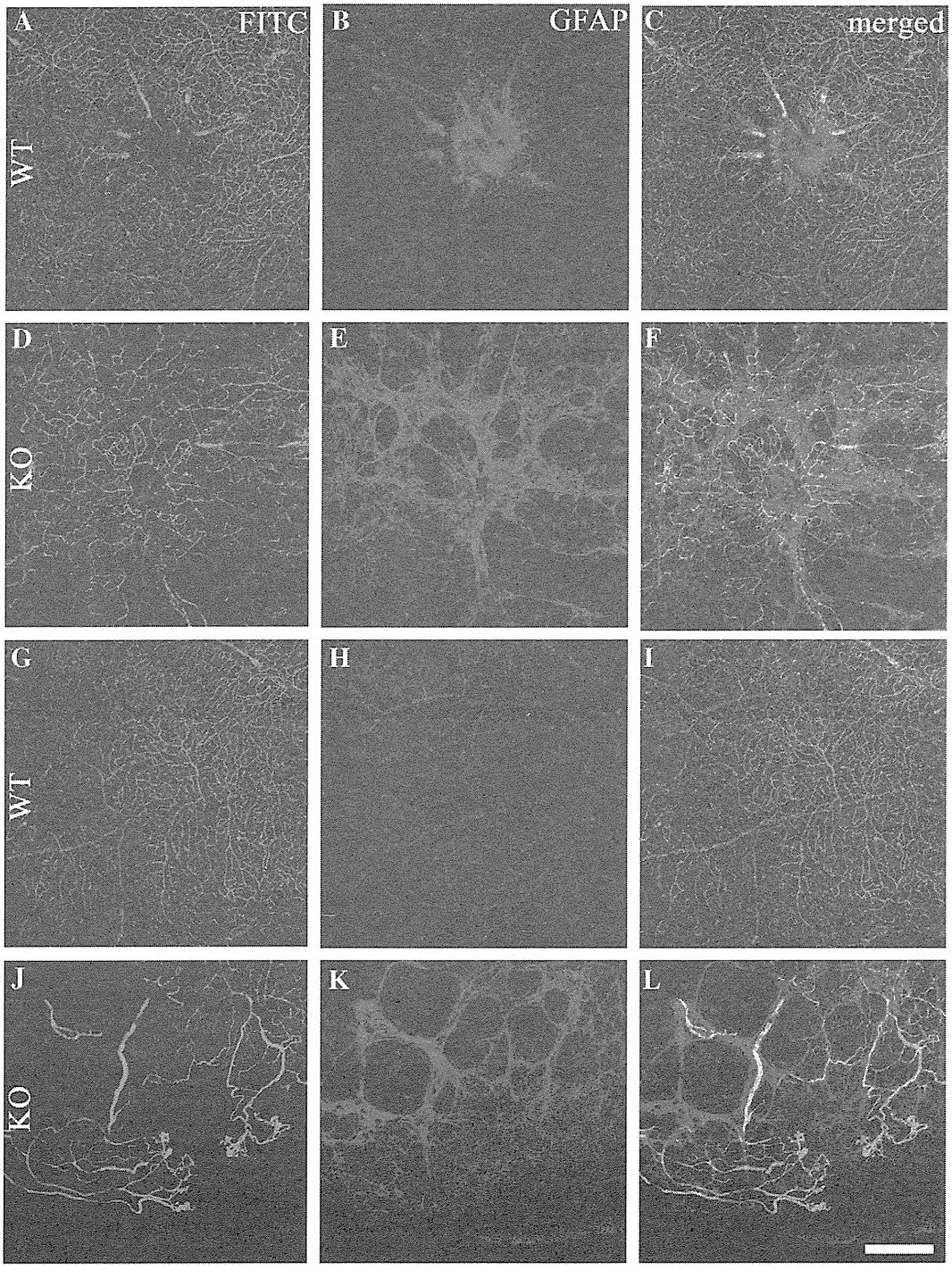

Fig. 7. Vascular network of flat mounted retinas stained by FITC-dextran perfusion and immunohistochemical staining for GFAP from wild-type and POMGnT1-deficient mice. Each animals are 10-week-old. A. D. G. and J. Flat mount retinal preparations from wild-type and POMGnT1-deficient mice perfused with FITC-dextran. B. E. H. and K. GFAP staining of astrocytes in flat mount retinal preparations from wild-type and POMGnT1-deficient mice. C. F. I. and L. Merged images are shown. Regions of central retinas (A–F) and peripheral retinas (G–L) are shown respectively. GFAP staining of astrocytes in POMGnT1-deficient retina is highly irregular especially around retinal vasculature (Eand K). Retinal vascular staining derived from perfusion of FITC-dextran showed disorganization of normal pattern of vascular network (D and J). Double staining of GFAP and FITC-dextran showed that abnormal retinal vasculature was associated with highly irregular astrocytic processes (F and L). Scale bars represent 100 μm in A–L.

with hematoxylin and eosin (H&E). For statistical analysis, measurements of retinal thickness were made at peripheral retina ~1.0–1.2 mm from the optic nerve head (Chi et al., 2010). For immunohistochemical analyses, the enucleated eyes were embedded in optimal cutting temperature compound (OCT, Miles Inc.) and frozen in liquid nitrogen before 6 μm sections were cut. After blocking the sections with 5% normal goat serum, sections were incubated overnight with rabbit polyclonal anti-glial fibrillary acidic protein (GFAP; Dako), anti-vimentin antibody (V9; Dako), anti-PKC antibody (MC5; Sigma), anti-Syntaxin antibody (HPC-1; Sigma), anti-alpha-dystroglycan antibody (VIA4-1; Upstate Biotechnology), or anti-dystrophin antibody (Dys2; Novocastra). The secondary antibodies were anti-mouse antibody conjugated with Alexa Fluor 488 (Molecular Probes) for vimentin, Syntaxin, alpha-dystroglycan, dystrophin, and anti-rabbit antibody

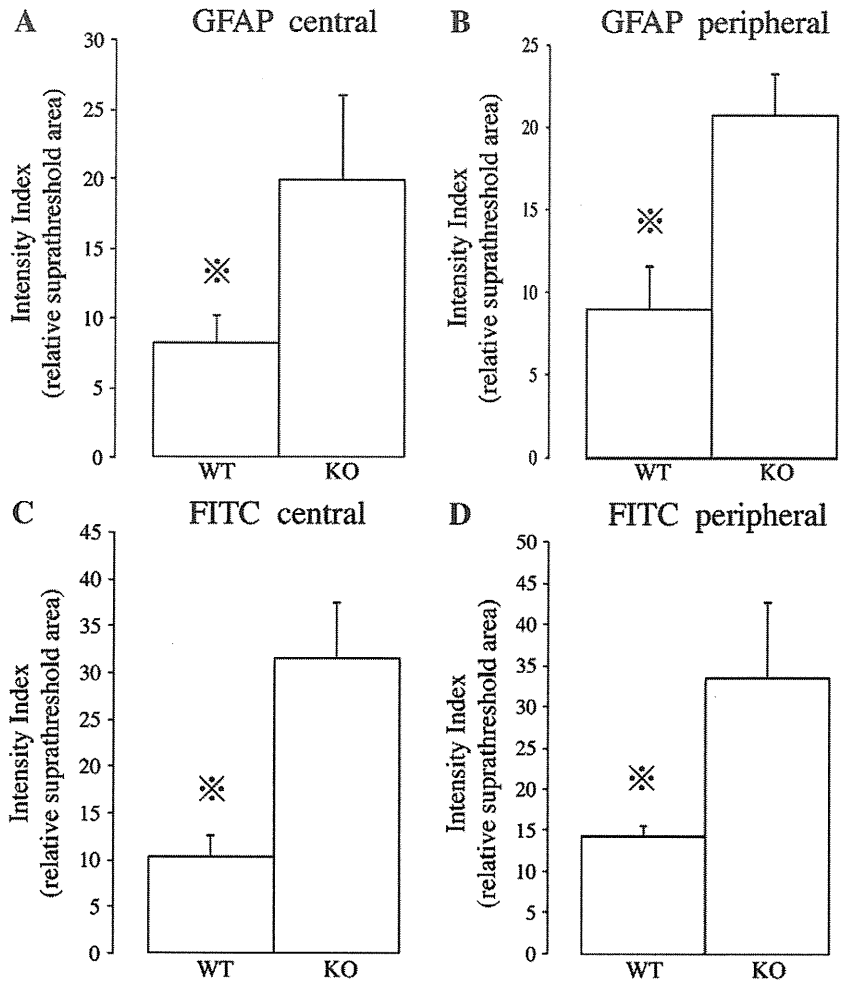

Fig. 8. Quantification of GFAP immunoreactivity and vascularization stained by FITC-dextran perfusion in flat mounted retinas from wild-type and POMGnT1-deficient mice. Animals used for this study ranged from 10-week-old to 12-week-old. The average of each 3 animals is plotted. A. and B. Intensity Index for GFAP immunoreactivity was significantly elevated in the central retina of POMGnT1-deficient mice compared to wild-type mice (8.33 ± 2.00 [SD] vs. 20.23 ± 6.22; $P = 0.003$) (A) and also in the peripheral retina of POMGnT1-deficient mice compared to wild-type mice (9.02 ± 2.54 [SD] vs. 20.70 ± 2.55; $P = 0.005$) (B). C. and D. Intensity Index for vascularization stained by FITC-dextran perfusion was significantly elevated in the central retina of POMGnT1-deficient mice compared to wild-type mice (10.50 ± 2.39 [SD] vs. 32.18 ± 6.04; $P = 0.004$) (C) and also in the peripheral retina of POMGnT1-deficient mice compared to wild-type mice (14.60 ± 1.35 [SD] vs. 34.29 ± 9.30; $P = 0.002$) (D).

conjugated with Alexa Fluor 488 (Molecular Probes) for GFAP, PKC. The immunostained sections were photographed with a confocal laser scanning microscope (TCSSP™, Leica Microsystems Japan).

Electroretinograms (ERGs)

ERGs were recorded from mice anesthesized with an intramuscular injection of ketamine (80 mg/kg) and xylazine (10 mg/kg), and the pupils were dilated with a mixture of tropicamide and phenylephrine. After overnight dark adaptation (>12 h), white light-emitting diodes embedded contact lens electrode was placed on the cornea under dim red light, this electrode was translucent to diffuse the stimulus and background lights. The stimulus intensity and duration were controlled by an electronic stimulator (LS-W; Mayo Co., Nagoya, Japan). The indifferent electrode was a needle inserted subcutaneously on the nasal bone, and a needle electrode on the neck served as the ground electrode. During recording, body temperature was kept to 33 °C with small animals heat controller (ATC-101B, Unique Medical, Tokyo, Japan).

