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

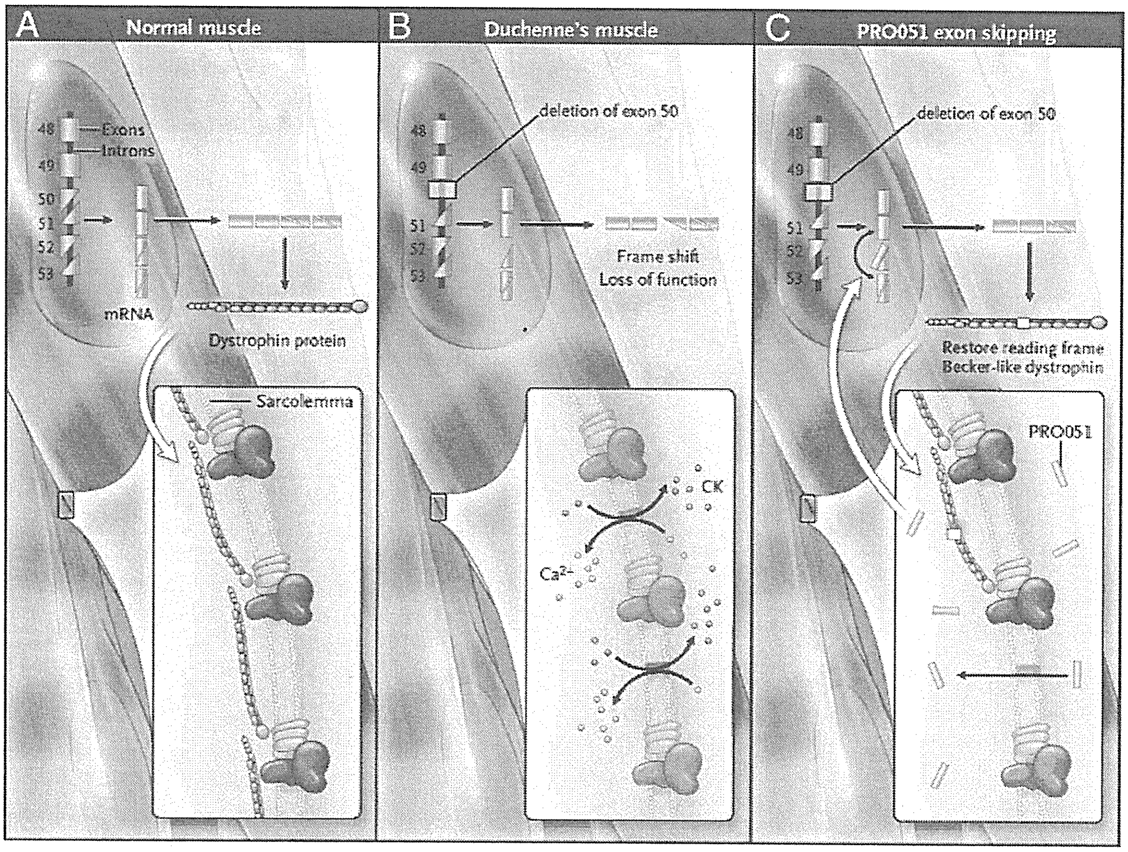

Figure 1. Mechanism of action of AO exon-skipping drugs. **A:** Dystrophin gene splicing in healthy muscle, in which all 79 exons are precisely spliced together to maintain the protein translational reading frame (only exons 48 to 53 are shown). **B:** A patient with DMD with a deletion of exon 50. The 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.

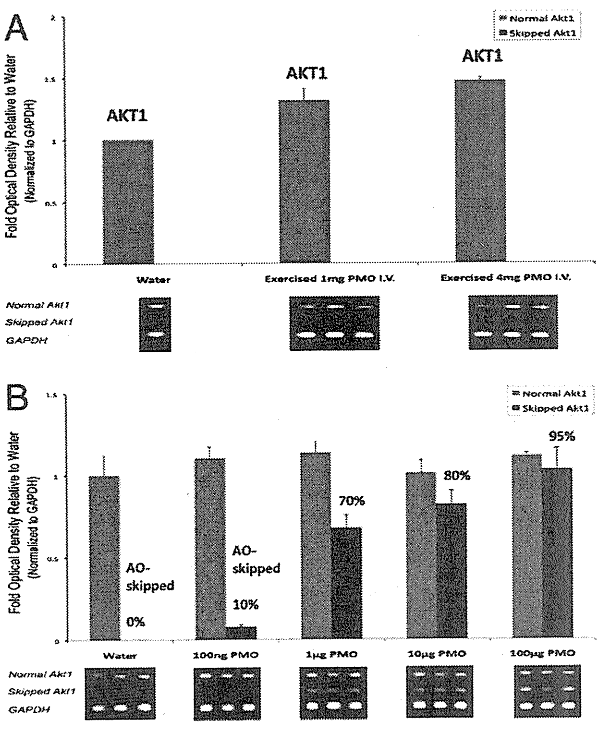

Figure 2. Morpholino AOs achieve myofiber delivery through bulk flow across unstable plasma membranes. Many publications have shown that morpholinos delivered i.v. achieve unexpected efficacy for modulating splicing within dystrophic myofibers, presumably through bulk flow across unstable dystrophic 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-

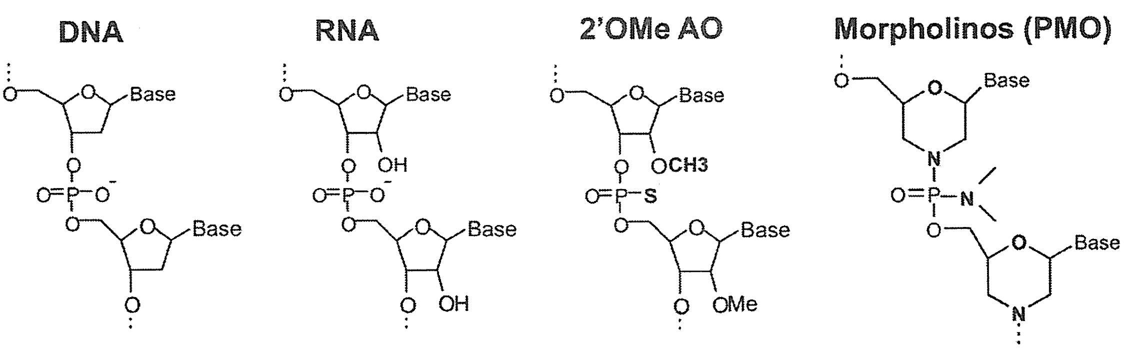

Figure 3. Backbone chemistries of nucleic acids and antisense drugs. Normal DNA and RNA has ribose rings (sugar moieties) attached by phosphodiester linkages, and one of four bases (G, A, T, C for DNA and G, A, U, C for RNA) is attached to the ribose and participates in sequence-specific base pairing 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.

date AO drugs developed for several conditions.[30–34] Some of these studies have used modification of the 5′ and 3′ ends, with 2′-O-methoxy ethyl–modified ribose to make the drugs more resistant to degradation by nucleases. AOs can prolong the intrinsic clotting pathway (activated partial thromboplastin time) and increase complement split products in the monkey, but this appears to be dose dependent, with clinically significant levels occurring at relative high plasma peak concentrations (>50 μg/mL).[35] Human phase 1 safety studies have shown concentration-dependent effects on coagulation and complement, with the maximum tolerated dose by 24-hour infusion being approximately 20 mg/kg.[36] The observed adverse effects appear to be transient. Similar to other 2′-substituted AOs, the most prominent end-organ finding for phosphorothioate AOs in the monkey has been the presence of granules in the proximal tubular epithelial cells of the kidney, most likely from the uptake by phagocytosis of filtered oligonucleotide.[37] Regarding applications to DMD, phosphorothioate chemistries (2′OMe) have the great advantage of extensive preclinical and clinical experience.

Morpholino

This is the chemistry of choice in the AVI BioPharma DMD program (AVI BioPharma, Bothell, WA). The key advantage of the morpholino chemistry compared with phosphorothioate is the superior therapeutic window. Morpholino AOs have been dosed i.v. in monkeys to 320 mg/kg per week and in rodents to 960 mg/kg per week, with no evidence of dose-limiting toxicities.[38] As noted later, the 2′OMe drug PRO051 showed proteinuria at 6 mg/kg per week using s.c. doses in patients with DMD, whereas a similar morpholino drug showed no proteinuria at doses to 320 mg/kg per week using i.v. delivery in monkeys.

The major disadvantages are the much lower clinical experience with morpholino chemistry. There have been three clinical trials completed involving 39 patients with morpholino antisense, compared with 40 trials and 2000 patients in completed trials with other antisense AO chemistries (*http://www.clinicaltrials.gov*).

Phosphorodiamidate morpholino oligomers (PMOs) are a class of backbone modification that has a morpholino ring as a replacement for the furanose, with phosphorodiamidate linkage connecting the morpholino nitrogen atom with the hydroxyl group of the 3′ side residue (Figure 3). This backbone modification sets this class of AOs apart from most other modifications, and the synthesis of these AOs is unique. Until recently, this chemistry was not in the public domain for therapeutic applications. As a result, only modest progress has been made in improving the purity, capacity, and cost of goods for these AOs.

AOs synthesized from morpholinos retain high sequence specificity and strong binding to the target RNA. They are sufficiently dissimilar from native RNA and DNA in that they are not recognized by host RNA or DNA or degrading enzymes, thus making them more stable. In animal models, AOs synthesized from morpholinos (PMOs) do not cause complement activation at high serum concentrations after repeated (weekly) i.v. administration (approximately 1 g/kg per week i.v.; AVI BioPharma poster, *http://www.avibio.com/wp-content/uploads/2010/10/AVI-4658-WMS-Preclin-Poster-101510.pdf*, last accessed March 1, 2011). PMOs are highly water soluble, are not subject to metabolic degradation, and do not activate the toll-like receptors, the interferon system, or the NF-κB–mediated inflammation response.[39]

Toxicity studies have been performed in both mouse (12 weekly i.v. or s.c. injections to 960 mg/kg per dose) and monkey (12 weekly i.v. or s.c. injections to 320 mg/kg per dose). No evidence of liver or kidney dysfunction was seen, although there was histological evidence of accumulation in the proximal renal tubules, a finding seen with most AOs. Clinical trials of PMOs are under way in the UK and are about to begin in the US; thus, clinical safety data for DMD are limited.

Additional Chemistries and Technologies for Exon Skipping

Although the approaches previously described are promising, alternative strategies are being developed to address some potential limitations. Alternatives include the development of methods and chemistries to i) increase potency to reduce the amount of drug that will need to be manufactured and delivered to patients; ii) permit delivery to nonskeletal muscle target tissues, such as the heart; and iii) mitigate the need for repeated parenteral administration (eg, weekly or monthly i.v.).

