Review

Current Translational Research and Murine Models For Duchenne Muscular Dystrophy

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Abstract. Duchenne muscular dystrophy (DMD) is an X-linked genetic disorder characterized by progressive muscle degeneration. Mutations in the \textit{DMD} gene result in the absence of dystrophin, a protein required for muscle strength and stability. Currently, there is no cure for DMD. Since murine models are relatively easy to genetically manipulate, cost effective, and easily reproducible due to their short generation time, they have helped to elucidate the pathobiology of dystrophin deficiency and to assess therapies for treating DMD. Recently, several murine models have been developed by our group and others to be more representative of the human \textit{DMD} mutation types and phenotypes. For instance, \textit{mdx} mice on a DBA/2 genetic background, developed by Fukada et al., have lower regenerative capacity and exhibit very severe phenotype. \textit{Cmah}-deficient \textit{mdx} mice display an accelerated disease onset and severe cardiac phenotype due to differences in glycosylation between humans and mice. Other novel murine models include \textit{mdx52}, which harbors a deletion mutation in exon 52, a hot spot region in humans, and \textit{dystrophin/utrophin} double-deficient (\textit{dko}), which displays a severe dystrophic phenotype due the absence of utrophin, a dystrophin homolog. This paper reviews the pathological manifestations and recent therapeutic developments in murine models of DMD such as standard \textit{mdx} (C57BL/10), \textit{mdx} on C57BL/6 background (C57BL/6-\textit{mdx}), \textit{mdx52}, \textit{dystrophin/utrophin} double-deficient (\textit{dko}), \textit{mdx}\textsuperscript{hDMD}, \textit{Dmd-null}, humanized DMD (h\textit{DMD}), \textit{mdx} on DBA/2 background (DBA/2-\textit{mdx}), \textit{Cmah-\textit{mdx}}, and \textit{mdx/mTRKO} murine models.

Keywords: Duchenne muscular dystrophy (DMD), exon skipping, \textit{mdx}, \textit{mdx52}, h\textit{DMD}, \textit{dko}, C57BL/6-\textit{mdx}, DBA/2-\textit{mdx}, \textit{Cmah-\textit{mdx}}, D\textit{md-null}

INTRODUCTION

Duchenne muscular dystrophy (DMD) is the most common and fatal form of muscular dystrophies with an incidence of 1 in 5,000 boys [1, 2]. It is characterized by progressive muscle wasting and degeneration [3]. Mutations in the \textit{DMD} gene result in the absence of a protein, dystrophin in the sarcolemma [3]. The \textit{DMD} gene, the largest known gene in humans, consists of 79 exons and a 14 kb long dystrophin mRNA [4]. Dystrophin has four domains: N-terminal domain, 24 spectrin-like rod-shaped domain, cysteine rich domain and C-terminal domain [5]. The N-terminal domain of dystrophin binds to actin, and the cysteine rich and C-terminal domains of dystrophin bind to dystrophin-glycoprotein complex (DGC), a multimeric protein complex found at the plasma membrane (sarcolemma) of muscle fibers (aka myofibers) [5, 6]. Along with DGC, dystrophin crucially links the actin cytoskeleton of the sarcolemma to the extracellular basement membrane, as...
illustrated in Figure 1 [5, 7]. In the presence of dystrophin, DGC maintains muscle membrane integrity by serving as a signalling center, and a shock absorber to reduce contraction-induced damage [7]. Mutations in many protein components of DGC (such as dystrophin, sarcoglycans or dystroglycans) lead to various forms of muscular dystrophy and murine models with various dystrophic phenotypes, partly because certain components of DGC are more crucial in function than others [7].

In the absence of dystrophin, almost all components of DGC is either lost or mislocalized, the DGC is rendered dysfunctional and, the sarcolemma is highly susceptible to damage during muscle contraction [8]. Normal skeletal muscles regenerate following injury via satellite cells, which are resident muscle stem cells found beneath the basement membrane of myofibers [9, 10]. However, since dystrophic skeletal muscles undergo rapid degeneration followed by regeneration, these chronic cycles of degeneration and regeneration progressively lead to exhaustion of satellite cell pools [9, 11]. As regeneration slows down and can no longer keep up with rapid degeneration, damaged myofibers are replaced with adipose and fibrotic tissues instead of new muscle tissue [9, 11]. The exhausted regenerative capacity along with chronic inflammation exacerbates the dystrophic phenotype.

The clinical onset and diagnosis of DMD occur between 3–5 years of age. During this period, the affected children display walking difficulties, and elevated creatine kinase levels [3, 12, 13]. Dystrophic muscles of DMD patients display muscle necrosis, invasion of inflammatory cells, impaired regeneration due to exhausted satellite cell pools, and progressive fibrosis and adiposis [6]. As the disease progresses, the affected individuals are wheelchair bound at around 11 years, require ventilation support and, death ensues due to respiratory or cardiac failure between ages 20 to late 30 [1, 14, 15].

Although there is no cure for DMD right now, the current treatment for DMD has increased the lifespan of patients by 7 years since the 1980s [15]. Current treatments of DMD include steroids, surgery and assisted ventilation. Steroids, such as prednisone and deflazacort, are administered at daily doses of 0.75mg/kg and 0.9mg/kg respectively to prolong ambulation in children with DMD [16–20]. Continuing steroid treatment into adulthood (after the loss of ambulation) aims to achieve the benefits of the treatment (respiratory muscle strength and delay in scoliosis) with fewer side-effects (weight gain and bone fragility), via an alternative dosing regimens (e.g. alternate day, high-dose weekend, or a 10-day “on” cycling with 10 or 20 days “off”) [20]. Surgery can be considered to correct for lower limb contractures (joint, ankle and knee contractures) and scoliosis [21]. Assisted ventilation has increased the lifespan of DMD patients by 10 years or more [22]. Non-invasive ventilation forces air into the lungs and is used to assist coughing, nocturnal hypoventilation and later during daytime hypoventilation [21]. Non-invasive ventilation is usually preferred over tracheostomy as it ensures a better quality of life while prolonging survival [21, 23, 24].

