PHARMACOLOGICAL STRATEGIES FOR MUSCULAR DYSTROPHY

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Duchenne muscular dystrophy (DMD) is a fatal, genetic disorder whose relentless progression underscores the urgency for developing a cure. Although Duchenne initiated clinical trials roughly 150 years ago, therapies for DMD remain supportive rather than curative. A paradigm shift towards developing rational therapeutic strategies occurred with identification of the *DMD* gene. Gene- and cell-based therapies designed to replace the missing gene and/or dystrophin protein have achieved varying degrees of success. However, pharmacological strategies not designed to replace dystrophin *per se* appear promising, and can circumvent many hurdles hampering gene- and cell-based therapy. Here, we will review present pharmacological strategies, in particular those dealing with functional substitution of dystrophin by utrophin and enhancing muscle progenitor commitment by myostatin blockade, with a view toward facilitating drug discovery for DMD.

DUCHENNE MUSCULAR DYSTROPHY
(DMD) A common, genetic neuromuscular disease associated with progressive deterioration of muscle function. In about a third of cases DMD is also associated with impairment of cognitive ability.

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The muscular dystrophies are a heterogeneous group of neuromuscular disorders, the most common being DUCHENNE MUSCULAR DYSTROPHY (DMD). The disease is named after the French neurologist Duchenne de Boulogne, who described the disorder c. 150 years back. Duchenne also devised a special harpoon-like needle (*emporte-pièce histologique*) and procedure (the muscle biopsy) to unequivocally demonstrate the devastating and progressive nature of the disease[1]. Meryon described similar cases from England, which reflects the global and common nature of the disease. A major breakthrough in the study of DMD came with the identification of the *DMD* gene by positional cloning[2]. The disease (and its milder allelic variant, Becker muscular dystrophy) is caused by mutations in the *DMD* gene that lead to quantitative and qualitative disturbances in the expression of the dystrophin protein[3]. Dystrophin is a member of the spectrin superfamily of proteins[4], which includes the spectrins, the α-actinins, and dystrophin and its related proteins. Three close relatives of dystrophin form the DYSTROPHIN-RELATED PROTEIN family: the chromosome-6 encoded dystrophin-related protein utrophin, or DRP[5–7], the chromosome-X encoded DRP2 (REF. 8) and the chromosome-18 encoded

dystrobrevin[9–11]. Dystrophin is biochemically associated with the membrane-bound dystrophin–glycoprotein complex (DGC), which forms an important link with laminin, a constituent of the extracellular matrix (FIG. 1). The DGC is part of a larger complex of proteins associated with dystrophin, which includes dystroglycan, sarcoglycan, neuronal nitric oxide synthase (nNOS), the syntrophins, dystrobrevin and utrophin[12–14]. Mutations in the genes encoding various members of the DGC (and proteins binding members of the complex, for example, α2 laminin) disrupt SARCOLEMMAL integrity and result in a variety of muscular dystrophies (TABLE 1). The reader is referred to the Further Information at the end of this review for more details on the clinical applications of basic research into DMD.

DMD patients seem able to compensate for the disease process until around five years of age when they present, typically with difficulties in climbing up stairs and keeping up with their peers at play. Ongoing muscle damage, tissue inflammation, attempts at REGENERATION, progressive muscle loss and replacement of healthy muscle with fibro-fatty tissue, and, ultimately, muscle wasting with contractures are the unfortunate consequences of this disease[1,15,16].
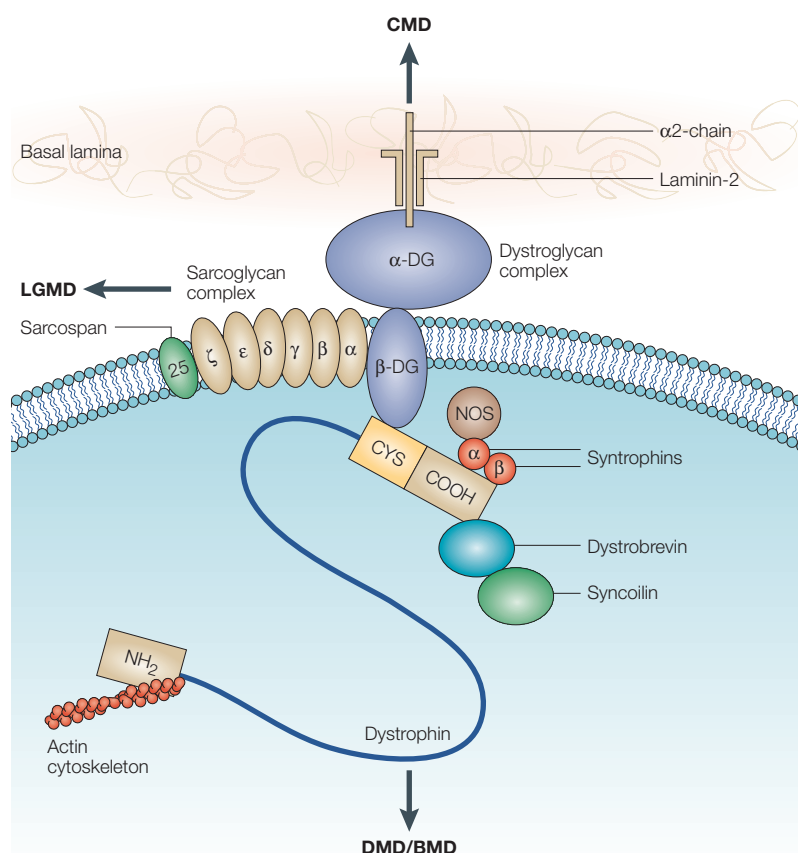
Figure 1 | **The muscular dystrophies and organization of the dystrophin–glycoprotein complex.** Schematic representation of the organization of the dystrophin–glycoprotein complex (DGC) and aetiology of the muscular dystrophies. Dystrophin interacts with cytoplasmic, transmembrane and extracellular proteins in skeletal muscle, reminiscent of the manner in which spectrin interacts with integral and peripheral members of the cytoskeleton in red blood cells. Mutations in dystrophin and other members of the DGC give rise to a variety of muscular dystrophies (TABLE 1). BMD, Becker muscular dystrophy; CMD, congenital muscular dystrophy; CYS, cysteine; DG, dystroglycan; DMD, Duchenne muscular dystrophy; LGMD, Limb girdle muscular dystrophy; NOS, nitric oxide synthase.

