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Chapter

Duchenne Muscular Dystrophy Animal Models

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Abstract

Duchenne muscular dystrophy is a complex and severe orphan disease. It develops when the organism lacks the expression of dystrophin - a large structural protein. Dystrophin is transcribed from the largest gene in the human genome. At the moment, there is no cure available. Dozens of groups all over the world search for cure. Animal models are an important component of both the fundamental research and therapy development. Many animal models reproducing the features of disease were created and actively used since the late 80's until present. The species diversity spans from invertebrates to primates and the genetic diversity of these models spans from single mutations to full gene deletions. The models are often non-interchangeable; while one model may be used for particular drug design it may be useless for another. Here we describe existing models, discuss their advantages and disadvantages and potential applications for research and therapy development.

Keywords: Duchenne muscular dystrophy, DMD, dystrophin, animal models, *mdx*, genome editing, exon-skipping, gene therapy

1. Introduction

Duchenne muscular dystrophy (DMD) was primarily described in 1834–1836 by Neapolitan physicians Giovanni Semmola and Gaetano Conte. Dr. Guillaume Duchenne de Boulogne made a significant contribution to the description of the disease in 1860s [1]. DMD is considered a rare, or orphan, disease but it is definitely one of the most frequent among muscular dystrophies. About one male in 3500 is diagnosed with DMD. DMD is an X-linked recessive disease so women are affected with a frequency of 1 case per 50 million [2–4]. Many attempts of various groups and organizations are set towards the search for the cure. Different strategies such as genome editing, replacement therapy, anti-inflammatory and antioxidative drug treatment are developed [5]. These therapies target different components of an extremely complex scheme of DMD pathogenesis. So animal models are important for study of the disease, research and development of the therapies. Many animal models were created or, in some cases, adapted from natural sources.

It is important to understand the mechanism of DMD pathogenesis and progression in order to discuss origins, purposes and potential uses of animal disease models. DMD develops when the organism lacks dystrophin expression. Dystrophin is encoded by the largest gene in the genome (*DMD*) that consists of more than 2.3

megabases (Mb). The gene contains 7 promoters and two polyadenylation signal sequences which orchestrate expression of 17 known isoforms. Three large isoforms are produced by three distant promoters. These isoforms are brain isoform Dp427c, muscle isoform Dp427m and Purkinje isoform Dp427p [6]. Each of them consists of 79 exons which include the first unique exon and 78 common exons. Several smaller isoforms are operated by 4 internal promoters and some of them have alternatively spliced variants. These isoforms are retinal Dp260, Dp140 which is prevalent in central nervous system (CNS) and kidney, Dp116 which is expressed in Schwann cells and ubiquitously expressed Dp71 and Dp40 [6, 7]. Muscle isoform Dp427m is the most characterized and widely studied due to its crucial role in DMD manifestation. Most of the mutations that lead to DMD progression are large insertions, exon deletions or duplications which lead to the shift of the reading frame in the Dp427m [8]. Usually these mutations produce preliminary stop codon leading to the complete absence of the protein [8]. Point mutations (deletions, insertions or substitutions) are responsible for a small portion of all DMD cases [8]. Other isoforms are studied less. The deficiency of most of them is usually linked to CNS and behavioral disorders while Dp260 deficiency is linked to retinal impairment [6, 7].

Muscle dystrophin is a very complicated molecular machine. The function of muscle dystrophin is formation of dystrophin-associated protein complex (DAPC) and absorption of mechanical tensions which occur due to muscle constriction [9]. Muscle dystrophin is 427 kDa protein that consists of 3685 amino acids [10]. The protein is usually divided in four functional and structural superdomains. The N-terminal superdomain consists of two calpain-homology domains and provides binding of the protein to actin. The second superdomain is called rod domain. It is the largest domain that includes 24 spectrin-like repeats and 4 unstructured hinge domains. It acts as a spring that adsorbs mechanical tensions. The third superdomain (referred to as cysteine-rich domain, or CR) includes WW-motif, two EF motifs and ZZ-motif. This domain binds dystrophin to the sarcolemmal proteins being the central driver of DAPC formation. C-terminal domain binds to several proteins performing mostly signal functions [10].

DAPC is located in sarcolemma and provides the linkage between dystrophin and external proteins such as laminin and collagen. The complex includes α- and β-dystroglycans, α-,β-, γ-,δ-,ε-sarcoglycans which interact with CR domain of dystrophin; and dystrobrevin, α1, β1, and β2-syntrophins, neuronal nitric oxide synthase (nNOS) and several other proteins which interact with C-terminal domain. The deficiency of these proteins also induces several pathologies such as limb-girdle muscular dystrophy, myotonia and some others [9].

The loss of dystrophin leads to several consequences. The initial one is the loss of membrane integrity and toughness. This causes membrane damage during muscle contractions and consequent membrane leakage. The homeostasis of extra- and intracellular components (calcium ions being the most important of all) is disrupted. This leads to calcium signaling imbalance, mitochondrial dysfunction (as mitochondria acts as calcium depo), proinflammatory and apoptotic signaling activation and other damaging consequences [11]. Finally, this results in muscle cell death and its replacement by new muscle cells originating from satellite predecessor cells that finally leads to depletion of the pool of satellite cells. Damaged and regenerating muscle tissue is characterized by central nuclei. The fraction of central nucleated myofibers is a quantitative marker of DMD progression and therapeutic treatment [12]. Normal muscular tissue is also replaced by connective tissue (fibrosis) and adipose tissue in addition to regeneration. Neutrophil and macrophage infiltration also accompanies the disease progression [13].

The first symptoms of DMD usually arise at the age of 16–18 months. The children may experience issues with walking, running or rising, toe walking or Gower's

sign. At the age of 2–3 years old the muscles of lower limbs begin to degrade. The children suffer from extensive weakness and obtain specific gait patterns. Scoliosis and flexion contractures of the limbs also develop in DMD patients. At the age of 10–12 years old children begin to use a wheelchair. Later, at 14 y.o., some patients develop dilated cardiomyopathy and arrhythmia. Patients usually die at 20 years due to heart failure or respiratory distress in absence of proper treatment. Female carriers do not suffer from severe symptoms; they usually have cardiomyopathy, mild respiratory issues, creatine kinase (CK) level enhancement and pseudo hypertrophy of the backside of the shin [14].

If any suspicious symptoms are observed CK level estimation is the first diagnostic procedure. This is a cheap and fast but not selective test as CK growth is a symptom of various muscle and nonmuscle (i.e. liver) diseases. So further diagnostics is required. If the CK is elevated the screening for exon deletion or duplication should be performed. About 30% of mutations may not be identified by these techniques (multiplex ligation-dependent probe amplification or comparative genomic hybridisation array) and full sequencing of the gene is required. The mutation location and character may help to predict the type and severity of the disease. If the mutation is still unidentified the muscle biopsy sample should be tested for dystrophin protein presence by immunohistochemistry or western blot [14].

In some cases, mutations in *DMD* gene do not lead to reading frame shift or do not cause severe instability or protein dysfunction. If the function of the protein is slightly affected the milder form of muscular dystrophy develops. This disease is referred to as Becker muscular dystrophy (BMD). BMD is characterized by a very wide spectrum of symptoms. In some cases disease may be almost as severe as DMD while in other cases it may develop comparatively mild phenotype [15]. In 1990 a patient with a large part (>50%) of the *DMD* gene deletion was discovered [16]. The patient was active at 61-year-old and demonstrated mild myodystrophy phenotype further described as BMD. The analysis of the mutant gene and its product revealed extremely valuable data on the mechanism of dystrophin molecular action. The deletion of the part of the gene did not lead to reading frame shift and functional protein was expressed. This protein lacked most of the rod domain while N-terminal, cysteine rich and C-terminal domains remained intact. Obviously the rod domain which is the largest part of the protein may be truncated without complete function loss. The second important outcome is the frameshift rule formulation. The restoration of the reading frame may lead to the synthesis of truncated but still partly functional protein and shift the DMD type to BMD type. These findings set the initial point for development of several antiDMD therapies [5].

Currently no ultimate cure for DMD exists. Several treatment strategies are currently applied and many approaches are waiting for approval or being developed [5]. Most of the approved treatments target the farther consequences of dystrophin loss [5, 11]. Glucocorticosteroids suppress fibrosis and inflammation and mechanical ventilation helps patients with respiratory deficits. Anti-inflammatory and antioxidant drugs are also used or being tested [11]. But these approaches do not target the primary issue and are capable of lengthening the lifespan for about a decade. Several more complex approaches are now being developed. One of the most promising candidate therapies is the gene replacement therapy [17]. The idea is the delivery of a shortened but still functional gene copy to the muscles lacking its natural variant. The delivery may be provided via various types of vectors such as viral vectors, nanoparticles or even plasmids [18]. Several difficulties complicate the path to success. These are extremely high research and production costs, immune response and comparatively large size of the protein and corresponding genetic construct. Another class of therapies being developed is restoration of the reading frame [19]. This may be achieved by introduction of antisense

oligonucleotide, genome editing or some other techniques. The next class of therapies is utrophin modulation. Utrophin is an autosomal paralog of dystrophin which shares almost similar domain organization and high sequence correlation with dystrophin. In embryonic muscles utrophin localizes similarly to dystrophin and performs the same functions. In muscles utrophin is replaced by dystrophin in early childhood and in adults it is present in such non-muscle tissues as renal epithelia. In the adult organism utrophin expression is extremely low. In the case of dystrophin deficiency the expression of utrophin starts to increase but its level is still insufficient for dystrophin replacement in humans. Several approaches such as transcription modulators may potentially increase utrophin expression and slow down the disease progression [20]. Interestingly, several species such as mice are able to increase utrophin expression to sufficient level without any modulators [21]. This may provide fundamental data about dystrophy compensation mechanisms. However, it questions the adequacy of the DMD model based on these species. Other strategies include cell-based therapies which are being developed for a long time and interesting exosome-based approach which originated from cell-based one [22].

As can be seen from the above, the existing and potent strategies for DMD therapy include genome editing, pre-mRNA splicing and cell modification, gene or cell delivery, and others [5]. All of them require animal models to be tested. In most cases these models are not interchangeable. For example, if one develops an exon-skipping strategy for a rare mutation, they will need an animal model with a corresponding mutation. So ideally a unique model is essential for every single mutation (at least for most common of them). The type and location of mutation is also important as, despite almost all mutations lead to absence of three major isoforms, the presence or absence of short isoforms depends on mutation location and type. So different mutations on similar backgrounds may have different phenotypes and may be valuable both for research and drug development. Many animal (mostly mouse) models with different specific mutations were developed both for fundamental studies of the gene and protein function and role of short isoforms and for proof-of-concept and preclinical studies of potential therapies.

Despite mouse models of DMD being the most common due to their relative cheapness they possess a significant disadvantage. All dystrophin-deficient animals have dystrophic symptoms but the severity of them does not often correlate with the disease severity in DMD patients. For example the lifespan of classic mouse model *mdx* is about 80% of normal [23, 24] while the lifetime of a human with DMD is not more than one third of healthy. To circumvent these demerits, several other species were used to reproduce the phenotype of DMD in animals. These are large animal models (dogs, pigs, primates) or mouse models with mutations in additional genes, or crossbreed models. In some cases the genetic structure of these models does not correspond to any known DMD mutation in humans but similar phenotype makes them useful for studies of the disease and several symptomatic therapies.

