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CRISPR applications for Duchenne muscular dystrophy: From animal models to potential therapies

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Abstract

CRISPR gene-editing technology creates precise and permanent modifications to DNA. It has significantly advanced our ability to generate animal disease models for use in biomedical research and also has potential to revolutionize the treatment of genetic disorders. Duchenne muscular dystrophy (DMD) is a monogenic muscle-wasting disease that could potentially benefit from the development of CRISPR therapy. It is commonly associated with mutations that disrupt the reading frame of the *DMD* gene that encodes dystrophin, an essential scaffolding protein that stabilizes striated muscles and protects them from contractile-induced damage. CRISPR enables the rapid generation of various animal models harboring mutations that closely simulates the wide variety of mutations observed in DMD patients. These models provide a platform for the testing of sequence-specific interventions like CRISPR therapy that aim to reframe or skip DMD mutations to restore functional dystrophin expression.

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CRISPR/Cas9, CRISPR therapy, Duchenne muscular dystrophy, animal models, mice models

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1 | INTRODUCTION

Duchenne muscular dystrophy (DMD) is a progressive and fatal muscle-wasting disease that affects, among others, striated muscles. It is a childhood-onset monogenic disorder associated with loss-of-function mutations to the X-linked *DMD* gene, which encodes the dystrophin protein (Hoffman et al., 1987). DMD mainly affects boys, at an estimated incidence of 1 in 5000 live male births (Crisafulli et al., 2020). Due to the lack of functional dystrophin expression in skeletal muscle, DMD patients begin experiencing muscle weakness and motor delay at 3–5 years of age, which progressively worsens to a loss of independent ambulation in their early teens (Vuillerot et al., 2010). They also subsequently develop respiratory insufficiency owing to the gradual loss of respiratory muscle strength, as well as develop dilated cardiomyopathy (LoMauro et al., 2015). Currently, most patients die prematurely due to heart failure (Matsumura et al., 2011). In addition to striated muscle pathophysiology, DMD is also associated with a variable degree of neurodevelopmental co-morbidities, intellectual disability, and cognitive impairment that does not affect all patients (Doorenweerd, 2020). This is due to the absence of one or more brain-specific dystrophin isoforms. Advances in the symptomatic treatment and long-term supportive care of DMD patients, such as the chronic use of gluco-corticosteroids, non-invasive ventilation, spinal surgery and cardio-protective medication, has improved their life expectancy up to 30–40 years (Birnkrant, Bushby, Bann, Alman, et al., 2018; Birnkrant, Bushby, Bann, Apkon, Blackwell, Brumbaugh, et al., 2018; Birnkrant, Bushby, Bann, Apkon, Blackwell, Colvin, et al., 2018; Broomfield et al., 2021; Landfeldt et al., 2020). However, DMD remains a universally fatal disease as curative treatments are not yet available.

Mutations to the *DMD* gene are also associated with a less common and symptomatically milder dystrophinopathy, Becker muscular dystrophy (BMD). In contrast to DMD, mutations that cause BMD result in the expression of internally truncated but partially functional dystrophin at various levels (Aartsma-Rus et al., 2006; England et al., 1990). BMD patients exhibit a broader clinical severity spectrum than DMD patients, but their symptoms are generally milder and slower progressing (Koenig et al., 1989; Monaco et al., 1988).

Early-stage treatments to manage the onset of muscle degeneration include gluco-corticosteroid regimens that slow the progression of muscle weakness and delay loss of ambulation (Birnkrant, Bushby, Bann, Apkon, Blackwell, Brumbaugh, et al., 2018). However, routine administration of gluco-corticosteroids has significant side effects ranging from weight gain and abnormal behavior to acute adrenal insufficiency, the latter of which is potentially life-threatening in stress situations (Bowden et al., 2019; Kinnett & Noritz, 2017; Matthews et al., 2016). There are several emerging therapies currently being developed for DMD, including (i) gene replacement therapies, which aim to deliver exogenous genes encoding micro-dystrophin, an internally truncated but likely partially functional version of the dystrophin protein (Duan, 2018), (ii) exon-skipping therapies, which uses antisense-oligonucleotide (AON)-based splice modulators to restore expression of BMD-like dystrophins (Aartsma-Rus et al., 2017), and (iii) small molecule therapies that upregulate utrophin, an autosomal paralog that could substitute dystrophin's function (Guiraud et al., 2018). However, due to the transient nature of these therapeutic approaches, they are non-curative and would thus require a lifetime of repeated administration.

Clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR-Cas9) gene-editing technology enables precise and efficient modification of DNA and has emerged as a potential therapeutic tool for long-lasting correction of disease-causing variants. Given that DMD is a monogenic disease, there is considerable scope for the development of CRISPR-Cas9 therapeutics for DMD to manage or prevent the progression of muscle wasting, which is currently supported by proof-of-concept studies in cell culture systems and in vivo animal models. Preclinical in vivo evaluation of these promising gene-editing strategies, especially in sequence humanized animal models, will be crucial for building the evidence base for clinical translation and will inform key aspects of clinical trial design, including the type of vehicle, dose, frequency and route of administration, as well as the target level of functional dystrophin recovery (Aartsma-Rus & van Putten, 2019). In this review, we explore the various DMD animal models available for DMD research, including those generated using CRISPR gene-editing technology, and discuss CRISPR's potential for DMD treatment as well as current challenges and concerns.

2 | *DMD* GENE AND THE DYSTROPHIN PROTEIN

DMD is located at Xp21 and is one of the longest human genes, spanning 2.2 megabases (Min, Bassel-Duby, & Olson, 2019). It contains at least seven tissue-specific promoters, and is alternatively spliced to produce four different

full-length isoforms (muscle isoform Dp427m, cortical isoform Dp427c, and Purkinje isoforms Dp427p1 and Dp427p2) as well as several other shorter isoforms (Doorenweerd et al., 2017). Apart from the well-characterized muscle isoform Dp427m, most of the other isoforms are mainly expressed in the central nervous system (CNS), and their role in brain development and function is generally less well understood.

The complete coding sequence of the muscle Dp427m isoform is about 11 kb long, encompasses 79 exons and encodes for a 427 kDa sarcolemmal protein called dystrophin (Figure 1a; Koenig et al., 1988). In muscle, dystrophin is a component of the dystrophin–glycoprotein complex (DGC; Figure 1b). The DGC is localized at the muscle cell membrane (sarcolemma) and connects the intracellular filamentous-actin (F-actin) cytoskeleton to the extracellular basal lamina. It functions to stabilize and reinforce the sarcolemma during muscle contraction (Danialou et al., 2001; Petrof et al., 1993). The role of dystrophin in the DGC is to tether F-actin of the outermost layer of myofilaments to the inner surface of the myofiber sarcolemma (Gao & McNally, 2015). In the absence of functional dystrophin, the sarcolemma is damaged by contraction-induced mechanical stress and cytotoxic inflammatory responses, leading to myofiber necroptosis (Morgan et al., 2018). Dystrophin has been found to be expressed in muscle satellite cells, where it is important for defining cell polarity and determining asymmetric cell division. Impaired satellite cell function and myoblast maturation in dystrophic muscle slow the regeneration of contractile units, and together with chronic inflammation lead to the progressive replacement of muscle with fibrous and fatty tissue (Chang et al., 2016; Ribeiro et al., 2019).

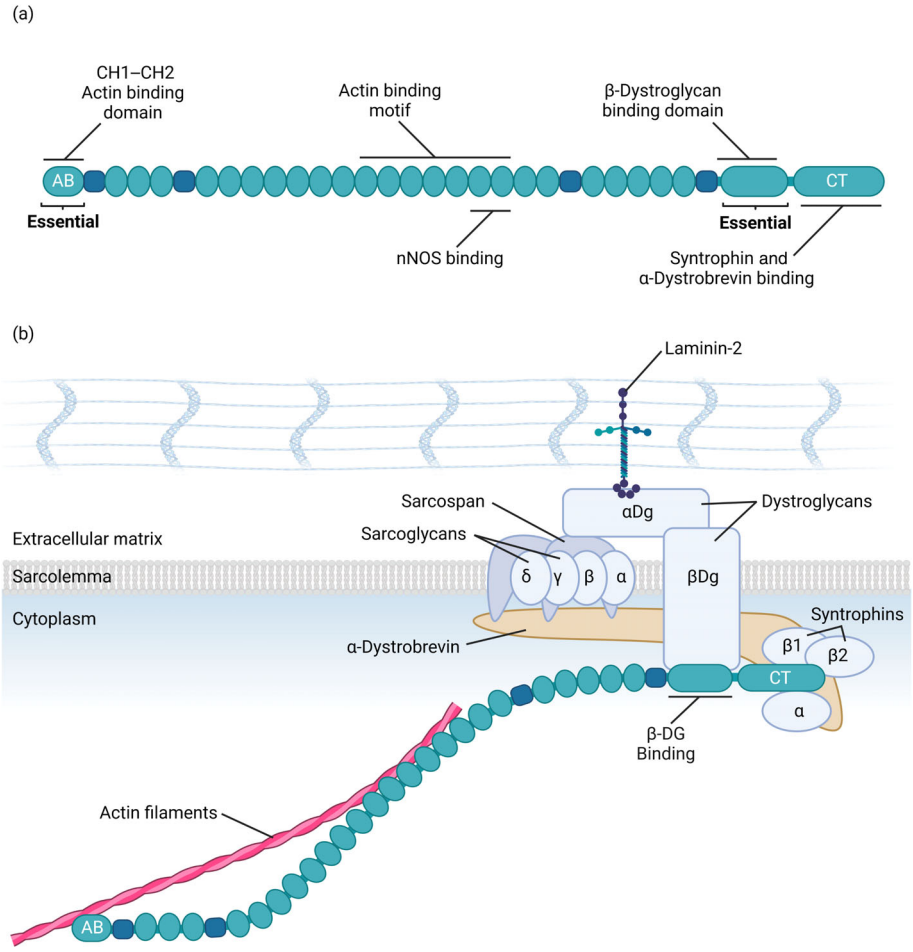

FIGURE 1 Structure and function of the dystrophin protein. (a) The essential N-terminal actin-binding and C-terminal (CT) β-dystroglycan binding domains are separated by a rod domain containing 24 spectrin-like repeats, intercalated by four hinge regions (dark blue). A secondary actin-biding motif and an nNOS binding region are located toward the center of the rod domain. The CT domain contains additional binding sites for other components of the dystrophin–glycoprotein complex (DGC). (b) In muscle, dystrophin connects intracellular actin filaments to other components of the DGC located on the sarcolemma, linking the intracellular cytoskeleton to the extracellular matrix which provides mechanical stability to the sarcolemma in response to muscle contractions.