DYSTROPHIN-RELATED PROTEINS
A family of proteins that are structurally and functionally related to dystrophin, consisting of utrophin or DRP, DRP2 and dystrobrevin. Utrophin seems capable of functionally substituting for the missing dystrophin and improving muscle pathology when experimentally overexpressed.

SARCOLEMMA
A thin membrane enclosing a striated muscle fibre.

All skeletal muscles are involved, except the extraocular muscles (EOM)[17–19]. Typically, DMD patients are wheelchair bound in their early teens and usually die of cardiac or respiratory causes by the third decade of their life, underscoring the importance of developing cures for this disorder.

The natural history of this disease, which seems depressingly relentless, does, however, offer a glimmer of hope from a therapeutic perspective. The delayed onset of disease, the central role of inflammation, the attempts and failure to regenerate muscle sufficiently, the existence of dystrophin-related proteins and the sparing of EOM provide clues to the pathways and mechanisms that can be pharmacologically targeted, either in combination with, or independently of, gene- and cell-based therapies designed to correct the primary molecular lesion.

Animal models of DMD

A number of natural and engineered animal models of DMD exist, and provide a mainstay for preclinical studies[20]. Although the mouse, cat and dog models all have mutations in the *DMD* gene[21–23] and exhibit a biochemical dystrophinopathy similar to that seen in humans, they show surprising and considerable variation in terms of their phenotype (TABLE 2). Like humans, the canine (Golden retriever muscular dystrophy and German short-haired pointer) models[22,24] have a severe phenotype; these dogs typically die of cardiac failure. Dogs offer the best phenocopy for human disease, and are considered a high benchmark for preclinical studies. Unfortunately, breeding these animals is expensive and difficult, and the clinical time course can be variable among litters.

The *mdx* mouse is the most widely used model due to availability, short gestation time, time to mature and relatively low cost[21]. Additional mutations (*mdx* 2-5[cv]) have also been described[25]. Unfortunately, the *mdx* mouse has a mild phenotype; muscle is histologically normal at birth, despite exhibiting high creatine kinase (CK) levels. The first wave of NECROSIS occurs around the third week after birth and continues for about a month. Active regeneration seems able to compensate for ongoing muscle damage in *mdx* skeletal muscle and the necrosis wanes, in contrast to the case in humans in which the muscle is progressively damaged and often replaced by fibro-fatty tissue in advanced cases. Indeed, apart from the diaphragm in aged mice, *mdx* skeletal muscles do not resemble muscle from advanced stages of DMD[26]. Although there is no 'standardized' method to evaluate the efficacy of an intervention in DMD, a consensus is emerging that a combination of anatomical, biochemical and physiological assays offer reasonably objective evaluation[27–30]. In particular, an increase of muscle strength and resistance to damage by lengthening or ECCENTRIC CONTRACTIONS are considered good prognostic indicators, in part because DMD patients have prominent deficits according to these measures. Including a cohort of normals along with control and treated dystrophic animals offers the advantage of being able to peg the degree of improvement achieved to the degree of improvement needed for achieving normalcy; information that can even be expressed numerically as a 'recovery score'[31]. So, the mild phenotype of the *mdx* mouse does not preclude its use; rather it simply necessitates appropriate study designs that maximize the information content of a potentially therapeutic intervention.

Two other animal models are noteworthy: first, the *utr*^{−/−} *mdx* mouse[32,33] which, although strictly speaking is not a true genetic model of DMD (as patients do not lack utrophin), is nonetheless a good phenocopy of DMD; and second, mice created by breeding the *mdx* mutation onto immunodeficient backgrounds, such as the *nu*^{−/−} *mdx* mice[34]. These offer the possibility of testing therapeutic reagents without some of the confounding immune responses.

Utrophin

In 1989, a paralogue of dystrophin was identified on human chromosome 6 (REF. 5). This dystrophin-related gene was later called utrophin[7] because, in contrast to dystrophin, it is ubiquitously transcribed. This created a

Table 1 | **Genetic classification of muscular dystrophies**

Disease	Inheritance pattern	OMIM number	Locus	Gene product
Dystrophinopathies				
Duchenne/Becker MD	Chromosome X	310200	Xp21.2	Dystrophin
Emery Dreifuss MD	Chromosome X	310300	Xq28	Emerin
Limb girdle MD (LGMD)				
Type 1A	Autosomal dominant	159000	5q31	Myotilin
Type 1B	Autosomal dominant	159001	1q21.2	Laminin A/C
Type 1C	Autosomal dominant	601253	3p25	Caveolin 3
Type 1D	Autosomal dominant	603511	7q	Not known
Type 1E	Autosomal dominant	603511	7q	Not known
Type 2A	Autosomal recessive	253600	15q15.1–q21.1	Calpain 3
Type 2B (Miyoshi myopathy)	Autosomal recessive	253601	2p13.3–p13.1	Dysferlin
Type 2C	Autosomal recessive	253700	13q12	γ-Sarcoglycan
Type 2D	Autosomal recessive	600119	17q12–q21.33	α-Sarcoglycan (adhalin)
Type 2E	Autosomal recessive	600900	4q12	β-Sarcoglycan
Type 2F	Autosomal recessive	601287	5q33	δ-Sarcoglycan
Type 2G	Autosomal recessive	601954	17q12	Telethonin
Type 2H	Autosomal recessive	254110	9q31–q34.1	E3-ubiquitin ligase
Type 2I	Autosomal recessive	606596	19q13.3	Fukutin-related protein
Type 2J	Autosomal recessive	Not available	2q24.3	Titin
Congenital MD (CMD)				
Merosin positive				
Rigid spine syndrome	Autosomal recessive	602771	1p36–p35	Selenoprotein N1
Ullrich syndrome	Autosomal recessive	254090	21q22.3	Collagen VI subunit α2
Merosin deficient without brain involvement				
Type 1A	Autosomal recessive	156225	6q22–q23	α2-Laminin (merosin)
Type 1B	Autosomal recessive	604801	1q42	Not known
Type 1C	Autosomal recessive	606596	19q13.3	Fukutin-related protein
CMD with brain involvement				
Fukuyama CMD	Autosomal recessive	253800	9q31	Fukutin
Walker-Warburg syndrome	Autosomal recessive	236670	9q34	O-Mannosyltransferase
Muscle–eye–brain disease	Autosomal recessive	253280	1p34–p33	POMGNT1

MD, muscular dystrophy; OMIM, Online Mendelian Inheritance in Man (http://www.ncbi.nlm.nih.gov/omim/); POMGNT, protein *O*-mannosyl-β-1,2-*N*-acetylglucosaminyltransferase.

