Molecular Therapy
Review

CRISPR technologies for the treatment of Duchenne muscular dystrophy

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The emerging clustered regularly interspaced short palindromic repeats (CRISPR)-mediated genome editing technologies have progressed remarkably in recent years, opening up the potential of precise genome editing as a therapeutic approach to treat various diseases. The CRISPR-CRISPR-associated (Cas) system is an attractive platform for the treatment of Duchenne muscular dystrophy (DMD), which is a neuromuscular disease caused by mutations in the *DMD* gene. CRISPR-Cas can be used to permanently repair the mutated *DMD* gene, leading to the expression of the encoded protein, dystrophin, in systems ranging from cells derived from DMD patients to animal models of DMD. However, the development of more efficient therapeutic approaches and delivery methods remains a great challenge for DMD. Here, we review various therapeutic strategies that use CRISPR-Cas to correct or bypass *DMD* mutations and discuss their therapeutic potential, as well as obstacles that lie ahead.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is a severe, X-linked recessive disease with an average incidence of ~1 in 5,000 live male births.[1] Most DMD patients exhibit progressive muscle degeneration associated with severe muscle weakness, loss of ambulation, cardiac or respiratory complications, and eventually death, in their 20s.[2]

The *DMD* gene consists of 79 exons that encode dystrophin, which is a cytoskeletal protein that plays an important role in a complex that connects the cytoskeleton of muscle fibers with the extracellular matrix and is present throughout the cell membrane.[3–5] Different types of mutations in *DMD* exons and introns cause various forms of dystrophinopathies.[3] Approximately 60% of DMD patients harbor a large deletion in the *DMD* gene, often affecting exons 45–55, a region that represents a mutational hotspot.[6] Deletion of a *DMD* exon can result in a shift in the reading frame and the formation of a premature stop codon, causing either expression of a truncated version of dystrophin that does not function properly or a complete lack of dystrophin expression.

In Becker muscular dystrophy (BMD), a relatively benign type of MD compared to DMD, a semi-functional dystrophin protein is expressed, compensating for the muscle loss.[2] Mild BMD symptoms include a relatively slow disease progression, which have little effect on lifespan.[7] Thus, alleviating symptoms in DMD patients by expressing a semi-functional protein to mimic a BMD-like disease phenotype could be an efficient strategy for treating DMD. It is notable that a 4% increase in normal dystrophin expression was sufficient to improve muscle function.[8–10]

Various pharmacologic therapeutic approaches have focused on converting the DMD phenotype to a BMD-like phenotype by restoring the disrupted *DMD* reading frame. In 2016, eteplirsen (Exondys 51), an antisense oligonucleotide drug with phosphorodiamidate morpholino oligomer chemistry, became the first medication with such a mechanism to be approved by the US Food and Drug Administration (FDA) for the treatment of DMD. It induces exon 51 skipping in the *DMD* gene, restoring the expression of semi-functional dystrophin and resulting in BMD-like mild symptoms in eteplirsen-treated DMD patients.[11] All of the patients treated with eteplirsen showed an increase in the frequency of dystrophin-positive fibers, by an average of 15.5-fold over untreated controls. In addition, the therapeutic efficacy of golodirsen (Vyondys 53) and viltolarsen (Viltepso), other phosphorodiamidate morpholino oligomer drugs, are under evaluation in clinical trials for treating DMD patients.[12,13] Treatment with golodirsen in a Phase I trial resulted in exon 53 skipping and a ~16-fold increase in dystrophin protein expression over baseline, with 1.02% of normal dystrophin protein expression at week 48. In another study, a 4-week randomized Phase II clinical trial, treatment with viltolarsen caused exon 53 skipping and transcript levels that were 42.4% of normal levels, which in turn led to significant dystrophin production, at 2.8% of normal levels.[12–14] These approaches have reduced disease symptoms, but none have yet eliminated the disease-causing mutation to allow long-term dystrophin expression.

Recently, therapeutic applications of genome editing have been explored for treating various genetic diseases. The clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated

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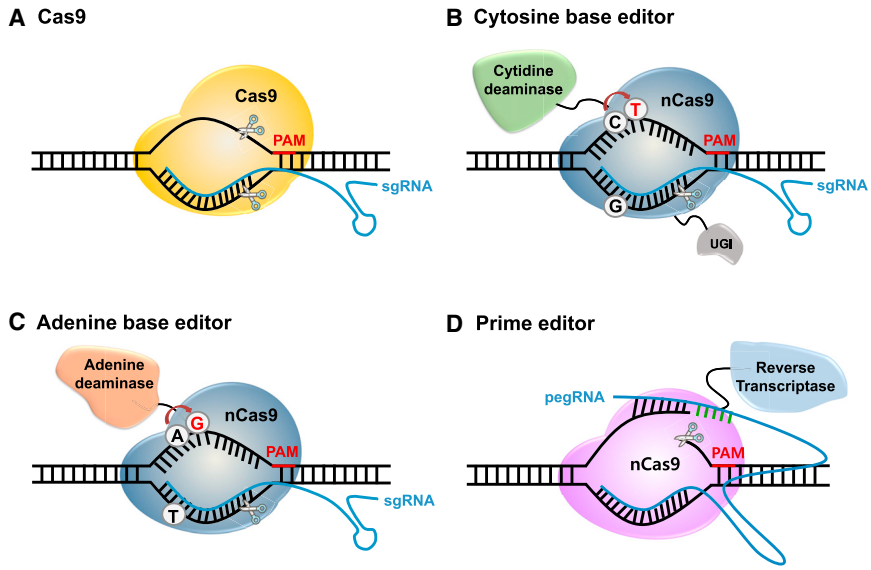

Figure 1. CRISPR-Cas genome editing tools
(A) Cas9-sgRNA complexes bind to DNA targets in the genome, generating DSBs 3 bp upstream of the PAM. (B) Cytosine base editors (CBEs), composed of cytidine deaminase fused to nCas9 (D10A) and UGI, enable direct conversion of targeted C:G base pairs to T:A base pairs. (C) Adenine base editors (ABEs), which consist of adenine deaminase fused to nCas9 (D10A), convert targeted A:T base pairs to G:C base pairs. (D) Prime editors consist of nCas9 (H840A) fused to an engineered Moloney murine leukemia virus reverse transcriptase domain. These editors can generate any desired sequence contained in the associated prime editing guide RNA (pegRNA).

