Development of CRISPR-Mediated Systems in the Study of Duchenne Muscular Dystrophy

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Duchenne muscular dystrophy (DMD) is a severe type of X-linked recessive degenerative muscle disease caused by mutations in the dystrophin (DMD) gene on the X chromosome. The DMD gene is complex, consisting of 79 exons, and mutations cause changes in the DMD mRNA so that the reading frame is altered, and the muscle-specific isoform of the dystrophin protein is either absent or truncated with variable residual function. The emerging CRISPR-Cas9-mediated genome editing technique is being developed as a potential therapeutic approach to treat DMD because it can permanently replace the mutated dystrophin gene with the normal gene. Prenatal DNA testing can inform whether the female fetus is a carrier of DMD, and the male fetus has inherited a mutation from his mother (50% chance of both). This article summarizes the present status of current and future treatments for DMD.

Keywords: CRISPR-Cas9, Duchenne muscular dystrophy (DMD), gene therapy, dystrophin

INTRODUCTION

Duchenne muscular dystrophy (DMD) is one of the most common X-linked recessive diseases and causes muscle degeneration with severe consequences. It is caused by mutations in the dystrophin (DMD) gene on the X chromosome, and affects 1 in 3,600–6,000 live male births. Dystrophin is a cell-membrane scaffolding protein that binds to dystrophin-associated protein complex (DAPC), including dystroglycans, sarcoglycans, sarcospan, dystrobrevins, and syntrophin. This complex is thought to play a structural role in ensuring membrane stability and to force transduction during muscle contraction. DMD is typically manifested as progressive muscle weakness associated with muscle wasting. The condition can be observed clinically from when the male child takes his first steps, and his ability to walk is progressively lost by 10–12 years old, with death occurring most commonly in early to mid-20s due to respiratory failure and, less commonly, heart failure. The pathogenesis of DMD is still unclear, and the optimal treatment for this disease has not been reported. Currently, glucocorticoids are available that reduce the risk of progressive scoliosis and stabilize pulmonary function in patients with DMD, but as the disease cannot be cured, long-term use may cause side effects.

Becker muscular dystrophy (BMD) is a related but less common and relatively benign type of X-linked muscular dystrophy, also caused by mutations in the DMD gene, which results in partial loss of functionality or a decreased level of dystrophin protein expression. The mutations include in-frame deletions, insertions, or amino acid substitutions. Compared to DMD, BMD has milder symptoms, slower disease progression, and fewer effects on life-span, and this potentially allows for additional strategies to restore the function of the dystrophin protein. Many therapeutic approaches have focused on shifting the severe DMD phenotype to be milder and more BMD-like by restoring the expression of the dystrophin gene having internal deletions. The three leading therapeutic methods (ataluren, eteplirsen, and gene therapy) are described further below.

ATALUREN

Ataluren (PTC12; Translarna) is a pharmaceutical chemical (oxadiazole) drug developed by PTC
Therapeutics for treatment of DMD to promote read-through of nonsense mutations (premature stop codons) in the dystrophin gene for patients who can walk and are $>2$ years old in Europe. The drug was approved by the European Medicines Agency (EMA) in 2014 for conditional treatment of DMD. The Phase IIa clinical trial begun in 2004 reported an increase in dystrophin levels in some participants. However, the 2017 Phase III trial showed no significant difference in the 6-minute walk test (6MWT) between the ataluren-treated and placebo-controlled groups. Nevertheless, the EMA concluded that "the non-clinical data available were considered sufficient to support the proposed mechanism of action and selectivity." 

ETEPLIRSEN

Eteplirsen (Exondys 51) is a nucleic acid drug (morpholino antisense oligomer) used to treat DMD. It promotes dystrophin production in defective genetic variants by restoring the translational reading frame of the DMD gene via specific skipping of exon 51. The dystrophin protein made is abnormal (truncated) but functional, and increased amounts of functional protein should slow or prevent the progression of DMD. In 2016, the U.S. Food and Drug Administration approved this treatment for approximately 13% of DMD patients. Although studies have demonstrated the safety of eteplirsen, one study did not find it to be clinically effective. A recent report showed that after 3 years of follow-up, patients treated with eteplirsen had a slower rate of decline in walking speed compared to previous data.

OTHER TREATMENTS FOR DMD

Other new clinical approaches for DMD, such as small molecule drugs, receptor agonists, and inhibitors, are still at the preclinical stage and require more development. Progress achieved in recent DMD clinical trials is reviewed in Mah.