REGENERATION
Muscle has limited ability to regenerate and repair itself when damaged. Cells contained within mature muscle known as satellite cells (sometimes referred to as committed stem cells), proliferate in response to damage and attempt to repair it.

NECROSIS
The death of a cell due to external damage or the action of toxic substances. Distinct from programmed cell death (apoptosis), which is a normal part of the developmental process.

ECCENTRIC CONTRACTION
Skeletal muscle usually contracts when stimulated. Lengthening of muscle during an active contraction is known as an eccentric contraction (ECC). Dystrophic muscle is particularly susceptible to damage during ECC.

lot of interest, as it raised the possibility that utrophin might be able to replace dystrophin in DMD patients. Utrophin is slightly smaller than dystrophin, with a messenger RNA (mRNA) of 13 kb that corresponds to a protein of 3,433 amino acids and a predicted molecular weight of 395 kDa. The primary structure of utrophin is very similar to that of dystrophin, particularly in the N- and C-terminal ends that bind other proteins. The N terminus of utrophin is similar to actin-binding regions identified in dystrophin, spectrin and α-actinin. The crystal structure[35] and binding affinities[36] of this domain are similar for utrophin and dystrophin. However, utrophin lacks the additional actin-binding activity associated with the dystrophin rod domain, and the N terminus of utrophin contains a short extension not found in dystrophin, which contributes to its affinity for actin[37]. In view of the similarity in the primary structures of their C termini, it is not surprising to find that

utrophin binds members of the DGC[14,38]. However, dystrophin and utrophin might bind different isoforms of the associated proteins. For example, utrophin has been reported to co-localize with α-syntrophin and to be lost in α-syntrophin-null mutant mice, whereas dystrophin and other isoforms of syntrophin are unaltered[39].

Utrophin is widely expressed, and continues to be expressed in DMD patients at elevated levels[6,40]. Utrophin is expressed not only in skeletal, cardiac and smooth muscle cells, but also in vascular endothelia, retinal glial cells, platelets, Schwann cells of the peripheral nerves and several cell types within the kidney (for a review see REF. 38). Utrophin is enriched at the neuromuscular junction (NMJ)[41–43] and myotendinous junctions in normal adult skeletal myofibres[41]. At the NMJ, utrophin is found at the crests of the junctional folds, in contrast to dystrophin, which is found mainly

Table 2 | **Dystrophinopathy across different species**

	Human	**Dog**	**Cat**	**Mouse**
X-linked disorder	+	+	+	+
Gene(s)	*DMD*	*GRMD*, *GSHP*	*FHMD*	*mdx*, *mdx*2–5 cv
Mutations	Deletions, point, duplications and splicing	Splicing and deletions	Promoter	Point and deletions
Dystrophinopathy	+	+	+	+
Muscle weakness	++	++	–	–
Absolute force	–	–	?	+
Specific force	–	–	?	–
Myofibre hypertrophy	+	+	+	+
Muscle wasting (old)	+++	+++	–	–
Serum creatine kinase	+++	+++	+++	+++

in the troughs[44,45]. However, in developing muscle, utrophin is localized at the sarcolemma along the entire length[46], a distribution also seen in regenerating muscle[41,47]. Studies of utrophin in tissue culture support the view that expression of utrophin could precede expression of dystrophin in development[48]. Indeed, the necrosis of *mdx* limb muscle begins only when the high neonatal levels of utrophin in muscle reduce to adult levels. The developmental time course suggests that two- to threefold upregulation of utrophin could be sufficient to prevent or delay the onset of necrosis in *mdx* mice[41].

Surprisingly, utrophin-null mutant mice seem normal[49,50], and so it has been proposed that utrophin might play a role in stabilizing the acetylcholine receptor (AChR) complex at the NMJ[49,50]. Indeed, *utr*^{−/−} mice show reduced folding at the NMJ, perhaps as a consequence of the reduced density of AChRs. Further evidence that utrophin and dystrophin play complementary roles in muscle was provided by the generation of a mouse deficient in both utrophin and dystrophin (*utr*^{−/−} *mdx*)[32,33]. These mice suffer from a progressive muscular dystrophy similar to that seen in DMD and die very prematurely. Muscle disease, rather than a cardiomyopathy, seems to account for this, because expression of a utrophin transgene in skeletal muscle alone prevents it[51]. Though the morphology of the NMJ appears more abnormal in double mutants than in either of the single mutants, this does not seem to impair their electrophysiological properties. Fibre necrosis starts earlier in the double knockout mice compared with *mdx* mice[32,33]. The double null mutant phenotype is consistent with the suggestion that sufficient functional redundancy exists between utrophin and dystrophin for the small amount of sarcolemmal utrophin in dystrophin-deficient muscle to partially compensate for the absence of dystrophin. This compensation is lost when utrophin is also absent, resulting in a more severe phenotype.

The hypothesis that utrophin and dystrophin might be functionally redundant is further strengthened by the generation of several lines of *mdx* mice that harbour utrophin transgenes that can ameliorate the dystrophic phenotype[52,53]. These transgenes consist of either truncated utrophin (lacking a section of the rod domain) or full-length utrophin whose expression is driven by a human actin skeletal muscle PROMOTER. In these mice, utrophin localizes throughout the sarcolemma and the DGC is reconstituted. Even a modest two- to threefold increase of utrophin over wild-type levels was found to be sufficient for some degree of morphological and functional recovery; far better recovery was noted with higher levels of utrophin upregulation[52,53]. Similar results have been obtained using viral vectors to deliver utrophin[54]. These studies show that increasing utrophin levels over the normal adult levels significantly prevents pathology, as judged by histological, biochemical and physiological criteria[31,52]. The basis of the success of utrophin as surrogate for dystrophin comes from the ability of the former to play a structural role and restore the mechanical continuity between cytoskeletal actin, DGC and the extracellular matrix. This is highlighted by the recovery of resistance to mechanical stress. As 'activity-induced damage' is probably one of the factors initiating muscle necrosis, the protection conferred by utrophin is a fundamental benefit.