Here we describe animal models starting from classic *mdx* identified in 80's to the newest ones introduced in 2020. The list of model species includes species from such invertebrates as *D. melanogaster* and *C. elegans* to monkeys. The origin, genotype, phenotype and purpose of these models are very diverse. We basically divide the models into two large groups. Chapter 1 will focus mostly on phenotypic properties of the most common models, the comparison of their advantages and disadvantages and their use in research and drug development. Chapter 2 will focus on the models created for development of unique and precision therapies. These are mostly murine models with various spectrum of mutations suitable for targeted drug design such as exon skipping.

2. Animal models to study the pathogenesis of DMD

The most widely used and well described animal model for Duchenne muscular dystrophy (DMD) research is the *mdx* mouse. Spontaneous X chromosome-linked mutation arose in inbred C57BL/10 colony of mice and produced viable and fertile homozygous animals. Mutant mice exhibited specific features similar to human DMD such as elevated plasma pyruvate kinase and CK levels and histological lesions of skeletal muscles. Later, the nature of the mutation was established. Nonsense point mutation caused by a single base substitution of C for T within an exon 23 leads to a premature termination of the dystrophin translation [24]. In addition to the absence of dystrophin all proteins of the DAPC such as sarcoglycans, syntrophin, nNOS, dystrobrevin, α-dystroglycan are significantly reduced at the sarcolemma in *mdx* skeletal muscle [9]. The absence of dystrophin and destabilization of the DAPC complex are believed to make muscle cells susceptible to stretch-induced damage and increased intracellular calcium influx. These pathological processes lead to skeletal and cardiac muscle degeneration [9]. Despite the absence of full-length dystrophin, *mdx* mice have mild symptoms of muscular dystrophy compared to DMD patients or the golden retriever muscular dystrophy (GRMD) dog model [24]. The pathogenesis of muscular dystrophy, physiological, biochemical and histological characteristics have been well studied in *mdx* mice of various ages. Birth body weight and neonatal death rates do not differ from their wild type counterparts. Significant histopathological abnormalities begin to be observed in *mdx* muscles at 3–4 weeks. The occurrence of extensive necrosis followed by regeneration and involving skeletal muscles was documented in *mdx* mice as young as 16–17 days [25]. In humans DMD is characterized by muscle hypertrophy in the early ages and atrophy in the late stages of disease. Contrary, in *mdx* mice myofibers pass through progressive hypertrophy from week 24 till the end of life without atrophy signs. Myofiber branching increased with the age and contributed to the hypertrophy. Aged *mdx* myofibers are also hypernucleated. The "extra" nuclei are central nuclei which highlight that the muscle undergoes continuous cycles of degeneration-regeneration. The estimation of synapse number indicated significant myofiber loss in *mdx* mice with the age [26]. The damaged skeletal muscle fibers with impaired function lead to a 20–30% loss in maximum specific force depending on mice age. The weakness is more severe in muscles of old *mdx* than in younger mice and healthy control mice [27]. *Mdx* muscle also demonstrates high susceptibility to contraction-induced injury [28]. Except skeletal muscles the diaphragm is severely damaged in *mdx* mice showing progressive deterioration, as is also typical for affected humans [24]. Compared to the voluntarily moving limb muscles, diaphragm fibers in *mdx* mice are subjected to early contraction-induced membrane rupture due to continuous action in the absence of dystrophin [24]. Histopathological changes of *mdx* diaphragm start to be observed at 4 weeks and include myofiber degeneration, necrosis, mineralization and large areas of fibrosis. But in contrast to the limb skeletal muscles, which are constantly affected to cycles of degeneration and regeneration, diaphragm undergoes progressive degeneration. By 16 months of age the *mdx* diaphragm looks pale due to extensive myofiber necrosis and replacement fibrosis. Changes in the physiological properties of *mdx* diaphragm correlate to histopathological lesions. Another muscular organ that is affected in *mdx* mice as in DMD patients is the heart. Echocardiographic signs of cardiomyopathy arise after ~8 months of age, while histological evidence of interstitial cardiac fibrosis does not appear until about 17 months [29].

Similar to DMD patients, *mdx* mice have increased levels of CK, marker of muscle damage, wherein CK levels were shown to increase with age, exercise, and male gender [30].

Since the pathogenesis of DMD in the *mdx* mice is genetically, biochemically and histologically similar to DMD patients, they have been extensively used as a preclinical model for DMD over the last 20 years. These mice are used to study the mechanisms of disease occurrence and dystrophin function, to test pharmaceutical drugs and to establish proof-of-concept for gene and cell therapy focusing on restoration of dystrophin expression [24, 30, 31]. The efficacy of a large number of pharmacological agents such as prednisone, deflazacort and other immunosuppressive and anti-inflammatory drugs currently used in therapy of DMD patients was tested in *mdx* mice in preclinical trials [30]. Also *mdx* mice were used in preclinical trials of replacement gene therapy on adeno-associated viruses carrying the dystrophin microgene/minigene. This therapy is currently in clinical trials [17].

Although *mdx* mice are the most commonly used animal model for DMD, its main disadvantage is the mild phenotype compared to DMD patients. To enhance muscular dystrophy pathology a lot of animal models with a more severe phenotype were created. Several approaches were used to create new murine models with DMD symptoms: N-ethylnitrosourea (ENU) mutagenesis (mdx^{2Cv}, mdx^{3Cv}, mdx^{4Cv}, mdx^{5Cv} mice models), generation of humanized transgenic mice with yeast artificial chromosomes (YAC) (hDMD mice), CRISPR/Cas9 (Clustered Regularly Interspaced Palindromic Repeats/CRISPR associated protein 9) and homologous recombination in embryonic stem cells (different murine models with exons deletion/duplication), Cre-loxP (Cre is from gene name *cre* that means "causes recombination"; loxP is for Locus of Crossover in P1) recombination system (*Dmd*-null mice), breeding *mdx* mice with other backgrounds (DBA/2-*mdx* mice, albino-*mdx* mice, BALB/c-*mdx* mice, immune deficient *mdx* mice) or other knockout (KO) murine models (*hDMD*/*mdx* mice, *hDMD*/*Dmd* null mice, *mdx*/Cmah−/−, *hDMD*/*mdx*/*Utrn*−/−, *mdx*/*Utrn*−/−, *mdx*/a7−/−, *mdx*/*MyoD*−/−).

Four new mdx murine models (mdx^{2Cv}, mdx^{3Cv}, mdx^{4Cv}, mdx^{5Cv}) were generated with ENU chemical mutagenesis [32]. Nature of these mutations was characterized. It was established that mdx^{2Cv} allele results from mutation affecting mRNA splicing, and is located in the splice acceptor of intron 42 [33]. The mdx^{3Cv} allele arises from a mutant splice acceptor site in intron 65 [32]. Similar to the mdx^{2Cv} allele, the mdx^{3Cv} splice acceptor mutation generates a complex pattern of aberrant splicing that generates multiple transcripts. But, in contrast to the mdx^{3cv} mutation, alternative transcripts generated from mdx^{2Cv} allele do not preserve the normal open reading frame [33]. In the case of the mdx^{4Cv} allele, mutation is a C to T transition in exon 53, creating a stop codon (CAA to TAA). In the mdx^{5Cv} allele, the dystrophin mRNA contains a 53 base pairs deletion and a single A to T transversion in exon 10 which does not alter the encoded amino acid. But a new splice donor was created (GTGAG) that generates a frameshifting deletion in the processed mRNA [33]. Despite all four new mutants show elevated serum CK level and muscle pathology similar to original *mdx* mice [32], each strain of mutant mice has unique features. Although each strain of mutant mice has unique features. The mdx^{3Cv} mice exhibit abnormal breeding behavior and cognitive defects in addition to dystrophic muscle pathology. The levels of DAPC proteins and full-length dystrophin were decreased. So mdx^{3Cv} mice may act as a useful model for studying the effect of subtherapeutic level of dystrophin on DMD phenotype recovery. Surprisingly, skeletal muscle strength was only slightly reduced compared to wild type mice and muscles were partially protected from eccentric contraction-induced injury [34]. Histopathological analysis of skeletal muscles, heart and diaphragm of the mdx^{4Cv} and mdx^{5Cv} mutants indicates 10-fold fewer revertants than in the muscles of *mdx*

mice [24]. Also *mdx*^{5Cv} mice have a more severe skeletal muscle phenotype than *mdx* mice. These mice showed pronounced functional deficits and lower interindividual variability in motor activity tests compared with *mdx* mice which is a great advantage in studies with small numbers of animals [24, 30, 31]. Both of these murine models *mdx*^{4Cv} and *mdx*^{5Cv} are currently used in preclinical trials of gene therapy [35].

There are several models of mice obtained by crossing *mdx* mice with other genetic backgrounds such as albino mice [36], BALB/c mice, DBA2 mice [37], C57BL/6 mice, C3H mice [38], FVB mice and immune deficient mice [24]. In some cases background does not dramatically alter dystrophic phenotype of *mdx* mice (BALB/c-*mdx* mice, C57BL/6-*mdx* mice, FVB-*mdx* mice). But some murine models obtained during the crossing showed new phenotypic features and more severe phenotypes than *mdx* mice (albino *mdx* mice, DBA2/*mdx* mice). For example, albino-*mdx* mice combined signs of muscular dystrophy (histopathology of skeletal muscles, increased serum CK level, body and muscle weights) with signs of oculocutaneous albinism (skin, fur and eye depigmentation) [36]. In contrast to original black *mdx* mice, albino-*mdx* mice showed slow geotaxis, which can indicate a deterioration of neurological state of DMD [39], and increased circulating cytokines levels [40].

The most phenotypically relevant to the human DMD murine model was created on the DBA2 background. The DBA2 inbred mouse strain carries a naturally occuring in-frame deletion within the latent TGFβ-Binding Protein 4 (LTBP4) gene. This promotes enhanced inflammation and loss of ambulation in DMD patients [41]. The DBA2-*mdx* (D2-*mdx*) mice showed progressive development of muscular dystrophy. These mice had severe histopathological features, including the rapid progression of fibrosis in diaphragm and skeletal muscles. In addition, all muscles of these mice had zones of extensive calcification. In contrast to original *mdx* mice D2-*mdx* mice developed cardiomyopathy at an earlier age, moreover, more fibrous tissue was observed in the hearts of D2-*mdx* mice [37]. The more pronounced dystrophic phenotype and faster progression of the disease in D2-*mdx* mice compared to *mdx* mice on C57BL/10 background makes D2-*mdx* mouse strain more suitable for evaluation of treatment efficacy in preclinical trials [42]. Immune-deficient *mdx* mice are used to test cell therapies as one of the approaches to treating muscular dystrophy. These strains were created by crossing of *mdx* mice with different strains of mice with mutations in different genes (c-kit receptor gene, IL-2 receptor gene, DNA-dependent protein kinase catalytic subunit deficient and others) and deficiency of B cells, T cells and NK cells [43], cytokine signaling deficiency [43], hematopoietic cells deficiency [44] or with severe combined immunodeficiency [45]. Severity of phenotypical features in immune-deficient dystrophic mice are usually similar to *mdx* mice. But these murine strains are a good model for preclinical trials of cell transplantation therapies.

Mdx murine model lacking dystrophin expression demonstrates less pronounced degenerative changes in comparison with DMD in humans. This may be attributed to various species-specific compensatory mechanisms in mice, increased expression of other membrane proteins in murine muscles, or the characteristics of the skeletal and cardiac muscles themselves. To study the effect of compensatory mechanisms in mice, double-knockout (DKO) murine models and humanized murine models were created. Compensation for the lack of dystrophin with structurally related proteins possibly leads to a milder DMD phenotype in *mdx* mice than in DMD patients. In *mdx* mice, unlike humans, the expression of utrophin, in skeletal muscles, diaphragm, heart and non-muscular tissues persists throughout life [46]. The amino acid sequence of utrophin repeats largely dystrophin and can hypothetically substitute it on the sarcolemma and participate in muscle contraction [9].