One approach is to increase the charge of the AO through addition of residues along the backbone or at either end. Examples of modifications of the end of the AO include the peptide-modified PMO or morpholino[40] and guanidium dendrimer (vivo morpholino).[41] Another approach is to add targeting peptides (ie, small amino acid sequences that can interact with the muscle fiber membrane).[42,43]

Although each of these modifications to the backbone increases potency, the modifications also tend to bypass the holes in membrane delivery that unstable DMD membranes afford and, thus, lose this disease-specific advantage in DMD. They also tend to increase toxicity because they may bind to plasma proteins or cell surface proteins on nonmuscle cells (or vasculature or blood cells) and generate undesired off-target effects. Although alternative chemistries will be a continued focus for research, it is likely that efficacy in DMD will first be proved using the existing PMO and 2′OMe chemistries.

Another alternative approach is to perform exon skipping using gene therapy instead of AOs.[44–46] Herein, specific mRNA splicing molecules (ie, U7 or U1 RNA) are designed to splice out extra exons; these customized U7 drugs are coded within gene therapy viral vectors. The muscle is infected with the virus, the U7 drugs are expressed, and the drugs work efficiently at driving the desired in-frame spliced products. A critical advantage of the U7 approach is that one treatment may last a lifetime because the gene therapy vectors seem stable in muscle and continue to express the U7 RNA. A disadvantage of this approach is that it requires

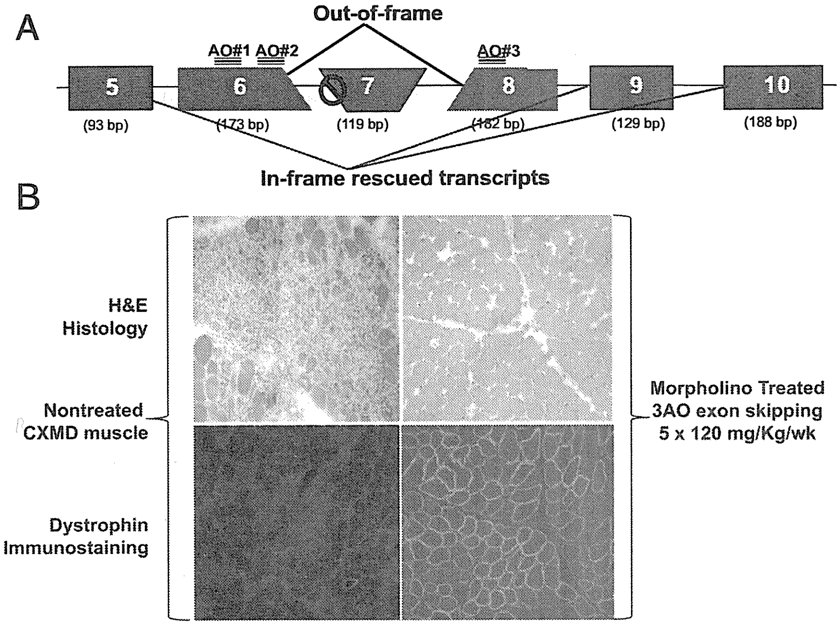

Figure 4. Delivery of multiple PMO drugs to a dog model of DMD skips multiple exons and results in *de novo* dystrophin production. **A:** Schematic of dog gene structure. The sporadic golden retriever dystrophin gene mutation is a splice-site mutation of exon 7 (red symbol). This forces the exclusion of exon 7, whereby the dystrophic dog muscle splices exon 6 to 8, but these exons do not share the same reading frame (out of frame). AOs covering exons 6 and 8 were designed (AOs 1, 2, and 3) to block inclusion of exons 6 and 8, leading to in-frame rescued transcripts (exons 5 to 9 or 5 to 10). **B:** Histological features and matched dystrophin immunostaining of AO-treated dystrophic dogs (**right**) and controls [nontreated canine X linked muscular dystrophy (CXMD) muscle; **left**]. Nontreated muscle shows necrosis of myofibers and inflammatory cell infiltration, whereas AO-treated muscle shows no inflammation or necrosis. Dystrophin protein is absent in the nontreated muscle, whereas the AO-treated muscle shows high amounts of membrane dystrophin, comparable to healthy muscle. Adapted with permission from Yokota et al (copyright 2009, John Wiley & Sons).[56]

viral gene therapy; as previously noted, gene therapy of DMD has faced persistent hurdles of immune response, viral production, and systemic delivery.[47]

Evidence for Efficacy of AO Exon Skipping: Preclinical and Clinical Studies

Animal Studies

The premise for exon skipping in DMD has been well studied in the *mdx* mouse model. In the early part of this decade, several laboratories established the fact that delivering sequence-specific AOs can induce exon skipping, which reestablishes the reading frame of dystrophin mRNA in myogenic cell cultures.[19,48–50] After these early findings, the AOs could be delivered via i.m. injection and could induce dystrophin expression to near–normal levels in most muscle fibers; this was accompanied by functional improvement.[51] Most recently, systemic delivery of AOs by i.v. injections can induce exon skipping and dystrophin expression up to levels found in healthy muscle. In addition, after three i.v. injections at weekly intervals, enhanced dystrophin expression was detected in every skeletal muscle examined.[52] Regarding dose-response and dosing schedules, single injections at a high dose (3 g/kg) show robust dystrophin expression and relatively long persistence of protein rescue.[53] These preclinical data suggest that i.v. delivery might show good efficacy at a frequency of three to four doses per year, rather than the weekly doses used in most current preclinical and clinical studies.

An oft-quoted adage is that academic medicine has generated thousands of highly efficacious mouse drugs and far fewer effective human drugs. Demonstration of efficacy in a large animal model typically engenders more confidence in human applications. Therefore, work[54,55] has been performed in the dog model of DMD that has a mutation in exon 7 of the dog dystrophin gene. Dogs with DMD represent a particularly stringent test of exon skipping, in that: i) they typically show rapidly progressive disease, often leading to death by 6 months; ii) the nature of the dog mutation requires skipping of two exons to bring the transcript back into frame; and iii) because the dog deletion is near the beginning of the dystrophin protein (actin binding site), this may be more biochemically disabling to the protein than more central deleted regions (Figure 4). In these studies, three morpholino AO drugs were codelivered to dogs with DMD to achieve exon skipping, using high doses of up to 200 mg/kg i.v. per week.[56] Given the size of the dogs, these studies required production of a large amount of AO drug.

Despite the stringency of the model, all of the three dogs tested showed stabilization or improvement of multiple functional, imaging, and histological parameters (Figure 4). Dystrophin production was increased to an average of approximately 20% in all skeletal muscles, and no toxicities were observed despite the high cumulative exposure. The dystrophin amounts varied considerably from muscle to muscle, and, consistent with murine studies, systemic delivery to the heart was poor.

Clinical Studies

The first human studies were published from a private/public partnership in Leiden, the Netherlands, between the university and Prosensa Therapeutics.[57] The AO drug, PRO051, was against exon 51 of the human dystrophin (*DMD*) gene and used phosphorothioate (2′OMe) chemistry (Figure 1). In a phase 1 safety study completed in 2007, single i.m. doses of PRO051 were safe and well tolerated in four patients with DMD who were aged 10 to 13 years and were selected on the basis of mutational status, muscle condition, and positive response to exon skipping 51 in their cultured cells *in vitro*. A biopsy specimen of the injection site that was obtained 4 weeks later showed evidence of *de novo* dystrophin expression.

Data from an investigator-initiated clinical trial in London, UK, using a single i.m. injection of morpholino AO (AVI BioPharma) were published in 2009.[58] The investigators used an AO sequence that was similar, but not identical, to that used in the previous Dutch trial but switched to the newer morpholino chemistry. In this phase 1 study, AVI-4658 was given to seven patients with DMD (aged 12 to 18 years) as an i.m. injection in the extensor digitorum brevis. Two boys received a low dose of 0.09 mg in 900 μL, and five boys received 0.9 mg in 900 μL. Each boy received a saline injection in the contralateral extensor digitorum brevis. Muscle biopsy specimens were obtained before treatment and at 3 or 4 weeks and examined for dystrophin production. AVI-4658 was well tolerated, and no dose-limiting toxicities were observed. Treated patients had evidence of induced dystrophin production in a dose-responsive manner.

In both i.m. studies, the amount of dystrophin in treated muscle, measured by immunoblot, was low (approximately 1% to 5%) versus levels in healthy muscle. Although immunoblotting is a good method for determining the average levels of dystrophin in the tissue, it has less sensitivity compared with dystrophin immunostaining, which is able to identify individual fibers expressing relatively low levels of dystrophin. Work is ongoing to evaluate and standardize the optimal methods for use in clinical trials. In addition, the amount of dystrophin expression that correlates with clinical response is not established. From early genotype-phenotype studies[59,60] of dystrophinopathies, dystrophin immunoblot levels >10% of normal may be necessary for clinical activity; neither i.m. study consistently reached this level.

An open-label dose-ranging study[61] of the PRO051 2′OMe drug in 12 patients was recently reported. Patients with DMD were given five weekly s.c. doses, ranging from 0.5 to 6 mg/kg, with muscle biopsy specimens obtained at both 2 and 7 weeks after the initiation of treatment. Both the 2- and 7-week biopsy specimens showed drug-induced dystrophin mRNA splicing and protein production, although the levels of dystrophin by immunoblot appeared lower than might be needed for altering clinical symptoms. There was no clear dose-response relationship between dystrophin immunostaining and drug doses. All patients were then enrolled into a 12-week extension study using the peak dose (6 mg/kg per week). At the conclusion of the extension study, patients seemed to perform better on a 6-minute walk test, suggesting clinical efficacy. Because biopsy specimens were not obtained after the 12-week extension study, it was not possible to correlate molecular efficacy with apparent clinical efficacy; and because the study was open label and not placebo controlled, the improvement in functional outcomes needs to be interpreted cautiously. Nevertheless, this study provided sufficient evidence for GlaxoSmithKline to initiate a 1-year, phase 3, blinded placebo-controlled study of 6 mg/kg per week s.c. dosing in 180 patients; the study enrolled patients at 14 sites in seven countries as this article was being written (*http://clinicaltrials.gov/ct2/show/NCT01254019?term=duchenne&rank=4*, last accessed March 1, 2011).

A key issue for success of high-dose antisense drug delivery is the achievement of a balance of toxicity and efficacy (therapeutic window). As previously described, there are well-documented toxicities that limit human dosing to approximately 20 mg/kg, yet both mouse and dog studies suggest that ≥40 mg/kg may be required for sufficient dystrophin production. In the GlaxoSmithKline/Prosensa dose-ranging study, all 12 patients enrolled experienced proteinuria and an elevated urinary α1-microglobulin level at week 12 of the extension period, suggestive of kidney toxicity. Renal proximal tubuli accumulate oligonucleotides through drug reabsorption, and it will be important to monitor kidney toxicity in the ongoing 12-month phase 3 study.

