# ADVANCES IN DUCHENNE MUSCULAR DYSTROPHY GENE THERAPY

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Since the initial characterization of the genetic defect for Duchenne muscular dystrophy, much effort has been expended in attempts to develop a therapy for this devastating childhood disease. Gene therapy was the obvious answer but, initially, the dystrophin gene and its product seemed too large and complex for this approach. However, our increasing knowledge of the organization of the gene and the role of dystrophin in muscle function has indicated ways to manipulate them both. Gene therapy for Duchenne muscular dystrophy now seems to be in reach.

EXTRACELLULAR MATRIX
In muscle, this is a thin layer (basal lamina) that contains collagen, elastin and fibronectin, which surrounds each muscle fibre. This might act as a semipermeable filter or a selective cellular barrier and is important in regeneration after damage.

TRANSDUCTION
The transfer of genetic material into a cell using a viral vector.

IMMUNOGENICITY
The properties of a virus, transgene, vector, compound or molecule that provoke an immune response.

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The X-linked dystrophin gene (*DMD*) is by far the largest of the 30,000 genes that encode proteins in the human genome: its 79 exons cover 2.6 million base pairs (bp). This large size makes the gene prone to rearrangement and recombination events that cause mutations. In most cases, the mutations are deletions of one or more exons (~60%); however, duplications (~6%)[1], translocations and point mutations have also been found (see Leiden muscular dystrophy pages in online links box). In general, mutations that disrupt the reading frame of the dystrophin transcript and lead to prematurely aborted dystrophin synthesis cause Duchenne muscular dystrophy (DMD). DMD is the most frequent lethal heritable childhood disease: 1 in every 3,500 boys are born with it[2].

Dystrophin is an important structural element in muscle cells that anchors proteins from the internal cytoskeleton to those in the fibre membrane. Loss of functional dystrophin causes fibre damage and membrane leakage[3,4], but the potential role of the protein in signal transduction[5,6] indicates that the disease symptoms might have a two-pronged source. Progressive muscle weakness leads to all DMD patients being wheelchair-bound before the age of 12, and patients generally die in their twenties.

Mutations in the dystrophin gene that do not disrupt the translational reading frame result in the milder Becker muscular dystrophy (BMD) phenotype, which is found in 1 in every 20,000 newborn boys[2]. In BMD patients, the aberrant transcript is in-frame and encodes a dystrophin that, despite being internally truncated and possibly present at reduced levels, is at least partially functional. BMD patients therefore show intermediate to mild phenotypes and have much longer life expectancies.

Many different strategies for DMD gene therapy have been studied and improved over the years (for an overview see TABLE 1). As the more conventional strategies have been reviewed extensively elsewhere and are unlikely to result in an effective treatment for DMD in the near future, we only briefly discuss these methods. Instead, we focus on recent advances in three relatively new strategies: the delivery of functional mini- and micro-dystrophins by recombinant adeno-associated viral (rAAV) vectors; therapeutic antisense-induced exon skipping; and dystrophin replacement by utrophin upregulation. These three innovative methods have several advantages compared with other approaches (see below), which mark them as the most likely strategies to lead to an effective treatment for DMD.

## Conventional gene-therapy strategies

The size of the dystrophin gene has been an important challenge for gene-therapy researchers. To replace a defective dystrophin gene, an artificial dystrophin cDNA construct must be transferred into the nuclei of muscle cells, where it must be expressed and regulated appropriately. So, to deliver the 14 kb dystrophin cDNA

CYTOTOXICITY
The properties of a virus, transgene, vector, compound or molecule that are toxic for cells.

ELECTROPORATION
The application of an electric current to the plasma membrane of a cell, to temporarily open pores or channels through which DNA might pass.

PRESSURIZED ISOLATED-LIMB PERFUSION
The introduction of therapeutic agents under pressure in a limb after isolation of the blood circulation by clamping.

MICROBUBBLES
Encapsulated gas microbubbles that can be used as drug or gene carriers, which are able to penetrate into the smallest membranes. When exposed to sufficiently high-amplitude ultrasound, the microbubbles rupture and release the drugs and genes that are contained in their encapsulating layer.

TRANSFECTION
The transfer of exogenous DNA into a cell.

MYOBLAST TRANSPLANTATION
The implantation of exogenous muscle-progenitor cells into muscle to generate new myofibres or to support existing myofibres.

(11.5 kb coding sequence), vectors with a large capacity were needed. The capacity of first generation adenoviral vectors (up to 8 kb) was too small. Later, high capacity (28 kb) 'gutless' vectors, from which all adenoviral genes had been removed, bypassed this restriction and delivered extra benefits in the form of reduced host immune response to the viral vector and improved persistence of transgene expression in muscle[7,8]. However, two crucial problems need to be overcome before adenoviral vectors can be used therapeutically: they are too large to easily cross the EXTRACELLULAR MATRIX that surrounds mature myofibres and there are not many adenoviral attachment receptors on the surface of myofibres (see REFS 9,10 for a potential solution to the latter problem).

In contrast to adenoviral vectors, herpes simplex virus type-1 (HSV-1) vectors can naturally carry large inserts. HSV-1 vectors have shown relatively high TRANSDUCTION levels *in vivo*, but, similar to gutless adenoviral vectors, this is only seen in newborn and regenerating muscle[11,12]. The IMMUNOGENICITY and CYTOTOXICITY of HSV-1 hampers the long-term expression of transgenes.

The size of the full-length dystrophin cDNA is not a problem for non-viral DNA plasmid vectors that can be engineered to contain large inserts. These vectors are synthetic and non-infectious, so they are highly suitable for use in the clinic (a phase I open clinical trial is now in progress[13]). However, this delivery strategy is inefficient in muscle tissue, so cationic lipid formulations, ELECTROPORATION, PRESSURIZED ISOLATED-LIMB PERFUSION or MICROBUBBLES and ultrasound are needed to enhance TRANSFECTION efficiencies[14–16]. Less invasive, and preferably systemic, methods are needed before plasmid vectors can be routinely used in the clinic.