Therefore, upregulation of utrophin may be one of the treatment options for DMD. To test such drugs as well as to study DMD pathogenesis, mice deficient in both dystrophin and utrophin were created. This double-knockout (*mdx/utrn*$^{-/-}$, u-dko) murine model was derived from breeding dystrophin deficient *mdx* mice with utrophin deficient mice [47]. In contrast to *mdx* mice u-dko mice were smaller and weaker and developed severe muscular dystrophy phenotype similar to phenotype in DMD patients. All clinical signs of the disease (pathohistology of skeletal and cardiac muscles, muscle functions) were more pronounced in u-dko mice than in *mdx* mice. These mice also started to show DMD symptoms at an earlier age [47]. This murine model is currently used in preclinical trials of gene therapy drugs based on adeno-associated or adenoviruses carrying shortened utrophin genes. Several studies have shown that utrophin-delivering therapy is equally effective as micro/minidystrophin-delivering therapies [48]. Another protein that can replace the absent dystrophin and perform complementary function in *mdx* muscles is the membrane protein integrin α7. Dystrophin and integrin α7 double knockout mice (*mdx*/α7$^{-/-}$), as well as u-dko mice, showed a more apparent dystrophic phenotype compared to original *mdx* mice [49]. Dystrophin- and integrin α7-deficient mice had reduced body mass compared to *mdx* mice and demonstrated early lethality (4 weeks after birth). Skeletal and cardiac muscles of double-knockout *mdx*/α7$^{-/-}$ mice were more severely affected and exhibited loss of membrane integrity, more prominent histopathological and functional characteristics [49].

Another explanation for the less pronounced dystrophic phenotype in *mdx* mice may be the increased regeneration of muscle fibers after necrosis which presents in formation of fibers with centrally located nuclei and muscle pseudohypertrophy [23]. To test this hypothesis, several murine models with reduced muscle regeneration were created. Since activated satellite cells are involved in the regeneration of skeletal muscle fibers, murine models with knockout of genes involved in the activation of satellite cells, were created to limit regenerative capacity. The first approach is a knockout of myogenic basic-helix–loop–helix transcription factors MyoD which plays an important role in myogenesis [50]. Mice lacking both MyoD and dystrophin (*mdx*/MyoD/$^{-/-}$) created by breeding of *mdx* mice with MyoD mutant mice developed a severe cardiomyopathy and muscle hypertrophy leading to premature death [51]. Phenotypically these mice are much closer to DMD patients. The second approach to enhance DMD phenotype in mice is a modeling of the telomerase RNA absence. It was established that telomere length in human dystrophic cardiomyocytes and skeletal muscles is shorter than in normal muscles [52]. To create such a murine model, *mdx* mice were crossed with mice lacking telomerase RNA (*mdx*/mTR KO) [52]. *Mdx*/mTR KO showed a severe dystrophic phenotype and significantly reduced lifespan compared to *mdx* or mTR KO controls. Also aged mice showed explicit skeletal deformity (kyphosis) [52]. Double knockout mice make a significant contribution to the study of DMD pathogenesis and the assessment of DMD drug therapy effectiveness, however, these murine models do not directly explain the differences in phenotype between mice and humans. Therefore, so-called humanized murine models were created.

Humanization makes phenotype of *mdx* mice closer to the phenotype of DMD patients. Mice have reduced inflammatory and immunologic reactivity compared to humans. For example, mice, unlike humans, evolutionally retained the cytidine monophosphate-sialic acid hydroxylase (*Cmah*) gene. Introduction of human-like inactivating deletion of Cmah gene into *mdx* mice prevented synthesis of the sialic acid N-glycolylneuraminic acid [53]. The *mdx*/Cmah/$^{-/-}$ mice had genotypic and phenotypic similarities to human DMD, enhanced DMD severity and shortened lifespan compared to *mdx* mice. Cardiac muscle of mutant mice shows large areas of fibrosis and mononuclear infiltration. These features make

mdx/Cmah$^{-/-}$ murine model suitable for evaluating effects of new DMD therapy on dystrophic cardiac muscle.

Dystrophin function, as well as pathogenesis and treatment strategies for DMD have been well studied in different murine models (*mdx*, *mdx/Utrn*$^{-/-}$ dko and many others). All these murine strains lack full-length dystrophin expression and show specific dystrophic features. However, the expression of small isoforms of dystrophin may remain in some models. To study the contribution of small isoforms to the DMD pathogenesis, a model with a completely deleted dystrophin gene was created. Using the Cre-loxP recombination system Dmd gene was completely removed in mice. The resulting mutants (*Dmd*-null mice) were viable, but the males were sterile. The mice showed an evident dystrophic phenotype and behavioral abnormalities [54].

Murine models are the most convenient and widely used for studying protein function, pathogenesis and treatment options for the disease. Many preclinical trials of drugs that are currently used or tested in clinical trials have been performed on DMD murine models. However, many laboratories use not only mice for their studies, but also other species of animals, including non-mammalian models, other rodents or large mammals. Non-mammalian DMD models were generated in zebrafish *Danio rerio*, *Drosophila melanogaster* and *Caenorhabditis elegans* [24, 30, 31, 55]. Non-mammalian DMD models have some advantages over mammals. Fishes, worms and insects are eukaryotic models and have some valuable features: small size, high reproduction rate, fast growth and development, a large number of offsprings and fully sequenced genomes. Dystrophin amino acid sequence and subcellular localization are highly conserved between humans and zebrafish. The zebrafish *dmdta222a* mutants (*sapje*) with dystrophin deficiency showed muscle degeneration which was more severe than in *mdx* mice and died at an early larval stage [56]. Zebrafish DMD model is a good model to test exon-skipping therapeutic strategy. For example, FDA approved drug Ataluren (Translarna) was tested on zebrafish and led to restoration of muscle contractile functions [57]. One more non-mammalian DMD model is dystrophin deficient *Drosophila melanogaster*. Muscle-specific RNAi-mediated knockdown of all dystrophin isoforms in flies led to severe muscle degeneration, cardiomyopathy phenotype and climbing deficits [58, 59]. Nematode worm *Caenorhabditis elegans* is also used for DMD model creation. These worms have dystrophin homolog gene dys-1. Loss-of-function in dys-1 resulted in worm hyperactivity and hypercontraction [55].

In addition to mice, larger animal models are now available. All DMD canine and feline models have been identified in natural populations. Porcine, rat, monkey and rabbit models were created with CRISPR/Cas9 technology [24, 31]. The most popular DMD models in large animals are canine models. Spontaneous mutations in the dystrophin gene causing the development of dystrophic phenotype have been identified in 14 dog breeds [60]. Some of them are currently bred in nurseries as a DMD canine model, others were discovered in natural populations as individual cases and described in the literature. The first group includes the well known golden retriever muscular dystrophy dog model (GRMD), Cavalier King Charles spaniel model [61], Welsh corgi model Australian Labradoodle model, German short-haired pointer and new labrador retriever model with inversion in dystrophin gene [60]. The most widely used and well described canine model of DMD is the GRMD model. The GRMD mutation was first reported in four animals in the early 1980s [62]. It was established that GRMD dogs had a splice site mutation (transition A > G) in intron 6 causing abnormal mRNA splicing and loss of exon 7 of dystrophin gene. GRMD dogs had severe dystrophic phenotype including elevated CK level, skeletal muscle atrophy with contractures, dyspnoea, dysphagia, dilated cardiomyopathy, large fibrosis and fat tissue areas. The GRMD dog population also showed heterogeneity

of dystrophic features between different individuals, what also makes this model similar to DMD in humans [60]. A clinical course of GRMD dogs is more similar to DMD patients in contrast to *mdx* mice. Large body size, severe muscular dystrophic phenotype, humoral and cellular immune response to viral vector and transgene, as well as transplanted cells similar to human, make GRMD dogs a more suitable model for preclinical trials to test pharmaceutical drugs, gene replacement therapies and cell therapies [31]. The GRMD dogs model was used in different preclinical trials of gene and cell therapies. The advantage of using GRMD dogs in these studies is the experiment design similar to clinical trials. For example, in clinical trials, the inclusion criterion is the intake of immunosuppressive drugs. In dogs, in contrast to mice, the immune reactivity is similar to that of humans, which makes it possible to reproduce this design as well as to study the obvious adverse reactions associated with the activation of host immunity [63]. The mutation of Cavalier King Charles spaniel (CKCS) model is a splice site mutation (transition G > T) in intron 50 causing the deletion of exon 50 [61]. CKCS dogs show elevated levels of serum CK and typical areas of necrosis and regeneration in skeletal muscles and heart. Dogs of this breed seem to be suitable for testing due to their small body mass and amiable temperament. CKCS canine model can be used to test exon 51 skipping, the therapy that may be suitable for many patients, as DMD mutation hotspot is located between exons 45 and 55 [61]. The mutation of Welsh corgi model, Australian Labradoodle model and German short-haired pointer model (GSHPMD) are LINE-1 insertion in intron 13, point mutation in exon 21 and whole *DMD* gene deletion respectively [60]. These dogs show severe dystrophic phenotype including muscle degeneration, mineralization and inflammatory infiltration. It is important to note that GSHPMD dog model with completely absent dystrophin is the most suitable preclinical model for the prediction of immune responses to gene therapy due to the lack of immunological tolerance to dystrophin [64]. One more interesting canine DMD model is the recently identified labrador retriever (LRMD) model with an inversion in dystrophin gene. 2.2-Mb spontaneous inversion disrupting the *DMD* gene within intron 20 was found in two young labrador retriever dogs. The clinical signs of disease included elevated CK level in serum, specific histopathological lesions of skeletal and cardiac muscles, myopathic electrodiagnostic profile, high neonatal lethality. The LRMD dogs had detected expression of Dp71 isoforms of dystrophin. But unlike the GRMD dogs with absent Dp71 isoform, the LRMD dogs have more severe dystrophic phenotype. This may indicate that the presence of the Dp71 isoform in muscles does not provide a functional advantage [60].

In addition to dystrophic dog colonies maintained in nurseries several cases of spontaneous mutations in dogs of different breeds have also been described. The interesting case is 7 base pair deletion in exon 42 in Cavalier King Charles spaniel, the second CKCS model with mutation in the *DMD* gene hotspot area. These dogs had generalized skeletal muscle atrophy of the temporal region, limbs and thoracolumbar spine [65]. One more case of spontaneous mutation in dystrophin gene was revealed in Miniature Poodle dog. Dogs had whole *DMD* gene deletion and showed all dystrophic clinical signs including muscle degeneration, lumbar kyphosis, stiff gait and abnormal posture. Neurological examination also revealed reluctance to exercise in these dogs [66]. One case of disease development was also recently detected in the Jack Russell Terrier population. The dog had deletion of exons 3–21 causing severe dystrophic phenotype and death at the young age [67]. Progressive muscle weakness was also detected in a male border collie dog. Its mutation was a single nucleotide deletion in canine DMD exon 20, minor DMD mutation hotspot, resulting in generalized muscle atrophy, muscle fatigue and dysphagia [68].