Utrophin and dystrophin are most likely to have arisen from a duplication early in vertebrate evolution[55], as their similarities extend beyond their primary structures. Utrophin too is encoded by multiple small exons arranged over a very large genomic region (about 1 Mb). The transcript is transcribed from several promoters (FIG. 2), and a variety of shorter C-terminal isoforms have also been described[56]. Two promoters (A and B) have been described at the 5′ end of the utrophin gene that give rise to full-length transcripts and protein[57,58]. The basal transcription of promoter A is also controlled by a downstream utrophin-enhancer element[59]. The transcripts expressed from promoters A and B produce proteins that differ at their N-termini with unique sections of 31 and 26 amino acids, respectively. Using antibodies specific for these regions, it has been demonstrated that it is utrophin A that is expressed at the NMJ and upregulated in regenerating muscle; the expression of utrophin B is mainly confined

PROMOTER
A region of a gene that controls expression of that gene. Its activity is controlled by transcription factors that in turn are activated or repressed by a number of extra- and intracellular mechanisms.
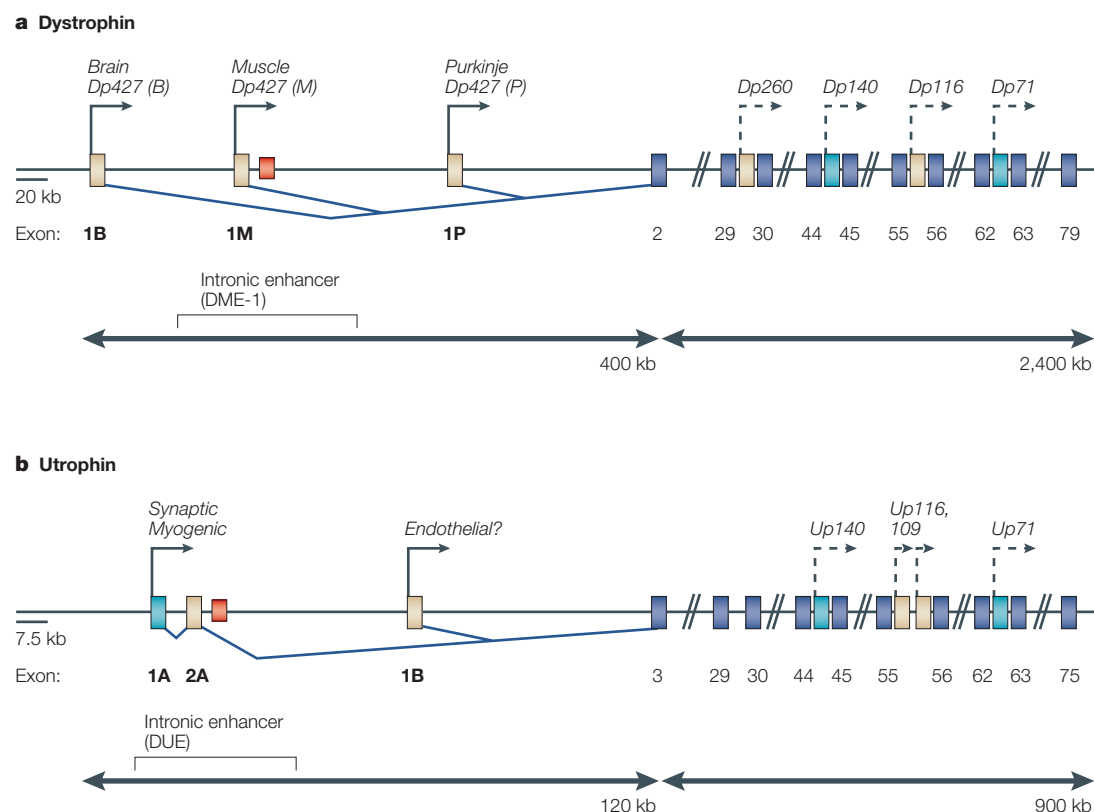
a Dystrophin

b Utrophin

Figure 2 | **Identified dystrophin and utrophin human isoforms and associated promoters.** The organization of the genes encoding dystrophin and utrophin are depicted schematically. Within the respective diagrams, common exons relative to full-length isoforms (blue), unique translated first exons (yellow), and unique untranslated first exons (green) are as indicated. Arrows indicate transcription start sites; red boxes (with captions) are characterized enhancer elements. The 5′ genomic regions between exons and enhancers to exon 2 (dystrophin) or 3 (utrophin) are to scale; all other regions are not to scale.

to the vascular endothelia[60]. Promoter A is CpG rich and, in part, responsible for the synaptic expression of the gene, because it contains an N-box motif, which is well established as the element contributing to the synaptic expression of genes encoding subunits of the AChR[61,62]. The N-box binds the ets-related transcription factor complex GA-binding protein (GABP) α/β and mediates transcriptional activation of the utrophin A promoter by the neurite-associated growth factor heregulin[63,64]. Heregulin-mediated activation of GABP α/β occurs through the extracellular signal-regulated kinase signalling cascade[64]. Utrophin A promoter activation by GABP α/β is further enhanced by the transcription factors Sp1 and Sp3, although the exact location of the Sp1-response element in the promoter is unclear[65–67]. The A promoter also drives the MYO-GENIC induction of utrophin expression, and this effect is mediated through the binding of myogenic regulatory factors to the evolutionarily conserved consensus E-box[66]. *In vitro* studies have shown that the B core promoter is transactivated by Ap1 (REF. 68) and ets/GABP[68,69] in a similar manner to that observed for other endothelial promoters. Functional synergism was found with GATA2 and c-jun for B promoter activation[68], although additional factors must confer the specific endothelial expression pattern, as GATA2 and

c-jun are widely expressed. These studies suggest that the systemic delivery of transcription factors, signalling molecules and peptide fragments encoding active domains of growth factors represents potential pharmacological approaches to upregulate utrophin *in vivo* (FIG. 3).

More recently, the use of nitric oxide donors or substrates of NOS, such as L-arginine, has been reported to enhance utrophin expression *in vitro*[70] and *in vivo*[71]. Increased expression of integrins[72] and T-cell GalNAc transferase[73] through the introduction of transgenes into *mdx* muscle has been shown to improve pathology as well as increase the levels of utrophin. It is not clear that these effects are due directly to the increased levels of utrophin, nor that the utrophin increase is due to transcriptional activation. Indeed, increases in utrophin can be observed as a result of post-transcriptional mechanisms, such as greater mRNA or protein stability[74,75].

