HHS Public Access

Author manuscript

Expert Opin Drug Discov. Author manuscript; available in PMC 2021 April 01.

Published in final edited form as:

Expert Opin Drug Discov. 2020 April ; 15(4): 443–456. doi:10.1080/17460441.2020.1718100.

Duchenne muscular dystrophy animal models for high-throughput drug discovery and precision medicine

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Abstract

Introduction—Duchenne muscular dystrophy (DMD) is an X-linked handicapping disease due to the loss of an essential muscle protein dystrophin. Dystrophin-null animals have been extensively used to study disease mechanisms and to develop experimental therapeutics. Despite decades of research, however, treatment options for DMD remain very limited.

Areas covered—High-throughput high-content screening and precision medicine offer exciting new opportunities. Here, the authors review animal models that are suitable for these studies.

Expert opinion—Nonmammalian models (worm, fruit fly, and zebrafish) are particularly attractive for cost-effective large-scale drug screening. Several promising lead compounds have been discovered using these models. Precision medicine for DMD aims at developing mutation-specific therapies such as exon-skipping and genome editing. To meet these needs, models with patient-like mutations have been established in different species. Models that harbor hotspot mutations are very attractive because the drugs developed in these models can bring mutation-specific therapies to a large population of patients. Humanized hDMD mice carry the entire human

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Declaration of Interest

D Duan is a member of the scientific advisory board for Solid Biosciences and an equity holder of Solid Biosciences. The Duan lab has received research supports unrelated to CRISPR editing from Solid Biosciences and Edgewise Therapeutics. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

Reviewer Disclosures

Peer reviewers on this manuscript have no relevant financial or other relationships to disclose.

dystrophin gene in the mouse genome. Reagents developed in the hDMD mouse-based models are directly translatable to human patients.

Keywords

adeno-associated virus; AAV; Becker muscular dystrophy; BMD; CRISPR; DMD; dystrophin; exon-skipping; genome editing; personized therapy

1. Introduction

Duchenne muscular dystrophy (DMD) is the most common lethal muscle disease affecting boys and young men [1]. DMD is caused by mutations in the dystrophin gene [2]. The dystrophin gene is one of the largest genes in the genome. The full-length dystrophin gene spans ~2.4 mb at the X chromosome locus Xp21. It contains 79 exons and transcribes into a 14 kb mRNA. The 427 kD full-length dystrophin protein can be divided into four major domains including the N-terminal, rod, cysteine-rich and C-terminal domain. The rod domain contains 24 spectrin-like repeats and four hinges. In DMD patients, dystrophin expression is abolished mostly by frame-shift mutations. Becker muscular dystrophy (BMD) is a mild allelic variant of DMD [3]. BMD patients carry in-frame mutations that result in the production of a truncated but partially functional dystrophin protein.

Dystrophin is located immediately underneath the sarcolemma in muscle cells. It interacts with the filamentous cytoskeleton through its N-terminal domain and connects to the extracellular matrix via the interaction between the cysteine-rich domain and transmembrane protein dystroglycan. Dystrophin also interacts with a variety of cytosolic proteins, including syntrophin, dystrobrevin and neuronal nitric oxide synthase (nNOS) via the C-terminal domain and rod domain. In addition to these direct interactions, dystrophin also associates with transmembrane protein sarcospan and sarcoglycans. Together, dystrophin and its associated proteins form the dystrophin glycoprotein complex (DGC) [4, 5]. The DGC stabilizes sarcolemma during contraction. The DGC also mediates muscle cell signaling. Loss of dystrophin compromises both mechanical and signaling function. As a consequence, muscle cells undergo a cascade of pathogenic events which eventually lead to degeneration, necrosis, inflammation and replacement of muscle by fibrofatty tissues [6].

Two different strategies have been used to develop drugs for DMD. One aims at restoring dystrophin expression using genetic approaches. The other aims at ameliorating downstream pathological changes with pharmaceutical drugs. Irrespective of the approach, rigorous preclinical testing in animal models is the critical first step. Below we review animal models used in DMD drug discovery with an emphasis on models that are suitable for high throughput and high content screening and precision medicine.

2. Naturally occurring DMD models have laid the foundation for DMD drug discovery

About one-third of DMD patients have no family history [7, 8]. The disease in these patients are due to spontaneous mutations. Similar to humans, naturally-occurring mutations also

present in animals. The mdx mouse and golden retriever muscular dystrophy (GRMD) dog were discovered in 1984 and 1981, respectively [9, 10]. Both models show elevated serum creatine kinase (CK) and muscle pathology. However, only the GRMD dog displays clinical signs similar to that of human patients. The mdx mouse and GRMD dog were later confirmed to carry spontaneous null mutations in the dystrophin gene [11, 12]. In the mdx mouse, a C to T transition in exon 23 creates a nonsense mutation that stops dystrophin protein translation [11]. In the GRMD dog, an A to G transition near the end of intron 6 disrupts the conserved splicing acceptor site. This results in erroneous splicing of exon 6 to exon 8 and subsequent frame shift [13].

Since mdx mice and GRMD dogs are the first confirmed dystrophin deficient animals, they become the most widely used DMD models in pre-clinical studies. Mdx mice are especially popular in research arena because they are fertile, have a near normal lifespan, easy to maintain, readily available, and importantly, affordable to most research laboratories [14]. The use of mdx mice has also benefited from ample reagents, tools and outcome measurement protocols that are already developed for murine studies. Finally, research in mdx mice is promoted by the extensive knowledge on the disease natural history in this model and the establishment of the standard operating procedures to study mdx mice [15, 16]. Indeed, mdx mice have been used to establish preliminary efficacy data for essentially every pharmaceutical drug that has been or is being tested in human patients, as well as many that are still in the early phase of the drug development pipeline (reviewed in [17–22]). Besides drug therapy, mdx mice have also served as the testbed to establish the proof-of-principle for various gene and cell therapies that are intended to restore dystrophin expression (reviewed in [23–33]). An excellent example in this regard is systemic micro-dystrophin gene therapy with vectors derived from adeno-associated virus (AAV) (reviewed in [22, 34–36]).

Mild clinical presentation is a major drawback for the mdx model [37]. This is largely due to the presence of various compensatory mechanisms. To overcome this hurdle, a number of double-knock out mice have been created by genetic inactivation of compensatory genes (such as utrophin, integrin, MyoD, telomerase RNA) in mdx mice (reviewed in [14, 38]). Alternatively, clinically more severe mice are obtained by breeding mdx mice from their original C57Bl/10 background to other backgrounds (such as DBA/2 and Cmah) (reviewed in [14, 38, 39]). Although these phenotypic mouse models resemble patients better, they have not become the mainstream model in DMD research yet. There are several reasons. Most of these models lack comprehensive natural history data. Some are difficult to establish a colony. In the case of double knock out mice, there are always concerns on data interpretation because human patients do not carry mutation in the second gene that is inactivated in mice.

In contrast to mdx mice, GRMD dogs show a clinical course much more similar to that of human patients (reviewed in [10, 14, 40]). Further the body size of the dog is much larger than that of the mouse. The larger body size is extremely appealing for scaling up gene therapy and cell therapy. Despite these advantages, the GRMD dog has only been used in a limited number of studies for testing drug, gene and cell therapy (reviewed in [10, 41, 42]). This is largely because of the difficulties in the generation and caring of affected dogs, the high cost-associated with the colony maintenance, the large dose of vectors needed for systemic

delivery, and the lack of expertise and dedicated personnel in most laboratories. Nevertheless, studies conducted in the canine model are considered much more informative in shaping the design of clinical trials. A carefully planned and sufficiently powered study in dystrophic dogs should be encouraged at the late stage of preclinical drug development.

