

Recent advances in Duchenne muscular dystrophy

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Abstract: Duchenne muscular dystrophy (DMD), an allelic X-linked progressive muscle-wasting disease, is one of the most common single-gene disorders in the developed world. Despite knowledge of the underlying genetic causation and resultant pathophysiology from lack of dystrophin protein at the muscle sarcolemma, clinical intervention is currently restricted to symptom management. In recent years, however, unprecedented advances in strategies devised to correct the primary defect through gene- and cell-based therapeutics hold particular promise for treating dystrophic muscle. Conventional gene replacement and endogenous modification strategies have greatly benefited from continued improvements in encapsidation capacity, transduction efficiency, and systemic delivery. In particular, RNA-based modifying approaches such as exon skipping enable expression of a shorter but functional dystrophin protein and rapid progress toward clinical application. Emerging combined gene- and cell-therapy strategies also illustrate particular promise in enabling ex vivo genetic correction and autologous transplantation to circumvent a number of immune challenges. These approaches are complemented by a vast array of pharmacological approaches, in particular the successful identification of molecules that enable functional replacement or ameliorate secondary DMD pathology. Animal models have been instrumental in providing proof of principle for many of these strategies, leading to several recent trials that have investigated their efficacy in DMD patients. Although none has reached the point of clinical use, rapid improvements in experimental technology and design draw this goal ever closer. Here, we review therapeutic approaches to DMD, with particular emphasis on recent progress in strategic development, preclinical evaluation and establishment of clinical efficacy. Further, we discuss the numerous challenges faced and synergistic approaches being devised to combat dystrophic pathology effectively.

Keywords: dystrophy, animal models, pharmacological, exon skipping, gene therapy, utrophin

Introduction

Duchenne muscular dystrophy (DMD) is the most common fatal genetic disorder diagnosed in childhood, with a sex-linked inheritance pattern of one in 3500 live male births.^{1,2} Affected individuals can be diagnosed at birth on the basis of elevated serum creatine kinase (CK), a biochemical marker of muscle necrosis,³ prior to visible difficulty in walking between 1 and 3 years of age. The clinical course of DMD is progressive; muscle weakness by age 5 years eventually leads to loss of independent ambulation by the middle of the second decade and death during the third decade, primarily as a result of respiratory or cardiac complications.² The genetic causation of DMD was established by localization of candidate complementary DNAs (cDNAs) to

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the short arm of the X chromosome (Xp band 21.2),⁴ which led to full characterization of the 2.5-Mb DMD locus and corresponding 427-kDa dystrophin protein.⁵ The sheer size of the resulting 14-kb dystrophin messenger RNA transcript served to explain how one-third of DMD cases arise from spontaneous new mutations.⁶ In terms of clinical manifestation, DMD results from failure to produce functional dystrophin protein as a result of nonsense or frame-shift DNA mutations,⁶ whereas those retaining the amino acid reading frame result in partially functional dystrophin and the milder allelic variant, Becker muscular dystrophy (BMD).

The genetic link to dystrophic pathology was elucidated by localization of dystrophin protein to the sarcolemma of skeletal and cardiac muscle, which is absent in DMD patients.⁵ Structural and functional studies illustrate that dystrophin is pivotal for maintaining structural integrity by linking the internal actin cytoskeleton of individual muscle fibers via F-actin binding of its N-terminus⁷ and C-terminal binding to the dystrophin-associated protein complex (DAPC) through β -dystroglycan (β -DG).⁸ The DAPC comprises several internal scaffold and transmembrane proteins, including α/β -DGs, sarcoglycans, sarcospan, and biglycan, by which linkage to collagen and laminin is achieved; while evidence suggests some of these links have functional signaling roles, their predominant purpose appears mechanical (reviewed in Davies and Nowak).⁹ In addition, the C-terminus of dystrophin interacts with neuronal nitric oxide synthase,¹⁰ dystrobrevin,¹¹ and the syntrophins.¹² At the molecular level, loss of dystrophin and consequential loss of the DAPC create sarcolemmal instability, enhancing susceptibility to mechanically induced damage and degeneration.¹³ Although the muscle initially responds through enhanced regeneration,¹⁴ successive rounds of necrosis eventually deplete the supply of muscle progenitor cells, which leads to infiltration of adipose and fibrotic connective tissue and exacerbates muscle wasting.¹⁵

Fragility of the DAPC also results in stretch-induced membrane permeability, leading to disruption of cellular homeostasis.¹⁶ The resultant elevation of intracellular calcium ($[Ca^{2+}]_i$) levels triggers increased production of reactive oxygen species (ROS) by the mitochondria,¹⁷ which contributes to the self-perpetuating cycle of increased oxidative stress, sarcolemmal damage, and eventual myofiber death.⁶ Chronic activation of signaling pathways involved in the inflammatory response further exacerbates dystrophic pathology by increasing myofiber expression of the major histocompatibility complex¹⁸ and the secretion of chemokines and cytokines.¹⁹ Although profibrotic signaling

is initially activated in an attempt to repair compromised myofibers, it is susceptible to upregulation by the unremitting nature of muscle damage, which triggers fibrosis and perpetuates inflammation.²⁰ As the cellular mechanisms that govern these secondary responses are intimately linked, it is difficult to ascribe hierarchical order to the molecular events that exacerbate DMD pathogenesis.

Although standards of care are improving, with better quality of life and prolonged survival,³ there is no cure for DMD. Clinical intervention is generally restricted to symptom management, such as ventilators for respiratory support and administration of glucocorticoids to stem progressive muscle damage. Long-term corticosteroid treatment purportedly extends functional ability for up to 2 years²¹ by modifying cellular events, including inflammation and Ca^{2+} homeostasis; however, their relative nonspecificity also causes unfavorable effects such as weight gain and loss of bone density.²² Nonetheless, established steroidal efficacy provides a basis for devising therapeutic strategies able specifically to target molecular defects underlying dystrophic pathology. Several promising approaches have emerged due to advances in experimental design, delivery, and efficacy for all three subgroups: gene therapy, cell therapy, and pharmacological therapy. In this review, we describe the current status of each approach, with particular emphasis on clinical application. Further, we discuss emerging combinatorial strategies that are most likely to provide future candidates for a definitive DMD therapy.

Mammalian models of Duchenne muscular dystrophy

Animal models have been an invaluable resource to elucidate the molecular basis of DMD pathogenesis and in assessing therapies that may carry substantial risk in humans (Table 1). As the dystrophin-deficient phenotype significantly differs between species, the suitability of each animal model is primarily based on phenotypical similarity to DMD, weighed against the extent of pathological characterization, scope for genetic manipulation, accessibility, and breeding costs.²³

Murine models of DMD

Mouse models are indispensable for developing therapeutic approaches for DMD, since they are easily and reliably reproduced. The widely used X-linked muscular dystrophy mouse *mdx* model²⁴ arises from a spontaneous nonsense mutation in exon 23²⁵ and absence of dystrophin protein.⁵ Although muscle necrosis and high CK levels are evident from 2 weeks, the *mdx* phenotype is most pronounced

between 3 and 4 weeks, when, in contrast to DMD patients, successive cycles of extensive necrosis are countered by regeneration,²⁴ eventually decreasing to chronic low-level damage by 8 weeks, permitting a near-normal lifespan.²³ Further, deterioration of skeletal and cardiac muscle (including fibrosis and inflammatory cell infiltration at later stages) in *mdx* is comparatively mild, where only the diaphragm is considered to recapitulate the severity of human disease.²⁶ This phenotypic disparity extends to *N*-ethyl-*N*-nitrosourea (ENU)-induced genetic variant *mdx* strains (*mdx2-5cv*)²⁷ that are not commonly used for therapeutic studies. Despite issues involving body size, genetic background, and pathological features, *mdx* is the established model for in vivo efficacy due to its desired transgenic and breeding capacity. For example, gene-based skipping of exon 51 (a strategy that is theoretically applicable to the highest percentage of DMD patients with out-of frame deletion mutations)²⁸ can be assessed using exon 52 knockout mice (*mdx52*).²⁹ Further, the development of “humanized” (hDMD) transgenic mice containing full-length human dystrophin has recently enabled direct preclinical screening of human-specific exon-skipping approaches.³⁰

Although inaccurate as genetic models, several double knockouts, including the myogenic transcription factor MyoD,³¹ the discriminant analysis of principal component (DAPC) α -DB,³² parvalbumin,³³ α 7 integrin,³⁴ cytidine monophosphate–sialic acid hydroxylase,³⁵ and the dystrophin autosomal paralogue utrophin³⁶ have been developed. The most clinically relevant and widely used are dystrophin/utrophin knockout mice (*mdx*; *utrn*^{−/−}, commonly referred to as *dko*),³⁶ which illustrate similar pathology to *mdx* at 4–5 weeks, after which this model progressively recapitulates DMD disease pathogenesis, resulting in a dramatically reduced lifespan.²³ As decreased survival of *dko* mice potentially hampers experimental design, a haploinsufficiency model (*mdx*; *utrn*^{+/-}) has been generated but is not widely used.³⁷

Canine X-linked muscular dystrophy

Spontaneous canine X-linked muscular dystrophy (CXMD) has been reported³⁸ in golden retriever (GRMD),³⁹ beagle (CXMD_J),⁴⁰ rottweiler,⁴¹ German shorthaired pointer,⁴² Japanese spitz,⁴³ and Cavalier King Charles spaniel (CKCS-MD)⁴⁴ breeds. Of these, the phenotypic progression of GRMD, resulting from an intron 6 splice acceptor mutation (leading to skipping of exon 7 and truncated dystrophin protein)³⁹ has been the most extensively characterized.²³ GRMD represents the most accurate animal model

for DMD in recapitulating phenotypic timing and severity, where muscle degeneration and generalized necrosis noted from birth onwards results in extensive fibrosis by 6 months and respiratory failure commensurate to human pediatric age.³⁹ Given the retriever’s suitability in respect of genetic background and body size, GRMD has been instrumental in predicting disease pathogenesis, severity, and treatment efficacy,⁴⁵ providing proof of concept for numerous cell- and gene-therapy approaches (see Table 1). However, the use of GRMD is restricted by dramatic phenotypical variation between sibs (causing difficulties in preclinical standardization),³⁹ welfare implications, and high costs of maintenance and treatment.²³ These concerns have been partially addressed by interbreeding GRMD dogs with smaller beagle sires (canine X-linked muscular dystrophy in Japan [CXMD_J]), resulting in a near-identical phenotype to GRMD but with an improved survival rate.⁴⁶

Although GRMD and CXMD_J⁴⁷ dogs have several advantages over *mdx* as an exon-skipping model, they also retain a similar disadvantage where the disease-causing mutation lies outside the region commonly affected in humans.⁴⁸ The recent characterization of severe CXMD in CKCS dogs is of particular clinical relevance given its genotypic causation (a donor splice acceptor mutation in exon 50 and predicted protein truncation).⁴⁴ Further, success in inducing exon 51 skipping in cultured CKCS-MD myoblasts⁴⁴ indicates the potential of CKCS-MD as a suitable in vivo model (see gene-therapy section).