Interestingly, dystrophin deficiency observed in Becker muscular dystrophy (BMD) patients show varying clinical symptoms, wherein many display a much milder phenotype than DMD patients, and some even display an asymptomatic phenotype [25–27]. The reading frame theory, which is well substantiated, explains that milder phenotypes observed in BMD are caused by in-frame mutations in the DMD gene. These in-frame mutations maintain the reading frame and result in the formation of truncated, internally deleted dystrophin protein. The reading frame theory explains the difference in phenotypes between DMD and BMD patients in 92% of cases [27]. However, in the remaining 8% of the cases, patients display severe phenotypes with in-frame deletions, duplications, and/or due to epigenetic and environmental factors [28].

Here, we will discuss the developments in therapeutic approaches and these include: Exon skipping, gene replacement therapy, stem cell therapy, utrophin up-regulation and read-through therapy using pharmacological agents. Table 1 provides a brief description of therapeutic approaches of DMD. Subsequently, we will focus specifically on murine models: The merits and caveats of each model and their applications in preclinical research. The mouse models discussed here are the standard mdx (with C57BL/10 background), mdx on C57BL/6 background (C57BL/6-mdx), mdx52, dystrophin/utrophin double-deficient (dko), mdx<sup>Bgeo</sup>, Dmd-null, humanized DMD (hDMD), mdx on DBA/2 background (DBA/2-mdx), Cmah-mdx, and mdx/mTR<sup>KO</sup> murine models.

**Therapeutic approaches**

**Exon Skipping:** Many consider exon skipping using antisense oligonucleotide (AONs) as one of the most promising therapeutic approaches [29–32]. This
Fig. 1. Dystrophin links actin cytoskeleton to the dystrophin glycoprotein complex. In normal muscles, the N-terminal domain of dystrophin binds to actin. Dystrophin then, subsequently interacts with the components of DGC: It interact with neuronal nitric oxide synthase (nNOS) at the region between exon 42 to exon 45, then, its cysteine rich domain binds to \( \alpha \)-dystroglycan, and lastly, its C-terminal domain binds to syntrophin and dystrobrevin.

| Table 1                                                                                       |
|------------------------------------------------------------------------------------------------|
| Overview of therapeutic approaches and its associated glossary of terms                        |
| **Exon skipping therapy**                                                                      |
| Antisense oligonucleotides are used to splice one or multiple exons in pre-mRNA to restore the reading frame |
| **Antisense oligonucleotides (AONs)**                                                          |
| Short synthetic nucleic acids that target specific sequences of pre-mRNA, modulating the splicing pattern to allow for in-frame dystrophin mRNA. Some of the AONs developed are 2′-O-methyl phosphorothioate (2′OMePS), phosphorodiamidate morpholino oligomers (PMOs), Vivo-morpholinos (vPMOs) and peptide-linked PMOs (PPMOs). Each of these AONs has different chemistries but the latter two have cell-penetrating moieties. |
| **Gene replacement therapy**                                                                  |
| Provides a substitute for dystrophin in a dystrophin-null background by packaging a truncated form of the dystrophin gene in vectors such as the non-pathogenic recombinant adeno-associated virus (rAAV) vector. |
| **Stem cell therapy**                                                                        |
| Involves stem cell transplantation, proliferation and differentiation into muscle cells and hence, contributes to increased muscle regeneration, preventing muscle wasting and fibrosis. |
| **Induced pluripotent stem cells (iPSC)**                                                      |
| Adult somatic cells that are genetically reprogrammed into an embryonic stem cell-like pluripotent state and hence, can differentiate into myofibers and increase muscle regeneration capacity. |
| **Utrophin upregulation therapy**                                                             |
| Aims to increase levels of utrophin, a protein similar to dystrophin, in dystrophic muscles to compensate for the absence of dystrophin. Pharmacological drugs, such as SMT C1100, SMT022357 and Biglycan, are shown to increase utrophin levels. |
| **Read-through therapy**                                                                     |
| Pharmacological agents, such as Ataluren (aka PTC124), are used to replace a premature stop codon (nonsense mutation) with a new amino acid, allowing for continued translation of dystrophin protein. |
| **Endonuclease-based gene repair**                                                            |
| DNA gene editing technique: Endonucleases used to create site-specific breaks in double-stranded DNA, which initiates DNA repair and gene correction. |
approach focuses on restoring the reading frame of dystrophin mRNA using AONs [33–35]. The quasi-dystrophin produced after exon skipping must be partially functional as it allows for milder phenotypes, similar to those seen in BMD patients [36–38]. However, exon-skipping is not without limitations: Dystrophin restoration induced by phosphorodiamidate morpholino oligomer (PMO or morpholino) AON exon-skipping lasts for only up to 8 weeks in dystrophic dogs and repeated AON administration is required to sustain its therapeutic effects, and issues with low exon skipping efficiency [39]. To overcome these limitations, developments in exon skipping include multiple exon skipping and, the use of various AON delivery systems to improve efficiency [40].

Using a cocktail of AONs allows for multiple (as opposed to single) exon splicing, thereby potentially increasing the applicability of the treatment to 90% (instead of 60%) of DMD patients [29, 41]. Skipping exon 45–55 can potentially treat 63% of DMD patients with deletion mutation [36]. Tricyclo-DNA (tcDNA), a new class of AON higher dystrophin levels in diaphragm (50%) and heart (40%) and, 3–4 fold higher skipping than 2’-O-methyl phosphorothioate (2’OMePS) and PMO at equimolar dosing regimens in mdx treated mice [42]. Moreover, new generation morpholinos such as octa-guanidine conjugated vivo-morpholinos (vPMOs) and peptide-linked PMOs (PPMOs), have a cell-penetration moiety and more effective AON chemistries than unmodified morpholinos [43]. Thereby, they are more efficiently delivered into various tissues and have a higher efficacy of dystrophin rescue [43]. Drisapersen, a 2’OMePS exon-skipping drug (ClinicalTrials.gov: NCT01254019), was unsuccessful at Phase III clinical trial as it did not yield statistically significant improvements in the 6 minute walking distance test (6MWT) compared to placebo [44, 45]. According to post-trial ad hoc analysis, drisapersen failure may be due to variation in patients’ age (large number of older participants), disease severity and standards of care among different countries [46]. Limitations in 6MWT arise when differences in age and height (which affects stride length) of patients’ are observed. According to Goemans et al., pooled analysis of two phase II trials suggested that drisapersen can slow down the disease when treated at younger ages and for an extended time [46, 47]. Currently, drisapersen continues to be developed by BioMarin. While 2’OMePS have ribose rings, a negative charge and are structurally similar to RNA, morpholinos are more stable, less toxic and have reduced off-target effects due to their 6-membered ring (lack of similarity to RNA) and neutral charge [48, 49]. Another clinical trial led by Sarepta Therapeutics is investigating the efficacy and safety of a PMO exon-skipping drug called eteplirsen, in advanced stage DMD patients who can undergo exon 51 skipping (ClinicalTrials.gov: NCT02286947) [50].