Unequivocally, canine models have a significant advantage over murine models due to their more pronounced dystrophic phenotype and possible immune response

to treatment. However, as well as *mdx* mice, GRMD and other dogs have some disadvantages associated with the high cost of keeping dog colonies and training of personnel caring for sick animals. In addition, due to a greater body weight than in mice, large amounts of drugs are required for dogs, which is essential for gene therapy based on viral delivery. Nevertheless, studies in dogs are considered more informative than studies in mice. The results of the dog trials provide a better indication of future clinical trials. In this regard, it is important to use not only widespread mice but also dogs in the design of preclinical trials.

The first case of hypertrophic feline muscular dystrophy (HFMD) in domestic cats was described in 1989 [69]. Spontaneous mutation causing dystrophic phenotype was established as a deletion of the dystrophin promoter and first exons corresponding to dystrophin from muscle and Purkinje cells. Dystrophic cats showed pronounced appendicular and axial muscle hypertrophy, involving of tongue and diaphragm, histopathological lesions in skeletal muscles, diaphragm and heart, including different fiber diameter and acute necrosis and cardiomyopathy [70]. The HFMD model is rarely used in DMD preclinical research because tongue hypertrophy and diaphragm defects lead to difficulties in feeding, animal welfare and early death.

The CRISPR/Cas9 technology has made it possible to create several more models of DMD in such animals as pigs, rats, rabbits and monkeys. Rats are the most convenient animals for biomedical research, therefore several rat models have been created. The first rat model was created using CRISPR/Cas9 gene editing [71] and had exon 3–6 deleted in dystrophin gene. Dystrophin deficient rats showed reduced muscle strength and specific dystrophic phenotype of skeletal muscles, diaphragm and heart. Also these rats showed age-dependent decline of cardiac functions similar to DMD patients [72]. Later, based on this model, another rat model with an in-frame mutation in the dystrophin gene was generated [73]. New mutant rats had reduced expression of truncated dystrophin and mild phenotype similar to BMD patients. These rats can be useful to study BMD pathogenesis and efficiency of dystrophin recovery. The third rat model was created using TALEN (Transcription activator-like effector nucleases) technology. Its mutation was a frame shifting 11 base pairs deletion in exon 23 generating premature stop codon [74]. Animals exhibited reduced muscle strength, cardiomyopathy, large muscle necrosis and fibrosis. This model can be used for preclinical research as a small DMD animal model.

Several mice models were created that may be suitable mostly for scientific use. One of them is the $Dmd^{mdx-bgeo}$ model [75]. It contains the beta-Geo marker inserted after exon 63. The protein product translated from the resulting allele lacks cysteine-rich and C-terminal domains and is not functional. The $Dmd^{mdx-bgeo}$ model mostly resembles the mdx^{3cv} model as both of them lack all dystrophin isoforms including Dp71 and Dp40. Hemizygous $Dmd^{mdx-bgeo}$ animals demonstrate phenotypic properties similar to other *mdx* models. LacZ (β-galactosidase-mediated) staining helps to visualize the expression of dystrophin in various tissues on different stages of development including embryonic. Nevertheless, the dysfunctionality of dystrophin-lacZ chimeric protein should always be taken into account.

Dmd^{EGFP} reporter mouse [76] lacks the disadvantage of $Dmd^{mdx-bgeo}$ model. The eGFP (enhanced green fluorescent protein) coding sequence was introduced behind the exon 79 and the chimeric protein remains functional. The transgenic mice did not show any signs of pathology. This approach allows us to observe almost all major dystrophin isoforms except for those having alternative C-terminal domain. The studies with this model may provide valuable data on dystrophin expression and localization in muscle and non-muscle tissues and shed the light on its functions.

In 1999 the Dp71-null mouse model was described [77]. The first and unique exon of Dp71 is located between exons 62 and 63 of the *Dmd* gene. It is replaced by promotorless b-geo gene in Dp71-null mice leaving all other dystrophin isoforms intact (except for Dp40). The resulting construction provided the expression of β-Galactosidase regulated by Dp71 promoter while the native product, Dp71, was absent. This model acts as a valuable tool for examination of the role and functions of Dp71 isoform both by Dp71 promoter activity estimation by LacZ staining and Dp71-null phenotype examination. The further experiments demonstrated that Dp71 deficiency causes retinal vascular inflammation, increases retinal vascular permeability. AAV-mediated delivery of Dp71 restored retinal homeostasis and prevented retinal oedema [78] and restored defective electroretinographic responses [79]. Dp71 expression in neurons plays a regulatory role in synapse organization, formation and function and inactivation of Dp71 may lead to increased severity of mental retardation and intellectual disability [80].

3. Animal models to test precision medicine approaches

Genetic testing revealed the incredible diversity of mutations in *DMD* gene. However, mutations are not equally presented throughout the gene. As much as 80% of all mutations are concentrated in exons 2–20 and 45–55 representing two hotspots. Mutations can be divided into two groups: frequent (one or more exons deletions and duplications) and rare (point substitutions in exons and introns, small deletions and duplications). [81]. Mutation-specific precision medicine approaches are mostly based on the reading frame rule and convert mutations from Duchenne to Becker type. In the presence of frameshift generating mutations additional removal of one or several exons can restore the reading frame and cause expression of shortened yet functional dystrophin protein. For exons removal during splicing process antisense oligonucleotides (ASO, AON) are used. AON binds specifically to the splice sites of selected exons hiding them from cellular splicing machinery and leading to their exclusion from mRNA. Different chemical structures are used for reduced AON cleavage, prolonged circulation, better cellular and nuclear penetration. The most popular backbones are presented by PMO (phosphorodiamidate morpholino oligomers), 2-OMePS (2'O-methylated phosphorothioate), vivo-morpholino (morpholino oligo covalently linked to octaguanidine dendrimer), LNA (locked nucleic acids), tcDNA (tricyclo-DNA). Indeed, AON can be delivered naked or in the lipid complex, fused with targeting peptides or other molecules enhancing biodistribution. In addition to AON, vectorized drug candidates are tested for exon skipping. Their design is based on U7 snRNA, naturally participating in histone pre-mRNA processing. Deletion of additional exons directly from genomic DNA (gDNA) is also proposed as a mutation-specific therapeutic strategy for DMD. For this purpose viral delivery of one or two single guide RNA (sgRNA) and Cas9 encoding sequences is tested. Targeted Cas9-induced double strand cleavage is also applied for indel generation in affected or neighborhood exons. Indels lead to +1 or − 1 frameshifts with a certain probability. This approach is known as reframing. Exon can be excluded from mRNA due to another DNA modification - base editing in conservative splice site sequence. For this approach Cas9 fused with base editing enzymes is utilized. Both for U7 snRNA (U7 small nuclear RNA) and Cas9 delivery viral vectors such as lentiviruses and AAV are used. Majority of experiments for mutation-specific approach examinations are conducted on patients-derived cell cultures and modified human embryonic stem (ES) cells. However, complexity of the disease and limitations of functional tests applicable *in vitro* force to generate and use genetically modified animal models.

Mice with various mutations in the dystrophin gene, replicating mutations found in individual patients or groups of patients are the most common among the genome-edited models. The timeline of disease progression and traits of new models are usually not well studied. Main DMD symptoms are similar to those found in *mdx* mice. The purpose of these "genetic" models is to test mutation-specific therapies and show not only restoration of dystrophin expression but also improvement in locomotor activity, illustrating the functionality of the shortened protein. The ease of maintenance and reproduction, extensive experience in obtaining and speed of reproduction, together with the high conservativeness of the dystrophin gene, make mice the optimal objects for such work, nevertheless, there are other, larger animal models with mutations often found in patients with DMD.

Deletions of one or more exons are the most common mutations in the DMD gene. They account for 68% of all mutations. Among them, deletions of single exon 44 (3%), 45 (4%), 50 (2%), 51 (3%), 52 (3%) are represented with approximately the same frequency [81]. Directed mutations in the dystrophin gene in laboratory animals were obtained for the purpose of selecting drugs for exon-skipping. Exon structures of popular models with deletions in mutation hotspot are shown on **Figure 1**. The first models were obtained by homologous recombination using embryonic stem cells. In 1997 a mouse model *mdx52* with a deletion of exon 52 was created [82], where this exon was replaced with a neomycin resistance cassette. This mouse model was used to test various drug candidates: PS-modified tcDNA (phosphorothioate-modified Tricyclo-DNA) based ASO for skipping of the exon 51 [83], PMO for exon 51 skipping [84, 85], AAV9-U7snRNA for exon 51 skipping [86],

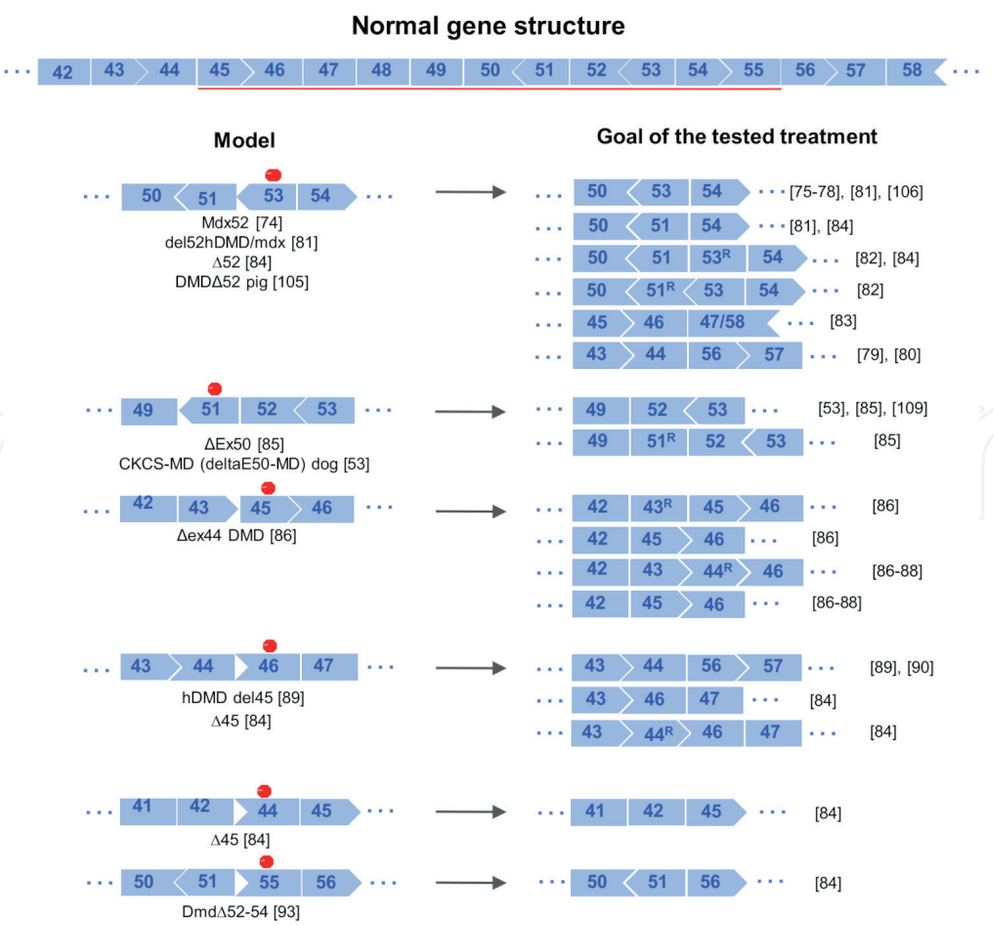

Figure 1.
Animal models representing DMD exon deletions in mutation hotspot. Gene fragment structures around exon with frameshift mutation are shown on the left. Currently tested therapeutic approaches and resulting exon structures are shown on the right.

mix of vivo-morpholinos for simultaneous skipping of the exons 45–55 [87, 88]. With the advent of effective genome editing techniques, frequent mutations were the first to be reproduced in animals. TALEN were used to create mice with exon 52 deletion resulting in **del52hdmd/***mdx* model [89]. This model is notable for the fact that the mutation was introduced into the sequence of the human gene. Thus, **del52hdmd/***mdx* model can be used to test drugs that are designed to target unique human sequences. The authors showed the effectiveness of AON for skipping exons 51 and 53 to the human sequence during intramuscular delivery [89]. This line was used to test CRISPR/Cas9 genome editing complexes for reframing in exons 51 and 53 during lentiviral delivery [90]. AAV9 double SaCas9 (*Staphylococcus aureus* Cas9 ortholog) and guide mix was tested for deletion with borders within exons 47 and 58 for himeric exon formation [91]. Later, another mouse model with a deletion of exon 52, **Δ52**, was obtained using CRISPR/Cas9 genome editing system [92]. This model was used to test CRISPR/Cas9-based drugs for exon 53 removal or reframing [92].

