4. Add 200 µL of chloroform (for RNA)
5. Shake well.
6. Wait 2 min.
7. You can see three layers including the RNA layer (top), DNA layer (middle), and protein layer (bottom).
8. Centrifuge at 12,000 ×*g* for 15 min at 4°C.
9. Take 400 mL carefully from top layer. Remove supernatant from the top layer, and put in another tube.
10. Add 500 mL isopropanol.
11. Keep in -80°C for O/N.
12. Centrifuge at 12,000 ×*g*, 10 min, 4°C.
13. Decant fluid. You can see a pellet of RNA in bottom.
14. Wash with 75% EtOH.
15. Centrifuge at 8,000 ×*g*, 5 min, 4°C.
16. Dry up, keep upside down for 15 min or O/N.
17. Add 15–30 µL water, then quantify RNA concentration.

3.6. RT-PCR

1. Make Reaction mix containing 1.5 µL 10 mM forward primer, 1.5 µL 10 mM reverse primer, 1 µL dNTP, 5 µL one-step PCR kit buffer, 0.7 µL RNAsin, 1 µL enzyme mixture from one-step PCR kit, and 200 ng RNA and add water to the total of 25 µL.
2. Perform RT-PCR in the thermocycler with 1 cycle of 50°C 30 min, 1 cycle of 95°C 15 min, 35 cycles of 94°C 1 min, 60°C 1 min and 72°C 1 min. Finally add 1 cycle of 72°C 10 min and then store PCR product in 4°C.

3.7. cDNA Sequencing

1. Use Qiagen gel extraction kit to excise the band of interest for subsequent cDNA sequencing according to manufacturer's instructions. Exon 6-9 skipped band (101 bp) is identified by electrophoresis using 2% agarose gel (Fig. 1).
2. Use BigDye® Terminator v3.1 cycle sequencing kit for cDNA sequencing with the same primers following manufacturer's instructions (Fig. 1).

3.8. Muscle Sampling from Necropsy of Dogs

1. Inject with thiopental sodium for induction of general anesthesia, then maintain anesthetic status by isoflurane.
2. Euthanize dogs by bleeding from the carotid artery.
3. Collect following muscles by necropsy of dogs 2 weeks after final injection of oligos. These muscles include TA, extensor digitorum longus (EDL), Gastrocnemius, soleus, biceps femoris, rectus femoris, biceps brachii, triceps brachii, deltoid, extensor carpi ulnaris (ECU), extensor carpi radialis (ECR),

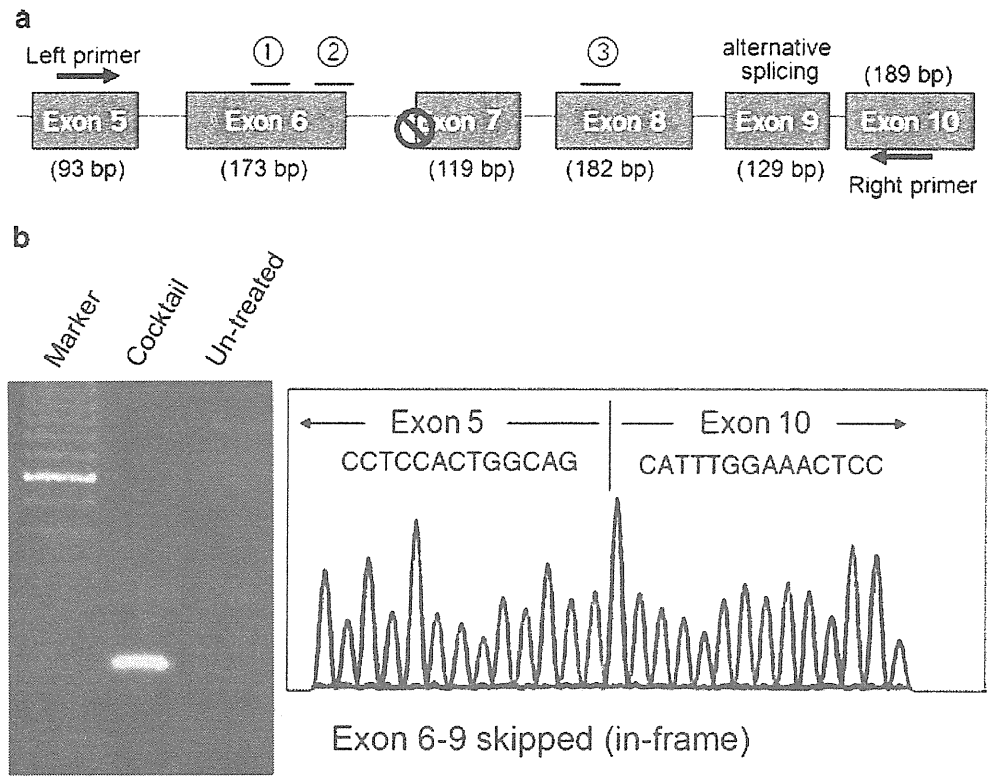

Fig. 1. Multiple exon skipping in dystrophic dogs. (**a**) Schematic outline of the protocol. A splice site mutation in intron 6 leads to deletion of exon 7 at mRNA level in dystrophic dogs. To restore the reading frame, two additional exons (exon 6 and exon 8) need to be skipped (removed) by three oligo cocktail of-antisense. Exon 9 is known as alternative splice site. (**b**) RT-PCR and cDNA sequencing after exon skipping in dystrophic dogs. *Left panel*; RT-PCR reveals exon 6–9 skipped in-frame products (101 bp) in dystrophic dogs after the treatment of cocktail oligos. Alternative splice site Exon 9 is also mostly removed from the resulting mRNA. *Right panel*; Exon-skipping patterns are further confirmed by cDNA sequencing.

flexor carpi ulnaris (FCU), flexor carpi radialis (FCR), gracilis, intercostal, abdominal muscles, diaphragm, lateral dorsi, esophagus, sternocleidomastoid, and the heart.

