

factor IX gene therapy trial in which rAAV was delivered to the liver, only short-term transgene expression was achieved and levels of therapeutic protein declined to baseline levels 10 weeks after vector infusion.⁴⁰ This was accompanied by elevation of serum transaminase levels and a CTL response toward specific AAV capsid peptides. To overcome this response, transient immunosuppression may be required until AAV capsids are completely cleared. Additional findings suggest that T-cell activation requires AAV2 capsid binding to the heparan sulfate proteoglycan (HSPG) receptor, which would permit virion shuttling into a DC pathway, as cross-presentation.⁴² Exposure to vectors from other AAV clades, such as AAV8, did not activate capsid-specific T-cells.

5. Challenges and limitations of related strategies

5.1. Design of Read-through Drugs

To suppress premature stop codon mutations, treatments involving aminoglycosides and other agents have been attempted. PTC124, a novel drug capable of suppressing premature termination and selectively inducing ribosomal read-through of premature, but not normal, termination codons, was recently identified using nonsense-containing reporters.⁴³ The selectivity of PTC124 for premature termination codons, its oral bioavailability and its pharmacological properties indicate that this drug may have broad clinical potential for the treatment of a large group of genetic disorders with limited or no therapeutic options.

5.2. Modification of mRNA Splicing

By inducing the skipping of specific exons during mRNA splicing, antisense compounds against exonic and intronic splicing regulatory sequences were shown to correct the open reading frame of the DMD gene and thus to restore truncated yet functional dystrophin expression *in vitro*.⁴⁴ Intravenous infusion of an antisense phosphorothioate oligonucleotide created an in-frame *dystrophin* mRNA via exon skipping in a 10-year-old DMD patient possessing an out-of-frame exon 20 deletion of the *dystrophin* gene.⁴⁵ Moreover, the adverse-event profile and local dystrophin-restoring effect of a single intramuscular injection of an antisense 2'-O-methyl phosphorothioate oligonucleotide, PRO051, in patients with DMD were explored.⁴⁶ Four patients received a dose of 0.8 mg of PRO051 in the TA muscle. Each patient showed specific

skipping of exon 51 of dystrophin in 64 to 97% of myofibers, without clinically apparent adverse side effects.

The efficacy and toxicity of intravenous injection of stable morpholino phosphorodiamidate (morpholino)-induced exon skipping were tested using CXMD₁ dogs, and widespread rescue of dystrophin expression to therapeutic levels was observed.⁴⁷ Furthermore, a morpholino oligomer with a designed cell-penetrating peptide can efficiently target a mutated *dystrophin* exon in cardiac muscles.⁴⁸

Long-term benefits can be obtained through the use of viral vectors expressing antisense sequences against regions within *dystrophin* gene. The sustained production of dystrophin at physiological levels in entire groups of muscles as well as the correction of muscular dystrophy were achieved by treatment with exon-skipping AAV1-U7.⁴⁹

5.3. Ex Vivo Gene Therapy

Transplantation of genetically corrected autologous myogenic cells is a possible treatment for DMD. Freshly isolated satellite cells transduced with lentiviral vectors expressing micro-dystrophin were transplanted into the TA muscles of *mdx* mice, and these cells efficiently contributed to the regeneration of muscles with micro-dystrophin expression at the sarcolemma.⁵⁰ Mesoangioblasts are vessel-associated stem cells and might be candidates for future stem cell therapy for DMD.⁵¹ Intra-arterial delivery of wild-type canine mesoangioblasts resulted in the extensive recovery of *dystrophin* expression, normal muscle morphology and function in the GRMD. Multipotent mesenchymal stromal cells (MSCs) are less immunogenic and have the potential to differentiate and display a myogenic phenotype.⁵²

6. Future perspectives

6.1. Pharmacological Intervention

The use of a histone deacetylase (HDAC) inhibitor would likely enhance the utility of rAAV-mediated transduction strategies in the clinic.⁵³ In contrast to adenovirus-mediated transduction, the improved transduction with rAAV induced by the HDAC inhibitor is due to enhanced transgene expression rather than to increased viral entry. The enhanced transduction was proposed to be related to the histone-associated chromatin form of the rAAV concatemer in transduced cells. Since various HDAC inhibitors are currently being tested in clinical trials for

many diseases, the use of such agents in rAAV-mediated DMD gene therapy is theoretically and practically reasonable.

6.2. Capsid modification

A DNA shuffling-based approach for developing cell type-specific vectors is an intriguing possibility to achieve altered tropism. Capsid genomes of AAV serotypes 1-9 were randomly reassembled using PCR to generate a chimeric capsid library.⁵⁴ A single infectious clone (chimeric-1829) containing genome fragments from AAV1, 2, 8, and 9 was isolated from an integrin minus hamster melanoma cell line previously shown to have low permissiveness to AAV.

7. Conclusions and Outlook

DMD remains an untreatable genetic disease that severely limits motility and life expectancy in affected children. The systemic delivery of rAAV to transduce truncated dystrophin is predicted to ameliorate the symptoms of DMD patients in the future. To translate gene transduction technologies into clinical practice, development of an effective delivery system with improved vector constructs as well as efficient immunological modulation must be established. A novel protocol that considers all of these issues would help improve the therapeutic benefits of DMD gene therapy.

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Efficacy of Systemic Morpholino Exon-Skipping in Duchenne Dystrophy Dogs

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Objective: Duchenne muscular dystrophy (DMD) is caused by the inability to produce dystrophin protein at the myofiber membrane. A method to rescue dystrophin production by antisense oligonucleotides, termed *exon-skipping*, has been reported for the *mdx* mouse and in four DMD patients by local intramuscular injection. We sought to test efficacy and toxicity of intravenous oligonucleotide (morpholino)-induced exon skipping in the DMD dog model.

Methods: We tested a series of antisense drugs singly and as cocktails, both in primary cell culture, and two in vivo delivery methods (intramuscular injection and systemic intravenous injection). The efficiency and efficacy of multiexon skipping (exons 6–9) were tested at the messenger RNA, protein, histological, and clinical levels.

Results: Weekly or biweekly systemic intravenous injections with a three-morpholino cocktail over the course of 5 to 22 weeks induced therapeutic levels of dystrophin expression throughout the body, with an average of about 26% normal levels. This was accompanied by reduced inflammatory signals examined by magnetic resonance imaging and histology, improved or stabilized timed running tests, and clinical symptoms. Blood tests indicated no evidence of toxicity.

Interpretation: This is the first report of widespread rescue of dystrophin expression to therapeutic levels in the dog model of DMD. This study also provides a proof of concept for systemic multiexon-skipping therapy. Use of cocktails of morpholino, as shown here, allows broader application of this approach to a greater proportion of DMD patients (90%) and also offers the prospect of selecting deletions that optimize the functionality of the dystrophin protein.