Responses were amplified by a preamplifier (MEG-5200, Nihon Koden, Tokyo, Japan) with band pass between 1 and 300 Hz, and eight responses were recorded with the Power Lab system (AD Instruments Japan Inc., Nagoya, Japan). Stimulus intensity was calibrated by photo sensor built in LS-W. The stimulus intensity to elicit scotopic ERGs was 0.009 cds/m^2, and that to elicit the mixed rod–cone ERGs was 3.0 cds/m^2. Photopic ERGs were recorded after 10 min of light adaptation with 31.6 cd/m^2 and the photopic ERGs were elicited with a stimulus intensity of 3.0 cds/m^2. The interstimulus interval was 10 s for scotopic ERGs, 15 s for combined rod–cone ERGs, and 2 s for the photopic ERGs.

Retinal flat mounts

Anesthetized mice were perfused with 40 ml of PBS through the heart followed by 5 ml of 4% paraformaldehyde in PBS. Then, 2 ml of a mixture of fluorescein-isothiocyanate (FITC)-conjugated high-molecular-weight dextran (molecular weights: 2×10^6 and 4×10^4 Da in a proportion of 2:1 and a concentration of 10 mg/ml; Sigma, St. Louis, MO) was perfused through the heart. The eyes were enucleated and placed in 4% paraformaldehyde overnight at 4 °C. The anterior segment was removed, and four radial incisions were made in the remaining sclera-choroid-neurosensory retina complex. The isolated retinas were placed in ice-cold methanol for 15 min and transferred to PBS. After two 15 min washes in PBS at room temperature (RT), the retinas were transferred into a blocking solution of 10% fetal bovine serum (FBS) and 10% normal goat serum (NGS) for 1 h at RT. The retinas were incubated with rabbit polyclonal anti-glial fibrillary acidic protein (GFAP; Dako) or reticular fibroblasts and reticular fibers antibody (ER-TR7; Santa Cruz

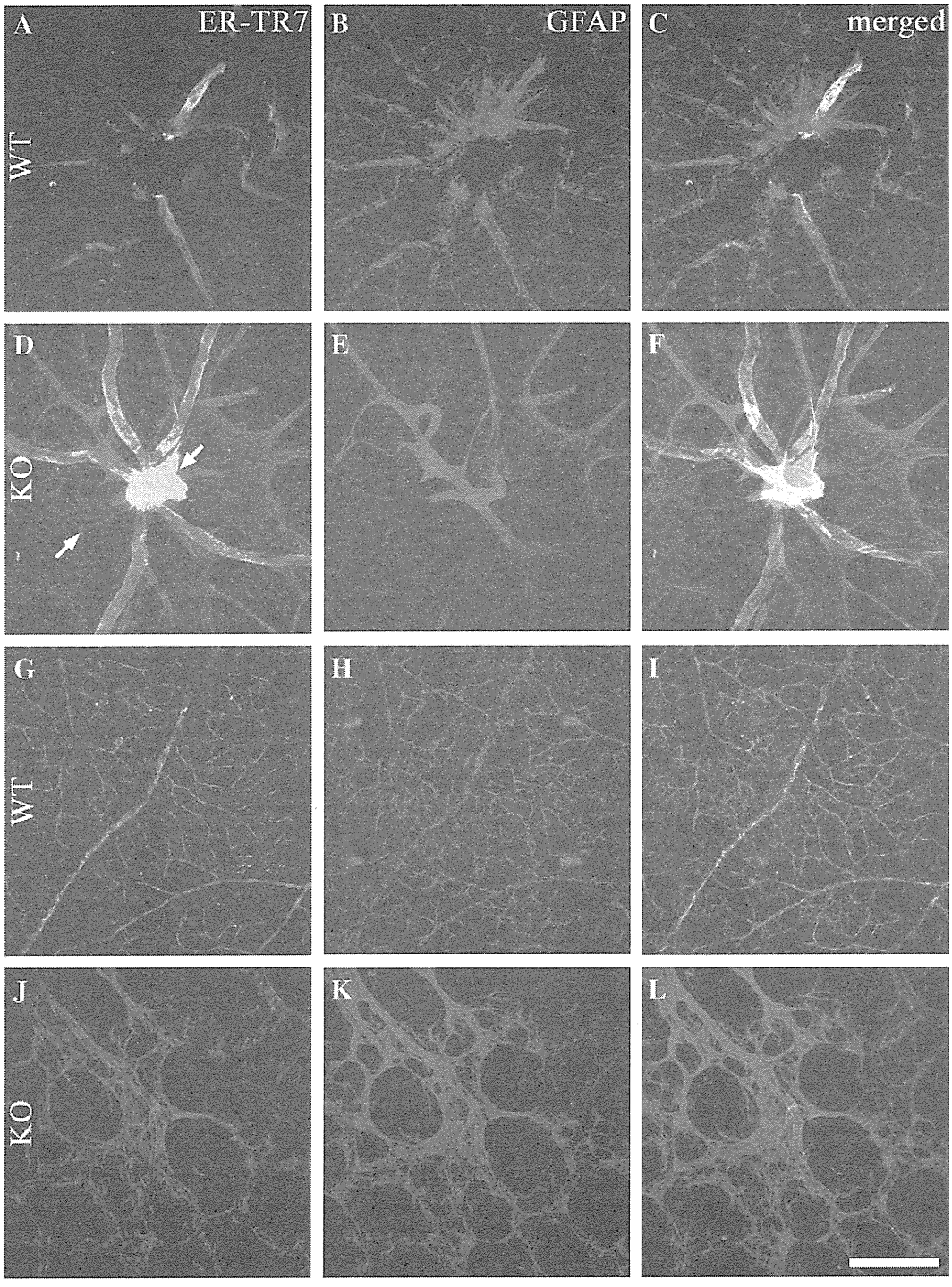

Fig. 9. Immunohistochemical staining for ER-TR7 antigen and GFAP in flat mount retinal preparations from wild-type and POMGnT1-deficient mice. Each animals are 10-week-old. A. D. G. and J. ER-TR7 staining in flat mount retinal preparations from wild-type and POMGnT1-deficient mice. B. E. H. and K. GFAP staining of astrocytes in flat mount retinal preparations from wild-type and POMGnT1-deficient mice. C. F. I. and L. Merged images are shown. Regions of central retinas (A–F) and peripheral retinas (G–L) are shown respectively. ER-TR7-positive fibrous tissues were found closely associated with reactive astrocytes around optic nerve head (D) and peripheral vasculature (J). Note that ER-TR7 and GFAP staining are not identical (arrows). Scale bars represent 100 μm in A–L.

Biotechnology) in 10% FBS and 10% NGS in PBS for 18 h at 4 °C. Retinas were incubated with anti-rabbit antibody conjugated with Alexa Fluor 568 (Molecular Probes) for GFAP, and anti-rat antibody conjugated with Alexa Fluor 488 (Molecular Probes) for ER-TR7 in 10% FBS and 10% NGS in PBS for 2.5 h at room temperature. The flat mounted retinas were photographed with a confocal laser scanning microscope system TCSSP™ (Leica).

Intensity index calculation

GFAP and FITC-dextran stained images were analyzed with ImageJ software (Version 1.44, NIH, Bethesda, MD). Each images were captured using the same camera settings for power, gain, iris aperture size. Data was obtained for the relative area of each region of interest with pixel intensity above a set threshold, and averaged across three

images for both of wild-type and mutant mice. Thus, the index of intensity is defined as the relative area of supra-threshold pixels in each region of interest averaged for both of wild-type and mutant mice (Feilchenfeld et al., 2008). All quantitation was performed with the experimenter blinded to the condition.

Statistical comparisons

All data were expressed as the mean ± standard deviation. For statistical comparisons of retinal thickness and amplitudes of a- and b-wave of ERGs, two-tailed Mann–Whitney nonparametric tests were used. For statistical comparisons of Intensity Index for GFAP immunoreactivity, unpaired t-tests were used. In all statistical comparisons, a P-value less than 0.05 was considered statistically significant.

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Current Status of Pharmaceutical and Genetic Therapeutic Approaches to Treat DMD

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Duchenne muscular dystrophy (DMD) is a genetic disease affecting about one in every 3,500 boys. This X-linked pathology is due to the absence of dystrophin in muscle fibers. This lack of dystrophin leads to the progressive muscle degeneration that is often responsible for the death of the DMD patients during the third decade of their life. There are currently no curative treatments for this disease but different therapeutic approaches are being studied. Gene therapy consists of introducing a transgene coding for full-length or a truncated version of dystrophin complementary DNA (cDNA) in muscles, whereas pharmaceutical therapy includes the use of chemical/biochemical substances to restore dystrophin expression or alleviate the DMD phenotype. Over the past years, many potential drugs were explored. This led to several clinical trials for gentamicin and ataluren (PTC124) allowing stop codon read-through. An alternative approach is to induce the expression of an internally deleted, partially functional dystrophin protein through exon skipping. The vectors and the methods used in gene therapy have been continually improving in order to obtain greater encapsidation capacity and better transduction efficiency. The most promising experimental approaches using pharmaceutical and gene therapies are reviewed in this article.

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INTRODUCTION

Muscular dystrophies are characterized by progressive degeneration and weakness of multiple muscle groups depending on the specific dystrophy. Duchenne muscular dystrophy (DMD) is an X-linked pathology due to the absence of dystrophin in muscle fibers.[1,2] The first symptoms of the disease appear during early childhood, usually before 3 years of age, and death occurs in the mid to late twenties.