Reframing in exon 51 was also tested in mouse models with deletion of exon 50 **ΔEx50** and **ΔEx50-Dmd-Luc** [93]. In the *Dmd* gene of **ΔEx50-Dmd-Luc** mice, in addition to the deletion of exon 50, the luciferase gene sequence is also introduced at the C-terminus, connected to the protein sequence via an autocatalytic 2A peptide. Thus, luminescence was observed during the restoration of the reading frame, which allowed to assess the effectiveness of drugs *in vivo* without resorting to invasive methods [93]. Bioluminescence was detected both after intramuscular and systemic delivery of Cas9 and sgRNA-51 by AAV9. The presence of bioluminescence was shown to correlate with dystrophin expression as verified by western blotting and immunohistochemistry (IHC) [93].

One of the most frequent deletions, the deletion of exon 44, was reproduced in mice **Δex44 DMD** [94]. Correction of exon 44 deletions by gene editing of surrounding exons could potentially restore the reading frame of dystrophin in ~12% of patients with DMD. Authors created AAV9-Cas9 and AAV9-sgRNA mix targeting 5′-end of exon 45 and tested them *in vivo* during intramuscular injections on this model. The most perspective guide sequence 6 (G6) was used for systemic delivery and selection of a better Cas9 to sgRNA AAV particles ratio. Selected conditions lead to force increase from 59% to 107% in the extensor digitorum longus (EDL) muscle of **ΔEx44 DMD** mice [94]. 20-fold lower dose of self-complementary adeno-associated virus (scAAV) bearing Cas9 + sgRNA was used for exon 45 skipping and reframing on the same model [95]. Weekly injection of (1,2-dioleoyl-3-trimethyl-ammonium-propane) LNPs (lipid nanoparticles) encapsulating Cas9/sgDMD RNPs (ribonucleoproteins) into Tibialis Anterior (TA) muscles was tested on **ΔEx44 DMD** mice. The expression of dystrophin in TA muscles was successfully restored after skipping or reframing of exon 45 induced by treatment, as demonstrated by immunofluorescence and western blot analysis. Quantitative analysis of the western blot result showed that 4.2% of dystrophin protein was restored [96].

CRISPR/Cas9 genome edited **hDMD del45** model represents deletion of exon 45 in human dystrophin gene in the presence of wild type *Dmd* gene while **hDMD del45** *mdx* **D2** has dystrophic phenotype due to *Dmd* gene knock-out [97]. In the same paper exons 45–55 deletion strategy (Cas9 + gRNAs to introns 44 and 55) aiming to help 60–65% of patients was tested [97, 98]. A more realistic approach from the clinical application point of view is multiple exon 45–55 skipping using U7 snRNAs [99]. It was tested on **hDMD/***mdx* model [100]. Similar multiple exon-skipping strategy using PMOs cocktail [98] was tested on **hDMD/Dmd Null** mice [85]. The **hDMD/Dmd Null** model compares favorably with the previous models, since it does not have a mouse dystrophin sequence and allows us to quantify the level of exon skipping and compare the effectiveness of different sequences and

drugs with each other, which is demonstrated by the example of exon 51 skipping [85]. Moreover, the presence of normal dystrophin in some models leads to the absence of the necessary symptoms for the delivery of oligonucleotides and viruses, such as inflammation in the muscles and intact cellular membranes. It's necessary to point out the crucial role of **hDMD** mouse model with full-length *DMD* gene integrated into chromosome 5 [100]. It is not very useful for any drug substances by itself due to simultaneous expression of wild type human and murine dystrophin proteins. But when crossing to *mdx* or other *Dmd* knockout mice (**hDMD/*mdx*, hDMD/Dmd Null**) it becomes an extremely important background for creation of new models. Any antisense or guide molecules designed and tested on subsequent animals can be transferred to human cells without sequence adaptation.

Models with single exon deletions **Δ43** (exon 43 deletion), **Δ45** (exon 45 deletion) were reported together with **Δ52** (exon 52 deletion) **DMD** mice [92]. These mouse models were used to test single guide genome editing procedures aiming at exon skipping or reframing. AAV9 and scAAV9 viruses with a guide and Cas9 sequences were used. Intramuscular delivery of guide RNA to exon 44 and Cas9 encoding viruses to TA muscle restored dystrophin expression in both **Δ43** and **Δ45 DMD** models. Interestingly, the selected guide generated both exon reframed and exon skipped transcripts in **Δ45 DMD** muscle, but only exon skipped transcripts in **Δ43 DMD** muscle. Restoration of dystrophin in **Δ45 DMD** muscle was more efficient than in **Δ43 DMD** muscle when using the same sgRNA for gene editing [92].

Deletions of several exons are quite common in patients, but to date only one mouse model with an extended mutation in the hotspot is known. Deletion of exons 52–54 was simulated in **Dmd Δ52–54** in which authors declare severe cardiac dysfunction in addition to common skeletal muscle symptoms. CRISPR-mediated single sgRNA exon skipping of exon 55 was tested on this model. Also another model with deletion of exons 52–55 was created to check potential benefit of the treatment [101]. CRISPR/Cas9 system popularity, easy to use and high efficiency allow to generate such 100% skipping (editing) models to check generated shortened dystrophin protein functionality.

Duplications of one or several exons are also highly widespread mutations, affecting 5–10% of all DMD patients [102]. The most common duplication in the patient population **Dup2** (duplication of exon 2) was recreated in the mouse model created in 2015 [103]. Correction of the mutation was shown on this model after intramuscular injections of AAV1.U7-ACCA [104]. Main attribute of this mutation and corresponding model is that precision skipping of a duplicated exon results in full-length dystrophin expression. Thus, it is the rare case when exon-skipping converts Duchenne type mutation to wild type rather than Becker type. At the same time it is a challenge to accurately skip only copy of exon 2 not affecting the main sequence.

Nonsense mutations are also very common in the human population affecting approximately 25% of the patients [102]. The most popular *mdx* mouse model representing nonsense mutation was found in the natural population [105]. A lot of treatment strategies targeting downstream disease mechanisms were tested on this model. Precision medicine approach on *mdx* model includes stop codon readthrough [106]. Targeted single nucleotide mutation was created in **DMD-KO** (D108) mouse [107] representing C-to-T conversion generating stop codon in exon 20 (Q871Stop). This mouse model was used for adenine base editing using guided SpCas9 (*Streptococcus pyogenes* Cas9 ortholog) nickase [108]. Created by ENU mutagenesis, *mdx*^{4cv} model also has C-to-T conversion in exon 53 generating TAA stop codon [33]. Adenine base editor was also tested on this mouse model with modified Cas9 recognizing relaxed minimal PAM (protospacer adjacent motif) sequence - NG [109]. Both *mdx* and *mdx*^{4cv} models were used for trans-splicing method

efficiency demonstration [110]. Intramuscular delivery of AAV vectors expressing trans-splicing template (PTM) allowed detectable levels of dystrophin in *mdx* and *mdx*^{4cv}, illustrating that a given PTM can be suitable for a variety of mutations.

Rare mutations found in patients were repeated in models to test precision gene editing methods. Those contain big deletion of exons 8–34 in **DmdDel8–34** mouse model (Egorova et al., 2019). Reading frame in this model can be restored by simultaneous skipping of exons 6 and 7. The fact that the N-terminal actin binding domain is partly encoded by these exons, gives the opportunity to better understand structure-functional interplay in dystrophin protein and its shortened forms. Several approaches including vivo-morpholino induced exon-skipping and CRISPR/Cas9 gene editing are tested on this model ([111]; unpublished data). Other variants of rare mutations generated by Koo and colleagues, represent small frameshift mutations [112]. In vivo treatment with AAV vectors encoding CjCas9 (*Campylobacter jejuni* Cas9 ortholog) and single guide to the affected exon restored the reading frame and enhanced muscle strength.

Large mammalian models have more pronounced DMD symptoms in comparison to murine models. But limited availability and less extensive experience in their genome modification led to reduced use in precision medicine approaches testing. Pig model with deletion of exon 52 **DMDΔ52** was created using somatic nuclear transfer from bacterial artificial chromosome (BAC)-edited cells [113]. AAV9 vector with intein splitted Cas9 and two guides around exon 51 was tested on these animals reaching widespread dystrophin expression, prolonged survival and reduced arrhythmogenic vulnerability [114]. Another example of CRISPR/Cas9 Dmd gene targeting in pigs resulted in indels in exon 27 which lead to premature piglet death at day 52 [115]. So this model was not tested yet for any treatment approach.

DMD KO rabbits represent different mutations in exon 51 which is within the mutation hotspot in human *DMD* gene [116]. Many of these mutations are small deletions or insertions, disrupting the reading frame of the *DMD* gene, resulting in frameshift and complete absence of dystrophin expression followed by main phenotypic features in skeletal muscles and cardiomyopathy. Animals could benefit from 50–51, 51–52 or 45–55 exons skipping, however this model animals were not used for any precision medicines testing.

Identified in natural population **CKCS-MD** (deltaE50-MD) dogs have a splice site missense mutation in intron 50 of the *DMD* gene, causing out-of-frame skipping of exon 50 and resulting in a lack of dystrophin and a severe dystrophic phenotype resembling DMD [61]. In the same paper authors show that additional skipping of exon 51 could restore dystrophin expression on cultured myoblasts [61]. Single guide genome editing aiming at exon 51 skipping or reframing shows big potential after intramuscular and intravenous delivery [117].

Naturally occurring intron splice site mutation that leads to the loss of exon 7 was identified in Golden retriever dogs leading to generation of **GRMD** (**CXMDJ**) canine model [62]. It is the most widespread canine model which is used in numerous studies including exons 6 and 8 skipping driven by PMO and 2'OMePS [118, 119], AAV1-U7snRNA [120], rAAV6-U7snRNA [121], AAV8-U7snRNA [122].

The next model stands out and its value is more in demonstrating the possibility of creating new models than in itself. The new mice obtained by transgenesis carry randomly embedded copies of the EGFP under the CAG promoter (strong synthetic promoter that consists of regulatory elements from **C**MV, chicken beta-**a**ctin gene and rabbit beta-**g**lobin gene), which are separated by exon 23 with the murine *mdx* mutation. EGFP expression is possible only in case of exon 23 skipping, which was demonstrated using PMO and LNA/2'-OMe based AONs [123]. Thus, the resulting mouse model allows noninvasive assessment of the effectiveness of exon skipping, as well as studying the bio-distribution of drugs. The creation of similar

mouse models for testing exon skipping, more applicable to patients, will allow more intensive studies of future drugs.