AVI BioPharma has performed a dose-escalation study in the UK with systemically administered AVI-4658. Although not yet published, data have been presented in press releases and at meetings. The study included six cohorts given 12 weekly i.v. doses, ranging from 0.5 to 20.0 mg/kg per dose. At the highest dose, one patient is reported to have *de novo* dystrophin production, with approximately 50% of fibers testing positive for dystrophin by immunostaining (AVI BioPharma news release, *http://investorrelations.avibio.com/phoenix.zhtml?c=64231&p-irol-newsArticle&ID=1433350&highlight=*, last accessed March 1, 2011); however, this likely translates to approximately 20% of total dystrophin muscle content by immunoblotting. The response of patients to a similar dose has been variable, and large interpatient variability may become a theme in exon skipping. There are at least two likely reasons for differences in interpatient response to a similar dose. First, i.v. doses are typically calculated based on weight of the patient (mg/kg); the peak serum dose, at which the drug can permeate through the leaky DMD myofiber membranes, may be more important. Thus, drug doses may need to be calculated more by body mass index or some other means of approximating blood volume, rather than simply by patient weight. Second, the *de novo* dystrophin produced by exon skipping is Beckerlike (not normal); researchers have observed that there can be remarkable interpatient variability in muscle dystrophin content, despite patients having the same in-frame deletion. For example, patients with Becker dystrophy who share a common exon 45 to 47 deletion can vary widely in the amount of dystrophin in their muscle by immunoblot and the severity of the histopathological features (Table 1).[62]

The preclinical and clinical data available thus far suggest that exon skipping may hold significant promise as a candidate treatment for DMD (although the response may be variable). However, these studies are early and clinical development is ongoing. Prosensa, in partnership with GlaxoSmithKline, has announced work on AO, targeting additional exons. AVI has an investigational new drug with the Food and Drug Administration and is expected to begin enrolling patients in trials in the US in 2011.

Table 1. Variability in Dystrophin Amount and Severity of Histopathological Features in Patients with Becker Muscular Dystrophy Who Share the Same In-Frame Deletion

Patient no.	Age at biopsy (years)	CPK level (U/L)	Histopathological features (severity of dystrophy)	Immunoblot (%)	Immunostaining
31	9	9760	Very mild	80	+++
32	7	NA	Moderately severe	5	++
33	1	3000	NA	50	++++
34	37	2844	Mild	20	+++
35	29	692	Mild	50	+++
36	38	NA	Severe	5	++
37	43	NA	Moderate	5	++
38	20	9543	Very mild	30	+++
39	13	NA	Moderately severe	80	++
40	59	NA	Moderate	30	++

Data are adapted from Kesari et al.[62] The gene mutation was an exon 45 to 47 deletion for all patients.

CPK, serum creatine phosphokinase; NA, not available; ++, moderate intensity; +++, moderately high intensity; ++++, high intensity (similar to normal controls).

Regulatory Pathway for AO Drugs

Exon skipping in DMD presents some unique challenges and may serve as a test case for personalized medicine, in which drugs are customized to a patient's genetic fingerprint. The exon 51 drug would only be applicable to relatively few patients with DMD. Indeed, drugs against five exons would be needed before even half of the patients with DMD could be treated with exon skipping. As each drug is developed, the number of patients available for that drug becomes smaller, for an already rare disorder. If each exon is considered a new drug requiring the full battery of toxicology and preclinical and clinical studies, then the time for development and costs represent a significant challenge. Some of the populations are so small that achieving statistical significance in a clinical trial will not be possible. Because some mutations will require simultaneous delivery of multiple drugs, as was the case with the dog model (Figure 4), the problem is compounded.

AO drugs in development for DMD have been granted Orphan Drug Designation by the Food and Drug Administration, which is designed to facilitate the development of these (and other) drug candidates.[63] This designation provides certain tax credit and marketing incentives to sponsors. Although Orphan Drug Designation does not change the requirements for drug approval, these drugs may also qualify for a 6-month priority review.[64] Although the challenges are significant (as previously described), at least two companies have launched clinical trials of AO products; these products will begin to define the regulatory path forward. Also, regulatory and scientific agencies, parent advocates, and academic researchers in the US and Europe are working to define the key issues and potential solutions in AO drug development for DMD.

One concept that has received some attention is based on an assumption that AOs of a given chemistry will have a common safety profile (preclinical and clinical) and that they will have a common pharmacokinetic profile. If this turns out to be the case, then cumulative data on the initial exon-specific drugs may allow a more streamline preclinical toxicology package. Also, if biomarkers, such as qualitative dystrophin expression, can be validated and correlated with clinical outcomes in initial trials, they could hypothetically be used in studies of later exon-specific drugs (particularly when a given mutation occurs in a few boys). After the first exon-specific drugs (eg, two drugs) are subjected to the standard battery of preclinical and clinical tests, using existing paradigms for drug approvals in rare life-threatening orphan diseases, subsequent exon-specific drugs (and perhaps multidrug combinations) would be approved, with a reduced battery of testing. This process reduces the cost and time to bring all exonic drugs to all patients with DMD. This concept is similar to the concept used in the annual release of the influenza vaccine. After approval of a given manufacturer's vaccine, in subsequent years, the seasonal vaccine (often with a composition that is different from that studied for initial approval) is released (approved) based on a smaller, but well-defined, set of parameters. Regardless of the pathway to approval, given that the number of boys with DMD available for study prelicensure will be limited, it is likely that postapproval studies and long-term follow-up of treated patients will be required.

Another issue in AO drug development for DMD is the selection of clinical trial end points based on an understanding of the natural history of DMD and (as previously discussed) standardized consensus methods for dystrophin protein measurement (biochemical outcome measures). The outcome measure that has previously been used for drug approval in other areas has been a 6-minute walk test. The TREAT-NMD European network has formed an international effort with the US Wellstone Center network to address clinical outcome measures in clinical trials, and publications are expected within the next year. One of the issues with the existing test is that it limits registration trials to ambulatory boys. Additional end points for boys in most need of treatment (nonambulatory) are needed, such that this group of patients can benefit from participation in clinical trials and so that nonambulatory boys will be included in the drug approval process.

Finally, approval of AO drugs for DMD will require refinements in production and potency. As previously mentioned, current estimations of the dose and regimen needed for treatment of a boy with DMD suggest that it may involve ≥10 i.v. injections per year, with a cumulative annual dose of >10 g of AO drug. If we assume that these doses will be tolerated, the current production costs of morpholino drugs are high and the GMP production capacity is limited. 2′O-methyl chemistries are more widely available and less expensive. For morpholinos, one approach to decrease the high cost of production of large amounts of drug is to increase potency so that less drug is needed per patient. Some promising approaches to increase potency have been reported in mouse models, in which the AOs are modified to more efficiently enter cells or by codelivery of small molecules or nanoparticles that enhance AO uptake or splicing efficiency.[65–69] However, these drugs show new toxicities relative to the naked unmodified morpholino backbone; and it may be challenging to achieve an appropriate therapeutic window, despite the higher potency.

Premature Stop Codon Read Through: Gentamicin and Ataluren (PTC124)

In approximately 10% to 15% of boys with DMD, the disease is caused by a point mutation that causes a change in a triplet codon, so that it no longer codes for an amino acid but instead codes for a stop signal (nonsense codons UAA, UAG, or UGA). Translation of the dystrophin protein is prematurely stopped, and the short fragment is nonfunctional and/or degraded. A promising therapy for nonsense mutation DMD is ataluren (PTC Therapeutics, South Plainfield, NJ), an orally delivered small molecule designed to selectively induce ribosomal read through of premature stop codons but not normal termination codons. Ataluren was developed after gentamicin, an aminoglycoside, promoted read through in mammalian models and in the *mdx* mouse model but presented lack of potency and potential toxicity and administration issues.[70] These proof-of-concept experiments led researchers to use high-throughput screening methods to identify compounds that suppressed the early, but not normal, termination codons; and did not present the potency, toxicity, and administration issues associated with gentamicin. In *mdx* mice and muscle cell cultures from patients, ataluren, a nonaminoglycoside, promoted dystrophin production in primary muscle cells in humans and in *mdx* mice expressing dystrophin nonsense alleles. In addition, ataluren restored striated muscle function in *mdx* mice within 2 to 8 weeks of drug exposure.[71]

PTC Therapeutics has completed phase 1 clinical trials with ataluren and is finishing data analysis of its phase 2 studies. In phase 1, ataluren, delivered as a single or multiple doses, was safe and well tolerated and supported the initiation of phase 2 trials. A total of 62 healthy adult male and female volunteers were treated in phase 1.[72] In phase 2, 38 patients with DMD were given ataluren at one of three dose levels for 28 days. The drug was safe and well tolerated, with infrequent adverse events. Plasma concentrations correlating to activity in preclinical models were found at the middle and high doses. In addition, patients receiving ataluren showed qualitative increases in muscle dystrophin expression and reductions in serum creatinine kinase levels. These patients are being followed up in an open-label long-term safety study. In April 2008, a phase 2b study was initiated; by February 2009, the study had full enrollment by 173 patients with nonsense mutation DMD at 37 sites in 11 countries. This randomized, double-blind, placebo-controlled study had three arms, with approximately 55 patients per arm: placebo, low dose (10 mg/kg), and high dose (20 mg/kg) (PTC Therapeutics, *http://www.parentprojectmd.org/site/DocServer/2010-04-16_Final_Summary_of_Ataluren_Data_at_AAN.pdf?docID=9461*, last accessed March 1, 2011). Inclusion criteria permitted both steroid- and non–steroid-treated patients, a broad age range, and patients showing both Duchenne and Becker phenotypes. As a result, there was considerable range in disease progression. Neither drug-treated arm reached significance for the primary clinical outcome measure (a 30-m increase in the 6-minute walk test), although the low-dose cohort showed a promising trend toward clinical improvement. Dystrophin data have not been reported, and there have been no formal announcements of if or how clinical testing will continue.

Ataluren is in clinical trials for three other genetic disorders: cystic fibrosis (phase 3), hemophilia A and B (phase 2), and methylmalonic acidemia (phase 2). However, no new trials are listed for DMD; and the future of the drug in patients with muscular dystrophy is uncertain.

Summary

Small-molecule drugs to coax dystrophin production from mutated genes in DMD have emerged as the most promising molecular therapeutics. Both exon skipping using AOs and stop-codon read through (PTC124) have entered clinical trials, and preliminary results are encouraging. Both approaches are mutation specific and can be thought of as personalized medicine. Should clinical efficacy be demonstrated for exon skipping, then it will be important to have an efficient path for approval of other exon-specific drugs in the same class (chemistry) to bring this to most patients with DMD.