Feline and porcine models

Hypertrophic feline muscular dystrophy (HFMD)⁴⁹ and the 238 tailored pig model (238-DMD)⁵⁰ represent two large animal models of DMD that substantially differ in their genetic derivation that are suitable candidates for therapeutic assessment. While HFMD represents spontaneous dystrophin deficiency as a result of an extensive promoter deletion,⁵¹ it is not widely used to limited pathological similarity to DMD.³⁸ In contrast, the exon 52–deleted 238-DMD pig, similar to *mdx52*, was engineered to assess exon 51 skipping methodologies,⁵⁰ and appears to be a bona fide model, as ascertained by absence of dystrophin protein, elevated serum CK levels, and early degenerative changes on muscle histology.⁵⁰ Further, porcine models have a number of practical advantages, such as the ability to circumvent numerous issues that currently preclude experimental transition from *mdx* into larger models (such as transgenic manipulation and breeding considerations), while retaining a similar size and physiology to humans.

Table 1 Animal models used in assessing therapeutic strategies for Duchenne muscular dystrophy (DMD)

model	Providence	Mutation	Pathology and comments	Gene and protein replacement	Gene repair and exon skipping	Cell/pharmacological therapy
<i>mdx</i> ^{24f} mouse	Spontaneous	3185C>T (exon 23) nonsense ²⁵	Muscle fiber degeneration/regeneration, myopathy less severe later in life, normal lifespan ²⁴ used for most strategies owing to breeding and experimental simplicity ²³	Gene replacement ^{66*} adenovirus: mini-(mDYS), micro-(μDYS) dystrophin. ⁷² Cell/gene replacement lentiviral: mDYS-MDSC ⁹¹ , plasmid: mDYS-MDSC ⁹⁵ - myoblast ^{86,97} DYS-HAC(FL)-iPS ¹⁰⁰ , MAB ¹⁰¹ Protein compensation utrophin (TAT-μUtr) ¹⁸⁰ , biglycan (rhBGN) ¹⁸⁵ , laminin (LAM-111) ^{193,194} utrophin upregulation	Gene repair ^{102,103} exon skipping 2'OMePS/PMO [†] , 2'OMePS/PMO MSTN ¹²⁷³ 2'OMePS MSTN/DYS ²⁷⁴	Cell: allogenic human and <i>mdx</i> myoblasts ³⁵⁵ allogenic murine and human MDSC ^{62-64*} Pharmacological: Dystrophin gene: negamycin, gentamicin ^{147,148,152} functional compensation/membrane stability: utrophin [†] , α7 integrin ^{191,192} , PA188 ²⁰⁰ , ROS inhibitors: antioxidants [†] , BGP-15 ²¹⁵ , Necrosis: PDE5 inhibitors ^{230,231} , NO-releasing agents [†] MPTP/calpain inhibitors [†] Fibrosis: TGF-β/BMP/RAS antagonists [†] MSTN inhibition ²⁵⁵ inflammation: prednisolone [†] , TNF-α/NF-κB inhibition ¹⁶² , IGF-1 [†] None reported
<i>mdx4Cv</i> ²⁷ mouse	ENU mutagenesis	7925C>T (exon 53) nonsense	10 times fewer fiber revertants than <i>mdx</i> -2Cv with essentially identical muscle pathology ²⁷	Gene replacement plasmid: mDYS ⁸⁶ , adenovirus: mDYS ⁷² Cell/gene replacement lentivirus: μDYS-SC ⁹²	Exon skipping 2'OMePS/PMO ¹¹⁷ Multiple	Pharmacological ROS inhibitors/antioxidants: melatonin ²⁰⁶ None reported
<i>mdx5Cv</i> ²⁷ mouse	ENU mutagenesis	1306a>t SD (exon 10) missense	Pathology as per <i>mdx4Cv</i> ²⁷	Cell/gene replacement lentivirus: μDYS-MDSC ⁹⁰	Gene repair ^{107,108}	Pharmacological ROS inhibitors/antioxidants: melatonin ²⁰⁶ None reported
<i>mdx52</i> ²⁹ mouse	Targeted disruption	Δexon 52	Similar pathology to <i>mdx</i> , with limb muscle hypertrophy ²⁹	None reported	Exon-skipping PMO ¹¹⁵	None reported
<i>mdx;utrn-/-</i> (<i>dko</i>) ³⁶ mouse	Targeted disruption	<i>mdx;utrn</i> tm1Jrs (exon 7) Nonsense	Considered a DMD phenocopy over <i>mdx</i> due to earlier onset of muscle dystrophy and premature death ³⁶	Gene replacement ^{66*} adenovirus: mini-(mDYS) and micro-(μDYS) dystrophin ⁷² Protein compensation utrophin (TAT-μUtr) ¹⁸⁰ , α7 integrin peptide ¹⁸⁹	Exon-skipping in vivo: PMO adenovirus U7snRNA ¹³¹	Cell Lentiviral systemic allogenic (donor derived) and autologous MAB ⁷³ Pharmacological Membrane stability: VPA ¹⁹¹ , ROS inhibitors: BGP-15 ²¹⁵ , fibrosis: RAS ²⁶⁷ , BMP ²⁶³ , TGF-β ²⁴⁹ antagonists, inflammation: IGF-1 ²⁷⁷

hDMD ³⁰ mouse	YAC insertion of full-length human dystrophin ³⁰	Telometric (chr 5) integration ¹³⁹	Complements dystrophic pathology in mdx (hDMD/mdx) ³⁰ and dko crosses (hDMD/dko) ¹³⁹	Not applicable	Exon-skipping in vivo: 2'OMePS single ¹¹⁵ multiple ³⁰ , 2'OMePS / PMO single ¹²⁴ , adenovirus - U7snRNA multiple ¹³²	Not applicable
GRMD ³⁹ golden Retriever	Spontaneous	739-2a>g ³⁹ SA (intron 6) deletion	Most similar to DMD pathology of all models ²³ limitation of phenotypic variation between sibs ³⁹	Gene replacement adenovirus: mini-(mDYS) dystrophin ^{7,81‡} , minitrophin (mUtr) ^{7‡80} Cell/gene replacement lentiviral: μ DYSMDSC ⁹¹ , MAB ²³	Gene repair ¹⁰⁴ exon-skipping in vitro: 2'OMePS/ PMO single ^{113‡} in vivo: AAVU7snRNA single ^{133,134}	Cell Systemic allogenic (donor-derived) canine MAB ²³ Pharmacological Membrane stability: PA188 ²⁰¹ calpain inhibitors: C10 ¹²³⁷
CXMD ⁴⁰ beagle	Spontaneous	739-2a>g ³⁹ SA (intron 6) deletion	Phenotype as per GRMD; smaller breed size ⁴⁰	Gene replacement adenovirus: mini-(mDYS) dystrophin ⁸²	Exon-skipping in vitro: PMO single ¹¹³ in vivo: 2'OMePS/PMO Multiple ^{47,116}	Cell Systemic/intramuscular allogenic (donor-derived) ⁷²
CKCSMD ⁴⁴ spaniel	Spontaneous	7294+5t>g SD (intron 50) deletion	Phenotype as per GRMD; smaller breed size ⁴⁴	None reported	Exon-skipping cell based: PMO single ⁴⁴	None reported

Notes: ¹Overview of experimental approach/pathology; ²multiple studies; refer to relevant sections; ³using mdx/severe combined immunodeficiency mice; ⁴human specific sequence. Approaches not reported for ⁵mouse: mdx variants: mdx-2cv²⁷ (6326+2a>t-3cv²⁷ (9772-16t>a); ⁶canine: (see⁸⁹), rottweiler⁴¹, German pointer⁴⁰ and Japanese spitz⁴³, other: feline HFMD model (del Dp427m+Dp427p⁴⁶) and pig DMD-238 (Lexon 52)³⁰.

Abbreviations: SASD, splice acceptor/splice donor; ENU, N-ethyl-N-nitrosourea; SC, satellite cell; 2'OMePS, 2'-O-methyl oligoribonucleotide; PMO, phosphorodiamidate morpholino oligomer; IPS, induced pluripotent stem cells; FL, full length; rhBGN, recombinant human biglycan; MSTN, myostatin; MDSC, muscle derived stem cells; MAB, mesoangoblasts; HAC, human artificial chromosome; PA188, polyaxamer188; TAT, HIV-1 trans activator of transcription protein; BMP, bone morphogenic protein; BGP-15, O-(3-piperidino-2-hydroxy-1-propyl) nicotinic amidoxime; TNF α , tumour necrosis factor alpha; TGF β , transforming growth factor beta; IGF-1, insulin growth like factor 1; RAS, Renin-Angiotensin system; PDES, phosphodiesterase 5; VPA, valproic acid.