(Cas) system is a powerful technology for genome editing, especially for correcting disease-causing mutations. Here, we review recent progress in the area of CRISPR-mediated genome editing to treat DMD with various strategies to eliminate pathogenic mutations in the *DMD* gene. These approaches could also be extensively applied for treating other neuromuscular disorders and other types of genetic diseases.

Genome editing tools: The CRISPR-Cas system

CRISPR-Cas was identified as an adaptive immune system in bacteria and archaea that functions to prevent the invasion of foreign genetic materials.[15] Upon viral DNA entry into a bacterium, the cell integrates segments of viral DNA into the CRISPR locus of a bacterial genome. When the same type of virus next invades, RNAs are transcribed from the CRISPR array and cooperate with Cas9 endonuclease to cleave the complementary viral DNA sequence. In the CRISPR system, two RNAs (CRISPR RNA [crRNA] and *trans*-activating CRISPR RNA [tracrRNA]) are transcribed for target searching. These two components can be linked together to generate a programmable single guide RNA (sgRNA), the form that is now widely used for efficient genome editing. Cas9 derived from the type II CRISPR system of *Streptococcus pyogenes* (SpCas9) is the most studied and generally used form of the endonuclease; it recognizes a 5′-NGG-3′ or 5′-NAG-3′ protospacer adjacent motif (PAM) and cleaves target DNA 3 bp upstream of the PAM, generating double-strand breaks (DSBs)[15–17] (Figure 1A). Over time, Cas proteins from various other species have been discovered. Among them, Cas12a endonuclease, also called CRISPR from *Prevotella* and *Francisella 1* (Cpf1), is derived from a type V (class II) CRISPR system.[18] It has been reported that Cpf1 endonuclease from *Acidaminococcus* sp. *BV3L6* and *Lachnospiraceae bacterium ND2006* recognize T-rich PAM motifs (5′-TTTV-3′) and cause staggered end cleavage with equal or greater efficiency than Cas9 orthologs.[19] Of special interest, Cpf1 can be used for multiplex genome editing, in which multiple sites are simultaneously edited. Other Cas9 orthologs derived from *Staphylococcus aureus* (SaCas9)[20] or *Campylobacter jejuni* (CjCas9),[21] which are smaller than SpCas9, make it possible to efficiently package the genes encoding these nucleases into small viral vector systems together with its sgRNA. The 2 Cas9 nucleases respectively recognize 5′-NNGRRT-3′ and 5′-NNNVRYAC-3′ PAM sequence before target DNA cleavage.

More recently, several base editing systems have been developed that allow single base conversion or base editing in cells and organisms in a guide RNA-dependent manner. For targeted base mutagenesis, fusion of a deaminase enzyme, activation-induced cytidine deaminase (AID) or rat APOBEC1, with a catalytically deficient D10A/H840A Cas9 (called dead Cas9 or dCas9) or Cas9 nickase (nCas9) and uracil DNA glycosylase inhibitor (UGI, to prevent base excision repair) enabled direct conversion of a targeted cytidine (C):guanine (G) base pairs to thymine (T):adenine (A) base pairs (Figure 1B).[22,23] Furthermore, A base editors (ABEs), which convert A:T base pairs to G:C base pairs, have been constructed using an evolved version of *Escherichia coli* tRNA adenosine deaminase TadA, TadA*; they consist of heterodimeric TadA-TadA* conjugated with nCas9 (D10A mutation)[24] (Figure 1C). These base editing tools convert target bases in a limited editing window located several nucleotide positions upstream of a PAM sequence in the non-target strand. Various approaches are under way to broaden the target window range[25–28] and to increase the efficiency by using various Cas orthologs in this system.[29–35] Although the CRISPR-Cas and base editing systems can precisely install or correct mutations, they have limitations; in particular, Cas9 activity can lead to transversions and random insertions or deletions (indels) at the target site. Moreover, base editors can generate undesired bystander mutations within the base editing window. Most recently, a new genome editing technology, prime editors, was developed with the potential to overcome the limitations of the current genome editing system.[36] These editors consist of nCas9 with an inactivated HNH domain (H840A) fused to an engineered Moloney murine leukemia virus reverse transcriptase domain, making it possible to edit the genome to generate any desired sequence[36]

Table 1. Summary of CRISPR-mediated therapeutic strategies to rescue the DMD phenotype