3. High-throughput high-content screening in nonmammalian DMD modes opens the door to novel therapeutic targets

Our understanding on disease mechanisms has constituted the foundation for traditional DMD drug discovery. With the recognition of dystrophin deficiency as the molecular culprit [2], a great deal of effort has been placed on dystrophin restoration with gene therapy. On the other side, pharmaceutic approaches for DMD have centered on downstream secondary pathogenic events such as inflammation, fibrosis, calcium dysregulation, oxidative stress, mitochondria dysfunction, ischemia, leaky sarcolemma, and muscle atrophy [19, 21]. While these target-defined strategies have been very fruitful in establishing the current landscape of DMD drug discovery, there are also important limitations. For example, we still do not have a crystal-clear understanding of all the molecular and cellular changes in DMD. In a small but significant group of patients, clinical manifestations remain unexplainable by the reading-frame rule [43–50]. For patients with genotype-phenotype disconnection, the lack of mechanistic insights poses an unsurmountable challenge for the traditional drug discovery approaches because there is no clear druggable target.

Current DMD drug discovery heavily depends on the mouse model. This is doable when dealing with one or two drug candidates. However, it becomes challenging or even impossible if one wants to test a broad range of doses of a particular drug or to find the most effective drug from dozens or hundreds of chemical variants. The time-consuming (often months) and labor-intensive (often multiple assays by multiple people) nature of the mouse study also eliminates the possibility of exhaustively evaluating all known drugs that belong to the same category of a lead drug.

The development of nonmammalian DMD models has opened the door for high-throughput, high-content screening of large libraries of chemical compounds with either known or unknown function (Table 1). Three most commonly used nonmammalian models are Caenorhabditis elegans (C. elegans), Drosophila melanogaster and zebrafish. These models carry many excellent features that make them highly desirable for high throughput, high content screening. These include but not limit to their easy maintenance, short life cycle, large progeny size, genetic tractability, and visible phenotype (Figure 1). The dystrophin gene is highly conserved between mammals and nonmammals [51–55]. The homolog of the human dystrophin gene has been identified in chromosome 1, 3, and 1 in C. elegans, Drosophila, and zebrafish, respectively (https://www.ncbi.nlm.nih.gov/gene?Db=gene&Cmd=DetailsSearch&Term=173038) [55, 56]. DMD models have also been generated in C. elegans, Drosophila, and zebrafish (reviewed in [14, 57–62]).

3.1. The C. elegans DMD model

The first C. elegans DMD model [dys-1(cx18), hlh-1(cc561ts)] was developed in 2000 in the Segalat lab [63]. This model carries a null mutation in the dystrophin gene [dys-1(cx18)] and a thermosensitive mutation in the MyoD gene [hlh-1(cc561ts)] [63]. The double mutant worms show a time-dependent locomotion impairment and muscle degeneration. Mobility change can be visualized by eyeballing. Muscle degeneration can be examined with phalloidin staining (Figure 1). Using these assays, the Segalat lab blindly screened ~1,000 approved drugs [64–68]. They identified more than 20 candidate drugs that are capable of blocking muscle degeneration and/or improving mobility. Interestingly, these drugs not only include the ones that were known previously (such as prednisone, cyclosporin A and calcium antagonist Nifedipine) [64, 67, 68], but also include many new drugs such as antidepressants (serotonin and tricyclic compounds) [65, 67] and carbonic anhydrase inhibitors (methazolamide and dichlorphenamide) (Table 1) [66]. Subsequent validation studies in mdx mice confirmed muscle protection effect of antidepressants and carbonic anhydrase inhibitors [66, 67].

Recently, Hewitt et al reported a new worm DMD model only deficient for dystrophin [dys-1(cx33)] [69]. The dys-1(cx33) mutant carries a different dystrophin null mutation and shows a more severe phenotype than the dys-1(cx18) mutant. Using an automatic force measurement system called NemaFlex, the authors found strength reduction in the dys-1(cx33), but not dys-1(cx18) mutant. The author further validated this new model as a drug screening platform using drugs that are known to improve muscle force in the DMD model (prednisone and melatonin). In conclusion, the authors suggest that future drug screening should use the dys-1(cx33) model [69].

3.2. The Drosophila DMD model

A large collection of Drosophila DMD models have been developed (reviewed in [59, 70–73]). According to the FlyBase website (https://flybase.org/reports/FBhh0000191.html#do_annotation_sub), at least 17 knockdown models have been generated using RNA interference [74, 75]. Besides the knockdown model, at least 7 genetic loss-of-function mutants are available including Dys^{8-2}, Dys^{E17}, Dys^{det-1}, Dys^{EP3397}, DysDf, Dyskx43, and DysExel6184[74, 76, 77]. The fly DMD models show progressive climbing deficits and muscle degeneration. Interestingly, dystrophin plays an important role in wing vein development in Drosophila [78]. As a result, morphological changes in the wing vein can be used as a visible phenotype for screening (Figure 1) [79, 80].

Dilated cardiomyopathy is a major health threat in DMD patients [81]. Two compound mutants Dyskx43/DysExel6184 and Dyskx43/Dys^{8-2} display cardiac dysfunction resembling dilated cardiomyopathy [76]. Surprisingly, heart disease in the fly DMD model can be rescued by Dp116, a naturally occurring truncated dystrophin isoform containing only spectrin-like repeats 22, 23, 24, hinge 4, the cysteine-rich domain and the C-terminal domain. Considering the fact that Dp116 worsens skeletal muscle disease in dystrophin-null mdx4cv mice [82], cautions should be taken when using Dyskx43/DysExel6184 and Dyskx43/Dys^{8-2} as a DMD cardiomyopathy model.

Although the fly DMD model has been proposed for large-scale, high-throughput small molecular screening (Table 1) [62, 70], no blind screening has been conducted using a large chemical compound library yet. Nevertheless, using a candidate drug approach, Pantoja et al found that drugs that can increase the level or signaling of sphingosine 1-phosphate, including 2-acetyl-4(5)-tetrahydroxybutyl imidazole (THI), THI-oxime (a derivative of THI), and FTY720 (gilenya), can suppress dystrophic phenotypes in several fly DMD models [77]. A subsequent study in mdx mice validated findings obtained from the fly model [83].

3.3. The zebrafish DMD model

The zebrafish model is the most frequently used DMD model for high-throughput, high-content drug screening (reviewed in [60, 61, 84, 85]). Similar to the fly model, both knockdown and genetic null DMD models are available in zebrafish. In the knockdown model, dystrophin expression was reduced by inhibiting mRNA translation with a morpholino oligomer [86]. A total of five dystrophin null zebrafish DMD models have been identified in the genetic screen following ethylnitrosourea (ENU) mutagenesis [87–90]. These include *sapje (dmd^{ta222a}), sapje-like (sap^{cl100}), dmd^{pc1}, dmd ^{pc2}, and dmd^{tm90c}*[87, 88, 91]. Four of these contains nonsense mutation in exons 4 (*sapje*), 21 (*dmd^{pc1}*), 32 (*dmd ^{pc2}*) and 53 (*dmd^{tm90c}*), respectively [88, 91]. The *sapje-like* model carries a mutation in the donor splice junction of exon 62 [87].

Dystrophin deficient zebrafish share many characteristic features of human patients. They die prematurely before reaching the reproductive age. Their muscles not only show histological lesions (degeneration/regeneration, necrosis, inflammation and fibrosis) but also are highly susceptible to mechanical strain [92]. Further, both absolute and specific muscle force are reduced [92, 93]. Importantly, the extent of force deficit is similar to what was reported in GRMD dogs [92].