The dystrophin gene, called *DMD* gene, extends over 2.4 megabases of the X chromosome, thus ~90 times the size of most genes. It contains 79 exons that code for a 14kb mRNA.[3,4] Its translation generates a large protein of 3,685 amino acids with a molecular size of 427 kDa[5] called dystrophin. This protein is localized beneath the sarcolemma of the muscle fibers.[6]

Dystrophin can be divided into four main regions (**Figure 1a**). The N-terminal domain interacts with actin filaments.[7] The central rod domain also links to actin filaments[8] and, in addition, to neuronal nitric oxide synthase (nNOS).[9] This enzyme is implicated in several physiological functions of the muscle such as its regeneration and its contraction.[10] The central domain also contains four hinge regions that provide flexibility.[11] The third region is the cystein-rich domain that interacts with the sarcolemmal β-dystroglycan, which in turn interacts with the transmembrane α-dystroglycan.[12] The dystrophin C-terminal region is associated with α-, β-, and γ-syntrophins.[13–15] Since dystroglycans and syntrophins are also linked to other proteins, dystrophin thus interacts with many proteins in a complex called dystrophin-associated glycoprotein complex (DGC) (**Figure 1b**).[16–19] The main function of dystrophin is to stabilize and link the muscle fiber cytoskeleton to the membrane. The lack of functional dystrophin results in the loss of the DGC, thereby rendering the muscle fibers less resistant to mechanical stress.[16,20]

In DMD, the *DMD* gene mutations almost always result in a premature stop codon due to frameshift mutations or nonsense mutations. There are >4,700 different mutations divided into three main categories: deletion of one or more exons, duplication of one or more exons and small mutations. Depending on the cohorts studied, the proportion of these categories varies from 60 to 80% for deletions, from 7 to 11% for duplications and from 10 to 30% for more subtle DNA changes including nonsense mutations, splice-site mutations, and small insertions/deletions that disrupt the reading frame.[21–26] As mentioned, most of the

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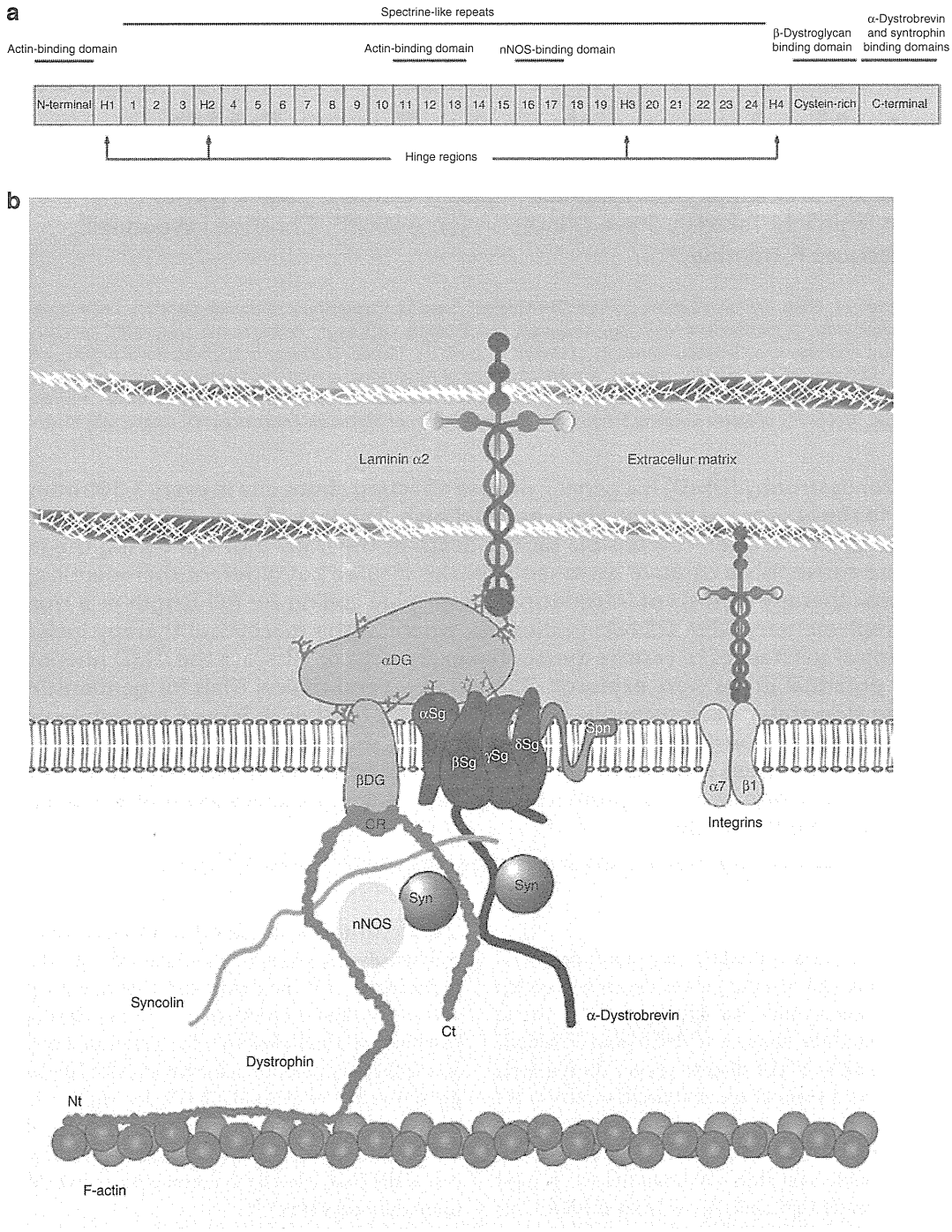

Figure 1 The dystrophin protein. (**a**) Schema representing the four main domains of dystrophin: the N-terminal part, central rod domain (containing 24 spectrin-like repeats and four hinge domains), cystein-rich region and the C-terminal part. The protein binding domains are also indicated. (**b**) Diagram of the dystrophin-associated glycoprotein complex (DGC). This complex includes dystrophin with its C-terminal (Ct), cysteine-rich (CR), and N-terminal (Nt) regions as well as proteins associated in this complex. DG, dystroglycan; nNOS, neuronal nitric oxide synthase; Sg, sarcoglycan; Syn, syntrophin. Modified from Odom *et al.*[19]

deletions in the *DMD* gene result in a frameshift.[27] Those that do not produce a frameshift result in the production of an internally deleted dystrophin and give rise to a dystrophy called Becker muscular dystrophy (BMD).[28] The BMD phenotype varies according to the functional loss of the missing exons but is generally less severe than DMD.[29–31] For example, a deletion in the rod domain will often be less severe than a deletion in N-terminal. The life expectancy of BMD patients is also variable: some may suffer life threatening complications in their late twenties and have a similar life expectancy as DMD patients whereas many live a normal lifespan beyond 50 years of age.

DMD symptoms are very severe. Thus, even if there are currently no curative treatments for this disease, the medical monitoring and the care coverage of these patients contribute to

prevention of some complications and to improvement in their quality of life. For that purpose, the follow-up of patients must be considered at various levels: rehabilitation, cardiac, pulmonary, orthopedic, psychosocial, and nutrition.[32,33]

Following the initial open-label trials of corticosteroids, the potential benefit of prednisone was clearly demonstrated >20 years ago in a double-blind randomized controlled trial for 6 months in a study of >100 boys.[34] Subsequent reports showed equal benefit using deflazacort, a sodium-sparing steroid.[35] These results were confirmed by other studies (see refs. 32,36,37 for an exhaustive list of these studies). Long-term follow-up of open-label administration of corticosteroids reveals prolonged ambulation for about 2 years. In addition, the lower prevalence of scoliosis through the use of long-term corticosteroid treatment represents a significant change in the natural progression of DMD.[38] Prednisone prescription to DMD patients is now openly authorized in many countries but many patients are forced to stop taking the drug because of unwanted side effects that include weight gain, bone demineralization, vertebral compression fractures, hypertension, and/or behavior disorders.

Besides the DMD patient's follow-up, different therapeutic approaches are currently in development to improve the DMD phenotype. This review focuses more specially on the current status of pharmaceutical and of gene therapy approaches in DMD. We have not reviewed the different potential cell therapies for DMD; however, some *ex vivo* gene therapies have been included.

PHARMACEUTICAL APPROACH

The great advantage of a pharmacological approach is that nearly all drugs can be delivered systemically (orally, intravenously, subcutaneously) and thus will reach and potentially treat all muscles which is critical for clinical success in DMD. However, the development and testing of new drugs for the DMD population is far from being a simple task.

Dystrophin restoration approaches

Stop codon read-through. About 10–15% of DMD patients have a mutation that converts an amino acid into a premature nonsense codon, while the rest of the mRNA is unaffected.[21–26] Some drugs have been shown to enable stop codon read-through by introducing an amino acid at the premature stop codon to continue the mRNA translation. This phenomenon called "stop codon read-through" has been intensively investigated.

Gentamicin: Gentamicin is an aminoglycoside antibiotic interacting with the translational machinery (40S ribosomal subunit) when it recognizes a stop codon.[39–41] This interaction induces the introduction of an amino acid at stop codons in the mRNA and thus allows the translational machinery to continue the mRNA translation.[42,43] It specially occurs in premature stop codons since the context of nucleotide sequences surrounding nonsense mutations and regular stop codons are different.[44] Gentamicin was tested as a therapeutic approach for DMD. When used in dystrophic (*mdx*) mice, this drug induced up to 20% dystrophin-positive fibers.[45] After this positive result, two clinical trials on DMD and BMD patients were undertaken. However, the results were moderate[46,47] as was also the case for some further studies in animals.[48,49] Recently, a clinical trial showed that a 6 months gentamicin administration resulted in up to 15% dystrophin expression in three DMD patients, lower percentages in three other patients, and no expression in the remaining patients.[50] The different results obtained in mouse and in human are probably due to the presence of different gentamicin isomers, which are not all equally potent in inducing read-through[41] and since each gentamicin batch consists of a mix of different isomers, some batches may be more effective than others.

Given that gentamicin has variable effects and exhibits some toxicity, less toxic effective derivatives of this drug need to be developed for an effective DMD treatment.

Ataluren: Ataluren (PTC124) is a new molecule recently identified by PTC Therapeutics (South Plain Field, NJ). It is presumed to work similarly to gentamicin except that PTC124 binds to the 60S ribosomal subunit.[51] Its efficiency is comparable to gentamicin in mouse: between 20 and 25% dystrophin-positive fibers were observed in treated *mdx* mice.[52] Three phase II clinical studies began on DMD and BMD patients but these studies were halted prematurely on March 2010 since the predetermined primary outcome (30 m improvement compared to placebo in the 6-minute walk test) was not reached[53] while ataluren was generally well tolerated in DMD patients.[54] No information is available concerning the dystrophin expression in treated muscles.

Even though gentamicin and ataluren have shown good efficiency in the *mdx* mouse model, the clinical studies that have been done up to date showed that these drugs still need further improvements before they can be used clinically in DMD patients.

Exon skipping. In BMD patients, dystrophin is internally deleted, but still partially functional due to the presence of the essential N- and C-terminal domains. Using antisense molecules which were able to interfere with splicing signals, the skipping of the targeted specific exons in the dystrophin pre-mRNA can restore the open reading frame and allow the expression of an internally deleted but functional dystrophin in DMD patients (**Figure 2**). These molecules are small synthetic modified RNAs or DNAs called antisense oligonucleotides (AOs) able to bind specific intronic or exonic sites of pre-mRNA. Annealing to selected splice motifs, the AO essentially masks the targeted exon from the splicing machinery, thereby promoting specific exon exclusion from the mature mRNA. Two types of AO are mainly used: 2′-O-methyl-phosphorothioate (2OMP) and phosphorodiamidate morpholino oligomer (PMO) (**Supplementary Figure S1**).