Structurally, dystrophin contains four functional domains (Figure 1a): (i) an N-terminal actin-binding (AB) domain which contains tandem calponin homology domains (CH1 and CH2) for directly anchoring dystrophin to F-actin (Norwood et al., 2000; Way et al., 1992); (ii) an elongated central rod domain consisting of 24 spectrin-like repeats, separated into three sub-regions by four proline-rich hinges (Le Rumeur et al., 2010); (iii) a cystine-rich (CYS) β-dystroglycan-binding (β-DgB) domain (Margulies, Blanchette, NISC Comparative Sequencing Program, Haussler, & Green, 2003); and (iv) a C-terminal domain that binds to α-dystrobrevin and syntrophins (Blake et al., 1995). The AB and β-DgB domains are essential for the mechanical linkage between F-actin and the DGC, respectively, and are thus critical to dystrophin function. Additionally, dystrophin contains neuronal nitric oxide synthase (nNOS) binding sites at its spectrin-like repeats 16 and 17 to localize nNOS to the sarcolemma, which catalyzes the production of nitric oxide (NO) that induces vasodilation to maintain sufficient blood perfusion to contracting muscles during exercise (Zhao et al., 2019).

Given the large genomic footprint of *DMD*, it has a higher than average mutation rate for a human gene (1×10^{-4} vs. 1×10^{-5} to 1×10^{-6}) (Aartsma-Rus et al., 2006). The various mutations identified in DMD patients are publicly available in databases such as the TREAT-NMD DMD global database (Bladen et al., 2015). Of these mutations, the most prevalent are large deletions spanning one or more exons, accounting for two-thirds of cases. Other mutations, such as exonic (large) duplications, small deletions and insertions, and nonsense mutations, account for about 11%, 10%–15%, and 10%–15% of cases, respectively. Exactly 80% of large deletions in *DMD* occur in mutational hotspots between exons 2–20 and 45–55, while half of the large duplications occur in a proximal hotspot between exons 2–20. Among these large deletions, the loss of exon 45 is most commonly observed (4%).

With some exceptions, most DMD mutations follow Monaco's reading frame rule: a shift in the *DMD* reading frame leads to a prematurely truncated and dysfunctional dystrophin due to the absence of the essential C-terminal β-DgB domain (Aartsma-Rus et al., 2006; Monaco et al., 1988). For example, deletion of exon 50 (exon 50Δ) causes exon 51 to be placed out-of-frame with preceding exons, resulting in an aberrant reading frame (wrong amino acid code) and the introduction of a premature termination codon (PTC) that truncates the dystrophin protein. A recent study has suggested that *DMD* transcript reduction due to the PTC occurs through an NMD-independent mechanism that is potentially epigenetically mediated through histone marks (García-Rodríguez et al., 2020). In contrast, mutations that give rise to BMD are often internal truncations that preserve the reading frame, which retains mechanical functionality. For instance, BMD patients with an in-frame deletion of exons 45–55 have been described to experience very mild or even no symptoms (Ferreiro et al., 2009; A. Nakamura et al., 2008). Therefore, allowing DMD patients to produce BMD-like dystrophins by reframing or skipping one or more exons appears to be a potential therapeutic strategy to reduce DMD severity. It is estimated that, in theory, up to 83% of DMD patient mutations can be corrected by skipping either one or two exons (Aartsma-Rus et al., 2009).

Sequence-specific targeting of the *DMD* gene can be achieved two different ways, either at the pre-mRNA level using AON-based exon-skipping drugs or by gene correction using CRISPR-Cas9 gene-editing technology. Ideally, sequence-directed strategies should be designed to apply to a large subset of patients as personalized strategies for each disease mutation are not feasible. For instance, skipping exon 51 has the potential to benefit 14% of DMD patients, which is the largest subset for a single-exon skipping strategy that includes patients with large deletions ending with exon 50 and large deletions starting with exon 52 (Bladen et al., 2015). There are currently four AON-based exon-skipping drugs that have received accelerated approval from the FDA: Eteplirsen 51, Golodirsen 53, Viltolarsen 53, and Casimersen 45 (Sheikh & Yokota, 2021). These drugs are splice modulators that bind and exclude target exons of the pre-mRNA *DMD* transcript during mRNA processing, resulting in shorter BMD-like mRNA with restored reading frames. The small, synthetic nature of these AONs enable them to be manufactured quickly and subsequently delivered systemically to patients using weekly intravenous infusions. However, as the underlying mutations that cause DMD disease remain, these drugs require repeated administration to maintain exon skipping. Thus, genetic "reframing" of causative mutations on the DNA level through CRISPR-Cas9 genome editing is an attractive strategy for DMD therapeutics.

3 | CRISPR-Cas9 GENE-EDITING

CRISPR-Cas9 is a revolutionary gene-editing tool derived from the anti-viral immune system of prokaryotes. It has enabled the relatively simple targeting, modification and regulation of DNA (Ran, Hsu, Wright, et al., 2013). Currently utilized extensively for molecular and biomedical research, CRISPR technology has the potential to be transitioned to the clinic as a therapy for presently incurable genetic disorders.

The two-part CRISPR-Cas9 system used for gene editing consists of a programmable guide RNA (gRNA) and a Cas9 nuclease (Cong et al., 2013). The gRNA contains a specific nucleotide sequence that directs Cas9 to a target locus for editing. It is a combination of a CRISPR RNA (crRNA), which contains the sequence complementary to the target DNA (guide sequence), and a trans-activating CRISPR RNA (tracrRNA), which folds into a multistem-loop structure to which Cas9 binds (Jinek et al., 2012; Mali et al., 2013). Cas9 is a bi-lobed endonuclease that contains a nuclease domain on each lobe; HNH cleaves the target DNA strand, and RuvC cleaves the non-target DNA strand. Together, these endonuclease domains create a DNA double-stranded break (DSB) (Jiang & Doudna, 2017; Nishimasu et al., 2014). The catalytic activity of the Cas9 protein is activated when bound to gRNA, which triggers a conformational change in its structure (Jinek et al., 2014). The protospacer adjacent motif (PAM) is an essential sequence feature of a target DNA locus that enables it to be recognized and bound to by Cas9 (Wright et al., 2016). The PAM is positioned on the non-target DNA strand and is located immediately downstream of a target sequence. Cas9 directly interacts with the PAM via its PAM-interacting domain at its C-terminal end. Active Cas9 searches for the target sequence by binding to PAMs and then checking the upstream DNA for complementarity to the guide sequence (Sternberg et al., 2014). Correct RNA–DNA pairing subsequently leads to the double-stranded cleavage of DNA by the HNH and RuvC domains.

There are several Cas9 variants available in the gene-editing toolkit—each with features that provide specific advantages for different applications. The most widely used is the wildtype (WT) SpCas9, a natural Cas9 variant derived from *Streptococcus pyogenes*. It recognizes a 5′-NGG-3′ PAM, uses a ∼20 nucleotide (nt) guide sequence, and creates a blunt or +1 nt overhanging DSB 3 nt upstream of the PAM (Mali et al., 2013; Ran, Hsu, Wright, et al., 2013). It is important to note that the complementarity recognition of SpCas9 is not very stringent—it can occasionally generate "off-target" mutations by aberrantly cleaving sequences similar to the intended guide sequence (Cho et al., 2014; Fu et al., 2013; Hsu et al., 2013; Tsai et al., 2015; X.-H. Zhang et al., 2015). Several engineered SpCas9 variants such as SpCas9-HF1, eSpCas9(1.1), and HypaCas9 have sequence modifications that reduce their tolerance for mismatches, improving target specificity and reducing off-target activity (J. S. Chen et al., 2017; Kleinstiver et al., 2016; Slaymaker et al., 2016; Tan et al., 2019). SpCas9 nickases (SpCas9n) have one inactivated endonuclease domain that limits cutting activity to only one DNA strand. Although a single Cas9n/gRNA will not generate on-target (or off-target) mutations, "staggered" DSBs can be generated using Cas9n and a pair of adjacent gRNAs on opposite DNA strands (Cho et al., 2014; Ran, Hsu, Lin, et al., 2013). This additional reliance on a second target site to generate DSBs by dual-nicking approaches improves targeting specificity and reduces off-targeting.