4. Conclusions

Here we described several dozens of Duchenne muscular dystrophy models. The list of species used to create these models includes worms, fruit flies, fishes, dogs, cats, mice, pigs, rats and monkeys. Some of them were found in natural populations, while the others were artificially created. The spectrum of genetic interventions spans from point mutations to complete deletion of the largest gene - *Dmd* gene, double knockouts and humanized constructions. The genetic and phenotypic diversity provides great opportunities for fundamental studies and drug development.

The correspondence of the model phenotype to human DMD phenotype is extremely important for drug testing. Some of the models, especially based on small animal species, could not represent DMD features correctly. The same goes for many mice models. The mouse model which has mutation identical to a certain DMD case may not correctly represent the DMD phenotype. Vice versa, several double knockout mice models reproduce the DMD phenotype much closer while being an inadequate genetic model. The models based on larger species are more useful as their phenotype is usually closer to DMD. But the creation, maintenance and cost of these animals complicates their use and restricts diversity. Indeed, no ideal DMD model is still created. However, the development of novel promising DMD treatment strategies requires both genetically similar models for precision drugs testing and phenotypically appropriate models for disease study and design of therapies. We should expect the expansion of the DMD-related animal models list in the nearest future.

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References

[1] Emery AEH, Emery MLH. The History of a Genetic Disease: Duchenne Muscular Dystrophy Or Meryon's Disease. OUP Oxford; 2011. 231 p.

[2] Moat SJ, Bradley DM, Salmon R, Clarke A, Hartley L. Newborn bloodspot screening for Duchenne muscular dystrophy: 21 years experience in Wales (UK). Eur J Hum Genet. 2013 Oct;21(10):1049-1053.

[3] Ryder S, Leadley RM, Armstrong N, Westwood M, de Kock S, Butt T, et al. The burden, epidemiology, costs and treatment for Duchenne muscular dystrophy: an evidence review. Orphanet J Rare Dis. 2017 Apr 26;12(1):79.

[4] Ishizaki M, Kobayashi M, Adachi K, Matsumura T, Kimura E. Female dystrophinopathy: Review of current literature. Neuromuscul Disord. 2018 Jul;28(7):572-581.

[5] Shieh PB. Emerging Strategies in the Treatment of Duchenne Muscular Dystrophy. Neurotherapeutics. 2018 Oct;15(4):840-848.

[6] Culligan KG, Mackey AJ, Finn DM, Maguire PB, Ohlendieck K. Role of dystrophin isoforms and associated proteins in muscular dystrophy (review). Int J Mol Med. 1998 Dec;2(6):639-648.

[7] Doorenweerd N, Mahfouz A, van Putten M, Kaliyaperumal R, T' Hoen PAC, Hendriksen JGM, et al. Timing and localization of human dystrophin isoform expression provide insights into the cognitive phenotype of Duchenne muscular dystrophy. Sci Rep. 2017 Oct 3;7(1):12575.

[8] Aartsma-Rus A, Ginjaar IB, Bushby K. The importance of genetic diagnosis for Duchenne muscular dystrophy. J Med Genet. 2016 Mar;53(3):145-151.

[9] Lapidos Karen A., Kakkar Rahul, McNally Elizabeth M. The Dystrophin Glycoprotein Complex. Circ Res. 2004 Apr 30;94(8):1023-1031.

[10] Le Rumeur E, Winder SJ, Hubert J-F. Dystrophin: more than just the sum of its parts. Biochim Biophys Acta. 2010 Sep;1804(9):1713-1722.

[11] Guiraud S, Davies KE. Pharmacological advances for treatment in Duchenne muscular dystrophy. Curr Opin Pharmacol. 2017 Jun;34:36-48.

[12] Folker ES, Baylies MK. Nuclear positioning in muscle development and disease. Front Physiol. 2013 Dec 12;4:363.

[13] Rosenberg AS, Puig M, Nagaraju K, Hoffman EP, Villalta SA, Rao VA, et al. Immune-mediated pathology in Duchenne muscular dystrophy. Sci Transl Med. 2015 Aug 5;7(299):299rv4.

[14] Birnkrant DJ, Bushby K, Bann CM, Apkon SD, Blackwell A, Brumbaugh D, et al. Diagnosis and management of Duchenne muscular dystrophy, part 1: diagnosis, and neuromuscular, rehabilitation, endocrine, and gastrointestinal and nutritional management. Lancet Neurol. 2018 Mar;17(3):251-267.

[15] Bushby KM, Gardner-Medwin D, Nicholson LV, Johnson MA, Haggerty ID, Cleghorn NJ, et al. The clinical, genetic and dystrophin characteristics of Becker muscular dystrophy. II. Correlation of phenotype with genetic and protein abnormalities. J Neurol. 1993 Feb;240(2):105-112.

[16] England SB, Nicholson LV, Johnson MA, Forrest SM, Love DR, Zubrzycka-Gaarn EE, et al. Very mild muscular dystrophy associated with the deletion of 46% of dystrophin. Nature. 1990 Jan 11;343(6254):180-182.

[17] Duan D. Systemic AAV Micro-dystrophin Gene Therapy for Duchenne Muscular Dystrophy. Mol Ther. 2018 Oct 3;26(10):2337-2356.

[18] Nance ME, Hakim CH, Yang NN, Duan D. Nanotherapy for Duchenne muscular dystrophy. Wiley Interdiscip Rev Nanomed Nanobiotechnol [Internet]. 2018 Mar;10(2). Available from: http://dx.doi.org/10.1002/wnan.1472

[19] Schneider A-FE, Aartsma-Rus A. Developments in reading frame restoring therapy approaches for Duchenne muscular dystrophy. Expert Opin Biol Ther. 2020 Oct 19;1-17.

[20] Guiraud S, Roblin D, Kay DE. The potential of utrophin modulators for the treatment of Duchenne muscular dystrophy. Expert Opinion on Orphan Drugs. 2018 Mar 4;6(3):179-192.

[21] Weir AP, Burton EA, Harrod G, Davies KE. A- and B-utrophin Have Different Expression Patterns and Are Differentially Up-regulated in mdx Muscle *. J Biol Chem. 2002 Nov 22;277(47):45285-45290.

[22] Sienkiewicz D, Kulak W, Okurowska-Zawada B, Paszko-Patej G, Kawnik K. Duchenne muscular dystrophy: current cell therapies. Ther Adv Neurol Disord. 2015 Jul;8(4):166-177.

[23] Chamberlain JS, Metzger J, Reyes M, Townsend D, Faulkner JA. Dystrophin-deficient mdx mice display a reduced life span and are susceptible to spontaneous rhabdomyosarcoma. FASEB J. 2007 Jul;21(9):2195-2204.

[24] McGreevy JW, Hakim CH, McIntosh MA, Duan D. Animal models of Duchenne muscular dystrophy: from basic mechanisms to gene therapy. Dis Model Mech. 2015 Mar;8(3):195-213.

[25] Muntoni F, Mateddu A, Marchei F, Clerk A, Serra G. Muscular weakness in the mdx mouse. J Neurol Sci. 1993 Dec 1;120(1):71-77.

[26] Massopust RT, Lee YI, Pritchard AL, Nguyen V-KM, McCreedy DA, Thompson WJ. Lifetime analysis of mdx skeletal muscle reveals a progressive pathology that leads to myofiber loss. Sci Rep. 2020 Oct 14;10(1):17248.

[27] Lynch GS, Hinkle RT, Chamberlain JS, Brooks SV, Faulkner JA. Force and power output of fast and slow skeletal muscles from mdx mice 6-28 months old. J Physiol. 2001 Sep 1;535(Pt 2):591-600.

[28] Dellorusso C, Crawford RW, Chamberlain JS, Brooks SV. Tibialis anterior muscles in mdx mice are highly susceptible to contraction-induced injury. J Muscle Res Cell Motil. 2001;22(5):467-475.

[29] Quinlan JG, Hahn HS, Wong BL, Lorenz JN, Wenisch AS, Levin LS. Evolution of the mdx mouse cardiomyopathy: physiological and morphological findings. Neuromuscul Disord. 2004 Sep;14(8-9):491-496.

[30] Spurney CF, Gordish-Dressman H, Guerron AD, Sali A, Pandey GS, Rawat R, et al. Preclinical drug trials in the mdx mouse: assessment of reliable and sensitive outcome measures. Muscle Nerve. 2009 May;39(5):591-602.

[31] Wasala NB, Chen S-J, Duan D. Duchenne muscular dystrophy animal models for high-throughput drug discovery and precision medicine. Expert Opin Drug Discov. 2020 Apr;15(4):443-456.

[32] Chapman VM, Miller DR, Armstrong D, Caskey CT. Recovery of induced mutations for X chromosome-linked muscular dystrophy in mice. Proc Natl Acad Sci U S A. 1989 Feb;86(4):1292-1296.

[33] Im WB, Phelps SF, Copen EH, Adams EG, Slightom JL, Chamberlain JS. Differential expression of dystrophin isoforms in strains of mdx mice with different mutations. Hum Mol Genet. 1996 Aug;5(8):1149-1153.

[34] Li D, Yue Y, Duan D. Preservation of muscle force in Mdx3cv mice correlates with low-level expression of a near full-length dystrophin protein. Am J Pathol. 2008 May;172(5):1332-1341.

[35] Ramos JN, Hollinger K, Bengtsson NE, Allen JM, Hauschka SD, Chamberlain JS. Development of Novel Micro-dystrophins with Enhanced Functionality. Mol Ther. 2019 Mar 6;27(3):623-635.

[36] Krivov LI, Stenina MA, Yarygin VN, Polyakov AV, Savchuk VI, Obrubov SA, et al. A new genetic variant of mdx mice: study of the phenotype. Bull Exp Biol Med. 2009 May;147(5):625-629.

[37] van Putten M, Putker K, Overzier M, Adamzek WA, Pasteuning-Vuhman S, Plomp JJ, et al. Natural disease history of the D2-mdx mouse model for Duchenne muscular dystrophy. FASEB J. 2019 Jul;33(7):8110-8124.

[38] Schmidt WM, Uddin MH, Dysek S, Moser-Thier K, Pirker C, Höger H, et al. DNA damage, somatic aneuploidy, and malignant sarcoma susceptibility in muscular dystrophies. PLoS Genet. 2011 Apr;7(4):e1002042.

[39] Thiessen DD, Lindzey G. Negative geotaxis in mice: effect of balancing practice on incline behaviour in C57BL-6J male mice. Anim Behav. 1967 Jan;15(1):113-116.

[40] Stenina MA, Krivov LI, Voevodin DA, Yarygin VN. Phenotypic differences between mdx black mice and mdx albino mice. Comparison of cytokine levels in the blood. Bull Exp Biol Med. 2013 Jul;155(3):376-379.

[41] Flanigan KM, Ceco E, Lamar K-M, Kaminoh Y, Dunn DM, Mendell JR, et al. LTBP4 genotype predicts age of ambulatory loss in Duchenne muscular dystrophy. Ann Neurol. 2013 Apr;73(4):481-488.

[42] Hammers DW, Hart CC, Matheny MK, Wright LA, Armellini M, Barton ER, et al. The D2.mdx mouse as a preclinical model of the skeletal muscle pathology associated with Duchenne muscular dystrophy. Sci Rep. 2020 Aug 21;10(1):14070.

[43] Vallese D, Negroni E, Duguez S, Ferry A, Trollet C, Aamiri A, et al. The Rag2^{-}Il2rb^{-}Dmd^{-} mouse: a novel dystrophic and immunodeficient model to assess innovating therapeutic strategies for muscular dystrophies. Mol Ther. 2013 Oct;21(10):1950-1957.

