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. Many DMD patients exhibit progressive muscle degeneration associated with severe muscle weakness, loss of ambulation, cardiac or respiratory complications, and eventually death, in their 20s. 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. Different types of mutations in DMD exons and introns cause various forms of dystrophinopathies. 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. 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. Mild BMD symptoms include a relatively slow disease progression, which have little effect on lifespan. 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.

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. 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. 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. 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
(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. 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)

(5’-TTTV-3’) and cause staggered end cleavage with equal or greater efficiency than Cas9 orthologs. Of special interest, Cpf1 can be used 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).

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) (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 and to increase the efficiency by using various Cas orthologs in this system.

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. 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.

![Figure 1. CRISPR-Cas genome editing tools](https://example.com/figure1.png)
| Subject | Strategy | Nuclease | DMD mutation | Therapeutic target gene region(s) | Model(s) | Delivery | Reference |
|---------|----------|----------|--------------|----------------------------------|----------|----------|-----------|
|         | exon reframing |         |              |                                  |          |          |           |
|         | exon deletion |         |              |                                  |          |          |           |
|         | exon skipping |         |              |                                  |          |          |           |
|         | homology-directed repair |         |              |                                  |          |          |           |
|         | base editing (exon skipping) |         |              |                                  |          |          |           |
|         | base editing (correction) |         |              |                                  |          |          |           |
|         | transcriptional modulation |         |              |                                  |          |          |           |

**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 |
|---------|----------|----------|--------------|----------------------------------|----------|----------|-----------|
|         | exon reframing |         |              |                                  |          |          |           |
|         | exon deletion |         |              |                                  |          |          |           |
|         | exon skipping |         |              |                                  |          |          |           |
|         | homology-directed repair |         |              |                                  |          |          |           |
|         | base editing (exon skipping) |         |              |                                  |          |          |           |
|         | base editing (correction) |         |              |                                  |          |          |           |
|         | transcriptional modulation |         |              |                                  |          |          |           |

(Continued on next page)
(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. 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. 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. 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 vector (AAV) for delivery. Of note, 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. In human genome editing, deletion of a mutation hotspot spanning DMD exons 45 to 55 could treat ~60% of DMD patients. 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%. 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. 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. 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. In evaluations of this approach in vivo, skipping of exon 51 was induced, leading to ORF reframing and restoration of dystrophin expression, in mouse and canine models of DMD that lack exon 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, 31,32 exon 53, 33 and DMD exon 44. 34,35 As one example of this approach, the nonsense mutation in exon 53 in mdx 34 mice was repaired by intramuscular injection of AAV6 carrying Cas9, sgRNA, and donor template sequences into TA muscles. 33 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. 33 In addition, the nonsense mutation in Dmd exon 23 in the mdx mouse was corrected by SpCas9 with a 180-nt ssODN 34 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. 34 The ssODNs used in this study included the correct 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; 62 hence, G1-arrested cells (post-mitotic cells) such as mature myofibers and cardiomyocytes are not corrected efficiently by HDR-mediated gene editing. 63 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. 62 Several studies have shown that the upregulation of utrophin could rescue DMD phenotypes. 27,53,60,70,75 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
used to target the *Lama1* promoter, leading to increased laminin-111 expression. In addition, *klotho*, a transmembrane protein that is epigenetically silenced in muscle cells of *mdx* mice, has been targeted for upregulation by CRISPRa. 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. 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 (Figure 2G). The CRISPRi system used here consists of dCas9 fused to a repressive Krüppel associated box (KRAB) domain and an appropriate sgRNA. 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. Several studies have reported that CRISPR-induced knockout of *Mstn* or *MSTN* increased muscle mass and myotube formation. However, it has also been reported that the *MSTN* knockout is only slightly beneficial for inducing excessive muscle growth but causes impaired force generation. 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, rat, rabbit, pig, and monkey (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. 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, 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\(^5\, 6\) (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.\(^7\) 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.\(^8\) 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.\(^9\) 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.\(^11\) 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.\(^12\) 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.\(^13\) DMD pig models have also been successfully generated.\(^14\) 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.\(^15\) 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\(^{+/−}\) pigs.\(^16\) It is notable that previously generated DMD\(^{−/−}\) or DMD\(^{−/−}\) pigs generated using CRISPR/Cpf1 could not survive >3 months, whereas the heterozygous female DMD pig mentioned above survived for >1 year.\(^17\) 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.\(^18\) CRISPR has been successfully used to generate DMD mutant rhesus monkeys by inducing frameshift mutations in exon 4 and/or exon 46 \(^19\) (Figure 3C). After the injection of Cas9 mRNA and sgRNA, 46.47% of embryos carried different indel mutations in DMD.\(^20\) 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%.\(^21\) 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 biologics 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-OffFinder,\(^22\) CCTop,\(^23\) and CT-Finder,\(^24\) have been developed to predict potential off-target sites in the whole genome. In particular, CIRCLE-seq,\(^25\) Digencode-seq,\(^26\) and GUIDE-seq,\(^27\) 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\(^10\)), evoCas9,\(^10\) Hypa-Cas9,\(^10\) and high-fidelity SpCas9 (SpCas9-HF1,\(^10\) HeFSpCas9\(^10\)), and the use of truncated guide RNA.\(^10\) In addition, the use of the small anti-CRISPR protein AcrII could regulate Cas expression and thereby reduce indiscriminate cleavage.\(^10\)

**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.\(^10\, 10\) AAV vectors have 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.\(^10\, 10\) A severe cellular immune response also occurred in the Phase I/II clinical study of recombinant AAV gene therapy for hemophilia.\(^10\)

In addition, CRISPR components can generate an immune response in vivo because they are derived from foreign bacteria.\(^10\) 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.\(^10\) 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.\(^10\) Thus, there is an urgent need to develop highly efficient AAV-CRISPR systems that are effective at low doses 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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