Additionally, the availability of different Cas9 variants expands the available genomic sites targetable for CRISPR editing. For example, engineered SpCas9 variants such as SpCas9-VQR and SpCas9-NG recognize 5′-NGA-3′ and 5′-NG-3′ PAM sequences, respectively (Hu et al., 2018; Kleinstiver et al., 2015; Nishimasu et al., 2018). Also, native Cas9s derived from other bacterial species recognize a range of different PAM sequences. Of particular interest is the *Staphylococcus aureus* derived Cas9 (SaCas9), which recognizes the 5′-NNGRRT-3′ PAM (Ran et al., 2015; H. Xie et al., 2018). SaCas9 is attractive for therapeutic applications as its coding sequence, gRNA, and heterologous promoters are within the packaging limit (∼4.7 kb) of adeno-associated virus (AAV) vectors (Cebrian-Serrano & Davies, 2017).

Following CRISPR-induced DNA cleavage, the cell's endogenous DNA DSB repair machinery can introduce modifications at the break site. Knock-in mutations utilize the homology-directed repair (HDR) pathway and allow for integration of an exogenous single stranded or double stranded DNA repair template (Jasin & Rothstein, 2013). However, HDR is generally inefficient, restricted mainly to mitotic cells, and occurs during the cell cycle's later stages (S and G2). The second modification type does not require a donor template and arises from the non-homologous end joining (NHEJ) or microhomology-mediated end joining (MMEJ) pathways (Hailong Wang & Xu, 2017). Unlike HDR, these pathways are active in all cell cycle phases. NHEJ involves the direct ligation of broken DNA ends. However, this process is error-prone and can introduce small insertions and deletions (InDels) that potentially inactivate the target gene. In contrast, MMEJ makes predictable and reproducible modifications that rely on microhomologies (between 5 and 25 bp) flanking a DSB, resulting in the intervening deletion of bases between the microhomologies and the subsequent retention of only one microhomology region (McVey & Lee, 2008). The heterogenous InDels arising from the template-free repair of CRISPR-induced DSBs is a combination of repair outcomes from the NHEJ and MMEJ processes. While end-joining repair appears random and "noisy," it is now understood to be a controlled process and is thus predictable (Allen et al., 2018; Shen et al., 2018; van Overbeek et al., 2016). Researchers can thus exploit this to identify gRNAs that generate precise InDel outcomes from end-joining repair for specific gene editing applications. Additionally, if two cuts are performed simultaneously to DNA, it can result in a large intervening deletion of the fragment flanked by the DSBs. Dual-cuts do not always result in intervening deletions, but can also result in localized InDels at both target sites. The introduction of multiple cuts can also result in larger deletions up to the scale of an entire chromosome (Adikusuma et al., 2017).

Furthermore, NHEJ can also be used to create insertions using the homology-independent targeted integration (HITI) strategy, that enables knock-in of double stranded donor DNA in non-dividing cells (Suzuki et al., 2016).

The CRISPR toolbox also includes gene-editing strategies utilizing Cas9 fusion proteins. Base editors perform single-nucleotide conversions without DSBs, through the activity of DNA base deaminases linked to Cas9n or deactivated Cas9 (dCas9). Commonly used cytosine (CBE) and adenine base editors (ABE) catalyze C→G to T→A and A→T to G→C transitions, respectively (Figure 2b,c), while more recently generated base editors enable the C-to-G base transversion in mammalian cells (Gaudelli et al., 2017; Komor et al., 2016; Molla et al., 2020). Another gene-editing

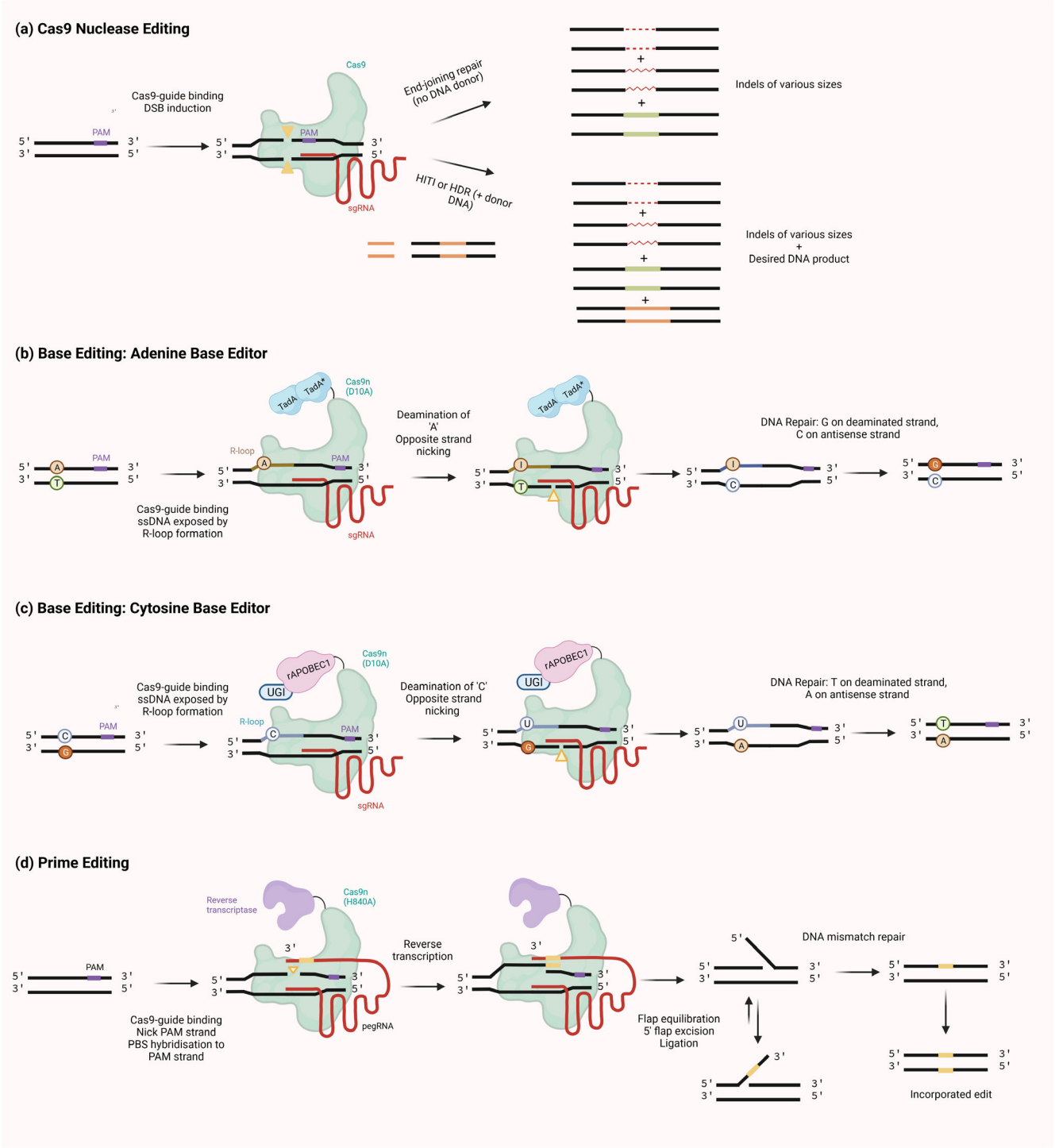

FIGURE 2 Legend on next page.

approach is PRIME editing, a newly developed strategy consisting of a Cas9n fused to an engineered reverse transcriptase (RT) (Figure 2d). This technology enables more precise control over repair outcomes by specifying the intended substitution, insertion, or deletion on the extended prime editing gRNA (pegRNA), which acts as a repair template for the fused RT domain (Anzalone et al., 2019). However, its activity is strongly influenced by cellular context, where it works better in some cell lines than others (Adikusuma et al., 2021; Anzalone et al., 2019).

4 | CRISPR-GENERATED ANIMAL MODELS OF DMD

4.1 | Mouse models

CRISPR's powerful utility to directly modify DNA has better enabled researchers to generate genetically modified animal disease models, including for DMD research (Table 1). Before CRISPR was available, DMD research used *Dmd* knockout (KO) mouse models with spontaneous mutations such as the *mdx* mouse model harboring a nonsense point mutation in *Dmd* exon 23 (Bulfield et al., 1984), or generated by *N*-ethyl-*N*-nitrosourea-induced mutagenesis such as the mdx^{4cv} mouse model which has a nonsense point mutation in *Dmd* exon 53 (Chapman et al., 1989). These mouse models are neither reflective of the diverse array of natural patient DMD mutations nor are genocopies of human disease (Bladen et al., 2015; Tuffery-Giraud et al., 2009). Genetic engineering of mouse embryonic stem (ES) cells enabled generation of a *Dmd* null mouse model with the entire *Dmd* gene deleted (Kudoh et al., 2005) as well as a *Dmd* KO model containing a gene trap cassette (Wertz & Füchtbauer, 1998). Gene targeting was also used to generate disease-relevant mouse models with mutations commonly found in human DMD patients, like *mdx52*, which lacks *Dmd* exon 52 (Araki et al., 1997) and the *Dup2* mouse that carries a *Dmd* exon 2 duplication (Vulin et al., 2015). Although these models have proven to be useful, the techniques behind their generation are time consuming and labor-intensive, as chimeras containing modified ES cells are required to transmit the *Dmd* mutation through the germ line.