**Gene replacement:** This therapy aims to restore dystrophin expression by replacing the mutant DMD gene with a synthetic substitute using recombinant adeno-associated virus (AAV) vectors [51–57]. AAV is non-pathogenic, and infects non-dividing cells [33, 58]. However, the AAV vector cannot carry the whole DMD gene due to its small packaging size [33, 59]. In order to accommodate for the small packaging size of the vector, less essential regions of the DMD gene are removed to form micro-dystrophin, a truncated but functional form of dystrophin [56, 59–63]. Interest in AAV therapy arose from its transduction ability in quiescent satellite cells, persistent expression of delivered transgenes and non-pathogenicity [56, 64–67]. While AAV vectors display low immunogenicity than other vectors, the host’s humoral and cellular immune responses remain a major concern [68]. Dystrophin epitopes from rare ‘revertant’ (truncated dystrophin-positive) fibers (RFs) could sensitize autoreactive T cells and mount an immune response against the transgene product [69]. However, the potential for an immune response can be reduced by intramuscular administration, doses ranging from 2E11 vg/kg to 1.8E12 vg/kg, pre-screening against vector specific neutralizing antibodies and by administering immunosuppressants [54, 70]. A Phase I clinical trial was recently conducted using AAV2.5 vectors (rAAV2.5-CMV-minidystrophin; ClinicalTrials.gov number: NCT00428935). Each of the two-dose (2.0E10 vg/kg and 1.0E11 vg/kg) cohort studies of three subjects were administered in the biceps of six DMD patients and was found to be safe and well tolerated [67, 71]. Currently, a Phase I clinical trial involves AAV1 vectors (rAAV1.CMV.huFS344; ClinicalTrials.gov number: NCT02354781) which is administered in quadriceps, tibialis anterior gluteal muscles to six DMD patients at a total dose of 2.4E12 vg/kg [72].

**Stem cell therapy:** Satellite cells are muscle stem cells that allow for muscle regeneration after injury and are located between the sarcolemma and basal lamina of myofibers [73, 74]. Dystrophic muscles undergo continuous cycles of degeneration and regeneration in the dystrophic muscles eventually reduces the ability of resident satellite cells to
regenerate injured muscle [73]. This leads to the loss of muscle mass and compensatory insertion of fibrofatty tissue [73]. A limitation of gene replacement and exon skipping therapies is that the stage of the disease determines the effectiveness of the treatment because fibrofatty tissue replaces muscle cells with the progression of the disease [33, 75]. Ideally, stem cell therapy can overcome this hurdle by allowing for increased muscle regenerative capacity in dystrophic muscles [33, 76]. However, the transplantation of satellite cells show limited migration and self-renewal capacity. Stem cell types such as mesoangioblasts and CD133+ cells are able to enter and self-renew satellite cell niches, contribute to muscle regeneration and, unlike satellite cells and myoblasts, they can be delivered systemically [75, 77]. Mesoangioblasts are blood vessel-associated stem cells, which can pass through the walls of blood vessels and differentiate into myofibers [78]. CD133+ cells are human-derived and can differentiate into muscle stem cells [79]. Other developments include, human induced pluripotent stem cells (iPSCs), which are derived by reprogramming adult somatic cells into a pluripotent state, and are similar to embryonic stem cells in morphology and gene expression [75, 80, 81]. The advantage of this therapy includes the production of large numbers of myogenic progenitors, the lack of ethical issues that surrounded embryonic stem cells, and the potential to devise patient-specific iPSCs, ideally preventing a host’s immune response [33]. Another kind of stem cells are mesenchymal cells, which are multipotent and can give rise to many tissues including skeletal and cardiac [82]. Aside from their regenerative properties and ability to be delivered systemically, mesenchymal stem cells are most advantageous for their anti-inflammatory properties [82]. Yet, stem cell therapy comes with challenges such as immune and inflammatory reactions, poor survival and limited migration of injected cells [83–87].

Utrophin upregulating is another viable therapy because utrophin is a protein very similar to dystrophin with 80% amino acid sequence homology and takes the functional role of dystrophin during foetal muscle development [88]. The advantage of induced utrophin expression is that it could potentially prevent an immune response against dystrophin [89]. A drug called Biglycan, is a proteoglycan found endogenously in mice and humans, which stabilizes the muscle membrane by recruiting utrophin to the sarcolemma [90]. SMT C1100 is another oral drug that upregulates utrophin and reduces muscular dystrophy in mdx mice [91]. However, phase 1a clinical trial showed low plasma levels of SMT C1100 and, a phase 1b clinical trial (which was recently completed) tested the safety and tolerability of SMT C1100 at higher doses (however, the results are not yet published) (ClinicalTrials.gov number: NCT02056808) [91]. SMT022357 is a second generation drug with better metabolic and physiochemical profile than SMT C1100 [92]. It shows increased utrophin expression in cardiac, respiratory, and skeletal muscles in mdx mice and decreases necrosis and fibrosis [92]. Utrophin upregulation cannot completely restore muscle function to normal, possibly due to its inability to bind to neuronal nitric oxide synthase (nNOS) and/or due its structural differences to dystrophin [93]. Nevertheless, utrophin upregulation improves muscle function and reduces muscular dystrophy, and is applicable to all patients regardless of their mutation type [93].

Read through therapy involves suppression of nonsense mutations in DMD patients [94–96]. Gentamicin, an antibiotic allows for read through of premature termination codon (PTC) mutations, i.e. nonsense mutation, by replacing a stop codon with a new amino acid to continue translation [95, 97, 98]. However, it is not used clinically in DMD patients due to serious dose limiting toxicities including a hearing loss. PTC124 (also known as Ataluren) is a drug that appears more potent than gentamicin in restoring dystrophin expression although there exist some controversies regarding its read through ability [99]. Ataluren is currently being investigated in a phase III trial for its efficacy during a 6 minute walk test in DMD patients with nonsense mutations (ClinicalTrials.gov number: NCT01826487) [99, 100]. Generally, the applicability of read through therapies is limited to around 10–15% of DMD cases [101].