Subject	Strategy	Nuclease	DMD mutation	Therapeutic target gene region(s)	Model(s)	Delivery	Reference
Therapeutic approach	exon reframing	SpCas9	*DMD* exon 48–50 deletions *DMD* nonsense mutation in exon 51	*DMD* exon 51	human DMD myoblasts	electroporation	37
		SpCas9	*DMD* exon 45–52 deletions	*DMD* exon 53	human DMD myoblasts	adenovirus	38
		SpCas9	*DMD* exon 44 deletion	*DMD* exon 45	human iPSCs	electroporation	39
		CjCas9	*Dmd* nonsense mutation in exon 23	*Dmd* exon 23	DMD mice	All-in-one AAV9	40
		LbCpf1	*DMD* exon 48–50 deletions *DMD* nonsense mutation in exon 51	*DMD* exon 51	human iPSCs	nucleofection	41
	exon deletion	SpCas9	*Dmd* nonsense mutation in exon 23	*Dmd* intron 22 and 23	*mdx* mice	AAV9	42
		SpCas9	*DMD* exon 46–51 deletions *DMD* exon 46–47 deletions	*DMD* intron 44 and 55	human DMD myoblasts	nucleofection	43
		SaCas9	*Dmd* nonsense mutation in exon 23	*Dmd* intron 22 and 23	*mdx* mice	AAV8	44
		SaCas9	*Dmd* nonsense mutation in exon 23	*Dmd*exon 23	Ai9 *mdx* mice	AAV9	45
	exon skipping	SpCas9	*DMD* point mutation in intron 47, exon 51	*DMD* exon 47A, exon 51	human iPSCs	nucleofection	46
		SpCas9	*DMD* exon 44 deletion	*DMD* splice site of exon 43 or exon 45	human iPSCs	nucleofection	47
		SpCas9	*DMD* nonsense mutation in exon 53	*DMD* splice acceptor site of exon 53	human DMD myoblasts	adenovirus	48
		SpCas9	*Dmd* exon 50 deletion	*Dmd* splice acceptor site of exon 51	DMD mice	AAV9	49
		SpCas9	*DMD* exon 50 deletion	*DMD* splice acceptor site of exon 51	canine model of DMD	AAV9	50
	homology-directed repair	SpCas9	*Dmd* nonsense mutation in exon 23	*Dmd* exon 23	*mdx* mice	injection	51
		SpCas9	*Dmd* nonsense mutation in exon 23	*Dmd* exon 23	mouse muscle stem cells	adenovirus	52
		SpCas9	*Dmd* nonsense mutation in exon 53	*Dmd* exon 53	mdx^{4cv} mice	AAV6	53
		SpCas9	*DMD* exon 44 deletion	*DMD* exon 44	human iPSCs	electroporation	39
		SpCas9	*DMD* exon 7 skipping	*DMD* splice acceptor site of intron 6 and exon 7 boundary	canine model of DMD	injection	54
		LbCpf1	*Dmd* nonsense mutation in exon 23	*Dmd* exon 23	*mdx* mice	injection	41
	base editing (exon skipping)	TAM based on SaCas9 (Cytosine base editor)	*DMD* exon 51 deletion	*DMD* splice site of exon 50	human iPSCs	lipotransfection	55
	base editing (correction)	ABE7.10	*Dmd* nonsense mutation in exon 20	*Dmd* exon 20	DMD mice	trans-splicing AAV	56
	transcriptional modulation	CRISPRa (dCas9-VP160)	*DMD* exon 45–52 deletions	*UTRN* A, B promoter	immortalized DMD patient muscle cells	Electroporation	57
		CRISPRa (dCas9-VP160)	*Dmd* nonsense mutation in exon 23	*Lama1* promoter	mouse myoblasts, *mdx*/rag mice	transfection, injection, electroporation	58

(*Continued on next page*)

Table 1. *Continued*

Subject	Strategy	Nuclease	DMD mutation	Therapeutic target gene region(s)	Model(s)	Delivery	Reference
		CRISPRa (dCas9-VP64)	*Dmd* nonsense mutation in exon 23	*klotho* and *Utrn*	*mdx* mice	AAV9	59
		SaCas9	*DMD* exon 46–51 deletions	3′ UTR of *UTRN* inhibitory miRNA target region	human iPSCs	electroporation	60
		CRISPRi (dCas9-KRAB)	epigenetic dysregulation of *DUX4*	*DUX4* promoter or *DUX4* exon 1	human FSHD myocytes	lentivirus	61

AAV, adeno-associated viral vector; ABE, adenine base editor; CRISPRa, CRISPR activator; CRISPRi, CRISPR interference; FSHD, facioscapulohumeral muscular dystrophy; hiPSC, human induced pluripotent stem cell; TAM, targeted AID mediate mutagenesis.

(Figure 1D). Permanent DNA edits occur when the non-edited strand is replaced by the DNA repair system of the cell using a reverse transcriptase template containing the edit.[36] These various CRISPR-Cas systems show great potential for precise genome editing. Here, we summarized the leading strategies for CRISPR-mediated *DMD* gene editing below (Table 1).

Therapeutic approach: Exon reframing

Approximately 51% of DMD patients have deleterious frameshifting exon deletion mutations that interrupt the *DMD* open reading frame (ORF) based on the Leiden DMD mutation database.[62] In the case of DMD-causing frameshift mutations, small indels generated by non-homologous end joining (NHEJ)-mediated repair upstream of the premature stop codon have a 1 in 3 probability of reframing the ORF (Figure 2A). Several groups have demonstrated successful *DMD* exon reframing with this strategy.[37–41] As one example of this approach, in a DMD mouse model, CjCas9 and its *Dmd* exon 23-specific sgRNA were used to target a site upstream of a premature stop codon caused by a frameshift mutation in *Dmd* exon 23. After CjCas9-induced cleavage, NHEJ at the cleaved site reframed the ORF. Compared to other Cas9 nucleases, CjCas9 is notable for having the smallest known size to date. In this study, sequences encoding CjCas9 and its sgRNA were packaged into an all-in-one adeno-associated viral (AAV) vector serotype 9, maximizing the delivery efficiency to target muscles.[40] This treatment resulted in indel formation at the target site, with a frequency of up to 8%, which in turn led to dystrophin expression in 28%–39% of muscle fibers and improved muscle strength, demonstrating the possibility of applying the CRISPR system to correct the *DMD* ORF *in vivo*.[40]