In 2013, CRISPR was used to efficiently generate genetically modified mice through zygotic microinjection of CRISPR components, followed by the implantation of the injected zygotes to pseudopregnant females (Haoyi Wang et al., 2013). This technique is relatively simple, quick and efficient, unlike the classical vector-mediated gene targeting approach that is laborious and prone to failure. Since then, it has been widely adopted for the generation of mutant mouse models, including *Dmd* mouse models with single and multi-exon deletions mimicking hotspot mutations observed in DMD patients. CRISPR-engineered single exon deletions in *Dmd* include exon 43Δ, exon 44Δ, exon 45Δ, exon 50Δ, exon 51Δ, and exon 52Δ (Amoasii et al., 2017; Chemello, Bassel-Duby, & Olson, 2020; Min et al., 2020; Min, Li, et al., 2019), all of which are frameshifting, leading to the subsequent loss of functional dystrophin expression. These exon deletions were created using two gRNAs to simultaneously cut within the introns flanking the target exon, generating a large intervening deletion. Using the same approach, two models with multi-exon deletions have also been made, a *Dmd* exons 8–34Δ

FIGURE 2 CRISPR-Cas9 genome editors and their mechanism of action, applied in both DMD animal model generation and subsequent mutant model correction. (a) Cas9 nuclease (green) is directed by its guide RNA (red) to generate blunt-end DSBs, within the protospacer three base pairs upstream of the PAM (purple). The double stranded break generated by CRISPR-Cas nuclease cleavage can be repaired by alternative pathways. End-joining repair (NHEJ, MMEJ/alt-EJ) produces uncontrolled, yet predictable InDels typically for gene disruption. In the presence of a donor DNA template, the less-efficient homology-directed repair (HDR) pathway operates primarily in dividing cells to install targeted mutations whereas homology independent targeted integration (HITI) can be used to knock-in large fragments of DNA into the genome of non-dividing cells. (b) Adenine base editors (ABEs) mediate A-to-G conversion by using a Cas9 nickase nCas9 (green) fused to an evolved adenosine deaminase (TadA*, blue) to catalyze the deamination of adenosine (A) to inosine (I) within the R-loop. Inosine is recognized as guanine (G) by the cell polymerase after DNA repair. (c) Cytosine base editors (CBEs) mediate a C-to-T conversion by using nCas9 fused to a cytidine deaminase (APOBEC1, pink) and uracil glycosylase inhibitor (UGI, blue) to catalyze the deamination of cytosine (C) to uracil (U) within the R-loop. The resulting U–G pair is repaired through the cell's mismatch repair mechanism converts the resulting C–G into T–A. The presence of the UGI blocks the uracil DNA glycosylase from initiating base excision repair on uracil, favoring the formation of T–A base pairs. (d) Prime editing utilizes an engineered reverse transcriptase (RT, purple) fused to a Cas9 nickase (H840A, green) to nick the non-target strand and a prime editing guide RNA (pegRNA, red), which has an extended 3′ RT template (yellow) with the desired edit. Once the non-target DNA (PAM) strand is nicked, it hybridizes to the pegRNA where the RT can copy the template RT template (within 3′ of the pegRNA) to incorporate the desired edit into the nicked strand. After 5′ flap excision, DNA mismatch repair ensures the desired mutation is copied across to result in both strands being modified.

TABLE 1 Summary of CRISPR-generated DMD animal models

Animal	Strain	Model	Technology (generation)	Summary	Therapies tested	References
Mouse	C57BL/6J and ICR	Dmd Ex20 nonsense pm	Base editing (BE3)	~73% of blastocysts genotyped for the exon 20 mutation.	CRISPR BE of Ex20 point mutation (Ryu et al., 2018)	Kim et al. (2017)
	C57BL/6J	Dmd Ex50Δ	CRISPR-SpCas9	Dystrophin absent in skeletal muscle. Typical dystrophic histopathology and reduced grip strength at 8 weeks.	CRISPR single cut-reframing or Ex51 skipping	Amoasii et al. (2017)
	C57BL/6J	Dmd Ex23 frameshift	CRISPR-SpCas9 (RNP)	Dystrophin absent in skeletal muscle.	CRISPR-CjCas9 single cut-mediated NHEJ	Koo et al., 2018
	C57BL/6J	Dmd Ex44Δ	CRISPR-SpCas9	Dystrophin absent in skeletal and cardiac muscles histopathology at 4 weeks, decreased EDL-specific force and forelimb grip strength.	CRISPR single cut-reframing or skipping of Ex51/Ex53 or Ex45 (Zhang et al., 2020)	Min, Bassel-Duby, and Olson (2019)
	Not outlined	Dmd Ex50Δ-P2A-Luc	CRISPR-SpCas9	Similar to Amoasii et al. (2017), with capability of in vivo non-invasive monitoring of dystrophin levels via luciferase expression.	CRISPR single cut-reframing or Ex51 skipping	Amoasii et al. (2019)
	C57BL/6J x CBA	Dmd Ex8-34Δ	CRISPR-SpCas9	Dystrophin and DAGC members absent in skeletal muscles, histopathology at 12 weeks, decreased TA force parameters and wire hanging test performance across age (2–12 months).	N/A	Egorova et al. (2019)
	C57BL/6J	Dmd Ex43Δ, Ex45Δ, Ex52Δ	CRISPR-SpCas9	Dystrophin absent in skeletal and cardiac muscle, typical dystrophic myopathology at 4-weeks, elevated serum CK levels.	CRISPR single cut-reframing for Ex44, Ex53 skipping	Min et al. (2020)
	C57BL/6N	Dmd Ex51Δ	CRISPR-SpCas9	Absent dystrophin expression. Muscle has fibrotic tissue and inflammatory infiltration. Reduced forelimb grip strength and increased serum CK levels at 4 weeks of age.	CRISPR Adenine BE to disrupt Ex50 splice donor site, inducing Ex 50 skipping (Chemello et al., 2021)	Chemello, Wang, et al. (2020)
	C57BL/6J	Dmd Ex18-30 Duplication	CRISPR-SpCas9	Reduced dystrophin expression in skeletal and cardiac muscles, typical histopathology at 15 weeks, decreased performance in grip strength and locomotor function.	CRISPR single cut at intron 21 of duplicated junction sites (Maino et al., 2021)	Maino et al. (2021)
Humanized Mouse	Primarily C57BL/10 and Primarily DBA2 background	hDMDTgΔEx45/*mdx* and hDMDTgΔ45/*mdxD2*	CRISPR-SpCas9	Both models have absent dystrophin expression. Muscle of hDMDTgΔ45/*mdxD2* mice display fibrosis, inflammation and calcium deposits.	CRISPR deletion of Ex45-Ex55	Young et al. (2017)
	Presummably primarily C57BL/10	hDMDTgΔEx52/*mdx*	CRISPR-SpCas9	Absent dystrophin expression, increased serum creatine kinase marker.	CRISPR HITI-mediated integration of Ex52 or superexon Ex52-79.	Pickar-Oliver et al. (2021)

TABLE 1 (Continued)

Animal	Strain	Model	Technology (generation)	Summary	Therapies tested	References
	C57BL/6J	hEx45KI-*mdxΔ44*	CRISPR-SpCas9	Absent dystrophin expression, increased serum creatine kinase marker.	CRISPR deletion of Ex45	Kenjo et al. (2021)
Canine	Mixed Breed (unspecified)	Dmd Ex6 Internal 6Δ	CRISPR-SpCas9	Reduced dystrophin expression, increased utrophin expression, increased serum creatine kinase marker. Typical histopathology by 5- to 6-months with increased fat and visible muscle atrophy by MRI.	N/A	Oh et al. (2022)
Rat	Wistar-Imamichi	Dmd Ex3-16Δ	CRISPR-SpCas9	Dystrophin absent in skeletal muscle, histopathology at approximately 4 or 13 weeks for skeletal muscle and at 13 weeks for the heart, decreased wire hanging test performance.	N/A	Nakamura et al. (2014)
Porcine	Diannan miniature pigs	DMD Ex27 Indel	CRISPR-SpCas9	Hotspot deletion. Marked utrophin upregulation. Cas9-mediated on-target mosaic mutations were 60%–70% of dystrophin alleles in muscle. Founders survived up to 52 days.	N/A	Yu et al. (2016)
	Bama miniature pig #1	DMD Ex13 nonsense pm	BE3, hA3A-BE3, SCNT	No dystrophic phenotype recorded. Transmission of biallelic mutations to single healthy DMD ± female pup.	N/A	Xie et al. (2019)
	Bama miniature pig #2	DMD Ex51 Indel	CRISPR-SpCas9, SCNT	100% mortality by 12 weeks. Typical histopathology in skeletal muscles.	N/A	Zou et al. (2021)
Rabbit	New Zealand rabbits	DMD Ex51 fs	CRISPR-SpCas9	50% survival reduction by 20 weeks, dystrophin absent in skeletal, histopathology at 20 weeks, reduced mobility at 8–12 weeks, elevated serum CK, and possibly dystrophic-related cardiomyopathy at 16 weeks.	N/A	Sui et al. (2018)
Monkey	Rhesus Monkey	DMD Ex4 and Ex46 Indels	CRISPR-SpCas9	Stillborn monkeys analyzed. Dystrophin absent in muscle tissues, typical muscle atrophy pathology observed.	N/A	Chen et al. (2015)

model and a *Dmd* exons 52–54Δ mouse model (Egorova et al., 2019; Wong et al., 2020). A *Dmd* mouse model that enables quantification of dystrophin expression in live animals was also generated using CRISPR, whereby a 2A-luciferase (Luc) reporter was inserted at the C-terminal of the endogenous *Dmd* gene (Amoasii et al., 2019). The subsequent deletion of exon 50 using CRISPR generated Dmd exon 50Δ-Luc mutant mice, which allows for the real-time assessment of exon 51 reframing on dystrophin restoration, based on the luciferase signal intensity detected by non-invasive bioluminescence imaging. More recently, CRISPR has also been used to edit mouse ES cells to generate a multi-exon tandem duplication mouse model, *Dup18-30* (Maino et al., 2021). Finally, base editing via zygote microinjection of a CBE has been used to engineer a *Dmd* mouse model with a nonsense mutation within *Dmd* exon20 (Kim et al., 2017).