Endonuclease-based gene repair: Nuclease-mediated genome editing creates site-specific double stranded breaks in DNA [102, 103]. This cellular DNA repair mechanisms, such as homologous recombination (HR) or non-homologous end joining (NHEJ) mechanisms, result in insertions or deletions at break points that may lead to wild-type sequence correction [104]. The four engineered endonucleases recently developed include meganucleases, zinc-finger nucleases, transcription activator-like effector nucleases (TALEN) and, clustered regularly interspaced short palindromic repeat/Cas9 (CRISPR/Cas9) [102, 104–106]. This therapy is able to restore the normal reading frame of the dystrophin gene, delete a nonsense codon and knockout a
gene [103]. This therapy recently emerged in DMD studies, allowing permanent gene correction (by precise modifications at the target locus), and hence, overcomes the hurdle of transient mRNA correction (which calls for continuous drug administration) associated in AON-exon skipping and pharmacological read through therapies [106]. The advantage of this therapy is that it creates precise modifications at the target locus, and hence, yields a specific protein product with predictable functionality [105].

**MURINE MODELS OF DMD**

To name a few, among the many different animal models of DMD, are zebrafish, dog and pig models. Homozygous sap mutant zebrafish have a nonsense mutation at the N-terminal domain of sapje (sap) locus (an orthologue of DMD locus), resulting in the loss of dystrophin, muscle degeneration and, extensive fibrosis and inflammation [107]. The zebrafish model is useful for screening small-molecule drugs and visualizing molecular processes in vivo as the embryos and larvae are translucent [107]. However, these non-mammalian zebrafish models are phylogenetically far apart from humans. The commonly studied, Golden Retriever muscular dystrophy (GRMD) dog model harbours a mutation in intron 6, leading to a premature stop codon in exon 8, and are more similar to DMD patients in disease severity than mouse models [108–110]. Beagle-based canine X-linked muscular dystrophy (CXMD) dogs are crossed to GRMD to contain the same mutation but are smaller and easier to handle than GRMD [111]. However, dogs with identical mutations can show large differences in dystrophic phenotype, which can blur end points and confound data interpretation [112–115]. Pigs are more similar in anatomy, physiology, and genetics to humans than dogs and mice, but the newly developed pig models are not yet used in preclinical studies. Transgenic pig with a mutation in DMD exon 52 show symptoms similar to DMD patients, such as, elevated serum creatine levels, lack of functional dystrophin, and progressive fibrosis [111, 116, 117]. However, it also displays upregulation of utrophin (dystrophin homologue) as observed in mouse models [116, 117]. While the spontaneous substitution of arginine to tryptophan, in exon 41 results in dystrophinopathy, the affected pigs display a BMD-like (and not a DMD) phenotype [118, 119].

Murine models are often used to lay the groundwork for DMD studies including the pathogenesis of DMD and, the efficacy and toxicity of therapeutics [6]. However, murine models also have limitations such as lack of host immune responses to therapeutic agents (e.g.: Vector capsids) and, small size (compromising the ability to produce and deliver scaled-up amount of vectors to large volumes of muscles) [120]. Nevertheless, murine models are valuable animal models for research as they can be bred and genetically engineered with relative ease, and they are less expensive than other large animal models such as dogs and pigs. Many mouse models such as hDMD, Cmah-mdx, mdx/mTRKO and DBA/2 background have been recently developed. Table 2 provides a brief summary of the dystrophic features of murine models discussed in this review paper.

**Mdx on C57BL/10 background**

**Features of mdx mice**: Mdx, a commonly used classic mouse model, harbors a spontaneous point mutation at exon 23 of the Dmd gene, leading to the loss of dystrophin. Mdx arose from an inbred strain of C57BL/10. Mdx pathogenesis involves increase in creatine kinase levels, muscle degeneration, variation of fiber size, and centrally nucleated fibers (CNFs) indicative of muscle regeneration [6, 121]. While young mdx mice display mild cardiomyopathy, older mdx mice (especially female mice between ages 20 to 22 months) show severe dilated cardiomyopathy, frequent premature ventricular contractions, and cardiac fibrosis [122, 123]. Mdx has a much milder phenotype and normal lifespan compared to DMD patients: It does not exhibit impaired regeneration, accumulation of fibrofatty tissue, reduced myofiber number, except for in the diaphragm [124, 125]. The mild phenotype of mdx mice can be explained by (1) high regenerative capacity: The satellite cell pools of C57BL/10 were able to renew themselves even after 50 cycles of severe degeneration-regeneration (2) upregulation of utrophin, a dystrophin homologue, throughout their lifespan (unlike DMD patients), attenuating the effects of dystrophin deficiency [126].

**Involvement in therapeutic approaches**: The mdx (C57BL/10 background, C57BL/10-mdx) mouse is the most widely used model of DMD [127, 128]. In an effort to reduce the mild dystrophic phenotype of mdx mice, high dose irradiation of mdx muscles were employed to block muscle regeneration [129, 130]. For instance, one study irradiated hind limb muscles of mdx mice which prevented the expansion of revertant fibers (RFs), and showed that RF expansion depends on muscle regeneration [131]. Another study genetically labelled (LacZ reporter)
myofibers which were then transplanted in irradiated hindlimb muscles of mdx mice, resulting in self renewal of satellite stem cell pools [132]. Mdx mice on various immunodeficient backgrounds, such as mdx-null and recombinase-activating gene (Rag)2-γ chain-/C5- mice (which is required for V(D) rearrangement), were created to evaluate gene and cell therapies, without the compounding effects of an immune response [120]. Meng et al. reported that the efficiency of transplanting human muscle stem cells (pericytes and CD133 + cells) into mouse muscles depends on the environment and the mouse strain [133]. They reported that there were more myofibers and satellite cells of donor origin in (Rag)2-γ chain-/C5- mice than mdx-nude mice and, that cryoinjured muscles provided a more permissive environment for transplantation than irradiated muscles [133]. Mdx mice have also been used in developing pharmacological treatments of DMD, such as VBP15. VBP15, a synthetic corticosteroid oral drug, inhibits NF-κB and doesn’t lead to side effects associated with currently used steroids (e.g. prednisolone) since it doesn’t stimulate glucocorticoid-responsive element (GRE) transactivation [134]. Mdx mice treated with VBP15 (15 mg/kg) showed increase force in extensor digitorum longus (EDL) muscles by 12% and 16% in the two preclinical trials, while prednisolone showed no increase in force [134]. For maximal force exerted by forelimb muscles of mdx mice, VBP15 showed increase in force while prednisolone showed a decrease compared to non-treated mdx mice likely because the mdx mice treated with