References

1. Hoffman EP, Brown RH Jr, Kunkel LM: Dystrophin: the protein product of the Duchenne muscular dystrophy locus. Cell 1987, 51:919–928
2. Hoffman EP, Hudecki MS, Rosenberg PA, Pollina CM, Kunkel LM: Cell and fiber-type distribution of dystrophin. Neuron 1988, 1:411–420
3. Koenig M, Hoffman EP, Bertelson CJ, Monaco AP, Feener C, Kunkel LM: Complete cloning of the Duchenne muscular dystrophy (DMD) cDNA and preliminary genomic organization of the DMD gene in normal and affected individuals. Cell 1987, 50:509–517
4. Hoffman EP, Fischbeck KH, Brown RH, Johnson M, Medori R, Loike JD, Harris JB, Waterston R, Brooke M, Specht L, Kupsky W, Chamberlain J, Caskey CT, Shapiro F, Kunkel LM: Characterization of dystrophin in muscle-biopsy specimens from patients with Duchenne's or Becker's muscular dystrophy. N Engl J Med 1988, 318:1363–1368
5. Koppanati BM, Li J, Reay DP, Wang B, Daood M, Zheng H, Xiao X, Watchko JF, Clemens PR: Improvement of the mdx mouse dystrophic

phenotype by systemic in utero AAV8 delivery of a minidystrophin gene. Gene Ther 2010, 17:1355–1362

6. Kornegay JN, Li J, Bogan Jr, Bogan DJ, Chen C, Zheng H, Wang B, Qiao C, Howard JF Jr, Xiao X: Widespread muscle expression of an AAV9 human mini-dystrophin vector after intravenous injection in neonatal dystrophin-deficient dogs. Mol Ther 2010, 18:1501–1508
7. Tremblay JP, Skuk D, Palmieri B, Rothstein DM: A case for immunosuppression for myoblast transplantation in duchenne muscular dystrophy. Mol Ther 2009, 17:1122–1124
8. Torrente Y, Belicchi M, Marchesi C, Dantona G, Cogiamanian F, Pisati F, Gavina M, Giordano R, Tonlorenzi R, Fagiolari G, Lamperti C, Porretti L, Lopa R, Sampaolesi M, Vicentini L, Grimoldi N, Tiberio F, Songa V, Baratta P, Prelle A, Forzenigo L, Guglieri M, Pansarasa O, Rinaldi C, Mouly V, Butler-Browne GS, Comi GP, Biondetti P, Moggio M, Gaini SM, Stocchetti N, Priori A, D'Angelo MG, Turconi A, Bottinelli R, Cossu G, Rebulla P, Bresolin N: Autologous transplantation of muscle-derived CD133+ stem cells in Duchenne muscle patients. Cell Transplant 2007, 16:563–577
9. Skuk D, Goulet M, Roy B, Piette V, Côté CH, Chapdelaine P, Hogrel JY, Paradis M, Bouchard JP, Sylvain M, Lachance JG, Tremblay JP: First test of a "high-density injection" protocol for myogenic cell transplantation throughout large volumes of muscles in a Duchenne muscular dystrophy patient: eighteen months follow-up. Neuromuscul Disord 2007, 17:38–46
10. Kunkel LM, Hejtmancik JF, Caskey CT, Speer A, Monaco AP, Middlesworth W, et al: Analysis of deletions in DNA from patients with Becker and Duchenne muscular dystrophy. Nature 1986, 322:73–77
11. Monaco AP, Bertelson CJ, Liechti-Gallati S, Moser H, Kunkel LM: An explanation for the phenotypic differences between patients bearing partial deletions of the DMD locus. Genomics 1988, 2:90–95
12. Matsuo M, Masumura T, Nishio H, Nakajima T, Kitoh Y, Takumi T, Koga J, Nakamura H: Exon skipping during splicing of dystrophin mRNA precursor due to an intraexon deletion in the dystrophin gene of Duchenne muscular dystrophy kobe. J Clin Invest 1991, 87:2127–2131
13. Hagiwara Y, Nishio H, Kitoh Y, Takeshima Y, Narita N, Wada H, Yokoyama M, Nakamura H, Matsuo M: A novel point mutation (G-1 to T) in a 5′ splice donor site of intron 13 of the dystrophin gene results in exon skipping and is responsible for Becker muscular dystrophy. Am J Hum Genet 1994, 54:53–61
14. Ginjaar IB, Kneppers AL, v d Meulen JD, Anderson LV, Bremmer-Bout M, van Deutekom JC, Weegenaar J, den Dunnen JT, Bakker E: Dystrophin nonsense mutation induces different levels of exon 29 skipping and leads to variable phenotypes within one BMD family. Eur J Hum Genet 2000, 8:793–796
15. Hoffman EP, Morgan JE, Watkins SC, Partridge TA: Somatic reversion/suppression of the mouse mdx phenotype in vivo. J Neurol Sci 1990, 99:9–25
16. Lu QL, Morris GE, Wilton SD, Ly T, Artem'yeva OV, Strong P, Partridge TA: Massive idiosyncratic exon skipping corrects the nonsense mutation in dystrophic mouse muscle and produces functional revertant fibers by clonal expansion. J Cell Biol 2000, 148:985–996
17. Sherratt TG, Vulliamy T, Dubowitz V, Sewry CA, Strong PN: Exon skipping and translation in patients with frameshift deletions in the dystrophin gene. Am J Hum Genet 1993, 53:1007–1015
18. Hoffman EP: Skipping toward personalized molecular medicine. N Engl J Med 2007, 357:2719–2722
19. Dunckley MG, Manoharan M, Villiet P, Eperon IC, Dickson G: Modification of splicing in the dystrophin gene in cultured Mdx muscle cells by antisense oligoribonucleotides. Hum Mol Genet 1998, 7:1083–1090
20. Mann CJ, Honeyman K, Cheng AJ, Ly T, Lloyd F, Fletcher S, Morgan JE, Partridge TA, Wilton SD: Antisense-induced exon skipping and synthesis of dystrophin in the mdx mouse. Proc Natl Acad Sci U S A 2001, 98:42–47
21. van Deutekom JC, Bremmer-Bout M, Janson AA, Ginjaar IB, Baas F, den Dunnen JT, van Ommen GJ: Antisense-induced exon skipping restores dystrophin expression in DMD patient derived muscle cells. Hum Mol Genet 2001, 10:1547–1554
22. Zhao X, Pan F, Holt CM, Lewis AL, Lu JR: Controlled delivery of antisense oligonucleotides: a brief review of current strategies. Expert Opin Drug Deliv 2009, 6:673–686
23. Bennett CF, Swayze EE: RNA targeting therapeutics: molecular mechanisms of antisense oligonucleotides as a therapeutic platform. Annu Rev Pharmacol Toxicol 2010, 50:259–293
24. Juliano R, Bauman J, Kang H, Ming X: Biological barriers to therapy with antisense and siRNA oligonucleotides. Mol Pharm 2009, 6:686–695
25. Mokri B, Engel AG: Duchenne dystrophy: electron microscopic findings pointing to a basic or early abnormality in the plasma membrane of the muscle fiber. Neurology 1975, 25:1111–1120
26. Heemskerk H, de Winter C, van Kuik P, Heuvelmans N, Sabatelli P, Rimessi P, Braghetta P, van Ommen GJ, de Kimpe S, Ferlini A, Aartsma-Rus A, van Deutekom JC: Preclinical PK and PD studies on 2′-O-methyl-phosphorothioate RNA antisense oligonucleotides in the mdx mouse model. Mol Ther 2010, 18:1210–1217
27. Vollmer J, Jepsen JS, Uhlmann E, Schetter C, Jurk M, Wader T, Wüllner M, Krieg AM: Modulation of CpG oligodeoxynucleotide-mediated immune stimulation by locked nucleic acid (LNA). Oligonucleotides 2004, 14:23–31
28. Sheehan JP, Lan HC: Phosphorothioate oligonucleotides inhibit the intrinsic tenase complex. Blood 1998, 92:1617–1625
29. Henry SP, Giclas PC, Leeds J, Pangburn M, Auletta C, Levin AA, Kornbrust DJ: Activation of the alternative pathway of complement by a phosphorothioate oligonucleotide: potential mechanism of action. J Pharmacol Exp Ther 1997, 281:810–816
30. Chi KN, Siu LL, Hirte H, Hotte SJ, Knox J, Kollmansberger C, Gleave M, Guns E, Powers J, Walsh W, Tu D, Eisenhauer E: A phase I study of OGX-011, a 2′-methoxyethyl phosphorothioate antisense to clusterin, in combination with docetaxel in patients with advanced cancer. Clin Cancer Res 2008, 14:833–839
31. Hau P, Jachimczak P, Bogdahn U: Treatment of malignant gliomas with TGF-beta2 antisense oligonucleotides. Expert Rev Anticancer Ther 2009, 9:1663–1674
32. Fidias P, Pennell NA, Boral AL, Shapiro GI, Skarin AT, Eder JP Jr, Kwoh TJ, Geary RS, Johnson BE, Lynch TJ, Supko JG: Phase I study of the c-raf-1 antisense oligonucleotide ISIS 5132 in combination with carboplatin and paclitaxel in patients with previously untreated, advanced non-small cell lung cancer. J Thorac Oncol 2009, 4:1156–1162
33. Plummer R, Vidal L, Griffin M, Lesley M, de Bono J, Coulthard S, Sludden J, Siu LL, Chen EX, Oza AM, Reid GK, McLeod AR, Besterman JM, Lee C, Judson I, Calvert H, Boddy AV: Phase I study of MG98, an oligonucleotide antisense inhibitor of human DNA methyltransferase 1, given as a 7-day infusion in patients with advanced solid tumors. Clin Cancer Res 2009, 15:3177–3183
34. Geary RS: Antisense oligonucleotide pharmacokinetics and metabolism. Expert Opin Drug Metab Toxicol 2009, 5:381–391
35. Henry SP, Beattie G, Yeh G, Chappel A, Giclas P, Mortari A, Jagels MA, Kornbrust DJ, Levin AA: Complement activation is responsible for acute toxicities in rhesus monkeys treated with a phosphorothioate oligodeoxynucleotide. Int Immunopharmacol 2002, 2:1657–1666
36. Advani R, Lum BL, Fisher GA, Halsey J, Geary RS, Holmlund JT, Kwoh TJ, Dorr FA, Sikic BI: A phase I trial of aprinocarsen (ISIS 3521/LY900003), an antisense inhibitor of protein kinase C-alpha administered as a 24-hour weekly infusion schedule in patients with advanced cancer. Invest New Drugs 2005, 23:467–477
37. Monteith DK, Levin AA: Synthetic oligonucleotides: the development of antisense therapeutics. Toxicol Pathol 1999, 27:8–13
38. Sazani P, Weller DL, Shrewsbury SB: Safety pharmacology and genotoxicity evaluation of AVI-4658. Int J Toxicol 2010, 29:143–156
39. Summerton J, Weller D: Morpholino antisense oligomers: design, preparation, and properties. Antisense Nucleic Acid Drug Dev 1997, 7:187–195
40. Wu B, Moulton HM, Iversen PL, Jiang J, Li J, Li J, Spurney CF, Sali A, Guerron AD, Nagaraju K, Doran T, Lu P, Xiao X, Lu QL: Effective rescue of dystrophin improves cardiac function in dystrophin-deficient mice by a modified morpholino oligomer. Proc Natl Acad Sci U S A 2008, 105:14814–14819
41. Wu B, Li Y, Morcos PA, Doran TJ, Lu P, Lu QL: Octa-guanidine morpholino restores dystrophin expression in cardiac and skeletal muscles and ameliorates pathology in dystrophic mdx mice. Mol Ther 2009, 17:864–871
42. Yin H, Betts C, Saleh AF, Ivanova GD, Lee H, Seow Y, Kim D, Gait MJ, Wood MJ: Optimization of peptide nucleic acid antisense oligonucle-

otides for local and systemic dystrophin splice correction in the mdx mouse. Mol Ther 2010, 18:819–827