As there is no definitive DMD animal model, GRMD and *mdx* currently represent the most appropriate model for preclinical testing by consensus.²³ It is tempting to speculate reliance on the *mdx* mouse model has hampered therapeutic progress, as the mild clinical phenotype results in difficulty in assessing certain issues, such as devising gene or cell therapies for larger muscle.⁵² Nonetheless, the genetic tractability, reproducibility, and convenient size make mouse models invaluable tools in DMD research, provided their physiological differences are acknowledged. This reliance on smaller animals is largely due to practical difficulties imposed by larger models such as GRMD, which more closely represent DMD patients in size and pathological expression, but are never likely to supersede *mdx* in high-throughput studies. However, the future use of dog (and possibly pig) models to hone mouse-developed technologies on a more comparable phenotype is unclear, given a number of proof-of-concept strategies developed in *mdx* and human DMD cell lines have circumvented their use to successfully progress to human safety trials.

Cell-based therapeutic approaches

Cell-based therapies involve transplantation or transduction of allogeneic (donor-derived) or autologous (patient-derived) cells to engraft with existing myofibers or repopulate the cellular niche to promote functional muscle regeneration.⁵³

Myoblast transplantation

Allogeneic myoblast transfer was the first cell-based strategy to be assessed in immunologically tolerant mice, providing evidence of host–donor fusion and stimulating myofiber development;⁵⁴ parameters that were subsequently recapitulated in *mdx* mice (reviewed in Mouly et al).⁵⁵ Although allogeneic cell transplantation can circumvent the need for genetic manipulation to reintroduce functional dystrophin, the risk of graft rejection remains.⁵⁷ In addition, several unfavorable characteristics of using donor myoblasts, including (1) poor intramuscular migration, (2) low efficiency of dystrophin production, (3) limited cell survival, and (4) immune complications in *mdx*, were mirrored in early clinical trials assessing allogeneic implantation of immunohistocompatible myoblasts in DMD patient muscle.⁵⁶ Further, this approach leads to massive central ischemic necrosis in nonhuman primates.⁵⁷

Satellite cells and muscle-derived stem cells

As a result of the various pitfalls encountered with myoblast transfer, stem cell transplantation was deemed a more

attractive option due to their differentiation potential and self-renewal capacity.⁵⁸ Among the first to be assessed were satellite cells (SCs), a quiescent and committed population of myogenic precursors that actively divide and differentiate in response to myofiber growth or damage.⁵⁹ When SCs remain attached to single myofibers for transplantation, they illustrate self-renewal and self-sufficiency as a regenerative source.⁶⁰ At present, direct SC engraftment faces two major hurdles: (1) the rapid decline of their autologous isolation potential, especially in the later stages of muscle degeneration, and (2) their individual isolation, in particular as in vitro expansion drastically reduces their engraftment and regeneration capacity.⁶¹ It also remains unclear whether SCs derive from precursors resident in muscle or from circulating progenitors.⁶⁰ A number of these parameters can be alleviated through the use of muscle-derived stem cells (MDSCs), which are commonly thought to represent a predecessor of the SC.⁵⁹ As MDSCs represent a multipotential cell population, they are considered distinct from the myogenically committed SCs. Further, MDSCs have a number of advantages over SCs, including (1) increased engraftment ability, (2) expression of specific stem cell markers that allow specific isolation, and (3) expansion and maintenance in an in vitro progenitor state.^{20,59,62} Systemic delivery of allogeneic murine or human MDSCs^{63,64} can restore dystrophin expression and ameliorate dystrophic pathology in immunotolerant *mdx*/severe combined immunodeficiency (SCID) mice. Further, autologous transplantation of MDSCs in DMD patients during a Phase I clinical safety study did not result in local or systemic side effects.⁶² Despite these encouraging results, the typically heterogeneous nature of MDSCs may affect their efficacy, depending on their isolation and culturing conditions.⁵⁹

Pluripotent and non-muscle-derived progenitor cells

Several non-muscle cell types such as embryonic stem (ES) cells can be converted to myogenic precursors after coculturing with skeletal myoblasts or by myogenic induction.⁶⁵ To circumvent ethical and legal restrictions associated with deriving ES cells,⁶⁶ allogeneic pluripotent human cells have been successfully isolated from early-age amniotic fluid (human AF-amniotic fluid stem cells) and umbilical cord (human umbilical cord-derived mesenchymal stem cells [hUC-MSCs]). Both of these donor cell populations were able to fuse with host myofibers after intramuscular or intravascular delivery, respectively, in immunosuppressed mice, although not within a dystrophic (ie, *mdx*) genetic background.^{67,68}

However, given that hUC-MSC engraftment demonstrates effective elevation of muscle proteins in dysferlin-deficient dystrophic mice (an animal model for limb-girdle muscular dystrophy type 2B and Miyoshi myopathy, both caused by mutations of the dysferlin gene),⁶⁸ a planned Phase I/II trial is currently recruiting to assess their safety and efficacy in DMD patients.⁶⁹

In recent years, a number of tissue-specific adult stem cells, which maintain, generate, and replace terminally differentiated cells within their resident organ, have demonstrated myogenic potential.⁵³ Among the most promising are adult MSCs, which can differentiate to form myogenic cells in situ.²⁰ In contrast to other DMD cell-based therapies, MSCs also possess distinct anti-inflammatory activities and represent an ethical alternative to ES cells.⁷⁰ For example, intramuscular injection of bone marrow-derived MSCs was successful in regenerating muscle cells and repairing muscle degeneration in *mdx*/SCID mice.⁷¹ Intramuscular or interarterial injection of myogenically induced canine wild-type allogeneic (dog leukocyte antigen matched with an unaffected littermate donor) bone marrow MSCs was able to establish long-term, widespread muscle engraftment and differentiation in CXMD_J dogs without requiring immunosuppression.⁷²

Another promising MSC-based approach is the use of vessel-associated mesoangioblasts (MABs), multipotential progenitors with the ability to differentiate into many mesodermal phenotypes, including myotubes.⁷⁰ Interarterial delivery of donor wild-type MABs in GRMD dogs illustrates impressive engraftment capability, leading to extensive recovery of muscle morphology and function.⁷⁰ Encouragingly, similar parameters can be achieved in *dko* mice, where proliferating MABs illustrated the ability to form new myofibers and promote expression of dystrophin and its autosomal paralogue – utrophin.⁷³ These results establish MABs as a feasible candidate for DMD stem cell therapy, and an interarterial Phase I/IIa DMD clinical trial using MABs from healthy donors has been initiated.⁷⁴

From myoblast transfer to the use of stem and progenitor cells, a common hurdle remains in the effective use of cell therapy in DMD patients. For cell transplantation to be successful, hurdles such as immune rejection must be overcome, or advances in developing methods to manipulate autologous cells to reexpress dystrophin must be made. The most promising cell-based approach is thus likely to involve ex-vivo expansion of pluripotent patient-derived myogenic precursors (such as MABs) with gene therapy to enable autologous, genetically corrected cell engraftment. Recent progress in

the rapidly expanding combined cell–gene therapy field is outlined in the next section.

Gene-based therapeutic approaches

As DMD is caused by recessive and monogenic genetic mutations, therapeutic strategies can be devised to correct or improve muscle function by (1) exogenous delivery of functionally engineered dystrophin gene constructs or (2) repair/augmentation of the endogenous locus (Figure 1). Encouragingly, both approaches benefit from either the rapid progress in RNA-based technology or by combination with cell-based therapies.

Gene-replacement therapy

Delivery of exogenous functional dystrophin is an attractive prospect to benefit all DMD patients (given the inconsequential nature of the endogenous mutation), and gene replacement is traditionally divided into viral and naked (nonviral) categories. The major challenge common to viral and nonviral approaches involves developing suitable delivery vectors and gene cassettes while avoiding a destructive immune response. Further, the large size of the dystrophin gene, coupled with the limited carrying capacity of vectors such as recombinant adeno-associated virus (rAAV) prompted construction of internally deleted but highly functional “mini”-dystrophin (mDYS)⁷⁵ and “micro”-dystrophin (μDYS)⁷⁶ constructs to facilitate gene transfer.

Historically, studies using systemic and intramuscular rAAV-mediated delivery of mDYS and μDYS in *mdx* demonstrated promising efficacy in a number of parameters, including successful formation of sarcolemmal mDYS-/μDYS-associated protein complexes and improved muscle function while reducing fibrosis (reviewed in Bowles et al).⁷⁷ Although in vivo rAAV-mediated gene transfer has been effective in reducing dystrophic pathology in both GRMD and *dko* animal models (reviewed in Seto et al),⁷⁸ the immune reaction against rAAV particles and dystrophin protein itself has been readily apparent in *mdx* and is particularly severe in GRMD.⁷⁹ Recent efforts to improve immune tolerance and transduction efficiency have led to increasing use of rAAV8- and AAV9-modified serotypes. For example, intramuscular rAAV2/9-μDYS⁸⁰ and rAAV9-mDYS⁸¹ gene transfer in *mdx* and systemic injection of rAAV8-μDYS in CXMD_J⁸² and rAAV8-mDYS in GRMD⁸¹ not only illustrate widespread transgene expression but also increase tropism in cardiac and skeletal muscle. However, lingering immune concerns continue to limit clinical assessment of rAAV-mediated gene transfer. This was evident in a 2010 Phase I dose-escalation