| Murine models | Molecular types and phenotypic features of murine models of Duchenne muscular dystrophy | References |
|---------------|--------------------------------------------------------------------------------|------------|
| mdx (C57BL/10 genetic background) | Spontaneous point mutation in exon 23 of the Dmd gene. | Skeletal muscle degeneration-regeneration, necrosis, little fibrosis, utrophin upregulation and, greater regenerative capacity than DMD patients. | (121) |
| mdx (C57BL/6 genetic background) | Spontaneous point mutation in exon 23 of the Dmd gene. | Similar to C57BL/10-mdx, used for comparative studies, greatest regenerative capacity than other inbred strains of mdx. | (135) |
| Mdx2cv | Introns 42 point mutation | C57BL/6 background and the chemically induced mutation creates a new splice acceptor site. | (137) |
| Mdx3cv | Introns 65 point mutation | C57BL/6 background and the chemically induced mutation creates a new splice acceptor site. | (137) |
| Mdx4cv | Nonsense mutation at exon 53 | C57BL/6 background and harbours a chemically induced nonsense mutation. | (137) |
| Mdx5cv | Point mutation at exon 10 of Dmd | C57BL/6 background and the chemically induced mutation causes a new splice site in exon 10. | (137) |
| mdx52 (C57BL/6 genetic background) | Deletion mutation in exon 52 of the Dmd gene | Variation in myofiber size, skeletal muscles are hypertrophic, muscle degeneration-regeneration cycles, necrosis, lower RFs than C57BL/6-mdx | (140) |
| dko | Double deficient of the Dmd and Utr genes | Severe and progressive muscle wasting, weight loss after weaning, abnormal breathing rhythms, early onset of joint contractures, short life span and kyphosis by 20 weeks | (150) |
| mdxβgeo | Insertion of ROSA β-geo gene trap vector in exon 63 | Loss of most dystrophin isoforms (including Dp71), cardiac hypertrophy, abnormally dilated esophagus. (Note: The cysteine rich and C-terminal domains are lost in these mice) | (159) |
| Dmd-null | Deletion of the entire dystrophin gene | Produced by Cre-loxP technology. Lacks revertant fibers and all dystrophin isoforms. Displays muscle hypertrophy, behavioural abnormality and infertility. | (162) |
| hDMD | Knock-in of the complete human DMD gene in chromosome 5 of mouse genome. | No dystrophic phenotype | (163) |
| mdx (DBA/2 genetic background) | Spontaneous point mutation in exon 23 of the Dmd gene. | Lower muscle mass, greater fibrosis and fatty tissue accumulation, and lower regenerative capacity of satellite cells than C57BL/10-mdx mice. | (138) |
| Cmah-mdx (C57BL/10 genetic background) | Deletion mutation in the Cmah gene and spontaneous point mutation in exon 23 of the Dmd gene | Nearly 50% mortality at 11 months of age, loss of ambulation by 8 months, greater fibrosis than mdx (C57BL/10) mice in skeletal muscles like diaphragm and quadriceps, and necrosis in the heart by 3 months | (174) |
| mdx/mTRKO | Exon 23 point mutation and deletion of RNA component TERC (mTR) of telomerase | Severe dystrophic phenotype: Impaired self-renewal capacity, severe muscle wasting, accumulation of fibrosis and calcium deposits, increase creatine kinase levels, kyphosis, dilated cardiomyopathy, heart failure and shortened lifespan (12 months). | (181) |
prednisolone displayed growth retardation [134]. Aside for improvements in muscle strength, VBP15 (15 mg/kg) treated mice showed a 38% reduction in inflammatory foci compared to non-treated [134]. VBP15 in currently undergoing a randomized, double-blinded and placebo-controlled phase I clinical trial in healthy adults, to evaluate the safety of VBP15 after a single dose and after 14 daily doses of VBP15 (ClinicalTrials.gov Identifier: NCT02415439). Arginine pyruvate is another pharmacological drug and was shown to protect mdx mice against cardiac hypertrophy by 25%, ventricular dilation by 20%, and kyphosis by 94% [128].

**Mdx mice on C57BL/6 background**

*Features of C57BL/6-mdx mice: Mdx on C57BL/6 background (C57BL/6-mdx) is a novel murine model that is valuable in comparative studies, involving the use of mouse models such as mdx52 [135]. The C57BL/10 genetic background of mdx mice poses as a barrier to analyze and compare the phenotype of other mouse models such as mdx52 (which possesses a C57BL/6 genetic background). C57BL/10 inbred strain is akin to and shares a common origin with C57BL/6 but differs in allelic variants at H9, Igh2 and Lv loci [136], C57BL/6 genetic background was employed in mdx<sup>2cv</sup>, mdx<sup>3cv</sup>, mdx<sup>4cv</sup> and mdx<sup>5cv</sup>, which were created by treating the mice with chemical mutagens (ethylnitrosourea (ENU)) (see Table 2) [137].* Mdx<sup>2cv</sup> and mdx<sup>3cv</sup> mice both harbor a point mutation at the splice acceptor site in intron 42 and in intron 65, respectively. Mdx<sup>4cv</sup> mice harbor a nonsense mutation in exon 53. A point mutation in mdx<sup>5cv</sup> mice causes a new splice site in exon 10 [67]. The different mutation locations in these mdx strains relative to the seven different promoters in the Dmd gene leads to a wide array of dystrophin isoforms and hence, these mutants might be useful in studies involving dystrophin function and expression [67]. Aside from being useful in comparative studies involving mouse models with similar genetic background, C57BL/6-mdx mice cannot recapitulate the DMD phenotype any better than mdx mice.