43. Yin H, Moulton HM, Betts C, Seow Y, Boutilier J, Iverson PL, Wood MJ: A fusion peptide directs enhanced systemic dystrophin exon skipping and functional restoration in dystrophin-deficient mdx mice. Hum Mol Genet 2009, 18:4405–4414
44. Goyenvalle A, Vulin A, Fougerousse F, Leturcq F, Kaplan JC, Garcia L, Danos O: Rescue of dystrophic muscle through U7 snRNA-mediated exon skipping. Science 2004, 306:1796–1799
45. Incitti T, De Angelis FG, Cazzella V, Sthandier O, PinnarÒ C, Legnini I, Bozzoni I: Exon skipping and duchenne muscular dystrophy therapy: selection of the most active U1 snRNA antisense able to induce dystrophin exon 51 skipping. Mol Ther 2010, 18:1675–1682
46. Goyenvalle A, Babbs A, van Ommen GJ, Garcia L, Davies KE: Enhanced exon-skipping induced by U7 snRNA carrying a splicing silencer sequence: promising tool for DMD therapy. Mol Ther 2009, 17:1234–1240
47. Lorain S, Gross DA, Goyenvalle A, Danos O, Davoust J, Garcia L: Transient immunomodulation allows repeated injections of AAV1 and correction of muscular dystrophy in multiple muscles. Mol Ther 2008, 16:541–547
48. Mann CJ, Honeyman K, McClorey G, Fletcher S, Wilton SD: Improved antisense oligonucleotide induced exon skipping in the mdx mouse model of muscular dystrophy. J Gene Med 2002, 4:644–654
49. Aartsma-Rus A, Bremmer-Bout M, Janson AA, den Dunnen JT, van Ommen GJ, van Deutekom JC: Targeted exon skipping as a potential gene correction therapy for Duchenne muscular dystrophy. Neuromuscul Disord 2002, 12(Suppl 1):S71–S77
50. Dickson G, Hill V, Graham IR: Screening for antisense modulation of dystrophin pre-mRNA splicing. Neuromuscul Disord 2002, 12(Suppl 1):S67–S70
51. Lu QL, Mann CJ, Lou F, Bou-Gharios G, Morris GE, Xue SA, Fletcher S, Partridge TA, Wilton SD: Functional amounts of dystrophin produced by skipping the mutated exon in the mdx dystrophic mouse. Nat Med 2003, 9:1009–1015
52. Lu QL, Rabinowitz A, Chen YC, Yokota T, Yin H, Alter J, Jadoon A, Bou-Gharios G, Parteridge T: Systemic delivery of antisense oligoribonucleotide restores dystrophin expression in body-wide skeletal muscles. Proc Natl Acad Sci U S A 2005, 102:198–203
53. Wu B, Lu P, Benrashid E, Malik S, Ashar J, Doran TJ, Lu QL: Dose-dependent restoration of dystrophin expression in cardiac muscle of dystrophic mice by systemically delivered morpholino. Gene Ther 2010, 17:132–140
54. Cooper BJ, Winand NJ, Stedman H, Valentine BA, Hoffman EP, Kunkel LM, Scott MO, Fischbeck KH, Kornegay JN, Avery RJ, Williams JR, Schmickel RD, Sylvester JE: The homologue of the Duchenne locus is defective in X-linked muscular dystrophy of dogs. Nature 1988, 334:154–156
55. Sharp NJ, Kornegay JN, Van Camp SD, Herbstreith MH, Secore SL, Kettle S, Hung WY, Constantinou CD, Dykstra MJ, Roses AD, et al: An error in dystrophin mRNA processing in golden retriever muscular dystrophy, an animal homologue of Duchenne muscular dystrophy. Genomics 1992, 13:115–121
56. Yokota T, Lu QL, Partridge T, Kobayashi M, Nakamura A, Takeda S, Hoffman E: Efficacy of systemic morpholino exon-skipping in Duchenne dystrophy dogs. Ann Neurol 2009, 65:667–676
57. van Deutekom JC, Janson AA, Ginjaar IB, Frankhuizen WS, Aartsma-Rus A, Bremmer-Bout M, den Dunnen JT, Koop K, van der Kooi AJ, Goemans NM, de Kimpe SJ, Ekhart PF, Venneker EH, Platenburg GJ, Verschuuren JJ, van Ommen GJ: Local dystrophin restoration with antisense oligonucleotide PRO051. N Engl J Med 2007, 357: 2677–2686
58. Kinali M, Arechavala-Gomeza V, Feng L, Cirak S, Hunt D, Adkin C, Guglieri M, Ashton E, Abbs S, Nihoyannopoulos P, Garralda ME, Rutherford M, McCulley C, Popplewell L, Graham IR, Dickson G, Wood MJ, Wells DJ, Wilton SD, Kole R, Straub V, Bushby K, Sewry C, Morgan JE, Muntoni F: Local restoration of dystrophin expression with the morpholino oligomer AVI-4658 in Duchenne muscular dystrophy: a single-blind, placebo-controlled, dose-escalation, proof-of-concept study. Lancet Neurol 2009, 8:918–928
59. Hoffman EP, Kunkel LM, Angelini C, Clarke A, Johnson M, Harris JB: Improved diagnosis of Becker muscular dystrophy by dystrophin testing. Neurology 1989, 39:1011–1017
60. Hoffman EP, Arahata K, Minetti C, Bonilla E, Rowland LP: Dystrophinopathy in isolated cases of myopathy in females. Neurology 1992, 42:967–975
61. Goemans NM, Tulinius M, van den Akker JT, Burm BE, Ekhart PF, Heuvelmans N, Holling T, Janson AA, Platenburg GJ, Sipkens JA, Sitsen JM, Aartsma-Rus A, van Ommen GJ, Buyse G, Darin N, Verschuuren JJ, Campion GV, de Kimpe SJ, van Deutekom JC: Systemic administration of PRO051 in Duchenne's muscular dystrophy. N Engl J Med 2011, 364:1513–1522
62. Kesari A, Pirra LN, Bremadesam L, McIntyre O, Gordon E, Dubrovsky AL, Viswanathan V, Hoffman EP: Integrated DNA, cDNA, and protein studies in Becker muscular dystrophy show high exception to the reading frame rule. Hum Mutat 2008, 29:728–737
63. Braun MM, Farag-El-Massah S, Xu K, Coté TR: Emergence of orphan drugs in the United States: a quantitative assessment of the first 25 years. Nat Rev Drug Discov 2010, 9:519–522
64. McNeil DE, Davis C, Jillapalli D, Targum S, Durmowicz A, Coté TR: Duchenne muscular dystrophy: drug development and regulatory considerations. Muscle Nerve 2010, 41:740–745
65. Hu Y, Wu B, Zillmer A, Lu P, Benrashid E, Wang M, Doran T, Shaban M, Wu X, Lu QL: Guanine analogues enhance antisense oligonucleotide-induced exon skipping in dystrophin gene in vitro and in vivo. Mol Ther 2010, 18:812–818
66. O'Leary DA, Sharif O, Anderson P, Tu B, Welch G, Zhou Y, Caldwell JS, Engels IH, Brinker A: Identification of small molecule and genetic modulators of AON-induced dystrophin exon skipping by high-throughput screening. PLoS One 2009, 4:e8348
67. Ferlini A, Sabatelli P, Fabris M, Bassi E, Falzarano S, Vattemi G, Perrone D, Gualandi F, Maraldi NM, Merlini L, Sparnacci K, Laus M, Caputo A, Bonaldo P, Braghetta P, Rimessi P: Dystrophin restoration in skeletal, heart and skin arrector pili smooth muscle of mdx mice by ZM2 NP-AON complexes. Gene Ther 2010, 17:432–438
68. Goyenvalle A, Babbs A, van Ommen GJ, Garcia L, Davies KE: Enhanced exon-skipping induced by U7 snRNA carrying a splicing silencer sequence: promising tool for DMD therapy. Mol Ther 2009, 17:1234–1240
69. Rimessi P, Sabatelli P, Fabris M, Braghetta P, Bassi E, Spitali P, Vattemi G, Tomelleri G, Mari L, Perrone D, Medici A, Neri M, Bovolenta M, Martoni E, Maraldi NM, Gualandi F, Merlini L, Ballestri M, Tondelli L, Sparnacci K, Bonaldo P, Caputo A, Laus M, Ferlini A: Cationic PMMA nanoparticles bind and deliver antisense oligoribonucleotides allowing restoration of dystrophin expression in the mdx mouse. Mol Ther 2009, 17:820–827
70. Malik V, Rodino-Klapac LR, Viollet L, Wall C, King W, Al-Dahhak R, Lewis S, Shilling CJ, Kota J, Serrano-Munuera C, Hayes J, Mahan JD, Campbell KJ, Banwell B, Dasouki M, Watts V, Sivakumar K, Bien-Willner R, Flanigan KM, Sahenk Z, Barohn RJ, Walker CM, Mendell JR: Gentamicin-induced readthrough of stop codons in Duchenne muscular dystrophy. Ann Neurol 2010, 67:771–780
71. Welch EM, Barton ER, Zhuo J, Tomizawa Y, Friesen WJ, Trifillis P, et al: PTC124 targets genetic disorders caused by nonsense mutations. Nature 2007, 447:87–91
72. Hirawat S, Welch EM, Elfring GL, Northcutt VJ, Paushkin S, Hwang S, Leonard EM, Almstead NG, Ju W, Peltz SW, Miller LL: Safety, tolerability, and pharmacokinetics of PTC124, a nonaminoglycoside nonsense mutation suppressor, following single- and multiple-dose administration to healthy male and female adult volunteers. J Clin Pharmacol 2007, 47:430–444

Current Status of Pharmaceutical and Genetic Therapeutic Approaches to Treat DMD

Christophe Pichavant[1], Annemieke Aartsma-Rus[2], Paula R Clemens[3], Kay E Davies[4], George Dickson[5], Shin'ichi Takeda[6], Steve D Wilton[7], Jon A Wolff[8], Christine I Wooddell[8], Xiao Xiao[9] and Jacques P Tremblay[1]

[1]*Department of Neurosciences, CHUL Research Center, Quebec City, Quebec, Canada;* [2]*Department of Human Genetics, Leiden University Medical Center, Leiden, The Netherlands;* [3]*Department of Neurology, University of Pittsburgh, Pittsburgh, Pennsylvania, USA;* [4]*MRC Functional Genomics Unit, Department of Physiology, Anatomy and Genetics, University of Oxford, Oxford, UK;* [5]*School of Biological Sciences, Royal Holloway-University of London, Egham, UK;* [6]*Department of Molecular Therapy, National Institute of Neuroscience, National Center of Neurology and Psychiatry (NCNP), Tokyo, Japan;* [7]*Centre for Neuromuscular and Neurological Disorders, University of Western Australia, Perth, Western Australia, Australia;* [8]*Roche Madison, Madison, Wisconsin, USA;* [9]*University of North Carolina School of Medicine, Division of Molecular Pharmaceutics, Chapel Hill, North Carolina, USA*

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.