Due to premature death, the zebrafish model is maintained as heterozygous. Dystrophin-null zebrafish are generated by crossing heterozygous animals. Zebrafish do not have defined X and Y chromosomes [94]. Hence, 25% of the offspring will be dystrophin deficient due to recessive inherence of the mutated dystrophin gene. Dystrophic signs can be readily identified using a birefringence assay at 3 to 4 days post-fertilization in affected zebrafishes (Figure 1). The birefringence assay is a sensitive, inexpensive noninvasive assay to quickly evaluate muscle integrity in translucent zebrafish larvae [95, 96]. Normal larval zebrafish show a bright, highly ordered array of birefringence under polarized light. In contrast, affected larvae display reduced birefringence in areas corresponding to damaged muscle (Figure 1). The recently developed automated system and software have further streamline the assay for screening chemically treated larvae in 96 and even 384-well plates [97–99]. Besides the birefringence assay, the dystrophic phenotype can also be evaluated by quantifying survival, muscle force, and the touch-evoked escape response [96].

High throughput, high content small molecule screening in the zebrafish DMD model has greatly benefited from the birefringence assay (Figure 1). To date, at least five chemical libraries have been screened in dystrophic zebrafish [61]. These include Prestwick collection 1 (1,280 chemicals), Prestwick collection 2 (1,120 chemicals), NINDS 2 compound library

(1,040 chemicals), ICCB Known Bioactives 2012 (480 chemicals), and FDA Approved Drugs (640 chemicals) (Table 1) [61]. These screens have yielded at least 25 lead compounds [100–102]. The details of these 25 compounds have been reported elsewhere and will not be discussed here [102].

4. Developing personalized therapy using hotspot mutation mammalian models

More than 7,000 mutations have been reported in DMD patients [46, 103, 104]. Among these, ~68% are large deletions (≥ 1 exon), ~11% are large duplications (≥ 1 exon), and ~20% are small mutations (small deletions, small duplications, splice site mutations, nonsense and missense mutations) [103]. About 67 to 75% of all deletion mutations occur between exons 45–55 [50, 105]. This region is termed the major mutation hotspot (Figure 2A). Approximately 20% of all deletion mutations are found between exons 2 to 20 [105]. This region is termed the minor mutation hotspot (Figure 2B). Duplication mutations are clustered between exons 2 to 20 [45, 46, 103].

The great mutation spectrum creates a significant challenge for sequence-specific therapies, such as exon-skipping and genome editing. It is unrealistic to make a model for each mutation in light of the huge number of mutations found in patients (several thousands) [46, 47, 103]. However, if models can be generated to harbor mutations in hotspot regions, majority of patients will benefit from sequence-specific drugs developed in these models. For example, 13% patients can benefit from CRISPR-mediated exon 51 deletion or antisense oligonucleotide (AON)-mediated exon 51 skipping [103, 106–108]. Removing or skipping exons 45, 53, 44, 46, 52, 50, and 43 can benefit 8.1%, 7.7%, 6.2%, 4.3%, 4.1%, 4.0% and 3.8% DMD patients, respectively [103, 106–108]. Patients with large in-frame deletion of exons 45–55 often show very mild clinical disease [106, 109, 110]. About two-third of DMD patients can benefit from a variant dystrophin protein that lacks exons 45 to 55 [103, 106–108] (Figure 3A).

4.1. Major hotspot mutation mouse models

The major hotspot is located between exons 45 to 55 (Figure 2A) [50, 105]. The mdx4cv mouse is the first model harboring a mutation in the major hotspot. This mouse is generated by ENU mutagenesis in 1989 and it carries a nonsense mutation in exon 53 [111]. This model has been used to test CRISPR-mediated deletion of exons 52 and 53 from the genome [112], or AON-mediated skipping of exons 52 and 53 from the transcript [113]. In both cases, the reading frame was restored. The next major hotspot model is the mdx52 mouse. This model was created by targeted deletion of exon 52 using traditional gene knockout technique [114]. In this model, not only the full-length dystrophin protein (Dp427) but also two smaller isoforms (Dp260 and Dp140) are eliminated [114]. The mdx52 mouse has been used to demonstrate the proof-of-principle for exon 51 skipping [115] and exon 45 to 55 multi-exon skipping [116–118].

Two more major hotspot mouse models, the ΔEx44 and ΔEx50 model, were developed using the CRISPR technology recently [119, 120]. In ΔEx44 and ΔEx50 mice, dystrophin expression is aborted by exon 44 deletion and exon 50 deletion, respectively [119, 120]. These two models

have been used to test CRISPR-mediated gene repair therapy [119, 120]. Amoasii et al designed a single guider RNA (gRNA) to target an exonic splicing enhancer (ESE) near the 5'-end of exon 51. They then delivered the gRNA and SpCas9 to ΔEx50 mice using AAV vectors. SpCas9 created a double strand break at the ESE of exon 51. Subsequent repair by nonhomologous end joining (NHEJ) should disrupt the ESE and prevent inclusion of exon 51 in the transcript. Interestingly, only ~10% of the transcript showed the expected exon 51 skipping. On the other hand, ~70% of the transcript were re-framed due to insertion of an adenosine at the double strand break site in exon 51 [119]. A similar strategy was tested in the ΔEx44 model by Min et al [120]. This time, the authors co-delivered SpCa9 and a single gRNA targeting exon 45 with AAV vectors. They observed exon 45 skipping and re-framing in ~7% and ~42% of the transcript (Figure 3A) [120].

Above mentioned models carry mutations in the mouse genome. These models are good for proof-of-principle studies. However, due to species-specific differences in the genome sequence, reagents developed in these models cannot be directly used in human patients. To overcome this hurdle, a series of new hotspot mouse models were developed in the hDMD mouse, a transgenic strain carrying the full-length human dystrophin gene [121]. These include a human exon 45 deletion model and two human exon 52 deletion models [122–124]. The application of these models in DMD drug discovery is discussed in section 4.6.

4.2. Major hotspot mutation models in larger species

Besides mice, major hotspot mutation models have also been created in other species (Figure 2A). These include a rabbit model in which exon 51 is disrupted by partial deletion [125], a dog model with a point mutation at the 5'-end of intron 50 which leads to the exclusion of exon 50 in the mRNA, two independent pig models in which exon 52 is deleted [126, 127], and a monkey model in which exon 46 (or both exons 4 and 46) is disrupted by small deletions and insertions [128]. As dystrophic manifestations seem to be proportional to the size of the animal, it is likely that these newly developed models will show more severe clinical disease than mouse models do [129, 130].

The rabbit model was characterized by Sui et al recently [125]. Affected rabbits showed characteristic serological, histological and physiological features resembling those of human patients, including significant elevation of serum CK, aspartate aminotransferase, and alanine aminotransferase, variable myofiber size, centrally localized myonuclei (~30%), muscle inflammation, fibrosis, muscle atrophy, growth delay, mobility reduction, and premature death (~20% die within 2 weeks after birth and ~43% die within the first six months) [125]. A unique feature of the rabbit model is the early onset of cardiomyopathy. The left ventricular ejection fraction (EF) and fraction shortening (FS) were significantly reduced by 4 months of the age. Consistently, the authors observed myocardial inflammation, fibrosis, and fatty cell infiltration [125]. The rabbit model will be very useful for testing sequence-specific therapies, for example removing or skipping of exons 45–55.