Therapeutic approach: Exon deletion

Deletion of one or more exons can be used to restore disrupted ORF when it is shifted to be out-of-frame by frameshift mutations or the deletion or duplication of exon(s). In addition, the effects of certain point mutations can be rescued by removal of the mutated exon (Figure 2B). As examples of this approach, 2 sgRNAs were designed to remove the mutated exon by targeting intronic regions flanking the mutated *Dmd* exon 23 in *mdx* mice, which contain a nonsense mutation.[42,44,45] Of note, Ai9 *mdx* mice treated with AAV9-expressing SaCas9 and appropriate 2 sgRNAs targeting intronic regions flanking the exon 23 exhibited exon 23 deletion at a frequency of 39% in *tibialis anterior* (TA) muscles, demonstrating the therapeutic potential of this approach *in vivo*.[45] In human genome editing, deletion of a mutation hotspot spanning *DMD* exons 45 to 55 could treat ~60% of DMD patients.[37] Several groups have demonstrated deletion of this hotspot from the human *DMD* gene with therapeutic effects. In a study performed in human myoblasts from DMD patients, 2 sgRNAs, one targeting the 5′ end of exon 45 and the other targeting the 3′ end of exon 55, resulted in deletion from the genome of 336 kb that contained exons 45–55. This approach led to dystrophin expression with an edited allele efficiency of 5%–10%.[37] Similarly, use of 2 sgRNAs targeting regions flanking exons 45–55 resulted in the deletion of up to 725 kb from both cardiomyocytes and skeletal cells generated from human induced pluripotent stem cells (hiPSCs) derived from a DMD patient.[43] NSG-*mdx* scid mice engrafted with these exon 45- to 55-deleted DMD hiPSCs showed dystrophin expression and colocalization with the dystrophin-associated transmembrane protein, β-dystroglycan at the sarcolemma, suggesting that the deletion of a mutation hotspot would be clinically relevant.

Therapeutic approach: Exon skipping

Exon skipping induced by abolishing conserved RNA splice sites is a powerful strategy for restoring the *DMD* ORF. The strategy of causing one or more exons to be skipped in the process of RNA splicing could be useful for treating up to 83% of DMD patients.[62] The introduction of indels by NHEJ at an RNA splice site or deletion of the splice site of an out-of-frame exon abolishes splice site function, leading to targeted exon skipping (Figure 2C). Several CRISPR-mediated exon skipping approaches, involving splice site targeting, have been demonstrated. In particular, SpCas9-mediated NHEJ at 5′ or 3′ splice sites containing 5′-NAG-3′ or 5′-NGG-3′ PAM motifs can remove essential splice donor or acceptor sequences for skipping of the corresponding target exon. With this strategy, skipping of exons 43, 51, and 53 induced by NHEJ-induced disruption of splice acceptor (SA) sites was demonstrated in hiPSCs and human myoblasts.[46–48] In evaluations of this approach *in vivo*, skipping of exon 51 was induced, leading to ORF reframing and restoration of dystrophin expression, in mouse[49] and canine models of DMD that lack exon 50.[50] In these studies, splicing acceptor sites adjacent to exon 51 were modified such that exon 51 was skipped, resulting in the juxtaposition of exon 49 and 52 in the mRNA and ORF reframing.

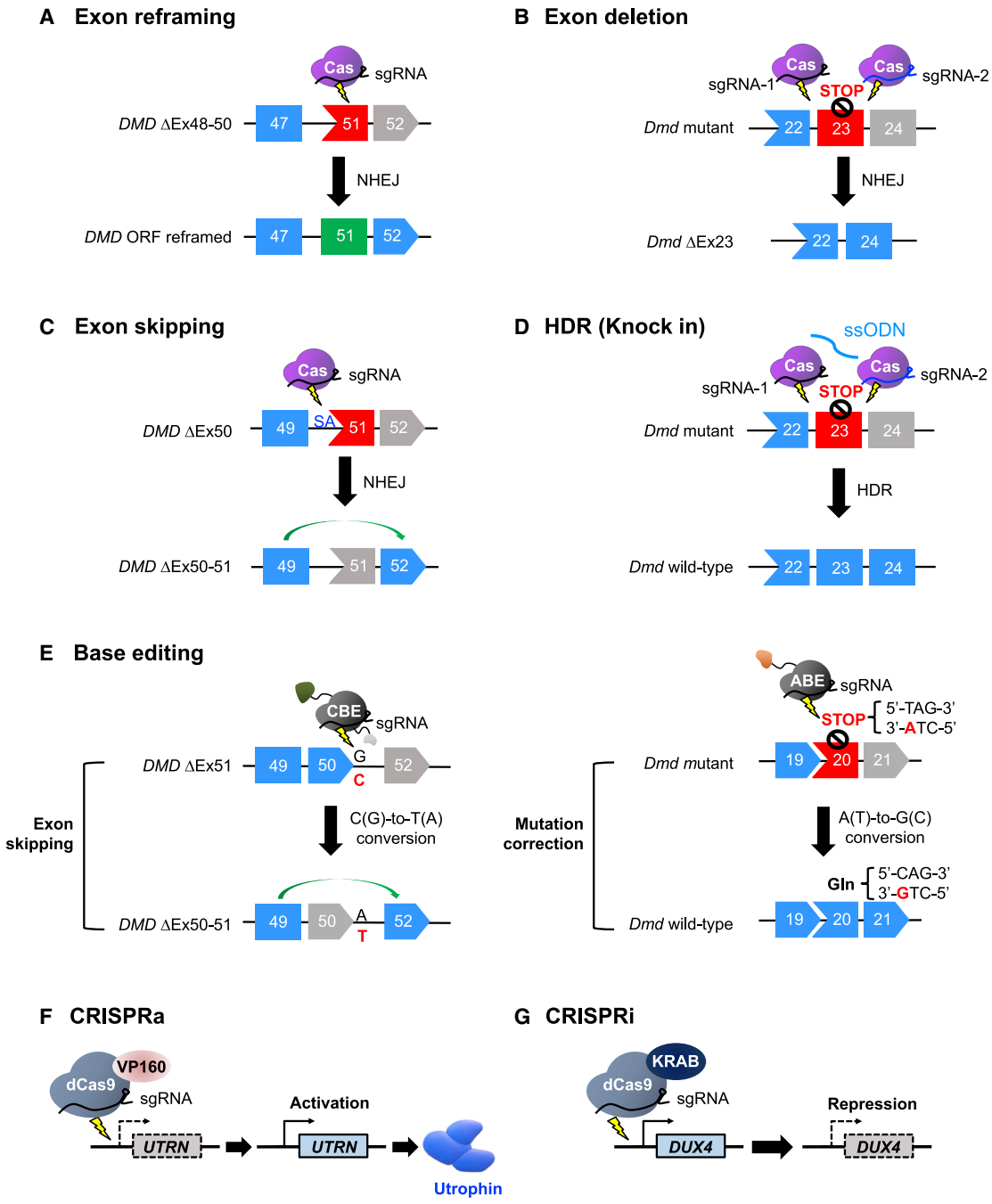

Figure 2. Mechanisms of CRISPR-mediated genome editing to correct mutations in the *DMD* gene or ameliorate the effects of such mutations