Although all KO models of endogenous *Dmd* lack dystrophin expression, they do not exhibit the severe phenotypes of muscle weaknesses and premature death observed in human DMD patients. However, these *Dmd* KO mice still exhibit DMD features such as muscle necrosis, elevated serum CK levels, increased centrally nucleated fibers and reductions in forelimb grip strength, which are useful indicators when assessing treatments and can therefore serve as in vivo models for therapeutic development. Since it was thought that the mild phenotype observed in *Dmd* KO mice was due to the compensatory effect of utrophin, the *Dmd* KO was generated on a Utrophin (*Utr*) null background (Grady et al., 1997; Yucel et al., 2018). Consistent with the hypothesized functional overlap of Dystrophin and Utrophin, *Dmd/Utr* double-KO mice exhibit a more severe DMD-like phenotype including premature death, muscle degeneration and cardiomyopathy (Grady et al., 1997).

4.2 | Humanized DMD mouse models

Since mouse and human *DMD* sequences are not identical, in vivo studies assessing sequence-dependent frame-restoring therapies that target mouse *Dmd* sequences may not be directly translated for use in human patients. The pre-clinical assessment of clinically-relevant sequence-dependent therapies therefore generally require sequence-humanized DMD mouse models, unless there is a fortuitous perfect match to the human target sequence in the mouse *Dmd* gene. However, even a perfectly matched target sequence in mice may not produce an accurate reflection of targeting outcomes, as shown in the testing of an AON targeting exon 44 that is fully homologous in mice and humans, but only worked in the human transcript (Heemskerk et al., 2009). Therefore, the genetic context surrounding the target sequence is likely to also be important.

Researchers from Leiden University Medical Center generated the first full-length hDMD transgenic mouse model, hDMDTg, by the integration of a 2.7 Mb yeast artificial chromosome (YAC) containing an intact (2.3 Mb) *hDMD* gene into mouse chromosome 5 ('t Hoen et al., 2008). The *hDMD* transgene (Tg) is complete with exonic and intronic sequences; splicing of its pre-mRNA produces full-length *hDMDTg* transcripts that encode human dystrophin. The expression pattern of *hDMDTg* transcripts recapitulates the tissue-specific expression of *mDmd* transcripts, although the expression levels of specific isoforms differ. Also, hDMDTg functionally rescues the dystrophic phenotype in Dmd mice, as *Dmd* KO Mice carrying the hDMDTg transgene (such as *mdx* mice or dystrophin–utrophin double-KO mice) are phenotypically normal ('t Hoen et al., 2008).

Several groups have generated humanized KO hDMDTg mice by creating exon deletions in *hDMDTg* that represent common exonic deletions in DMD patients. The first hDMDTg KO model was the hDMDTgEx52Δ/*mdx* model, generated using TALEN-mediated gene targeting (homologous recombination) in hDMDTg/*mdx* mouse ES cells (Veltrop et al., 2018). Another hDMDTgEx52Δ/*mdx* model has also been independently generated by CRISPR microinjection of hDMDTg/*mdx* zygotes to remove exon 52 of *hDMDTg* (Pickar-Oliver et al., 2021). CRISPR was also used to generate hDMDTgEx45Δ/*mdx* and hDMDTgEx45Δ/*mdxD2* mice by first inducing the deletion of exon 45, by zygotic microinjection of Cas9 mRNA with two pairs of gRNAs targeting the introns flanking exon 45 (Young et al., 2017). Crossing hDMDTgEx45Δ mice with *mdxD2* mice generated a dystrophic phenotype that is more severe than *mdx* mice, owing to their DBA/2J background (Heydemann et al., 2005; van Putten et al., 2019).

Notably, a recent study characterizing the initial hDMDTgEx52Δ/*mdx* model revealed that this strain contains a tail-to-tail duplication of the hDMD transgene (Yavas et al., 2020). The authors also noted that the parental hDMDTg strain also contains the duplication because the original characterization of hDMDTg/*mdx* mice reported a higher observed C/T ratio than expected in pyrosequencing genotyping ('t Hoen et al., 2008; Yavas et al., 2020).

CRISPR has also been used to generate mice with partially humanized sequences by replacing *Dmd* exon 45 and its surrounding introns with the corresponding human sequences (hEx45KI) using CRISPR-directed HDR in mouse ES cells (Kenjo et al., 2021). Subsequently, exon 44 was deleted by CRISPR to generate the hDMDTgEx45KI-*mdx*Ex44Δ

disease model, that enabled in vivo testing of CRISPR gRNAs targeting human exon 45 for the treatment of DMD mutations amenable to exon 45 skipping.

4.3 | Large animal models

Large DMD animal models such as dogs, pigs, and monkeys can also be used to study DMD, and they are generally thought to better resemble human physiology and anatomy. There are examples of spontaneous DMD mutations in canines including the Golden Retriever Muscular Dystrophy (GRMD) and Cavalier King Charles Spaniels Muscular Dystrophy (CKCS-MD) models that harbor splice site mutations, excluding exon 7 and exon 50 in the mRNA, respectively (Kornegay, 2017; Walmsley et al., 2010).

Other dog breeds have also been reported with spontaneous DMD variants in the form of exon insertions (Kornegay et al., 2012), deletions, point mutations (Shrader et al., 2018), gene inversions (Atencia-Fernandez et al., 2015; Barthélémy et al., 2020), and repetitive element insertions (Smith et al., 2007; Smith et al., 2011) within the DMD gene, all of which result in the loss of dystrophin in heart and skeletal muscle tissue. Dogs lacking dystrophin generally exhibit clinical and pathological features analogous to human DMD including respiratory muscle weakness and atrophy, abnormal gait and premature death. However, the variability of disease progression in canine models along with their small sample sizes make pre-clinical studies for comprehensive analyses of DMD pathology a great challenge.

Recently, CRISPR was used to generate a dystrophin-deficient dog, through transplantation of a gene-edited donor cell via somatic cell nuclear transfer (SCNT). Oh et al. (2022) targeted a minor deletion hotspot within exon 6, leading to an in-frame 57 bp deletion of the canine *DMD* gene (Oh et al., 2022). However, there was no further analysis done at the RNA level to confirm the nature of this internal deletion or its potential consequence on exon 6 skipping through splice site disruption. A single surviving pup was generated from 26 reconstructed oocytes. This pup exhibited severe dystrophic pathology that was validated through immunohistochemical staining, Western blot, magnetic resonance imaging and electrocardiogram analysis through to 10-months of age. However, the limited data (i.e., from $n = 1$ dog) makes it difficult to make phenotypic comparisons with the well documented GRMD model. As such, the challenge remains on how to use CRISPR-Cas9 to generate and breed a genetically and physiologically relevant dog model for therapeutic development.

Porcine models can also be used for preclinical therapeutic assessment, as they have similar genetic and anatomical characteristics to humans. Klymiuk et al. (2013) deleted DMD exon 52 in cultured male porcine kidney cells by gene targeting using homologous recombination, and used cell clones to generate mutant offspring by SCNT (Klymiuk et al., 2013). The male piglets lacked dystrophin expression and closely resembled the pathology of human disease including premature lethality, which prevented transmission of the mutation (Matsunari et al., 2018). Subsequent efforts using the same method in female cells successfully generated female carriers, which enabled establishment of a breeding cohort of *DMD* exon 52Δ pigs (Stirm et al., 2021). A *DMD* exon 52Δ Yucatan miniature pig model has also been generated by adeno-associated virus-mediated gene targeting and SCNT, generating seven piglets (Echigoya et al., 2021). From this batch, five piglets survived less than a week succumbing to the rapid disease progression, and another two survived longer for up to 6.5 and 7 months. These two pigs expressed exon 52Δ mRNA and had absent dystrophin expression in skeletal and cardiac muscle. Moreover, the authors noted poor growth in these mutant pigs from 2 months of age, accompanied by severe dystrophic phenotypes akin to the GRMD model such as forward pelvic limb positioning and postural instability.