2′-O-methyl-phosphorothioates: 2OMPs contain around 20 nucleotides and are obtained by modifying the classic synthesis of oligonucleotides.[55] The first modification is the replacement of the negatively charged oxygen by sulfur. The second one is the methylation of the hydroxyl group at the 2nd position of ribose. These modifications make the AOs more resistant to nucleases, improve their affinity for RNA, provide favorable pharmacokinetic properties and prevent RNase H to induce cleavage of RNA:RNA hybrids.[56–58]

Several 2OMPs designed to target several human *DMD* exons were tested with success in DMD patient-derived myotubes.[59,60] In

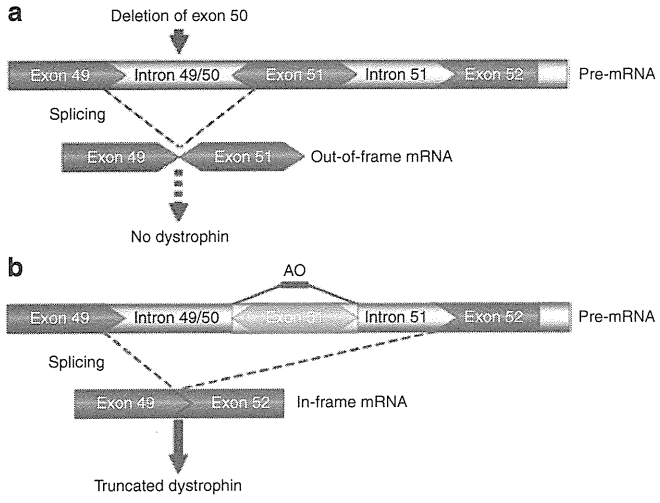

Figure 2 Example of exon skipping in Duchenne muscular dystrophy (DMD) patient who has a deletion of exon 50. (**a**) The absence of exon 50 in the dystrophin gene leads to an out-of-frame mRNA creating a premature stop codon in exon 51, thus aborting dystrophin synthesis during translation. (**b**) Using an antisense oligonucleotides (AO) targeting exon 51, this exon is skipped during splicing. This restores the open reading frame of the transcript and allows the synthesis of an internally deleted dystrophin. Modified from Van Deutekom *et al.*[65]

parallel, 2OMPs were designed to target the exon 23 of the mouse *DMD* gene since the nonsense mutation of *mdx* mouse is localized in this exon. Intramuscular administration of an AO targeting the exon 23 donor splice-site in these mice induced the restoration of dystrophin (without the exon 23) in the treated muscles.[61] These AOs were also intravascularly injected in *mdx* mice. Treated mice showed dystrophin restoration in many muscles.[62] However, low levels of dystrophin restoration were detectable in the heart.[63] A study demonstrated that repeated 2OMP injections increased the AO efficiency without increasing its toxicity.[62] A subcutaneous 2OMP injection has also been tested and this type of injection showed better pharmacokinetics and pharmacodynamics than intramuscular or intravenous injections.[64]

After these positive results in the *mdx* mouse model, a clinical trial on four DMD patients with the PRO051/GSK2402968 (2OMP targeting exon 51) was done. The muscle injected with 0.8 mg of this 2OMP showed 64–97% dystrophin-positive fibers (not corrected for positive muscle fibers in saline-injected contralateral muscle) with a level of dystrophin expression between 17 and 35%.[65] No adverse effects were found in the treated muscles. A phase I/II clinical trial, in which this same AO was injected subcutaneously, was recently completed and showed that this AO was well tolerated in all patients and that novel dystrophin expression was detected in each treated patient in a dose dependent manner.[66] A phase III study has started with this AO on DMD patients.[67]

Despite the fact that long-term toxicity studies in animal models with 2OMP are lacking, this approach seems promising.

Phosphorodiamidate morpholino oligomer: Similar to 2OMPs, PMOs (commonly referred to as morpholinos) are obtained by modifying the classic synthesis of oligonucleotides. Their ribose is replaced by a morpholine ring and the oxygen present in the phosphodiester link (the one that is not negatively charged) is replaced by a nitrogen atom. These modifications allow morpholinos to be biologically stable[68] and have antisense properties.[69]

Exon 23 of the mouse *DMD* gene was the first target of morpholinos. Restoration of dystrophin was observed in the treated *mdx* mouse muscles when morpholinos were intramuscularly injected[70] and in many muscles when intravenously[71] or intraperitoneally injected.[72] A partial restoration of dystrophin in the heart of *mdx* mice was also shown but the morpholino dose used was 50 times superior to the one used to treat skeletal muscles.[72] Recent studies of long-term repeated systemic treatment of *mdx* mice over a year with naked PMO at doses of 5 and 50 mg/kg have shown significant improvement in pathology and complete normalization of locomotor behavior without signs of renal or hepatic toxicity.[73] A morpholino designed to restore dystrophin expression in dystrophic (golden retriever muscular dystrophy) dogs was also synthesized and intravenously injected in these dogs. Five months later, treated dogs showed about 25% dystrophin-positive fibers throughout the body with a global improvement in muscle pathology in PMO-treated dogs compared to pretreated and untreated control dogs.[74] No significant signs of toxicity were found.

To enhance the cellular uptake of PMOs, they can be conjugated to peptides or other conjugates. The delivery of a morpholino conjugated with a dendrimeric octaguanidine (Vivo-Morpholino) was efficient to induce dystrophin expression in *mdx* mouse muscles.[75] Indeed, repeated injections at biweekly intervals achieved near 100% dystrophin-positive fibers in many skeletal muscles without eliciting a detectable immune response; the dystrophin restoration in the cardiac muscle reached up to 40%. PMOs conjugated with arginine-rich cell-penetrating peptides,[76] called pPMOs, also produced excellent restoration of dystrophin expression in *mdx* mice.[77,78] A pPMO targeting exon 23 was applied as well in utrophin$^{-/-}$ *mdx* mice by intraperitoneal injection. Whereas untreated animals typically died by 15 weeks of age, treated animals showed few signs of weakness, improved histopathology and appeared essentially normal at 1 year of age.[79] A muscle-targeting heptapeptide (MSP) fused to an arginine-rich cell-penetrating peptide (B-peptide) and conjugated to a PMO, called B-MSP-PMO, was also shown to be efficient for restoring dystrophin in *mdx* muscles.[80] Indeed, using an intravenous dose of 6 mg/kg of B-MSP-PMO administered biweekly over the course of 12 weeks, the dystrophin expression was found at a level of 100% in several muscles except for the heart. These pPMO seem well tolerated in *mdx* mice. Indeed, a pPMO targeting the exon 23 of the mouse *DMD* gene exhibited no toxic effects in kidneys at either 20 mg/kg weekly injection to the wild-type mice for 6 weeks or 30 mg/kg biweekly injection to *mdx* mice for 3 months. However, the same peptide conjugated to the PMO targeted to human exon 50 (AVI-5038) was found to cause mild tubular degeneration in the kidneys of nonhuman primates at 9 mg/kg weekly injections for 4 weeks.[81]

To target more dystrophin mutations occurring in DMD patients, other exons such as the exon 51 in *mdx*52 mice were targeted.[82] In addition, it is possible to remove in-frame exons from the dystrophin pre-mRNA and induce specific internally deleted dystrophin by using AOs. This has been done for exons 19/20 and 52/53 in wild-type mice.[83]

After these positive results in animal models, a clinical trial in seven DMD patients was undertaken to skip exon 51 and thus to restore the reading frame of their dystrophin mRNA using unmodified morpholinos. The morpholino (AVI-4658) was intramuscularly injected and biopsies were taken 3–4 weeks later. Two patients were treated with a low dose of this morpholino (0.09 mg) and five patients with a higher dose (0.9 mg). Only the patients receiving the higher dose produced dystrophin although exon skipping was observed in all patients by reverse transcriptase PCR. In the five patients receiving the higher dose, the muscles injected with the AO showed 44–79% dystrophin-positive fibers (corrected for positive fibers in saline-injected contralateral muscle) with a level of dystrophin expression between 22 and 32%.[84] No signs of toxicity were observed. After these encouraging results, a systemically delivered morpholino phase Ib/II clinical trial was undertaken. According to a press release from AVI Biopharma (Bothell, WA),[85] 19 DMD patients were enrolled in six dose cohorts (0.5, 1, 2, 4, 10, or 20 mg/kg) and treated during 12 weeks by weekly intravenous infusion. Some patients expressed dystrophin-positive fibers; those treated with the higher doses of morpholino had more uniform and widespread dystrophin-positive fiber distribution than patients who received lower doses. The morpholino was well tolerated in all patients. A phase II clinical trial is currently in preparation to evaluate higher weekly doses of AVI-4658 (50 and 100 mg/kg).[85]

Although pPMO seems to cause some toxicity in nonhuman primates, there are other ways to modify the peptide conjugate, which are hopefully less toxic, to allow clinical development for DMD patients.

Modification of the DMD gene with meganucleases or zinc finger nucleases. A new alternative treatment for DMD relies on the restoration of the dystrophin reading frame by inducing a micro-deletion or a micro-insertion in the *DMD* gene.[86] This can be done by inducing double strand breaks at the end of the exon, which precedes a deletion, or at the beginning of an exon, which follows a deletion. These double strand breaks can be induced with specially engineered meganucleases or zinc finger nucleases. They are spontaneously repaired by a process called nonhomologous end-joining, which introduces a micro-insertion or a micro-deletion. Alternatively, double strand breaks can be repaired by homologous recombination by providing a donor plasmid containing the coding sequence that is deleted in the patient's genome.