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Duchenne muscular dystrophy (DMD) and its milder form, Becker muscular dystrophy (BMD), are caused by mutations in the *DMD* gene.¹ DMD is a progressive and fatal X-linked myopathy arising from the absence of functional dystrophin at the myofiber plasma membrane.² Most DMD mutations are caused by out-of-frame (frameshift) or nonsense gene mutations, whereas the majority of BMD mutations are in-frame, and thus compatible with production of a messenger RNA (mRNA) transcript that can be translated into a partly functional quasi-dystrophin (reading frame rule).³ Some BMD patients with deletions as large as 33 exons (46% of the gene) can show little or no clinical symptoms, with only increased serum creatine kinase concentration.⁴ This raises the possibility of using

antisense-mediated removal of exons carrying nonsense mutations, or whose presence disrupts the open reading frame at the site of the mutation, so as to restore the translational reading frame and thus to convert DMD to a milder BMD phenotype.⁵ Recently, intramuscular injection of 2'-O-methylated phosphorothioate (2'-O-MePs) has been shown to induce limited dystrophin expression in four DMD boys.⁶ These studies, and extensive *mdx* mouse model systemic intravenous delivery reports, have rescued dystrophin expression by targeting a single exon. However, many DMD patients would require skipping of two or more exons to restore the reading frame. The ability to use cocktails of antisense oligonucleotides targeting multiple exons would permit designing resulting dystrophin proteins that re-

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mal control (wild-type) or dystrophic (CXMD) myoblasts (1.5×10^5 cells) were cultured in growth medium containing F-10 growth media containing 20% fetal calf serum, basic fibroblast growth factor (2.5 ng/ml), penicillin (200 U/ml), and streptomycin (200 μ g/ml) for 72 hours, followed by antisense oligonucleotide (2'-O-MePs) administration (0.25–5 μ g/ml, 30–600 nM) for 3 hours with lipofectin (Invitrogen, La Jolla, CA) following manufacturer's instructions (AOs/lipofectin ratio = 1:2). The cells were cultured in differentiation medium containing Dulbecco's minimum essential medium with HS (2%), penicillin (200 U/ml), and streptomycin (200 μ g/ml) for 4 to 5 days before analyses for RNA and protein. Morpholino antisense oligonucleotides carry no charge and cannot be transfected into cells efficiently, so only 2'-O-MePs chemistry was utilized for *in vitro* studies.

Intramuscular Injections

Animals were anesthetized with thiopental sodium induction and maintained by isoflurane for all intramuscular injections and muscle biopsies. Skin was excised over the site of injection, muscle exposed, and the injection site marked with a suture in the muscle. Antisense oligonucleotides were delivered as intramuscular injection using 1 ml saline bolus into the tibialis anterior or extensor carpi ulnaris (ECU) muscles using a 27-gauge needle. Antisense oligonucleotides were delivered either singly or in mixtures. Both 2'-O-MePs and morpholino chemistries were tested. Muscle biopsies were obtained at 2 weeks after antisense injection.

Intravenous Systemic Delivery

Three dogs were treated and all were given an equimolar mixture of morpholinos Ex6A, Ex6B, and Ex8A at 32 mg/ml each. Between 26 to 62 ml was injected into the right saphenous vein using a 22-gauge indwelling catheter, leading to a cumulative (combined) dose of 120 to 200 mg/kg per injection. Morpholinos were injected 5 to 11 times at weekly or biweekly intervals (see the Supplementary Table). Tissues were examined at 2 weeks after the last injection.

Reverse Transcriptase Polymerase Chain Reaction and Complementary DNA Sequencing

Total RNA was extracted from myoblasts or frozen tissue sections using TRIzol (Invitrogen). Then reverse transcriptase polymerase chain reaction (RT-PCR) was performed on 200 ng of total RNA for 35 cycles of amplification using One-Step RT-PCR kit (Qiagen, Chatsworth, CA) following manufacturer's instructions with 0.6 mM of either an exon 5 (CTGACTCTGGTTTGATTTGGA) or an exon 3/4 junction (GGCAAAACTGCCAAAAGAA) forward primer. Reverse primers were either exon 10 (TGCTTCGGTCTCTGTCAATG) or exon 13 (TTCATCGACTACCACACCA). The resulting PCR bands were extracted by using Gel extraction kit (Qiagen). BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) was used for complementary DNA sequencing with the same primers.

Hematoxylin and Eosin and Immunostaining

Eight micrometer cryosections were cut from flash-frozen muscle biopsies at an interval of every 200 μ m, then placed on poly-L-lysine-coated slides and air-dried. Anti-dystrophin

rod (DYS-1) or C-terminal monoclonal Ab (DYS-2) (Novocastra) were used as primary antibodies. Rabbit anti-neuronal nitric oxide synthase (anti-nNOS) (Zymed Laboratory, San Francisco, CA) was used for nNOS staining. Alexa 468 or 594 (Invitrogen) was used as secondary antibody. 4',6-diamidino-2-phenylindole containing mounting agent (Invitrogen) was used for nuclear counterstaining (blue). The number of positive fibers for DYS-1 was counted and compared in sections where their biggest number of the positive fibers was as described previously.¹⁵ Hematoxylin and eosin staining was performed with Harris hematoxylin and eosin solutions. Images were analyzed and quantified by using ImageJ software.¹⁶

Western Blotting Analysis

Muscle proteins from cryosections were extracted with lysis buffer containing 75 mM Tris-HCl (pH 6.8), 10% sodium dodecyl sulfate, 10 mM EDTA, and 5% 2-mercaptoethanol. Four to 40 μ g proteins were loaded onto precast 5% resolving sodium dodecyl sulfate polyacrylamide gel electrophoresis gels following manufacturer's instructions. The gels were transferred by semidry blotting (Bio-Rad) at 240 mA for 2 hours. DYS-2 (Novocastra) antibody against dystrophin, rabbit polyclonal antibody against α -sarcoglycan, and rabbit polyclonal antibody against desmin were used as primary antibodies.¹⁷ Horseradish peroxidase-conjugated anti-mouse or anti-rabbit goat immunoglobulin (Cedarlane Laboratories, Hornby, Ontario, Canada) was used as secondary antibodies. Enzyme chemiluminescence kit (GE) was used for the detection. Blots were analyzed by ImageJ software.

Blood Tests

Creatine phosphokinase activity, blood counts, serum biochemistry, and toxicology test in canine serum were assayed with the Fuji Drychem system and Sysmex F-820 according to manufacturer's instruction.

Magnetic Resonance Imaging

For imaging studies, animals were anesthetized with thiopental sodium and maintained by isoflurane. We used a superconducting 3.0-Tesla magnetic resonance imaging (MRI) device (MAGNETOM Trio; Siemens-Asahi Medical Technologies, Japan) with an 18 cm diameter/18 cm length human extremity coil. The acquisition parameters for T2-weighted imaging were TR/TE = 4,000/85 milliseconds, slice thickness = 6 mm, slice gap = 0 mm, field of view = 18 \times 18 cm, matrix size = 256 \times 256, and number of acquisitions = 3 during fast spin echo.

Functional Testing

Clinical evaluation of dogs was performed as described in our previous report.¹⁸ Grading of clinical signs in CXMD dogs was as follows: gait disturbance: grade 1 = none, grade 2 = sitting with hind legs extended, grade 3 = bunny hops with hind legs, grade 4 = shuffling walk, and grade 5 = unable to walk; mobility disturbance: grade 1 = none, grade 2 = lying down more than normal, grade 3 = cannot jump on hind legs; grade 4 = increasing difficulty moving around, and grade 5 = unable to get up and move around; limb or temporal muscle atrophy: grade 1 = none, grade 2 = sus-

pect hardness, grade 3 = can feel hardness or apparently thin, grade 4 = between grades 3 and 5, and grade 5 = extremely thin or hard; drooling: grade 1 = none, grade 2 = occasionally dribbles saliva when sitting, grade 3 = some drool when eating and drinking, grade 4 = strings of drool when eating or drinking, and grade 5 = continuous drool; macroglossia: grade 1 = none, grade 2 = slightly enlarged, grade 3 = extended outside dentition, grade 4 = enlarged and slightly thickened, and grade 5 = enlarged and thickened; dysphagia: grade 1 = none; grade 2 = takes time and effort in taking food, grade 3 = difficulty in taking food from plate, grade 4 = difficulty in chewing, swallowing, or drinking, and grade 5 = unable to eat.