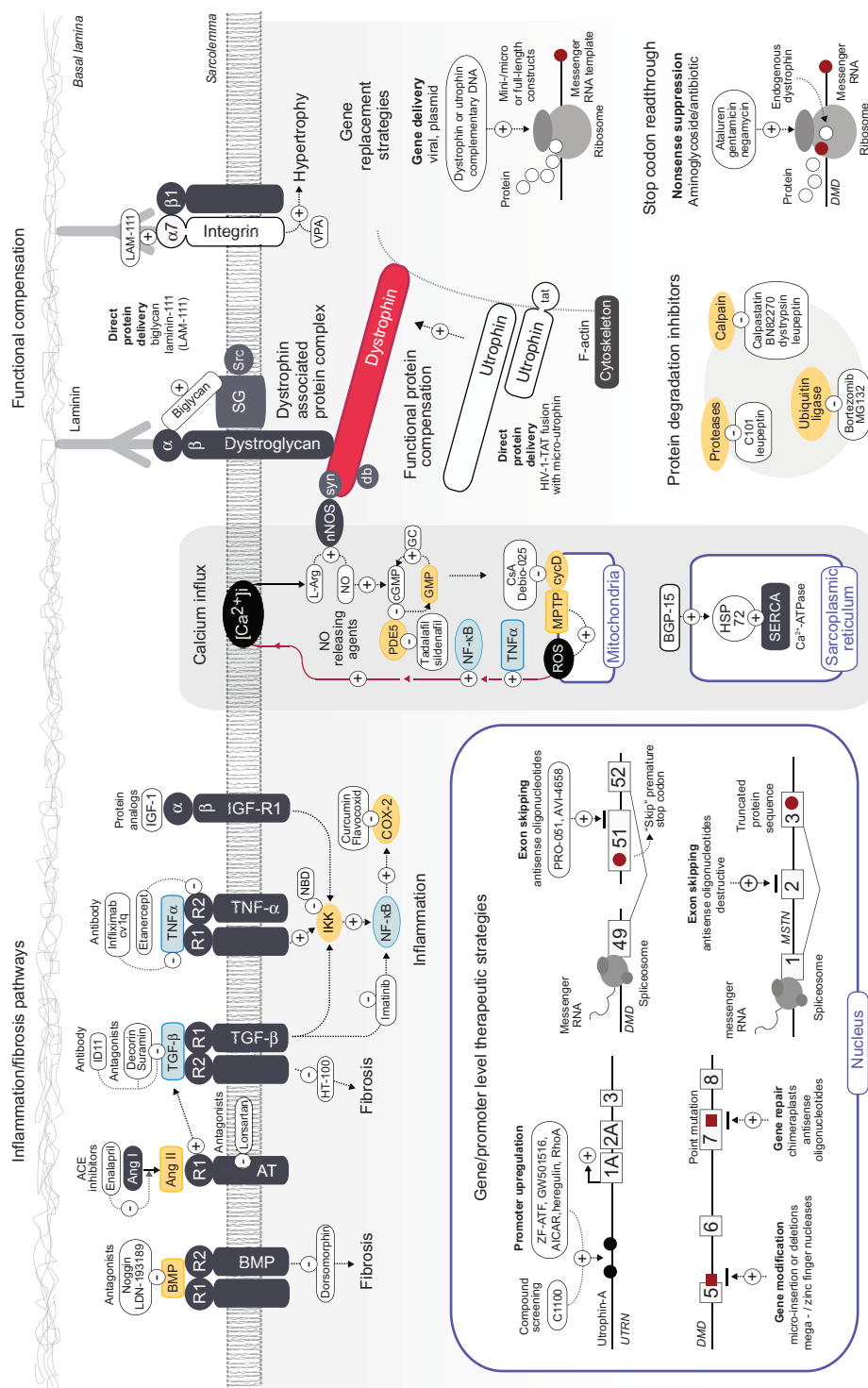


Figure 1 Current genetic and pharmacological targets of dystrophic pathology.

Notes: Receptor or structural protein components at the skeletal muscle sarcolemma targeted for therapeutic purposes are represented in dark grey. Components of signaling pathways specifically targeted for intentional downregulation are represented in yellow boxes, with two key regulators of dystrophic pathology NFκB, and TNFα, highlighted in light blue. Associated white boxes contain the therapeutic compound(s) used to modulate either a positive (+) or negative (–) effect on a particular protein/receptor. Arrow-headed lines represent a simplified version of the signaling pathways involved in inflammatory, fibrotic, and hypertrophic responses, symbolizing only regulatory proteins that are covered in the text. The background-shaded section represents cellular process affected by calcium influx, with the red line representing the feedback mechanism with ROS, TNFα, and NFκB. The interactions delineated between and within signaling pathways are not an exhaustive representation and pharmacological compounds that act in a nonspecific or undetermined mode of action have been excluded. Red dots, representative mRNA sequence leading to translation stop codon; black dots, promoter elements; red squares, representative of gene modification by repair or insertion/deletion by zinc finger or meganucleases.

Abbreviations: PA, polyaxamer 188; TGF-β, transforming growth factor beta; PI3K, phosphatidylinositol 3-kinase; AKT, protein kinase B (PKB); IKK, IκB kinase; NF-κB, nuclear factor kappa-light-chain enhancer of activated B cells; BMP, bone morphogenic protein; AngII, angiotensin II; R1/R2, receptor I/II; AT, angiotensin; ACE, angiotensin-converting enzyme; NBD, NEMO binding domain; IGF-1, insulin growth factor 1; LAM-III, laminin-III protein; VPA, valproic acid; ALK-4, activin receptor-like kinase; AcR1L, activin receptor type II; BCL/ABL, breakpoint cluster region/Abelson murine leukemia viral oncogene homologue 1; Akt, acutely transforming retrovirus AKT8 in rodent T-cell lymphoma; [Ca²⁺]_i, intracellular calcium; L-Arg, L-arginine; NO, nitric oxide; nNOS, neuronal nitric oxide synthase; cGMP, cyclic guanosine monophosphate; GMP, guanosine monophosphate; GC, guanylate cyclase; BGP-15, O-(3-piperidino-2-hydroxy-1-propyl) nicotinic amidoxime; PDE5, cGMP-specific phosphodiesterase type 5; TNF-α, tumour necrosis factor alpha; CsA, cyclosporine A; ROS, reactive oxygen species; MPTP, mitochondrial permeability transition pore; cycD, cyclophilin D; HSP72, heat shock protein 72; SERCA, sarco/endoplasmic reticulum Ca²⁺-ATPase; SG, sarcoglycans; Src, sarcoaspan; syn, syntrophin; db, dystrobrevin; RDO, RNA/DNA oligonucleotide; AON, antisense oligonucleotide; 2'OMePS, 2'-O-methyl oligoribonucleotide; PMO, phosphorodiamide morpholino oligomer; AICAR, 5-amino-1-β-D-ribofuranosyl-imidazole-4-carboxamide; UTR/DMD/MSTN, utrophin/DMD/myostatin gene; DAPC, dystrophin-associated protein complex; LAM, laminin; Utr, utrophin minigene construct; cDNA, complementary DNA.

study using intramuscular rAAV2.5-mDYS injection, which elicited failure in long-term transgene expression and severe T-cell reaction in a small cohort of DMD patients.⁸³ However, it is likely that AAV-mediated gene replacement will be subject to future trials, given recent improvements in translation optimization of the rAAV2.5 capsid has led to vastly improved immune tolerance in a Phase I follow-up safety study.⁷⁷

Nonviral gene-transfer methodologies have been explored by intramuscular injection of naked full-length and mDYS plasmid into *mdx* hindlimb muscle,^{84,85} mDYS in *mdx4cv* diaphragm,⁸⁶ and more recently by electrotransfer of full-length canine dystrophin into GRMD hind limb.⁸⁷ These studies indicate that plasmid-based gene transfer has greater potential for long-term expression compared to rAAV-mediated approaches but is hampered by lower comparative efficacy. Due perhaps to the latter, an initial clinical report detailing success of intramuscular delivery of full-length/mDYS plasmids in DMD patients to express functional protein within the injection site⁸⁸ has not been repeated. Further, commercial development of a plasmid-based therapy (Myodys; Transgene) was assessed in a Phase I trial in 2008; however, no data has subsequently been released.⁸⁹

An increasingly promising alternative strategy to deliver functional dystrophin involves the ex vivo combination of cell and gene therapies. This approach involves the use of genetically modified cells as autologous delivery vehicles to circumvent immune challenges and reduce the risk of implant rejection.⁹⁰ Systemic efficacy of combined cell–gene therapy was originally established by successful interarterial delivery of MDSC cells transduced with lentivirus to regenerating *mdx5cv* muscle.⁹⁰ This study was extended by lentiviral-mediated transduction of canine mDYS in human and GRMD MDSCs prior to transplantation into *mdx* and GRMD by either intramuscular injection or electrotransfer.⁹¹ Lentiviral vectors have also been used to demonstrate that μ DYS-transduced autologous *mdx4cv* SC⁹² and GRMD MABs⁹³ can regenerate dystrophin-positive myofibers in vivo. However, it is important to note that although the level of μ DYS-expressing fibers was sufficient in treated GRMD dogs to ameliorate dystrophic morphology (5%–50%), their clinical performance remained poor, in direct contrast to the phenotypical improvement observed using systemic delivery of unmodified donor cell MABs⁹³ (see section on cell-based therapeutics.).

Alternative viral delivery vehicles such as retrovirus also demonstrate transduction efficacy, although the risk of immunogenic graft rejection is increased.^{94,96} Nonetheless,

interarterial administration of isogenic MSCs containing retroviral-induced μ DYS enabled persistent, long-term (12-week) dystrophin restoration in *mdx* muscle fibers and satellite cells.⁹⁴ Although plasmid-based mDYS transduction in MDSC⁹⁵ and *mdx*/DMD myoblasts^{96,97} induces high in situ expression, mini-gene approaches have been superseded by development of a human artificial chromosome (HAC) containing the entire dystrophin gene (DYS-HAC).⁹⁸ DYS-HAC has a number of distinct advantages over plasmid-based approaches, such as stable episomal maintenance and ability to carry large genomic regions (including regulatory elements). DYS-HAC transduction via microcell-mediated chromosome transfer enables complete genetic correction of engraftable induced pluripotent stem (iPS) cells⁹⁹ from *mdx* and DMD patients.¹⁰⁰ Further, correct tissue expression of human dystrophin isoforms was evidenced in chimeric mice generated from DYS-HAC ES cell transfer.¹⁰⁰ Efficacy has recently been established in vivo using systemic delivery of DYS-HAC–transduced MABs, which illustrated morphological and functional amelioration of dystrophic pathology for up to 8 months posttransplantation.¹⁰¹ These approaches have led to planned trials of DYS-HAC–transduced MDSCs for autologous transplantation in patients.⁷⁴

Gene editing

An alternative approach to exogenous delivery of functional dystrophin by gene replacement or cell-based therapies is to induce de novo dystrophin production. Gene editing aims to repair or modify the underlying genetic defect (gene repair) or to modulate RNA processing (by selectively “skipping” exons of the dystrophin gene) to ameliorate effects of the underlying gene mutation.