Other approaches

Myostatin. A potential therapeutic method to improve muscle strength is to block myostatin. Myostatin is a member of the transforming growth factor-β family implicated in muscle size regulation. Indeed, in the myostatin gene knockout mouse, robust muscular hypertrophy and hyperplasia are observed.[87] Antibodies against myostatin were produced and intraperitoneally injected in *mdx* mice. The treated mice showed muscular hypertrophy, muscle strength increase, and histological improvement.[88] There are also other methods to block the myostatin pathway such as the use of follistatin[89] or of myostatin propeptide.[90] Another approach is to directly mutate the myostatin receptor, the activin type-II receptor[91] or to inject a soluble form of this receptor.[92] All these approaches led to improvements of the treated mouse phenotype similar to that observed in myostatin$^{-/-}$ mice. Recently, the use of destructive exon skipping of the myostatin pre-mRNA induced by 2OMP and PMO has been described to induce skeletal muscle hypertrophy, which along with dystrophin exon skipping (see above) may thus provide a potential combined antisense strategy to simultaneously reactivate dystrophin expression and increase muscle bulk.[93] In a recent clinical trial, the use of an antibody against myostatin (MYO-029) was undertaken. Although the antibody was well tolerated, no muscle strength improvements were detected perhaps due to a lower dose of antibody.[94] Other clinical trials with myostatin inhibitors are currently undertaken by at least four biotechnology and pharmaceutical companies.[95]

Utrophin. Utrophin shares 80% sequence identity with dystrophin and is expressed in the muscles during embryonic development.[96] However, in adult myofibers, it is located only at the neuromuscular junction and at the myotendinous junctions. Utrophin is over-expressed in muscle fibers of dystrophic mice and of DMD patients.[97,98] Since it has sequence homology with dystrophin, it was suggested that its upregulation could slow down DMD development. When its expression is increased three- to fourfold in transgenic *mdx* mice, their phenotype is similar to wild-type mice.[99] Therefore, an increase of the utrophin expression may be a potential therapy to improve DMD patients. The injection of heregulin in *mdx* mice increased utrophin expression by two to threefold and led to histological improvements.[100] The injection of L-arginine or nitric oxide also allowed utrophin upregulation in *mdx* mice.[101] Recently, the intraperitoneal injection of a TAT-utrophin protein in *mdx* mice increased their muscle strength.[102] A drug developed by Summit PLC (C110/BM195) to upregulate the utrophin expression was carried out by BioMarin pharmaceuticals in a phase I clinical trial with normal individuals. No adverse effects were reported but the pharmokinetics of the drug did not allow them to continue the development of this drug. Summit PLC is currently working on a new formulation, which may improve the pharmokinetics. Further investigation in increasing utrophin expression is required since the molecules tested so far in *mdx* mice did not increase utrophin expression sufficiently to completely suppress the symptoms due to the dystrophin deficiency in *mdx* mice.[103,104] Moreover, utrophin does not seem to anchor nitric oxide synthase at the sarcolemma like dystrophin does, thus leading to a premature muscle ischemia.[105] However, the levels of utrophin upregulation may be sufficient to alleviate most of the DMD symptoms.

GENE THERAPY

Since the first clinical trial of gene therapy in 1990,[106] there has been a strong interest for this therapeutic approach. However in 1999, a major setback occurred due to the death of a patient treated with an adenovirus for ornithine transcarbamylase deficiency.[107] This death is believed to have been triggered by a severe innate immune response to the adenoviral vector. In 2002, another death occurred in a clinical trial for severe combined immunodeficiency with the use of a retrovirus where one of the treated

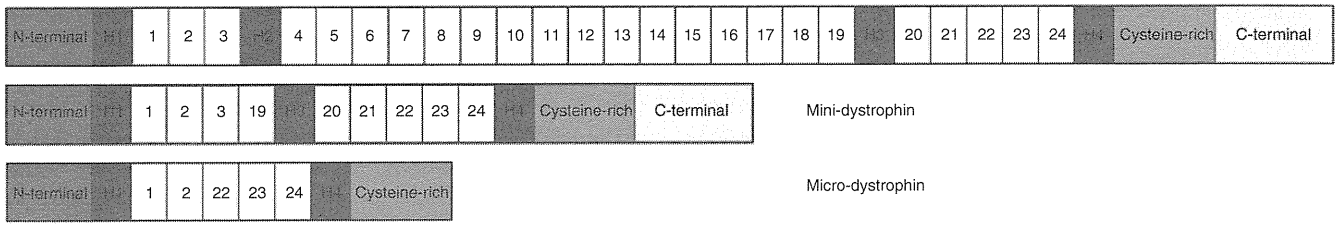

Figure 3 Dystrophin versions. The full-length dystrophin cDNA (11 kb) is represented at the top. The middle schema represents an example of a mini-dystrophin cDNA with an H2-R18 deletion; the approximate size of mini-dystrophins is about 6 kb. The bottom representation is a schema of a micro-dystrophin cDNA (around 4 kb) with an R3-R21 and C-terminal deletion.

patients died due to the activation of an oncogene.[108] However, the fatality rate of gene therapy is still much lower than that of the standard bone marrow transplantation treatment for severe combined immunodeficiency patients.[109] Moreover, >45 patients have now been treated via gene therapy, resulting in one death and >40 cures. Gene therapy is thus an appealing approach to cure many hereditary diseases such as DMD.

Gene therapy in DMD consists of the introduction of a functional copy of the *DMD* gene in muscle fibers with the aim of restoring muscle function including force generation and resistance to muscle contraction induced damage. The concept of dystrophin internally deleted genes that would fit the packaging capacity of small viral vectors came from clinical observations that some BMD patients with internally deleted dystrophins could maintain ambulation for many decades. This gave rise to the concept of mini-dystrophin (mDYS) or micro-dystrophin (μDys). Gene therapy is divided in two distinct categories: those using viral vectors to transfer the gene are referred to as "viral gene therapy" and those employing naked DNA as "nonviral gene therapy".

Internally deleted dystrophin genes

In gene therapy, the transgenes generally contain complementary DNA (cDNA) corresponding only to coding regions of a gene, *i.e.*, exons without introns. The dystrophin cDNA size is about 11 kb and is called full-length dystrophin (FLDYS). Apart from this FLDYS, several mDYS and μDys internally deleted versions exist (**Figure 3**). Indeed, a BMD patient with a deletion of the exons 17–48 in the *DMD* gene was reported to have only a mild dystrophic phenotype.[30] The missing region was located in the spectrin-like repeats of the rod domain resulting in an internally deleted dystrophin with only eight of these repeats instead of 24. The corresponding transgene was thus constructed[110] and other, even smaller, truncated versions were designed subsequently.[111,112] These constructions were called mDYS, or μDys when the C-terminal part is also missing.

Several transgenic mice expressing these internally deleted dystrophins were generated and analyzed[112–115]; all these mice showed the restoration of the DGC. The simple fact of restoring the DGC improves the muscle histology as well as the reduced leukocyte infiltration and the decreased number of centro-nucleated muscle fibers. The muscle strength is also increased but does not reach wild-type levels. However, the observed improvements vary depending on which exons are deleted. The use of internally deleted dystrophins is attractive but the best phenotypic restorations are still obtained with the use of FLDYS.

Viral gene therapy

Different viral vectors could be used for DMD gene therapy. Adenoviral vectors show poor efficiency in adult animal models compared to newborns. Moreover, the use of adenoviral vectors is complicated since half of the human population already has neutralizing antibodies against the adenoviral capsid and also tends to be far more immunogenic than adeno-associated viral vectors (AAV) and retroviral vectors. Due to these limitations, only AAV and lentiviral vectors are described below.

AAV. There are many different AAV, *i.e.*, >100 different sequences are available. Some of the differences lead to different serotypes. The serotypes 1, 2, 6, 8, and 9 are more frequently used for muscle gene therapy. The AAV vector is the only efficient vector for local or systemic delivery to the skeletal muscle and heart[116,117] but its packaging capacity limits the size of the dystrophin transgene.

AAV1[118] and AAV2[111] carrying transgenes encoding for μDYS were injected in *mdx* mouse muscles with success. Indeed, up to 80% dystrophin-positive fibers were found in the treated muscles. These AAV injections also restored the DGC. The results on the *mdx* mouse model being conclusive, experiments using AAV vectors were done in larger animal models. AAV6 and AAV8 coding for μDYS were injected in the dog model. Although dystrophin expression was observed, cytotoxic immune response against the viral capsid was detected,[119,120] which has also been observed for other transgenes delivered by AAV vectors in the dog model.[121] The AAV vector was also tested in nonhuman primates. Five months after the intramuscular injection of an AAV8 coding for μDYS, the transgene expression reached 80% in the treated muscle but this percentage decreased to 40% when the animal already had pre-existing antibodies against the AAV.[122] In small rodent studies, AAV vectors rarely cause cellular immune responses against either the capsid proteins or the transgene products. But in large animal and human studies, variable immunological outcomes have been observed.

Recently, a clinical trial was undertaken on six DMD patients with an AAV vector coding for a functional μDYS. Of the six treated patients, two showed pre-existing T-cells recognizing the rare dystrophin-positive revertant fibers that presented peptide epitopes deemed by the host as nonself. This was detected in ELISpots of peripheral blood mononuclear cells before and after intramuscular injection of the AAV.[123] Another patient had T-cells recognizing an epitope that encoded the transgene product but absent in the revertant fibers. Although the clinical trial was safe and muscle biopsies from the gene vector-treated arms and the contralateral control arms showed no difference in lymphocytes infiltration,

these intriguing findings strongly suggest that additional work is required to determine how many patients have T-cells to dystrophin epitopes and whether those T-cells will prevent successful gene therapy in DMD. In addition, choices of AAV vector serotypes and promoters may also make an impact on the clinical outcome.

Exon skipping was also investigated in combination with AAV vectors. AAV1 coding for the U7 snRNA or U1 small nuclear RNA (snRNA) genes modified to target the mouse dystrophin exon 23 were injected in *mdx* mice. The expression of the internally deleted dystrophin was observed up to 3 months following the injection of an AAV1 coding for the U7 snRNA[124] and for at least 1 year and half with an AAV1 coding for the U1 snRNA.[125] These results are encouraging but this approach has to be further investigated in larger animals such as nonhuman primates or dogs.

AAV vectors were also used to interfere with the myostatin pathway. An AAV vector coding for the myostatin propeptide, a myostatin inhibitor, was designed and injected in *mdx* mice. Muscle hypertrophy leading to phenotypic improvements was observed in the treated mice.[126] Dogs were also treated with the same vector. Unfortunately, few parameters were studied in this experiment and only the hypertrophy of some muscles was noted.[127] In contrast to the other dog studies using AAVs coding for μDYS, no immune responses against the AAV capsid were observed in this study. In the mouse, a recent experiment used an AAV coding for the activin type-II receptor to block the myostatin pathway. The effects of this AAV injection were similar to those observed in the mouse following the injection of the purified activin type-II receptor alone.[128]

The results obtained with AAV vectors are interesting for the development of a DMD therapy. Nevertheless long-term studies of the transgene expression and the immune response against the capsid will be required before this can be considered as potential treatment for DMD.