The dog model was initially discovered in a Cavalier King Charles Spaniel and named CKCS-MD [131]. Subsequently, the dog was crossed to the beagle background and renamed as deltaE50-MD [129, 132]. The CKCS-MD dogs are characterized by marked elevation of serum CK, early onset dysphagia (2 to 3 months of age), exercise intolerance, and characteristic

pathology changes in skeletal muscle (inflammation, variable fiber size, degeneration and regeneration) [131]. However, myocardial pathology appeared mild in a 2-year-old CKCS-MD dog [131]. The beagle background deltaE50-MD dogs are currently being characterized [133–135]. Preliminary conference reports revealed skeletal muscle atrophy, inflammation and fibrosis [134]. The deltaE50-MD dogs were also more susceptible to eccentric contraction-induced force drop [133]. In 6-minute walk test, affected dogs significantly underperformed normal dogs. The distance walked by affected dogs plateaued at ~ 6 months. By 18 months, the averaged distance walked by normal and affected dogs were 359 and 97 meters, respectively [135]. The deltaEx50-MD dog was recently used to test local and systemic CRISPR gene editing therapy [132, 136, 137]. Removing or skipping exon 51 should restore the reading frame in the deltaEx50-MD dog. To achieve this goal, Amoasii et al targeted SpCas9 to an ESE adjacent to the exon 51 splice acceptor with AAV vectors. This strategy will not remove exon 51 from the genome. However, it should result in exon 51 exclusion in the mRNA via exon-skipping. Alternatively, it may result in re-framing by adding one nucleotide (adenine) to exon 51. AAV vectors were delivered to four 1-m-old deltaEx50-MD dogs via intramuscular (2 dogs) or intravenous (2 dogs) injection. Robust dystrophin restoration was observed at 6 to 8 weeks after injection. Similar to what was found in ΔEx44 and ΔEx50 mice [119, 120], the authors observed much more re-framing events than exon-skipping events in deltaEx50-MD dogs [132].

Two independent exon 52-deleted pig models have been generated [126, 127]. In the model reported by Klymiuk et al, affected pigs showed CK elevation, characteristic skeletal muscle histopathology (such as excessive fiber size variation, myofiber degeneration and necrosis, centrally localized myonuclei, interstitial fibrosis, fatty replacement, and inflammation), muscle weakness, reduced mobility, and premature death by 3 months of age due to respiratory muscle failure [126]. The other pig model has not been published in a peer reviewed journal yet [127].

A non-human primate DMD model was reported in 2015 by Chen et al [128]. The authors co-delivered the Cas9 mRNA and two gRNAs (one targeting exon 4 and the other targeting exon 46) to the fertilized rhesus monkey eggs and obtained 14 live monkeys and four stillborn. Among live birth, frame-shifting mutations were identified in eight monkeys including two males and six females. Four had mutations in exon 4, two had mutations in exon 46, and two had mutations in both exons. At the time of publication, these monkeys were between 4 to 6 months of age and none developed obvious clinical signs of muscular dystrophy. For the remaining live birth, five did not have mutation, one animal had a small in-frame deletion in exon 4. Among four stillborn monkeys, two had normal dystrophin expression, one had greatly reduced dystrophin expression, and one more had no dystrophin expression. In two dystrophin-deficient stillborn monkeys, the authors found mosaic frame-disrupting mutations in 87% of the dystrophin gene alleles. They also found histological evidence of muscle disease such as the presence of hypertrophic myofibers with centrally localized myonuclei.

4.3. Minor hotspot mutation mouse models

The minor hotspot is located between exons 2 to 20 (Figure 2B) [105]. The mdx5cv mouse is the first model harboring a mutation in the minor hotspot. This mouse is generated by ENU mutagenesis in 1989 [111]. In mdx5cv mice, an A to T transversion in the middle of exon 10 creates a new splice donor. Aberrant splicing results in a 53 bp deletion in the mRNA and reading frame shift [138]. Interestingly, compared to that of mdx mice, mdx5cv mice show more severe muscle function deficits although both strains display similar histopathology [139].

Base editing is a newly developed genome editing tool. It replaces a single nucleotide without breaking the genome [140]. Kim et al created an exon 20 nonsense mutation mouse model using the cytidine base editor [141]. Specifically, a stop codon (TAG) was created by converting cytidine in a glutamine codon (CAG) in exon 20 to thymidine. This model was subsequently used to test the adenine base editor as a tool to treat nonsense mutation in DMD [142]. In this case, adenine in the antisense strand of the TAG stop codon (CTA) was substituted with guanine to become CTG. As a result, the TAG stop codon in the sense strand was reverted to the original glutamine codon (CAG) in exon 20 [142].

The first minor hotspot region deletion mouse model was reported by Egorova et al [143]. Using the CRISPR technology, the authors removed a 430 kb fragment of the mouse dystrophin gene between introns 7 and 34. The resulting $DMD^{del8-34}$ model lost ~12% of the dystrophin coding sequence spanning exons 8 to 34 and displayed a phenotype similar to that of mdx mice. The reading frame of the $DMD^{del8-34}$ model can be restored by skipping or deleting exons 6 and 7. Removing exons 6 and 7 is predicted to treat ~3% DMD patients [107]. Interestingly, variable clinical manifestations, from the mild BMD type to the severe DMD type, have been reported in patients with in-frame deletions containing exons 6 and 7 [143]. The $DMD^{del8-34}$ model offers an excellent platform to experimentally test exons 6/7 removal therapy.

The Dup2 mouse is one more mouse model with mutation located between exons 2 to 20 [144]. This mouse carries an exon 2 duplication mutation and is discussed in section 4.5.

4.4. Minor hotspot mutation models in larger species

A rat model with mutations in the minor hotspot was created in 2014 (Figure 2B) [145]. The CRISPR technology was used to induce reading-frame disrupting indels in exons 3 and/or 16. The resulting founder rats and their first-generation progeny displayed motor function deficiency and characteristic histological lesions in muscle. It is worth pointing out that the rat model does not have the acute necrotic phase, a unique phase only seen in the mouse model [14]. Further, DMD-mutated rats showed early onset cardiac disease, an observation confirmed in another rat model that carries a small deletion in exon 23 [146].

Several canine DMD models carry mutations between exons 2 and 20 (Figure 2B). These include the GRMD (point mutation in intron 6) [10], beagle-background canine X-linked muscular dystrophy in Japan ($CXMD_J$) (point mutation in intron 6 as in GRMD dogs) [147], Welsh corgi muscular dystrophy dog (repetitive element insertion in intron 13) [148], Labrador retriever muscular dystrophy dog (repetitive element insertion in intron 19) [14], and border

collie muscular dystrophy dog (a single nucleotide deletion in exon 20) [149]. GRMD dogs have been used to test multi-exon skipping with the AAV U7 approach [150, 151]. $CXMD_J$ dogs have been used to test multi-exon skipping with AON [152]. The Welsh corgi and Labrador retriever models are unique because a full-length dystrophin protein can be produced following genomic deletion of the inserted repetitive element with the CRISPR or other genome editing technology. Repetitive element insertion has been seen in introns in the minor hotspot in human patients [153]. The Welsh corgi and Labrador retriever models are thus ideal to develop personalized therapy for these patients. The border collie model can be used to test exon 19/20 or 20/21 skipping and removing therapies. Such treatments will meet the need of 1.6% DMD patients [107].

Several dystrophin-deficient monkeys carry indel mutations in exon 4 [128]. Although exon 4 skipping/removing therapies can restore the reading frame in 0.2% DMD patients [107], it is uncertain whether testing such therapies is the best use of the precious non-human primate model.