(A) Exon reframing induced by NHEJ. Small indels that are generated upstream of the premature stop codon in *DMD* exon 51 have a 1 in 3 probability of reframing the ORF. (B) Exon deletion using 2 sgRNAs targeting intronic regions flanking the mutated *Dmd* exon 23. (C) *DMD* exon 51 skipping induced by disruption of a splice acceptor (SA) site to juxtapose exons 49 and 52 in the mRNA and reframed the ORF. (D) Precise, HDR-mediated mutation correction using Cas9, 2 guide RNAs targeted to sites flanking the mutated *Dmd* exon 23, and ssODNs. (E) Base editor-mediated exon skipping using a CBE or mutation correction using an ABE. (F) CRISPRa-mediated epigenetic editing to upregulate utrophin expression. (G) CRISPRi-mediated epigenetic editing to inhibit *DUX4* expression.

Therapeutic approach: HDR-mediated gene correction

Homology-directed repair (HDR)-mediated genome editing can restore full-length dystrophin gene expression, whereas NHEJ-mediated exon reframing results in a truncated form of dystrophin. To induce HDR, Cas9, a guide RNA targeting the mutated region, and single-stranded oligodeoxynucleotides (ssODNs) or a donor template with the correct sequence are required (Figure 2D). Several studies have corrected the *Dmd* gene by knockin strategies targeting *Dmd* exon 23,[41,51,52] exon 53,[53] and *DMD* exon 44.[39] As one example of this approach, the nonsense mutation in exon 53 in *mdx*^{4cv} mice was repaired by intramuscular injection of AAV6 carrying Cas9, sgRNA, and donor template sequences into TA muscles.[53] Successful HDR occurred in 0.18% of the total genomes, which led to full-length dystrophin expression that was 1.8%–8.4% of that seen in wild-type (WT) mouse muscles.[53] In addition, the nonsense mutation in *Dmd* exon 23 in the *mdx* mice was corrected by SpCas9 with a 180-nt ssODN[51] or LbCpf1 with a 180-nt ssODNs,[41] together with a corresponding gRNA, resulting in correction rates of 17% to 41%[51] and 8% to 50%, respectively.[41] HDR-mediated genome editing has also been demonstrated in a large animal model. The defect of golden retriever MD dog, which contains splice site mutations that lead to exon 7 skipping, was repaired by CRISPR-induced HDR.[54] The ssODNs used in this study included the correct *DMD* sequence at the intron 6 acceptor splice site.[54] With this HDR-mediated repair, examination of muscle biopsies showed that *DMD* mRNA expression was increased and dystrophin expression was restored to 6% to 16% of normal levels.[54]

There are several limitations to HDR-based DMD therapy. First, the length of the donor DNA template is limited, so the technique is not applicable to large *DMD* deletion mutations. Second, HDR is restricted to the S and G2 phases of the cell cycle, when sister chromatids are available to accept the template DNA[63]; hence, G1-arrested cells (post-mitotic cells) such as mature myofibers and cardiomyocytes are not corrected efficiently by HDR-mediated gene editing.[53] Third, unwanted DNA fragments may be integrated into the *DMD* locus, resulting in an altered dystrophin expression. Lastly, because NHEJ is dominant in mammalian cells, HDR occurs at a much lower frequency than NHEJ. To overcome the low efficiency of HDR in muscles, the recently developed CRISPR-prime editing system has great potential for repairing the target *DMD* locus with direct reverse transcription of the desired sequence.

Therapeutic approach: Base editing

Therapeutic application of base editing in DMD is a promising strategy because the precise editing of a single base in the targeted site is possible without the generation of DNA DSBs. This method can correct point mutations in the *DMD* gene, which account for ~27% of DMD cases.[62] CRISPR-Cas-mediated base editing for the treatment of DMD has been demonstrated using two strategies: modulation of splicing or correction of a nonsense mutation (Figure 2E). Using a cytosine base editor (CBE; AID fused to nSpCas9 or nSaCas9), the G in the 5′ splice site of *DMD* exon 50 was targeted, disrupting the splice site and thereby leading to the skipping of exon 50 during mRNA splicing.[55] Approximately 90% of the genome acquired the intended G > A conversion, leading to exon 50 skipping in 99.9% of the *DMD* transcripts in cardiomyocytes differentiated from hiPSCs.[55] Another demonstration of *in vivo* base editing was the ABE-mediated correction of a nonsense mutation in a DMD mouse model.[56] To correct this mutation, located in *Dmd* exon 20, sequences encoding ABE7.10 (a TadA-TadA*-nSpCas9 fusion) were delivered to the TA muscles of the DMD mice. The ABE7.10-encoding construct was split into two parts to overcome the packaging limitations of AAV using a *trans*-splicing AAV (tsAAV) vector system. The two ABE7.10 sequence segments were packaged into independent AAV vectors and then delivered into TA muscles intramuscularly. The AAV vectors were combined via recombination between the two inverted terminal repeat sequences in each AAV vector during AAV concatemerization in a cell. The tsAAV-ABE-treated mouse muscles showed conversion of the stop codon (TAG) into a Gln codon (CAG) with a frequency of 3.3%, leading to increased dystrophin expression (up to 17% of the WT level) and colocalization with the nNOS protein at the sarcolemma, demonstrating the feasibility of ABE-mediated *in vivo* base editing for DMD.[56]