For timed running tests, each dog was encouraged to run down a hallway (15m), and elapsed time was recorded. Single tests were done because of easy tiring of dystrophic dogs.

Results

In Vitro Screening of Antisense Oligonucleotides in Dog Primary Myoblasts

The CXMD dog harbors a splice-site mutation of exon 7, leading to an out-of-frame mRNA transcript fusing exons 6 to 8 (see Fig 1). Both exons 6 and 8 must be excluded (skipped) from the mRNA to restore the reading frame. Four antisense oligonucleotides were designed against exons 6 and 8. Ex6A and Ex8A were designed to bind exonic splicing enhancers, and Ex6B and Ex8B were directed against the 5' splice boundaries of each exon (see Fig 1). The four AOs were transfected as 2'-O-MePs either singly or in mixture (cocktails; 5µg/ml each or 600nM each) into cultures of primary myoblasts isolated from the skeletal muscle of neonatal CXMD dogs. Four days after differentiation into myotubes, RNA was isolated and tested for specific exon skipping by RT-PCR. A cocktail of all 4 AOs produced a single 101bp band with 100% of RT-PCR product corresponding to a desired in-frame splice product of exons 5 to 10 (Fig 2A). It is of interest that exon 9, known to be an alternatively spliced exon in the dystrophin mRNA, was consistently skipped, although no antisense oligonucleotide was used against this exon (see Fig 2; see Supplemental Fig 1).¹⁰ Each of the four sequences was also transfected individually, and either Ex6A or Ex6B successfully induced skipping as a single AO (100% in-frame product) (see Fig 2A). The precise skipping of exons was confirmed by complementary DNA sequencing (see Fig 2B). We found a dose-response relation where use of 30 and 60nM of either Ex6A or Ex6B induced multiexon skipping of exons 5 to 10, although with less efficiency and more intermediate out-of-frame products (see Supplemental Fig 1A). In contrast, the Ex8A AO alone induced skipping of mainly exons 8 and 9, an out-of-frame transcript, whereas the Ex8B AO induced no detectable exon skipping (see Fig 2A). Dystrophin protein production from in-frame mRNA was con-

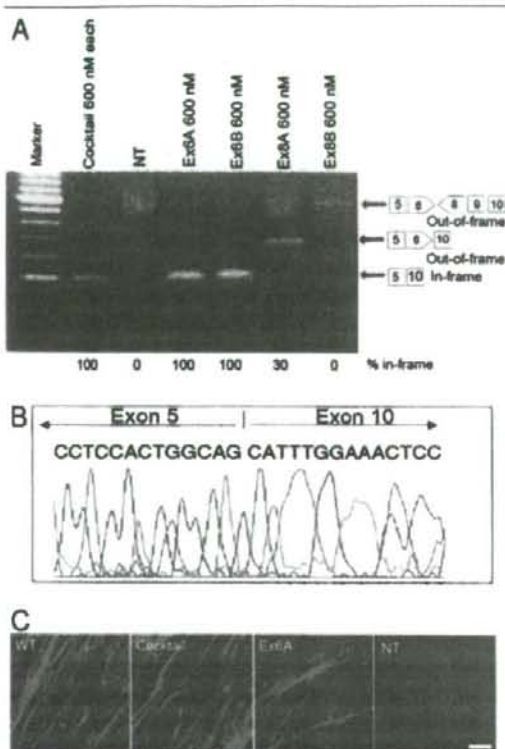


Fig 2. *In vitro* screening of antisense oligonucleotides and recovery of dystrophin expression by single antisense oligos in dog primary myoblasts. (A) Detection of exons 6 to 9 skipped in-frame products (101bp) using reverse transcriptase polymerase chain reaction (RT-PCR) at 4 days after the transduction of 5µg (600nM) each AOs of single (Ex6A or Ex6B) or cocktail AOs (Ex6A, Ex6B, Ex8A, and Ex8B) as indicated. The faint 585bp out-of-frame band is detected in Ex8B-treated myotubes. Nontreated myotubes (NT) show little RT-PCR product, likely because of nonsense-mediated decay. (B) Complementary DNA (cDNA) sequencing after antisense oligonucleotide treatment at 4 days after the transduction of Ex6A alone, showing the desired in-frame exons 5 to 10 skip. (C) Immunocytochemistry with dystrophin C-terminal antibody (Dys-2; red) and nuclear counterstaining (blue) for primary myotubes from canine X-linked muscular dystrophy (CXMD) cells at 4 days after transfection with cocktail or single antisense 2'-O-methylated phosphorothioate (2'-O-MePs) targeting exons 6 and 8 (5µg each/ml, or 600nM), nontreated wild-type (WT) cells, and CXMD cells. Scale bar = 50µm.

firmed by immunocytochemistry using either the four-sequence cocktail or Ex6A alone (see Fig 2C).

We tested the same sequences in normal (wild-type) control beagle cells (see Supplemental Fig 1B). When transfected into wild-type beagle myoblasts, exon 9 was again routinely removed from the transcripts. The antiexon 8 AO pair excised exon 8, similar to dystrophic

cells, whereas the exon 6-specific AO pair excised exon 6, as well as exon 9, but left exons 7 and 8 in place.

Efficient Skipping In Vivo Requires a Three-AO Cocktail

Given that Ex8B appeared ineffective by all in vitro assays, we did not continue with this sequence. Intramuscular injections were done using Ex6A alone, and equimolar mixtures of Ex6A, Ex6B, and Ex8A. Intramuscular injections into the tibialis anterior or ECU muscles of 0.5- to 5-year-old CXMD dogs were done for both 2'-O-MePs and morpholino chemistries, at a dose of 0.12 to 1.2mg of each sequence (Fig 3; see Supplemental Fig 2). Biopsies were performed 2 weeks later at injection sites, marked by suture threads. Dystrophin-positive fibers were concentrated around the injection site, and the absolute number of dystrophin-positive fibers was counted in cross section.

In contrast with the skipping patterns observed with in vitro cell transfections, injection of Ex6A alone induced skipping of only exon 6 in experiments using either morpholinos or 2'-O-MePs chemistries (see Fig 3; see Supplemental Fig 2). By contrast, the cocktail of Ex6A, Ex6B, and Ex8A induced robust dystrophin expression in a highly dose-dependent manner, with 1.2mg per each morpholino showing areas of complete dystrophin rescue, and high levels of dystrophin by immunohistochemical analysis and immunoblot (see Figs 3A, B, D; see Supplemental Fig 2). Intramuscular injections using the same cocktail with 2'-O-MePs chemistry showed similar results, with greater dystrophin rescue using the three AO cocktail compared with Ex6A alone (see Fig 3C). Two pairwise combinations of Ex8A with either Ex6A or Ex6B were tested with morpholino chemistry, and neither combination proved as efficient as the three-sequence cocktail (see Fig 3B).

