



# New therapies for Duchenne muscular dystrophy: challenges, prospects and clinical trials

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**Muscular dystrophies primarily affect skeletal muscle. Mutations in a large number of genes, mainly encoding cytoskeletal proteins, cause different forms of dystrophy that compromise patient mobility and quality of life, and in the most severe cases lead to complete paralysis and premature death. Although muscular dystrophies still lack an effective therapy, several novel strategies are entering or are ready to enter clinical trials. Here we review the main experimental strategies, namely drug, gene and cell therapies, outlining their goals and limitations. We also provide an update of ongoing or planned clinical trials based on these strategies.**

## Treatment strategies for muscular dystrophies

Muscular dystrophies are a group of diseases with clinical and molecular heterogeneity, characterized by the primary wasting of skeletal muscle, which compromises patient mobility. In the most severe disease, Duchenne muscular dystrophy (DMD), weakness of respiratory muscles and lack of dystrophin (see Glossary) protein in the heart lead to respiratory or cardiac failure (or both) and premature death [1].

In many cases, the mutation affects proteins that form a link between the cytoskeleton and the basal lamina. Absence of one protein often causes the disassembly of the whole multiprotein complex associated with dystrophin, leading to increased fragility of the sarcolemma, especially during intense contractile activity. This, in turn, results in increased calcium entry (although the molecular mechanisms have not been elucidated in detail) and focal or diffuse damage to the fiber [2]. Damaged or dead fibers can be repaired or replaced by satellite cells [3]. These cells, which were originally identified because of their location between the basal lamina and the membrane of the muscle fiber, are now considered the resident 'stem-like' cells in skeletal muscle. They are responsible for muscle growth and regeneration in postnatal life [4]. However, dystrophic satellite cells share the same molecular defect and produce fibers that are also prone to degeneration. With time, the population of satellite cells is exhausted and the muscle tissue is progressively replaced by connective and adipose tissue.

Muscular dystrophies are among the most difficult diseases to treat, although the underlying pathogenesis is well understood. Skeletal muscle is the most abundant tissue of the body and is composed of large multinucleated fibers, whose nuclei cannot divide. Consequently, any cell or gene replacement strategy must restore proper gene expression in hundreds of millions of postmitotic nuclei, which are embedded in a highly structured cytoplasm and surrounded by a thick basal lamina. Similarly, most pharmacological approaches must impinge on the complex and partly unknown biochemical mechanism of fiber degeneration that involves pathways such as calcium fluxes and protease activity, inhibitors of which are usually

## Glossary

**Adeno-associated viral vectors:** Vectors derived from adeno-associated virus, a small DNA, nonintegrating virus that requires adenoviral proteins for assembly.

**Adenoviral vectors:** Vectors derived from adenovirus, a large DNA, nonintegrating virus.

**AON:** Antisense oligonucleotides that target the donor or acceptor (or both) splice sites during pre-mRNA splicing.

**CD133<sup>+</sup> cells:** A type of stem cell or progenitor cell that expresses the CD133 antigen.

**CSA:** Cyclosporin A, an immunosuppressive drug that blocks T-lymphocyte activation.

**Dystrophin:** A large protein linking the actin cytoskeletal components to the plasma membrane.

**Exon skipping:** A mechanism that excludes one or more exons from RNA processing.

**HDACs:** Histone deacetylases, responsible for transcriptional repression.

**HLA:** Human leukocyte antigens, which define the immune phenotype for transplantation.

**MDSC:** Muscle-derived stem cells, isolated from late pre-plating mononucleated muscle cells.

**mdx mouse:** A naturally occurring mouse carrying a mutation in exon 23 of the dystrophin gene.

**Mesoangioblasts:** Stem or progenitor cells associated with the blood vessel wall.

**Microdystrophins, minidystrophins:** Truncated versions of dystrophin that are missing part of the central spectrin-like domains.