Transcriptional modulation using CRISPR-Cas

The CRISPR system has been engineered to regulate gene expression by fusing inactivated dCas9 with a transcriptional activator or repressor, generating tools called CRISPR activator (CRISPRa)[64–66] or CRISPR interference (CRISPRi), respectively.[67] Modulation of the expression of a gene related to the disease process, but different from the classic disease-associated gene, could be a new therapeutic approach for DMD and other diseases. Because dystrophin dysfunction has been considered to be the major cause of DMD, compensating for its lack of function with a different protein that functions similarly could be a novel treatment strategy. One advantage of such epigenetic editing is that it could be applicable to any of the DMD-associated mutations; furthermore, no DNA DSB is required to regulate gene expression in this approach. Utrophin, the cytoskeletal protein with a name that is a contraction of "ubiquitous dystrophin" is a homolog of dystrophin that is encoded by the autosomal *UTRN* gene. This protein is expressed in the myotendinous and neuromuscular junctions of adult skeletal muscles.[68,69] Several studies have shown that the upregulation of utrophin could rescue DMD phenotypes.[57,59,60,70–73] Treatment with dCas9-VP160 (dead SpCas9 fused to 10 tandem repeats of the transcriptional transactivator VP16) to target the *UTRN* A or B promoters, respectively, resulted in 1.7- to 2.7-fold or 3.8- to 6.9-fold increase in utrophin upregulation[57] (Figure 2F). Furthermore, SaCas9-mediated deletion of 5 inhibitory microRNA target regions within the *UTRN* 3′ untranslated region (UTR) resulted in 2-fold higher levels of utrophin in DMD-hiPSCs.[60]

The laminin protein is another potential compensatory molecule for DMD; the laminin complex links the extracellular matrix to integrin α7β1 in the sarcolemma, and thus could compensate for a loss of dystrophin in dystrophic muscles. Because injection of laminin-111 to *mdx* mouse muscles stabilized the sarcolemma, dCas9-VP160 was

Table 2. Summary of animal models of DMD generated by the CRISPR-Cas system

Subject	Animal	Strategy	Nuclease	DMD mutation	Target gene region(s)	Strain	Delivery	Reference
Disease modeling	mouse	exon deletion	SpCas9	*DMD* exon 45 deletion	*DMD* intron 44 and 45	C57BL/10 and DBA/2	microinjection	81
			SpCas9	*Dmd* exon 50 deletion	*Dmd* intron 49 and 50	C57BL/6J	not indicated	49
			SpCas9	*Dmd* exon 44 deletion	*Dmd* intron 43 and 44	C57BL/6J	microinjection	47
			SpCas9	*Dmd* exon 8–34 deletion	*Dmd* intron 7 and 34	C57BL/6J xCBA	microinjection	83
			SpCas9	*Dmd* exon 50 deletion	*Dmd* intron 49 and 50	not indicated	not indicated	84
			SpCas9	*Dmd* exon 43, 45, and 52 deletion	*Dmd* intron 42 and 43 *Dmd* intron 44 and 45 *Dmd* intron 51 and 52	C57BL/6	microinjection	85
		frameshift mutation	CjCas9	*Dmd* frameshift mutation in exon 23	*Dmd* exon 23	C57BL/6J	microinjection	40
		base editing	BE3	*Dmd* nonsense mutation in exon 20	*Dmd* exon 20	C57BL/6J and ICR mice	microinjection and electroporation	82
	rat	exon deletion	SpCas9	*Dmd* exon 3–16 deletions	*Dmd* exon 3 and exon 16	Wistar-Imamichi rats	microinjection	86
	rabbit	frameshift mutation	SpCas9	*DMD* frameshift mutation in exon 51	*DMD* exon 51	New Zealand rabbits	microinjection	87
	pig	base editing	BE3, hA3A-BE3	*DMD* nonsense mutation in exon 13	*DMD* exon 13	Bama miniature pigs and large white pigs	microinjection	88
	monkey	frameshift mutation	SpCas9	*DMD* frameshift mutation in exon 4 and/or exon 46	*DMD* exon 4 and/or exon 46	*Macaca mulatta* rhesus monkeys	microinjection	89

BE3, base editor 3; hA3A-BE3, hAPOBEC3A base editor 3; hDMD, humanized DMD.

used to target the *Lama1* promoter, leading to increased laminin-111 expression.[58] In addition, *klotho*, a transmembrane protein that is epigenetically silenced in muscle cells of *mdx* mice,[74] has been targeted for upregulation by CRISPRa.[59] Systemic injection of AAV9 encoding a *klotho*-targeting sgRNA and dCas9-VP64 to neonatal *mdx* mice restored *klotho* expression in muscle tissue and ameliorated DMD phenotypes.[59] Expression of DMD inhibitory molecules can be repressed by CRISPRi that inhibits their transcriptional start sites by catalyzing repressive chromatin modifications. In a study of facioscapulohumeral muscular dystrophy (FSHD), which results from epigenetic dysregulation of the *DUX4* gene in muscle, CRISPRi was used to downregulate the expression of DUX4 and potential *DUX4* activators[61] (Figure 2G). The CRISPRi system used here consists of dCas9 fused to a repressive Krüppel associated box (KRAB) domain and an appropriate sgRNA.[61] Another potential target for CRISPRi to treat DMD is myostatin (MSTN), a cytokine that is secreted by skeletal muscle cells and is a well-known cause of muscle atrophy with muscle wasting.[75] Several studies have reported that CRISPR-induced knockout of *Mstn* or *MSTN* increased muscle mass and myotube formation.[76,77] However, it has also been reported that the *MSTN* knockout is only slightly beneficial for inducing excessive muscle growth but causes impaired force generation.[78–80] Therefore, further extensive investigations of this approach are required before it can be applied in humans therapeutically.