4. Dissect muscles into small portion to stand on cork disks (1.2 cm diameter) labeled with the ID of the animal and muscle name on the back side.
5. Mix a portion of tragacanth gum (10–20 mL) well with equal amount of water until it becomes soft and sticky. Put them into 10 mL or 25 mL syringes. Unused gum in the syringe can be stored in freezer.
6. Put tragacanth gum to fix the muscle specimen on cork disks.
7. Put liquid nitrogen in a metal container and isopentane in a smaller metal container.
8. Lower the isopentane with the container into the liquid nitrogen. Wait for a couple of minutes until it becomes slushy and ready for freezing.
9. Put a portion of gum on the cork.

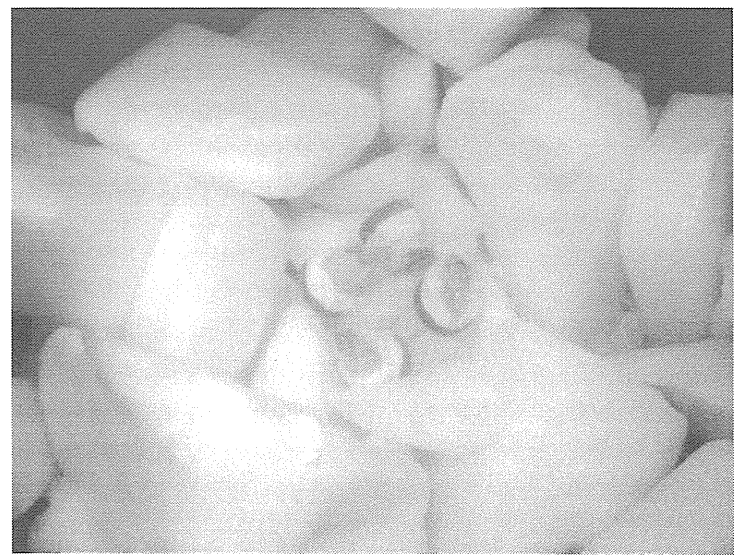

Fig. 2. Frozen muscle samples from dystrophic dogs.

10. Dissect out muscles and put it on the cork at RT. Place it on the cork longitudinally and put some gum blob around the bottom of them so that the longitudinal axis of the muscle is perpendicular to the cork and stable.
11. Place the muscle on cork into the cooled isopentane and shake vigorously for 1 min.
12. Place it on dry ice (Fig. 2).
13. Put samples in a glass vials, and store at –80°C.

3.9. Immunostaining for Dog Muscles

1. Set up cryostat for sectioning. The working temperature should be –25°C. Set the section thickness at 8 μm for immunohistochemistry and 12 μm for HE staining. Put in a blade.
2. Place muscle blocks on dry ice for transportation.
3. Label slide glasses in pencil with animal IDs, cut date, and muscle name.
4. Mount cork with muscle sample block and fix in place with water. Attach the chuck with tissue specimen onto the holder.
5. Start slicing the muscle until approximately one fourth of the way in the muscle.
6. Touch and transfer individual sections onto RT slide glass and leave at RT to dry.
7. Place every sixth section on the same slide (sections 1, 6, 11 on slide #1; sections 2, 7, 12 on slide #2) and cut them at interval of every 200 mm until you have five sections collected per slide. Keep sections clustered as closely as possible to reduce the amount of antibody solutions required.

8. When finished, allow slides to dry at RT for at least an additional 90 min. Slides can be stored at –80°C.
9. For immunohistochemistry, put slides in moisture chamber (and dry them for 30 min if they were stored in a freezer).
10. Blocking; 2 h in PBS with 15% goat serum at RT.
11. Incubate with a primary antibody; antidystrophin rod (DYS-1) or C-terminal monoclonal antibody (DYS-2) for dog dystrophin staining (1:150 dilutions) for overnight at 4°C.
12. Wash with PBS 5 min × 3 times.
13. Incubate with a secondary antibody, Alexa 594 goat antibody against mouse IgG_1 or IgG_2 (highly cross-absorbed) (1:2,500) for 30 min at RT.
14. Wash with PBS 5 min × 5 times.
15. Wipe off excess liquid and mount with DAPI-containing mounting agent for nuclear staining and then put cover glasses.
16. Count the number of positive fibers for DYS1 under fluorescent microscope and compare in sections where their biggest number of the positive fibers were as previously described (see Note 4) (11).
17. Immunohistochemistry is also applicable for myotubes. Use slide glasses with chambers to culture them and fix them by 4% PFA for 10 min (Fig. 3).

3.10. Western Blotting from Dog Muscles

1. Collect the 30–40 of cryo-sections of 15 μm in 1.5 mL tube on dry ice.
2. Add 150 mL of sample buffer and homogenize on ice.
3. Boil them for 3–5 min and centrifuge for 15 min at 16,500 × *g*.
4. Collect supernatant and keep the aliquot at –70°C.