**Sarcoglycans:** Small membrane proteins associated with the dystrophin complex.

**Sarcolemma:** The plasma membrane of the muscle fiber.

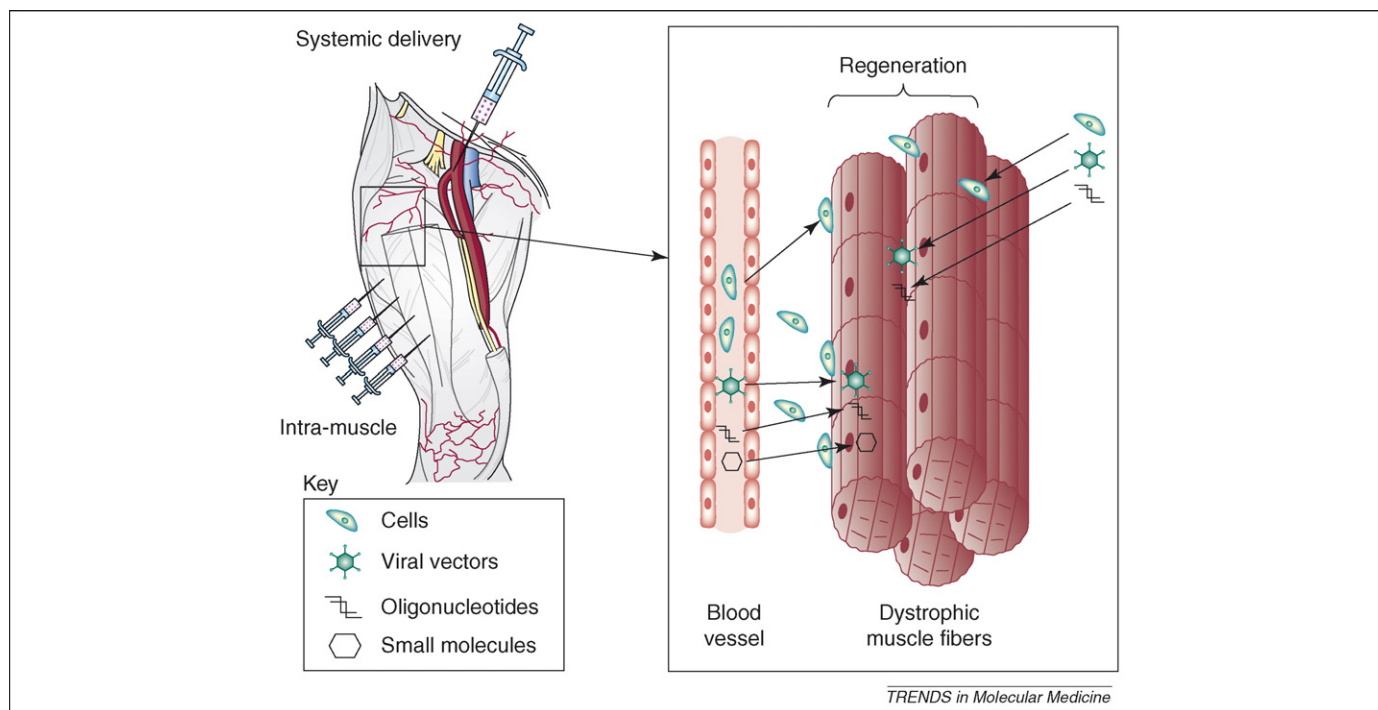
**Satellite cells:** Resident myogenic cells located underneath the basal lamina of muscle fibers.

**snRNPs:** Small nuclear ribonucleoproteins; these can target the donor or acceptor (or both) splice sites during pre-mRNA splicing.

**Side population (SP) cells:** A fraction of bone marrow mononucleated cells that are characterized by dye exclusion.

**Utrophin:** A large cytoskeletal protein, similar to dystrophin and able to compensate for its absence.

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**Figure 1.** Different delivery strategies for cells, viral vectors and small molecules. Intramuscular injection will require multiple injections for delivery to only a small volume of a given muscle. Systemic delivery would overcome this limitation, but crossing the vessel wall and the muscle basal lamina could be a problem. Small molecules will usually be administered orally and thus will reach the muscle through the circulation.

characterized by systemic toxicity. Nevertheless, the results that have accumulated in recent years have opened new perspectives for all these different approaches – drug, gene and cell therapies – and clinical experimentation has begun. It is important to underline that each strategy has advantages and limitations: for example, strategies that repair the dystrophin gene might be suitable only for a subset of mutations, whereas cell and gene therapies are limited by costs related to cell or vector production and can be available only for a limited number of patients [5]. A second consideration is that local delivery of the therapeutic agent is necessary as proof of principle but real clinical benefit can only follow systemic delivery. Similarly, possible adverse events will only be apparent following systemic delivery (Figure 1).