CRISPR-Cas9 has also been used to genetically modify porcine zygotes to generate DMD pigs. An attempt to generate a *DMD* exon 27Δ Chinese Diannan pig model by zygotic microinjection generated two offspring that died shortly after birth, one of which carried mutations in the *DMD* gene (Yu et al., 2016). The efficiency of generating a productive frame-disrupting mutation was 60% in skeletal muscles, with this mutant pig displaying typical dystrophic features and lower dystrophin levels compared with wild-type. A CRISPR base edited compound heterozygous female pig has been generated by SCNT, carrying a C to T substitution in exon 13 on one allele and an 18 bp deletion on the other, introducing a premature stop codon in the DMD transcript (J. Xie et al., 2019). More recently, Bama miniature pigs with InDels in *DMD* exon 51 were generated by CRISPR editing of fetal fibroblasts and SCNT. Nine of 15 live piglets carried *DMD* mutations but died within 12 weeks of age (Zou et al., 2021).

CRISPR has also been used to generate rhesus monkey DMD models through CRISPR-Cas9 targeting of *DMD* exon 4 and dual targeting of exon 4 and exon 46, in hopes of functional gene disruption by InDel generation across these two DMD hotspot regions (Y. Chen et al., 2015). From 179 Cas9-injected embryos, 14 live monkeys were born, 9 of which were identified to carry frame shifting mutations in exon 4 and/or exon 46 of the *DMD* gene. Two stillborn monkeys were found to have disrupted and hypertrophic muscle structure with reduced dystrophin expression. Phenotypic

analysis, however, was not performed for any of the live monkeys, although two of them were assessed by WGS for off-target mutations which were not detected (S. Wang et al., 2018).

4.4 | Other animal models

Rabbits are an attractive DMD model as they are relatively easy to breed and cost effective compared with larger animal models, and unlike most murine *Dmd* models they develop pathological hallmarks of muscular dystrophy and cardiomyopathy. Sui et al. (2018) developed a DMD rabbit model by co-injecting Cas9 mRNA and gRNAs targeting *DMD* exon 51 into 128 zygotes and produced 33 live rabbits, where 26 of which carried mutations in at least one allele of the *DMD* gene (Sui et al., 2018). The *DMD* KO rabbits had greatly reduced dystrophin expression and impaired physical ability evidenced by a lower number of steps per hour compared with WT rabbits. 20% of the DMD KO rabbits died prematurely by 2 weeks of age, increasing to 42.6% dead by 20 weeks of age. Cardiomyopathy was detectable at approximately 5 months in these rabbits, with echocardiography readings displaying decreased ejection fraction and fraction shortening.

Nakamura et al. (2014) generated inbred Wistar-Imamichi rats that harbor *Dmd* mutations using CRISPR through the zygotic microinjection of Cas9 mRNA and two sgRNAs targeting exon 3 and exon 16 (K. Nakamura et al., 2014). Interestingly, 90% of male founders had mutations in the *Dmd* gene, varying from 1 to 577 bp deletions to 1 to 4 bp insertions in one or both exons. Rats with frameshifting mutations lacked dystrophin while in-frame mutants expressed reduced dystrophin. By 13 weeks of age, the TA of mutant rats showed typical dystrophic histopathology (myofiber degeneration, elevated adipose levels, and increased fibrosis), although changes in the heart and diaphragm were less characterized.

5 | THE POTENTIAL OF CRISPR FOR DMD TREATMENT

In addition to generating DMD animal models, CRISPR has the potential to be developed as treatment to delay or prevent further muscle wasting in DMD patients. The advantage of editing the endogenous *DMD* is that the gene would remain under its own promoter, so all regulatory aspects driving its expression would remain the same. Due to the difficulty in controlling NHEJ repair outcomes and the inefficiency of HDR in mature cell types, conventional CRISPR-Cas9 gene editing may not be able to efficiently install precise edits to revert common DMD mutations to their WT sequences. However, the ability of CRISPR to efficiently cut DNA can be leveraged to reframe the *DMD* gene, such as introducing InDels to shift the reading frame or deleting the out-of-frame exon to induce exon skipping (Figure 3a,b). Since CRISPR modifies genomic DNA, it offers the possibility of a long lasting, and possibly "one-and-done" therapy that targets the underlying cause of DMD, that is, the primary transcript generated from the CRISPR-modified gene will be in-frame, precluding the need for repeated treatment. In contrast, current AON-based exon skipping therapy targets the primary mRNA transcript and must be administered throughout a patient's lifetime.

Exon skipping using CRISPR-Cas9 was initially demonstrated using a dual-cut strategy to create large intervening deletions through the simultaneous delivery of two gRNAs with close targeting proximity in the *mdx* mouse model that has a nonsense mutation in exon 23 of the mouse *Dmd* gene (Long et al., 2016; Nelson et al., 2016; Tabebordbar et al., 2016). These gRNAs were programmed to create DSBs in the introns flanking exon 23 to delete it. Since exon 23 is in frame, removing it bypasses the mutation, while allowing production of functional dystrophin.

Inducing single exon skipping has also been demonstrated using two exonic guides within the same exon-guides that bind close to the splice acceptor and splice donor sequences of *hDMD* exon 45 induced exon 45 skipping that is detectable at the transcript level (Kenjo et al., 2021). Larger multi-exon deletions using dual-cut approaches have also been shown to reframe dystrophin transcripts in *mdx* mice by exons 21–23 deletion (Xu et al., 2016), and in *mdx*^{4cv} mice (which harbors a nonsense mutation in exon 53) by deleting exons 52–53 (Bengtsson et al., 2017). Strategies that attempt to delete entire mutational hotspots such as exons 45–55 (Young et al., 2017) or exons 47–58 (Duchêne et al., 2018) are also feasible in cultured cells and are potentially applicable to >40% of DMD patients. However, deleting vast stretches of DNA is likely to be inefficient in vivo.

Researchers have also developed single-cut strategies to skip exons by targeting and disrupting the splice sites of a target exon with NHEJ-generated InDels. Interestingly, these InDels can also achieve frame restoration if created prior to the PTC, as specific InDel sizes can shift and restore the reading frame. This reframing strategy has been shown in

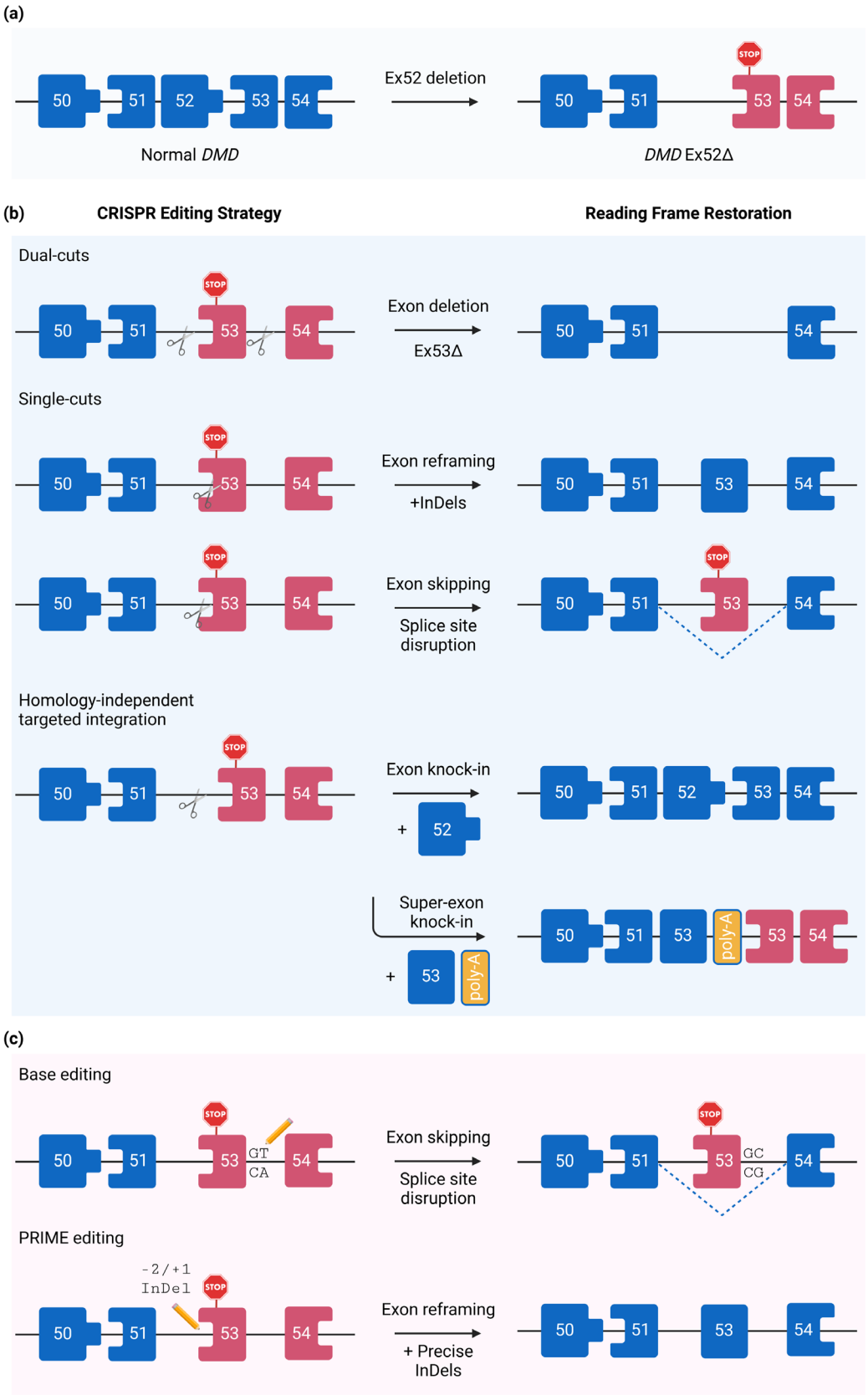

FIGURE 3 CRISPR strategies for *DMD* gene correction. (a) Schematic illustrating the reading frame-disruption and subsequent introduction of a premature termination codon (stop signal) in *DMD* exon 53 caused by an exon 52 deletion mutation. Reading frames are shown by exon shapes. In-frame exons are blue and out-of-frame exons are red. (b) Examples of double-stranded-break (DSB)-dependent CRISPR strategies to delete, reframe, skip and knock-in exons that restores the reading frame. Exon skipping is indicated by the dotted blue line. (c) Examples of CRISPR-based editing strategies that do not require DSBs.

