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ScienceDirect

Neuromuscular Disorders 25 (2015) 827–834

www.elsevier.com/locate/nmd

The first exon duplication mouse model of Duchenne muscular dystrophy: A tool for therapeutic development

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Received 26 March 2015; received in revised form 5 August 2015; accepted 6 August 2015

Abstract

Exon duplication mutations account for up to 11% of all cases of Duchenne muscular dystrophy (DMD), and a duplication of exon 2 is the most common duplication in patients. For use as a platform for testing of duplication-specific therapies, we developed a mouse model that carries a *Dmd* exon 2 duplication. By using homologous recombination we duplicated exon 2 within intron 2 at a location consistent with a human duplication hotspot. mRNA analysis confirms the inclusion of a duplicated exon 2 in mouse muscle. Dystrophin expression is essentially absent by immunofluorescent and immunoblot analysis, although some muscle specimens show very low-level trace dystrophin expression. Phenotypically, the mouse shows similarities to *mdx*, the standard laboratory model of DMD. In skeletal muscle, areas of necrosis and phagocytosis are seen at 3 weeks, with central nucleation prominent by four weeks, recapitulating the "crisis" period in *mdx*. Marked diaphragm fibrosis is noted by 6 months, and remains unchanged at 12 months. Our results show that the Dup2 mouse is both pathologically (in degree and distribution) and physiologically similar to *mdx*. As it recapitulates the most common single exon duplication found in DMD patients, this new model will be a useful tool to assess the potential of duplicated exon skipping.

Keywords: Duchenne muscular dystrophy; Duplication; Mouse model; Exon skipping

1. Introduction

The dystrophinopathies Duchenne and Becker muscular dystrophy (DMD and BMD) result from mutations in the *DMD* gene, which encodes the subsarcolemmal protein dystrophin. DMD typically results from mutations that disrupt the reading frame, leading to a complete absence of dystrophin. The milder BMD, in contrast, typically results from mutations that preserve an open reading frame. Most often, such mutations are deletions of portions of the central rod domain that preserve N-terminal and C-terminal domains, resulting in a partially functional protein. This clinical observation has led to the therapeutic approach of exon skipping for boys with DMD, using modified antisense oligonucleotides (AONs) – including 2′-*O*-methyl phosphorothioate and phosphorodiamidate morpholino oligomers – that are currently in trials as a method of inducing altered pre-mRNA splicing of the *DMD*. The expected result is that out-of-frame DMD mutations will be converted instead to transcripts that have larger yet in-frame deletions, with a predicted milder phenotype.

Deletions of one or more *DMD* exons account for around 65% of all dystrophinopathies [1]. Exon duplications, in contrast, account for up to 11% of disease-causing mutations in dystrophinopathy patients [1–4]. Although less common, selected duplications may represent a compelling target for exon skipping, as skipping of only one copy of a duplicated exon would be expected to restore an entirely normal DMD messenger RNA (mRNA) resulting in restoration of wild-type dystrophin protein. This would be predicted to result in a much more favorable response than the BMD-like response predicted for exon skipping of deletion mutations.

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http://dx.doi.org/10.1016/j.nmd.2015.08.005
0960-8966/

The development of this and other strategies to specifically target duplication mutations has been hindered by the absence of any animal model with a *DMD* exon duplication. The standard dystrophinopathy *mdx* mouse model carries a nonsense mutation in exon 23, other mouse models carry a variety of non-duplication mutations [5,6], and the most commonly utilized dog model, the golden retriever (GRMD) line, carries a splice site mutation that results in out-of-frame splicing of the mRNA transcript [7].

In order to create a new tool for translational studies of duplication exon skipping strategies, we created a mouse that carries a duplication of single *Dmd* exon. We chose to duplicate exon 2, which is the most common single exon duplication in DMD patients, accounting for 10% of all duplications [8]. Making use of a recombination breakpoint in intron 2 identified in DMD patients [8], we knocked in a complete copy of the murine exon 2 and flanking intronic sequence. Molecular studies on Dup2 mouse muscle confirm the duplication in mRNA, and show an almost complete absence of dystrophin by immunoblot. Histopathologically, the Dup2 mouse recapitulates features found in *mdx*, including an early phase of degeneration, necrosis, and phagocytosis, with later prominent central nucleation and endomysial fibrosis. Together, these data suggest that the Dup2 mouse is a valid dystrophinopathy model that will be useful in developing therapies directed toward duplication mutations.

2. Methods

2.1. Vector design

The knock-in (KI) vector was made by Vega Biolab (Philadelphia, PA). All three *Dmd* genomic DNA fragments (the 4 kb upstream homologous arm, the inserted exon 2 sequence, and 6 kb downstream homologous arm) were amplified from C57/Bl6 BAC clone RP23-278C20 DNA using high-fidelity Phusion DNA Polymerase (New England Biolabs, Ipswich, MA) and cloned into a knock-in backbone vector containing LoxP and Frt sites, along with a neomycin (neo) cassette and tyrosine kinase (TK) cassette. The orientation of these elements is shown on the KI design map (Fig. 1a).