MYOBLAST TRANSPLANTATION, which is another dystrophin-gene-delivery strategy, also has problems that have prevented its use in the clinic: specifically immune rejection, limited cell spreading and poor survival of the myoblasts immediately post-transplantation[17–19]. However, strategies are being developed to address these problems[20–23]. Recently, similar cell-therapy strategies, which use stem cells that are derived from bone marrow, muscle or blood vessels, have had notable successes in dystrophic mouse models (such as *mdx*; TABLE 2) and DMD muscle[18,24,25].

## Alternative genetic strategies

As well as conventional strategies, several alternative approaches to DMD gene therapy are being developed. One promising new avenue is the use of chimeroplasts — double-stranded DNA–RNA oligonucleotides — to repair genes *in situ* through the cellular DNA repair machinery[26]. This strategy allowed the repair of point mutations in exon 23 of the dystrophin gene in the *mdx* mouse and in intron 6 of the CXMD golden retriever dog (another model of DMD; TABLE 2)[27–29]. This method is still inefficient and highly dependent on the rate of repair activity in the host cells of interest. Nonetheless, the approach could be powerful because its effects are cumulative and permanent. The optimization of oligonucleotide chemistry, repair frequency and delivery strategy might further increase correction efficiencies up to therapeutic levels.

Another alternative strategy is to use AMINOGLYCOSIDE treatment in an attempt to suppress the premature stop codons that cause DMD. For example, gentamicin restored the expression of functional dystrophin in the

Table 1 | **Overview of strategies for Duchenne muscular dystrophy gene therapy**

| Strategy | Action/effect | Advantages | Disadvantages | Prospects |
|---|---|---|---|---|
| Adenoviral vectors | Full-length dystrophin cDNA transfer | High transduction levels in regenerating muscle, expression of fully functional dystrophin | Viral immune response, limited persistence of transgene expression, maturation dependent | ++ |
| Herpes simplex viral vectors | Full-length dystrophin cDNA transfer | High transduction levels in regenerating muscle, expression of fully functional dystrophin | Viral toxicity and immune response, limited persistence of transgene expression, maturation dependent | + |
| Plasmid vectors | Full-length dystrophin cDNA transfer | Synthetic, non-infectious, relatively safe, flexible, simple engineering | Large molecule, delivery requires efficient transfection method | ++ |
| Myoblast transplantation | Introduce dystrophin-producing cells | Non-infectious, relatively safe | Low efficiencies, immune suppression required | + |
| Stem-cell therapy | Introduce dystrophin-producing cells | Conventional treatment, relatively safe | Low efficiencies, immune suppression required | ++ |
| Chimeric oligonucleotides | Correction of mutation at the DNA level | Cumulative, permanent effect | Low *in vivo* efficiencies | + |
| Gentamicin therapy | Ribosomal read-through of stop codons in mRNA | Conventional drug | Low reproducibility, risk of nonspecific adverse effects | + |
| rAAV vectors* | Mini- or micro-dystrophin cDNA transfer | High transduction efficiencies in muscle, non-pathogenic minimal immune responses | Unable to deliver full-length dystrophin, laborious production systems | +++ |
| Antisense oligonucleotides* | Splicing modification of pre-mRNA | Synthetic, small-molecule drug, relatively safe, restores all isoforms | Repeated administrations and (targeting) delivery reagent needed, mutation specific | +++ |
| Utrophin upregulation* | Replacement of dystrophin | Small-molecule drug, no immune response, relatively safe | No effective specific compound identified as yet | ++ |

*These three relatively new strategies are most likely to lead to an effective treatment for Duchenne muscular dystrophy. The symbols in the prospects column indicate a subjective assessment of the probability of a particular strategy leading to an effective treatment, ranging from low (+) to high (+++). rAAV, recombinant adeno-associated virus.

Table 2 | **Overview of animal models for Duchenne muscular dystrophy gene-therapy studies**

| Model | Mutation | Effect | Pathologic/physiologic symptoms | Gene-therapy studies |
|---|---|---|---|---|
| *mdx* mouse | Nonsense mutation in exon 23 (3185C>T) | Stop codon introduced, dystrophin synthesis aborted prematurely | Fibre degeneration (particularly from 2–8 weeks), replacement by centrally nucleated regenerating fibres, myopathy less severe later in life, normal lifespan | Used for most strategies owing to its relative experimental simplicity |
| CXMD/GRMD golden retriever dogs | 3′ splice-site point mutation in intron 6 (739–2a>g) | Exon 7 skipped from transcript, frame shift, dystrophin synthesis aborted prematurely | Severely affected, massive muscle degeneration, resembles human DMD best compared with other models | Chimeroplasts, adenovirus-mediated (mini-)dystrophin/ utrophin transfer |
| HFMD | Deletion of Dp427m and Dp427p promoter regions | No muscle dystrophin expression | Large areas of muscle-fibre degeneration and regeneration, mononuclear infiltraton, hypercontracted fibres | None |

Note that further mouse mutants (*mdx*$^{2-5cv}$) that have been generated by 5 *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis are not often used for therapeutic studies[131].
CXMD, canine x-linked muscular dystrophy; DMD, Duchenne muscular dystrophy; GRMD, golden retriever muscular dystrophy; HFMD, hypertrophic feline muscular dystrophy.

AMINOGLYCOSIDES
A group of antibiotics (such as gentamicin) that inhibit bacterial protein synthesis and are particularly active against Gram-negative bacteria.

skeletal muscle of *mdx* mice[30]. As a result, several human trials with this conventional drug were initiated in selected DMD patients. However, in the one trial that has been fully reported, gentamicin treatment in humans was not as successful as it was in the mouse model. Also, a follow-up study in *mdx* mice was unable to reproduce the encouraging initial results[31]. Further experiments will be necessary to evaluate the feasibility of this strategy for DMD gene therapy.