If small molecules can be used to increase the levels of utrophin, the problems of toxicity and timing of delivery become important issues. Studies of mice expressing utrophin transgenes under the control of a ubiquitin C promoter suggest that increased expression in multiple tissues is not toxic[76]. Analysis of a utrophin transgene under the control of an inducible promoter indicated that delivery at birth prevented

MYOGENIC
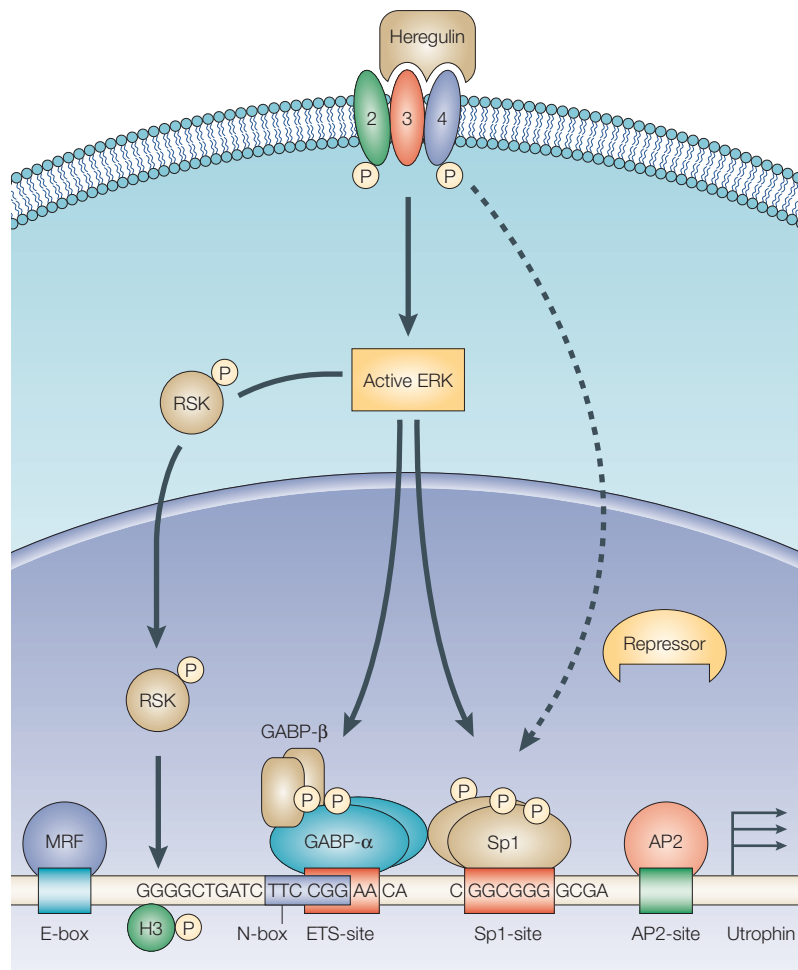Originating in or produced by muscle cells.

Figure 3 | **Model for transcriptional activation of the utrophin promoter A.** The model shows mechanisms of transcriptional activation of the utrophin promoter in muscle. In unstimulated muscle culture (and adult muscle), utrophin is transcribed at low levels, possibly because of transcriptional repressors, such as Ets2 repressor factor (ERF) or ERF-like molecules, acting on the N-box[136]. Transcription can be activated at multiple levels: extracellular growth factors, intracellular signalling cascades and transcription factors in the nucleus. AP2, activating enhancer-binding protein-2; ERK, extracellular signal-regulated kinase; ETS, erythroblastosis virus oncogene homologue ; GABP, GA-binding protein; H3, histone H3; MRF, myogenic regulatory factor; RSK, ribosomal S6 kinase; Sp1, transcription factor.

MYOBLAST
An undifferentiated cell in the mesoderm of the vertebrate embryo that is a precursor of a muscle cell.

CALCIUM HOMEOSTASIS
The ability of a cell to maintain the requisite intracellular calcium concentration. The body and individual cells have homeostatic mechanisms that can compensate or buffer the lowering or excess levels of intracellular calcium to some degree.

pathology, and even that induction at later stages had a therapeutic benefit, but less than that noted with delivery at birth[77]. All of these data were generated in mice that are able to regenerate efficiently, which is not the case in humans. Nevertheless, there is reason for some optimism, as the delivery of utrophin using adenovirus did improve the pathology in the dystrophic dog model[78], which is considered to be a better benchmark for preclinical studies.

Present therapeutics

In the less than 20 years since the *DMD* gene was discovered, varying degrees of success in the treatment of DMD have been achieved in preclinical animal studies, some of which are being followed up in humans. Present therapeutic strategies can be broadly divided into three groups: first, gene therapy approaches; second,

cell (MYOBLAST/stem cell) therapy; and last, pharmacological therapy. Gene- and cell-based therapies offer the fundamental advantage of obviating the need to separately correct secondary defects/ pathology (for example, contractures), especially if initiated early in the course of the disease. Unfortunately, these approaches face a number of technical hurdles. Immunological responses against viral vectors, myoblasts and newly synthesized dystrophin have been reported, in addition to toxicity, lack of stable expression and difficulty in delivery. However, steady progress is being made in overcoming these hurdles and, at the time of writing, the first gene therapy trials in DMD patients have been initiated at the Hopital Pitie-Salpetriere, Paris[79,80]. We refer the reader to BOX 1 for an overview of gene- and cell-based therapies. In this review, we will focus on pharmacological strategies that illustrate some of the breadth and future scope of development for this class of DMD therapies.

Pharmacological approaches

Pharmacological approaches for the treatment of muscular dystrophy differ from gene- and cell-based approaches in not being designed to deliver either the missing gene and/or protein. In general, the pharmacological strategies use drugs/molecules in an attempt to improve the phenotype by means such as decreasing inflammation, improving CALCIUM HOMEOSTASIS, upregulating compensatory proteins such as utrophin, and increasing muscle progenitor proliferation or commitment. With the exception of gentamicin, present pharmacological approaches are not even designed to correct the primary defect. These strategies offer the advantage that they are easy to deliver systemically and can circumvent many of the immunological and/or toxicity issues that are related to vectors and cell-based therapies. The major disadvantage is that if used singly, pharmacological approaches might improve only a specific and limited component of secondary pathology, rather than the overall phenotype (gentamicin and utrophin upregulation are exceptions, as these strategies would be predicted to achieve broader therapeutic effects). The combinatorial use of multiple agents could help broaden the therapeutic benefit; however, combinations might increase the chances of adverse drug interactions and complicate the evaluation of improvement, especially in clinical studies.