RT-PCR analyses of injected muscles showed the Ex6A/Ex6B/Ex8A morpholino cocktail to drive efficient skipping of exons 6 to 10 skipped products, with between 61 and 83% of RT-PCR products showing the desired in-frame product in the three muscles tested (see Fig 3D). Additional out-of-frame products were observed with Ex6A alone, and as a minority of products in the cocktail-treated muscles (see Fig 3D). Histological analyses of the muscle injected with the three-morpholino cocktail (1.2mg/each) showed significant histological improvement of the dystrophy, relative to uninjected muscle, using (see Fig 3A; bottom panels).

By immunoblotting, intramuscular injection of the optimal cocktail dystrophin induced dystrophin to 50% normal levels in a 2-year-old dog but only to 25% in a more clinically severe 5-year-old dog (see Supplemental Fig 2A). This result implies that the

muscle quality influences the amount of dystrophin that can be produced.

Intravenous Systemic Delivery of a Morpholino Cocktail Induces Body-Wide Dystrophin Expression

AOs must be deliverable systemically to be of therapeutic value. Accordingly, we undertook intravenous infusion of the three morpholino-cocktail showing the most success in the intramuscular experiments (Ex6A, Ex6B, and Ex8A). Three CXMD dogs were studied using intravenous doses similar to that used in mdx mouse studies (30–40mg/kg per injection), with weekly or biweekly dosing. The first dog received 120mg/kg morpholinos (40mg/kg per each sequence) in weekly intravenous injections, with five doses per kilogram over 5 weeks. The second dog was given the same dose 11 times at 2-week intervals over the course of 5.5 months. The third dog received a greater dose: 200mg/kg (66mg/kg of each morpholino) seven times at weekly intervals (see the Supplementary Table). All dogs were killed 2 weeks after the last injection, and multiple muscles were examined.

All skeletal muscles of each treated dog showed evidence of de novo dystrophin expression by immunofluorescence of cryosections, although the degree of rescue was variable (Fig 4A). Histopathology was markedly improved in regions showing high dystrophin expression (see Fig 4A). Immunoblotting confirmed expression up to approximately 50% of normal levels, but some muscles expressed only trace amounts (see Figs 4C, D). Dystrophin expression was also detected in cardiac muscles but, as in the mdx mouse,¹⁹ less than in skeletal muscles and concentrated in small patches (see Fig 4A). Of the three dogs, the average dystrophin protein expression level was greatest in the dog given seven weekly doses of 200mg/kg PMO, with an average of 26% dystrophin levels.

Selected muscles were studied for quantitative rescue of histopathology and for biochemical rescue of dystrophin-interacting proteins (dystrophin-associated glycoproteins and nNOS). A commonly utilized quantitative marker for muscle pathology is central nucleation of myofibers, where increased central nucleation is reflective of increased degeneration and regeneration. Quantitation of central nucleation in treated dogs compared with untreated littermates showed that intravenous antisense treatment reduced central nucleation in all five muscle groups examined (see Fig 4B).

Both nNOS and α -sarcoglycan are dystrophin-associated proteins that colocalize with dystrophin in normal muscle and are reduced in DMD muscle. nNOS immunofluorescence and α -sarcoglycan immunoblotting were done on a series of muscles from treated and control dogs. By immunoblot, α -sarcoglycan was seen to be increased in all muscles examined (Fig 5B). Likewise, nNOS was seen to relo-

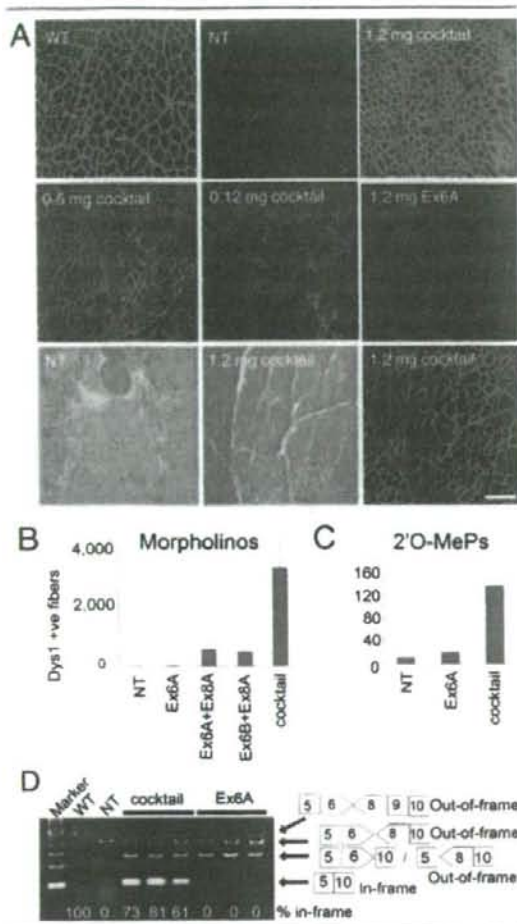


Fig 3. Recovery of dystrophin expression by *in vivo* intramuscular injection of a three-AO cocktail but not Ex6A alone. (A) Restoration of dystrophin expression in tibialis anterior (TA) at 14 days after single injection with 1.2mg Ex6A only, or cocktail containing 0.12mg each, 0.6mg each, or 1.2mg each of antisense morpholino Ex6A, Ex6B, and Ex8A are shown. Age-matched nontreated (NT) canine X-linked muscular dystrophy (CXMD) and wild-type (WT) dogs are shown as control animals. Hematoxylin and eosin (HE) staining at 14 days after 1.2mg each of the cocktail injection and age-matched nontreated control (NT) with consecutive cryosection stained with dystrophin (DYS-1) and 4',6-diamidino-2-phenylindole show histological correction of the dystrophy. Bar = 100 μ m. (B, C) The number of DYS-1-positive fibers in TA or extensor carpi ulnaris (ECU) at 14 days after a single injection with cocktail (Ex6A+Ex6B+Ex8A), or indicated combinations, at 1.2mg each (morpholino; B), or 120 μ g each 2'O-methylated phosphorothioate (2'O-MePs) (C). Values are mean \pm standard error of the mean. (D) RT-PCR analysis at 2 weeks after intramuscular injection of cocktail or Ex6A morpholino at 1.2mg each. The percentage of the in-frame exon 5 to 10 skip is shown under the gel image for treated muscle; normal control (WT) muscle shows the normal full-length in-frame transcript at the expected 100%.

calize to the membrane in dystrophin-positive regions in systemically treated dogs (see Fig 5A).

Muscle Imaging and Clinical Grading Scores Are Improved by Systemic Antisense

A global improvement in muscle pathology was further supported by the T2-weighted MRI examination (Fig 6). The high-intensity T2 signal, indicative of inflammation and increased water content, was diminished in PMO-treated dogs compared with pretreated and untreated control dogs in most muscles (see Fig 6).

Functional improvement of treated dogs was also assessed by a 15m timed running test and by a combined clinical grading score, as we have previously published.¹⁸ Three dogs treated with intravenous morpholinos were compared with three untreated, at the ages of both 2 or 5 months (pretreatment) and 4 or 7 months (posttreatment) (Figs 7B, C). The untreated littermates became slower over the treatment time, whereas all treated dogs ran faster after treatment. The single dog treated at an older age with more advanced symptoms showed greater improvement relative to untreated littermates (see Fig 7B).