[44] Walsh S, Nygren J, Pontén A, Jovinge S. Myogenic reprogramming of bone marrow derived cells in a W^{41}Dmd(mdx) deficient mouse model. PLoS One. 2011 Nov 28;6(11):e27500.

[45] Farini A, Meregalli M, Belicchi M, Battistelli M, Parolini D, D'Antona G, et al. T and B lymphocyte depletion has a marked effect on the fibrosis of dystrophic skeletal muscles in the scid/mdx mouse. J Pathol. 2007 Oct;213(2):229-238.

[46] Pons F, Robert A, Marini JF, Léger JJ. Does utrophin expression in muscles of mdx mice during postnatal development functionally compensate for dystrophin deficiency? J Neurol Sci. 1994 Apr;122(2):162-170.

[47] Deconinck AE, Rafael JA, Skinner JA, Brown SC, Potter AC, Metzinger L, et al. Utrophin-dystrophin-deficient mice as a model for Duchenne muscular dystrophy. Cell. 1997 Aug 22;90(4):717-727.

[48] Odom GL, Gregorevic P, Allen JM, Finn E, Chamberlain JS. Microutrophin

delivery through rAAV6 increases lifespan and improves muscle function in dystrophic dystrophin/utrophin-deficient mice. Mol Ther. 2008 Sep;16(9):1539-1545.

[49] Rooney JE, Welser JV, Dechert MA, Flintoff-Dye NL, Kaufman SJ, Burkin DJ. Severe muscular dystrophy in mice that lack dystrophin and alpha7 integrin. J Cell Sci. 2006 Jun 1;119 (Pt 11):2185-2195.

[50] Rudnicki MA, Braun T, Hinuma S, Jaenisch R. Inactivation of MyoD in mice leads to up-regulation of the myogenic HLH gene Myf-5 and results in apparently normal muscle development. Cell. 1992 Oct 30;71(3):383-390.

[51] Megeney LA, Kablar B, Perry RL, Ying C, May L, Rudnicki MA. Severe cardiomyopathy in mice lacking dystrophin and MyoD. Proc Natl Acad Sci U S A. 1999 Jan 5;96(1):220-225.

[52] Mourkioti F, Kustan J, Kraft P, Day JW, Zhao M-M, Kost-Alimova M, et al. Role of telomere dysfunction in cardiac failure in Duchenne muscular dystrophy. Nat Cell Biol. 2013 Aug;15(8):895-904.

[53] Wood CL, Suchacki KJ, van 't Hof R, Cawthorn WP, Dillon S, Straub V, et al. A comparison of the bone and growth phenotype of mdx, mdx:Cmah−/− and mdx:Utrn +/− murine models with the C57BL/10 wild-type mouse. Dis Model Mech [Internet]. 2020 Jan 10;13(2). Available from: http://dx.doi.org/10.1242/dmm.040659

[54] Kudoh H, Ikeda H, Kakitani M, Ueda A, Hayasaka M, Tomizuka K, et al. A new model mouse for Duchenne muscular dystrophy produced by 2.4 Mb deletion of dystrophin gene using Cre-loxP recombination system. Biochem Biophys Res Commun. 2005 Mar 11;328(2):507-516.

[55] Chamberlain JS, Benian GM. Muscular dystrophy: the worm turns to genetic disease. Curr Biol. 2000 Nov 2;10(21):R795–R797.

[56] Bassett DI, Bryson-Richardson RJ, Daggett DF, Gautier P, Keenan DG, Currie PD. Dystrophin is required for the formation of stable muscle attachments in the zebrafish embryo. Development. 2003 Dec;130(23):5851-5860.

[57] Li M, Andersson-Lendahl M, Sejersen T, Arner A. Muscle dysfunction and structural defects of dystrophin-null sapje mutant zebrafish larvae are rescued by ataluren treatment. FASEB J. 2014 Apr;28(4):1593-1599.

[58] Taghli-Lamallem O, Akasaka T, Hogg G, Nudel U, Yaffe D, Chamberlain JS, et al. Dystrophin deficiency in Drosophila reduces lifespan and causes a dilated cardiomyopathy phenotype. Aging Cell. 2008 Mar;7(2):237-249.

[59] Shcherbata HR, Yatsenko AS, Patterson L, Sood VD, Nudel U, Yaffe D, et al. Dissecting muscle and neuronal disorders in a Drosophila model of muscular dystrophy. EMBO J. 2007 Jan 24;26(2):481-493.

[60] Barthélémy I, Calmels N, Weiss RB, Tiret L, Vulin A, Wein N, et al. X-linked muscular dystrophy in a Labrador Retriever strain: phenotypic and molecular characterisation. Skelet Muscle. 2020 Aug 7;10(1):23.

[61] Walmsley GL, Arechavala-Gomeza V, Fernandez-Fuente M, Burke MM, Nagel N, Holder A, et al. A duchenne muscular dystrophy gene hot spot mutation in dystrophin-deficient cavalier king charles spaniels is amenable to exon 51 skipping. PLoS One. 2010 Jan 13;5(1):e8647.

[62] Kornegay JN. The golden retriever model of Duchenne muscular dystrophy. Skelet Muscle. 2017 May 19;7(1):9.

[63] Lorant J, Larcher T, Jaulin N, Hedan B, Lardenois A, Leroux I, et al. Vascular Delivery of Allogeneic MuStem Cells in Dystrophic Dogs Requires Only Short-Term Immunosuppression to Avoid Host Immunity and Generate Clinical/Tissue Benefits. Cell Transplant. 2018 Jul;27(7):1096-1110.

[64] VanBelzen DJ, Malik AS, Henthorn PS, Kornegay JN, Stedman HH. Mechanism of Deletion Removing All Dystrophin Exons in a Canine Model for DMD Implicates Concerted Evolution of X Chromosome Pseudogenes. Mol Ther Methods Clin Dev. 2017 Mar 17;4:62-71.

[65] Nghiem PP, Bello L, Balog-Alvarez C, López SM, Bettis A, Barnett H, et al. Whole genome sequencing reveals a 7 base-pair deletion in DMD exon 42 in a dog with muscular dystrophy. Mamm Genome. 2017 Apr;28(3-4):106-113.

[66] Sánchez L, Beltrán E, de Stefani A, Guo LT, Shea A, Shelton GD, et al. Clinical and genetic characterisation of dystrophin-deficient muscular dystrophy in a family of Miniature Poodle dogs. PLoS One. 2018 Feb 23;13(2):e0193372.

[67] Brunetti B, Muscatello LV, Letko A, Papa V, Cenacchi G, Grillini M, et al. X-Linked Duchenne-Type Muscular Dystrophy in Jack Russell Terrier Associated with a Partial Deletion of the Canine DMD Gene. Genes [Internet]. 2020 Oct 8;11(10). Available from: http://dx.doi.org/10.3390/genes11101175

[68] Mata López S, Hammond JJ, Rigsby MB, Balog-Alvarez CJ, Kornegay JN, Nghiem PP. A novel canine model for Duchenne muscular dystrophy (DMD): single nucleotide deletion in DMD gene exon 20. Skelet Muscle. 2018 May 29;8(1):16.

[69] Carpenter JL, Hoffman EP, Romanul FC, Kunkel LM, Rosales RK, Ma NS, et al. Feline muscular dystrophy with dystrophin deficiency. Am J Pathol. 1989 Nov;135(5):909-919.

[70] Blunden AS, Gower S. Hypertrophic feline muscular dystrophy: diagnostic overview and a novel immunohistochemical diagnostic method using formalin-fixed tissue. Vet Rec. 2011 May 14;168(19):510.

[71] Nakamura K, Fujii W, Tsuboi M, Tanihata J, Teramoto N, Takeuchi S, et al. Generation of muscular dystrophy model rats with a CRISPR/Cas system. Sci Rep. 2014 Jul 9;4:5635.

[72] Sugihara H, Kimura K, Yamanouchi K, Teramoto N, Okano T, Daimon M, et al. Age-Dependent Echocardiographic and Pathologic Findings in a Rat Model with Duchenne Muscular Dystrophy Generated by CRISPR/Cas9 Genome Editing. Int Heart J. 2020 Nov 28;61(6):1279-1284.

[73] Teramoto N, Sugihara H, Yamanouchi K, Nakamura K, Kimura K, Okano T, et al. Pathological evaluation of rats carrying in-frame mutations in the dystrophin gene: a new model of Becker muscular dystrophy. Dis Model Mech [Internet]. 2020 Sep 28;13(9). Available from: http://dx.doi.org/10.1242/dmm.044701

[74] Larcher T, Lafoux A, Tesson L, Remy S, Thepenier V, François V, et al. Characterization of dystrophin deficient rats: a new model for Duchenne muscular dystrophy. PLoS One. 2014 Oct 13;9(10):e110371.

[75] Wertz K, Füchtbauer EM. Dmd(mdx-beta geo): a new allele for the mouse dystrophin gene. Dev Dyn. 1998 Jun;212(2):229-241.

[76] Petkova MV, Morales-Gonzales S, Relizani K, Gill E, Seifert F, Radke J, et al. Characterization of a Dmd (EGFP) reporter mouse as a tool to investigate dystrophin expression. Skelet Muscle. 2016 Jul 5;6:25.

[77] Sarig R, Mezger-Lallemand V, Gitelman I, Davis C, Fuchs O, Yaffe D, et al. Targeted inactivation of Dp71, the major non-muscle product of the DMD gene: differential activity of the Dp71 promoter during development. Hum Mol Genet. 1999 Jan;8(1):1-10.

[78] Vacca O, Charles-Messance H, El Mathari B, Sene A, Barbe P, Fouquet S, et al. AAV-mediated gene therapy in Dystrophin-Dp71 deficient mouse leads to blood-retinal barrier restoration and oedema reabsorption. Hum Mol Genet. 2016 Jul 15;25(14):3070-3079.

[79] Barboni MTS, Vaillend C, Joachimsthaler A, Liber AMP, Khabou H, Roux MJ, et al. Rescue of Defective Electroretinographic Responses in Dp71-Null Mice With AAV-Mediated Reexpression of Dp71. Invest Ophthalmol Vis Sci. 2020 Feb 7;61(2):11.

[80] Miranda R, Nudel U, Laroche S, Vaillend C. Altered presynaptic ultrastructure in excitatory hippocampal synapses of mice lacking dystrophins Dp427 or Dp71. Neurobiol Dis. 2011 Jul;43(1):134-141.

[81] Bladen CL, Salgado D, Monges S, Foncuberta ME, Kekou K, Kosma K, et al. The TREAT-NMD DMD Global Database: analysis of more than 7,000 Duchenne muscular dystrophy mutations. Hum Mutat. 2015 Apr;36(4):395-402.

[82] Araki E, Nakamura K, Nakao K, Kameya S, Kobayashi O, Nonaka I, et al. Targeted disruption of exon 52 in the mouse dystrophin gene induced muscle degeneration similar to that observed in Duchenne muscular dystrophy. Biochem Biophys Res Commun. 1997 Sep 18;238(2):492-497.

[83] Aupy P, Echevarría L, Relizani K, Zarrouki F, Haeberli A, Komisarski M, et al. Identifying and Avoiding tcDNA-ASO Sequence-Specific Toxicity for the Development of DMD Exon 51 Skipping Therapy. Mol Ther Nucleic Acids. 2020 Mar 6;19:371-383.