### Gene therapy

#### Strategies to replace the mutated gene

Gene therapy for muscle diseases has faced problems common to other genetic diseases, such as immune response to the vector, and problems specific to muscular dystrophy, such as gene delivery to the majority of post-mitotic muscle fibers. Adenoviral vectors [6] raised much hope in the past but because of their great immunogenicity and large size (limiting diffusion in the muscle tissue) they are no longer used. Adeno-associated vectors are characterized by reduced size and lower, albeit significant, immunogenicity and are currently the vector of choice [7,8]. Because of their limited capacity they cannot accommodate the full-size cDNA of dystrophin (14 kb) although they can be used for smaller genes such as the sarcoglycans. A Phase I and II clinical trial for limb girdle muscular dystrophy 2C – by intramuscular injection in the radial muscle of  $\gamma$ -sarcoglycan-expressing adeno-associated viral (AAV)

vectors – has already started in France; more details are available on the website of the Association Française contre les Myopathies (<http://www.afm-france.org/>).

In the case of dystrophin two strategies are currently being tested in the dystrophic dog and have already entered or are ready to enter clinical experimentation: exon skipping and expression of dystrophin variants of reduced size. For the exon-skipping experiments, adeno-associated type 1 viral vectors were engineered by Luis Garcia's group to produce small nuclear U7 RNA targeting exons 6–8, thus excluding the mutation of dog dystrophin from a slightly shorter but in-frame transcript that is translated in a 'quasi' normal dystrophin protein (see 'Exon skipping' below for more details). Systemic delivery was achieved by high-pressure intravenous delivery. Scaling up AAV production, total body delivery and transient immune suppression to enable reinfusion are being validated to enable the design of a Phase I and II clinical protocol in patients.

In an alternative strategy, minigenes were constructed with reduced number of centrally located spectrin-like domains and preserved amino- and carboxy-terminal domains. The minigenes had been shown to substitute for the wild-type gene when expressed as transgenes in *mdx* mice, a model for DMD [9]. However, the resulting microdystrophins could be less effective in protecting muscle fiber integrity in larger animals, as indicated by dog studies, where human microdystrophin was expressed in dog dystrophic mesoangioblasts (see below) that were later transplanted into the arterial circulation of these animals; these cells failed to maintain force of contraction despite widespread microdystrophin expression [10]. Moreover, AAV injection caused an immune reaction against the viral proteins, although recent experiments

in the dog have indicated a strategy to overcome this immune response [7]. Based on these results, Phase I clinical trials have started in several centers in the USA to establish whether AAV directly injected into skeletal muscle produces functional microdystrophin in human muscle; details are posted on the website of the Muscular Dystrophy Association ([http://www.mdausa.org/research/view\\_trial.aspx?id=174](http://www.mdausa.org/research/view_trial.aspx?id=174)).

In addition to viral vectors, non-viral delivery has also entered clinical experimentation, despite the overall minor efficacy. Direct intramuscular injection of plasmids expressing human dystrophin resulted in protein expression in the site of injection [11]. However, systemic delivery of plasmids still faces significant technical hurdles.

## Cell therapy

### Strategies to replace affected cells

Pioneering experiments in mouse models of DMD demonstrated that myoblasts could be transplanted into dystrophic muscle and give rise to dystrophin-expressing myofibers [12]. This led to several clinical trials in the early 1990s that demonstrated safety but absence of evident functional benefit in the injected muscles. Failure was mainly the result of poor survival and migration of myoblasts and also possibly an immune response to donor myoblasts [13]. Subsequent experimentation has been devoted to optimization of this technique, and a Phase I clinical trial has been completed [14]. Although encouraging results have been obtained, local administration is still limited by the inability to deliver myoblasts systemically through the circulation.