Gene repair

Initial approaches to gene editing were aimed at correcting point mutations in the dystrophin gene using synthetic RNA/DNA “chimeraplasts” (RDOs), which enter the cell and attach to the target gene. The DNA strands of the chimeraplast and the gene complement each other with the exception of the nucleotides that require editing, which are then targeted by DNA repair enzymes, allowing the permanent replacement of the DNA target sequence with that of the chimeraplast.¹⁰² Although direct injection of RDOs into *mdx* muscle resulted in sarcolemmal localization of full-length dystrophin, myofiber conversion rates were poor (ranging from 1% to 15%).^{102,103} Similarly, direct intramuscular injection of RDOs illustrated sustained (over 48 weeks) in vivo repair of the GRMD point mutation and production of full-length dystrophin; resulting

levels of dystrophin protein were similarly low, and almost exclusively restricted to the injection site.¹⁰⁴

Consequently, RDO-mediated editing has been superseded by antisense oligodeoxynucleotide (AON) approaches due to considerations such as modification ability, cost, scale, and experimental variability.¹⁰⁵ Although RDO-induced point mutations can successfully enable *mdx* exon-skipping in vitro,¹⁰⁶ it is notable that AON-mediated gene editing in vitro and in vivo using the *mdx5cv* mouse model^{107,108} was originally devised to prevent not encourage exon skipping. The emergence of the latter as one of the most promising therapeutic approaches for DMD has led to a decline in using traditional gene-editing methodologies.

Exon skipping

Exon skipping is the most frequent alternative splicing mechanism known in mammals, and as such is a major contributor to protein diversity.¹⁰⁹ AONs aim to mimic exclusion (or “skipping”) of specific exons by hybridizing and thus blocking targeted pre-mRNA motifs involved in normal splicing to synthesize an internally truncated, semi-functional dystrophin protein.⁴⁸ Exon skipping has immense clinical potential, as 60% of DMD patients harbor deletions in exons 45–55 and sole targeting of exon 51 can address the majority of patients by addressing deletions of exon 50, exon 52, exons 48–50, or exons 49 and 50.⁴⁸ Further, a small-molecule “cocktail” approach enabling multiple exon skipping can feasibly be marketed as a single drug (reviewed in Benchaouir and Goyenvale).¹¹⁰

Preclinical in vitro proof of concept for AON-mediated exon skipping was established in primary cultured *mdx* myoblasts using targeted 2'-O-methyl oligoribonucleotides to exclude exon 23 and restore the dystrophin reading frame.¹¹¹ This result was recapitulated in vivo by intramuscular injection in *mdx*, which showed efficient AO nuclear uptake and sarcolemmal localization of dystrophin in treated muscle fibers.¹¹² As a result, two different AON chemistries have been under extensive study for clinical application: 2'-O-methyl-phosphorothioate (2'OMePS) and phosphorodiamidate morpholino oligomers (PMOs). Both 2'OMePS and PMO-induced exon-skipping approaches have been evaluated in cultured muscle cells from DMD patients, GRMD/CXMD_J dogs, and H2K-*mdx* and human explants (reviewed in Arechavala-Gomez et al).¹¹³ Further, systemic delivery of exon 23–skipping antisense compounds in *mdx* has been successful in restoring up to 50% of dystrophin expression in various muscle groups, improved muscle force, and reduced CK levels without tissue toxicity.¹¹⁴ Specific exon 51 skipping was established using intramuscular

injection of PMOs in *mdx52*¹¹⁵ and for human exons 44, 46, and 49 by 2'OMePS in hDMD mice,³⁰ which restored dystrophin expression in whole-body skeletal muscles in addition to improving muscle function.^{30,115} Use of multiexon-skipping “cocktails” in vivo was first achieved by systemic PMO delivery in CXMD_J,⁴⁷ and subsequently validated using 2'OMePS and PMO combinations in CXMD_J¹¹⁶ and *mdx4cv*.¹¹⁷ This approach appears more successful in increasing dystrophin expression in CXMD_J dogs (26% of normal levels)¹¹⁶ compared to *mdx4cv* (5%–7%)¹¹⁷ and trigger improvement in whole-body canine skeletal muscle (with the exception of heart) without adverse effects.⁴⁷ Further, PMOs generally illustrate in vivo superiority to 2'OMePS in the consistent and sustained induction of dystrophin protein.¹¹⁸

Encouragingly, 2'OMePS and PMO exon 51–skipping technologies have progressed to clinical trials, with early indications of success at the biochemical and clinical level. Proof of concept for PRO-051, a 2'OMePS AON developed by Prosensa,¹¹⁹ was established by intramuscular injection into DMD patients, which restored local sarcolemmal dystrophin in 64%–97% dystrophin-positive fibers and expression between 17% and 35% with no adverse effects.¹²⁰ This result is impressive, considering that a dystrophin expression level of 30% is postulated to avoid human pathogenesis,¹²¹ although the precise level required to induce clinical and functional improvement remains unclear.⁷⁵ A follow-up Phase I/IIa clinical trial using systemic administration of PRO-051 was also well tolerated, with dose-dependent molecular efficacy (60%–100% positive fibers and up to 15.6% expression) accompanied by modest clinical improvement after 12 weeks' extended treatment.¹²² PRO-051 is currently licensed by GlaxoSmithKline (GSK2402968), who have initiated three clinical trials, including a large international Phase III study.¹¹⁹ Prosensa have also opened a Phase I/IIa clinical study of PRO-044 (targeting exon 44) after assessment in DMD cultured cells, with preclinical trials of PRO-045 and PRO-053 (targeting exons 45 and 53, respectively) planned.¹¹⁹

AVI-4658 (Eteplirsen) is a splice-switching PMO developed by Sarepta Therapeutics,¹²³ identified by exon 51–specific AON screening in two different chemical forms in cultured human muscle cells and explants (wild type and DMD) and by local in vivo administration in hDMD mice.¹²⁴ AVI-4658 has also been tested in cultured myoblasts of the CKCS-MD dog, which restored the reading frame and protein.⁴⁴ A single-blind, placebo-controlled, dose-escalation study illustrated encouraging local dystrophin induction,¹²⁵ leading to a systemic intravenous Phase IIb dose-escalation study to assess further the safety, tolerability,

and pharmacokinetic properties of AVI-4658.¹²⁶ The initial 12-week study proved disappointing compared to PRO051, with 0%–55% positive fibers and up to 18% expression, with no significant improvement in clinical outcomes (even at higher doses), despite restoration of both components of the DAPC and neural nitric oxide synthase (nNOS) to the sarcolemma.¹²⁶ Although a subsequently longer clinical regime (24 weeks) has recently purported to improve dystrophin induction (averaging 22.5% dystrophin-positive fibers),¹²³ data on other parameters are currently unavailable. It is likely that additional trials to reoptimize delivery and dosage of AVI-4658 are planned by Sarepta Therapeutics, who have several other exon-skipping candidates, in particular AVI-5038 (targeting exon 50), which is currently in preclinical development.¹²³

An alternative exon-skipping methodology involves masking splicing sites using the endogenous targeting capacity of modified small nuclear RNAs (snRNAs), in particular U7 snRNA, to shuttle coupled AONs after rAAV vectorization (reviewed in Benchaouir and Goyenville).¹¹⁰ Proof of principle was established in exons 48–50 deleted DMD patient cell lines, where U1/U7 snRNA successfully altered dystrophin pre-mRNA splicing to rescue synthesis,¹²⁷ confirmed by exon 23 skipping in mouse C2C12 cells.¹²⁸ In vivo systemic rescue of *mdx* dystrophic muscle by single intravenous (IV) administration of exon 23–targeted rAAV-U7 constructs induced sustained muscle expression and correction of dystrophic pathology;¹²⁹ parameters confirmed in rAAV-U1 and -U7 transduced *mdx* muscle after local injection.¹³⁰ The remarkable potential of systemic IV rAAV-U7–mediated therapeutics follows recent, single treatment of self-complementary rAAV-U7–mediated exon skipping in *dco*, which restored near-normal dystrophin levels and improved function in all muscles examined, including heart.¹³¹ Human-specific multiexon skipping has also been achieved using rAAV-U7 in DMD cell lines and hDMD mice.¹³² Combined with the recent success of using rAAV-U7–mediated exon 7 skipping in long-term restoration of dystrophin expression in GRMD cardiac muscle,^{133,134} this approach illustrates significant potential in effectively targeting DMD cardiomyopathy.

Increasing emergence of proof-of-principle studies in gene-based dystrophin replacement and endogenous augmentation provide significant promise for treatment of DMD pathology, including the recent use of meganucleases and zinc-finger nucleases to induce endogenous microdeletion or -insertions in the endogenous gene.¹³⁵ Despite the lack of long-term toxicology studies, multiple AON-mediated exon

skipping potentially provides an applied therapeutic strategy for up to 83% of DMD patients.¹³⁶ However, difficulties in establishing long-term correction and circumventing immune challenges remain problematic, especially the inability of gene-replacement and PMO/2'OMePS-mediated exon skipping to effectively target cardiac tissue in *mdx* at doses corresponding to those required for clinical application.^{137,138} Several other issues, such as the timing of repeated administration, optimization of systemic delivery, and addressing poor cellular uptake, represent major hurdles in alleviating numerous chemical, clinical, and ethical issues. Moreover, further studies are required to clarify the mechanism through which AONs interfere with RNA splicing to optimize target sequences in humans.^{139,140} Recent studies to address these issues link inhibition of cell-cycle progression to enhance exon skipping,¹⁴¹ exonic sequences as better exon-skipping targets, and enhanced efficacy by repeated intraperitoneal delivery over intramuscular or IV injection.¹³⁷ Encouragingly, significant progress has been made in improving systemic delivery (especially in cardiac muscle) and lowering dosage of AONs in a number of animal models (including *mdx*, *dco*, and GRMD) by conjugation to nanoparticles, cell-penetrating peptides, or enhanced delivery using artificial vesicles (reviewed in Arechavala-Gomez et al¹¹³ and Moulton).¹⁴² It is therefore likely the first definitive DMD therapy will result from combining optimized multiexon-skipping methodologies with developing cell and pharmacological approaches.