## New strategies: mini- and micro-dystrophins

***Reduction of the dystrophin transgene size.*** A large range of deletions in dystrophin cause only mild phenotypes in BMD patients (for a notable example, see REF. 32). So, large parts of the gene seem not to be vital for function (FIG. 1). To map the regions that are crucial for dystrophin function, several transgenic *mdx* mice were engineered to carry different deletions throughout the four dystrophin domains. Deletions in the N-terminal domain were associated with relatively mild phenotypes, which indicates that this region might be important but not essential for attachment to actin and the cytoskeleton[33,34]. By contrast, deletions in the cysteine-rich domain cause severe dystrophy, owing to disruption of the entire dystrophin–glycoprotein complex[35]. The C-terminal domain, with its various alternative splicing patterns, seems not to be required for the assembly of this complex[35,36]. A series of large deletions that were evaluated in the central rod domain[37–40] indicated that although the rod structure is indispensable, the number of repeats can be markedly reduced. However, the proper positioning of the repeats and the presence and configuration of the hinge regions is crucial. A 6.2 kb mini-construct (ΔH2–R19)[39] that contained 8 repeats and hinge regions 1, 3 and 4, was engineered to mimic the exon 17–48 deletion in a BMD patient described by England and colleagues[32] (FIG. 1). This construct was found to be completely functional: transgenic *mdx* mice that carried this construct showed non-dystrophic muscle morphology and normal force generation.

Other mini-dystrophin constructs can also ameliorate dystrophic pathology in *mdx* mice[38]. These studies indicated that five repeats and two hinges were sufficient to provide crucial length and flexibility for the rod domain. Further reduction is feasible, as shown by several micro-constructs (3.6–4.2 kb) that were highly effective in supporting almost normal muscle structure and function, at least in mice[37,39–41]. The smallest effective micro-construct (ΔR4–R23)[39] was only 3.6 kb in size and carried 4 repeats and hinge regions 1, 2 and 4 (FIG. 1).

***Transgene delivery with rAAV vectors.*** Besides increasing our knowledge of the dystrophin protein, these domain-mapping studies also provided functional gene constructs with which the limited insert capacity of certain gene-transfer delivery vectors could be addressed. In particular,

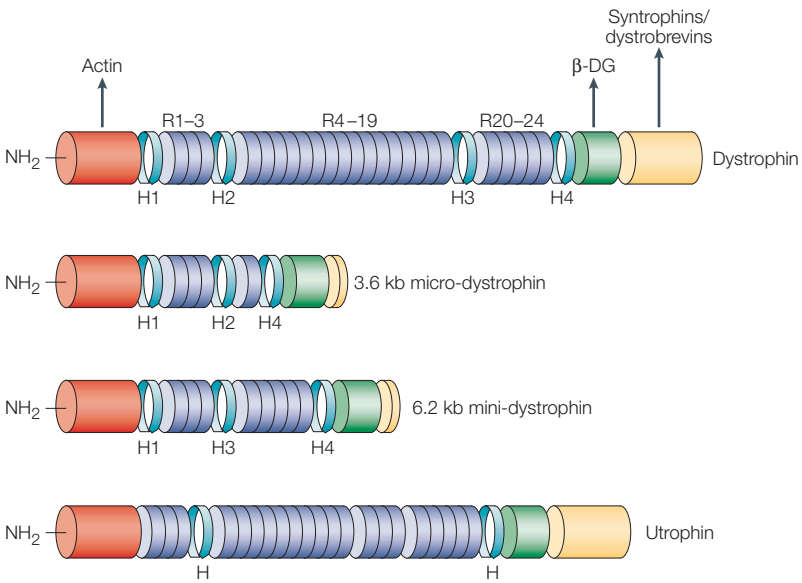


Figure 1 | **Structural domains of the human full-length dystrophin, Becker mini- and micro-dystrophins, and utrophin.** The full-length dystrophin consists of four structural domains[128–130]. The N-terminal domain (red) binds to F-ACTIN, the cysteine-rich domain (green) binds to β-DYSTROGLYCAN (β-DG) and the C-terminal domain (yellow) binds to DYSTROBREVINS and SYNTROPHINS. The central coiled-coil rod domain (blue) contains 24 SPECTRIN-like repeats (R1–R24) and 4 'hinge' regions (turquoise; H1–H4). In the mini- and micro-dystrophins, the rod domain is truncated by the deletion of multiple repeats and a hinge region. Note that the constructs that were cloned into recombinant adeno-associated viral vectors (rAAVs) did not contain the C-terminal domain. Utrophin also contains an actin-binding N-terminal domain, a central coiled-coil rod domain (lacking repeats 15 and 19, and 2 hinge regions, compared with dystrophin) and a C-terminal domain that interacts with the dystrophin–glycoprotein complex.

F-ACTIN
A protein that is involved in the contractile apparatus and the maintenance of the cytoskeleton of myofibres.

β-DYSTROGLYCAN
The α- and β-dystroglycans are the laminin-binding components of the dystrophin–glycoprotein complex, which provides a linkage between the subsarcolemmal cytoskeleton and the extracellular matrix.

DYSTROBREVINS
The components of the dystrophin–glycoprotein complex that bind to syntrophin and (indirectly) to the C-terminal of dystrophin. Dystrobrevin-α recruits signalling proteins, such as neuronal nitric oxide synthase.

SYNTROPHINS
Peripheral membrane proteins that bind to the C-terminal of dystrophin, which might have a role in the process of synaptogenesis.

the use of rAAV vectors became possible (BOX 1). Different mini- and micro-dystrophin gene constructs were cloned into rAAV type-2 vectors and tested in *mdx* mice[38,39,41,42]. These studies showed that the rAAV delivery of constructs carrying four, five or eight repeats, in combination with either two or three hinge regions, was an effective means of treating DMD symptoms in this model. Reversal of the *mdx*-associated morphological abnormalities was observed up to at least six months post-injection, independent of the choice of promoter (CMV, MCK or CK6), the injected muscle (gastrocnemius or tibialis anterior) and the age of the mice at the time of injection. Overall, there was widespread high expression of the mini- and micro-dystrophins, with correct localization at the fibre membranes and a restored dystrophin–glycoprotein complex. The therapeutic effect was characterized by the correction of several pathophysiological parameters that are associated with the *mdx* phenotype, such as variable fibre diameters, myofibres with central nuclei, reduced membrane integrity and necrosis. These data indicate that the rAAV-mediated delivery of effective mini- or micro-dystrophin gene constructs can slow or even halt the progression of the dystrophy on a long-term basis.