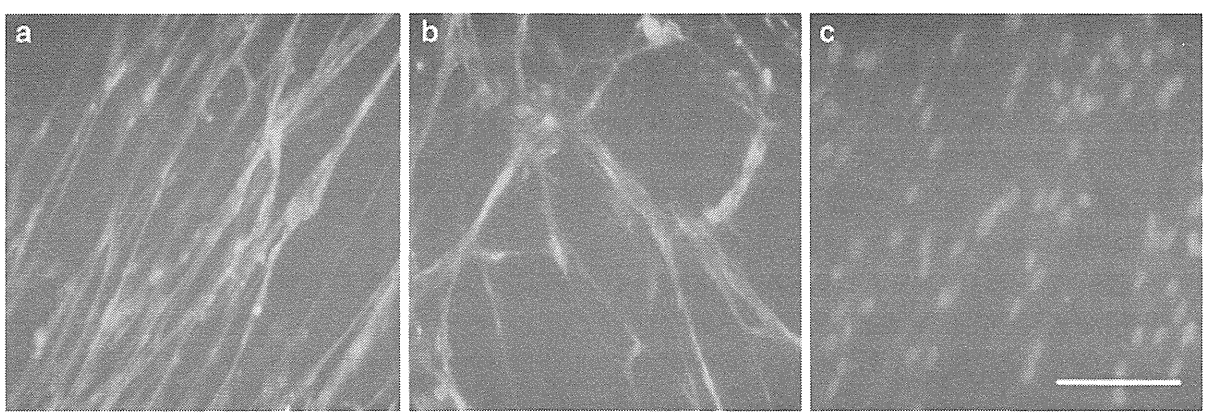

Fig. 3. Recovery of dystrophin expression after cocktail antisense transfection in dystrophic dog myoblast. Dystrophin expression and DAPI nuclear double staining of wild-type myotubes (**a**), cocktail of Ex6A, Ex6B, and Ex8A 2'OMePS transfected myotubes (**b**), and nontreated CXMD myotubes (**c**). Dystrophin C-terminal antibody DYS-2 is used. Bar: 50 μm.

5. Dilute an aliquot of protein 100-fold with distilled water to reduce final SDS concentration less than 0.1%. Measure protein concentration of the diluted protein sample with Bradford protein assay. Specifically, record the absorbance at 570 nm using a photospectrometer and calculate the concentration from standard curve.
6. For SDS-PAGE, set the glass plates for readymade mini-gel (5%).
7. Mix the samples with 2× Laemmli SDS-loading buffer.
8. Boil samples for 3 min, then load 20 mg of samples in each lane.
9. Run the gel at 150 V for approximately 3 h.
10. After running the gel, incubate the gel for 20 min in transfer buffer + 0.1 % SDS (optional for transferring high molecular weight proteins).
11. Wet four pieces of sponge and Whatman paper with ddH_2O, then soak them in the transfer buffer, and soak PVDF membrane using methanol for 1 min to prewet it, and then pour-off methanol and add H_2O, make sure that the membrane does not float. Leave it in water for 3 min.
12. Set the gel and membrane as shown in the manual.
13. Run 40–50 V o/n in cold room.
14. For blotting, prepare 2,000 mL of 0.05% PBS/Tween 20 (PBST). Wash the membrane briefly with 20 mL PBS.
15. Prepare 100 mL of PBST/5% milk powder, and incubate in 50 mL PBST/5% milk powder for 2 h.
16. Incubate the blot with primary antibody in the appropriate dilution with PBS/5% milk powder (1:100 dilution for Dys1 dystrophin antibody) for 1 h or O/N.
17. Wash the blot for 15 min each with 3× 100 mL PBST, then incubate the blot with the HRP conjugated secondary antibody.
18. Wash the blot for 20 min each with 3× 200 mL PBST.
19. Use ECL plus kit for detection. Mix two solutions at 40:1, and incubate with membrane for 1 min. Then use film and developer for the detection.
20. To preserve for the spare blot, rinse the blot with PBST, and store it in PBST at 4°C for a few weeks. Desmin antibody is used to normalize intersample loading amount. Signals are analyzed and quantified using Adobe Photoshop and ImageJ software (Fig. 4).

3.11. Clinical Grading of Dogs

1. Let a dog walk and evaluate gait disturbance: grade 1 = none, grade 2 = sitting with hind legs extended, grade 3 = bunny

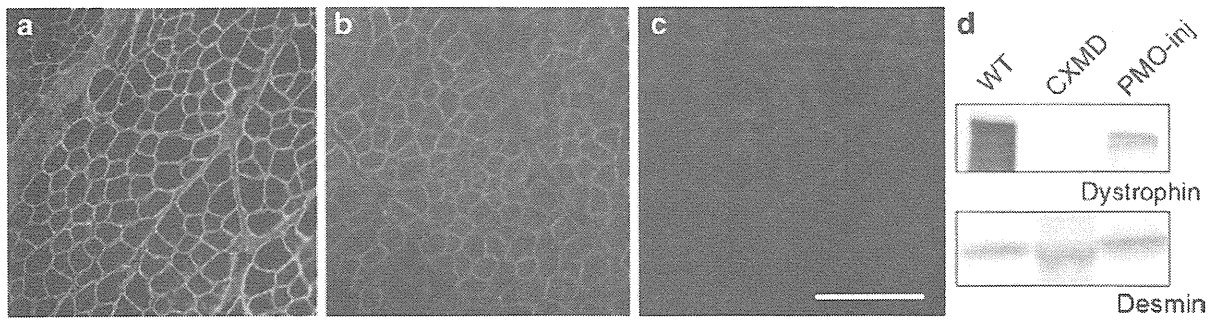

Fig. 4. Recovery of dystrophin expression after 7 × 200 mg/kg intravenous cocktail morpholino injections. Dystrophin expression of wild-type dog muscle (**a**), cocktail of Ex6A, Ex6B, and Ex8A PMOs injected dog muscle (**b**), nontreated CXMD dogs (**c**), Western blotting analysis with dystrophin antibody (**d**). Bar: 100 μm.

hops with hind legs, grade 4 = shuffling walk, and grade 5 = unable to walk (12).