Corticosteroids. The beneficial effects of corticosteroids on DMD patients were first noted about 40 years ago[81,82]. The exact mechanism by which steroids help remains unclear, perhaps as a result of their broad range of effects on cellular metabolism. Anabolic effects, reduction of tissue inflammation/ immunosuppression of cytotoxic cells, improved calcium homeostasis, utrophin upregulation, and stimulation of myoblasts are probable mediators for steroids, and perhaps act in combination. A number of trials have demonstrated improvement, particularly in the short-term, using prednisone and/or its derivative prednisolone on

defined DMD patients[83,84]. However, the long-term benefits are less obvious. More recently, a number of groups have achieved encouraging results with deflazacort, an oxazoline derivative of prednisolone[85]. Additional issues related to the use of steroids concern the type of side effects caused by different classes of steroids, and the exact dosage regimen that can reduce these side effects while preserving the therapeutic benefits[86].

Maintaining calcium homeostasis. A number of lines of evidence suggest a dysregulation of calcium homeostasis in dystrophic muscle. DMD patients accumulate abnormal amounts of intracellular calcium within affected muscles[87], but not in EOM[19], which are clinically and histologically spared. Furthermore, the EOM seem better able to maintain their calcium homeostasis, as evidenced by their ability to resist the necrosis induced by pharmacologically elevating intracellular calcium using calcium ionophores and/or blockers of the SARCOPLASMIC RETICULUM calcium re-uptake pump[19]. Expression profiling of EOM also indicates gene expression patterns consistent with a greater homeostatic ability[88]. The increased numbers of stretch-sensitive voltage-independent calcium channels noted in dystrophic muscle might also contribute to elevated calcium flux, which in turn is thought to result in an increased rate of protein degradation due to activation of calcium-dependent enzymes[89,90]. However, the effect of increased calcium-channel activity on increasing the cytosolic calcium concentration has been challenged[91], and in transgenic mice that display moderate utrophin overexpression a complete correction of the abnormal behaviour of these channels has been obtained without abolition of the dystrophic process[77]. It remains to be established whether the loss of calcium homeostasis is the primary initiating event of fibre dystrophy, or a secondary one resulting from fibre microlesions. Clinical trials using the calcium-channel blockers nifedipine and diltiazem have proved equivocal. Dantrolene, a drug that inhibits calcium release from sarcoplasmic reticulum, has, however, been reported to have some beneficial effect in a pilot study[92]. Downstream targets of the calcium homeostasis dysregulation have been successfully targeted using the broad-spectrum calpain inhibitor leupeptin (*N*-acetyl-leucyl leucyl-argininal) in *mdx* mice[93]. A reduction of

necrosis has also been demonstrated by breeding *mdx* mice with mice that transgenically overexpress calpastatin[94], a specific inhibitor of calpain 1 and calpain 2. These studies identify calcium homeostasis as an important pathway with potential to be manipulated[95]; the challenge lies in developing pharmacological reagents and regimens that specifically correct the homeostatic dysregulation, rather than simply reducing or chelating intracellular calcium. This is essential to avoid disrupting or adversely effecting the essential role(s) that calcium plays in normal cellular metabolism.

Decreasing inflammation. For a long time, the inflammation noted in DMD muscle was thought of only in terms of a nonspecific secondary response. However, Arahata and Engel's analyses of cellular infiltrates in myopathic muscle nearly 20 years ago suggested that this was an oversimplification[96–98]. Inflammation is recognized as playing a crucial role in DMD pathogenesis on the basis of a number of lines of evidence: dystrophin-deficient tissue seems to be invaded early in the disease process; the infiltration associated with dystrophic muscle seems distinct, rather than non specific, with prominent representation of cytotoxic T-cell, macrophage and mast-cell populations[96–99]; and expression profiling of DMD muscle demonstrates distinct patterns of immune/ immune modulatory pathway genes[100,101]. Additionally, DMD patients have also been reported to have elevated levels of cytokines, such as transforming growth factor-β (TGF-β), in the serum, presumably as a consequence of the tissue inflammation[102]. Immune responses have been found to modulate the dystrophic phenotype in a number of experimental situations. Apart from the promising results with steroids, antibody-mediated depletion of CD4^{+} and CD8^{+} T cells in *mdx* mice has been found to result in a reduction in muscle pathology[103]. Improvement of pathology in the diaphragm has also been reported in the *nude⁻/mdx⁻* mouse[34]. A worsening of phenotype has been demonstrated by breeding *mdx* mice with *Tsk* mice that have elevated mast-cell activity[104]. Conversely, mast-cell stabilizers such as cromoglycate, which is typically used in the management of asthma, have been shown to increase the strength of *mdx* mice[105] and are at present the subject of trials in DMD patients.

Increasing muscle strength. DMD patients have drastically reduced muscle strength, and so attempts have been made to increase muscle strength using a variety of reagents. Although results with anabolic steroids in DMD patients are equivocal, benefits have been reported in *mdx* mice using clenbutarol[106], a nonsteroid β_2 adrenoceptor agonist with marked anabolic effects on muscle. This class of compounds has been reported to increase protein production, decrease proteolysis and stimulate satellite-cell proliferation in skeletal muscle, and also to improve strength after experimental denervation and disuse atrophy in animals and humans.

SARCOPLASMIC RETICULUM
A meshwork of internal membranes in muscle cells or fibres.

MYOTUBE
A muscle fibre precursor formed by the fusion of myoblasts.