However, a greater immune response against rAAV-delivered (foreign) transgene products was observed in dystrophic muscle compared with normal muscle[43,44]. This was attributed to the inflammatory *mdx*-muscle environment and the effect of co-infecting antigen-presenting cells (APCs) that activate cytotoxic T lymphocytes against the transgenic products, which causes the destruction of transduced myofibres. Also, NEO-ANTIGENS were released from leaky dystrophic myofibres and presented by myoblasts and regenerating myofibres[44]. To minimize the immune responses in rAAV-based DMD gene-therapy studies, it will be necessary to use immunosuppressing drugs, muscle-specific promoters to avoid the activation of APCs and fully functional micro- and mini-dystrophin constructs to protect fibres from degeneration and the release of neo-antigens.

*Perspectives.* Micro- and mini-dystrophin constructs are effective in *mdx* mice, but considering that the human pathophysiology is notably more severe, it does not necessarily follow that they will be equally effective in DMD patients. The minimal functional requirements for the human dystrophin protein and its crucial domains might differ from the mouse homologue. Furthermore, it is still an open question whether micro- and mini-dystrophins fully restore muscle strength (as well as preventing muscle damage), as this might require larger dystrophins. Although rAAV has shown potential for muscle therapy, future clinical applications will require further optimization. AAV vectors that are based on alternative capsid serotypes have shown better transduction efficiencies in muscle[45,46]. Also, muscle-targeting vectors need to be developed to allow systemic treatment of DMD. Finally, as a fraction of the rAAV vectors integrate into the host genome, there will be a risk of insertional mutagenesis. However, this risk is difficult to assess compared with that of spontaneous mutation and recombination events in chromosomal DNA, and might be significantly outweighed by the therapeutic benefit for DMD patients.

## New strategies: antisense-induced exon skipping

The relatively mild BMD phenotypes that are caused by some large deletions or nonsense mutations have also pointed to another possible gene-therapy strategy: skipping an exon during PRE-mRNA SPLICING to enlarge a DMD deletion so that it becomes its nearest in-frame BMD counterpart (FIG. 2). Although creating a larger deletion for therapeutic purposes might seem counter-intuitive, this frequently occurs in nature. Some BMD patients have mutations that would normally cause a DMD-like phenotype. The crucial difference is that these mutations involve splicing motifs or interfere with splice-site selection and induce the spontaneous skipping of (further) exons. The resulting transcripts are in-frame and allow the synthesis of internally deleted dystrophins that can, to a varying extent, lead to a milder phenotype[47–50]. Such splicing anomalies are also thought to cause isolated clusters of 'revertant'(dystrophin-positive) myofibres in many DMD patients. Although rare, these revertant fibres increase in number with age, and their putative correlation with the severity of DMD is debated[47,48,51,52].

So, it was clear that skipping exons could have some therapeutic value, but the question remained: how could this be artificially induced? The answer came from a study on a Japanese DMD patient, in which a 52-bp frame-disrupting deletion in exon 19 was found to cause exon 19 skipping from the dystrophin transcript[53]. It was proposed that this region might contain an exon-recognition site (ERS) — also known as an exon-splicing enhancer (ESE) — which is a purine-rich sequence that is required for the correct splicing of exons with weak splice-site consensus sequences[54,55]. A small antisense oligodeoxyribonucleotide (ODN) was tested and found

### Box 1 | Recombinant adeno-associated viral vectors


**Recombinant adeno-associated viral (rAAV) vectors seem to be the best for gene delivery to muscle, despite their insert limit of only 5 kb. The virus is a non-pathogenic DEPENDOVIRUS that cannot reproduce without the presence of a helper virus and seems to largely evade the immune system. It is able to induce long-term transgene expression, mostly by forming stable, transcriptionally active monomeric and concatameric EPISOMES (a small fraction also integrates into the host-cell genome)[119,120]. Moreover, notable transduction efficiencies can be obtained in mature muscle tissue given the high expression of the heparan sulphate proteoglycan AAV type-2 receptor. More importantly, the rAAV particle is small enough to easily penetrate the extracellular matrix of muscle fibres. In several gene-therapy studies, rAAV has already shown its potential by efficiently delivering genes for muscle disorders, such as limb-girdle muscular dystrophy[43,121,122], and for metabolic diseases, such as haemophilia IX (REFS 123–125), Pompe disease[126] and diabetes type 1 (REF. 127).**

**Transgenes (for example, the mini- or micro-dystrophins) are cloned in between the AAV inverted terminal repeat (ITR) sequences, under the control of a promoter of choice (for example, CMV, MCK or CK6). For virus production in HEK-293 PRODUCER CELLS, these expression cassette-containing plasmids are then co-transfected with plasmids that contain AAV replication and capsid genes, and adenoviral helper genes (E2A, E4 and VA).**

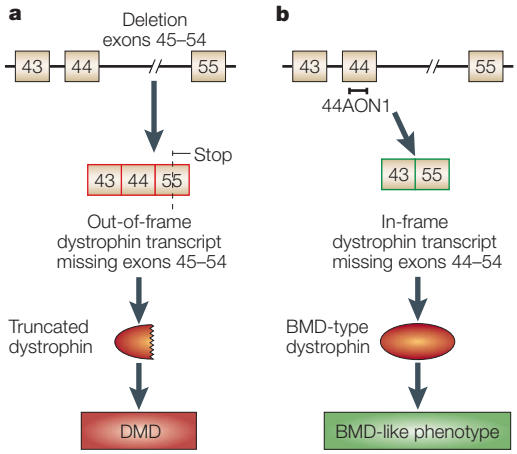


Figure 2 | **Schematic representation of the exon-skipping strategy. a** | In Duchenne muscular dystrophy (DMD) patients with a deletion of exons 45–54, an out-of-frame transcript is generated in which exon 44 is spliced to exon 55. Owing to the frame shift, a stop codon occurs in exon 55, which prematurely aborts dystrophin synthesis. **b** | Using an exon-internal antisense oligonucleotide (AON) in exon 44, the skipping of this exon can be induced in cultured muscle cells. Accordingly, the transcript is back in-frame and a Becker muscular dystrophy (BMD)-like dystrophin can be synthesized (FIG. 3).

to block this ERS sequence, as judged from the precise skipping of exon 19 following transfection of this ODN into human lymphoblastoid cells[56].