Lentivirus. The lentivirus encapsidation size is limited to carry the mDYS. Thus, a lentiviral vector carrying this internally deleted *DMD* gene was intramuscularly injected in adult and newborn *mdx* mouse muscles. The best results were obtained in younger mice where 65% of muscle fibers expressed the transgene.[129] In addition, better strength and protection against contraction induced injury were observed in the treated muscles. The lentivirus injection also transduced satellite cells.[130] Despite favorable results in small animals, no studies are available for larger animal models. Moreover, the random integration of lentiviral vectors, according to the target tissues and the enhancers used in a construct, predisposes to induction of tumors (insertional mutagenesis) even though they have not been observed to date in the described experiments.

Lentivirus can also be used to genetically modify cells, which can be transplanted or injected in animal models or eventually in patients. This technique is called *ex vivo* gene therapy. A lentiviral vector coding for μDYS was used to integrate this gene in the genome of side population cells, which were then intravenously injected in *mdx* mice. Only 1% of muscle fibers expressed the transgene in the treated muscles,[131] though this percentage was increased to 5% when these cells were intra-arterially injected.[132] Dystrophic dog mesoangioblasts were also transduced with a lentiviral vector coding for the human μDYS and intra-arterially injected in the same dogs.[133] The treated dogs showed good expression of human μDYS but two of the three treated dogs died of pneumonia during the experiment. The cause of this death was not explained by the investigators but the accumulation of the injected cells in the lungs could be involved in this mortality. Other cell types such as human and nonhuman primate myoblasts were transduced with human μDYS and transplanted with success in immunodeficient mouse and in nonhuman primate muscles respectively.[134] A lentiviral vector coding for dog μDYS was also used to transduce human and dystrophic dog myoblasts. Subsequently, these cells were transplanted in mouse muscles and transgene-positive fibers were observed in the treated muscles.[135]

In addition to the possibility of delivering an internally deleted dystrophin, the lentiviral vector may be used to induce exon skipping as well. A lentiviral vector coding for the U7 snRNA gene modified to induce the skipping of human dystrophin exon 51 was designed. Myoblasts of DMD patients having a deletion of exons 49 and 50 were transduced with this lentivirus and transplanted in immunodeficient mouse muscles. One month later, the expression of internally deleted dystrophin (without the exons 49–51) was detected in the treated muscles.[134] This approach was also used successfully with $AC133^+$ cells.[136]

The use of lentiviral vector is promising for DMD but its efficacy and the risk of tumorigenicity from cells transduced by direct injection of a lentiviral vector or by *ex vivo* genetic modification need to be evaluated in clinical trials.

Nonviral gene therapy

Nonviral gene therapy allows the introduction of a transgene into a tissue without using a viral vector. Thus, the main advantage of this method is to avoid any immune response due to viral capsids or other viral proteins. There are also no limitations concerning the transgene size but the transfection efficiency of nonviral gene therapy is progressively reduced with the increasing plasmid size.

Naked DNA. The simplest method to deliver a plasmid into muscle is its direct injection. Plasmids coding for μDYS and for FLDYS were injected in *mdx* mice[110]; however, the transfection efficiency was very low. Nevertheless, there is a possibility for prolonged transgene expression in muscles since muscle fibers are postmitotic. A phase I clinical trial was undertaken in 2004 on nine dystrophic patients[137] that were intramuscularly injected with a plasmid coding for human FLDYS. The three treated DMD patients just showed rare dystrophin-positive fibers. In the six treated BMD patients, the average level of dystrophin expression was slightly higher (about 3%). Although the application of naked DNA is appealing since this method is fast and the plasmids are easy to produce, the efficiency of direct intramuscular injection is currently too low to be clinically relevant. To improve gene delivery, chemical and physical methods can be used. However, due to the low effectiveness of chemical methods *in vivo*, only physical approaches are included in the present review.

Physical approach

Hydrodynamic pressure: Good expression levels were obtained following a rapid injection of a large quantity of plasmid DNA coding for luciferase or β-galactosidase.[138] This intravenous injection of a

large volume while using a tourniquet to occlude blood flow allows good dissemination of the naked DNA in muscles.[139] Indeed, the intravascular pressure induced the formation of transient pores in the endothelium of blood vessels allowing macromolecules, such as plasmids, to leak into the surrounding muscle and thereby access the muscle fibers.[140] The safety of this method was demonstrated in mice and in nonhuman primates.[139,141] The hydrodynamic limb vein injection used in *mdx* mice with a plasmid coding for FLDYS resulted in dystrophin expression in up to 20% of muscle fibers for >1 year.[142] The phenotype of the treated mice was also improved. Golden retriever muscular dystrophy dogs were also treated with this technique. The procedure appeared safe in the treated animals and enabled to obtain dystrophin expression but further work is required to determine the exact level of dystrophin expression.[143] This approach seems thus promising to introduce naked DNA in muscles.

Electroporation: A second method to improve the efficiency of muscle transfection is electroporation. The electric field used in this method enhanced the uptake of a plasmid previously injected in the muscle.[144,145] Indeed, the electric pulses permeabilized the cellular membrane, creating transient pores that facilitated the plasmid entry into the cell. However, these pores also increased calcium entry and activated proteases.[146] Therefore, it is important to select voltage settings, which allow maximal efficiency with the least amount of damage. As with the hydrodynamic pressure method, the electroporation of naked DNA in muscles resulted in transgene expression for >1 year.[147] The heart can also be treated by electroporation according to a recent research article.[148] A study showed that satellite cells can be transfected with this technique.[149] However, this study has not been confirmed. According to Schwann's equation, the threshold intensity of the applied electric field necessary to obtain membrane permeabilization is inversely proportional to the cell radius.[150] Since the radius of satellite cells is smaller than that of muscle fibers, the satellite cells and the muscle fibers cannot be electroporated simultaneously.

Since its first use in a clinical trial in 1991,[151] plasmid electroporation has proven to be safe and effective for transgene delivery to several tissues.[152–154] In the DMD context, a plasmid coding for mouse FLDYS was electroporated in *mdx* mouse muscles. The electroporated muscle fibers expressed the transgene for at least 1 month and exhibited a reduced number of centro-nucleated muscle fibers as well.[155,156] Dog FLDYS was also introduced with success in dystrophic dog muscle.[157] In this case, a specific immune response was observed in the treated dog muscle. Further studies are thus required to determine whether this immune response was against dystrophin or against the product of another transgene also present in the plasmid.

DISCUSSION

DMD is a devastating pathology leading to severe muscle weakness. This disease is due to the lack of dystrophin in smooth, cardiac, and skeletal muscles. Although there are currently no curative treatments for DMD, several therapeutic approaches are undergoing clinical evaluation such as pharmaceutical approaches and gene therapy.

Pharmaceutical approaches

The stop codon read-through is one of pharmaceutical approaches. The last clinical trial with ataluren showed that it was unable to achieve its primary outcome for improved muscle function. The long-term gentamicin clinical trials gave mixed results and showed too many toxicity issues to consider this antibiotic as a feasible approach to treat DMD patients having nonsense mutation. Moreover, stop codon read-through would only be relevant to only about 10 to 15% of DMD patients.

Exon-skipping can in theory be applied to 80% of DMD patients.[25] This method has shown its efficiency in mouse and dog models. Clinical trials using 2OMPs and morpholinos were also undertaken on DMD patients. In both cases, dystrophin expression was observed in the treated muscles and no significant adverse effects have been encountered. Only the results of intramuscular exon skipping trials have been published so far with results restricted to the site of delivery. However, the first results on the clinical trials using a morpholino (AVI-4658) or a 2OMP (PRO051/GSK2402968) systemically delivered showed good dystrophin expression.[66,85] Even though there are no long-term toxicity studies (>6 months) available on 2OMPs and morpholinos in nonhuman primate, these two compounds are promising for DMD.

Currently, no molecules upregulate utrophin expression sufficiently to restore the phenotype of dystrophic mouse models. Therefore, utrophin upregulation must be further improved before applying it in DMD.

Gene therapy

Another method to obtain a functional dystrophin is to introduce a cDNA in muscle fibers using gene therapy. The most promising viral vector to introduce a micro-dystrophin cDNA in muscle fibers is currently the AAV vector. The results obtained with this vector in mice, dogs, and nonhuman primates are good despite the fact that antibodies against the AAV capsid were sometimes found in the treated animals (humans also have pre-existing antibodies against AAV and adenovirus). However, a recent clinical trial using an AAV coding for micro-dystrophin did not demonstrate significant transgene expression in the treated DMD patient muscles. Moreover, this study detected lymphocytes reacting with dystrophin in response to transgene expression.[123]

One way to eventually avoid the potential toxicity following the dissemination of viral vectors throughout the body[158] is to transplant autologous cells, which have been genetically modified *ex vivo*. This *ex vivo* gene therapy has shown positive results in mice and nonhuman primates but is nevertheless limited by the same problems as myoblast transplantation, *i.e.*, the difficulty of reaching small muscles and the high number of injection trajectories necessary to obtain a high percentage of dystrophin-positive fibers. Exon skipping can also be induced by viral vectors carrying the U7 snRNA gene modified to target a specific exon. Since no results are yet available in large animals with this gene, the AO technology currently remains the most efficient and most frequently used method to induce exon skipping in DMD.

An alternative to *ex vivo* gene therapy is the use of naked plasmid delivered by hydrodynamic pressure or by electroporation. These two techniques have shown good efficiency to deliver dystrophin cDNA or internally deleted versions of it in mouse

model, although these physical methods are less efficient than systemic injection of viral vectors. Moreover, only a few preliminary results are available in larger animal models, such as dogs and nonhuman primates. The main limiting factor for electroporation is that at this time only a small number of muscle fibers can be treated with this technique since it requires penetration with electrodes into each muscle. The hydrodynamic method can be applied only to arm and leg muscles but not to muscles of the head and trunk.

Response to dystrophin in clinical trials

During clinical trials on DMD patients, anti-dystrophin antibodies were observed following nondystrophic myoblast transplantation[159] and dystrophin-specific T-cells were detected following the injection of AAV coding for micro-dystrophin. The presence of dystrophin-specific T-cells was also detected in one patient after treatment with gentamicin.[123] No anti-dystrophin antibodies were found in the DMD patients treated with AVI-4658 or with PRO051 but the presence of dystrophin-specific T-cells was not investigated. Apparently, there were no T-cell responses, or if there were, it was not effective enough to hamper dystrophin expression. This seems to indicate that if a therapeutic approach is effective to restore dystrophin in muscle fibers, some DMD patients may have to be under a sustained immunosuppression treatment.