Several adult-derived stem cells have been isolated, including bone marrow-derived stem cells, blood- and muscle-derived CD133<sup>+</sup> cells, muscle-derived stem cells (MDSC), side population (SP) cells (see below) and mesoangioblasts [15–20]. These cells have been characterized and used in animal transplantation experiments, and the results have opened up new possibilities for cell therapy in muscular dystrophy [21]. A few promising examples of stem cells are described below, and a list of adult stem cells used in preclinical animal models of muscular dystrophy is shown in Table 1.

### Bone marrow-derived stem cells

Several studies have demonstrated that wild-type (wt) total bone marrow-derived or side population (BM-SP) cells are incorporated into regenerating skeletal muscle fibers when transplanted into dystrophic mice [15,19,22,23]. However, in some cases transplanted cells failed to restore expression of the wt protein, suggesting that under standard conditions they have little therapeutic potency [24,25].

In other cases results have been more encouraging. For example, bone marrow mesenchymal stem cells, which show little myogenic differentiation, became highly myogenic when engineered to express raised levels of intracellular Notch protein, and produced dystrophin in many fibers when transplanted into *mdx* mice [19].

Another cell population, isolated from blood and from skeletal muscle as a result of expression of the stem cell CD133 antigen, has been shown to give rise to dystrophin-positive fibers when transplanted into *scid* (severe

combined immunodeficiency)/*mdx* mice [15]. Given the demonstrated safety of CD133-positive cells following their intramuscular transplantation [26], future clinical trials with this cell type are now feasible. Clinical trials could also be planned with other types of mesodermal stem cells, but we are currently unaware of preclinical work *in vivo*.

### Mesoangioblasts

So far, systemic delivery in dystrophic mice and dogs has been performed only with mesoangioblasts, which are vessel-associated progenitor cells [16]. Intra-arterial transplantation of donor mesoangioblasts ameliorated defective muscle structure and function in dystrophic mice [27] and dogs [10]. Recently, similar results were achieved with mesoangioblasts transplanted into *mdx*/utrophin null mice [28]. Moreover, isolation and characterization of the human counterparts of mouse mesoangioblasts from adult skeletal muscle revealed that these cells comprise a subpopulation of pericytes, cells associated with the endothelium that give rise to the vessel smooth muscle layer. Pericytes, which probably represent the adult human equivalent of mesoangioblasts, can be expanded *in vitro* and differentiate at elevated frequency into multinucleated muscle cells [20]. Because of these encouraging results, a clinical trial, using donor stem cells from an HLA (human leukocyte antigen)-identical donor, is planned for 2008 at San Raffaele Hospital, Milan. However, controversy recently arose regarding the experiments in dogs, with commentators criticizing the absence of control dystrophic dogs treated only with ciclosporin A (CSA), the general experimental design and the possible overinterpretation of results [29,30]. The possible beneficial effect of CSA in muscular dystrophy is currently being tested in a specific clinical trial in Germany [31]. Indeed, one paper reports beneficial effects of the drug in dystrophic mice [32] whereas three other papers clearly show a deleterious long-term effect, resulting from inhibition by CSA of the calcineurin pathway that is essential for muscle regeneration [33–35]. Regarding the experimental design, studies in dogs necessarily involve a restricted number of animals, which show great variability in the progression of the disease. However, in dogs receiving mesoangioblast transplantation there was extensive expression of dystrophin in up to 70% of the muscle fibers, which showed normal force of contraction and consequent amelioration of defective mobility; this ruled out interindividual variability and suggested that a similar treatment would benefit human patients. No adverse events related to mesoangioblast infusion have been observed in a total of 16 dogs treated [10] or currently under treatment.