***Exon skipping in the mdx mouse model.*** The feasibility of applying antisense oligonucleotides (AON) to induce potentially therapeutic exon skipping from the DMD pre-mRNA was shown in PRIMARY MUSCLE-CELL CULTURES from the *mdx* mouse model[57]. *mdx* mice are dystrophin deficient, owing to a nonsense mutation in the in-frame exon 23 (REF. 58; TABLE 2). In this study, 2′O-methyl-phosphorothioate AONs were not directed against an exon-internal ERS, but rather against both splice sites of exon 23. Skipping of this exon would bypass the nonsense mutation and generate an in-frame transcript with a small internal deletion. The splicing was successfully modulated using an AON that was specific for the 3′ splice site of intron 22. However, exon 22 was found to be spliced to exon 30, which indicated that the splicing machinery might be more profoundly affected. This transcript, which was also in-frame, was observed at low levels and might have allowed the synthesis of the dystrophin protein that was detected in ~1% of transfected *mdx* myotubes. In follow-up studies, the design of the AONs directed at the splice sites of exon 23 was further optimized[59–61]. Subsequently, AONs that were complementary to the 5′ splice site of intron 23 (that is, downstream of exon 23) were found to be more specific and efficient, with minimal effective doses *in vitro* that were as low as 5 nM[61]. At higher doses of up to 300 nM, an alternative splicing pattern was produced, which skipped both exons 22 and 23 (an out-of-frame combination). Notably, this area is known to have various alternative splicing patterns both in control cells and *mdx* cells[62], and AONs can enhance such local patterns[63]. This might be explained by the AON-induced disturbance of the local pre-mRNA configuration, which, in turn, might stall the SPLICEOSOMAL COMPLEX and force it to resolve the blockade by ligating non-sequential splice sites. Nonetheless, skipping of the single exon 23 produced considerable levels of dystrophin in the treated *mdx* myotubes. Furthermore, a recent study of *mdx* mice showed that after intramuscular injections of an efficient 5′ splice-site AON, nearly normal levels of an almost full-length dystrophin were produced in many myofibres, which improved muscle function[64]. Although this effect was optimal at 2–4 weeks, dystrophin was still detectable at 3-months post-injection.

***Exon skipping in human DMD muscle cells.*** The first evidence for human therapeutic exon skipping in muscle cells from DMD patients came from a study targeting exon 46 (REF. 65). A single exon 45 deletion is (at ~7%) the single most frequent DMD-causing mutation reported in the DMD database (see Leiden muscular dystrophy pages in online links box). Specific AON-induced skipping of exon 46 was achieved in muscle cells from two exon 45 deletion patients, by targeting exon-internal ESE-like sequences that were pre-selected by an *in vitro* binding assay. With skipping efficiencies of only ~15%, the reading frame was restored and dystrophin synthesis induced in more than 75% of transfected myotubes. Moreover, the significant dystrophin levels indicated accumulation of the protein. As BMD patients with an exon 45–46 deletion have a relatively mild phenotype, such a protein would probably be therapeutic in DMD patients with exon 45 deletions.

An assessment of the range of mutations that cause DMD shows that the skipping of any given exon could be an effective gene therapy for many different mutations (TABLE 3). For instance, skipping exon 51 would restore the reading frame in patients that carry a deletion of exons 45–50, 47–50, 48–50, 49–50, 50, 52 or 52–63, which adds up to a total of 17.5% of all DMD patients. Indeed, the skipping of only 12 different exons could theoretically correct almost 75% of all deletions (TABLE 3). In subsequent studies, we targeted 21 DMD exons that were primarily selected for their capacity to correct most known DMD deletions[63] (J.C.T.D., unpublished data). Two AONs were designed per exon — all directed to exon-internal sequences (but not necessarily all ESE-like) — which were predicted to have a partially open secondary RNA structure at 37°C. Their binding to the target sequence *per se* was proposed to alter the secondary exon pre-mRNA structure sufficiently to prevent exon selection for inclusion (see below). Pilot experiments in normal human muscle cells yielded a set of AONs that efficiently mediated the skipping of 20 of the 21 exons targeted: exons 2, 8, 17, 19, 29, 40–46, 48–53, 55 and 59. This indicates that most exons in the dystrophin gene are 'skippable', and that exon skipping might be applicable to most mutations (>75%), including deletions, duplications and nonsense mutations of in-frame exons.

SPECTRIN
A large contractile submembrane protein that, similar to dystrophin, contains an actin-binding domain and a long repeat domain.

DEPENDOVIRUS
A single-stranded DNA virus from the family parvoviridae (subfamily parvovirinae), which is dependent on a co-infection with helper adenoviruses or herpes viruses for efficient replication.

EPISOMES
DNA that can replicate autonomously in the cytoplasm of host cells.

HEK-293 CELLS
Host cells that generate viral particles following transfection with the rAAV plasmid and the helper plasmid.

NEO-ANTIGEN
A foreign (transgene) product that is able to stimulate an immune response.

PRE-mRNA SPLICING
The removal of introns from the precursor mRNA molecule; the remaining exons are spliced together.

PRIMARY MUSCLE-CELL CULTURES
Cells that are taken into culture directly from a tissue biopsy. In contrast to cell lines that only contain immortalized cells, these cultures contain heterogeneous cell populations.

SPLICEOSOMAL COMPLEX
A large dynamic complex that consists of small nuclear RNA molecules and protein components. It mediates the two catalytic steps of the splicing reaction: the excision of introns from the pre-mRNA and the ligation of the two exon termini.