Conclusion

Even though the *DMD* gene was discovered 23 years ago, there are still no curative treatments for DMD although the use of steroids and assisted ventilation have greatly improved the quality of life and extended life span by nearly 50%.[33]

When a therapeutic approach is found to restore dystrophin in the DMD patient's muscles, the problems of fat infiltration or fibrosis in the muscles will still need to be resolved, as well as the existing muscle weakness or bone deformation. An approach to improve muscle strength is to block the myostatin pathway. Indeed, myostatin inhibition leads to muscle hypertrophy and muscle strength increases in animals. The process of fat infiltration and fibrosis in DMD patient's muscles is not well understood and needs to be further investigated. The best approach will thus be to treat DMD patients when they are still young to avoid most of the consequences due to the absence of dystrophin. Moreover, all muscles (or a large proportion of them) will need to be treated to obtain a curative treatment.

SUPPLEMENTARY MATERIAL

Figure S1. AOs used in DMD.

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Review

Restoring Dystrophin Expression in Duchenne Muscular Dystrophy Muscle

Progress in Exon Skipping and Stop Codon Read Through

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The identification of the Duchenne muscular dystrophy gene and protein in the late 1980s led to high hopes of rapid translation to molecular therapeutics. These hopes were fueled by early reports of delivering new functional genes to dystrophic muscle in mouse models using gene therapy and stem cell transplantation. However, significant barriers have thwarted translation of these approaches to true therapies, including insufficient therapeutic material (eg, cells and viral vectors), challenges in systemic delivery, and immunological hurdles. An alternative approach is to repair the patient's own gene. Two innovative small-molecule approaches have emerged as front-line molecular therapeutics: exon skipping and stop codon read through. Both approaches are in human clinical trials and aim to coax dystrophin protein production from otherwise inactive mutant genes. In the clinically severe dog model of Duchenne muscular dystrophy, the exon-skipping approach recently improved multiple functional outcomes. We discuss the status of these two methods aimed at inducing *de novo* dystrophin production from mutant genes and review implications for other disorders. ***(Am J Pathol 2011, 179:12–22; DOI: 10.1016/j.ajpath.2011.03.050)***

Dystrophin Replacement: From the Outside, or Inside?

Duchenne muscular dystrophy (DMD) is the most common neuromuscular disease and affects all world populations equally. The cause of this genetic disease is loss of a single protein, dystrophin, in all types of muscle (ie, skeletal, cardiac, and smooth) and in neurons.[1,2] The loss of protein function is the consequence of mutations in the large *DMD* gene. The gene contains 79 exons distributed over 2.3 million bp of genetic real estate on the *X* chromosome; however, only approximately 14,000 bp (<1%) is used for translation into protein (coding sequence).[3] The 99.5% of intronic junk must be spliced out of the 2.3 million bp initial heteronuclear RNA transcript to lead to the mature 14,000 bp mRNA that includes all key information for dystrophin protein production. Patients with DMD have mutations in the gene that prevent the appropriate construction of the mRNA and/or the production of the dystrophin protein, and all patients with DMD show marked dystrophin deficiency in their muscle.[4]

During the past 25 years since gene and protein identification, dozens of innovative experimental therapeutic approaches for DMD have emerged; many are transitioning to clinical trials. These include slowing the progression of the disease by immune modulators (eg, steroids

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and transforming growth factor-β inhibitors), inducing or introducing proteins that may compensate for dystrophin deficiency in the myofiber (eg, utrophin, biglycan, and laminin), or bolstering the muscle's regenerative response (eg, myostatin and activin 2B). A parallel approach places dystrophin back into patient muscle.

There are two general tactics to introducing dystrophin back into dystrophin-deficient muscle: introducing a new more functional gene into the patient or repairing the patient's own gene in some manner. Gene therapy using viral vectors[5,6] and stem cell transplants[7] has been used for exogenous gene delivery. Despite extensive research, including limited clinical trials,[8,9] these approaches have failed to produce clinically significant levels of dystrophin in the muscle of patients with DMD. Key obstacles include delivery problems [ie, getting the stem cell or viral vector to the right place in the large target organ (muscle)], immunological barriers, and production issues (obtaining adequate amounts of cells or viruses to treat a patient). Therefore, clinical progress in gene therapy and cell transplantation has been slow.

On the other hand, approaches to coax dystrophin production out of the patient's own disabled gene have been more impressive. A key to the more rapid advance is the development of small-molecule drugs for gene repair that overcome problems with target organ delivery, production, and immune response.

In this review, we discuss progress and the remaining hurdles in small-molecule drug approaches for gene repair in DMD.

Turning Duchenne into Becker: Exon Skipping

With the characterization of the dystrophin gene, it was quickly recognized that patients with a clinically milder dystrophy, Becker muscular dystrophy, showed mutations of the same dystrophin gene as boys with Duchenne dystrophy.[10,11] The molecular explanation for the often dramatic clinical differences was framedness. Although the muscle of patients with DMD could not put together what was left of the dystrophin gene into a serviceable (translatable) mRNA (it was out of frame), patients with Becker dystrophy had mutations in which the rest of the gene could still be used effectively and produce translatable mRNA (in frame).

A model for therapeutics emerged in which a patient diagnosed as having clinically severe DMD might be converted to having the milder Becker dystrophy at the molecular level, by restoring the framedness [eg, turning an out-of-frame mutation into an in-frame (multiple of three) mutation]. This occurred spontaneously in some patients with DMD who appeared to have a frameshift nonsense mutation on genomic DNA but were able to rescue some dystrophin production by skipping an additional exon, bringing the resulting mRNA back into frame.[12–14] The same spontaneous exon-skipping process is observed in many muscle biopsy specimens from patients with DMD and in *mdx* mouse muscle in the form of revertant fibers [ie, a small proportion (<1%) of strikingly positive myofibers in a background of complete dystrophin deficiency].[15–17]

The therapeutic strategy using this concept was dubbed exon skipping, in which antisense oligonucleotides (AOs) were designed to modulate the splicing of the dystrophin gene of a patient with DMD, resulting in mRNA transcripts that are Becker-like (ie, able to make some level of functional dystrophin) (Figure 1). AOs are short nucleic acid sequences designed to selectively bind to specific mRNA or pre-mRNA sequences to generate small double-stranded regions of the target mRNA. By binding to these critical regions and forming double strands at key sites where the spliceosome or proteins of the spliceosome would normally bind, the mutated (frameshifting) exons are skipped and the remainder of the pre-mRNA is edited correctly in frame, albeit shorter. AOs were designed to target specific exons (eg, exon 51 drug PRO051 in Figure 1) and tested in the *mdx* mouse model[19,20] and then in cultures of muscle from patients with DMD.[21] In these systems, they diffused into the dystrophic myofibers and then into the nucleus, where they bound the unspliced pre-mRNA, modulated splicing, and restored dystrophin expression.

Why Do AOs Work Better in DMD Compared with Other Previous Clinical Applications?

Antisense drug development for human disease has been pursued for approximately 20 years, and AOs have been tested clinically in >90 clinical trials (*http://www.clinicaltrials.gov/ct2/results?term=antisense,* last accessed March 1, 2011). Of these trials, 40 have been completed, involving >2000 patients, targeting cancer, inflammatory disease, and other indications.[22,23] Despite this impressive effort, only one AO has been approved by the Food and Drug Administration (Vitravene, an intraocular injection to inhibit cytomegalovirus retinitis in immunocompromised patients; Isis Pharmaceuticals, Carlsbad, CA), and this drug is no longer marketed.

Why have so many of the AO drug programs failed, and why might AO treatment in DMD work better? Excellent literature reviews have indicated the significant biological barriers to antisense efficacy, including uptake and sequestration in the reticuloendothelial system, significant barriers to achieving sufficiently high intracellular concentrations in target cells because of endothelial, basement membrane, and cell membrane barriers, and intracellular sequestration in phagolysomes or in oligoprotein complexes. In addition, there is the challenging requirement that to produce pharmacological activity, a large fraction of many RNA targets needs knocking down (>90%) before biochemical efficacy is realized.[24] For DMD, the disease itself seems to have navigated some of these major hurdles, with a dramatic improvement in biochemical efficacy relative to other indications. There are two key differences with AO applications to DMD, and these result in an approximate 100-fold improvement in efficacy compared with previous AO applications. First, AO drugs in all other indications are designed to knock down (inhibit) the target, whereas the goal in DMD is to