## Strategies to repair the endogenous gene

### Exon skipping

Exon skipping of dystrophin gene exons containing a mutation is a promising potential therapy for DMD and other recessive muscular dystrophies [36]. Skipping specific exons would be expected to restore the reading frame and result in the production of internally deleted, but essentially functional dystrophin as observed in the milder Becker muscular dystrophy, thus providing signifi-

**Table 1. Stem cells for cell therapy of muscular dystrophy in preclinical animal models**

Stem cell type	Markers	Animal model	Year	Refs
Bone marrow-derived cells	CD34 <sup>+</sup> , CD45 <sup>+</sup>	<i>mdx</i> mouse	1999	[15]
			2004	[23]
		GRMD	2004	[24]
MABs (mesoangioblasts)	CD44 <sup>+</sup> , CD34 <sup>+</sup> , CD45 <sup>-</sup> , SCA1 <sup>+</sup>	$\alpha$ -SG KO	2003	[27]
		GRMD	2006	[10]
		<i>mdx</i> /utrn (-/-)	2007	[28]
CD133 <sup>+</sup> cells	CD133 <sup>+</sup>	<i>scid</i> / <i>mdx</i>	2004	[18]
MAPCs (multipotent adult progenitor cells)	CD34 <sup>-</sup> , CD45 <sup>-</sup> , CD117 <sup>-</sup> , CD133 <sup>+</sup>	No	2002	[17]
MSCs (mesenchymal stem cells)	CD34 <sup>-</sup> , CD45 <sup>-</sup> , CD44 <sup>+</sup>	<i>scid</i> / <i>mdx</i>	2005	[19]

Abbreviations: GRMD, golden retriever muscular dystrophy; SCA, stem cell antigen; utrn, utrophin;  $\alpha$ -SG KO,  $\alpha$ -sarcoglycan -/-.

cant functional improvement of DMD. Because some deletions and duplications of the dystrophin gene are more common than others, it has been estimated that skipping 12 exons would treat 73.3% of deletions. Among these possibilities, skipping exon 51 was the first choice because it could theoretically be therapeutic for ~20% of dystrophin deletions.

Exon skipping can be achieved by antisense oligonucleotides (AONs) or adeno-associated vectors expressing small nuclear ribonucleoproteins (snRNPs, see above). Both target one or more of the donor splice site, acceptor splice site or exonic sequences essential for exon definition during pre-mRNA splicing of specific exons. Upon binding of the AON or snRNP to a target exon, the exon will be spliced out with its flanking introns and the disrupted open reading frame will be restored. Preclinical studies have produced encouraging results in patient-derived muscle cells *in vitro* and in mice *in vivo* [36–41]. Following preclinical proof-of-concept, the Department of Human Genetics in Leiden has set up a first study in humans in collaboration with Pro-sensa B.V. (<http://prosensa.eu/>). The aim is to obtain clinical proof-of-concept and assess safety and tolerability of a single, local intramuscular injection of PRO051, a 2'-O-methyl RNA with a phosphorothioate backbone that targets exon 51 in the tibialis anterior muscle of DMD patients; more details are posted on the website of the Netherlands Trial Register (<http://www.trialregister.nl/trialreg/admin/retview.asp?TC=712>).

In parallel, the UK MDEX Consortium in collaboration with AVI BioPharma (<http://www.avibio.com/>) has recently started a clinical trial involving intramuscular injection of morpholino AONs targeting exon 51 into the extensor digitorum brevis (EDB) muscle of nonambulant DMD patients. The trial will be a dose escalation study with cohorts of three patients biopsied one month after a single administration, with the contralateral EDB serving as a control; more information is posted on the ClinicalTrials.gov website of the US National Institutes of Health (<http://clinicaltrials.gov/ct/show/NCT00159250;jsessionid=ED5806BC32E7EFF11F42859C2F990C9A?order=12>). In the absence of adverse events, these first intramuscular studies will be followed by systemic trials in patients in The Netherlands and the UK.

To prepare for these systemic trials, research now focuses on different systemic delivery routes and dosing strategies. Strategies to improve delivery of AONs to skeletal muscle and heart are being investigated in the hope of reducing the required dose, which is currently large (and expensive).