The combined clinical grading score similarly showed improvement or stabilization of disease progression after antisense treatment, relative to natural history controls (see Fig 7A). Videos documenting running ability of treated dogs and untreated littermates are available as supplemental data (see Supplemental Movies 1–5).

Serum creatine kinase was assessed before and after intravenous treatment, and compared with natural history controls (see Supplemental Fig 3). Although serum creatine kinase was variable, posttreatment creatine kinase levels were consistently less than natural history controls.

Intravenous High-Dose Morpholino Cocktail Shows No Evidence of Toxicity

No local inflammatory reactions or organ dysfunctions were recorded in the morpholino-treated dogs. Twice-weekly serum toxicology screens of the three systemically treated dogs showed no evidence of liver or kidney dysfunction (see Supplemental Fig 4). Levels of urea nitrogen, α -glutamyl transpeptidase, and creatinine all remained within the reference ranges. In addition, no significant changes were observed in amylase, total protein, total bilirubin, C-reactive protein, sodium, potassium, or chloride. Growth of body weight was also within reference range in all treated dogs (data not shown).

Discussion

This is the first report of widespread induction of dystrophin expression to therapeutic levels in the dog model of DMD. Overall, our findings provide a prom-

ising message for DMD patients. Specifically, we show that intravenous morpholino antisense (PMOs) can generate body-wide production of functional dystrophin in a model clinically more severe than DMD, resulting in stabilization or improvement of the clinical disease. Beneficial effects were documented by histology, MRI, and functional tests (running and combined clinical grading scores).

We encountered some unexpected findings that raise important questions as to how to pursue this promising approach into human clinical trials. Clearly, the choice of specific antisense sequences is a crucial determinant of the ultimate success of targeted exon skipping. To date, specific AO sequences have been assessed for efficiency of exon skipping using cell-based experimental (in vitro) systems, with the optimal sequences then used for in vivo experiments. In studies presented here, antisense oligonucleotides directed against exon 6 were able to efficiently induce the desired exon 5 to 10 splicing in vitro but not in vivo. Our observations of discrepant outcomes for ex vivo and in vivo in the dystrophic dog tell us that we do not currently possess a reliable means of screening for sequences that induce

efficient skipping of a particular exon in a particular mutational context. Data obtained from application of sequences as 2'-O-MePs in primary myogenic cells or as PMOs incubated ex vivo with muscle fragments failed to predict the effects when PMO sequences were tested in vivo. The high percentage of in-frame products here might be related to nonsense-mediated decay of out-of-frame products or quality of RNA from cell culture; however, the in vitro experiments were consistent using three different concentrations (600, 60, and 30nM) with two different sequences (Ex6A and Ex6B). The results were confirmed by RT-PCR, immunohistochemistry, and complementary DNA sequences (see Fig 2; see Supplemental Fig 1). The in vitro effect of the exon 6-specific sequence was, in addition, context dependent. For when transfected into wild-type beagle myoblasts, the exon 8 AO pair again excised exons 8 and 9, whereas the exon 6-specific AO pair excised exons 6 and 9, leaving exons 7 and 8 in place (see Supplemental Fig 1). Thus, excision of exon 8 by the exon 6-specific sequences occurs only in the context of

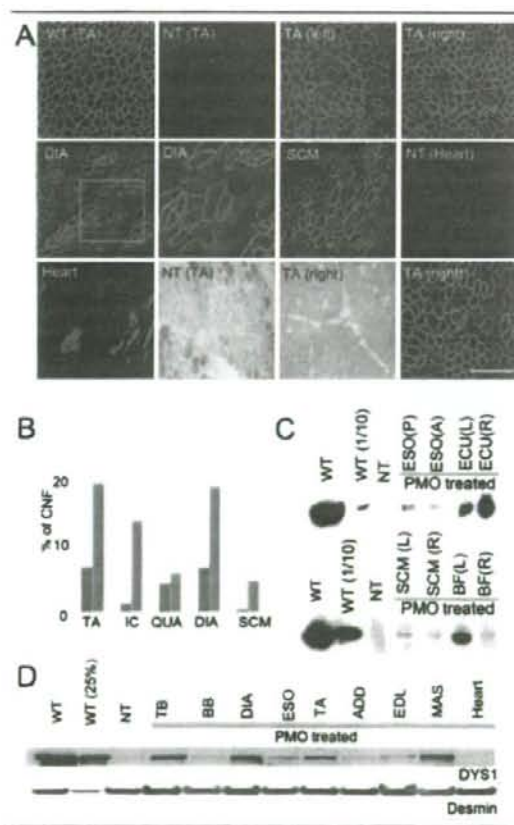


Fig 4. Widespread dystrophin expression and improved histology by intravenous systemic delivery of cocktail morpholinos in canine X-linked muscular dystrophy (CXMD) dogs. (A) Dystrophin (DYS-1) staining and histology in bilateral tibialis anterior muscles (TA), diaphragm (DIA), sternocleidomastoid (SCM), and heart at 2 weeks after final injection after five weekly intravenous injections of 120mg/kg cocktail morpholinos containing Ex6A, Ex6B, and Ex8A (2001MA). Comparisons were made with TA from normal control animal (wild type [WT]) and from nontreated CXMD littermate (NT) tibialis anterior (TA) and heart. Intravenous morpholino treatment resulted in extensive, though variable, dystrophin production in multiple muscles, but with only limited evidence of rescue in heart (isolated cardiocytes). Paired dystrophin immunostaining and histology from treated dog (TA, bottom panels) showed improved histopathology relative to untreated littermate (NT TA) histology. Bars = 200µm, except for higher magnification picture of DIA and hearts (100µm). (B) Quantitation of centrally nucleated fibers (CNFs) in TA, intercostal (IC), quadriceps (QUA), diaphragm (DIA), and sternocleidomastoid (SCM) in treated dog (blue bars; 2001MA) and untreated dog (red bars; 2008MA). (C) Western blotting analysis for detection of dystrophin at 2 weeks after final injection after 5 × weekly intravenous injections of 120mg/kg cocktail morpholinos containing Ex6A, Ex6B, and Ex8A (2001MA). Dystrophin rescue is variable with high expression in right extensor carpi ulnaris [ECU(R)] and left biceps femoris [BF(L)], and less in posterior [ESO(P)] or anterior esophagus [ESO(A)] and sternocleidomastoid (SCM). (D) Immunoblot analysis of dystrophin in intravenous morpholino-treated dog (2703MA; 7 × weekly dosing) and control animals (normal control [WT], nontreated [NT]). Desmin immunoblot is shown as a loading control. Dystrophin shows high levels (>25% control levels) in triceps brachii (TB), DIA, and masseter (MAS). ADD = adductor; BB = biceps brachii; EDL = extensor digitorum longus; MAS = masseter.