Table 3 | **Overview of therapeutic exon skipping for a series of DMD-causing deletions**

| Skippable exon | Therapeutic for DMD deletions (exons) | Percentage of deletions in LDMD database |
|---|---|---|
| 2 | 3–7, 3–19, 3–21 | 2.9 |
| 8 | 3–7, 4–7, 5–7, 6–7 | 4.5 |
| 17 | 12–16, 18–33, 18–41, 18–44 | 1.8 |
| 43 | 44, 44–47, 44–49, 44–51 | 3.7 |
| 44 | 14–43, 19–43, 30–43, 35–43, 36–43, 40–43, 42–43, 45, 45–54 | 7.8 |
| 45 | 12–44, 18–44, 44, 46–47, 46–48, 46–49, 46–51, 46–53, 46–55 | 11.2 |
| 46 | 21–45, 45, 47–54, 47–56 | 5.6 |
| 50 | 51, 51–53, 51–55 | 5.2 |
| 51 | 45–50, 47–50, 48–50, 49–50, 50, 52, 52–63 | 17.5 |
| 52 | 51, 53, 53–55 | 4.0 |
| 53 | 10–52, 45–52, 46–52, 47–52, 48–52, 49–52, 50–52, 52 | 7.5 |
| 55 | 45–54, 48–54 | 1.8 |
| Total | 12 AONs | 73.5 |

This series of Duchenne muscular dystrophy (DMD)-causing deletions were reported in the Leiden DMD (LDMD) database. Antisense oligonucleotide (AON)-induced skipping of just 1 of the 12 skippable exons listed would (theoretically) restore the reading frame in a series of DMD patients that were affected by different deletions.

The broad therapeutic potential of AONs was shown in cultured muscle cells from DMD patients that were affected by several different deletions[66] (for example, exons 45–54) (FIG. 2). In all cases, the targeted exon was specifically skipped and at relatively high levels of up to ~90% (such as in FIG. 3a), which induced the synthesis of significant levels of dystrophin in more than 75% of treated cells (FIG. 3b,c). These dystrophins located appropriately to the sarcolemma and restored the dystrophin–glycoprotein complex, which was a strong indication of functional restoration.

In a parallel study that targeted the splice sites of exon 51, a more permanent bypass of the deletion of exons 48–50 was achieved[67]. Here, the antisense sequences were cloned into small nuclear RNAs (snRNAs), which were delivered to cultured muscle cells by a recombinant retrovirus. Small integrated 'factories' were therefore introduced, which stably and efficiently produced the antisense sequences. The construct targeting both exon 51 splice sites was especially effective in inducing skipping in more than 60% of cells, which introduced the synthesis of a new shorter dystrophin.

*Perspectives.* Although exon skipping is a mutation-specific therapy, an important intrinsic advantage compared with conventional gene therapy is that it simultaneously corrects all dystrophin isoforms. It also maintains the original tissue-specific gene regulation. Moreover, AONs are small, sequence specific and synthetic, and so are relatively safe therapeutic agents. Although the results so far seem to support short-term therapeutic potential, several parameters need to be optimized for clinical application.

One such parameter is the choice of target sequence. As an exon will only be included in the mRNA when the spliceosomal complex recognizes both splice sites, these seem obvious targets for AONs. However, the *mdx* studies indicate that the design of AONs that target splice sites might be crucial. By contrast, a strategy that targets

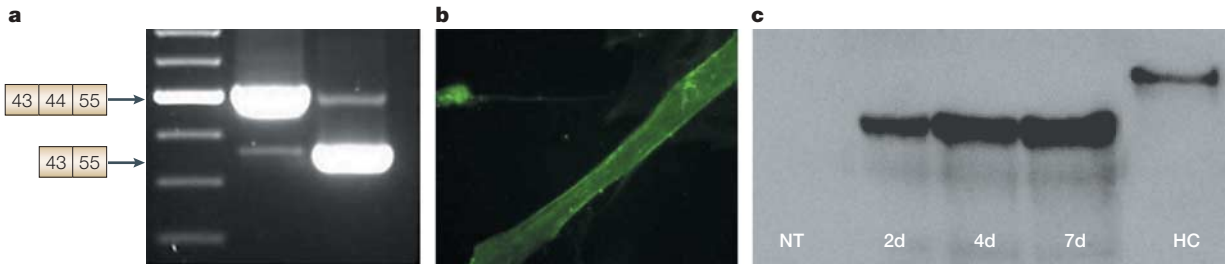


Figure 3 | **Therapeutic exon skipping in cultured myotubes from a DMD-patient (DL363.2) with a deletion of exons 45–54.** After transfection with an exon-internal antisense oligoribonucleotide (AON) in exon 44 (44AON1), exon 44 skipping was induced such that most transcripts were in-frame. **a** | RT-PCR of the region spanning exons 43–55 shows that in the untreated myotubes almost all of the transcripts are out-of-frame and contain exon 44 (lane 2), whereas in AON-treated myotubes most transcripts (~90%) are in-frame and are missing exon 44 (lane 3). **b**,**c** | Exon skipping also led to substantial dystrophin synthesis *in situ*, which was detected by the immunohistochemical analysis of treated myotubes (**b**) and by Western blot analysis of protein samples from treated myotubes (**c**) using the Dys2 antibody (raised against the distal exons 77–79). The dystrophin was located at the membrane at two days (2d) and accumulated up to seven days (7d) post-transfection. No dystrophin was observed in untreated samples (NT). As expected from the deletion, the dystrophin that was produced was shorter than the full-length dystrophin from a (1:10 diluted) human control sample (HC). Parts **b** and **c** reproduced with permission from REF. 66 © (2003) Oxford Univ. Press.

exon-internal sequences interferes with exon selection for inclusion before splicing: a process that is not yet well understood. We suggest that the secondary structure of an exon in the pre-mRNA has a crucial role in marking that exon for inclusion. There is increasing evidence that exonic signals other than ESEs are involved in splicing[55]. If this theory is correct, internally targeted AONs might disrupt this structural inclusion signal at an earlier stage, without hampering the actual splicing process. The efficacy of most of the AONs that were designed for 20 different exons indicates that targeting partially open structures in exons offers more choice for the design of AONs, with fewer restrictions on length and base composition. Targeting exon-internal sequences might also provide a higher level of specificity than splice-site consensus sequences, and might reduce the risk of nonspecific splicing aberrations. Moreover, in a recent comparative study, an ESE in exon 19 was shown to be a more sensitive target for AON-induced exon skipping than the flanking splice sites[68]. The lower concentrations of AON that are therefore required for inducing exon skipping also reduce this risk.