Received 22 December 2010; accepted 3 March 2011; published online 5 April 2011. doi:10.1038/mt.2011.59

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 427kDa[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

---

Correspondence: *Jacques P Tremblay, Unité de Neurosciences (RC 9300), Centre de recherche du CHUL, 2705 boulevard Laurier, Québec, QC, Canada, G1V 4G2. E-mail: Jacques-P.Tremblay@crchul.ulaval.ca*

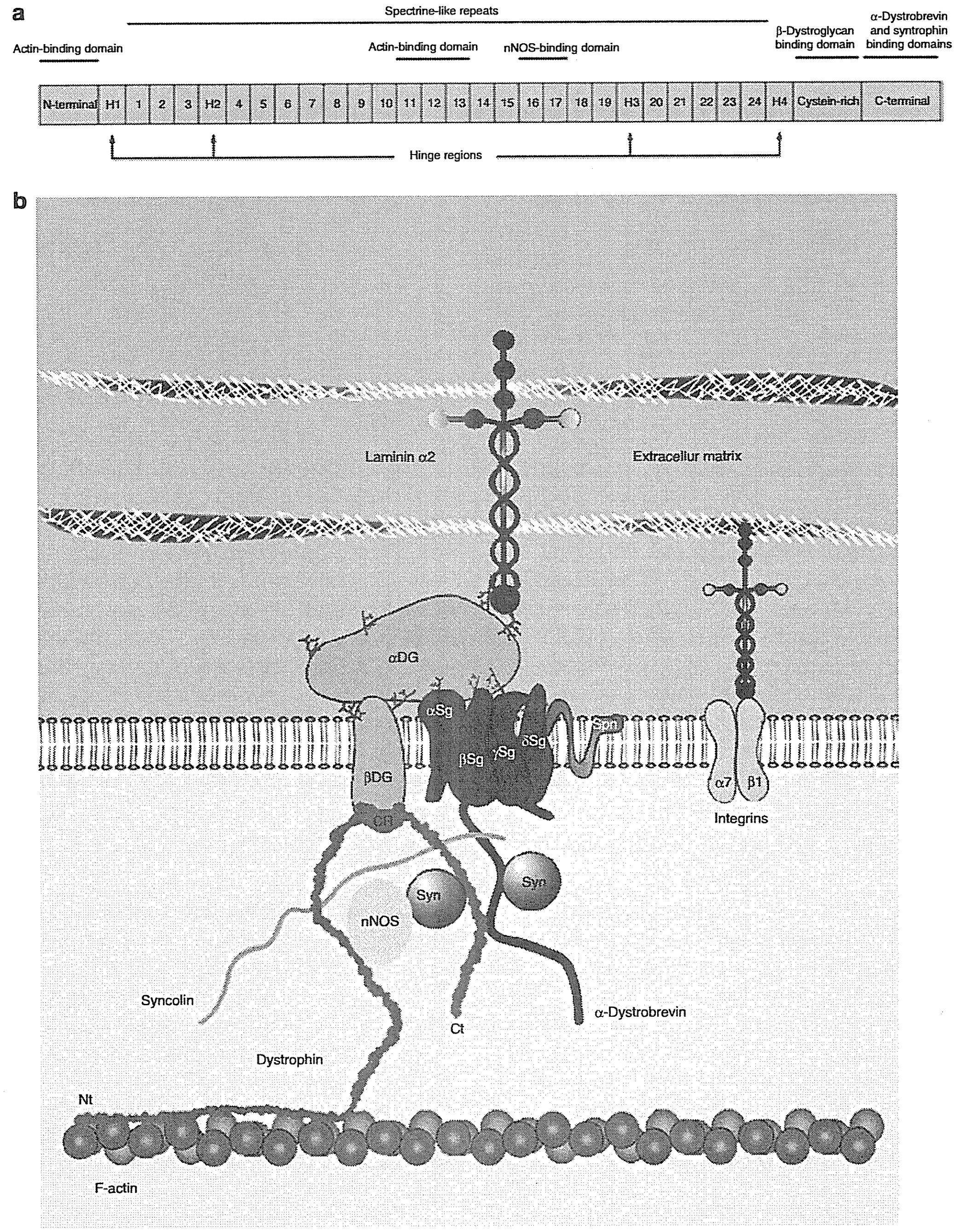

Figure 1 The dystrophin protein. **(a)** Schema representing the four main domains of dystrophin: the N-terminal part, central rod domain (containing 24 spectrin-like repeats and four hinge domains), cystein-rich region and the C-terminal part. The protein binding domains are also indicated. **(b)** 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

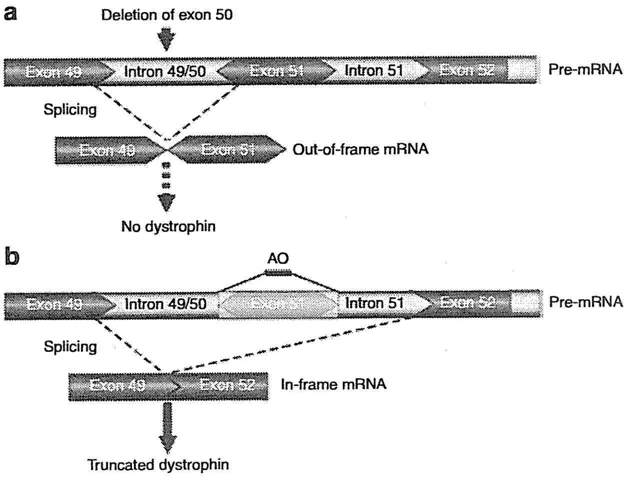

Figure 2 Example of exon skipping in Duchenne muscular dystrophy (DMD) patient who has a deletion of exon 50. (a) The absence of exon 50 in the dystrophin gene leads to an out-of-frame mRNA creating a premature stop codon in exon 51, thus aborting dystrophin synthesis during translation. (b) Using an 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

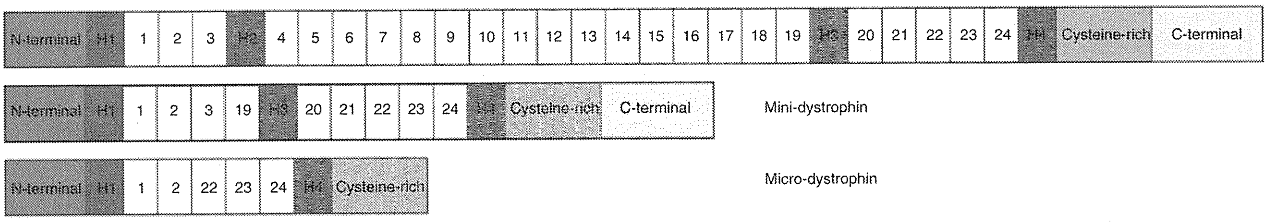

Figure 3 Dystrophin versions. The full-length dystrophin cDNA (11 kb) is represented at the top. The middle schema represents an example of a mini-dystrophin cDNA with an H2-R18 deletion; the approximate size of mini-dystrophins is about 6 kb. The bottom representation is a schema of a micro-dystrophin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

ACKNOWLEDGMENTS

We thank Jerry R Mendell for his helpful suggestions. J.P.T. is the president and hold shares in CellGene, a biotech company involved in the development of cell and gene therapies.