[84] Aoki Y, Nakamura A, Yokota T, Saito T, Okazawa H, Nagata T, et al. In-frame dystrophin following exon 51-skipping improves muscle pathology and function in the exon 52-deficient mdx mouse. Mol Ther. 2010 Nov;18(11):1995-2005.

[85] Echigoya Y, Lim KRQ, Trieu N, Bao B, Miskew Nichols B, Vila MC, et al. Quantitative Antisense Screening and Optimization for Exon 51 Skipping in Duchenne Muscular Dystrophy. Mol Ther. 2017 Nov 1;25(11):2561-2572.

[86] Aupy P, Zarrouki F, Sandro Q, Gastaldi C, Buclez P-O, Mamchaoui K, et al. Long-Term Efficacy of AAV9-U7snRNA-Mediated Exon 51 Skipping in mdx52 Mice. Mol Ther Methods Clin Dev. 2020 Jun 12;17:1037-1047.

[87] Aoki Y, Yokota T, Wood MJA. Development of multiexon skipping antisense oligonucleotide therapy for Duchenne muscular dystrophy. Biomed Res Int. 2013 Jul 31;2013:402369.

[88] Echigoya Y, Aoki Y, Miskew B, Panesar D, Touznik A, Nagata T, et al. Long-term efficacy of systemic multiexon skipping targeting dystrophin exons 45-55 with a cocktail of vivo-morpholinos in mdx52 mice. Mol Ther Nucleic Acids. 2015 Feb 3;4:e225.

[89] Veltrop M, van Vliet L, Hulsker M, Claassens J, Brouwers C, Breukel C, et al. A dystrophic Duchenne mouse model for testing human antisense oligonucleotides. PLoS One. 2018 Feb 21;13(2):e0193289.

[90] Lyu P, Yoo KW, Yadav MK, Atala A, Aartsma-Rus A, van Putten M, et al. Sensitive and reliable evaluation of single-cut sgRNAs to restore dystrophin by a GFP-reporter assay. PLoS One. 2020 Sep 24;15(9):e0239468.

[91] Duchêne BL, Cherif K, Iyombe-Engembe J-P, Guyon A, Rousseau J, Ouellet DL, et al. CRISPR-Induced Deletion with SaCas9 Restores Dystrophin Expression in Dystrophic Models In Vitro and In Vivo. Mol Ther. 2018 Nov 7;26(11):2604-2616.

[92] Min Y-L, Chemello F, Li H, Rodriguez-Caycedo C, Sanchez-Ortiz E, Mireault AA, et al. Correction of Three Prominent Mutations in Mouse and Human Models of Duchenne Muscular Dystrophy by Single-Cut Genome Editing. Mol Ther. 2020 Sep 2;28(9):2044-2055.

[93] Amoasii L, Li H, Zhang Y, Min Y-L, Sanchez-Ortiz E, Shelton JM, et al. In vivo non-invasive monitoring of dystrophin correction in a new Duchenne muscular dystrophy reporter mouse. Nat Commun. 2019 Oct 4;10(1):4537.

[94] Min Y-L, Li H, Rodriguez-Caycedo C, Mireault AA, Huang J, Shelton JM, et al. CRISPR-Cas9 corrects Duchenne muscular dystrophy exon 44 deletion mutations in mice and human cells. Sci Adv. 2019 Mar;5(3):eaav4324.

[95] Zhang Y, Li H, Min Y-L, Sanchez-Ortiz E, Huang J, Mireault AA, et al. Enhanced CRISPR-Cas9 correction of Duchenne muscular dystrophy in mice by a self-complementary AAV delivery system. Sci Adv. 2020 Feb;6(8):eaay6812.

[96] Wei T, Cheng Q, Min Y-L, Olson EN, Siegwart DJ. Systemic nanoparticle delivery of CRISPR-Cas9 ribonucleoproteins for effective tissue specific genome editing. Nat Commun. 2020 Jun 26;11(1):3232.

[97] Young CS, Mokhonova E, Quinonez M, Pyle AD, Spencer MJ. Creation of a Novel Humanized Dystrophic Mouse Model of Duchenne Muscular Dystrophy and Application of a CRISPR/Cas9 Gene Editing Therapy. J Neuromuscul Dis. 2017;4(2):139-145.

[98] Echigoya Y, Lim KRQ, Melo D, Bao B, Trieu N, Mizobe Y, et al. Exons 45-55 Skipping Using Mutation-Tailored Cocktails of Antisense Morpholinos in the DMD Gene. Mol Ther. 2019 Nov 6;27(11):2005-2017.

[99] Goyenvalle A, Wright J, Babbs A, Wilkins V, Garcia L, Davies KE. Engineering multiple U7snRNA constructs to induce single and multiexon-skipping for Duchenne muscular dystrophy. Mol Ther. 2012 Jun;20(6):1212-1221.

[100] 't Hoen PAC, de Meijer EJ, Boer JM, Vossen RHAM, Turk R, Maatman RGHJ, et al. Generation and characterization of transgenic mice with the full-length human DMD gene. J Biol Chem. 2008 Feb 29;283(9):5899-907.

[101] Wong TWY. Utilization of CRISPR/Cas9-mediated gene editing for correction of deletion mutations in DMD [Internet]. 2020 [cited 2021 Jan 21]. Available from: https://tspace.library.utoronto.ca/handle/1807/103696

[102] Flanigan KM, Dunn DM, von Niederhausern A, Soltanzadeh P, Gappmaier E, Howard MT, et al. Mutational spectrum of DMD mutations in dystrophinopathy patients: application of modern diagnostic techniques to a large cohort. Hum Mutat. 2009 Dec;30(12):1657-1666.

[103] Vulin A, Wein N, Simmons TR, Rutherford AM, Findlay AR, Yurkoski JA, et al. The first exon duplication mouse model of Duchenne muscular dystrophy: A tool for therapeutic development. Neuromuscul Disord. 2015 Nov;25(11):827-834.

[104] Wein N, Simmons T, Gumienny F, Huang N, Heller K, Yurkoski J, et al. A single neonatal injection of an AAV9.

U7snRNA virus mediating skipping of dmd exon 2 allows dystrophin expression preventing apparition of pathologic features in the Dup2 mouse one year post injection. Neuromuscul Disord. 2017 Oct 1;27:S187.

[105] Bulfield G, Siller WG, Wight PA, Moore KJ. X chromosome-linked muscular dystrophy (mdx) in the mouse. Proc Natl Acad Sci U S A. 1984 Feb;81(4):1189-1192.

[106] Malik V, Rodino-Klapac LR, Viollet L, Mendell JR. Aminoglycoside-induced mutation suppression (stop codon readthrough) as a therapeutic strategy for Duchenne muscular dystrophy. Ther Adv Neurol Disord. 2010 Nov;3(6):379-389.

[107] Kim K, Ryu S-M, Kim S-T, Baek G, Kim D, Lim K, et al. Highly efficient RNA-guided base editing in mouse embryos. Nat Biotechnol. 2017 May;35(5):435-437.

[108] Ryu S-M, Koo T, Kim K, Lim K, Baek G, Kim S-T, et al. Adenine base editing in mouse embryos and an adult mouse model of Duchenne muscular dystrophy. Nat Biotechnol. 2018 Jul;36(6):536-539.

[109] Xu L, Zhang C, Li H, Wang P, Gao Y, Mohler PJ, et al. Efficient precise in vivo base editing in adult dystrophic mice [Internet]. Cold Spring Harbor Laboratory. 2020 [cited 2021 Jan 21]. p. 2020.06.24.169292. Available from: https://www.biorxiv.org/content/10.1101/2020.06.24.169292v1

[110] Lorain S, Peccate C, Le Hir M, Griffith G, Philippi S, Précigout G, et al. Dystrophin rescue by trans-splicing: a strategy for DMD genotypes not eligible for exon skipping approaches. Nucleic Acids Res. 2013 Sep;41(17):8391-8402.

[111] Egorova T, Reshetov D, Polikarpova A, Vassilieva S, Vlodavets D, Deikin A. DMD TREATMENT: ANIMAL MODELS: P.203Exons 6 and 7 skipping test on new murine model of Duchenne muscular dystrophy. Neuromuscul Disord. 2018 Oct 1;28:S94.

[112] Koo T, Lu-Nguyen NB, Malerba A, Kim E, Kim D, Cappellari O, et al. Functional Rescue of Dystrophin Deficiency in Mice Caused by Frameshift Mutations Using Campylobacter jejuni Cas9. Mol Ther. 2018 Jun 6;26(6):1529-1538.

[113] Klymiuk N, Blutke A, Graf A, Krause S, Burkhardt K, Wuensch A, et al. Dystrophin-deficient pigs provide new insights into the hierarchy of physiological derangements of dystrophic muscle. Hum Mol Genet. 2013 Nov 1;22(21):4368-4382.

[114] Moretti A, Fonteyne L, Giesert F, Hoppmann P, Meier AB, Bozoglu T, et al. Somatic gene editing ameliorates skeletal and cardiac muscle failure in pig and human models of Duchenne muscular dystrophy. Nat Med. 2020 Feb;26(2):207-214.

[115] Yu H-H, Zhao H, Qing Y-B, Pan W-R, Jia B-Y, Zhao H-Y, et al. Porcine Zygote Injection with Cas9/sgRNA Results in DMD-Modified Pig with Muscle Dystrophy. Int J Mol Sci [Internet]. 2016 Oct 9;17(10). Available from: http://dx.doi.org/10.3390/ijms17101668

[116] Sui T, Lau YS, Liu D, Liu T, Xu L, Gao Y, et al. A novel rabbit model of Duchenne muscular dystrophy generated by CRISPR/Cas9. Dis Model Mech [Internet]. 2018 Jun 4;11(6). Available from: http://dx.doi.org/10.1242/dmm.032201

[117] Amoasii L, Hildyard JCW, Li H, Sanchez-Ortiz E, Mireault A, Caballero D, et al. Gene editing restores dystrophin expression in a canine model of Duchenne muscular dystrophy. Science. 2018 Oct 5;362(6410):86-91.

[118] Yokota T, Hoffman E, Takeda S ‘ichi. Antisense oligo-mediated multiple exon skipping in a dog model of duchenne muscular dystrophy. Methods Mol Biol. 2011;709:299-312.

[119] Nakamura A, Aoki Y, Tsoumpra M, Yokota T, Takeda S ‘ichi. In Vitro Multiexon Skipping by Antisense PMOs in Dystrophic Dog and Exon 7-Deleted DMD Patient. Methods Mol Biol. 2018;1828:151-63.

[120] Vulin A, Barthélémy I, Goyenvalle A, Thibaud J-L, Beley C, Griffith G, et al. Muscle function recovery in golden retriever muscular dystrophy after AAV1-U7 exon skipping. Mol Ther. 2012 Nov;20(11):2120-2133.

[121] Bish LT, Sleeper MM, Forbes SC, Wang B, Reynolds C, Singletary GE, et al. Long-term restoration of cardiac dystrophin expression in golden retriever muscular dystrophy following rAAV6-mediated exon skipping. Mol Ther. 2012 Mar;20(3):580-589.

[122] Le Guiner C, Montus M, Servais L, Cherel Y, Francois V, Thibaud J-L, et al. Forelimb treatment in a large cohort of dystrophic dogs supports delivery of a recombinant AAV for exon skipping in Duchenne patients. Mol Ther. 2014 Nov;22(11):1923-1935.

[123] Hara Y, Mizobe Y, Inoue YU, Hashimoto Y, Motohashi N, Masaki Y, et al. Novel EGFP reporter cell and mouse models for sensitive imaging and quantification of exon skipping. Sci Rep. 2020 Jun 22;10(1):10110.