Pharmacological approaches

DMD pharmacotherapy strategies involve the systemic delivery of small compounds that aim to (1) provide sarcolemmal-based compensation to directly address loss of the DAPC or (2) modify dysfunctional signaling pathways implicated in secondary pathology (Figure 1). A number of pharmacological strategies show efficacy in circumventing immunological and delivery hurdles that currently hamper gene- and cell-based therapies.¹⁴³ However, as pharmaceuticals frequently target molecules involved in complex signaling pathways, their development is far from simple. Here, we summarize pharmacological approaches according to their intended molecular targets and discuss their progress, pitfalls and promise as treatment strategies for DMD.

Targeting primary DMD pathology by functional compensation or restoration of the DAPC

In conjunction with medicinal chemistry, a number of pharmacological approaches have been devised to

specifically address the primary defect in DMD. These include (1) specific restoration of the DAPC by suppressing nonsense mutations in the dystrophin gene; (2) upregulation of its autosomal paralogue, utrophin, to provide a scaffold on which components of the DAPC can be restored to the sarcolemma; or (3) compensatory formation of integrin–laminin complexes, which have mechanosignaling similarities to the DAPC.

Aminoglycoside-mediated suppression of nonsense mutations

Approximately 10%–15% of DMD mutations convert an amino acid into a premature stop codon, while the rest of the mRNA is unaffected.¹⁴⁴ Nonsense mutation read-through strategies use aminoglycosides or small molecules to modify ribosomes to produce full-length functional protein by specifically targeting premature stop codons through contextual recognition of surrounding nucleotide sequences that differ between nonsense mutations and regular stop codons.¹⁴⁵ The most studied aminoglycoside for DMD therapeutic use is gentamicin, which acts via the 40S ribosomal subunit.¹⁴⁶ Proof-of-concept studies of gentamicin in *mdx* have been promising: a 2-week course of subcutaneous injection successfully enabled full-length dystrophin production with correct localization properties in both skeletal muscle¹⁴⁷ and the vascular system.¹⁴⁸ Further, these studies indicated improvement in a number of physiological parameters, including protection against contractile injury, normalization of blood flow and increased cardiac response to shear stress.^{147,148} However, following Phase I DMD/BMD clinical outcomes were highly variable, from promising^{143,149} to disappointing.¹⁵⁰ Potency issues due to batch consistency¹⁵¹ or dosage regimes may contribute to such conflicting outcomes, although the latter is less likely, given follow-up *mdx* studies could not replicate benefits described in one clinical trial.¹⁵² In an attempt to combat gentamicin toxicity and increase target specificity, a drug-delivery system using hybrid liposomes has been developed,¹⁵³ and it will be of interest if this approach results in future clinical assessment.

The small-molecule ataluren (PTC124), which also acts via the 60S ribosomal subunit,¹⁵⁴ exhibits similar efficiency in *mdx* to gentamicin at a lower concentration.¹⁵⁵ Although PTC124 was well tolerated in patients, three Phase II DMD/BMD clinical studies were halted as predetermined primary outcomes were not met.⁶⁹ Therefore, despite the favorable pharmacodynamic response of both gentamicin and ataluren, their clinical development remains problematic, making their

path to regulatory approval for DMD therapy a difficult one.¹⁴³ To circumvent toxicity concerns, an alternative approach is use of less toxic antibiotic peptides, such as negamycin, which inhibits eukaryotic RNA decoding.¹⁵⁶ Encouragingly, prolonged (4-week) intraperitoneal delivery in *mdx* enabled restoration of cardiac and skeletal muscle dystrophin levels comparable to those achieved with gentamicin,¹⁵⁶ making negamycin a promising therapeutic candidate.

Utrophin upregulation

A compensatory approach aimed at restoring components of the DAPC involves increasing levels of utrophin, the autosomal paralogue of dystrophin.¹⁵⁷ Although spatially restricted in adult myofibers to neuromuscular and myotendinous junctions,¹⁵⁸ extrajunctional utrophin is upregulated during embryonic development in *mdx* and DMD patients.¹⁵⁹ Utrophin-based upregulation therapy has a number of favorable attributes, notably (1) the ability to circumvent immunological challenges that accompany introduction of functional dystrophin protein; (2) in principle, effectiveness for all DMD patients, regardless of gene defect; and (3) amenability to systemic administration, given whole-body overexpression in *mdx* appears nontoxic.¹⁶⁰ Extensive proof-of-principle studies in *mdx* establish that a three- to fourfold increase in utrophin expression can enable functional restoration by formation of an alternative to the DAPC: the utrophin-associated protein complex (UAPC) complex (reviewed in Moorwood et al).¹⁶¹ Historically, a number of endogenous transcriptional/posttranscriptional effectors of utrophin have been evaluated in *mdx*, including direct injection of the active Ras homologue gene family, member A (RhoA), heregulin, NO, and L-arginine, but none of these approaches has been able to reproducibly increase utrophin levels (extensively reviewed in Fairclough et al).¹⁶²

The observation that endogenous utrophin is elevated in slow-twitch muscle¹⁶³ led to investigation of how key regulators of muscle oxidative metabolism can be augmented to obtain therapeutic levels of utrophin. Targeted upregulation of either peroxisome proliferator-activated receptor cofactor 1- α (PGC-1 α),^{164,165} its downstream effector peroxisome proliferator-activated receptor β/δ (PPAR β/δ), GA-binding protein (GABP) α/β , active calcineurin (CnA*), or associated nuclear factors of activated T cells (NFAT) in *mdx* mice illustrate a twofold increase in utrophin mRNA levels (reviewed in Fairclough et al).¹⁶² Encouragingly, many of these targets illustrate promoter-based synergism,^{166,167} indicating a multitargeting utrophin approach is feasible. However, it is important to note that, at

present, pharmacological optimization of individual targets holds varying promise. For example, the biological benefit of activating PPAR β/δ using the histone deacetylase inhibitor valproic acid (VPA) and its derivatives may be outweighed by concerns over their developmental toxicity.¹⁶⁸ Similarly, synthetic PPAR ligands are under evaluation for non-DMD therapies,¹⁶⁹ but complications including off-target kinase activation¹⁷⁰ and severe side effects have led in some cases to recall and reformulation.¹⁷¹ A more promising approach is administration of the adenosine monophosphate analog 5-aminoimidazole-4-carboxamide ribotide (AICAR). AICAR activates PGC-1 α and PPAR β/δ via AMP-activated protein kinase (AMPK),¹⁷² potentially affording greater therapeutic effect by enhancing synergism between PGC-1 α with the α subunit of GABP.¹⁶⁶ Encouragingly, AICAR administration elevates sarcolemmal utrophin and β -DG protein levels and fast-to-slow muscle-fiber transition¹⁷³ in *mdx* similar to using PPAR β/δ agonist GW501516¹⁶⁷ and AAV-mediated PGC-1 α delivery.^{164,165} As AICAR is in frequent clinical use,¹⁷⁴ its future clinical assessment in DMD patients thus seems likely.

An alternate promoter-based utrophin strategy involves artificial zinc-finger proteins fused with effector domains (ZF-ATF), which is currently being evaluated in *mdx* (see Passananti et al).¹⁷⁵ However, the current favored strategy is identification of orally deliverable compounds with utrophin upregulation capabilities (reviewed in Moorwood et al).¹⁶¹ Proof of principle was established by functional screening of chemical scaffold candidates, resulting in optimization of an orally bioavailable 2-arylbenzoxazole derivative – SMT-C1100 (BMN195).¹⁷⁶ Daily SMT-C1100 administration in *mdx* improved membrane integrity and demonstrated synergism with prednisolone.¹⁷⁶ However, a move into Phase I safety trials in healthy individuals was discontinued due to insufficient levels of SMT C1100 in plasma,¹⁷⁷ a difficulty being addressed by reformulation. However, the lack of safety issues with SMT-C1100 is encouraging, evidenced by complementary studies using compound libraries of FDA-approved and natural substances,¹⁶¹ pharmacological interest (Zalicus and PTC Therapeutics),¹⁵⁴ and development of improved screening assays.¹⁷⁸

Protein-based therapy: TAT-utrophin and biglycan

Direct protein replacement of utrophin in dystrophin-deficient muscle uses deliverable chimeras constructed by fusing the transactivator of transcription (TAT) protein transduction domain (PTD) of human immunodeficiency virus (HIV-1)¹⁷⁹ with micro-utrophin (μ Utr) protein (TAT- μ Utr).¹⁸⁰

Intraperitoneal injection of TAT- μ Utr in *mdx* established functional sarcolemmal μ Utr–glycoprotein complexes, leading to improved membrane integrity and contractile function.¹⁸⁰ As similar levels of functional improvement in *dko* mice establish this approach as an attractive therapeutic possibility,¹⁸¹ rigorous optimization is being performed prior to preclinical safety and toxicology studies.¹⁸² Pending results, clinical TAT- μ Utr trials (Retrophin, compound RE-001)¹⁸² are anticipated in late 2012.