#### Skipping premature termination (PTC124)

Nonsense mutations that induce premature stop codons cause premature translational termination in ~15% of the individual cases of DMD [42]. To address the need for a drug capable of suppressing premature termination, PTC124, a new, orally bioavailable agent that selectively induces ribosomal readthrough of premature but not normal termination codons, was identified and studied. PTC124 promoted dystrophin production in primary muscle cells from humans and *mdx* mice within 2–8 weeks of drug exposure [43]. PTC124 was well tolerated in animals at plasma exposures substantially in excess of those required for nonsense mutation suppression [44]. The selectivity of PTC124 for premature termination codons, its well-characterized activity profile, oral bioavailability and pharmacological properties all suggest that this drug has broad clinical potential for the treatment of a large group of genetic disorders with currently limited or no therapeutic options. July 2007 marked the completion of a Phase I trial in healthy volunteers using different doses of the drug, which was well tolerated up to 100 mg/kg. The pharmacokinetics suggested dosing three times a day (10, 10, 20 mg/kg). Three US centers have also completed a Phase II trial for DMD patients and final analysis will be available by the end of 2007; more details are available on the website of the Muscular Dystrophy Association ([http://www.mdausa.org/research/view\\_ctrial.aspx?id=153](http://www.mdausa.org/research/view_ctrial.aspx?id=153)).

#### Drug therapy

##### Compensating for absence of dystrophin

**Utrophin upregulation** Utrophin is a large protein, similar to dystrophin. It is expressed abundantly during muscle development but is later replaced by dystrophin in the muscle, with expression restricted to the neuromuscular junction [45]. In the absence of dystrophin, utrophin is upregulated, but not to a level sufficient to compensate functionally for the loss of dystrophin and prevent the progression of muscular dystrophy. Previous work has shown that increasing the expression of utrophin in the muscles of *mdx* mice by three- to fourfold is sufficient to prevent or dramatically reduce muscular dystrophy pathology [46].

After a detailed analysis of the utrophin promoter, a high-throughput screen was carried out by Summit plc (formerly VASTox plc; <http://www.summitplc.com>), searching for a chemical drug that would upregulate utrophin in a muscle cell line. Potential candidate drugs were validated by testing their ability to upregulate the endogenous protein *in vitro* and eventually *in vivo* in the *mdx* mouse. At the same time cognate molecules with greater activity and reduced side effects were synthesized. Several different classes of compounds showed varying degrees of activity; subsequent optimization of a favored class yielded a preclinical development candidate that passed the var-

ious *in vivo* efficacy tests, including reduction of eccentric contraction damage, fibrosis and inflammation in dystrophic muscle. Preclinical development is in progress and a first trial in humans is planned for 2008; more information is available from the Summit plc website (<http://www.summitplc.com/uploads/rns.pdf>).

#### *Improving function in dystrophic muscle*

Attempts to replace or correct the mutated gene might result in a definitive solution for muscular dystrophy but, as discussed above, this is not easy to achieve. Alternative strategies that prevent or delay muscle degeneration, reduce inflammation or promote muscle metabolism or regeneration might all benefit patients and, in the future, synergize with gene or cell therapy. Steroids that reduce inflammation are currently the only therapeutic tool used in the majority of DMD patients [47,48]. Many different strategies have been developed and the most promising will be described here in detail. More extensive recent reviews can be consulted [49–51].

#### *Promoting muscle hypertrophy and reducing muscle wasting*

Experiments to upregulate the expression levels of specific genes involved in muscular growth have been successful in ameliorating the pathology of dystrophic-cultured myotubes and dystrophic mice. Examples agents that upregulate gene expression are nitric oxide synthetase (NOS) [52], L-arginine (which is a NOS substrate),  $\beta_7\beta_1$ -integrin [53], synaptic cytotoxic T-cell GalNAc (acetyl galactosamine) transferase (Galgt2) [54], insulin-like growth factor 1 (IGF-1) [55], calpastatin [56] and the metalloprotease ADAM12 (a disintegrin and metalloprotease 12) [57]. Interestingly, following the overexpression of many of these genes, there was an increase not only in the levels of many dystrophin-associated proteins, but also in the amount of utrophin.

Myostatin is a negative regulator of muscle mass, and several studies have revealed the therapeutic potential of antagonizing the myostatin pathway. The use of antibodies specifically to block the action of myostatin [58] has led to functional improvement of dystrophic muscle in mice.