*Involvement in therapeutic approaches: There are not many therapeutic studies that involve the use of C57BL/6-mdx mice. Wang et al. reported that induced pluripotent stem cells (iPSCs) from muscle fibroblasts of 14 month C57BL/6-mdx mice (14m-MuF-iPSCs), showed lower proliferation and reprogramming activity than younger C57BL/6-mdx mice [135]. They also showed that the inhibition of TGF-β and BMP signalling stabilized the 14m-MuF-iPSCs, which differentiated into skeletal muscles as efficiently as iPSCs from younger C57BL/6-mdx mice [135]. Fukada et al. report that C57BL/6 strain has the best self-renewal capacity among four inbred strains of mdx mice: C57BL/6, DBA/2, BALB/c, and C3H/HeN [138]. C57BL/6-mdx mice are observed to have a significantly higher count of RFs than mdx52 at all age groups (2, 6, 12 and 18 months) examined [139]. Since the background of these murine model were identical, the results suggest that age, the type and the location of the mutation in the Dmd gene influences the expression and expansion of RFs in skeletal muscles [139].

**Mdx52 mice**

*Features of mdx52: Mdx52 mice, developed in 1997 by Araki and colleagues, contain a deletion of exon 52 of the Dmd gene, resulting in the absence of full-length dystrophin [140]. These mice exhibit muscle necrosis, regeneration and hypertrophy, and more importantly, lacks the expression of two of the four shorter dystrophin isoforms, Dp140 and Dp260 (Fig. 3) [140]. Since the mouse models of that time (except for mdx<sup>3cv</sup>) expressed all dystrophin isoforms, mdx52 was developed to study how deficiency in these isoforms influences the disease phenotype. While mdx52 mice display skeletal muscle pathology similar to mdx mice, the location of its deletion mutation, advantageously corresponds, to the hot spot region (exons 45–55) of mutations in DMD patients. Approximately 70% of DMD deletion mutations are located in this central region [141, 142]. Additionally, absence of Dp260 isoform in mdx52 mice causes abnormal electoretinograms (ERG) similar to DMD and BMD patients, who lack Dp260 due to mutations in exon 44–53 [143, 144]. Figure 2A shows that mdx52 mice have lower RF expansion (low RFs numbers within a single cluster) than age-matched mdx mice (which amounts to a 58% lower RF expansion at 12 months of age as reported by Echigoya et al., 2013) [139]. Hence, it is thought to be a better mouse model at evaluating dystrophin restoring therapies because naturally existing RF might prevent accurate assessment of a therapeutic efficacy.*

*Involvement in Therapeutic Approaches: Exon 51 skipping is the most common target for single exon skipping therapies and is applicable to 13% of all DMD patients [34, 38]. Skipping exon 51 using PMOs restored bodywide expression of in-frame dystrophin (20%–30% of normal levels) in mdx52 mice...*
along with improved muscle function [145]. Exon 51 skipping induced by intramuscular PMO injection in mdx52 mice was recently shown to have the highest percentage of dystrophin positive fibers at 5 weeks of age, when muscle regeneration was very active [146]. PMO uptake into muscle cells of mdx52 seems effective during myogenic differentiation to myotube formation; specifically PMO and 2’OMePS were most efficiently delivered in dystrophic muscles at early stages of C2C12 myotube formation [146].

Multiple exon skipping of exons 45–55 in whole body skeletal muscles using vPMOs restored dystrophin expression up to 15% and ameliorated skeletal muscle pathology in mdx52 mice [145, 147]. This multiple exon skipping therapy is theoretically applicable to 63% of DMD patients with out-of-frame deletion mutations [34, 38]. In addition, this specific mutation is associated with exceptionally mild BMD patients or asymptomatic individuals [148, 149]. Mdx52 is a valuable model for evaluating exon skipping therapies as its deletion mutation is associated with the hot spot region of the human DMD gene.

**Dko mice**

*Features of dko mice:* Dko is a double deficient mouse model that lacks dystrophin and utrophin [150]. Dko was developed to reflect the absence of utrophin protein observed in adult DMD patients, and thereby devise a more severe phenotype than mdx mouse model. Dystrophic features of dko mutants include severe and progressive muscle wasting, weight loss after weaning, abnormal breathing rhythms, early onset of joint contractures and kyphosis leading to slack posture and premature death between 4 to 20 weeks [150, 151]. Although respiratory failure appears to be the primary cause of death in dko mutants, cardiomyopathy and swallowing difficulties due to weak tongue muscles might be contributing factors [150, 151]. However, since dko mice die prematurely (mostly around 10 weeks), they are hard to generate and maintain [152]. Dko mice have more severe dystrophic phenotype than mdx because they lack compensatory utrophin expression that is present in mdx mice [150, 151]. Recent studies suggest that as little as 5% dystrophin expression...
levels can extend the lifespan of dko mice [153, 154]. Clinical symptoms such as waddling gait, kyphosis and short life span observed in dko mice are similar to those observed in DMD patients [150, 151]. Dko mice also express higher levels of immunoproteasome than mdx and display severe atrophy [155]. Mdx (C57BL/10 background) and utrophin-deficient (C57BL/6 background) mice were crossed multiple times to obtain dko mice with hybrid genetic background [150]. It might be more useful to mate C57BL/6-mdx with utrophin-deficient mice to rule out differences in genetic background.

**Involvement in Therapeutic Approaches:** Dko mutants have been used in gene therapies testing, such as exon skipping, and gene replacement using virus vectors. PPMO targeting exon 23 restored dystrophin expression in almost all skeletal muscles and restored expression of dystrophin associated protein such as glycosylated dystroglycan and neuronal nitric synthase in all age groups of dko mutants [156]. It was found that early treatment of PPMO (i.e. during 20–29 days of age) restored dystrophin expression in almost all skeletal muscles of dko mice and resulted in delayed disease progression, prevented severe kypho-