2. Evaluate 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.
3. Palpate limb or temporal muscle atrophy: grade 1 = none, grade 2 = suspect hardness, grade 3 = can feel hardness or apparently thin, grade 4 = between grades 3 and 5, and grade 5 = extremely thin or hard.
4. Evaluate 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.
5. Evaluate 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.
6. Evaluate 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.
7. Add up the total score.
8. For running test, encourage each dog to run one time for 15 m, and record elapsed time.

4. Notes

1. Alternatively, one can also use 2'O-MePs (Eurogentec) against exons 6 and 8 of the dog dystrophin gene. These include Ex6A (GUUGAUUGUCGGACCCAGCUCAGG),

Ex6B (ACCUAUGACUGUGGAUGAGAGCGUU), and Ex8A (CUUCCUGGAUGGCUUCAAUGCUCAC).

2. The efficacy of antisense oligos is highly unpredictable, and hence several antisense oligos should be designed for each target exon. A preferred antisense sequence contains 40–60% of GC, does not have more than three consecutive guanine, and does not lead to self dimers or hetero dimers when injected as a cocktail. We have designed more than ten antisense sequences against exon 6 and exon 8 of dogs, and optimized the most efficient combination of cocktail antisense oligos both in vitro and in vivo (Saito et al., Unpublished).
3. For 2′OMePS, U (uracil) is used instead of T (thymidine).
4. Occasionally dystrophin-positive revertant fibers can be detected in dystrophic dog muscles (5, 12). Revertant fibers cannot be distinguished from antisense-mediated dystrophin expression by immunohistochemistry unless an epitope-specific antibody is used. Therefore, the expression level should be carefully compared with untreated controls.

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The frontier of antisense oligonucleotide-induced therapy

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Abstract

Duchenne muscular dystrophy (DMD) is a severe muscle disorder characterized by mutations in the *DMD* gene that mainly disrupt the reading frame, leading to the absence of functional protein. The concept of using antisense oligonucleotides to manipulate pre-mRNA splicing, an approach known as 'exon skipping', offers a novel therapeutic strategy for the treatment of DMD. Recently, this approach was proved both promising and safe in clinical trials on DMD patients conducted in Europe, targeting exon 51. Moreover, this approach could be applied to the treatment of other neuromuscular diseases in which the disease mechanism involves splicing defects or aberrant alternative splicing. The purpose of this review is to summarize recent progress in exon skipping therapy for DMD and give an overview of other antisense oligonucleotide-based therapeutic applications.

Key words : antisense oligonucleotides, RNA splicing, *DMD* gene, Duchenne muscular dystrophy (DMD), dystrophin, exon skipping.

Abbreviations : ALS = amyotrophic lateral sclerosis ; AON = antisense oligonucleotides ; BMD = Becker muscular dystrophy ; $CXMD_J$ = canine X-linked muscular dystrophy in Japan ; DGC = dystrophin-glycoprotein complex ; DM1 = type 1 myotonic dystrophy ; DMD = Duchenne muscular dystrophy ; GRMD = golden retriever muscular dystrophy ; MBNL1 = muscleblind-like 1 ; 2'-O-methyl-modified phosphorothioate = 2'OMePS ; PMO = phosphorodiamidate morpholino ; PPMO = peptide-tagged PMO ; SMA = spinal muscular atrophy ; SMN1 = survival motor neuron 1 ; SOD1 = superoxide dismutase 1.

1. Introduction

The muscular dystrophies are a group of inherited primary myopathies characterized by progressive skeletal muscle weakness and atrophy. Duchenne muscular dystrophy (DMD) or the milder, allelic Becker muscular dystrophy (BMD), are X-linked diseases resulting from a genetic mutation in the *DMD* gene, which leads to a loss or severe reduction of functional dystrophin protein. As a result, the dystrophin-glycoprotein complex (DGC) that links the actin cytoskeleton of muscle fibers to their extracellular matrix collapses, leading to sarcolemmal fragility. DMD is typically diagnosed between the ages of 2 and 5 years mainly due to hyper-CKemia or gait disturbance and patients become wheelchair-bound and require ventilatory assistance during the second decade of life, demonstrating the severe progressive course of the disease. Thus far, there is no cure for DMD, and standard management for patients includes corticosteroid therapy and supportive measures. Although the drug initially improves muscle function and strength, there are side effects associated with the drug and limitations to their therapeutic value over time.

During the last two decades, several therapeutic strategies for the treatment of DMD have been under intense investigation : gene therapy using micro-dystrophin with an adeno-associated virus vector, stem cell transplantation using muscle satellite cells or bone marrow stromal cells, and read-through therapy for nonsense mutations. However, there is no established curative approach to DMD. Exon skipping using antisense oligonucleotides (AONs) is emerging as a very promising therapeutic approach for DMD. The strategy is to convert the severe DMD phenotype into the mild-to-moderate BMD phenotype by correcting the exon(s) that disrupt the reading frame, allowing the synthesis of the partly functional, internally deleted Becker-like dystrophin protein. This can be brought about by AONs that target specific exons and subsequently block the binding of the spliceosome to pre-mRNA, resulting in the skipping of exon(s). Here, we describe the recent progress in exon skipping for DMD and give an overview of other AON-based therapeutic applications.