several studies by Eric Olson's group (Amoasii et al., 2017; Grady et al., 1997; Min et al., 2020; Min, Li, et al., 2019). In one example using mice harboring an exon 50 deletion in mouse *Dmd* that causes a $3n - 1$ frameshift, their selected gRNA targeting exon 51 generated abundant +1 insertions that restore the correct reading frame (Amoasii et al., 2017). They restored up to 90% of dystrophin protein expression following the systemic injection of two AAV-9s carrying (i) CK8e muscle-specific promoter-driven SpCas9 and (ii) a single gRNA targeting exon 51 expressed by three cassettes with different PolIII promoters.

In addition, there is increased appreciation that the spectrum of InDels generated by non-templated DSB repair is non-random and largely predictable based on the sequences surrounding the cut site. Candidate gRNAs can therefore be selected based on their predicted ability to generate a high proportion of reframing InDels (Shen et al., 2018). The use of a single gRNA also reduces the potential for off-target mutations. Single cut strategies may also be more efficient than dual cut strategies since they do not rely on generating simultaneous DSBs which, instead of creating the desired intervening deletions, can also create localized InDels with no therapeutic benefit.

In vivo testing has also been performed in large animal DMD models such as in dogs and pigs. Amoasii et al. (2018) utilized CRISPR-Cas9 to correct the genetic defect in the E50-MD canine model, through single-cut guide targeting adjacent to the splice acceptor site to reframe and induce skipping of the out-of-frame exon 51, achieving up to ~80% dystrophin restoration in some muscles and up to 92% restoration in heart muscle as assessed by Western blot (Amoasii et al., 2018). However, some weaknesses in this study include the small sample size of dogs tested ($n = 2$ for intramuscular delivery and $n = 2$ for systemic delivery, only one with an AAV dose sufficient for body-wide dystrophin restoration) as well as the short period of analysis following CRISPR administration, so it is not known if the rescued dystrophin expression is sustained in these dogs. Moretti et al. (2020) co-delivered a split-intein Cas9 and a pair of gRNAs to excise exon 51 and restore the reading frame in the DMD exon 52Δ pigs, enabling modest dystrophin protein expression in peripheral muscles, diaphragm, and heart in 4-week-old piglets, as well as moderate phenotypic amelioration (Moretti et al., 2020). However, CRISPR intervention in these pigs only improved their maximum survival by ~30 days.

Alternative CRISPR-based strategies using Cas9-directed fusion proteins that do not rely on DSBs and endogenous DSB repair pathways have also been explored (Figure 3c). Adenine base editing has been demonstrated to correct nonsense mutations in mouse models with a PTC in *Dmd* exon 20 and in *mdx*^{4cv} mice, as well as to induce exon 52 skipping in *Dmd* exon 51Δ mice (Chemello et al., 2021; Ryu et al., 2018; Xu et al., 2021). PRIME editing has been used to correct the exon 52 reading frame of cultured human exon 51Δ induced pluripotent stem cell-derived cardiomyocytes (Chemello et al., 2021). Another approach for DMD treatment using CRISPR-Cas9 is to restore the full-length dystrophin coding sequence using HITI of a DNA donor. Circumventing inefficient HDR in non-dividing cells, donor DNA fragments can be knocked-in at a DBS site by NHEJ-mediated integration to replace missing exons and restore the complete *DMD* gene. Pickar-Oliver et al have evaluated SaCas9-initiated HITI of human exon 52 or a super-exon encoding exons 52–79. Although the insertion efficiencies are low, these strategies would allow the complete restoration of full-length dystrophin and would apply to a large subset of patients (Pickar-Oliver et al., 2021).

While CRISPR therapy in theory has the potential to prevent and delay further muscle degeneration in DMD, it is important to recognize the limitations, uncertainties and challenges associated with its application and development. Specifically targeting and correcting the *DMD* gene in muscle is not expected to alleviate neurological comorbidities as *DMD* in the CNS is not currently the primary target for reframing. Also, given how little we understand about the function of the other dystrophin isoforms in the nervous system, it would be difficult to predict the effects of restoring functional dystrophin expression in the brain. Furthermore, as DMD is typically diagnosed after a patient starts displaying symptoms associated with muscle weakness, at which time, the patient will already have accumulated irreversible loss of muscle tissue. CRISPR therapy would not be able to reverse or restore any existing damage to muscle such as fat and fibrous tissue at the time of intervention. As patients would likely have greater benefit from earlier intervention, neonatal screening for DMD will be important to maximize therapeutic outcomes. There are also concerns that patients might experience T-cell immune responses toward the restored dystrophin protein (Mendell et al., 2010). However, since patients would typically have a small percentage revertant fibers that are dystrophin positive due to spontaneous alternative splicing or secondary mutation events that bypasses the mutation, their immune systems would have already been exposed to a residual level of dystrophin (Min, Bassel-Duby, & Olson, 2019).

Off-targeting is a concern that must be addressed before DMD CRISPR therapy can be tested in the clinic. Off-target editing at genomic sites with high sequence similarity with the target sequence may lead to unintended and potentially deleterious effects such as the disruption of normal gene function and induction of chromosomal rearrangements, and

may even cause cancer (Sheridan, 2021). Further complicating this, off-targets would be difficult to evaluate when editing is performed systemically, since editing events at each cell nucleus would be independent from one another. Therefore, to minimize off-targeting, it will be crucial to develop CRISPR strategies that are highly specific (such as by selecting target sequences that do not have a high degree of sequence relatedness to potential off-target sites) and by using high-fidelity Cas9s. There will also be a need for putative patient-specific off-targets by whole genome sequencing- this should be a relatively inexpensive exercise given the decreasing cost of next-generation sequencing technology.

6 | DELIVERY OF CRISPR THERAPY IN VIVO

A significant challenge facing the delivery of CRISPR components to striated muscle systemically is the scale at which CRISPR editing has to be carried out. As skeletal muscle accounts for approximately 40% of total body weight in humans, delivery methods for body-wide CRISPR editing have to be designed to be able reach a large number of cells in a tissue-specific manner in patients. It is important to note however that, since skeletal muscle fibers are multinucleated, restoring *DMD* in only a few of these nuclei may be sufficient to restore dystrophin expression along the length of a muscle fiber. Furthermore, complete correction of all nuclei may not be necessary as data from BMD patients indicate that a 4%–50% restoration of dystrophin expression may be sufficient to confer clinical benefit (Chemello, Wang, et al., 2020; Hoffman et al., 1989).

For the systemic delivery of CRISPR components to muscle tissue, AAV serotype 9 is commonly used due to its high muscle tropism. Cas9 and gRNA encoded in its DNA payload can be driven by muscle-specific promoters, for example, CK8 for muscle-restricted expression (Amoasii et al., 2018). However, due to the limited packaging capacity of AAV (~4.7 kb) and the relatively long coding sequence of SpCas9, CRISPR therapeutics employing SpCas9 would require co-delivery of two AAV vectors encoding Cas9 and gRNA separately or as intein-split Cas9, doubling the viral dose required for therapy. Some improvements to AAV-9 delivery strategies for DMD have been described, such as the delivery of gRNA using double-stranded self-complementary AAV that improves its stability and transduction efficiency, as well as capsid optimization for improved potency and tissue selectivity via directed capsid evolution (Tabebordbar et al., 2021; Y. Zhang et al., 2020). Split AAV systems can also be used to deliver the larger coding sequences of Cas9-fusion proteins used in base and prime editors. *Dmd* editing has also been explored using the more compact SaCas9 (Bengtsson et al., 2017; El Refaey et al., 2017; Nelson et al., 2016) and *Campylobacter jejuni* (Cj) Cas9 (Koo et al., 2018) which can be accommodated in a single AAV vector. More recent work with AAV-9 have also found that AAV-9 is able to transduce muscle satellite cells (stem cells responsible for the regeneration of skeletal muscle), albeit at low efficiency. Nevertheless, editing of these cells would allow the sustained replenishment of dystrophin positive myofibers after muscle damage (Kwon et al., 2020; Nance et al., 2019).

