx* mice

1. In our hands, *mdx* mice can be successfully put through the wire hang exercise with reproducible results between 6–8 weeks of age.
2. After assembly of the custom wire hang apparatus (*see* Note 1), place the *mdx* mouse on the metal cage top and have the mouse home cage beneath to catch the mouse after exhaustion.
3. Simply invert the metal cage top when the mouse is equidistant from all edges, so that the mouse cannot use anything else for support when suspended upside down (they will attempt to use their tail). Once the cage top is *fully* upside down, begin recording the time. Again, since *mdx* mice are incredibly weak, it is important that the cage top is inverted slowly so they can adequately grab on for the initial hold.
4. We calculate the final “latency to fall” time as an average of 3 consecutive trials per mouse.

3.5. Grip strength measurement of *mdx* mice

1. In our hands, *mdx* mice can be successfully put through the grip strength exercise with reproducible results between 6–8 weeks of age. While grip strength can be performed serially at younger ages, there is significantly more variability in the data prior to 6 weeks of age in a large part due to relative differences in body weight.
2. Once the grip strength meter is set up, change the settings to record the force pulled by the mouse to grams and *peak* force.
3. Restrain the mouse at the base of the tail and hover the mouse gently over the tension bar until both forelimbs have grasped it completely (*see* Note 6). Gently pull the mouse backward away from the bar by the base of the tail until the mouse can no longer grip the bar.
4. We record the maximal force generated. Each mouse is given a trial of 5 consecutive pulls. Grip strength is calculated by taking the average of the 3 highest measurements and then normalized to body weight [9].
5. It is imperative that the technique is standardized and performed in an identical manner for each mouse. The most reliable and reproducible data will come from a standardized operator performing this test in a manner that is blind to genotype and experimental group.

⁵It is unlikely that many of the mice will sustain the higher speeds, unless of course they are provided with ergogenic manipulations.

⁶It may be useful to adhere a piece of food in front of apparatus so the mouse is more inclined to grab the bar.

3.6. Evans blue dye extravasation

1. Evans blue dye (EBD) is an auto-fluorescent, non-toxic compound that can be safely given to *mdx* mice in order to determine the extent of skeletal muscle cell damage and myofiber permeability at the microscopic level [23].
2. EBD is injected intraperitoneally at a dose of 1% volume to gram body weight (e.g. 250 μ L for a 25 g mouse) 16 hours prior to harvesting the muscle [24,25].
3. Desired muscles (we use quadriceps and gastrocnemius) should be isolated and freshly frozen in OCT for creating 6 μ m frozen sections.
4. Once the sections have been mounted on coverslips, tissue can be analyzed for dye extravasation into the myocytes by fluorescence microscopy and semi-quantitative imaging.
5. Alternatively, anatomically matched whole muscle tissue can be processed for total Evan's blue dye content (via spectrophotometric absorbance at 620 nm) normalized to input muscle mass as previously published [26].

3.7. RNA extraction from quadriceps muscle and processing for microarray studies

1. Homogenize quadriceps tissue from *mdx* mice that has been preserved in RNA Later stabilization reagent in Qiazol with the Tissue-lyzer using stainless steel beads at 30 Hz for a total of 4 minutes. Use chloroform to extract the aqueous phase of the solution.
2. Purify RNA from the aqueous phase using the Aurum Total RNA Mini Kit using on-column DNase treatment per the manufacturer's instructions.
3. Once an adequate amount of quality RNA has been collected, RNA can either be prepared for microarray (see 4–7) or RNA-seq studies. The same RNA can be used for qRT-PCR with use of the iScript kit and the TaqMan method using the Roche Universal Probe Library System (see [10]), or simply stored at -80°C .
4. For microarray studies, the procedures for RNA preparation, labeling, and array hybridizations that we use are performed according to standard protocols set up by the Dana Farber Cancer Institute Gene Expression Core. While we discuss microarray analyses here, note that RNA can also be prepared for global gene expression analysis by RNA coupled with next generation DNA sequencing (RNA-seq), another widely used transcriptomic approach in the DMD field [27].
5. cDNA is generated using the WT Expression Kit and is labeled using the GeneChip WT Terminal Labeling and Hybridization protocol.
6. Labeled and fragmented cDNA is hybridized to Mouse 430A 2.0 arrays for 16–18 hours in the GeneChip Hybridization oven 645 at 45°C with rotation at 60 rpm.
7. Hybridized array samples are washed and stained using the Fluidics Station 450. After staining, the GeneChip arrays can be immediately scanned using the GeneArray Scanner 3000 7G Plus. Bioconductor (bioconductor.org) packages

affyQCReport and affy are used to perform quality control and normalization of the arrays.

3.8 Microarray and differential expression analysis

1. Custom BrainArray Entrez Gene probe set annotations (brainarray.mbni.med.umich.edu/Brainarray/Database/CustomCDF/19.0.0/entrezg.asp) can be used to summarize expression by Robust Multichip Average [28].
2. Probe sets that are called “present” in at least one sample by affy mas5call and having an interquartile range greater than 10% are selected and examined for differential expression analyses.
3. A one-way ANOVA linear model is used on the data sets to fit to the comparisons and to estimate the false discovery rate (FDR) and adjusted *P* value (family-wise error rate, FWER) for each gene. These analyses are carried out using functions in the R package limma in Bioconductor [29,30]. Genes with a fold change greater than 2 and false discovery rate less than 0.05 can then be retained for further analysis.

3.9. Functional Annotation of Genesets and Gene Set Enrichment Analysis (GSEA)

Genome-wide analysis has become an increasingly valuable tool for understanding the detrimental effects of Dystrophin deficiency and the salutary effects of glucocorticoid therapy in *mdx* mice and in humans [10,31,32]. For example, we have been able to determine that the gene signature of *Klf15* gain and loss of function in skeletal muscle is highly enriched for a metabolic gene signature that is dysregulated in *mdx* skeletal muscle [10]. Functional annotation of gene sets has also been useful in elucidating broader pathways involved in DMD pathogenesis, as we have demonstrated for *Klf15*-dependent transcriptional programs that govern amino acid catabolism.

1. Lists of up- and down-regulated genes can be submitted online to the Database for Annotation, Visualization, and Integrated Discovery (DAVID) server (<https://david.ncifcrf.gov/>) for functional annotation and pathway-based categorization of transcripts as described by Huang and colleagues [33].
2. Microarray gene sets of differentially expressed genes from various experimental designs acquired from tissues can be compiled using gene IDs as identifiers and compared on the basis of Entrez Gene IDs after collapsing multiple probe sets for a given gene, if necessary, based on maximal interquartile range.
3. For GSEA, collections of gene signatures are downloaded from the Molecular Signatures Database (MSigDB, <http://www.broadinstitute.org/gsea/msigdb>) and supplemented with manually curated signatures derived from published reports.
4. We use GSEA software (version 2.07, www.broad.mit.edu/GSEA) to determine the significance of correlation between gene sets and degree of expression.
5. The signal-to-noise ratio of *linear* (not log2) data are used as the metric with gene set permutation.

6. When publishing results, microarray data with adherence to annotation standards should be deposited in the Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo>).

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