2. DMD and BMD

The *DMD* gene is the largest known human gene, spanning ~2,500 kb of the X chromosome and occupying ~0.1% of the genome ; it is composed of 79 exons[1]. Its main protein product, dystrophin, was first characterized in 1987[2]. Dystrophin is a rod-shape

structure that consists of four domains : (i) the N-terminal actin-binding domain ; (ii) a rod domain composed of 24 spectrin-like rod repeats and four hinges ; (iii) a cysteine-rich domain that interacts with the dystroglycan and sarcoglycan complexes ; and (iv) the C-terminal domain that interacts with the syntrophin complex and dystrobrevin. Dystrophin is localized at the subsarcolemma and forms the DGC with dystroglycan, sarcoglycan, and the syntrophin/dystrobrevin complexes. One of the roles of the DGC is to stabilize the plasma membrane and to protect muscle fibers from damage. The absence of dystrophin makes muscle fibers vulnerable to stretch-induced damage, subsequently increasing intracellular calcium influx, and is followed by muscle degeneration. Mutations of the *DMD* gene that disrupt the open reading frame result in no dystrophin expression. This leads to muscle fiber damage and the loss of muscle fibers, impaired muscle function, and eventually the severe phenotype observed in DMD patients. DMD patients suffer from severe, progressive muscle wasting leading to loss of ambulation and respiratory weakness. Cardiac complication is observed in up to 90% of DMD patients[3].

In contrast, mutations that maintain the open reading frame enable the production of internally deleted, but partially functional dystrophin. These mutations are associated with BMD, a much milder form of muscular dystrophy. Patients with BMD exhibit a large phenotypic spectrum, ranging between severe childhood-onset muscular diseases to asymptomatic cases. Cardiac complications (leading to cardiomyopathy and heart failure) are frequent, age-dependent, and unpredictable. In general, onset of neuromuscular symptoms precedes cardiac involvement. Patients generally remain ambulant until later in life and have near normal life expectancies[4].

While current treatment for DMD patients is limited almost completely to glucocorticosteroids, there are several promising new therapeutic approaches that have been investigated extensively : gene therapy using micro-dystrophin with an adeno-associated virus vector, stem cell transplantation using muscle satellite cells or bone marrow stromal cells, and read-through therapy for nonsense mutations[5]. Possibly the most promising of all novel therapies for DMD is exon skipping, whereby AONs splice out specifically targeted exon(s) of the pre-mRNA of *DMD* to generate the shortened but functional dystrophin protein seen in BMD (**Fig. 2**)[6].

3. Exon skipping for DMD

The first experiments involving AON-mediated splicing were performed by Kole, et al[7].