A second parameter is the design of the antisense drug. Most of the studies mentioned applied 2′O-methyl-phosphorothioate oligoribonucleotides. Chemical modifications of the antisense molecules render them resistant to cellular endonuclease and unable to induce RNaseH-activity. However, phosphorothioate modification of the phosphate backbone might cause toxic and/or non-antisense side effects in cells[69,70]. Recent advances in the technology of designing AONs include modifications to the sugar or backbone that might improve their sequence-specificity, resistance to endonucleases, target RNA affinity, cellular uptake and PHARMACOKINETIC PROFILE. So, alternatively modified AON analogues, such as locked nucleic acids (LNAs)[71], peptide nucleic acids (PNAs)[72] and morpholinos phosphorodiamidate oligomers[73], are now under evaluation[74].

A third parameter is the design of a safe and efficient delivery system. Delivery-system design depends on the chemical characteristics of the chosen antisense drugs. Proof-of-principle studies, such as that of Lu and colleagues[64], initially apply intramuscular injections of the AONs. However, for clinical applications, less invasive systemic delivery is preferred. Studies of the systemic delivery of AONs in mice have indicated that only 5% of the original dose reaches the muscle tissue[75,76]. So, high doses or muscle-specific targeting will be necessary. Extensive studies in mice are needed to determine toxicity, dosage and the frequency of administrations required.

## New strategies: utrophin upregulation

Dystrophin has a homologue called utrophin[77]. The utrophin gene (*UTRN*) maps to chromosome 6q24 and contains 74 exons that are dispersed over ~1 Mb[78]. Although the total genomic length of the utrophin gene is only approximately one-third of that of the dystrophin gene, its transcript is (at 13 kb) almost as large (FIG. 1). Dystrophin and utrophin are highly similar and one probably originated from a duplication of the other[79]. Similar to dystrophin, utrophin consists of an actin-binding N-terminal domain, a central coiled-coil rod domain and a C-terminal domain that interacts with the dystrophin–glycoprotein complex[80] (FIG. 1). The most prominent difference is that it lacks the spectrin-like repeats 15 and 19, and 2 hinge regions of dystrophin[81].

Utrophin is ubiquitously expressed in most tissues — most prominently in lungs, blood vessels and the nervous system. In muscle, its local expression is maturation dependent: in fetal muscle it is initially dispersed over the SARCOLEMMA, during development it is gradually replaced by dystrophin[82] and in mature muscle it is located only at the neuromuscular and myotendinous junctions[83]. At the post-synaptic membrane of the neuromuscular junctions, utrophin co-localizes with the ACETYLCHOLINE receptors and is thought to have a structural and functional role in the differentiation and maintenance of postsynaptic membrane domains[84,85]. By contrast, in the regenerating muscle of DMD patients, *mdx* mice and dystrophin-deficient cats, utrophin was found to be both upregulated and redistributed to the sarcolemma[86–88]. This latter observation led to the hypothesis that utrophin might have a complementary, as well as a protective, role in dystrophic muscle. Further support for this hypothesis comes from utrophin–dystrophin double-deficient mice, which show severe progressive muscle weakness, neuromuscular and myotendinous-junction abnormalities, and die prematurely[89,90]. In fact, they provide a better model for DMD gene-therapy studies than *mdx* mice, which are physically less affected.

***Utrophin to treat dystrophin deficiency.*** Studies in *mdx* mice showed the feasibility of utrophin upregulation to treat DMD. High expression of a truncated utrophin transgene at the sarcolemma notably reduced dystrophic pathology and intracellular calcium homeostasis, and improved mechanical muscle performance[91,92]. Furthermore, adenoviral delivery of mini-utrophin restored the dystrophin–glycoprotein complex, reduced the number of centrally nucleated fibres and so rescued the dystrophic phenotypes of three animal models of DMD[93–97]. The expression of full-length utrophin mRNA in transgenic *mdx* mice produced even better results, despite expression levels that were at most 50% of the normal endogenous levels[98,99]. Coupled with data that showed that nonspecific overexpression of full-length utrophin had no toxic effects on a broad range of tissues[100], these results indicate that utrophin upregulation need not be strictly controlled and tissue specific.

***Targeting the utrophin promoters.*** Two promoters (A and B) apparently control the expression[101–103] of full-length utrophin A and B, respectively (FIG. 4). The upstream promoter A is located in a CpG ISLAND at the 5′ end of the gene and contains several putative binding sites for the transcription factors Sp1, Sp3 and Ap2, which drive constitutive transcription from this promoter[101,102]. It also contains an E-box that binds

RNaseH
Ribonuclease H. An enzyme that cleaves RNA/DNA complexes.

PHARMACOKINETIC PROFILE
The characteristics of a drug that determine its absorption, distribution and elimination in the body.

SARCOLEMMA
The membrane that encloses a striated muscle fibre.

ACETYLCHOLINE
A neurotransmitter ($C_7H_{17}NO_3$) that is released at autonomic synapses and neuromuscular junctions. It is active in the transmission of nerve impulses and is formed enzymatically in tissues from choline.

CpG ISLAND
Genomic regions that are rich in the CpG pattern, are resistant to methylation and are often associated with promoter activity.