Animal modeling of DMD

Generating animal models of DMD makes it possible to study the pathophysiology of the disease and to evaluate the efficacy of biodrugs before clinical trials. With this aim, CRISPR-Cas-mediated DMD animal modeling has been demonstrated in mouse,[40,47,49,81–85] rat,[86] rabbit,[87] pig,[88] and monkey[89] (Table 2). A DMD mouse model was generated using CBE, namely Base Editor 3 (BE3): a nonsense mutation (CAG [Gln] to TAG [stop codon]) was introduced in exon 20 of the *Dmd* gene in the mouse genome (Figure 3A). In this study, BE3 (rat APOBEC1-nCas9 (D10A)-UGI)-encoding mRNA and sgRNAs were introduced into a mouse embryo by microinjection or electroporation, respectively, resulting in 73% and 81% of blastocysts containing the *Dmd* exon 20 mutation.[82] This mouse model was further used to correct nonsense mutations using ABE. as described above in the Therapeutic approach: Base editing section. In addition, the humanized DMD mouse model,[90] in which the entire human *DMD* sequences were integrated into mouse chromosome 5, was used for

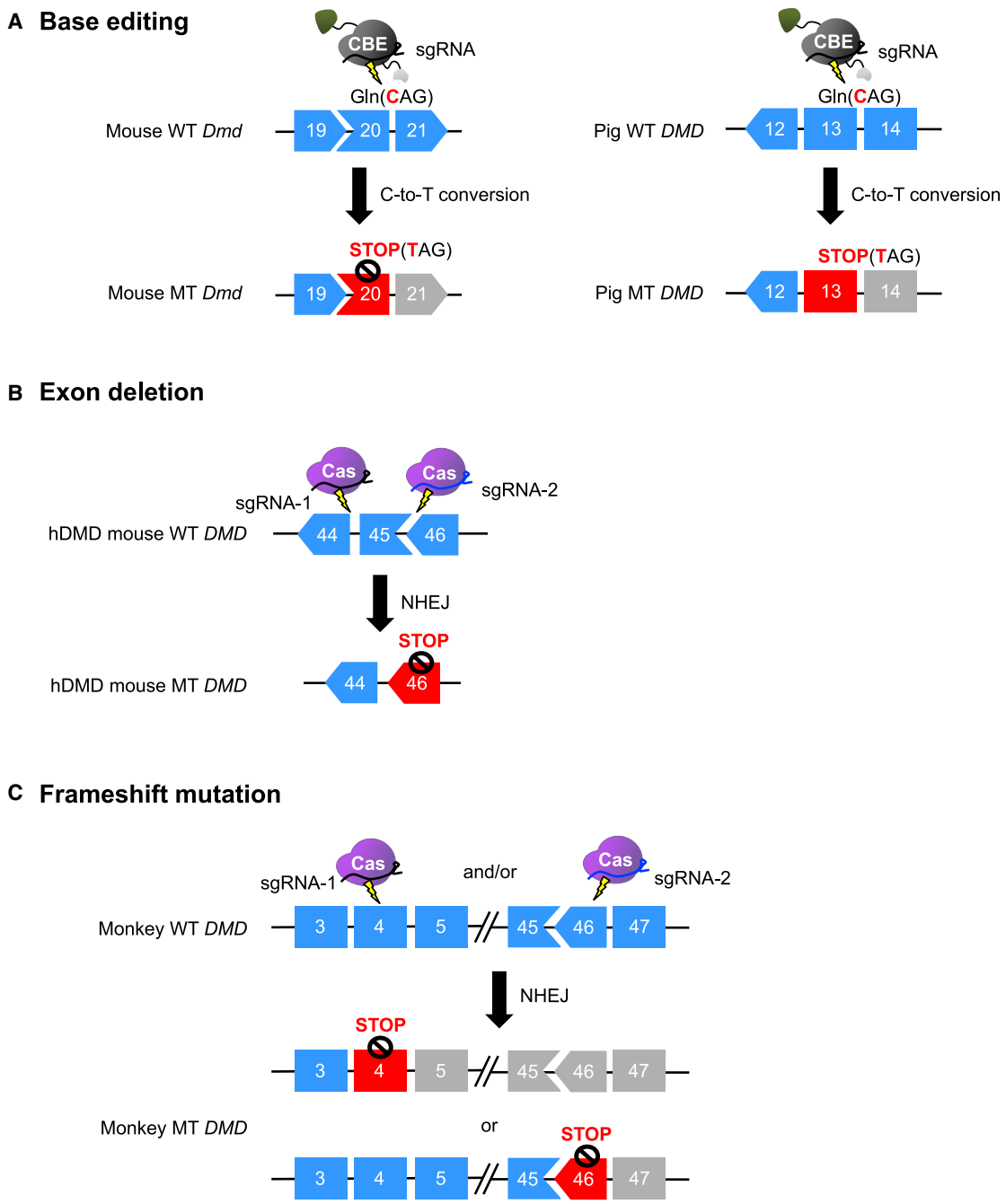

Figure 3. CRISPR-mediated animal modeling of DMD

(A) DMD mice (left panel) and DMD pigs (right panel) with a nonsense mutation in *Dmd* exon 20 and *DMD* exon 13, respectively, generated by the CBE BE3. (B) Humanized DMD mice in which *DMD* exon 45 has been deleted using SpCas9 and 2 sgRNAs targeting the sites flanking the region to be deleted. (C) DMD monkeys with frameshift mutations in exon 4 and/or exon 46 induced by CRISPR-mediated NHEJ. WT, wild type; MT, mutant type.

evaluating the efficiency of CRISPR tools that target the human gene. To place *DMD* out-of-frame, an exon 45 deletion was induced using Cas9 together with 2 sgRNAs targeting intron 44 and intron 45 of the human *DMD* gene in a humanized DMD mouse zygote[81] (Figure 3B). Treatment of CRISPR targeting intronic regions flanking *DMD* exons 45–55 in these mice resulted in the rejoining of introns 44 and 55, which in turn led to dystrophin expression at the sarcolemma.[81] However, humanized DMD mice are less than ideal for generating knockout models. They contain 2 copies of the *DMD* transgene, integrated in a tail-to-tail orientation, and both must be modified to result in the desired phenotype.[91] A DMD rat model was also generated by targeting both exon 3 and exon 16 to induce a deletion of the region spanning these 2 exons.[86] DMD rodent models mimic the defective and pathological features of the disease, yet they do not fully represent the phenotype of DMD patients due to the compensatory effect of utrophin and robust muscle regeneration in these models.[92] Thus, therapeutic strategies may benefit from examining more severe mammalian models of DMD that better mimic the pathology of DMD patients.