4.5. Animal models for duplication mutation

Duplication is the second most common mutation type in DMD patients [103]. However, there is only one duplication model. Using a targeted knock-in strategy, the Flanigan laboratory inserted exon 2 and its flanking sequence into intron 2 at a location corresponding to the duplication mutation hotspot in human patients [144]. The resulting Dup 2 mouse carries a duplicated copy of exon 2 (Figure 2B and 3B). This disrupts the open reading frame in the transcript and aborts dystrophin expression in muscle. The Dup 2 mouse exhibits a phenotype similar to that of the mdx mouse except that (i) the Dup 2 mouse rarely has revertant fibers, (ii) the Dup 2 mouse has a significantly less pronounced CK elevation in the first 12 weeks, and (iii) the Dup 2 mouse has trace amount (≤ 2%) of dystrophin [144, 154].

Exon 2 duplication is the most common duplication mutation in DMD patients [155]. Removing exon 2 is expected to treat 12.7% of single exon duplication patients (1.9% of all DMD patients) [107]. Deletion of one copy of exon 2 restores the full-length protein (Figure 3B). However, deletion of both copies of exon 2 leads to reading frame shift (Figure 3B). Surprisingly, patients without exon 2 display a very mild BMD phenotype instead of the predicted severe DMD phenotype [154]. Subsequent mechanistic studies revealed activation of an internal ribosome entry site in exon 5 and production of a slightly truncated dystrophin protein missing only the first half of the N-terminal actin-binding domain. Approximately 6% of DMD patients can benefit from this N-terminal shortened dystrophin protein. To test exon 2 deletion as a personalized therapy for patients who carry mutations at the 5’-end of the dystrophin gene, Wein et al treated the Dup 2 mouse with an AAV-U7 vector to skip both copies of exon 2 in the Dup 2 mouse. Although the treatment created a frame-shift mutation, it induced the expression of the N-terminal shortened dystrophin protein (Figure 3B). This counter-intuitive therapy significantly improved muscle force and attenuated muscle pathology [154].

4.6. Humanized mouse models for precision medicine

The ultimate goal of preclinical evaluation of a personalized therapy is to apply the animal-validated medication to human patients. Despite the highly conserved nature of the dystrophin gene, there exist significant differences in the DNA sequence among different species. As a result, custom-designed genetic medication for one species often cannot treat the same mutation in a different species. Ideally, sequence-specific DMD therapy should be developed in muscles that carry the human dystrophin gene.

To address this unmet need, ‘t Hoen et al generated the hDMD mouse [121]. Specifically, they introduced the full-length human dystrophin gene to murine embryonic stem cells with a yeast artificial chromosome [121]. The hDMD mouse generated from these cells contained a single copy of the stably integrated human dystrophin gene in mouse chromosome 5. The hDMD mouse maintained the tissue-specific expression pattern of the smaller isoforms of human dystrophin (Dp427m, Dp427c, Dp427p, Dp260, and Dp71). Both human dystrophin and mouse dystrophin were expressed in the hDMD mouse. Subsequent crossing with the mdx mouse resulted in the hDMD/mdx mouse which only expressed human dystrophin [121]. Although the expression of mouse dystrophin is abolished in the hDMD/mdx mouse, this mouse still carries the full-length mouse dystrophin gene. Given the high homology between the human and mouse dystrophin gene sequence, there is a possibility that a therapeutic reagent designed for the human sequence may cross-react with the mouse sequence. To overcome this hurdle, Echigoya et al crossed hDMD mice with DMD-null mice [156]. In DMD-null mice, the entire mouse dystrophin gene is deleted [157]. The resulting hDMD/Dmd-null mice only carry the human dystrophin gene [156].

Although the hDMD mouse, hDMD/mdx mouse, and hDMD/Dmd-null mouse are useful to determine whether human sequence-specific drugs can result in expected changes in the DNA sequence and/or the RNA transcript of the human dystrophin gene, these models are not suitable for studying therapeutic efficacy because they do not have muscle disease. To truly capitalize these models for preclinical development of patient-ready precision therapies, several groups introduced deletions in the major mutation hotspot region of the human dystrophin gene in the hDMD mouse or hDMD/mdx mouse [122–124]. Using the CRISPR technology, Young et al removed exon 45 from the human dystrophin gene in the hDMD mouse and created the hDMD del45 mouse [122]. The hDMD del45 mouse was subsequently crossed with the mdx mouse and the DBA/2-background mdx mouse to generate the hDMD del45 mdx mouse and hDMD del45 mdxD2 mouse, respectively. Both human and mouse dystrophin were absent in these two newly generated strains. Consequently, these mice exhibited dystrophic pathology. The hDMD del45 mdx mouse and hDMD del45 mdxD2 mouse are now ready for in vivo evaluation of personalized genetic interventions that are designed to treat human patients. Such personalized therapies may include but not limit to skipping/removal of exon 44 (treat 6.2% DMD patients), exon 46 (treat 4.3% patients), or exons 45 to 55 (treat 60% DMD patients) [107, 158]. As a proof-of-principle, the authors showed restoration of human dystrophin after CRISPR-mediated deletion of exons 46 to 55 from the hDMD del45 mdxD2 mouse [122, 158].

Using the transcription activator ligand effector nuclease (TALEN) technology, Veltrop et al deleted exon 52 from the human dystrophin gene in embryonic stem cells derived from the

hDMD/mdx mouse [159]. The hDMD del52/mdx mouse derived from these embryonic stem cells showed dystrophic muscle pathology and functional impairment [123]. Similar to the humanized models generated by Young et al [122], the hDMD del52/mdx mouse is also ready for preclinical evaluation of personalized therapies developed for treating human patients. Examples of such therapies include skipping/removal of exon 51 (treat 13% DMD patients), exon 53 (treat 7.7% patients), or exons 45 to 55 (treat 60% DMD patients) [107]. To validate the utility of the model, the authors tested human sequence-specific AONs for exons 51 and 53 skipping in the hDMD del52/mdx mouse [159]. The exon 51 AON had two mismatches with the mouse sequence and the exon 53 AON had four mismatches with the mouse sequence. Direct muscle injection resulted in pronounced skipping of the human dystrophin transcript and restoration of human dystrophin. While the exon 53 AON only induced human-specific skipping, the exon 51 AON also induced low-level skipping of the mouse dystrophin transcript. These results suggest that species-specific sequence differences indeed greatly influence the outcome of precision medicine.

5. Conclusion

DMD is the most common inherited muscle-wasting disease. Currently, there is no cure. Animal models are essential for therapy development. Naturally occurring mdx mice and GRMD dogs have been the primary animal models in DMD drug discovery in the last three decades. High-throughput high-content screening provides a means to rapidly evaluate large collections of chemicals and small molecules for potential drug hits without prior knowledge of the mechanism of action. A series of nonmammalian DMD models have been developed in nematode, fruit fly and zebrafish. Easy to use methods that are based on dystrophin-deficiency related phenotype are now available for conducting large-scale screening. Many interesting hits have been discovered in these screens. Some of these hits may eventually become marketed drugs after preclinical validation in mammalian models and clinical evaluation in DMD patients.

Precision medicine is an emerging approach for developing customized therapy to meet the need of individual patient. DMD is caused by mutations that disrupt the open reading frame. Sequence-specific therapy such as exon skipping and genome editing restores the reading frame at the transcript and gene level, respectively. In DMD, most mutations are deletions and duplications clustered at two hotspots. Many hotspot mutation models were created in the mouse, rat, rabbit, pig and monkey in last several years. Several naturally occurring hotspot mutation models have also been discovered in dogs. Together, 21 hotspot mutation DMD models are now available. Many of these have been used in proof-of-principle studies to establish the feasibility of novel exon-skipping therapies and CRISPR therapies. Importantly, several mouse models were generated on the background of transgenic mice that carry the full-length human dystrophin gene. These humanized mouse models are especially valuable for in vivo evaluation of drugs that are intended for exon-skipping and genome editing in human patients.