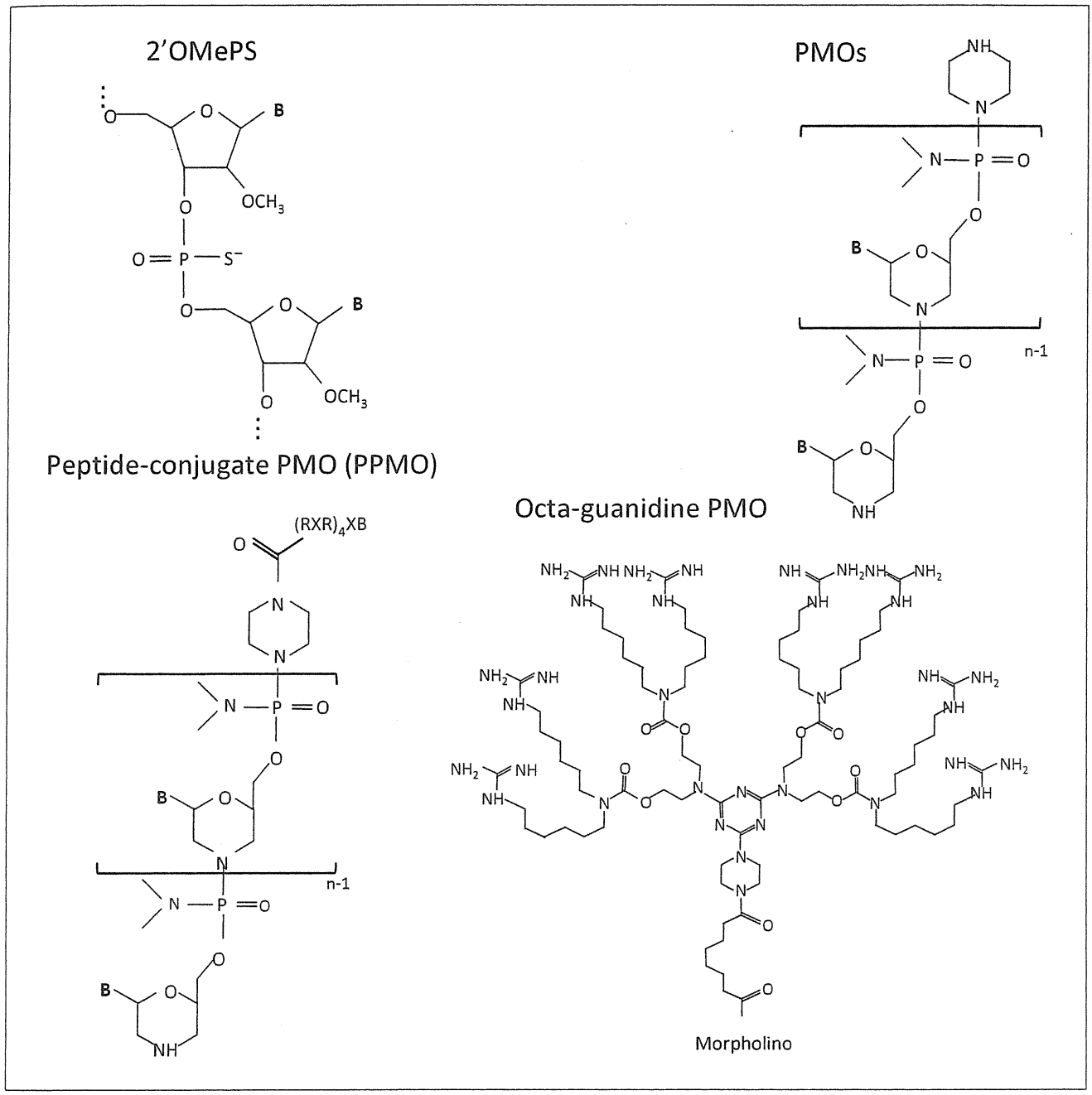

Fig. 1 Chemical structures of antisense oligonucleotides

(a) 2'-O-methyl-modified phosphorothioate (2'OMePS) ; (b) phosphorodiamidate morpholino oligomers (PMOs) ; (c) AcHN-(RXRRBR) 2XB peptide-tagged PMO (R, arginine, X, 6-amino-hexanoic acid and B, R-alanine) (PPMO) ; (d) octa-guanidine PMO.

To restore normal splicing in β-thalassemia, they masked the cryptic splice sites that arose from intronic mutations in the β-globin gene using 2'-O-methyl-modified phosphorothioate antisense oligonucleotides (2'OMePS). 2'OMePS have a chemically modified RNA structure with methylation at the 2'-OH position of the ribose ring (**Fig. 1**). This modification increases the half-life and distribution to tissues. This experiment revealed new

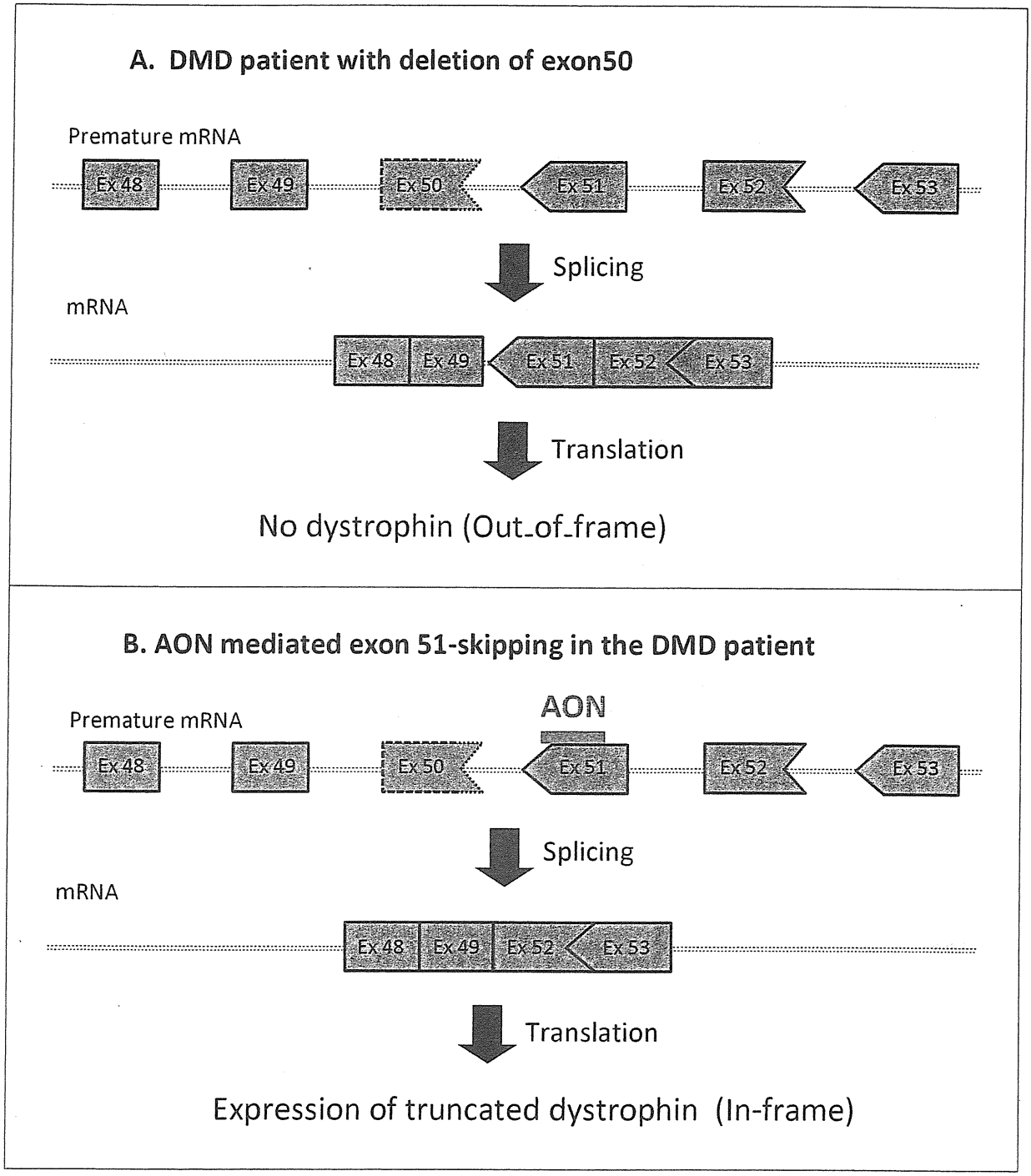

Fig. 2 Antisense oligonucleotide-mediated exon 51-skipping in a DMD patient
(a) The DMD patient exhibits a deletion of exon 50 resulting in an out-of-frame mRNA and lack of dystrophin expression. (b) AONs are used to induce skipping of exon-51 to restore the reading frame back.

therapeutic avenues for diseases caused by abnormal splicing[7].