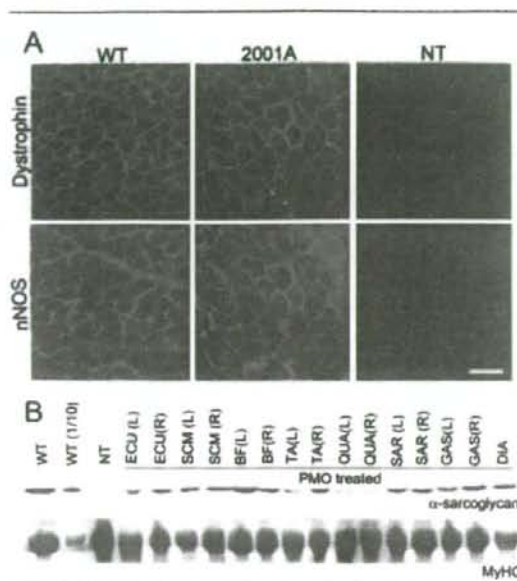


Fig 5. Recovery of localization and expression of dystrophin-associated proteins after systemic delivery of cocktail morpholinos to canine X-linked muscular dystrophy (CXMD) dogs. Neuronal nitric oxide synthase (nNOS) (A) and α -sarcoglycan (B) expression at 2 weeks after $5 \times$ weekly 120mg/kg cocktail (2001MA) or $7 \times$ weekly 200mg/kg cocktail morpholino injections (2703MA) to CXMD dogs. Recovery of nNOS expression at sarcolemma was observed by double immunofluorescence against dystrophin (DYS-1) and nNOS. Scale bar = $50 \mu\text{m}$. By immunoblot (B), α -sarcoglycan levels are increased in treated dog muscles, compared with untreated dystrophic controls (NT). Myosin heavy chain (MyHC) shown as a loading control. WT = wild-type normal control animals; WT(1/10) = wild-type diluted samples, ie, 4 μg loaded; NT = nontreated CXMD muscles (tibialis anterior); ECU = extensor carpi ulnaris; SCM = sternocleidomastoid; BF = biceps femoris; TA = tibialis anterior; QUA = quadriceps; SAR = sartorius; GAS = gastrocnemius; DIA = diaphragm; L = left side; R = right side.

the mutant exon 7 splice site. Together, the differences between patterns of skipping in vivo versus in vitro and between wild-type versus mutant genotypes tell us that efficiency of skipping during transcription is dominated by variables other than the availability or otherwise of specific local sequence. Thus, it is prudent to consider testing of selected sequences in multiple systems with human dystrophin mRNA as the target before committing to a specific sequence for clinic trials.

We observed efficient skipping targeted its removal in both wild-type and CXMD (see Figs 2 and 3), which is known as alternative splice site.²⁰ AOs targeting exon 8 have been reported to induce skipping of both exon 8 and 9 in human and in dog studies (see Figs 2 and

3).^{10,21} It appears likely that the small size of intron 8 compared with intron 7 (1.1 vs 110Kb) predisposes to splicing of exon 8 to exon 9 before splicing to exon 7.

In the systemically treated dogs, we found widespread expression of dystrophin in all muscles analyzed but with considerable variation (see Fig 4). No difference in dystrophin expression between fiber types was evident (data not shown). Even contralateral muscles differed from one another, suggesting that variation in efficacy of dystrophin production is a reflection of transient sporadic events such as myopathic episodes or changes in vascularization or circulation rather than any intrinsic muscle-specific properties. Pathological stages of degeneration/regeneration may also be in-

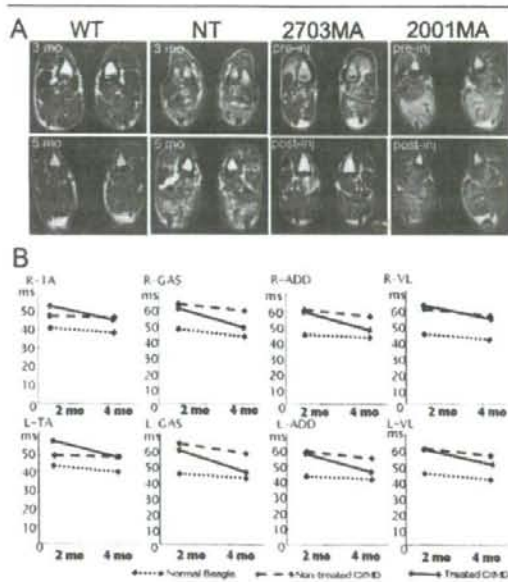


Fig 6. Amelioration of pathology and reduced inflammation signal in magnetic resonance imaging (MRI). T2-weighted MRI of hind legs at 1 week before initial injection (pre-inj) and at 2 weeks after final injection (post-inj) of $7 \times$ weekly intravenous (IV) injection of 200mg/kg cocktail morpholinos (2703MA) or $5 \times$ weekly IV injection of 120mg/kg cocktail morpholinos (2001MA). Age-matched untreated dogs (wild type [WT]; normal control) and nontreated dystrophic control (NT) are shown for comparison. (B) Changes of T2 value examined by MRI at 2 weeks after $7 \times$ weekly 200mg/kg cocktail morpholino injections. Changes of T2 values in hind legs at 1 week before initial injection and at 2 weeks after final injection are shown. Intravenous morpholino treatment resulted in decreased T2 signal in all muscles examined. TA = tibialis anterior; GAS = gastrocnemius; ADD = adductor; VL = vastus lateralis. Dotted lines represent normal beagle; dashed lines represent nontreated canine X-linked muscular dystrophy (CXMD); solid lines represent treated CXMD.

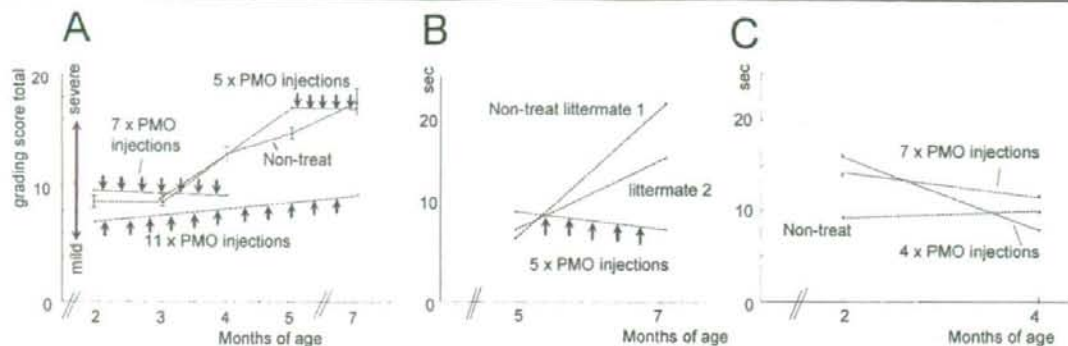


Fig 7. Stabilization of clinical symptoms by systemic morpholino treatment. (A) Combined clinical grading scores before (black lines) and after starting treatment (red lines) of the three treated dogs. Clinical grades of gait disturbance, mobility disturbance, limb or temporal muscle atrophy, drooling, macroglossia, and dysphagia are scored as described in Materials and Methods. A series of untreated dogs ($n = 6-13$) was studied for comparison (dashed line, standard error bars). (B, C) Fifteen-meter timed running tests (2001MA) of age showed decreased timed 15m run after treatment, whereas untreated littermates showed slowed running ability (B). Similarly, two littermate dogs treated at 2 to 4 months of age (2703MA; 2702FA) showed quicker 15m times after treatment compared with nontreated littermate (C).

involved. Overall, most studies to date suggest that 10 to 20% normal dystrophin levels are needed to improve muscle function,^{22,23} and the data on systemic morpholino-induced exon skipping presented here imply that some, but not all, muscle groups reached this therapeutic level.