6. Expert opinion

DMD drug discovery has followed two independent but intervening pathways. One pathway aims at restoring dystrophin expression in muscle using gene and/or cell therapies. The other pathway aims at ameliorating secondary and/or downstream defects such as inflammation and fibrosis via pharmaceutical interventions. In either case, animal models are essential in identifying the leads, demonstrating the proof-of-principle, and establishing preclinical efficacy and safety data for investigative new drug (IND) application before moving to human trials.

Naturally occurring mdx mice and GRMD dogs have served as the primary workhorse in DMD drug discovery in last several decades. Although these models are still very useful, they cannot fully meet all the needs of DMD drug discovery due to their inherent limitations. For example, the cost and workload have prevented the use of mammalian models in large-scale chemical screening. Most common mutations in DMD patients are large exon deletions and duplications. However, both mdx mice and GRMD dogs carry point mutation and hence are not ideal for precision medicine which aims at developing patient-tailored therapies.

High-throughput high-content library screening offers a great opportunity for new drug discovery. It also provides an economical and expedite way to repurpose medications that have already been approved for other indications. In this regard, simple nonmammalian animal models such as worm, fruit fly and zebrafish are particularly useful. These animals have a small body size, large progeny number, short life cycle, and are easy to breed and maintain. Importantly, the dystrophic phenotype in these models can be easily quantified using tools compatible with high-throughput high-content screening (Figure 1). The availability of nonmammalian DMD models enables unbiased phenotypic screening beyond what can be offered from in vitro models such as patient cells, induced pluripotent stem cells, engineered tissues, and organoids. Fruitful results have been obtained from screens performed in the C. elegans and zebrafish DMD models. Of interest, these screening have identified drugs that are currently in use in DMD patients (such as prednisone) and drugs that have been tested in clinical trials in DMD patients (such as cyclosporin A, nifedipine and sildenafil citrate). These findings suggest that high throughput screening in nonmammalian models may represent a useful approach in DMD drug discovery. Despite immense potential of the nonmammalian models in speeding up new drug discovery, it should be recognized that these animals are evolutionarily very distant from humans, the dose response and physiological consequences could be significantly different between non-mammals and mammals. The effect observed in nonmammalian models could be irrelevant for humans. For this reason, the hit identified in nonmammalian model screening has to be carefully validated in mammalian DMD models before clinical translation.

One of the most important goals of animal model development is to generate models that are suitable for testing novel therapies that can be directly applied to human patients. Hotspot deletion mutation is a characteristic feature in DMD. Unfortunately, mutation in most of the existing DMD models does not fit this profile. New animal models are in urgent need for developing therapeutic interventions that can treat hotspot deletion mutation. To fill the gap, a series of new mammalian DMD models were established in last several years. The

currently available major hotspot models include the exon 44 deletion mouse model [120], exon 45 deletion mouse model [122], exon 46 mutation monkey model [128], exon 50 deletion mouse model [119], exon 50 exclusion dog model [131], exon 51 mutation rabbit model [125], exon 52 deletion mouse model [123], and exon 52 deletion pig models [126, 127] (Figure 2A). The currently available minor hotspot models include the exon 2 duplication mouse model [144], exon 3 and/or 16 mutation rat model [145], exon 4 mutation monkey model [128], exon 8 to 34 deletion mouse model [143], intron 13 and 19 mutation dog models [14, 148], exon 20 nonsense mutation mouse model [141], and exon 20 mutation dog model [149] (Figure 2B). Among these models, the ones with deletion mutation in the human dystrophin gene are particularly useful for evaluating sequence-specific precision medicine such exon-skipping and genome editing [122, 123]. The humanized mouse model provides a unique opportunity to study interactions between the candidate drug and the human sequence in a dystrophic mammal. The pharmacokinetic and pharmacodynamic information learnt from these studies would be of great value for clinical trial design.

Technology advances, in particular targeted genome editing with CRISPR, have significantly simplified the process of animal model creation. Before 2000, there are only two major hotspot models (exon 52 deletion mice [114] and exon 53 nonsense mutation mdx4cv mice [111]) and two minor hotspot models (exon 10 mutation mdx5cv mice [111] and exon 7 exclusion GRMD dogs [12]). Since 2010, the number of major and minor hotspot models has increased to 11 and 10, respectively. However, model characterization has fallen behind model development. Research colonies remain to be established for many new models (e.g. the rabbit, pig and non-human primate models) in order to establish the natural history. A better understanding of onset and progression of skeletal muscle disease and cardiomyopathy in these new models will help us select the most appropriate models for a particular study. For example, one may want to use the rat and rabbit models in studies designed to treat DMD-associated cardiomyopathy because these two models display early onset heart disease [125–127]. An alternative option for cardiomyopathy related studies is the recently established Australian Labradoodle dog model because this model also shows prominent cardiac disease [160]. If the survival is the primary readout, one may want to use the pig (affected pigs die prematurely before 3 months of age) or rabbit (~43% affected rabbit die before 6 months of age) model [125, 126].

It is worth pointing out that the newly developed animal models can also be used to investigate some puzzling clinical observations. Results from such studies may have important implications for personalized therapy in DMD. A good example is the use of the Dup 2 mouse to elucidate the molecular mechanism underlying the paradox genotype-phenotype miscorrelation seen in exon 2 deletion patients [144, 154]. The discovery of the novel ribosome entry site in exon 5 reveals a highly promising new approach to treat patients that have mutations at the 5’-end of the dystrophin gene [154]. By the same token, the hDMD del 45/mdx mouse will be very useful to determine whether in-frame exon 45–47 or 45–48 deletion can indeed yield better outcome than in-frame exon 45–46 deletion [161].

In summary, with newly developed nonmammalian models and hotspot mutation models, investigators can now conduct studies that are not possible with traditional mdx and GRMD models, for example, high-throughput high-content screening, in vivo evaluation of human

sequence-specific AONs for exon skipping and human sequence-specific guide RNAs for CRISPR editing. These new studies will no doubt accelerate DMD drug discovery. However, it does not mean we have solved all animal model-related issues. Several important models are still to be generated such as the hDMD/mdx mouse with human exon duplication mutation and the hDMD/mdx mouse with deletion mutation in the minor hotspot region of the human dystrophin gene. Nearly all current therapies aim to covert the severe DMD phenotype to a relatively mild BMD phenotype. But BMD is not disease-free. Currently, very few models exist for BMD [162, 163]. If we were to further improve DMD therapy, we also need to develop more BMD models in preparation for BMD drug discovery in the future.

Last but not least, we should always bear in mind that no model is perfect. Depending on the question asked and the stage of drug discovery, different models should be used. Nonmammalian models should only be reserved for library screening. Large animal models should be used for late-stage scale-up studies after the candidate drug has been validated in the murine model. The hDMD mouse-based models are excellent for on-target effect evaluation when developing human-ready personalized therapy. However, these models cannot be used to study off-target effect because the hDMD mouse does not carry the entire human genome, it only carries the human dystrophin gene. In this case, human cells may be a better platform to study off-target editing in the human genome.

Funding

The authors thank the support from the National Institutes of Health (NIH) (AR-69085, AR-70517 and NS-90634 to D Duan, GM-063732 and GM-117059 to S Chen), the Department of Defense (MD150133 to D Duan) and the Jackson Freel DMD Research Fund (to D Duan).