REFERENCES

1. Zatz, M, Vianna-Morgante, AM, Campos, P and Diament, AJ (1981). Translocation (X;6) in a female with Duchenne muscular dystrophy: implications for the localisation of the DMD locus. *J Med Genet* **18**: 442–447.
2. Davies, KE, Pearson, PL, Harper, PS, Murray, JM, O'Brien, T, Sarfarazi, M *et al.* (1983). Linkage analysis of two cloned DNA sequences flanking the Duchenne muscular dystrophy locus on the short arm of the human X chromosome. *Nucleic Acids Res* **11**: 2303–2312.
3. Monaco, AP, Neve, RL, Colletti-Feener, C, Bertelson, CJ, Kurnit, DM and Kunkel, LM (1986). Isolation of candidate cDNAs for portions of the Duchenne muscular dystrophy gene. *Nature* **323**: 646–650.
4. Koenig, M, Hoffman, EP, Bertelson, CJ, Monaco, AP, Feener, C and Kunkel, LM (1987). Complete cloning of the Duchenne muscular dystrophy (DMD) cDNA and preliminary genomic organization of the DMD gene in normal and affected individuals. *Cell* **50**: 509–517.
5. Koenig, M, Monaco, AP and Kunkel, LM (1988). The complete sequence of dystrophin predicts a rod-shaped cytoskeletal protein. *Cell* **53**: 219–228.
6. Zubrzycka-Gaarn, EE, Bulman, DE, Karpati, G, Burghes, AH, Belfall, B, Klamut, HJ *et al.* (1988). The Duchenne muscular dystrophy gene product is localized in sarcolemma of human skeletal muscle. *Nature* **333**: 466–469.
7. Levine, BA, Moir, AJ, Patchell, VB and Perry, SV (1990). The interaction of actin with dystrophin. *FEBS Lett* **263**: 159–162.
8. Amann, KJ, Renley, BA and Ervasti, JM (1998). A cluster of basic repeats in the dystrophin rod domain binds F-actin through an electrostatic interaction. *J Biol Chem* **273**: 28419–28423.
9. Lai, Y, Thomas, GD, Yue, Y, Yang, HT, Li, D, Long, C *et al.* (2009). Dystrophins carrying spectrin-like repeats 16 and 17 anchor nNOS to the sarcolemma and enhance exercise performance in a mouse model of muscular dystrophy. *J Clin Invest* **119**: 624–635.
10. Stamler, JS and Meissner, G (2001). Physiology of nitric oxide in skeletal muscle. *Physiol Rev* **81**: 209–237.
11. Koenig, M and Kunkel, LM (1990). Detailed analysis of the repeat domain of dystrophin reveals four potential hinge segments that may confer flexibility. *J Biol Chem* **265**: 4560–4566.
12. Ishikawa-Sakurai, M, Yoshida, M, Imamura, M, Davies, KE and Ozawa, E (2004). ZZ domain is essentially required for the physiological binding of dystrophin and utrophin to β-dystroglycan. *Hum Mol Genet* **13**: 693–702.
13. Adams, ME, Butler, MH, Dwyer, TM, Peters, MF, Murnane, AA and Froehner, SC (1993). Two forms of mouse syntrophin, a 58 kd dystrophin-associated protein, differ in primary structure and tissue distribution. *Neuron* **11**: 531–540.
14. Ahn, AH and Kunkel, LM (1995). Syntrophin binds to an alternatively spliced exon of dystrophin. *J Cell Biol* **128**: 363–371.
15. Adams, ME, Mueller, HA and Froehner, SC (2001). *In vivo* requirement of the alpha-syntrophin PDZ domain for the sarcolemmal localization of nNOS and aquaporin-4. *J Cell Biol* **155**: 113–122.
16. Ervasti, JM and Campbell, KP (1991). Membrane organization of the dystrophin-glycoprotein complex. *Cell* **66**: 1121–1131.
17. Ervasti, JM (2007). Dystrophin, its interactions with other proteins, and implications for muscular dystrophy. *Biochim Biophys Acta* **1772**: 108–117.
18. Pilgram, GS, Potikanond, S, Baines, RA, Fradkin, LG and Noordermeer, JN (2010). The roles of the dystrophin-associated glycoprotein complex at the synapse. *Mol Neurobiol* **41**: 1–21.
19. Odom, GL, Gregorevic, P and Chamberlain, JS (2007). Viral-mediated gene therapy for the muscular dystrophies: successes, limitations and recent advances. *Biochim Biophys Acta* **1772**: 243–262.
20. Campbell, KP and Kahl, SD (1989). Association of dystrophin and an integral membrane glycoprotein. *Nature* **338**: 259–262.
21. Aartsma-Rus, A, Van Deutekom, JC, Fokkema, IF, Van Ommen, GJ and Den Dunnen, JT (2006). Entries in the Leiden Duchenne muscular dystrophy mutation database: an overview of mutation types and paradoxical cases that confirm the reading-frame rule. *Muscle Nerve* **34**: 135–144.
22. Scheuerbrandt, G (2006). Approaching therapies for boys with Duchenne muscular dystrophy. Parent Project Muscular Dystrophy. Annual conference in Cincinnati/Ohio, 13-16 July 2006. *Acta Myol* **25**: 77–97.
23. Cunniff, C, Andrews, J, Meaney, FJ, Mathews, KD, Matthews, D, Ciafaloni, E *et al.* (2009). Mutation analysis in a population-based cohort of boys with Duchenne or Becker muscular dystrophy. *J Child Neurol* **24**: 425–430.
24. Flanigan, KM, Dunn, DM, von Niederhausern, A, Soltanzadeh, P, Gappmaier, E, Howard, MT *et al.*; United Dystrophinopathy Project Consortium. (2009). Mutational spectrum of DMD mutations in dystrophinopathy patients: application of modern diagnostic techniques to a large cohort. *Hum Mutat* **30**: 1657–1666.
25. Aartsma-Rus, A, Fokkema, I, Verschuuren, J, Ginjaar, I, van Deutekom, J, van Ommen, GJ *et al.* (2009). Theoretic applicability of antisense-mediated exon skipping for Duchenne muscular dystrophy mutations. *Hum Mutat* **30**: 293–299.
26. Mendell, JR, Buzin, CH, Feng, J, Yan, J, Serrano, C, Sangani, DS *et al.* (2001). Diagnosis of Duchenne dystrophy by enhanced detection of small mutations. *Neurology* **57**: 645–650.
27. Monaco, AP, Bertelson, CJ, Liechti-Gallati, S, Moser, H and Kunkel, LM (1988). An explanation for the phenotypic differences between patients bearing partial deletions of the DMD locus. *Genomics* **2**: 90–95.
28. Becker, PE and Kiener, F (1955). [A new x-chromosomal muscular dystrophy]. *Arch Psychiatr Nervenkr Z Gesamte Neurol Psychiatr* **193**: 427–448.
29. Malhotra, SB, Hart, KA, Klamut, HJ, Thomas, NS, Bodrug, SE, Burghes, AH *et al.* (1988). Frame-shift deletions in patients with Duchenne and Becker muscular dystrophy. *Science* **242**: 755–759.
30. England, SB, Nicholson, LV, Johnson, MA, Forrest, SM, Love, DR, Zubrzycka-Gaarn, EE *et al.* (1990). Very mild muscular dystrophy associated with the deletion of 46% of dystrophin. *Nature* **343**: 180–182.
31. Beggs, AH, Hoffman, EP, Snyder, JR, Arahata, K, Specht, L, Shapiro, F *et al.* (1991). Exploring the molecular basis for variability among patients with Becker muscular dystrophy: dystrophin gene and protein studies. *Am J Hum Genet* **49**: 54–67.
32. Bushby, K, Finkel, R, Birnkrant, DJ, Case, LE, Clemens, PR, Cripe, L *et al.*; DMD Care Considerations Working Group. (2010). Diagnosis and management of Duchenne muscular dystrophy, part 1: diagnosis, and pharmacological and psychosocial management. *Lancet Neurol* **9**: 77–93.
33. Bushby, K, Finkel, R, Birnkrant, DJ, Case, LE, Clemens, PR, Cripe, L *et al.*; DMD Care Considerations Working Group. (2010). Diagnosis and management of Duchenne muscular dystrophy, part 2: implementation of multidisciplinary care. *Lancet Neurol* **9**: 177–189.