Compared to mice, rabbits show more similarities to humans in their physiology, anatomy, and genetics, making them suitable models for cardiac and metabolic diseases. With these advantages, rabbits are an appropriate focus for DMD modeling. CRISPR-Cas was used to induce a desired exon 51 knockout in the rabbit *DMD* gene; 78.8% of newborn pups carried a single *DMD* mutation and 84.6% carried biallelic *DMD* mutations.[87] This model sufficiently mimics the histopathological and functional defects of DMD patients, including impaired mobility and defects in muscle regeneration, suggesting it will be a useful model for preclinical studies.[87] DMD pig models have also been successfully generated.[88] A nonsense mutation was generated in the *DMD* gene in 75% of embryos injected with BE3 mRNA and an appropriate sgRNA (Figure 3A). A newer version of BE3 based on human APOBEC3A induced mutations in 50% of the embryos.[88] Ultimately, 1 live heterozygous piglet, which carried the C-to-T mutation in one *DMD* allele and an 18-bp deletion in the other was born after mating $DMD^{-/+}$ female pigs with $DMD^{+/y}$ pigs.[88] It is notable that previously generated $DMD^{-/-}$ or $DMD^{-/y}$ pigs generated using CRISPR/Cpf1 could not survive >3 months, whereas the heterozygous female DMD pig mentioned above survived for >1 year.[88] Nonhuman primates, represented by rhesus monkeys (*Macaca mulatta*), are essential for disease modeling given their strong similarities to humans across physiological, developmental, behavioral, immunologic, and genetic levels.[93] CRISPR has been successfully used to generate *DMD* mutant rhesus monkeys by inducing frameshift mutations in exon 4 and/or exon 46[89] (Figure 3C). After the injection of Cas9 mRNA and sgRNA, 46.47% of embryos carried different indel mutations in *DMD*.[89] After Cas9-injected embryos were transferred to surrogate rhesus monkey mothers, mosaic frameshifting *DMD* mutations were observed in 2 stillborn and 9 live monkeys, which represented a gene-targeting rate of 61.1%.[89] This monkey showed early muscle atrophy pathology, indicating that a monkey model of DMD was successfully generated.

Further extensive evaluations of pathophysiology in the animal models of DMD are required. Thereafter, these models could provide an alternative option as a new platform for investigating the therapeutic effects of biodrugs before clinical trials.

Challenges: Off-target effects of CRISPR-Cas

Off-target effects of the CRISPR system could lead to indels or base editing in non-targeted regions of the genome, causing unexpected genomic instabilities. Cancer or other diseases may be generated with such off-target nuclease activity, carrying serious risks that would outweigh the benefits of genome editing. Hence, the off-target effects of the CRISPR system must be carefully evaluated. A number of different approaches are being investigated to reduce such off-target activity. To date, several bioinformatic tools, including CRISPR-OFFinder,[94] CCTop,[95] and CT-Finder,[96] have been developed to predict potential off-target sites in the whole genome. In particular, CIRCLE-seq,[97] Digenome-seq,[98] and GUIDE-seq,[99] which are *in vitro*-based assays, are useful methods for identifying potential off-target sites. These methods account for off-target cleavage sites that may be affected by genetic variation. In addition, efforts to improve the specificity of Cas9 are under way, including the development of enhanced specificity SpCas9 (eSpCas9[100]), evoCas9,[101] HypaCas9,[102], and high-fidelity SpCas9 (SpCas9-HF1,[103] HeFSpCas9[104]), and the use of truncated guide RNA.[105] In addition, the use of the small anti-CRISPR protein AcrII could regulate Cas expression and thereby reduce indiscriminate cleavage.[106]

Challenges: Delivery efficiencies, toxicity, and immunogenicity

AAV is the most commonly used vector for DMD gene therapy because of its ability to target muscle tissue with high efficiency.[107–109] AAV vector has several benefits, including low pathogenicity, low immunogenicity, and the ability to provide long-term expression as a gene carrier. However, it is crucial to study the safety of this viral vector for the application of the CRISPR approach before clinical treatment. Several studies have demonstrated that systemic delivery of a high dose of AAV vector (2×10^{14} vg/kg) induces systemic and sensory neuronal toxicity in rhesus monkeys and piglets.[110,111] A severe cellular immune response also occurred in the Phase I/II clinical study of recombinant AAV gene therapy for hemophilia.[112]

In addition, CRISPR components can generate an immune response *in vivo* because they are derived from foreign bacteria.[113] If a patient had been previously exposed to Cas proteins from a given species and then re-exposed during CRISPR-based therapy, a sustained immune response could also lead to Cas endonuclease clearance, which would greatly reduce the effectiveness of treatment and could lead to organ impairment. It has been reported that 58% and 78% of healthy people had anti-Cas9 antibodies against SaCas9 and SpCas9, respectively.[114] This result shows the potential for numerous patients to exhibit an immune response following Cas9 treatment, ultimately resulting in reduced gene editing efficiency. It has been reported that such humoral and cellular immune response can be avoided by treating neonatal mice.[115] Thus, there is an urgent need to develop highly efficient AAV-CRISPR systems that are effective at low doses and to establish

a treatment protocol for the appropriate age group of DMD patients. In addition, modification of the AAV capsid to make it even less immunogenic could maximize the therapeutic effect.

Conclusions

The CRISPR-Cas system provides a powerful genome editing tool for highly efficient DMD therapy. Rapid progress in DMD genome editing is occurring, with the evaluation of the safety and efficacy of therapeutic strategies. When the current limitations of the CRISPR system for the treatment of DMD are overcome, CRISPR-based tools will offer the means to permanently correct DMD mutation or ameliorate their effects. Continued development of the CRISPR system as a means of DMD therapy indicates that it should be an alternative gene therapy technology in the near future.

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AUTHOR CONTRIBUTIONS

Both of the authors contributed to the writing of this review.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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