Systemic delivery of morpholinos in CXMD dogs as in mdx mice induced only modest dystrophin production in the heart (see Fig 4).¹⁹ The reason is not clear, but it has been suggested that dystrophic skeletal muscle fibers may give greater access to AOs because they have more "leaky" membranes than the smaller cardiac cells and because the syncytial structure of myofibers may permit wider diffusion of PMO molecules from each site of entry.²⁴ Cardiac ischemia, as indicated by abnormal Q-waves in CXMD,²⁵ may also limit access of AOs to cardiomyocytes. Some improvement in delivery has been reported with cell-penetrating peptide-tagged morpholinos, or use of microbubbles and ultrasound that may enhance uptake efficiency in the heart by facilitating penetration of cell membranes, although toxicity of these strategies is not clear.^{26,27}

Considerable evidence for functional and histological improvement was seen in the three systemically treated dogs (see Figs 4-7). All were stabilized compared with their untreated littermates in motor function tests, general clinical condition, and serum creatine kinase levels. MRI images also showed reduction of T2-weighted signal, interpreted as a sign of diminished inflammation, after morpholino delivery (see Fig 6). However, longer-term experiments are required to investigate whether AOs can reduce infiltration by fibrofatty tissue and to what extent functional loss can be recovered.

Many DMD patients require two or more exons to be skipped to restore the reading frame. The data reported here are the first demonstrations of efficient skipping of multiple exons systemically through intravenous delivery. The dog model required skipping of two to three exons to restore the reading frame, and we were able to show efficient skipping of three exons by both intramuscular and intravenous delivery methods. Multiexon skipping has also been shown in vitro cell cultures and in mdx mice by intramuscular injections.^{8,28-30} Multiexon skipping increases the range of potentially treatable DMD patients and also raises the prospect of selecting the most functionally favorable in-frame dystrophins,³¹ although skipping larger stretches of exons has yet not been achieved and currently may not be feasible. Specific morpholino cocktails able to treat a large proportion of DMD patients with optimized quasi-dystrophin production might be submitted for regulatory approval as a single "drug." For example, a cocktail of AOs targeting exons 45 to 55 would be applicable in up to 63% of patients with dystrophin deletions, and this specific deletion is associated with asymptomatic or mild BMD clinical phenotypes.^{31,32}

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Reduced proliferative activity of primary POMGnT1-null myoblasts in vitro

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ABSTRACT

Protein O-linked mannosyltransferase 1 (POMGnT1) is an enzyme that transfers N-acetylglucosamine to O-mannose of glycoproteins. Mutations of the POMGnT1 gene cause muscle-eye-brain (MEB) disease. To obtain a better understanding of the pathogenesis of MEB disease, we mutated the POMGnT1 gene in mice using a targeting technique. The mutant muscle showed aberrant glycosylation of α -DG, and α -DG from mutant muscle failed to bind laminin in a binding assay. POMGnT1^{-/-} muscle showed minimal pathological changes with very low serum creatine kinase levels, and had normally formed muscle basal lamina, but showed reduced muscle mass, reduced numbers of muscle fibers, and impaired muscle regeneration. Importantly, POMGnT1^{-/-} satellite cells proliferated slowly, but efficiently differentiated into multinuclear myotubes *in vitro*. Transfer of a retrovirus vector-mediated POMGnT1 gene into POMGnT1^{-/-} myoblasts completely restored the glycosylation of α -DG, but proliferation of the cells was not improved. Our results suggest that proper glycosylation of α -DG is important for maintenance of the proliferative activity of satellite cells *in vivo*.

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1. Introduction

POMGnT1 is the glycosyltransferase that catalyzes the transfer of N-acetylglucosamine (GlcNAc) to O-mannose of glycoproteins, the second step of Ser/Thr O-mannosylation (Yoshida et al., 2001; reviewed in Endo and Toda,

2003). Mutations in the POMGnT1 gene cause muscle-eye-brain (MEB) disease, a rare autosomal recessive disorder characterized by congenital muscular dystrophy with elevated serum creatine kinase (CK) levels, severe visual failure, and gross mental retardation (Yoshida et al., 2001).

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α -Dystroglycan (α -DG) is a heavily glycosylated glycoprotein and a well-known substrate of POMGnT1. Dystroglycan is encoded by a single gene (*DAG1*) and is cleaved into two proteins, α -dystroglycan (α -DG) and β -dystroglycan (β -DG), by posttranslational processing (Ibraghimov-Beskrovnaia et al., 1992). DGs are central components of the dystrophin-glycoprotein complex (DGC) at the sarcolemma, and α -DG was shown to serve as a cell surface receptor for laminin (Ibraghimov-Beskrovnaia et al., 1992), agrin (Gee et al., 1994; Campanelli et al., 1994), perlecan (Peng et al., 1998; Kanagawa et al., 2005), and neuexin (Sugita et al., 2001). In skeletal muscle, the laminin- α -DG linkage is thought to be critical for plasma membrane stability (recently reviewed in Kanagawa and Toda 2006). In MEB muscle, the α -DG core protein is preserved but hypo-glycosylated, and α -DG prepared from the muscle fails to bind laminin *in vitro* (Michele et al., 2002). Therefore, it is proposed that the disruption of the α -DG-laminin linkage is the main pathomechanism of dystrophic changes seen in MEB muscle.

To further elucidate the molecular pathogenesis of MEB disease, we generated POMGnT1-knockout mice using a gene targeting technique, and examined the mutant skeletal muscle. During our experiments, Liu et al. reported the generation of POMGnT1-deficient mice (Liu et al., 2006). The report showed severe muscle pathology, but the mechanism by which POMGnT1 deficiency causes muscle phenotype was not clearly shown. In this report, we report that POMGnT1-deficient mice show remarkably minimal signs of muscle degeneration and regeneration, but also show small muscle mass, reduced numbers of muscle fibers, and impaired muscle regeneration. POMGnT1-deficient myoblasts proliferate poorly *in vitro*. The proliferation was not improved by retrovirus vector-mediated POMGnT1 expression in POMGnT1^{-/-} myoblasts, suggesting that α -DG-laminin interaction *in vivo* is important for maintenance of the proliferative activity of satellite cells.

2. Results

2.1. Inactivation of the POMGnT1 gene in mice

We mutated the POMGnT1 gene by replacing exon 18 with a neomycin-resistance gene in mouse ES cells (depicted in Fig. 1). Two ES clones successfully entered the germline. Although there was no evidence of embryonic lethality, more than 60% of the homozygotes died within 3 weeks of birth. Survivors were smaller than their wild-type littermates (Fig. 3A) throughout life, but most of them had a normal life span. We confirmed that the POMGnT1^{-/-} mice completely lacked the POMGnT1 enzyme activity (Fig. 2A). A monoclonal antibody, VIA4-1, that reacts with the sugar moiety of α -DG gave no signal in either POMGnT1^{-/-} brain (Fig. 2B) or muscle (data not shown). A polyclonal antibody against α -DG core protein revealed that the POMGnT1^{-/-} brain expresses approximately 80 kDa α -DG protein, which is much smaller than that seen in the wild-type brain (ca. 110 kDa) (Fig. 2C). We next examined whether α -DG in POMGnT1^{-/-} brain binds laminin. Wheat germ agglutinin (WGA)-enriched brain protein from control and POMGnT1^{-/-} mice was separated on

SDS-PAGE gels, blotted onto a PVDF membrane, incubated with EHS laminin, and then bound laminin was detected by an anti-laminin antibody. α -DG in POMGnT1^{-/-} brain failed to bind to laminin (Fig. 2D).