DMD GENE EDITING APPROACHES

In recent years, multiple genetic editing systems with engineered nucleases have been developed, including meganuclease, zinc finger nucleases (ZNFs), transcription activator-like effector nucleases (TALENs), and the clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 system. CRISPR-associated enzyme protein 9 (Cas9) uses CRISPR sequences to recognize and cleave DNA or RNA complementary to the CRISPR sequence. All three systems have been used to explore the mechanisms of monogenetic diseases and to develop potential new treatment approaches. The TALEN and ZFN-based genomic engineering approaches, however, have not achieved the goal of quick and accurate genome editing for treatment of DMD.

CURRENT CRISPR-Cas9 TECHNOLOGY

The current CRISPR-Cas9 technology has been modified from that originally found in prokaryotic genomes. It makes human genome editing more accessible and thus may provide the possibility of curing DMD. CRISPR RNA structures (arrays) have an AT-rich leader sequences, followed by short (28–37 base pairs [bp]) repeats, which are separated by unique spacers (21–72 bp). The Cas9 endonuclease, together with another endonuclease Cpf1 (with different DNA cutting activity), are guided by single-guide RNAs (sgRNAs) to bind to a targeted genomic locus next to a protospacer adjacent motif (PAM) and generate double-strand breaks (DSBs). The DSBs can then undergo repair by the non-homologous end joining (NHEJ) or the homology-directed repair (HDR) systems. NHEJ is most common but leads to insertion/deletion mutations. In contrast, HDR can produce precise modification at the target locus in the S phase or G2 phase of the cell cycle. In the past 4 years, the CRISPR-Cas9 system has been rapidly developed into a number of alternative systems: CRISPR-Cpf1, CRISPR-dCas9, and CRISPR-C2c2 (CRISPR/Cas13a). The CRISPR systems have been widely used in the field of biomedical research and now show great potential for gene therapy of DMD, as summarized below.

NHEJ-mediated DMD gene therapy

Figure 1 shows three strategies used to restore the reading frame of variant DMD genes, as described further below. Classical exon skipping. Gene editing-mediated exon skipping is a powerful approach that can be used to skip the targeted exons in the pre-mRNA in order to repair point mutations and deleterious frame shifts. This method could restore muscle structure and function by removing the splicing acceptors of the targeted exons (Fig. 1). It was first described by Ousterout et al. who used ZFN to remove exon 51 in DMD patient myoblasts. This approach can be applied to treat DMD and other diseases caused by gene mutations, including gene deletions or insertions of frame mutations, exon repeats, and pseudogenes. Maggio et al. success-
proteins, one lacking exons 45–52 (DMD.D45–52) and a second lacking exons 48–50 (DMD.D48–50) in myoblasts. They designed “all-in-one” adenoviral vectors (AdVs) for delivering the CRISPR-Cas9 complex to the target genomic sequences located between the splice acceptor (SA) site of exon 51, and of exon 53, and their premature stop codon. Li et al.29 partially expressed dystrophin protein function in a DMD patient who lacked exon 44 by disrupting the SA site of exon 45 with both the TALENs and CRISPR-Cas9 systems. Direct exon deletion. Many DMD patients have gene deletions that disturb the dystrophin gene open reading frame (ORF). A relatively straightforward way exists to restore the reading frame: remove exons around the inherited deletion with sgRNA sequences directed at both ends of the target exon to guide double-target sgRNA-directed cleavage by the Cas9 protein. Exon 51 of the human DMD gene has been a primary target for this approach, as the removal of this exon would address about 13% of DMD patients.30 Recently, a large deletion of exons 45–55 has been tested to capture mutations in a much larger segment of the DMD gene and will apply for >60% of patients with deletion mutations.31–33 In mdx mice, an animal model of DMD with a nonsense mutation in exon 23 of the Dmd gene, the mouse dystrophin protein expression and muscle strength were increased by CRISPR-Cas9 cleavage of exon 23.34–36 The deletion of a single exon is limited for DMD treatment. However, studies have observed satisfactory results by generating multiple exon deletions of the Dmd gene exons 21–23, 52–53, and 45–55 regions in mdx mice. The mdx mice, however, do not mimic the clinical disease of DMD patients. DMD dogs are a better research model. Amoasii et al.37 used the deltaE50-MD dog model treated with adeno-associated virus serotype 9 (AAV9)-mediated SpCas9 and gRNA targeting exon 51. The expression of dystrophin was measured 6 weeks after intramuscular injection or 8 weeks after intravenous (i.v.) injection. Dystrophin restoration was observed (5–70%) after i.v. injection of different skeletal muscles. In the myocardium of the dog receiving the highest dose of treatment, dystrophin levels returned to 92% of normal. The muscle histopathology of deltaE50-MD dogs was also improved after treatment, and no obvious adverse reactions and off-target effects were observed. Good results in gene editing of mdx mice and DMD dogs in vivo have successfully demonstrated that CRISPR-Cas9 targeted exon knockout may provide an effective approach for human DMD gene therapy.