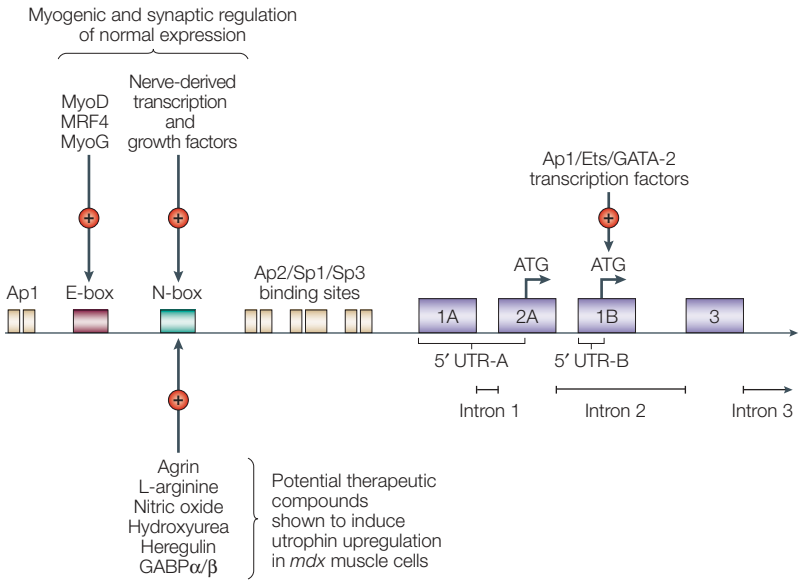


Figure 4 | **Schematic representation of the human utrophin A and B promoter regions and the factors that affect expression.** Several putative binding sites for the transcription factors Ap1, Ap2, Sp1 and Sp3 have been identified in promoter A. This promoter region also contains a myogenic regulatory E-box and a synaptic regulatory N-box that, through the binding of several myogenic factors (MyoD, MyoG and MRF4) and nerve-derived transcription and growth factors, respectively, regulate normal utrophin-A expression. For DMD therapeutic purposes, several compounds have been analysed and shown to interact with the N-box to induce utrophin upregulation in *mdx* muscle cells, both *in vitro* and *in vivo*. Promoter B is located in intron 2 and contains putative binding sites for various transcription factors, including Ap1, Ets and GATA-2. Exon 3 is the first common exon in the utrophin A and B transcripts.

myogenic factors such as MyoD, MyoG and MRF4 (REFS 104,105), which regulate utrophin in a muscle-specific manner.

Another important element in the promoter A region is the N-box. Similar to its function in acetylcholine subunit and esterase genes, this motif is responsible for the synapse-specific expression of utrophin[106,107]. Transcription from promoter A is regulated through interaction of this N-box with nerve-derived transcription and growth factors[108–111]. Indeed, the treatment of cultured myotubes with agrin, L-arginine, nitric oxide (NO) or hydroxyurea, both *in vitro* and *in vivo*, enhanced the levels of utrophin at the sarcolemma[18,112] (FIG. 4). Similarly, overexpression of the growth factor heregulin or the Ets-related transcription factor GA-binding protein (GABPα/β) in cultured myotubes caused an N-box-dependent increase in utrophin expression[109,110].

Studies like these not only contribute to the understanding of the regulatory mechanisms that control temporal and spatial utrophin expression, but also identify potential targets for (small) pharmacological compounds to increase utrophin expression. Using utrophin promoter–reporter gene constructs, Davies and colleagues have screened libraries in high-throughput quantifiable assays to identify compounds that might sufficiently upregulate utrophin-A expression, albeit through unknown pathways. However, despite several years of effort, these studies have not yet yielded any candidate drug. Conversely, glucocorticoids were recently shown to induce the elevation of utrophin protein levels[113].

The more distal promoter B is located in the large second intron of the gene[103] (FIG. 4). It does not contain an N-box, and is therefore not controlled by synaptic factors. Utrophin promoter B was thought to be responsible for the presence of the extrasynaptic utrophin transcripts that are observed in muscle[114]. In fact, using antibodies that are specific for utrophin A and B (raised against their unique N-termini), it was recently shown that utrophin B is expressed throughout different tissues specifically in vascular endothelial cells[115]. These studies also indicated that utrophin A (which is expressed at the neuromuscular junctions, choriod plexus, pia mater and renal glomerulus), and not utrophin B, accounts for the increased utrophin levels in dystrophin-deficient muscle[115]. Utrophin B is regulated by different mechanisms compared with utrophin A, and the involvement of different regulatory elements might provide alternative targets. The promoter region contains putative binding sites for various transcription factors, and, similar to other endothelial genes, it is transactivated by Ap1, Ets and GATA-2 (REFS 116,117). However, it remains to be shown whether utrophin B can replace dystrophin in dystrophic muscle, as all previous DMD gene-therapy studies in *mdx* mice used transgenes that were based on utrophin A.

*Perspectives.* The idea of replacing an absent or dysfunctional dystrophin with utrophin, both functionally and locally, is unique and provides promising therapeutic possibilities for DMD. Upregulation of utrophin has several advantages compared with dystrophin-based therapies. As utrophin is not a neo-antigen, it is unlikely to induce immune rejection. Furthermore, initial studies show no short-term deleterious effects of full-body overexpression, which indicates that a strict control of the therapeutic effect would not be necessary. Also, a twofold to threefold upregulation of utrophin has already been shown to be sufficiently therapeutic to alleviate the dystrophic muscle pathology. Finally, utrophin upregulation by pharmacological compounds would be relatively safe, and allow a more straightforward systemic treatment that reached all muscle tissue. As utrophin A, and not B, replaces dystrophin in skeletal muscle, the upstream promoter region seems a more obvious target for upregulation. Although several growth and transcription factors have been characterized as effective activators of promoter A, the challenge will be to identify a drug that is sufficiently utrophin-specific to circumvent putative adverse effects. Future research might identify further regulatory elements in the gene as potential targets for this strategy[118].

## The future of DMD therapy

As with other monogenic diseases, the initial high hopes that identifying the gene responsible for DMD would rapidly bring rational therapy have been

dampened. However, steady progress in understanding the gene and its function has pointed to several innovative therapeutic strategies. It now seems reasonable to expect that the next decade will see great advances in this field. Considering its efficiency and relative simplicity, the antisense approach seems the next candidate (and probably the most promising so far) for clinical trials. Indeed, would it not be fitting if the unique characteristics of DMD — the large size of the gene, the complexity of its processing and the intriguing milder BMD — were also to lead to an initial version of its therapy?


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