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Article Highlights

- Duchenne muscular dystrophy (DMD) is a lethal muscle disease with limited therapeutic options.
- Dystrophin-deficient mdx mice and golden retriever muscular dystrophy dogs are the most widely used animal models in the last three decades.
- Nonmammalian DMD models in worms, flies and zebrafishes open the door to high-throughput drug discovery.
- Hotspot mutation models are excellent platform for developing personalized therapy for a large population of DMD patients.
- The human dystrophin gene-containing mouse models allow accelerated translation of sequence-specific therapies to DMD patients.

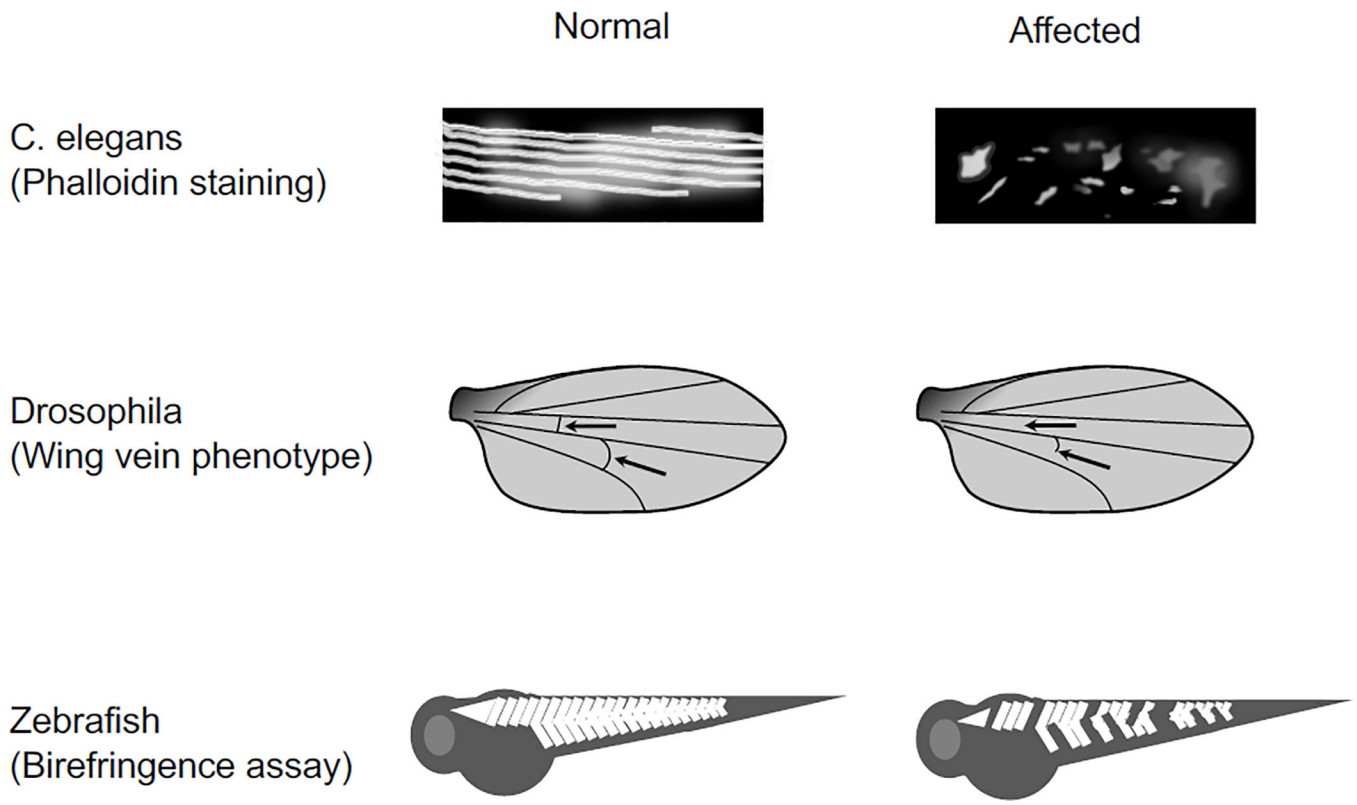

Figure 1. Methods for high-throughput phenotype screening in nonmammalian DMD models.

Top panel, Schematic cartoon illustration of phalloidin staining of the body wall muscle in normal and affected worms. In normal, phalloidin staining reveals a well-organized pattern of actin in muscle. In affected, phalloidin staining shows a patchy fragmented pattern. **Middle panel,** Schematic cartoon illustration of wing vein phenotype in fruit fly. Posterior cross veins are intact in a normal fly but is absent or defective in an affected fly. Arrow, location of the posterior cross vein. **Bottom panel,** Schematic cartoon illustration of birefringence assay in zebrafish. Normal zebrafish has a bright ordered birefringence pattern. Affected zebrafish has random pocketed region with reduced birefringence.