There remain, however, some challenges for the clinical application of AAV, such as the potential immunogenicity of the AAV capsid when administered systemically (Ronzitti et al., 2020). Furthermore, AAVs can integrate into the host genome with potential insertional mutagenesis. In a long-term study of dogs with hemophilia A administered with AAV expressing canine factor VIII, there were instances of clonal expansion of transduced liver cells where AAV integration occurred in genes associated with growth control (Nguyen et al., 2021). In addition, there are concerns for the delivery of CRISPR components by AAV in humans, including the induction of cellular immune responses toward AAV-mediated Cas9 expression or pre-existing immunity toward Cas9 that may attenuate editing efficacy, trigger inflammatory responses, and induce destruction of edited cells by the immune system (Hakim et al., 2021; Li et al., 2020). In canine models of DMD, AAV-9-delivery of CRISPR components has been recently shown to elicit humoral and cytotoxic T-cell responses against Cas9, which eliminates dystrophin corrected muscle fibers, reducing levels of dystrophin rescue (Hakim et al., 2021). Moreover, they found that editing in dystrophic dogs with pre-existing Cas9 immunity was significantly less efficient and resulted in an infiltration of immune cells in skeletal muscle. However, it is not known how many pediatric individuals have pre-existing Cas9 immunity and whether this will be a problem for genome editing in DMD. Also, the possibility of AAV integration can lead to the long-term expression of CRISPR components and increases the risk of unintended off-targeting (Hanlon et al., 2019).

Transient, virus-derived delivery approaches like virus-like particles (VLPs) and non-integrating lentiviruses (NILVs) can also potentially be developed for CRISPR DMD therapy. Since they carry non-DNA cargo, they can be administered without the risk of integrating CRISPR sequences leading to their prolonged expression, minimizing the potential for off-target editing. Delivery of Cas9 and gRNA ribonucleoprotein (RNP) using VLPs have been tested in

myoblasts and in patient induced pluripotent stem cell-derived skeletal muscle cells in vitro, as well as in muscle tissue via localized intramuscular injections in mice (Gee et al., 2020). However, as these VLPs have VSV-G envelope glycoproteins that have broad cell tropism, targeting specificity could be further increased by engineering VLPs with different envelope glycoproteins that confer muscle tropism.

Lipid nanoparticle (LNP) delivery of Cas9 mRNA and gRNA is being explored as a non-viral alternative for CRISPR DMD therapy, with lower immunogenicity than AAV. Kenjo et al. (2021) have recently described an LNP with an optimized formulation for targeting skeletal muscle that elicits a weaker anti-Cas9 immune response and allows for subsequent administrations of CRISPR components (Kenjo et al., 2021). Widespread delivery of the LNP was demonstrated by perfusion of a single isolated limb. However, as systemic delivery was not reported and non-muscle targeting was not assessed, the specificity of this formulation is undetermined. Further optimizations of LNP formulations that is highly muscle-specific, and able to target to both heart and skeletal muscle tissue would be highly desirable, since unlike DNA-delivered AAV, further target specificity cannot be delineated by muscle-specific promoters. Other non-viral delivery options that have been previously tested in a localized context include the electroporation of Cas9 and gRNA encoding plasmids into the Flexor digitorum longus muscle of adult *mdx* mice (Xu et al., 2016), and the delivery of Cas9 and gRNA RNP using gold nanoparticles conjugated to DNA and complexed with cationic endosomal disruptive polymers to gastrocnemius and tibialis anterior muscle of four-week-old *mdx* mice (Lee et al., 2017).

7 | CONCLUSION

The advent of CRISPR gene-editing technology has revolutionized DMD research. It has facilitated the generation of DMD animal models and can itself be potentially harnessed to treat DMD. Despite the progress it has enabled, there is still much work to be done before the potential of CRISPR therapies can be realized. Most of the published in vivo preclinical studies target the endogenous animal gene, which almost without exception cannot be directly translated to the clinic due to differences in the human *DMD* sequence. The availability of sequence-humanized hDMDTg mice has made it possible to perform in vivo testing of human-relevant CRISPR candidates. However, the apparent tandem inverted duplication of the *DMD* transgene is a significant limitation because it does not accurately simulate the single-copy nature of the endogenous *DMD* gene and may create additional editing outcomes that confound interpretation.

Although mice lacking dystrophin exhibit a milder clinical phenotype compared with human DMD, they still serve as a useful platform for in vivo testing of sequence-dependent therapies in a small animal model. The restoration of the dystrophin and the phenotypic markers such as CK serum levels and reduced grip strength could provide indicators of successful in vivo treatment. However, these mouse models could not be used to assess the safety and tolerability of treatments, and also address whether treatments could extend the shortened lifespan of human DMD patients. To address this, dystrophin–utrophin double-KO mice could be utilized since the double-KO present with more severe phenotypes and shorter lifespan.

Large animal models generally better reflect human physiology, anatomy, metabolism and clinical features of hereditary diseases. Pig models would be ideal for testing the safety and tolerability of DMD therapeutic candidates, but there are not many models available to date. With the advance of CRISPR pig zygote injection, the generation of more DMD models in pigs is expected. However, there are sequence differences between pig and human; the cDNA sequence of DMD exons 44–54 is only 91.5% identical between humans and pigs which could mean that many human CRISPR candidates cannot be tested in the pig model unless the CRISPR target regions in the pig are humanized.

Notwithstanding the significant ethical considerations, monkeys may be used as animal model for testing the therapeutic CRISPR candidates. Although thorough clinical phenotyping of live monkeys lacking dystrophin has yet to be performed, they likely resemble human DMD as muscle degeneration is evident at an early stage. Furthermore, DMD exons 44–54 cDNA sequences are 98% identical between humans and monkeys, meaning that most of the exonic human CRISPR candidates would match in monkeys for in vivo testing without having to humanize the monkey sequences. However, this would be increasingly costly if new monkey models have to be generated to test each different mutation-specific CRISPR gene-editing strategy, on top of the already mentioned ethical considerations.

Furthermore, while the occurrence of on-target editing can be assessed in humanized models or in models with sufficient homology, off-target effects can only be evaluated in human systems, such as for example using myogenically differentiated induced pluripotent stem cells (Young et al., 2016). There are currently many studies demonstrating the

utility of CRISPR to correct a wide variety of DMD mutations but given the extent of which edits would be performed systemically, additional focus needs to be placed on ensuring that therapeutic editing can be carried out as safely as possible in patients. Efforts can be made to optimize CRISPR strategies for improved specificity to eliminate unintended off-targeting, higher editing efficiency so that lower doses can be administered with therapeutic effect, and biasing editing outcomes to those with a reframing or skipping effect. Delivery methods can also benefit from more muscle-specific delivery, as well as more tissue-restricted and shorter duration of Cas9 and gRNA expression to reduce the likelihood of unintended targeting and improve immunogenicity. As current CRISPR strategies being explored strongly focus on correcting exonic deletions in DMD, efforts should also be broadened to tackle duplications, nonsense mutations and small InDels. Animal models carrying these mutations can be generated using conventional CRISPR-initiated HDR or using the newly developed Nuclease Prime Editor (Adikusuma et al., 2021).

CRISPR clinical trials have commenced for several diseases including the ex vivo correction of sickle cell disease and transfusion-dependent β-thalassemia, as well as the in vivo correction of Leber congenital amaurosis (Daich Varela et al., 2021; Frangoul et al., 2021). It is possible that CRISPR clinical trials for DMD will occur in the future. The development and translation of safe and effective CRISPR therapies for DMD would be a gamechanger for this devastating and currently incurable disease. Notably, some caution is needed here to avoid raising false hope within the patient community. CRISPR therapies will not be a cure; they will not allow the restoration of muscle tissue and function that has been lost at the time of intervention. As such, patients treated earlier are anticipated to benefit more if a CRISPR therapy ever becomes a reality. Furthermore, the treatment will allow production of a partially functional dystrophin, so treatment, if effective, will delay future disease progression rather than halt it. However, this would be a significant improvement and would help address the sizable unmet medical need currently facing the vast majority of DMD patients.

AUTHOR CONTRIBUTIONS

Yu C. J. Chey: Visualization (equal); writing – original draft (lead); writing – review and editing (equal). **Jayshen Arudkumar:** Visualization (equal); writing – original draft (lead); writing – review and editing (equal). **Annemieke Aartsma-Rus:** Writing – original draft (supporting); writing – review and editing (equal). **Fatwa Adikusuma:** Supervision (equal); writing – original draft (supporting); writing – review and editing (equal). **Paul Q. Thomas:** Supervision (equal); writing – review and editing (equal).

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest. Annemieke Aartsma-Rus has no conflicts related to this manuscript. For full transparency she discloses being employed by LUMC which has patents on exon skipping technology, some of which has been licensed to BioMarin and subsequently sublicensed to Sarepta. As co-inventor of some of these patents AAR is entitled to a share of royalties. AAR further discloses being ad hoc consultant for PTC Therapeutics, Sarepta Therapeutics, Regenxbio, Alpha Anomeric, BioMarin Pharmaceuticals Inc., Eisai, Entrada, Takeda, Splicesense, Galapagos, and Audentes. Past ad hoc consulting has occurred for: CRISPR Therapeutics, Summit PLC, Astra Zeneca, Santhera, Bridge Bio, Global Guidepoint and GLG consultancy, Grunenthal, Wave, and BioClinica. AAR also reports having been a member of the Duchenne Network Steering Committee (BioMarin) and being a member of the scientific advisory boards of Eisai, hybridize therapeutics, silence therapeutics, Sarepta therapeutics. Past SAB memberships: ProQR, Philae Pharmaceuticals. Remuneration for these activities is paid to LUMC. LUMC also received speaker honoraria from PTC Therapeutics and BioMarin Pharmaceuticals and funding for contract research from Italpharmaco, Sapreme, and Alpha Anomeric. Project funding is received from Sarepta Therapeutics.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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