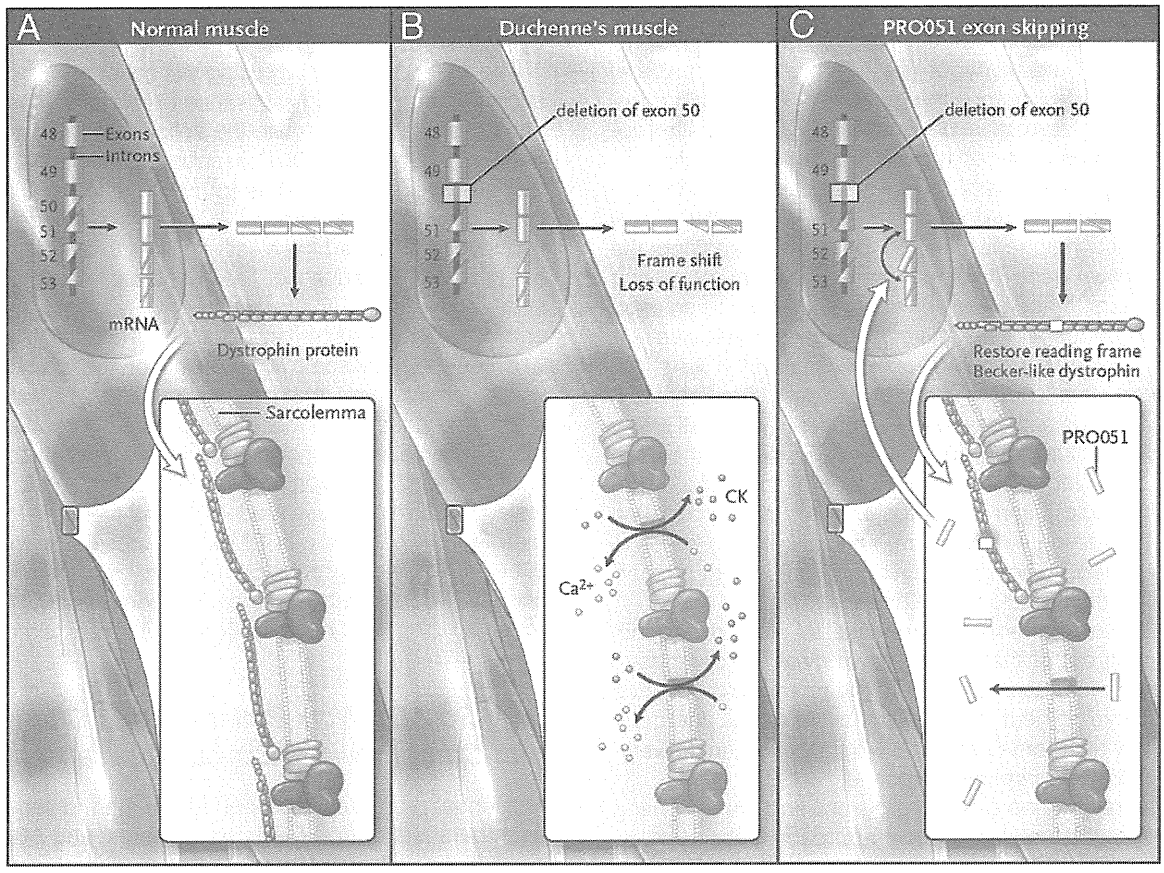

Figure 1. Mechanism of action of AO exon-skipping drugs. **A:** Dystrophin gene splicing in healthy muscle, in which all 79 exons are precisely spliced together to maintain the protein translational reading frame (only exons 48 to 53 are shown). **B:** A patient with DMD with a deletion of exon 50. The remaining exons are spliced together, but there is a disruption of the reading frame, disabling the ability of the mRNA to produce any dystrophin. Consequently, there is a dystrophin deficiency in muscle and unstable plasma membranes. CK indicates creatine kinase. **C:** The mechanism of action of PRO051, an AO drug targeting exon 51. The exon 51 sequence (adjacent to the missing exon 50 sequence) is skipped, so that the mRNA splices exon 49 to 52. The new deletion is able to be translated into semifunctional Becker-like dystrophin, resulting in partial repair of the myofiber plasma membrane. Reproduced with permission from Hoffman (copyright 2007, Massachusetts Medical Society).[18]

rescue (knock up or increase) the target. Second, the membranes of DMD muscle are leaky as a result of the underlying pathophysiological features, facilitating a route of entry for AOs into myofibers.[25] Indeed, i.v.delivered AO's show very poor delivery to normal muscle, while dystrophic muscle or i.m. injection in normal muscle shows excellent delivery (Figure 2).[26]

Regarding previous knockdown AO approaches, it is approximately 10 times harder to effectively knock down a target than it is to knock up a target (as in DMD). In a knockdown model, the goal is to take 100% of the protein down to approximately ≤10% to achieve the desired biochemical loss of function. For example, in cancer, where an oncogene is targeted by an AO, the AO would need to bind approximately 90% of the mRNA target to bring protein expression down to 10% and oncogenic transformations are generally not a single-gene disorder. For DMD, the goal is to restore expression of the target gene to >10%, but this translates into needing to hit only approximately 10% of mRNA targets with the drug (bringing protein expression from 0% to 10%). Thus, knockdowns need to hit 90% of targets, but DMD knock ups need to hit only 10% of targets (a 10-fold difference).

Then, there is an additional advantage of dystrophic muscle providing easier access for the AO into myofibers. All previous AO applications have had trouble achieving adequate concentrations of drug within the cell. The major barrier to AO drugs is the cell plasma membrane. AOs typically do not traverse membranes well, and efforts to make the drugs more cell permeable tend to increase toxicity. Patients with DMD have unstable plasma membranes in their muscle fibers, which effectively provide a leaky entry for drug delivery (Figure 1). Although it is challenging to quantify this delivery advantage in DMD muscle, the cell permeability defect may increase drug delivery by a factor of ≥10. Consistent with this model of unstable membrane delivery, systemic AOs delivered to healthy muscle do not show effective delivery, indicating that the dystrophic process is a requirement for sufficient drug delivery (Figure 2).

Taken together, the 10-fold increase in cell delivery because of unstable membranes and the 10-fold relaxed requirements for hitting mRNA targets cumulatively give AO used in DMD a 100-fold advantage compared with other clinical applications of antisense.

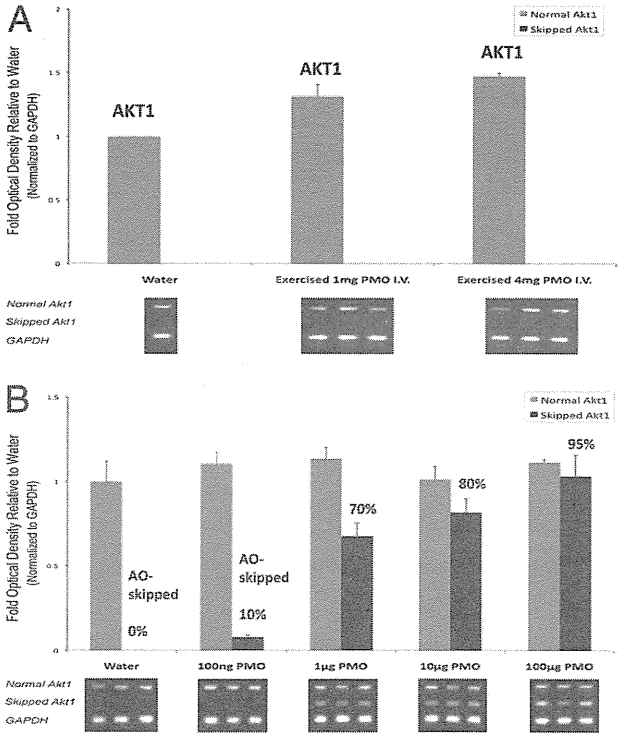

Figure 2. Morpholino AOs achieve myofiber delivery through bulk flow across unstable plasma membranes. Many publications have shown that morpholinos delivered i.v. achieve unexpected efficacy for modulating splicing within dystrophic myofibers, presumably through bulk flow across unstable dystrophic plasma membranes. Herein, we test this model using i.v. versus i.m. delivery of a morpholino in healthy murine muscle. **A:** 0 mg (water) or 1 or 4 mg morpholino was given in an i.v. bolus in healthy mice, and drug delivery to myofibers was assayed by exon skipping in the Akt1 mRNA (skipped Akt1). No detectable exon skipping was observed in healthy skeletal muscle (0%). **B:** As a positive control, the same morpholino was delivered by i.m. injection in saline (0, 0.1, 1, 10, and 100 µg). The saline destabilizes the myofiber membranes, and efficient dose-related exon skipping is observed (skipped Akt1). GAPDH indicates glyceraldehyde-3-phosphate dehydrogenase.

AO Medicinal Chemistry and Preclinical Safety

Organisms have fairly sophisticated inflammatory responses directed against exogenous DNA or RNA. Genetic material coming into the body from the outside is assumed to be infectious; as a result, DNA or RNA is immunostimulatory or proinflammatory. Oligonucleotides activate innate immunity, with single-stranded oligonucleotides binding to toll-like receptor 9 or other receptors of innate immunity. This binding tends to be both sequence and chemistry dependent.[27] Thus, AO drugs must be disguised in a way to circumvent surveillance and inflammatory responses. Typically, this is accomplished by avoiding CG motifs that are more common in bacterial DNA and by using medicinal chemistry that keeps the G, A, T, and C bases the same (so they can bind to the target sequence) but replacing the ribose-phosphodiester backbone with different chemistries (Figure 3) that evade immune surveillance. In addition, medicinal chemistry can be used to further enhance cell uptake. In general, increasing the charge of the backbone increases protein binding, including cell surface binding, making it more likely that the AOs get into cells. However, increased charge can also make AOs more toxic, often through facilitating interactions with other proteins (eg, the tenase complex of intrinsic clotting cascade[28] or factor H in the alternative complement cascade).[29] In DMD, the need to increase charge to enhance delivery is ameliorated (AOs do not have the same cell delivery problem as in other disorders) because there is already a leaky gateway into the cell. There are two commonly used backbone chemistries that are being used in the development of AO for DMD, one charged and the other uncharged (described later), and each has its pros and cons.

2′-O-Methyl Phosphorothioate

Candidate drugs using this chemistry keep the ribose ring intact but add moieties to both the ribose ring and the phosphodiester linkage between riboses in the AO chain. This is the chemistry of choice in the Prosensa/GlaxoSmithKline DMD drug development program (Prosensa Therapeutics, Leiden, the Netherlands). The toxicity and clinical safety of phosphorothioate oligonucleotides as a class have been well characterized in preclinical studies and human clinical trials of candi-

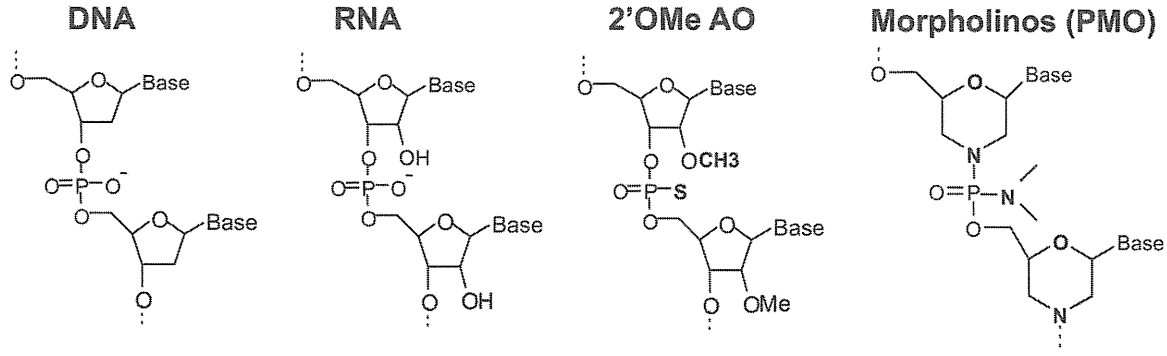

Figure 3. Backbone chemistries of nucleic acids and antisense drugs. Normal DNA and RNA has ribose rings (sugar moieties) attached by phosphodiester linkages, and one of four bases (G, A, T, C for DNA and G, A, U, C for RNA) is attached to the ribose and participates in sequence-specific base pairing with other nucleic acid strands. The AO drug chemistries modify the backbone to make the drugs more stable and less toxic. The 2′OMe AO adds a methyl group to the ribose ring and a sulfur residue to the phosphodiester linkage. The morpholino (PMO) chemistry makes many more changes, replacing the ribose with a nitrogenous morpholine ring; amine groups replace the phosphodiester linkage. Despite the relatively dramatic chemical changes to the PMO backbone, the spacing between the bases is similar to DNA and RNA and does not disrupt base pairing with other nucleic acid strands.