34. Mendell, JR, Moxley, RT, Griggs, RC, Brooke, MH, Fenichel, GM, Miller, JP *et al.* (1989). Randomized, double-blind six-month trial of prednisone in Duchenne's muscular dystrophy. *N Engl J Med* **320**: 1592–1597.
35. Biggar, WD, Gingras, M, Fehlings, DL, Harris, VA and Steele, CA (2001). Deflazacort treatment of Duchenne muscular dystrophy. *J Pediatr* **138**: 45–50.
36. Angelini, C (2007). The role of corticosteroids in muscular dystrophy: a critical appraisal. *Muscle Nerve* **36**: 424–435.
37. Manzur, AY, Kuntzer, T, Pike, M and Swan, A (2008). Glucocorticoid corticosteroids for Duchenne muscular dystrophy. *Cochrane Database Syst Rev*, **1**: CD003725.
38. King, WM, Ruttencutter, R, Nagaraja, HN, Matkovic, V, Landoll, J, Hoyle, C *et al.* (2007). Orthopedic outcomes of long-term daily corticosteroid treatment in Duchenne muscular dystrophy. *Neurology* **68**: 1607–1613.
39. Singh, A, Ursic, D and Davies, J (1979). Phenotypic suppression and misreading *Saccharomyces cerevisiae*. *Nature* **277**: 146–148.
40. Palmer, E, Wilhelm, JM and Sherman, F (1979). Phenotypic suppression of nonsense mutants in yeast by aminoglycoside antibiotics. *Nature* **277**: 148–150.
41. Yoshizawa, S, Fourmy, D and Puglisi, JD (1998). Structural origins of gentamicin antibiotic action. *EMBO J* **17**: 6437–6448.
42. Kaufman, RJ (1999). Correction of genetic disease by making sense from nonsense. *J Clin Invest* **104**: 367–368.
43. Aurino, S and Nigro, V (2006). Readthrough strategies for stop codons in Duchenne muscular dystrophy. *Acta Myol* **25**: 5–12.
44. Manuvakhova, M, Keeling, K and Bedwell, DM (2000). Aminoglycoside antibiotics mediate context-dependent suppression of termination codons in a mammalian translation system. *RNA* **6**: 1044–1055.
45. Barton-Davis, ER, Cordier, L, Shoturma, DI, Leland, SE and Sweeney, HL (1999). Aminoglycoside antibiotics restore dystrophin function to skeletal muscles of *mdx* mice. *J Clin Invest* **104**: 375–381.
46. Wagner, KR, Hamed, S, Hadley, DW, Gropman, AL, Burstein, AH, Escolar, DM *et al.* (2001). Gentamicin treatment of Duchenne and Becker muscular dystrophy due to nonsense mutations. *Ann Neurol* **49**: 706–711.
47. Politano, L, Nigro, G, Nigro, V, Piluso, G, Papparella, S, Paciello, O *et al.* (2003). Gentamicin administration in Duchenne patients with premature stop codon. Preliminary results. *Acta Myol* **22**: 15–21.
48. Dunant, P, Walter, MC, Karpati, G and Lochmüller, H (2003). Gentamicin fails to increase dystrophin expression in dystrophin-deficient muscle. *Muscle Nerve* **27**: 624–627.
49. Arakawa, M, Shiozuka, M, Nakayama, Y, Hara, T, Hamada, M, Kondo, S *et al.* (2003). Negamycin restores dystrophin expression in skeletal and cardiac muscles of *mdx* mice. *J Biochem* **134**: 751–758.
50. Malik, V, Rodino-Klapac, LR, Viollet, L, Wall, C, King, W, Al-Dahhak, R *et al.* (2010). Gentamicin-induced readthrough of stop codons in Duchenne muscular dystrophy. *Ann Neurol* **67**: 771–780.
51. PTC Therapeutics. Ataluren for genetic disorders. <http://www.ptcbio.com/3.1.1_genetic_disorders.aspx>.
52. Welch, EM, Barton, ER, Zhuo, J, Tomizawa, Y, Friesen, WJ, Trifillis, P *et al.* (2007). PTC124 targets genetic disorders caused by nonsense mutations. *Nature* **447**: 87–91.
53. Clinical Trials. <http://www.clinicaltrials.gov/ct2/results?term=ptc124>.
54. PTC Therapeutics (2010). Pivotal data presented at the world muscle society congress suggest ataluren slows the loss of walking ability in patients with nonsense mutation Duchenne/Becker muscular dystrophy. <http://ptct.client.shareholder.com/releasedetail.cfm?ReleaseID=518941>.
55. Dias, N and Stein, CA (2002). Antisense oligonucleotides: basic concepts and mechanisms. *Mol Cancer Ther* **1**: 347–355.
56. Stein, CA and Cheng, YC (1993). Antisense oligonucleotides as therapeutic agents–is the bullet really magical? *Science* **261**: 1004–1012.
57. Monia, BP, Lesnik, EA, Gonzalez, C, Lima, WF, McGee, D, Guinosso, CJ *et al.* (1993). Evaluation of 2′-modified oligonucleotides containing 2′-deoxy gaps as antisense inhibitors of gene expression. *J Biol Chem* **268**: 14514–14522.
58. Baker, BF, Lot, SS, Condon, TP, Cheng-Flournoy, S, Lesnik, EA, Sasmor, HM *et al.* (1997). 2′-O-(2-Methoxy)ethyl-modified anti-intercellular adhesion molecule 1 (ICAM-1) oligonucleotides selectively increase the ICAM-1 mRNA level and inhibit formation of the ICAM-1 translation initiation complex in human umbilical vein endothelial cells. *J Biol Chem* **272**: 11994–12000.
59. van Deutekom, JC, Bremmer-Bout, M, Janson, AA, Ginjaar, IB, Baas, F, den Dunnen, JT *et al.* (2001). Antisense-induced exon skipping restores dystrophin expression in DMD patient derived muscle cells. *Hum Mol Genet* **10**: 1547–1554.
60. Aartsma-Rus, A, Janson, AA, Kaman, WE, Bremmer-Bout, M, den Dunnen, JT, Baas, F *et al.* (2003). Therapeutic antisense-induced exon skipping in cultured muscle cells from six different DMD patients. *Hum Mol Genet* **12**: 907–914.
61. Lu, QL, Mann, CJ, Lou, F, Bou-Gharios, G, Morris, GE, Xue, SA *et al.* (2003). Functional amounts of dystrophin produced by skipping the mutated exon in the *mdx* dystrophic mouse. *Nat Med* **9**: 1009–1014.
62. Lu, QL, Rabinowitz, A, Chen, YC, Yokota, T, Yin, H, Alter, J *et al.* (2005). Systemic delivery of antisense oligoribonucleotide restores dystrophin expression in body-wide skeletal muscles. *Proc Natl Acad Sci USA* **102**: 198–203.
63. Heemskerk, HA, de Winter, CL, de Kimpe, SJ, van Kuik-Romeijn, P, Heuvelmans, N, Platenburg, GJ *et al.* (2009). *In vivo* comparison of 2′-O-methyl phosphorothioate and morpholino antisense oligonucleotides for Duchenne muscular dystrophy exon skipping. *J Gene Med* **11**: 257–266.
64. Heemskerk, H, de Winter, C, van Kuik, P, Heuvelmans, N, Sabatelli, P, Rimessi, P *et al.* (2010). Preclinical PK and PD studies on 2′-O-methyl-phosphorothioate RNA antisense oligonucleotides in the *mdx* mouse model. *Mol Ther* **18**: 1210–1217.
65. van Deutekom, JC, Janson, AA, Ginjaar, IB, Frankhuizen, WS, Aartsma-Rus, A, Bremmer-Bout, M *et al.* (2007). Local dystrophin restoration with antisense oligonucleotide PRO051. *N Engl J Med* **357**: 2677–2686.
66. Prosensa. Developing therapies for neuromuscular diseases. <http://prosensa.eu/technology-and-products/pipeline/pro051gsk2402968>.
67. Prosensa. GSK and Prosensa announce start of phase III study of investigational Duchenne muscular dystrophy medication.(2011) <http://www.prosensa.eu/press-release/gsk-and-prosensa-announce-start-phase-iii-study-investigational-dmd-medication>.
68. Hudziak, RM, Barofsky, E, Barofsky, DF, Weller, DL, Huang, SB and Weller, DD (1996). Resistance of morpholino phosphorodiamidate oligomers to enzymatic degradation. *Antisense Nucleic Acid Drug Dev* **6**: 267–272.
69. Partridge, M, Vincent, A, Matthews, P, Puma, J, Stein, D and Summerton, J (1996). A simple method for delivering morpholino antisense oligos into the cytoplasm of cells. *Antisense Nucleic Acid Drug Dev* **6**: 169–175.
70. Gebski, BL, Mann, CJ, Fletcher, S and Wilton, SD (2003). Morpholino antisense oligonucleotide induced dystrophin exon 23 skipping in *mdx* mouse muscle. *Hum Mol Genet* **12**: 1801–1811.
71. Alter, J, Lou, F, Rabinowitz, A, Yin, H, Rosenfeld, J, Wilton, SD *et al.* (2006). Systemic delivery of morpholino oligonucleotide restores dystrophin expression bodywide and improves dystrophic pathology. *Nat Med* **12**: 175–177.
72. Wu, B, Lu, P, Benrashid, E, Malik, S, Ashar, J, Doran, TJ *et al.* (2010). Dose-dependent restoration of dystrophin expression in cardiac muscle of dystrophic mice by systemically delivered morpholino. *Gene Ther* **17**: 132–140.
73. Malerba, A, Sharp, PS, Graham, IR, Arechavala-Gomeza, V, Foster, K, Muntoni, F *et al.* (2011). Chronic systemic therapy with low-dose morpholino oligomers ameliorates the pathology and normalizes locomotor behavior in *mdx* mice. *Mol Ther* **19**: 345–354.
74. Yokota, T, Lu, QL, Partridge, T, Kobayashi, M, Nakamura, A, Takeda, S *et al.* (2009). Efficacy of systemic morpholino exon-skipping in Duchenne dystrophy dogs. *Ann Neurol* **65**: 667–676.
75. Wu, B, Li, Y, Morcos, PA, Doran, TJ, Lu, P and Lu, QL (2009). Octa-guanidine morpholino restores dystrophin expression in cardiac and skeletal muscles and ameliorates pathology in dystrophic *mdx* mice. *Mol Ther* **17**: 864–871.
76. Moulton, HM, Nelson, MH, Hatlevig, SA, Reddy, MT and Iversen, PL (2004). Cellular uptake of antisense morpholino oligomers conjugated to arginine-rich peptides. *Bioconjug Chem* **15**: 290–299.
77. Jearawiriyapaisarn, N, Moulton, HM, Buckley, B, Roberts, J, Sazani, P, Fucharoen, S *et al.* (2008). Sustained dystrophin expression induced by peptide-conjugated morpholino oligomers in the muscles of *mdx* mice. *Mol Ther* **16**: 1624–1629.
78. Wu, B, Moulton, HM, Iversen, PL, Jiang, J, Li, J, Li, J *et al.* (2008). Effective rescue of dystrophin improves cardiac function in dystrophin-deficient mice by a modified morpholino oligomer. *Proc Natl Acad Sci USA* **105**: 14814–14819.
79. Goyenvalle, A, Babbs, A, Powell, D, Kole, R, Fletcher, S, Wilton, SD *et al.* (2010). Prevention of dystrophic pathology in severely affected dystrophin/utrophin-deficient mice by morpholino-oligomer-mediated exon-skipping. *Mol Ther* **18**: 198–205.
80. Yin, H, Moulton, HM, Betts, C, Merritt, T, Seow, Y, Ashraf, S *et al.* (2010). Functional rescue of dystrophin-deficient *mdx* mice by a chimeric peptide-PMO. *Mol Ther* **18**: 1822–1829.
81. Moulton, HM and Moulton, JD (2010). Morpholinos and their peptide conjugates: therapeutic promise and challenge for Duchenne muscular dystrophy. *Biochim Biophys Acta* **1798**: 2296–2303.
82. Aoki, Y, Nakamura, A, Yokota, T, Saito, T, Okazawa, H, Nagata, T *et al.* (2010). In-frame dystrophin following exon 51-skipping improves muscle pathology and function in the exon 52-deficient *mdx* mouse. *Mol Ther* **18**: 1995–2005.
83. Fletcher, S, Adams, AM, Johnsen, RD, Greer, K, Moulton, HM and Wilton, SD (2010). Dystrophin isoform induction *in vivo* by antisense-mediated alternative splicing. *Mol Ther* **18**: 1218–1223.
84. Kinali, M, Arechavala-Gomeza, V, Feng, L, Cirak, S, Hunt, D, Adkin, C *et al.* (2009). Local restoration of dystrophin expression with the morpholino oligomer AVI-4658 in Duchenne muscular dystrophy: a single-blind, placebo-controlled, dose-escalation, proof-of-concept study. *Lancet Neurol* **8**: 918–928.
85. AVI-4658 Data From Phase 1b/2 Study Presented at 15th International Congress of the World Muscle Society Supports Potential as Disease Modifying Therapy. AVI BioPharma's investigational drug candidate avi-4658 demonstrates broadly favorable profile of safety and tolerability, new dystrophin expression, stable clinical performance and inflammatory modulation in the treatment of Duchenne muscular dystrophy. <http://investorrelations.avibio.com/phoenix.zhtml?c=64231&p=irol-newsArticle&ID=1483148&highlight=>.
86. Chapdelaine, P, Pichavant, C, Rousseau, J, Pâques, F and Tremblay, JP (2010). Meganucleases can restore the reading frame of a mutated dystrophin. *Gene Ther* **17**: 846–858.
87. McPherron, AC, Lawler, AM and Lee, SJ (1997). Regulation of skeletal muscle mass in mice by a new TGF-β superfamily member. *Nature* **387**: 83–90.
88. Bogdanovich, S, Krag, TO, Barton, ER, Morris, LD, Whittemore, LA, Ahima, RS *et al.* (2002). Functional improvement of dystrophic muscle by myostatin blockade. *Nature* **420**: 418–421.
89. Gamer, LW, Wolfman, NM, Celeste, AJ, Hattersley, G, Hewick, R and Rosen, V (1999). A novel BMP expressed in developing mouse limb, spinal cord, and tail bud is a potent mesoderm inducer in Xenopus embryos. *Dev Biol* **208**: 222–232.
90. Böttinger, EP, Factor, VM, Tsang, ML, Weatherbee, JA, Kopp, JB, Qian, SW *et al.* (1996). The recombinant proregion of transforming growth factor β1 (latency-associated peptide) inhibits active transforming growth factor β1 in transgenic mice. *Proc Natl Acad Sci USA* **93**: 5877–5882.
91. Lee, SJ and McPherron, AC (2001). Regulation of myostatin activity and muscle growth. *Proc Natl Acad Sci USA* **98**: 9306–9311.
92. Lee, SJ, Reed, LA, Davies, MV, Girgenrath, S, Goad, ME, Tomkinson, KN *et al.* (2005). Regulation of muscle growth by multiple ligands signaling through activin type II receptors. *Proc Natl Acad Sci USA* **102**: 18117–18122.
93. Kang, JK, Malerba, A, Popplewell, L, Foster, K and Dickson, G (2011). Antisense-induced myostatin exon skipping leads to muscle hypertrophy in mice following octa-guanidine morpholino oligomer treatment. *Mol Ther* **19**: 159–164.
94. Wagner, KR, Fleckenstein, JL, Amato, AA, Barohn, RJ, Bushby, K, Escolar, DM *et al.* (2008). A phase I/IItrial of MYO-029 in adult subjects with muscular dystrophy. *Ann Neurol* **63**: 561–571.
95. Lee, SJ (2010). Speed and endurance: you can have it all. *J Appl Physiol* **109**: 621–622.