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**Fig. 3.** The promoters and isoforms of the dystrophin gene, and the location of mutations in murine models. (A) The location of different promoters (brain (B), muscle (M), Purkinje (P), retinal (R), brain-3 (B3), Schwann cell (S), and general (G)) of the dystrophin gene is displayed alongside with the location of mutations observed in some murine models (and also illustrates the insertion of the ROSAβgeo in 3' end of exon 63 in mdxβgeo). Yellow rectangles represent exons. (B) The promoters of Dp427 results in “full-length” dystrophin protein (consisting of the N-terminal actin-binding domain, rod domain, WW domain, cysteine rich domain (Cys) and C-terminal domain (CT)). The remaining promoters lead to shortened dystrophin isoforms.
sis and eye infection, and increased life span of dko mutants [156]. However, treatment of PPMO at an advanced stage of the disease had little effect on dko mice even in the presence of high levels of dystrophin [156]. The likely reasons for this finding in later stage are severe loss of muscle fibres and its replacement by fibrotic tissue, along with severe kyphosis [156]. Utrophin upregulation therapy is advantageous in immune response evasion against dystrophin. Dko mutants were also used to test the efficacy of utrophin minigene delivery using adenovirus vectors [157]. Utrophin minigene was found in nearly 95% of muscle fibers 30 days after injection along with a significant reduction in necrosis and an 85% reduction of centrally nucleated fibers (likely due reduced degeneration) was observed in TA muscles compared to non-treated dko mice [157]. Recently, small nuclear RNAs (U7snRNA) along with AONs were packaged into AV vector (scAAV9-U7ex23) and intravenously injected into dko mice [158]. This approach of using small nuclear RNA in antisense mediated-exon skipping therapy was employed to overcome hurdles such as, low efficacy in cardiac muscles, poor uptake and rapid clearance of the drug [158]. Treated dko mice displayed increased dystrophin levels (among 45% to 95%) in all muscles including cardiac muscle, improved muscle function, and increased lifespan (50.2 weeks compared to 10.2 weeks in non-treated dko mice) [158].

**Mdxgeo**

*Features of mdxgeo.* Mdxgeo contains an insertion of a gene trap vector (ROSAgeo) in exon 63 of the Dmd gene, resulting in the loss of cysteine rich and C-terminal domains (as illustrated in Figure 3A) [159]. This mouse model was developed by Wertz & Fuchtbauer in 1998 [159]. And unlike the spontaneous and ethynitrosourea (ENU)-induced mutant mice of that time, mdxgeo had all isoforms mutated and could detect the Dmd gene expression early in embryogenesis and in adult organs (such as the brain, liver, eye, pancreas and lung) by staining for α-galactosidase (LacZ reporter) [159]. Mdxgeo mice display a loss of dystrophin isoforms (including Dp71), abnormally dilated esophagus, cardiac hypertrophy, and other typical dystrophic features such as muscle degeneration, cellular infiltration, and regenerated fibers with centrally located nuclei [159]. Full-length dystrophin was absent in skeletal muscles, however, trace amounts of PCR product reflecting wild-type mRNA was detected in the brain [159]. Krasowska et al. used mdxgeo and inhibitory synaptic markers (such as neurexin2 and vesicular GABA transporter) to show that cognitive impairments in DMD patients might be due to aberrant clustering of receptors at inhibitory synapses in the hippocampus [160].

**Dmd-null**

*Features of Dmd-null mice.* Dmd-null mice contain a deletion of the entire Dmd gene on mouse chromosome X using a Cre-loxP recombination technique [161]. Dmd-null mice were developed to prevent the expression of all dystrophin isoforms (Fig. 3B illustrates dystrophin isoforms) [161]. While mdxgeo may express dystrophin isoforms, Dmd-null mice can express neither revertant fibers nor any of the isoforms as its alternative splicing (exon skipping) ability is lost due to the deletion of the entire gene [162]. Dmd-null mice display muscle hypertrophy, behavioural abnormality, infertility and other dystrophic features similar to mdx mice [161]. These mice are useful in transgenic studies that investigate the function of dystrophin isoforms [161].

**hDMD mice**

*Features of hDMD: Humanized DMD mouse model (B6.DBA2.129-hDMD∗/geo) has been engineered to carry the complete human DMD gene in chromosome 5 of the mouse genome (wild type) [163, 164]. This is not a disease model as it allows for the expression of full-length human dystrophin protein as well as intrinsic murine dystrophin. ‘t Hoen and colleagues designed the humanized DMD model (hDMD) to assess the efficacy and safety of human specific AONs in vivo for sequence specific therapies such as exon-skipping [163]. The hDMD mouse model might provide further insight into gene regulation, genomic stability, and frequency of mutations and recombination in the DMD gene [163]. The hDMD mouse model might potentially be engineered in future to carry mutations in the human DMD gene in a dystrophin-deficient, mdx background [163].

*Involvement in Therapeutic Approaches:* The hDMD murine model is advantageous to test sequence specific therapies such as exon skipping. Optimization of human specific AONs could only be previously conducted in vitro. hDMD mice are very useful as it allows for preclinical testing and optimization of human specific AONs in vivo [165, 166]. Goyenvalle et al. employed the hDMD mouse model...
model to evaluate the in vivo efficacy of 11 different U7 small-nuclear RNA in the splicing of exon 45–55 [167]. Their constructs, which were packaged in an AAV vector, could achieve an efficient multi-exon skipping of at least 3 exons in the DMD gene [167]. On the other hand, crossing hDMD mice with mdx or dko mouse models rescued the dystrophic phenotype as human dystrophin compensated for the lack of dystrophin in the mice [164]. Histological results showed normal fiber size, absence of CNFs and lack of fibrosis [164]. Ongoing experiments aim to induce deletions in the human DMD gene of the hDMD/mdx mouse. This would have great value in preclinical in vivo studies of muscle function, dystrophin expression and the overall success of a particular AON treatment.

Mdx on DBA/2 background

Features of DBA/2-mdx mice: Mdx on DBA/2 background (DBA/2, DBA/2-mdx) has a more severe dystrophic phenotype than mdx (C57BL/10 background) and shares more histopathological features with DMD patients. Fukada and colleagues developed the DBA/2-mdx mouse model which is available in Jackson laboratory and Central Institute for Experimental Animals (CIEA) Japan. The DBA/2 inbred strain is considered a challenging breeder and possesses many mutated genes: They are highly susceptible to hearing loss (Cdh23<sup>ahl</sup>), eye abnormalities reflective of glaucoma (Gpnmb<sub>R150X</sub> and Tyrp1<sup>iso</sup>), extremely intolerant to alcohol and morphine (Kldr1<sup>DBA/2f</sup>) [168, 169]. Unlike C57BL/6 strain, DBA/2 strain is susceptible to audiogenic seizures and resistant atherosclerotic aortic lesions [170–172]. Moreover, DBA/2 mice also display shorter life spans, more pronounced weight loss with age (sarcopenia) and significantly lower self-renewal efficiency of satellite cells than that of C57BL/6 [138]. Unlike mdx mice, mdx on a DBA/2 background show reduced muscle mass, increased fibrosis, and fatty tissue accumulation and reduced regeneration potential of satellite cells, resulting in prominent muscle weakness [138]. Figure 2B shows that DBA/2-mdx mice show a lower percentage of CNFs than mdx and mdx52 mice at 2 months (a 33% reduction of CNFs was shown from unpublished data). The self-renewal ability of satellite cells might explain the difference in phenotypes between mdx and DBA/2-mdx mice [61, 92].