A related protein-based pharmacological candidate is biglycan, a small leucine-rich proteoglycan found at elevated levels within the ECM of DMD patient skeletal muscle.¹⁸³ Biglycan is a critical regulator of sarcolemmal proteins such as nNOS, components of the DAPC, and utrophin in particular, during the muscular response to cell damage and apoptosis.¹⁸⁴ Single systemic administration of recombinant human biglycan (rhBGN) was well tolerated and sufficient to counteract *mdx* pathology by enhancing UAPC stabilisation, as an identical dose regime was ineffective in *dko* mice.¹⁸⁵ To mitigate off-target effects, active rhBGN is currently being manufactured without biglycan-associated complex carbohydrate side chains for preclinical evaluation (Tivorsan, compound TVN-102).¹⁸³

α 7-integrin upregulation/laminin-111

Integrin/laminin complexes act as mechanosignaling anchors, linking ECM laminin and fibronectin with intercellular cytoskeletal components.¹⁸⁶ Similar to the structural and signaling role of the DAPC, α 7 β 1-integrin/laminin-211 complexes act as crucial enablers of muscle development, repair, regeneration, and integrity in skeletal muscle.^{187,188} Indeed the degree of functional redundancy between integrin/laminin complexes and the DAPC coupled with endogenous elevation of sarcolemmal α 7 β 1 protein in DMD patients and *mdx* mice indicated that α 7-integrin upregulation may stem DMD muscle pathology.¹⁸⁷ Efficacy of functional compensation was established by transgenic overexpression of α 7-integrin in *dko* mice, which was effective in extending longevity (threefold), reducing kyphosis and increasing mobility as a result of increased sarcolemmal α 7 β 1 protein.¹⁸⁹ While not preventing initial degeneration, α 7-integrin upregulation appears to mediate sarcolemmal stability after subsequent regeneration by promoting SC proliferation, adherence, and activation.^{189,190} Favorably, inducing α 7-integrin overexpression does not demonstrate visible toxicity or affect in vivo global gene-expression profiles,¹⁹⁰ and, as a result, small-compound screening for α 7-integrin upregulators has been initiated.

Similar to utrophin, compound screening for $\alpha 7$ -integrin provides a relatively uncomplicated means to develop orally bioavailable molecules to complement other DMD therapies or benefit patients ineligible for strategies such as exon skipping. VPA was identified as an $\alpha 7$ -integrin upregulation compound using a cell-based assay, and intraperitoneal injection of VPA in *dko* mice results in decreased fibrosis, hypertrophy, and increases sarcolemmal integrity.¹⁹¹ However, contrary to observations from in vitro studies, $\alpha 7$ -integrin levels remain unchanged.¹⁹¹ This discrepancy may be explained by the observation that VPA and $\alpha 7$ -integrin both act, albeit independently, via the acutely transforming retrovirus AKT8 in rodent T-cell lymphoma (Akt)/mammalian target of rapamycin (mTOR) signaling pathway¹⁹¹ to positively regulate skeletal muscle hypertrophy.¹⁹² Thus, in vivo VPA administration alone appears sufficient to trigger Akt-mediated signaling independently of $\alpha 7$ -integrin.¹⁹¹ However, as previously outlined in the utrophin section, the potential toxicity of VPA required to achieve clinical benefit remains a concern.¹⁶⁸

An alternative candidate identified via small-compound screening is laminin-111 (LAM-111).¹⁹³ Intramuscular or systemic LAM-III injection in *mdx* has enabled the sufficient induction of $\alpha 7$ -integrin to achieve both sarcolemmal stabilisation¹⁹³ and increased regenerative capacity.¹⁹⁴ Although this study has been countered by the failure of enhancing heterodimer LAM-111 formation in improving dystrophic skeletal muscle morphology in *mdx* mice,¹⁹⁵ the use of validated $\alpha 7$ -integrin effectors has clinical promise. To preclude compounds with toxicity concerns, current pharmacological strategies are based on FDA-approved drug libraries (Prothelia)¹⁹⁶ or synergistic approved drug approaches (Zalicus).¹⁹⁷ Further, a number of lead compounds are currently at the preclinical (LAM-111/PRT-01 and PRT-20) and discovery (PRT-300) stages.¹⁹⁶

Targeting secondary DMD pathology resulting from dystrophin deficiency

Although the pathological presentation of dystrophin deficiency has been traditionally classified according to phenotypical and biochemical parameters such as fibrosis, necrosis, oxidative stress, and inflammation, it is increasingly apparent that the molecular processes that underlie these processes are intimately linked.¹⁹⁸ As a result, pharmacological intervention devised to alleviate one of these parameters may result in either assisting or even hindering one another. This is apparent with long-term corticosteroid treatment, which is thought to act by positively modifying

both inflammation and Ca^{2+} homeostasis.²¹ With this in mind, we categorize the progression of pharmacological intervention approaches according to their original aim of targeting a specific pathological or cellular defect, while outlining their links to others.

Reactive oxygen species and intracellular Ca^{2+} influx

Fragility of the DAPC leads to stretch-induced membrane permeability and Ca^{2+} influx, which activates proteases and enhances mitochondrial production of ROS,¹⁷ which in turn regulates the nuclear factor kappa-light-chain enhancer of activated B cells (NF- κ B) complex.¹⁹⁹ Although the synthetic membrane sealant polyaxamer 188 (PA188) shows promise in reducing membrane permeability in *mdx*²⁰⁰ and GRMD models,²⁰¹ the primary strategy to aid sarcolemmal integrity involves supplementing the antioxidant response in DMD patients to normalize redox balance and protect against oxidative stress (reviewed in Tidball and Wehling-Henricks).²⁰²

Dietary supplementation using glutamine, which is normally depleted in DMD patients,²⁰³ prevents glucocorticoid-mediated upregulation of the transforming growth factor beta (TGF- β) family member myostatin (see section on fibrosis)²⁰⁴ and protects against oxidative stress in *mdx*.²⁰⁵ However, similar to combinatorial approaches using creatine and alanine, reproducibility in clinical trials is modest at best (reviewed in Fairclough et al).¹⁶² In contrast, the commercial antioxidant melatonin improves muscle redox status and reduces inflammation in *mdx5cv*²⁰⁶ and DMD patients,²⁰⁷ while prenatal administration of epigallocatechin gallate effectively reduces ROS-mediated NF- κ B activation in *mdx*^{208,209} to the extent that Phase I/II DMD clinical trials are planned.²¹⁰

Another promising supplement is *N*-acetylcysteine (NAC), a direct ROS scavenger and indirect L-cysteine precursor.²¹¹ NAC treatment in postnecrotic *mdx*-decreased nuclear NF- κ B protine enhances sarcolemmal UAPC formation, reduces stretch-induced damage,²¹² and protects against damage and necrosis after only a week of treatment.²¹³ Further, linking antioxidants to lipophilic cations has human efficacy and improves effectiveness of antioxidants in disease models.²¹⁴ A recent study directly addressed calcium influx in *mdx* and *dko* muscle via oral gavage of BGP-15 (*O*-[3-piperidino-2-hydroxy-1-propyl]nicotinic amidoxime), increasing expression of heat shock protein 72, which binds and preserves sarcoplasmic reticulum Ca^{2+} -ATPase SERCA under cellular stress.²¹⁵ As BGP-15 administration has been effective in slowing progression, preserving strength, and improving muscle function in *dko*,²¹⁵ this methodology holds immense therapeutic potential for DMD.

Mitochondrial permeability transition pore

Sustained increase of $[Ca^{2+}]_i$ and the resultant redox imbalance in dystrophic muscle induces formation of the mitochondrial permeability transition pore (MPTP), leading to a self-perpetuating cycle of ROS, TNF- α , and NF- κ B and eventual cell death (reviewed in Lemasters et al).²¹⁶ Attempts to desensitize mitochondria using cyclosporine A (CsA)-mediated blockage of cyclophilin D^{217,218} in *mdx* decreased necrosis in some studies²¹⁹ but not others.²²⁰ This result was reflected in DMD clinical trials where a small study reported improvements in muscle-force generation,²²¹ but a following, larger randomized trial illustrated little benefit.²²² To circumvent the effect of long-term CsA treatment on both the immune system and calcineurin signaling, use of a nonimmunosuppressing CsA analog (Debio-025) was recently shown to enhance MPTP blockade over CsA or prednisolone in *mdx*, and is under assessment for other muscular dystrophies (reviewed in Fairclough et al).¹⁶²

Necrosis

Dysregulation of the NO/cyclic guanosine monophosphate (cGMP) pathway has been implicated in the loss of contractile performance and sarcolemmal integrity in dystrophic muscle.²²³ Increased $[Ca^{2+}]_i$ levels in dystrophic muscle leads to increased catalysis of NO (a free radical scavenger and regulator of the NO/cGMP signaling pathway) from L-arginine by NOS, whereas limiting $[Ca^{2+}]_i$ initiates NO-facilitated relaxation.²²⁴ Further, loss of the DAPC in *mdx* and DMD muscle prevents anchoring of nNOS at the sarcolemma, which also decreases NO levels and results in myofiber damage.¹⁰ Conversely, transgenic NOS restoration in *mdx* reduces ROS-mediated activation, decreases muscle damage/fibrosis and enhances formation of UAPC complexes,²²⁵ and NO-releasing agents such as the nonsteroidal anti-inflammatory drug HCT-1026 can prevent *mdx* muscle inflammation and damage to a greater extent than prednisolone.²²⁶ In addition, several NO donors including MyoNovin, isorbide dinitrate, or the analog guaifenesin dinitrate²²⁷ act to alleviate multiple aspects of *mdx* pathology by activation of SCs²²⁴ and/or via alleviating glucocorticoid side effects.²²⁸ Similar benefits are observed in *mdx* when catalysis of GMP to cGMP is either increased by transgenic means²²³ or by using phosphodiesterase 5 (PDE5) inhibitors to prevent degradation of cGMP.²²⁹ Further, PDE5 inhibitors deactivate hypertrophy signaling pathways triggered by pressure load, including those deregulated in DMD, such as calcineurin/NFAT, phosphoinositide-3 kinase/Akt, and extracellular signal-regulated kinase 1/2 cascades.²²⁹ Given that commercially

available cGMP-specific PDE5 inhibitors can prevent and even reverse contraction-induced myofiber damage (tadalafil)²³⁰ or cardiac hypertrophy (sildenafil)²³¹ in *mdx* mice, both are at the recruitment stage for DMD clinical trials.²³²