2.2. Generation of the mouse model

The targeting construct was electroporated into C57BL/6J-derived Bruce4 (Thy1.1 congenic C57BL/6 strain) [9] embryonic stem (ES) cells with a Gene Pulser II apparatus (Bio-rad). A total of 288 resistant clones were selected in the presence of G418 and diphtheria toxin. The ES cell clones were screened by RT-PCR, and a single positive clone identified. For chimera production, 3 week old Albino C57BL/6J-*Tyr*$^{c-2J}$ females were superovulated by administering 5 international units (IU) Pregnant Mare Serum Gonadotropin (PMSG) intraperitoneally, followed by 5 I.U. Human Chorionic Gonadotropin (HCG), 48 hours post PMSG. The mice were then mated with albino BL/6 males and from pregnant females, blastocysts were harvested at 3.5 days post coitus (d.p.c.). To generate chimeras, 5–10 ES cells from the correctly targeted clone were injected into the albino BL/6 blastocysts, which were subsequently transferred into the uterus of 2.5 d.p.c. pseudopregnant ICR strain females. Chimeric males recognized by the presence of black pigmented coat color were then bred with BL/6 females. Their pups were then screened for the presence of the duplication by RT-PCR, and positive males and females were then bred to establish a homozygous female and hemizygous male colony. All procedures were carried out in accordance with institutional policies (IACUC #02605AR).

2.3. Duplication screening

Animals were screened by RT-PCR instead of PCR on genomic DNA, as a specific primer set and conditions amenable to robust amplification of genomic DNA proved difficult to identify, which we attributed as possibly due to secondary structural features. Duplication screening was performed on RNA extracted from mice tail or ear clips using Tri-reagent (Sigma-Aldrich, St. Louis, MO) and the manufacturer's protocol. RT-PCR was performed on total RNA with the Maxima Universal First Strand cDNA Synthesis kit (Thermo Scientific) using a forward primer located in the 5′UTR within exon 1 (TACCTAAGCCTCCTGGAGCA) and a reverse primer located at the exon 3–exon 4 junction (CTT TTGGCAGTTTTTGCCCTGTA). Products were visualized on a 2% agarose gel.

2.4. Dystrophin expression

Sections (20 at 40 μm) or whole muscles were digested with 100μl of lysis buffer pH7.4 (150mM NaCl, 50mM Tris, 0.05%NP-40, 1% digitonin [Sigma, D141]) and protease and phosphatase inhibitors for 1 hour in ice after homogenization with beads. After centrifugation (14000g, 20min at 4°C), supernatant was quantified and mixed with a Laemmli buffer 4X (250mM Tris-HCl, 8% SDS, 40% glycerol, 8% beta-Mercaptoethanol, pH=6.8, 0.01% bromophenol blue). One hundred and fifty micrograms of protein were boiled for 5min at 100°C and then loaded on a 3–8% gel (Life Technology,

Fig. 1. **Features of the Dup2 mouse.** (a) Knock-in targeting strategy. Numbers indicate the relative positions of exons, cloning sites, and restriction sites. The mouse exon 2 sequence (62 nucleotides [nt]) and about ~150–200 nt of flanking intronic sequence are flanked by arms of homologous recombination, with the targeted insertion point at position c.93+32207_32208 in intron 2. (Upper row of numbers refer to relative position within intron 2, which consists of 209,572 nt in the mouse.) LoxP sites are represented by gray triangle and Frt sites by gray circles. (b) RT-PCR from muscle using primers in exon 1 (forward) and exon 3–4 junction (reverse) confirmed inclusion of the duplicated exon 2 in Dup2 but not Bl6 mice. No spontaneous skipping of a duplicated exon was detectable. (c) Dystrophin immunostaining performed in tibialis anterior muscles from Bl6, *mdx* and Dup2 using a dystrophin polyclonal C-terminal antibody. Dystrophin was present at the plasma membrane in the Bl6 muscle but was not detected in muscles from the Dup2 mouse. In the *mdx* mouse, rare revertant fibers (one marked by "*") can be detected. (d) Immunoblotting of protein extracted from gastrocnemius (gas) and tibialis anterior (TA) muscles from Bl6 and Dup2 was performed with a dystrophin polyclonal C-terminal antibody and showed a slight (< 2.5%) but variable amount of protein in all muscles analyzed when overexposed and quantified using an alpha-actinin labeling as a loading control. **(e)** Haematoxilin–eosin (HE) staining from Dup2 muscle at the age of 3 and 4 weeks. At 3 weeks, some inflammation can be observed, which leads to a process of degeneration and regeneration (marked by central nucleation) by 4 weeks of age.