Box 1 | **Gene and cell therapy for Duchenne muscular dystrophy**

Gene therapy strategies attempt to deliver coding regions of the gene encoding dystrophin[128] using specialized delivery vehicles (vectors). These vectors deliver the genetic material to target tissues, such as muscle and heart, and the missing protein is made in these cells using pre-existing cellular machinery. Direct (vector-less) delivery of small DNA[129,130] or DNA/RNA fragments[131,132] is also being used to repair certain types of mutations. Cell-based strategies attempt to transplant muscle precursors such as myoblasts[133] or stem cells[134,135] taken from healthy donors, into dystrophin-deficient muscle. Muscle stem cells also have the potential of being able to home in on muscle.
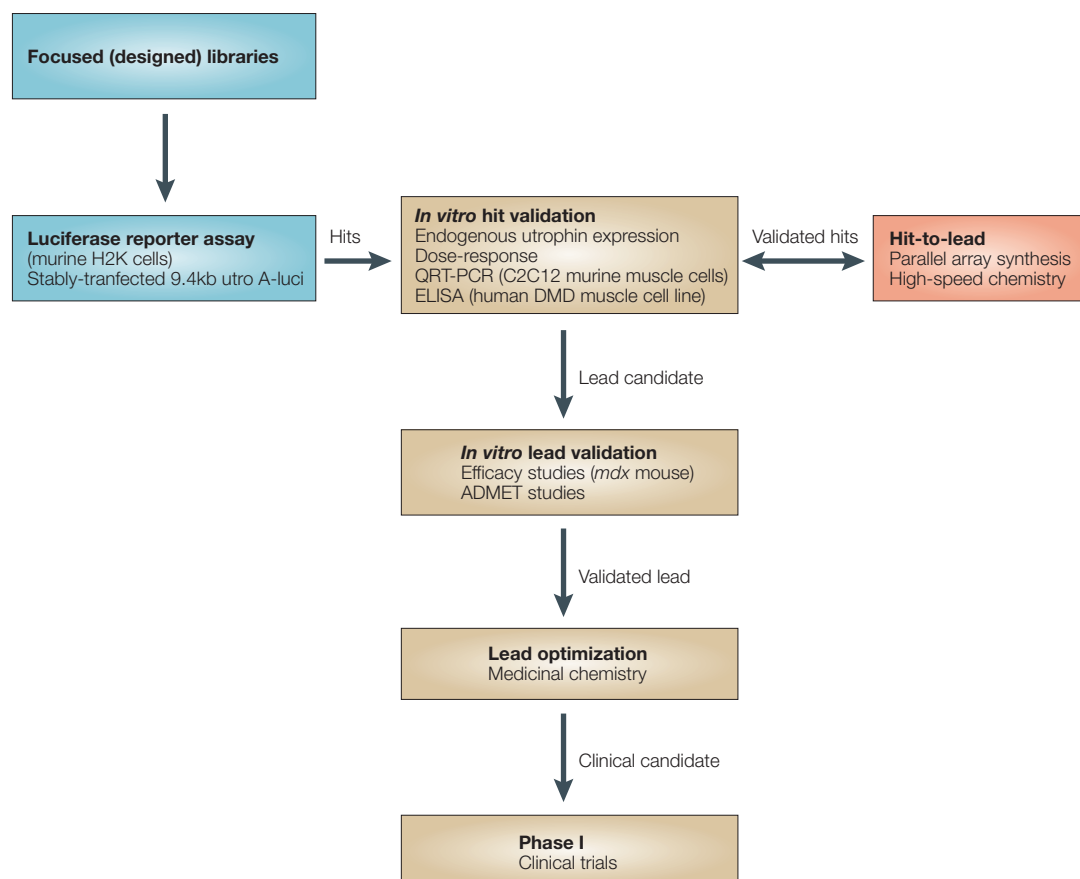
Creatine is a naturally occurring precursor of phosphocreatine. The energy content of phosphocreatine is used by muscle to meet its metabolic needs. Creatine is widely used by athletes as a performance-enhancing substance, particularly for high-intensity, short-duration work, where it seems beneficial. The treatment of cultured *mdx* muscle with creatine increases phosphocreatine and MYOTUBE survival[107], and trials of creatine monohydrate in human patients suffering from a variety of neuromuscular diseases have reported some degree of improvement of muscle strength[108].

Suppressing a stop codon: gentamicin. The *mdx* mouse and a minority (5–15%) of DMD patients have a premature stop codon as the disease-causing mutation in the *DMD* gene. An ingenious pharmacological approach uses the aminoglycoside gentamicin to act as a suppressor of the stop codon mutation and allow a read-through of the dystrophin complementary DNA. In *mdx* mice treated with gentamicin, dystropin levels increased to 10–20% of normal levels, and anatomical, biochemical and functional improvement was noted[109]. In a two-week pilot trial in four DMD patients, an initial reduction of CK was noted in all patients and sustained in two patients; however, no full-length dystrophin was detected[110]. Future studies are needed to fully evaluate this promising avenue of therapy, as differences exist between the formulations and dosage used in the *mdx* and DMD trials. Escalating the dose in humans to levels that were found to be beneficial in *mdx* mice might be fundamentally limiting due to adverse effects of higher dosage on auditory and renal functions.

High-throughput screening

Over the last decade, there has not only been an explosion in the identification of disease genes, but also important developments in synthetic chemistry. It is now possible to rapidly generate thousands of unique and distinct drug-like chemical entities in an automatic way, which can then be used in a biological screen against a specific target. Subsequent medicinal chemical optimization of hit compounds from the screen can then be carried out very rapidly, because of the automation of chemical synthesis. This approach is being used to screen for compounds that upregulate the expression of utrophin. FIGURE 4 shows a typical screen (S. G. Davies, K. E. Davies and A. W. Mulvaney, personal communication). The cell line used for the screen is an immortalized *mdx* mouse H2K cell line that has been transfected with a bacterial artificial chromosome containing both the A and B promoters

Figure 4 | **Schematic overview of high-throughput utrophin promoter screening.** ADMET, absorption, distribution, metabolism, excretion, toxicity; DMD, Duchenne muscular dystrophy; ELISA, enzyme-linked immunosorbent assay; QRT-PCR, quantitative reverse transcription-polymerase chain reaction.
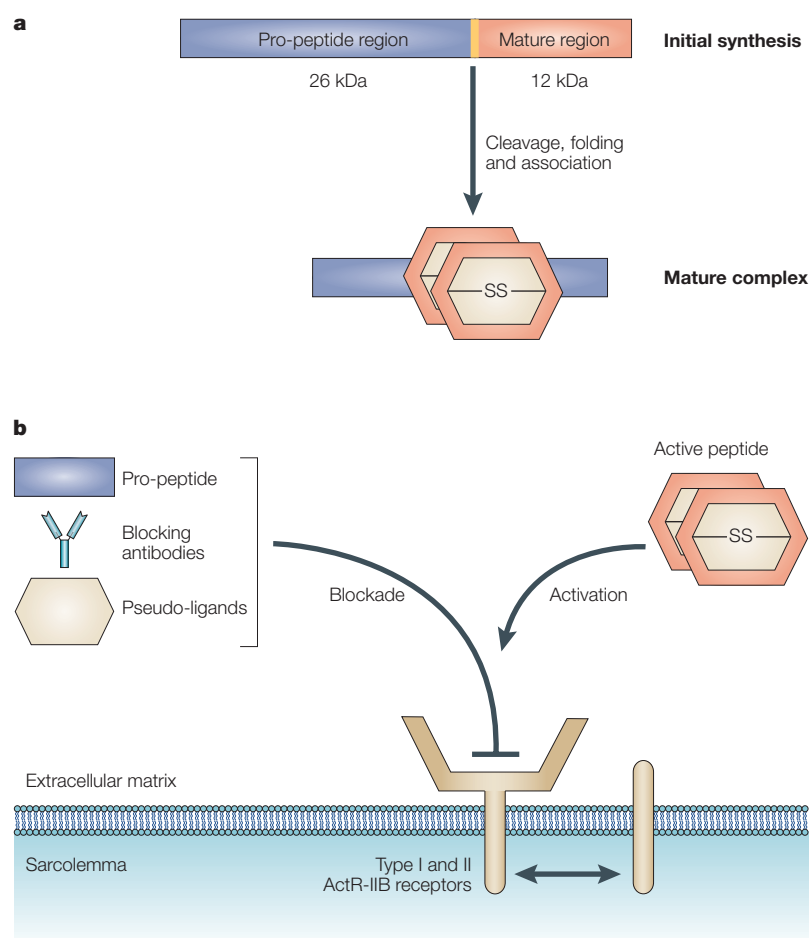
LUCIFERASE
The enzyme that catalyses the oxidation of luciferin, a reaction that produces bioluminescence.