Based on these results Wyeth Pharmaceuticals (<http://www.wyeth.com/>) developed neutralizing antibodies, among which MYO-029 has entered clinical trials for different forms of muscular dystrophy. No severe adverse events were observed in the short term, other than hypersensitivity to the antibody in some patients. Long-term safety and efficacy are currently being tested; further information is available on the website of the Muscular Dystrophy Association ([http://www.mdausa.org/research/view\\_trial.aspx?id=138](http://www.mdausa.org/research/view_trial.aspx?id=138)). In the future this therapy could be combined with gene or cell therapy.

**Histone deacetylase inhibitors** Pharmacological blockade of histone deacetylases (HDACs) leads to the upregulation of regeneration-activated genes and results in the formation of hypernucleated, larger-than-normal myotubes. One of the genes selectively upregulated by deacetylase inhibitors (DIs) in regenerating muscle progenitors is follistatin, which counters the activity of myostatin, mentioned above, which is a negative regulator of muscle

regeneration and postnatal growth. The therapeutic efficacy of DIs in mouse models of muscular dystrophy was tested by daily intraperitoneal injections of the DI trichostatin A (TSA) or MS27 (a selective inhibitor of class I deacetylases) in 3-month-old dystrophin-deficient (*mdx*) mice [59]. This treatment increased the size of skeletal muscles and alleviated the morphological and pathological consequences of the primary genetic defect. These data demonstrate that DIs counter the progression of muscular dystrophy by boosting muscle regeneration, and suggest their use in the pharmacological therapy of neuromuscular diseases [60].

**NO-releasing anti-inflammatory agents** A beneficial effect in muscle repair has been reported after the administration of nitric oxide (NO) using a NO-releasing derivative of flurbiprofen, a non-steroidal anti-inflammatory drug [61]. Long-term (one-year) oral treatment, with a nitric oxide-releasing derivative of flurbiprofen named HCT 1026, of murine models for limb girdle muscular dystrophy and DMD ( $\alpha$ -sarcoglycan null and *mdx* mice, respectively), significantly ameliorated the morphological, biochemical and functional phenotype in the absence of secondary effects. The drug efficiently slowed down disease progression by reducing inflammation, preventing muscle damage, and preserving the number and function of satellite cells. As an additional striking beneficial effect, HCT 1026 enhanced the therapeutic efficacy of arterially delivered donor stem cells, by increasing fourfold their ability to migrate and reconstitute muscle fibers.

#### **Concluding remarks**

Research into therapeutic approaches for DMD has moved rapidly in recent years. In 2000, we described initial experiments suggesting a future clinical translation as 'wishful thinking' [62]. Only four years later, we and others pointed out that experiments in animal models were paving the way to clinical experimentation [63–66]. Now several clinical trials have started and results will soon be available; these first studies are aimed at proof of concept and testing of safety and tolerability. Therefore, it would be unwise to expect a 'cure' from these first attempts. Nevertheless, the results of trials with PTC, myostatin neutralizing antibodies and AAV will be known soon and encouraging outcomes are expected. In any case, these studies will provide a basis for further, larger clinical trials, in which functional benefit will be assessed and the stage set for more improvements, possibly involving combinatorial therapies. It is important to emphasize that most current strategies cannot be applied to all patients: for example, exon skipping is only feasible for mutations that do not affect the essential parts of dystrophin; PTC will only be beneficial for nonsense mutations; AAV might not be administered to patients who are already immunized against the natural virus; and cell therapy could initially be applied only to a small cohort of patients because of its great cost, the laborious expansion of cells under clinical grade conditions and the potential risks associated with intravascular infusion of a large number of circulating nonhematopoietic cells.

Other molecules that upregulate utrophin or counteract muscle degeneration (HDAC inhibitors, myostatin blocking agents, NO donors), in addition to novel anti-inflammatory reagents, might contribute to slowing down or even halting progression of the disease. It is likely that several additional trials using these molecules will start in the near future. Although less specific, these treatments would be less expensive and feasible for all dystrophic patients.

Money and time are limiting factors for all approaches but particularly for gene and cell therapy. The current stringent regulatory constraints will delay significantly clinical translation of novel approaches and will raise the cost to a point that, even in successful cases, will make it impossible to apply them to all eligible patients. Therefore strategies to lower costs are required to develop treatments that are available for large numbers of patients.

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