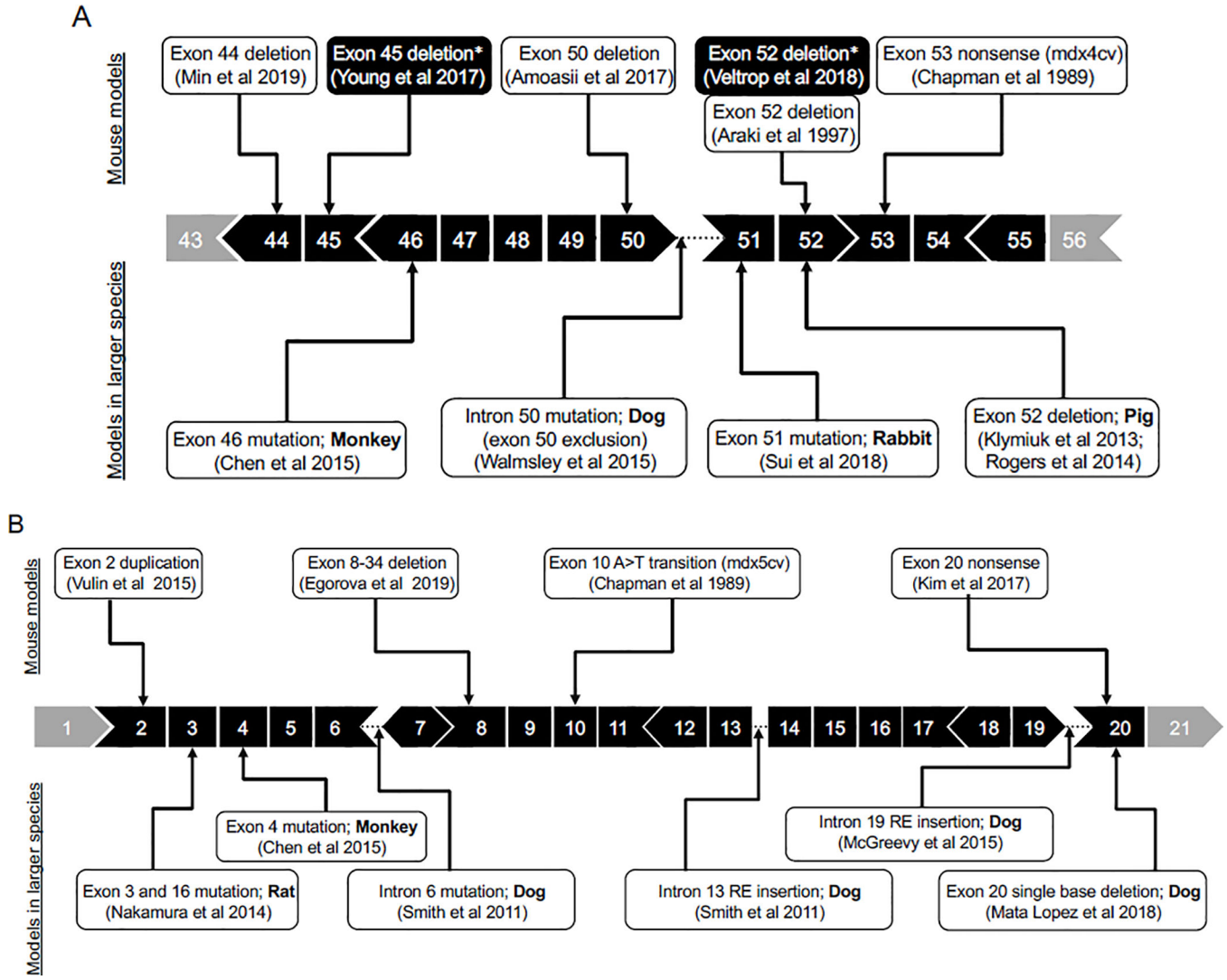

Figure 2. DMD animal models with mutations in regions of exon 45–55 and exon 2–20.

In human patients, about two-third of deletion mutations occur between exons 45 and 55 (the major mutation hotspot) and about one-fifth of deletion mutations occur between exons 2 and 20 (the minor mutation hotspot). A variety of animal models with mutations found in the regions corresponding to the major and minor hotspots of the human DMD gene have been reported. **A,** Models with mutations in the major mutation hotspot region. **B,** Models with mutations in the minor mutation hotspot region.

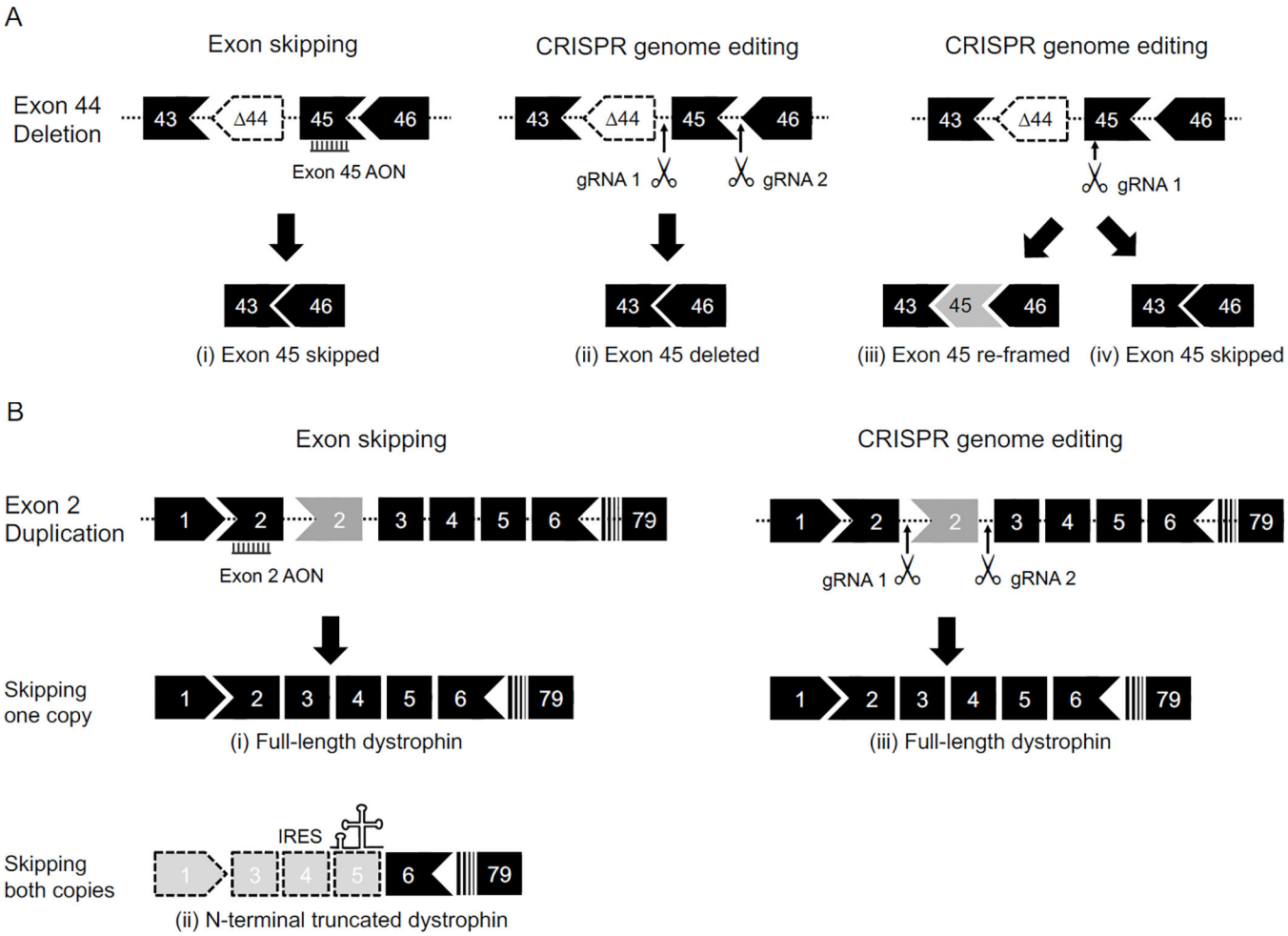

Figure 3. Strategies for treating hotspot mutation with personalized medicine.

A, Exon 44 deletion can be treated with (i) exon 45 skipping using antisense oligonucleotides, (ii) exon 45 deletion using CRISPR, (iii) exon 45 re-framing using CRISPR, and (iv) exon 45 skipping using CRISPR. **B,** Exon 2 duplication can be treated with (i) skipping one copy of exon 2 using antisense oligonucleotides, (ii) skipping both copies of exon 2 using antisense oligonucleotides to induce IRES-mediated translation of a N-terminal partially truncated dystrophin, and (iii) deleting one copy of exon 2 using CRISPR. AON, antisense oligonucleotides; gRNA, guide RNA; IRES, internal ribosome entry site.

Table 1.

DMD drug discovery with nonmammalian models

	C. elegans	Drosophila		Zebrafish
Compound screened	>1000 compounds	No library screened yet	3 candidate drugs	>4500 compounds
Screening Method	Phalloidin staining. Staining is continuous and uninterrupted in WT but discontinuous/disrupted in DMD.	Wing posterior cross vein. The vein is present in WT but absent in DMD.	Projectin immunostaining. Staining is strong and continuous in WT but weak and/or fragmented in DMD.	Birefringence assay. WT shows continuous birefringence but DMD shows fragmented birefringence.
Results	20 candidate drugs have been identified including prednisone, serotonin, carbonic anhydrous inhibitors, cyclosporin A		THI, THI-oxime, FTY720	25 lead compounds have been identified including epirizole, homochlorcyclizine dihydrochloride, conessine, aminophylline, equilin, pentetic acid, proscillaridin A, ergotamine, pergolide, fluoxetine, flunarizine, ropinirole, homochlorcyclizine, pentetic acid, nitromide, propantheline bromide, androsterone acetate, crassin acetate, pomiferin, sildenafil citrate, cerulenin, 9a-11b-prostaglandin F2
References	64 to 68	79	62,77	61, 100 to 102