Next, the Kobe group pioneered a new potential therapeutic application for DMD. This group attempted to skip exon 19 of the *DMD* gene in exon 20-deleted DMD patients, with

the idea based on dystrophin Kobe, in which exon 19 is naturally skipped due to a 52-bp deletion within the exon[8)9)].

Then, some experiments were carried out in cells from patients and a mouse model to demonstrate the feasibility of exon skipping. Two groups studied cultured muscle cells from the *mdx* mouse[10)11)], which carries a nonsense point mutation in the in-frame exon 23[12)13)]. In both studies, skipping of exon 23 maintained the reading frame, at least at the RNA level as demonstrated by reverse transcription PCR. Another group showed restoration of dystrophin at the protein level after exon 46 skipping in cultured muscle cells from two DMD patients with an exon 45 deletion[14)]. These seminal reports paved the way for many other studies conducted in patient-derived cell cultures[8)-14)]. These, and numerous *in vivo* studies in DMD animal models (described below), have convincingly demonstrated the therapeutic potential of exon skipping[15)-18),25)26)].

1) Mdx mice

In experiments performed by Mann, et al[15)] and Lu, et al[16)], 2OMePS with the non-ionic polymer F127 were injected into the tibialis anterior muscles of mdx mice. The sequences of the 2'OMePS were complementary to the mouse *Dmd* exon/intron 23 boundary region. Dystrophin expression, with its correct subsarcolemmal cellular localization, was restored in 20% of the muscle fibers. Moreover, this led to the expression of DGC components and improved isometric force. Furthermore, systemic administration of the 2'OMePS with F127 revealed that dystrophin was expressed in the skeletal muscle of the whole body except the heart. There was no toxicity arising from the 2'OMePS. However, there were still two hurdles remaining : firstly, F127 was required to administer 2'OMePS into the muscle ; and secondly, dystrophin expression did not reach a therapeutic level.

Later, phosphorodiamidate morpholino oligonucleotides (PMOs), the new generation of antisense oligonucleotides (**Fig. 1**), were administrated into mdx mice[17)18)]. PMOs have morpholine rings instead of deoxyribose rings in DNA or ribose rings in RNA, and the morpholine rings bind to each other through phosphorodiamidate instead of phosphoric acid. PMOs are non-ionic, which minimizes protein interactions and nonspecific antisense effects, and they have several advantages over 2'OMePS such as high solubility in water and a high binding capacity to mRNA. Systemic induction of dystrophin expression was observed by PMO administration and it reached a useful level in skeletal muscle throughout the whole body except the cardiac muscle. One limitation of PMO-mediated exon-skipping therapy is that cardiac muscle cannot easily take up PMOs. Recently, to improve the uptake of PMOs

by cardiocytes, peptide-tagged PMOs (PPMOs ; **Fig. 1**) and octa-guanidine PMOs (**Fig. 1**) were developed. PPMOs are covalent conjugates of PMOs with cell-penetrating peptides such as β-alanine (B), β-arginine (R), or 6-aminohexane (X)[19]. On the other hand, octa-guanidine PMOs are PMOs coupled with eight guanidinium head groups on dendrimer scaffolds that enable delivery into cells[20)-22)]. Both types of modified PMOs have been demonstrated to be more effective than native PMOs in inducing exon skipping in cardiac muscle after intravascular injection[19)21)]. However, there are potential concerns that PPMOs might elicit an immune response or demonstrate toxicity compared with PMOs because of the protein moiety.

2) Canine X-linked muscular dystrophy (CXMD$_J$)

Golden retriever muscular dystrophy (GRMD) dogs are characterized by progressive skeletal muscle weakness and atrophy, and carry a point mutation at the intron 6 splice acceptor site in the canine *DMD* gene. However, these dogs are too large to be maintained conveniently. Thus, we have developed a strain of medium-sized dystrophic Beagle dogs (canine X-linked muscular dystrophy in Japan ; CXMD$_J$) at the National Center of Neurology and Psychiatry, Tokyo, by artificial insemination of frozen GRMD semen[23)24)]. The mutation leads to skipping of exon 7, which leads to a premature stop codon in exon 8. As a result, no dystrophin is produced in the affected muscles. Recently, we reported systemic administration of a cocktail of three PMOs targeting exons 6 and 8 to restore in-frame dystrophin in CXMD$_J$ dogs[25)]. We showed that dystrophin was restored in the skeletal muscle of the entire body. The motor ability of treated dystrophic dogs was also improved with no side effects. This is the first report that multi exon skipping is feasible and effective in improving performance of dystrophic animals *in vivo*. However, the expression of dystrophin in cardiac muscle was not observed in CXMD$_J$ dogs, even at high doses, the same as in *mdx* mice[25)].

3) Mdx52 mice

The particular mutations found in the mdx mouse and CXMD$_J$ dog are very rare in humans. Since there is a significant genetic heterogeneity among patients with DMD, it will be necessary to target the mutational hotspots in the *DMD* gene for providing exon-skipping therapy to more patients. For instance, effective skipping of exon 51 could treat 13% of all DMD patients according to the Leiden muscular dystrophy database (http: //www.dmd.nl). Fortunately, the mdx52 mouse exists, in which exon 52 of the murine *Dmd* gene was deleted

by homologous recombination, leading to a lack of dystrophin and dystrophic changes with muscle hypertrophy. Using this mouse, we conducted a preclinical study of exon 51 skipping. We systemically delivered PMO into *mdx52* mice, seven times at weekly intervals. This induced 20–30% of wild-type dystrophin expression levels in the muscles of the whole body, and was accompanied by amelioration of the dystrophic pathology and improvement in skeletal muscle function[26].