2.2. POMGnT1^{-/-} muscle shows very mild dystrophic changes

Immunohistochemistry of cross-sections of tibialis anterior (TA) muscles showed that dystrophin and other members of the DGC complex were normally expressed at the sarcolemma of POMGnT1^{-/-} muscle (Fig. 3 and Table 1). Laminin α 2 chain was detected around POMGnT1^{-/-} muscle fibers. On H.E.-stained cross-sections, surprisingly, the POMGnT1^{-/-} muscle showed almost normal morphology. Central nucleation of myofibers indicates regeneration events in the past. In the TA muscles of 4-week-old wild-type mice, 0.25% of myofibers were centrally nucleated. In POMGnT1^{-/-} mice, 0.28% of the myofibers had central nuclei. In contrast, ca. 40–50% of myofibers of age-matched mdx mice were centrally nucleated. Even at 24 months of age, the percentage of centrally nucleated myofibers was lower (3.6%) in POMGnT1^{-/-} TA muscle, compared with age-matched wild-type TA muscle (9.0%). POMGnT1^{-/-} TA muscle also lacked other signs of degeneration and regeneration. Electrophoresis of muscle extracts on glycerol SDS-PAGE gels showed no difference in MyHC isoform composition of quadriceps and gastrocnemius muscles between POMGnT1^{-/-} and wild-type littermates (data not shown). In both wild-type and POMGnT1^{-/-} muscle, the muscle basal lamina was normally formed (Supplementary Fig. 1). Electron microscopy also showed that the sarcomere structures are almost normal in POMGnT1^{-/-} mice. We next examined the serum creatine kinase (CK) levels, an index of on-going muscle damage, in wild-type, POMGnT1^{-/-}, and age-matched mdx mice (Fig. 5). The serum CK levels of 5- to 20-week-old POMGnT1^{-/-} mice were slightly higher (av. 586 U/L, n = 10) ($p < 0.05$) than those of wild-type littermates (less than 100 U/L, n = 4), but were much lower than those of mdx mice (more than 5000 U/L, n = 3, $p < 0.01$). The serum CK levels of 2-year-old POMGnT1^{-/-} mice were still low (less than 300 U/L, n = 4).

2.3. Repetitive muscle injury causes more fibrosis and fatty infiltration in POMGnT1^{-/-} than in WT TA muscle

Dystroglycans expressed on the cell membrane of satellite cells are proposed to play an important role in muscle regeneration (Cohn et al., 2002). In addition, the average size of POMGnT1^{-/-} myofibers was smaller than those of wild-type myofibers (Fig. 4). Moreover, the number of myofibers is reduced in POMGnT1^{-/-} skeletal muscle of neonatal and adult POMGnT1 mice, suggesting proliferation defect of POMGnT1^{-/-} myoblasts (Fig. 4). To test the hypothesis, we damaged POMGnT1^{-/-} TA muscle by cardiotoxin (CTX) and examined their regeneration. After single cardiotoxin injection, POMGnT1^{-/-} muscle regenerated well like wild-type (data not shown). Next, we injected CTX into TA muscles of POMGnT1^{-/-} and heterozygous littermates three times at intervals of 2 weeks, or 1 week interval, and examined the muscle. We summarized the results in Fig. 6. POMGnT1^{-/-} muscle showed more fibrosis and

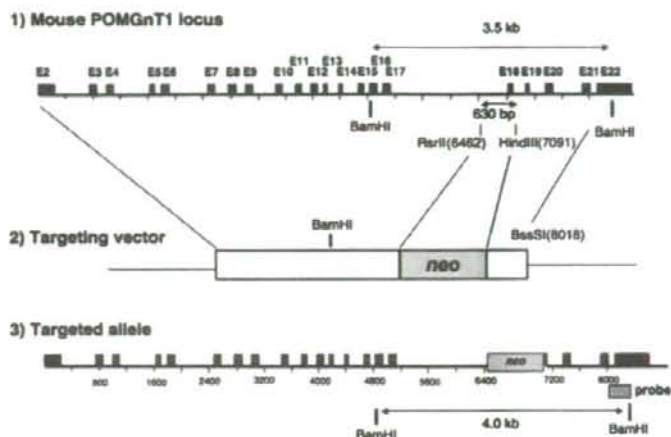


Fig. 1 – Targeted disruption of the mouse *POMGnT1* gene in embryonic stem (ES) cells. The successfully targeted allele lacks a 630 bp-genome fragment containing exon 18, and instead has a neo resistance gene. Recombination in ES cells was confirmed by Southern blotting with the probe shown by a shaded box. The nucleic acid numbers are from AB053221 in GenBank.

fatty infiltration, which is a sign of inefficient muscle regeneration, than *POMGnT1*^{-/-} muscle. Together with reduced numbers of myofibers in muscle, the results suggest that the function of satellite cells in *POMGnT1*^{-/-} skeletal muscle is impaired.

2.4. Defective proliferative activity of *POMGnT1*^{-/-} myoblasts

We next tested activation and proliferation of satellite cells on living myofibers isolated from wild-type and *POMGnT1*^{-/-}

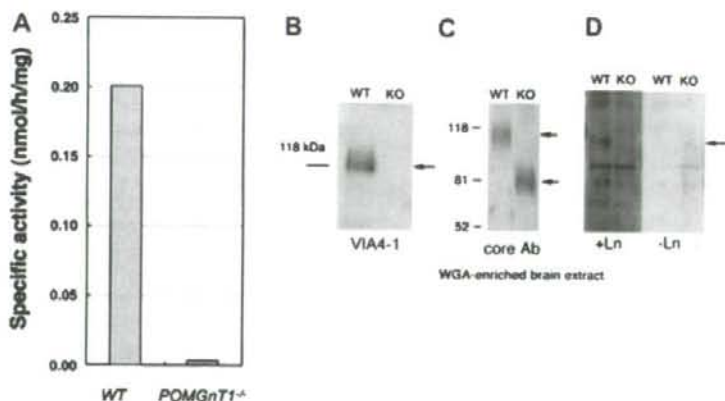


Fig. 2 – *POMGnT1*^{-/-} mice show undetectable *POMGnT1* enzyme activity and aberrant glycosylation of α -dystroglycan (α -DG) in *POMGnT1*^{-/-} mice. (A) The amount of *POMGnT1* activity is based on the amount of [³H]GlcNAc transferred from UDP-GlcNAc to mannosyl peptide. The reaction product was purified by reverse-phased HPLC, and the radioactivity was measured. (B) Wheat germ agglutinin (WGA) agarose-enriched brain extracts from wild-type (WT) or *POMGnT1*^{-/-} (KO) mice were resolved on a 7.5% SDS-PAGE gel, transferred to a PVDF membrane, and probed with anti- α -DG antibody, VIA4-1, which recognizes glycosylated α -DG. (C) The blot was incubated with polyclonal antibodies specific for α -DG core protein. The antibody detected ~110 kDa bands in wild-type brain extract, and 80 kDa bands in the brain extract of *POMGnT1*^{-/-} mice. (D) Laminin overlay assay showing that α -DG in *POMGnT1*^{-/-} brain does not bind laminin *in vitro*. +Ln, laminin was incubated with the blotted membrane. -LN, without laminin.