Figure 5 | **Model for myostatin processing and pharmacological blockade. a** | The amino terminal of unprocessed myostatin molecules encodes the pro-peptide region (AA 1–262, 26 kDa, blue). A solid bar marks the invariant RSRR cleavage site (AA 263–266, yellow). The mature, or active, peptide is encoded by the 12-kDa region (AA 267–375, red) that follows the proteolytic cleavage site. Cleavage and folding during biosynthesis are required to generate an active form. However, myostatin circulates as an inactive complex due to association with the pro-peptide in serum. **b** | Schematic showing strategies to achieve pharmacological myostatin blockade. ActR-IIB, activin receptor type IIB.

INCREASING MUSCLE MASS
Muscle mass can increase in response to a variety of physiological and pathological stimuli. Growth/developmental factors regulate muscle mass by complex mechanisms including regulation of the proliferation and differentiation of muscle precursor cells.

of utrophin linked to a LUCIFERASE promoter gene. These cells are incubated with the individual compounds from the chemical library, and any positive hits are verified by polymerase chain reaction after reverse transcription of RNA (RT-PCR). They are then tested on human cell lines from DMD patients. Any positive compounds are optimized using medicinal chemistry *in vitro*, before being tested *in vivo* in the *mdx* mouse. The advantage of this approach to DMD therapy is that it should result in a drug that can be delivered systemically and that can target many muscles in the body.

Increasing muscle mass

One of the most prominent signs seen in advanced DMD patients is extreme muscle loss. The degree of muscle loss is progressive, and reflects the inability of dystrophic muscle to adequately regenerate and/or repair itself in response to mechanical stresses and the ongoing disease process. Enhancing muscle-progenitor proliferation and/or progenitor commitment by increasing positive, or blocking negative, regulators of muscle growth offers potentially useful pharmacological therapeutic strategies. Examples of positive regulators include insulin-like growth factor-1 (IGF-1), which is known to activate satellite cells and lead to muscle hypertrophy. The delivery of adequate quantities of this class of growth factors to dystrophic muscle by a variety of means would be predicted to increase muscle mass. Indeed, IGF-1 has been shown to increase muscle mass along with anatomical, biochemical and physiological improvement of the disease phenotype in *mdx* mice[111–113].

Clues for negative regulators of muscle growth have been provided by a natural mutation that causes the 'double-muscled' phenotype in cattle (especially in the Belgian Blue breed) first identified roughly 200 years ago. These cattle have, on average, 20% more muscle mass, decreased body fat and an increased ability to convert feed into lean muscle. The genetic 'defect' responsible for the impressive musculature is a mutation in the gene encoding myostatin (*GDF8*)[114–116]. Myostatin is a member of the TGF-β superfamily, and has been engineered in mice to generate null[116] and dominant-negative mutants[117,118] with similar increases of muscle mass. In common with other members of the superfamily, myostatin consists of a 26-kDa inhibitory N-terminal pro-peptide region, a small invariant cleavage site followed by the region encoding a 12-kDa active/mature peptide[116]. The precursor undergoes proteolytic cleavage, folding and dimerization to form an active molecule (FIG. 5a). The pro-peptide can inhibit the biological effects of the active myostatin dimer when bound as a complex[119], and must dissociate for biological activity, which occurs after receptor binding. The mature myostatin peptide activates the type IIB activin receptor receptor[120] and initiates a signalling cascade that ultimately leads to transcriptional changes of muscle-specific genes that inhibit/prevent myoblast progression[121–124]. On the basis of common familial characteristics, a number of strategies can be conceived to inhibit/block the biological effects of myostatin (FIG. 5b). Recently, antibody-mediated myostatin blockade was used to achieve anatomical, biochemical and physiological improvement in *mdx* mice[124]. Consistent with this, genetically modified mice that lack both dystrophin and myostatin have an improved phenotype as well[125]. The nature of physiological improvement was similar to that seen with IGF-1, with improvement noted in absolute strength but no improvement in susceptibility to damage by lengthening contractions[111,124]. Future studies are needed to evaluate the long-term benefits of these strategies and their limitations. Additionally, humanized antibodies, stable human pro-peptide and pseudo-ligands would have to be developed/derived for use in DMD. INCREASING MUSCLE MASS is emerging as an important therapeutic strategy with the potential to be used in conjunction with other strategies for muscular dystrophy.

Conclusions

Tremendous progress is being made using rational pharmacological approaches for the treatment of dystrophin-deficiency in animal models of DMD. However, drug discovery for DMD ultimately depends on translating these encouraging leads into therapeutic trials with patients in the clinic. The Cooperative International Neuromuscular Research Group and European Neuromuscular Centre are examples of organizations that facilitate the translation of advances in basic science into benefits for DMD patients[126].

Indeed, at the time of writing, clinical trials are already underway on compounds first identified/verified by high throughput screening in *mdx* mice just three years ago[126,127]. Efficient networks such as these should help ensure interest and participation from academics and industry alike, which in turn should greatly enhance the process of drug development for DMD patients. The challenge is to continue to make advances in disease pathophysiology, applying the knowledge toward developing pharmacological therapies and translating it to DMD patients, efficiently.

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Acknowledgements

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