4. Proof-of-concept of exon skipping in DMD patients

Following these studies of AONs *in vivo*, which demonstrated dystrophin expression and functional restoration, two groups in Europe have started to demonstrate the proof-of-concept in DMD patients. However, to use this therapy in a clinical trial for DMD, there was an important issue that had to be addressed. Normally, a small group of healthy volunteers would be selected to assess the safety, tolerability, and pharmacokinetics. However, because this therapy produces out-of-frame dystrophin in normal individuals, a clinical trial in healthy volunteers was not possible from a safety point of view. Therefore, the two groups conducted phase I clinical trials of exon 51-skipping in DMD patients in whom skipping of this exon could restore an in-frame and functional dystrophin protein. The Dutch group chose a 20-mer 2'OMePS (PRO051)[27]. Four DMD patients received 0.8 mg of PRO051 injected into the tibialis anterior muscle. A biopsy performed 28 days later revealed restoration of subsarcolemmal dystrophin in 64–97% of the myofibers in each patient[27]. Furthermore, PRO051 did not evoke any clinically apparent side effects.

On the other hand, a British group collaborating with AVI Biopharma selected a 30-mer PMOs (AVI-4658)[28]. A single administration of AVI-4658 was injected unilaterally into the extensor digitorum brevis of seven patients, in a single-blind, dose-escalation study that included a placebo control administered to the contralateral same muscle. AVI-4658 successfully restored the expression of dystrophin at the higher dose (0.9 mg) received by five of the seven patients. No adverse events related to AVI-4658 were observed[28]. These two studies have demonstrated the proof-of-concept of exon skipping for DMD patients.

5. Application of AON therapy for other neuromuscular diseases

The attempts at antisense therapy in DMD provide a useful platform to develop

experimental therapies for other neuromuscular diseases, such as amyotrophic lateral sclerosis (ALS), spinal muscular atrophy (SMA), and myotonic dystrophy type 1 (DM1)[29]. Recently, ISIS Pharmaceuticals, a company focused on clinical applications for AONs, began a phase I study in familial ALS patients with a superoxide dismutase 1 (*SOD1*) mutation, attempting to knock down the mutant *SOD1* gene that causes disease[30].

1) Spinal muscular atrophy

SMA is an autosomal recessive neuromuscular disease characterized by degeneration of lower motor neurons, with resulting progressive muscle weakness. The clinical phenotype and disease severity can be varied. Absence of the survival motor neuron 1 (*SMN1*) gene is the cause of SMA ; however, its severity is mainly correlated with the copy number of the SMA modifier gene *SMN2*, which is nearly identical to *SMN1* but has a single nucleotide replacement[31]. One *SMN2* copy produces only 10–20% of full-length protein identical to SMN1, whereas the remaining *SMN2* transcripts aberrantly splice out exon 7 because of a silent mutation within an SF2/ASF-binding site, resulting in a truncated, unstable version of the SMA protein. Therefore, exon inclusion, not skipping, in exon 7 of *SMN2* would be required for a therapeutic effect in SMA[32)33)]. Enhancing exon 7 inclusion within the *SMN2* pre-mRNA is predicted to result in more full-length SMN protein being expressed. AON-mediated inhibition of the intronic splice silencer site in intron 7 of the *SMN2* gene did affect pre-mRNA processing, leading to exon 7 inclusion in the mRNA and resulting in increased production of normal SMN protein[34]. Thus, this approach has a therapeutic potential for SMA and other diseases caused by mutations that disrupt SF2/ASF-binding sites or induce exonic splicing silencers.

2) Myotonic dystrophy type 1

DM1 is the most common adult form of muscular dystrophy. Unlike any of the other muscular dystrophies, the muscle weakness is accompanied by myotonia (delayed relaxation of muscles after contraction) and by a variety of abnormalities in addition to those of muscle. DM1 is caused by an RNA gain-of-function due to an expanded CUG repeats in the 3'untranslated region of the dystrophia myotonica-protein kinase gene. Because the interaction of muscleblind-like 1 (MBNL1) protein with the expanded CUG repeat contributes to aberrant pre-mRNA splicing and pathogenesis in DM1 patients, Wheeler, et al. administered a 25-mer PMO (CAG25) complementary to the CUG repeat to HSA^{LR} mice, which express high amounts of expanded CUG_{250} repeats in the context of the skeletal α-

actin gene and show similar patterns of aberrant alternative splicing to those seen in DM1 patients. Treatment of skeletal muscle redistributed the MBNL1 protein from nuclear RNA foci and normalized the aberrant splicing regulation, resulting in improved ion channel function and minimization of skeletal muscle myotonia[35]. Thus, this approach has therapeutic potential for DM1 and possibly other diseases caused by triplet repeat expansion such as spinocerebellar atrophy or Huntington disease.

6. Conclusions

Approximately 140 years after the first description of DMD patients and almost 25 years after the identification of dystrophin, a promising therapy for DMD patients is now emerging. The results of two recent phase I clinical trials in DMD patients are highly encouraging. Over the next few years, treatments specific to DMD will likely become available. Although additional basic and translational studies are required to develop safer and more effective treatment strategies, this therapy may have broad application for other neuromuscular diseases.

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