Involvement in therapeutic approaches: DBA/2-mdx is a very new murine model and hence, there are not many therapeutic studies involving its use. Imatinib, a tyrosine kinase inhibitor, blocks the expression of PDGFRα (tyrosine kinase receptors) in skeletal muscle mesenchymal progenitors and reduces fibrosis in DBA/2-mdx mice [173]. Additionally, the therapeutic dose of imatinib does not influence the proliferation of myoblasts in vitro and its use may be promising for stem cell therapies [173].

Cmah-mdx mice

Features of Cmah-mdx: Cmah-mdx mice, developed by Chandrasekharan and colleagues, harbor two mutations: A deletion mutation in the Cmah gene (Cmah<sup>tm1Avrk</sup>) and a nonsense mutation in exon 23 of the Dmd gene (Dmd<sup>mdx</sup>) [174]. The CMAH gene is required for the expression of N-acetylneuraminic acid (Neu5Ac), a type of sialic acid that is incorporated in glycan structures such as glycoproteins and glycolipids [175, 176]. Mice lacking only the Cmah gene display impairments in humoral immune function, coordination, hearing and wound healing [177, 178]. While the Cmah gene is expressed in mice, it is naturally inactive in humans [179]. Knocking-out the Cmah allele eliminates Neu5Ac in all cells of the mdx mice and humanizes the glycan structures in mice [178, 180]. Chandrasekharan et al. reports that changing the sialylation in mdx mice, brought about by the Cmah gene deletion, enhances the disease severity in the mice [174]. In contrast to mdx mice, Cmah-mdx mice showed increased mortality, loss of ambulation, and increased cardiac and skeletal impairment at an earlier age and/or to a greater extent [174]. At 11 months of age, nearly 50% of the Cmah mice died [174]. In comparison to mdx, Cmah-mdx mice at 8 months showed a 70% reduction in constant speed (5 rpm) rotarod test (loss of ambulation), and a reduction in peak force by 88% and 66% for diaphragm and cardiac trabeculae, respectively [174]. Cmah-mdx mice also had increased fibrosis in the quadriceps at 6 weeks of age, increased regions of necrosis in the heart at 3 months of age and, increased fibrosis in the diaphragm relative to mdx mice at 6 months of age [174]. Chandrasekharan et al. discussed two mechanisms that leads to the accelerated and more severe dystrophic phenotypes in Cmah-mdx mice: 1) diminished function of dystrophin-glycoprotein complex including reduced binding of extracellular matrix proteins to α-dystroglycan and reduced utrophin upregulation, 2) increased activation of complement (C5b-9) driven by increased expression of antibodies specific to dietary Neu5Gc, a foreign gly-
can in Cmah-deficient mice [174]. Currently, there are no published therapeutic approaches involving Cmah-mdx mice, a mouse model recently developed in 2010.

**mdx/mTRKO**

Features of *mdx/mTRKO* mice: *mdx/mTRKO* was generated by crossing *mdx* mice with mice containing deletion in the RNA component TERC (mTR) of telomerase [181]. Telomerase is an enzyme that maintains the length of telomeres, which are DNA repeats that protect chromosomes from aberrant recombination, fusion and degradation [181]. *Mdx/mTRKO* was developed, as many studies showed that DMD patients progressively lose muscle regenerative capacity with age and, that telomere shortening increases with age in DMD patients and correlates with reduced regeneration [181]. Unlike *mdx* mice, *mdx/mTRKO* (with dystrophin deficiency and telomerase dysfunction) show a more severe dystrophic phenotype as seen in humans: impaired self-renewal capacity of stem cells, muscle wasting, accumulation of fibrosis and calcium deposits, increased creatine kinase levels, kyphosis, dilated cardiomyopathy, heart failure and shortened lifespan of around 12 months [181]. Mourkioti et al. suggest that dystrophin deficiency coupled with oxidative stress and metabolic demands of cardiac muscles leads to accelerated telomere shortening and progressive cardiomyopathy [182].

**CONCLUSIONS**

Although murine models differ in some respects to the clinical manifestations of DMD in humans, they are still valuable for basic and cost effective investigations involving pathogenesis, and in preclinical trials. Developments in murine models of DMD are essential for overcoming limitations of existing murine models such as *mdx* and for higher success in clinical trials. Modifications to *mdx* mice are useful for reducing the discrepancies in dystrophic phenotypes between mice and humans. For instance, inducing secondary mutations (e.g. *Cmah*-deficient *mdx* mice) that have important cellular effects (e.g. altering the form of glycosylation) or, modifying the genetic background (e.g. DBA/2-*mdx* mice), leads to increased severity of dystrophic phenotype observed in *mdx* (C57BL/10 genetic background) mice. Genetic background influences phenotype: DBA/2 inbred strain has a much lower regenerative capacity of satellite cells than C57BL/10 and C57BL/6 inbred strains, and DBA/2 inbred strain is shown to display reduced muscle weight and myofiber numbers than C57BL/6 inbred strain. *Mdx52* mice are similar to and have the same genetic background as C57BL/6-*mdx* mice, but provide an added value, since it carries a deletion mutation corresponding to the hot spot region (exons 45–55) of the *DMD* gene. DBA/2-*mdx*, *mdx/mTRKO* and *dko* mouse models provide a more severe dystrophic phenotype than *mdx*. *Mdxβgeo* and *Dmd-null* mice lack dystrophin isoforms (including Dp71) and revertant fiber expression, and hence, may be useful in assessing the efficacy of dystrophin amelioration in preclinical trials. The *hDMD* mouse model is useful for optimizing human specific sequences of AONs in preclinical trials. Overall, developments in murine models greatly help in their contributions to the therapeutic approaches for DMD in preclinical trials.

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