Frameshift induction. The NHEJ approach can be used to induce insertions and deletions. It is...
possible to restore dystrophin expression by resetting an ORF containing a premature stop codon, aimed at targeted exons. This is not efficient, however, and is still under development. Li et al.\textsuperscript{29} applied this strategy to restore dystrophin expression by terminating exon 45 expression in induced pluripotent stem cell (iPSC) lines induced from fibroblasts obtained from a DMD patient with a deletion of exon 44 in the dystrophin gene.

**HDR-mediated precise repair**

As with CRISPR-Cas9 mediated DMD exon skipping, the primary principle of HDR-mediated precise repair is to prevent exon splicing or to delete exons and then restore dystrophin protein expression by imprecise deletion mutations caused by NHEJ. This approach cannot be applied to mutations located within the exon coding for essential domains, in which case a HDR-mediated precise repair is required. Dystrophin gene mutations could be rescued by a knock-in strategy to restore full-length dystrophin protein expression. In this strategy, a donor template should be delivered as well as Cas9 and sgRNA.

The CRISPR-Cas9 technology successfully knocked in exon 44 at the 5' region of exon 45, and resulted in full-length dystrophin protein expression in differentiated myoblasts.\textsuperscript{29} In addition, it has been reported that the nonsense mutation in exon 23 of the Dmd gene was repaired in the mdx mouse model by HDR.\textsuperscript{38} HDR is restricted to the S and G2 phases of the cell cycle, when sister chromatids are available to serve as a repair template.\textsuperscript{39} HDR is not useful in G1-arrested cells, such as mature myofibers and cardiomyocytes, which are less likely to undergo HDR-mediated genome editing.\textsuperscript{40} One approach is to reduce NHEJ activity competing with HDR,\textsuperscript{41} while another is to create frameshift mutations by NHEJ to restore the reading frame. A small fragment was inserted by a nuclease (e.g., Cas9) that targets the locus next to the deletion region. A previous study\textsuperscript{42} showed that after mega-nucleases were used to create inserts, plasmids were then electroporated into myoblasts and restored dystrophin expression \textit{in vivo}, which was verified with TALENs and CRISPR-Cas9.\textsuperscript{29,43,44} The targeted insert created by this approach is random and inefficient, however, and a serious concern is the possibility that unintended fragments might be created that could create new antigens in the engineered dystrophin protein.

**Base editing**

Figure 2 shows two strategies for base editing to restore dystrophin protein expression. For some DMD patients with point mutations in the DMD gene, the traditional CRISPR-Cas9 system is not suitable and induces unnecessary insertions or deletions. The base editing approach can be useful. “Dead” Cas9 (dCas9), a variant of Cas9 nuclease with endonuclease activity removed, still retains the ability to produce a gRNA/Cas9 complex that binds the targeted DNA regions (Fig. 2).

A recent study\textsuperscript{45} linked the C → U editing enzyme APOBEC-1 to the N-terminus of dCas9. This

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**Figure 2.** Mechanisms of base editing mediated gene correction by CRISPR-Cas9. As for a point mutation in dmd/DMD gene, CRISPR-meditated base editing can mutate the invariant GU-AG sequences at splice sites to modulate mRNA splicing or generate a direct precise base repair in a targeted manner. Green letters represent normal base and red letters represent mutated base. Color images are available online.
induced C → T mutations and showed higher efficiencies than HDR. In addition, Gaudelli et al.\(^{46}\) mediated the conversion of A•T to G•C in genomic DNA without double-stranded cleavage by combining adenine base editors (ABEs) with a catalytically impaired CRISPR-Cas9 mutant. These approaches are suitable for treating point mutations in about 10% of DMD patients,\(^{47}\) and they also can induce exon skipping by disrupting premature stop codons or splice acceptor sites, which is termed CRISPR-SKIP.\(^{48}\) Yuan et al. demonstrated the feasibility of CRISPR-Cas9-mediated single base editing for DMD in vitro.\(^ {49}\) They used an activation-induced cytidine deaminase (AID) enzyme fused to dSpCas9 or dSaCas9 to target the splice site of exon 50, thus converting a guanine to adenine with a single base edit, eliminating GT. The splicing consensus sequence induced exon 50 skipping in human iPSCs with the deletion of exon 50, restoring the ORF and dystrophin function of DMD. There are other proteins that have similar functions as dystrophin, and DMD can be alleviated by upregulating the expression of these proteins. The cytoskeletal protein ubiquitous dystrophin (Utrophin), encoded by the gene UTRN (MIM: 128240), is an autosomal homologue of dystrophin that is abundant in many tissues. Upregulation of its transcription could provide an approach for treating DMD. Studies indicate that increasing the levels of UTRN mRNA in excess of twofold more than basal levels is sufficient to have a functional benefit.\(^ {51,52}\) Wojtal et al.\(^{53}\) utilized the CRISPR-dCas9-VP160-mediated therapeutic approach for upregulating utrophin and used sgRNAs to target two UTRN promoters, resulting in a 1.7- to 6.9-fold increase in utrophin protein expression, which restored the dystrophin-associated glycoprotein β-dystroglycan in myoblasts of DMD patients.