Protein-degradation inhibitors

Cysteine calpains are also activated in response to $[Ca^{2+}]_i$ in DMD,²³³ including the muscle-specific isoform absent in limb-girdle muscular dystrophy-2A.²³⁴ Interestingly, calpain modulation in dystrophic muscle primarily disrupts regulatory mechanisms influenced by calpains rather than increase proteolytic activity.²³⁴ Alleviating *mdx* muscle degeneration and necrosis by targeting calpain overactivity has been successful, using natural (calpastatin) or pharmacological means, including leupeptin, prodrug BN82270, dystrypsin (camostat mesylate) and enhanced-uptake cell-penetrating alpha-keto-amide calpain inhibitors (reviewed in Fairclough et al).¹⁶² However, despite links between calpain inhibition and TGF- β downregulation,²³⁵ calpastatin overexpression²³⁶ or administration of leupeptin-carnitine conjugate C101 was ineffective in *mdx*²³⁶ and GRMD²³⁷ models. Further, calpain inhibitors are also endogenously countered via elevated proteasome activity,^{164,236} which may preclude their pharmacological use. Alternatively, proteasomal targeting by systemic administration of nonspecific (MG-132) and specific (bortezomib) ubiquitin ligase inhibitors effectively reduces NF- κ B-mediated inflammation and restores DAPC components to the sarcolemma in *mdx*^{238–240} and DMD explants.²⁴¹ Therefore, their synergistic use with compounds that restore redox balance/provide functional compensation is possible if a balance between benefit and side effects from long-term use can be achieved.

Inflammation

Prior to the onset of visible muscle damage, increased TNF- α leads to induction of I κ B kinase (IKK) mediated NF- κ B signaling,²⁴² a major contributor to the inflammatory and necrotic response of DMD myofibers.²⁴³ Indeed, NF- κ B activation leads to aberrant signaling inexorably linked to increased ROS, and this synergism significantly contributes to the preliminary wave of inflammation in secondary DMD pathology.²⁴⁴ Further, glucocorticoids exert positive effects through NF- κ B inhibition,²⁴⁵ indicating that specific pharmacological targeting of NF- κ B may have therapeutic benefit.

Direct TNF- α inhibition using the anti-TNF- α antibody infliximab, or depletion of circulating TNF- α levels using

receptor decoy protein (etanercept) decrease fibrosis and necrosis and improve muscle function in *mdx* (reviewed in Fairclough et al).¹⁶² Blocking downstream targets of NF- κ B signaling such as cyclooxygenase-2 (COX-2) by curcumin improves sarcolemmal integrity and muscle strength in *mdx*, in addition to decreasing CK and levels of factors involved in the inflammatory process, including TNF- α and NF- κ B.^{246,247} Unfortunately, the progressive increase of NF- κ B levels in dystrophic muscle become increasingly resistant to curcumin,²⁴⁷ indicating that targeted COX-2 inhibition may not provide benefit in a clinical setting. Nevertheless, a DMD Phase I safety trial is planned for the COX-2 inhibitor Flavocoxid.⁶⁹ In contrast to curcumin, direct targeting of IKK using rAAV-mediated intramuscular administration of dominant negative IKK protein improves regeneration in older but not younger *mdx* mice.²⁴⁸ Impressively, systemic delivery of IKK inhibitory peptide (NF- κ B essential modulator binding domain [NBD]) increases regeneration, reduces necrosis, and improves contractile function in *mdx* and *dko* diaphragm.²⁴⁹ Optimizing intracellular delivery of NBD by fusion with a cationic cell-penetrating octalysine peptide²⁵⁰ (8K-NBD) leads to further improvement in *mdx* histology.²⁵¹ Further, the ability of 8K-NBD to enhance benefits provided by AAV9-mDYS delivery²⁵² indicates that IKK-mediated NF- κ B inhibition may assist in treating residual fibrosis and necrosis observed with gene-transfer approaches.

Fibrosis

Pathological fibrosis in DMD muscle correlates with increased TGF- β signaling,²⁵⁴ which hallmarks increased type I collagen production²⁵³ and the upregulation of several key intracellular markers of inflammation and oxidative stress.²⁵³ TGF- β antagonists suramin and decorin have shown efficacy in promoting muscle recovery by attenuating sarcolemmal damage, decreasing fibrosis, and enhancing muscle regeneration (reviewed in Burks and Cohn).²⁵⁵ Oral administration of nontoxic antifibrotics such as Bowman-Birk inhibitor and imatinib inhibit upstream and downstream TGF- β effectors, respectively, to affect a phenotype similar to direct antagonists in *mdx*.^{256–258} The plant alkaloid halofuginone (granted orphan drug status for DMD as HT-100)²⁵⁹ acts as a potent inhibitor of TGF- β profibrotic signaling to recapitulate these parameters in *mdx* by enhancing myotube fusion²⁵⁹ and function in initial²⁶⁰ and established fibrosis,²⁶¹ negating the necessity for accurate therapeutic timing. It is also interesting to note that the

TNF- α receptor decoy etanercept (see previous section) also reduces type I collagen and TGF- β mRNA,²⁶² indicating TNF- α blockade approaches may be effective in modulating TGF- β -mediated fibrosis.

TGF- β signaling can also be indirectly mediated by blocking bone morphogenic protein (BMP) ligands or the renin-angiotensin system (RAS), as both are continuously elevated in *mdx* and DMD skeletal muscle (reviewed in Burks and Cohn).²⁵⁵ BMP antagonists noggin, dorsomorphin, and LDN-193189 enhance differentiation in human myoblasts²⁶³ and intramuscular AAV delivery of the most potent and selective antagonist, noggin (“ad-noggin”), in *dko* mice enhances regeneration and alleviated dystrophic pathology.²⁶³ However, repressing BMP signaling may influence toxicity and severity of side effects, parameters that preclude long-term in vivo administration of dorsomorphin.²⁶³ RAS inhibition is considered a recent promising approach, where administration of angiotensin converting enzyme inhibitors (ACEi), antagonists of the angiotensin II (ATII type I) receptor, or TGF- β -neutralising antibodies (such as ID-11) demonstrate improvements in *mdx* pathology such as reduced fibrosis, increased muscle strength, and enhanced respiratory function.^{264–266} Further, ATII receptor agonists may counter effects mediated by NF- κ B, such as inflammation- and oxidative stress-related muscle damage.²⁶⁷ As early intervention using combined ACEi/ATII type I antagonists preserves muscle function in *dko* to an extent currently unparalleled by other pharmacological strategies,²⁶⁷ preclinical evaluation is highly anticipated.

Blocking secreted myostatin,²⁶⁸ a TGF- β -related negative regulator of muscle growth,²⁶⁹ also increases muscle-fiber size. Myostatin-null mice illustrate robust muscular hypertrophy and hyperplasia by deregulation of myoblast proliferation and differentiation.²⁶⁸ Similar improvements in *mdx* have been achieved using neutralizing antibodies, myostatin propeptide (MRPO) follistatin-derived peptides and the soluble extracellular form of the myostatin activin type-II receptor (reviewed in Burks and Cohn).²⁵⁵ Unfortunately, Phase II trials of recombinant ActRIIB decoy (ACE-031) were suspended due to safety issues,²⁷⁰ and Phase I/II clinical trials of antimyostatin MYO-029 antibody (stamulumab), although well tolerated, did not improve muscle strength.²⁷¹ These findings impact alternate approaches, as clinical trials are not planned for AAV8-mediated MRPO delivery validated in the GRMD model.²⁷² However, myostatin-blockade approaches have benefited from exon-skipping methodologies developed for dystro-

phin, where induction of *mdx* muscle hypertrophy using destructive 2OMP/PMO myostatin pre-mRNA targeting²⁷³ has advanced to 2'OMePS-based strategies simultaneously targeting myostatin and dystrophin.²⁷⁴

Muscle-growth strategies also involve exogenous delivery of insulin-like growth factor I (IGF-I), which stimulates SC proliferation and differentiation during muscle regeneration.²⁷⁵ Subcutaneous injection or viral expression of human IGF-I (rhIGF-I) or polyethylene glycol-modified IGF-I analogs (PEG-IGF-I) in *mdx* increase muscle strength and resistance to fatigue,^{276,277} but are ineffective against mechanical injury and myofiber degeneration.²⁷⁸ Further, PEG-IGF-I administration in *dko* and older *mdx* mice highlight somewhat limited potential to ameliorate severe or established pathophysiology, and the authors suggest delivery should be initiated only for mild muscle pathologies.²⁷⁷ Nevertheless, an IGF-I Phase I clinical trial is currently at the recruitment stage.⁶⁹

Conclusion

In recent years, significant progress has been made in the discovery of novel DMD therapeutic strategies and the continued development of established gene- and cell-based protocols. This is due, in part, to the continued understanding of molecular mechanisms that underlie DMD pathogenesis and the ability to establish efficacy using an increasing array of animal models. The development of a definitive DMD therapy is increasingly likely to involve synergism between adjunctive pharmaceuticals with gene-based approaches (such as exon skipping) to target multiple aspects of dystrophic pathology. Further, advances in cell-based technology show distinct promise in aiding efforts to correct endogenous dystrophin by their ability to act as autologous delivery vehicles. The successful move to clinical trials in each field has not only highlighted important aspects in the treatment and management of DMD but has also provided useful information for future design to accurately determine the age and state of the disease where treatment has clinically meaningful benefit. It is also increasingly apparent that accurate genetic diagnosis is key, given the increasing development of mutation-specific molecular therapies. As outlined in this review, many challenges lie ahead in the development and delivery of DMD therapeutics, and the specific approach(es) that will eventually result in success is unclear. However, it is clear that despite various hurdles, the incredible progress in therapeutic design in recent years has led to improved methodologies with immense translational potential.

Disclosure

KED is a consultant for Summit Plc. and is on the Scientific Advisory Board of Prosensa Plc. The authors report no other conflicts of interest in this work.

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