Increased expression of the laminin-111 protein complex is another approach for DMD therapy. Perrin et al.\(^{54}\) promoted its expression by targeting the promoter of laminin subunit alpha 1 (LAMA1) with one or several gRNAs and a dCas9 coupled with the VP160 transcription activation domain, and observed increased expression levels of LAMA1 mRNA and protein in mouse muscle. This approach would be suitable for all DMD patients and could avoid the risks associated with genomic cleavage using a functional Cas9 nuclease.

**CRISPR-Cas9 FOR THE GENERATION OF DMD MODELS**

CRISPR-Cas9 is capable not only of correcting gene mutations but also inducing permanent mutations for the purposes of creating new DMD models, which is of great significance for DMD disease mechanism research, testing genetic engineering efficacy and evaluating preclinical treatments for DMD.

**Cell models**

Cell models are commonly used for DMD research due to their many advantages, such as simple operation, the ability to analyze the structural and functional changes related to the disease microscopically, and ease of large-scale culture. Cell transplantation could be a reasonable approach for DMD treatment. For experimental studies in vitro, it is sometimes difficult to find a cell model with the appropriate mutations, and CRISPR-Cas9 can be used to generate desired DMD mutations in rhabdomyosarcoma cells,\(^ {55,56}\) human iPSCs,\(^ {57}\) or mouse cell models.\(^ {58}\)

**Animal models**

CRISPR-Cas9 can generate animal models of DMD by inducing permanent mutations in the DMD gene. The widely used mouse Dmd model was generated by this technique: Dmd Δ50 mice were created to mimic human DMD exon 51 deletions.\(^ {59}\) Dmd Δ45 humanized DMD (hDMD) mice\(^ {60}\) and Dmd-mutated rats with mutations in exons 3 and 16 were also induced with CRISPR-Cas9.\(^ {61}\) Due to the compensatory effect of utrophin, mouse models are less susceptible to the disease and do not fully reflect the muscle lesions of DMD.\(^ {62}\) In order to simulate the clinical manifestations and disease progression better seen in human DMD patients, large animals appear to be more suitable. DMD-modified pigs were successfully generated by co-injecting zygotes with Cas9 mRNA and sgRNA targeting DMD exon 27,\(^ {63}\) and showed the same pathology and clinical manifestations as DMD patients. In addition, the researcher\(^ {64}\) successfully inserted the Cas9 protein gene into a specific site of the pig genome (ROSA26) and added a locus of X-over P1 (LoxP) site capable of binding to Cre re-
combinase near the Cas9 gene to control the cutting function. By switching to gRNAs and recombinases, the pig’s genome can be directly edited to generate DMD animal models. However, there is still a difference between pig and human genes, and an animal genetically closer to humans should be selected. In 2015, Chen et al. used CRISPR-Cas9 to target exons 4 and 46 of the monkey DMD gene to create mutations and which caused DMD in the monkeys, thus creating an important primate model for the study of DMD pathogenesis and the development of novel clinical treatment approaches.

SAFETY CHALLENGES FOR CRISPR-Cas9 IN DMD GENE THERAPY

The CRISPR-Cas9 system is beneficial for DMD research because it offers an entirely new therapeutic approach. As CRISPR-based therapies advance toward human clinical trials, it is important to understand natural human genetic variation and the potential problems that could be introduced with this new “gene editing” technique for comprehensive safety evaluation.

AAV toxicity

AAV, which provides specific serotypes to target muscle tissue, serves as the most commonly used vector for DMD gene therapy. The toxicity caused by AAV is an important factor that restricts the application of the CRISPR-Cas9 approach in clinical treatment. Choosing the right effective dose of AAV is a problem that must be addressed before conducting clinical trials. For a long time, researchers agreed that AAV gene drugs had the advantages of low pathogenicity, low immunogenicity, and long-term expression of foreign genes in a wide range of hosts, and were the most promising gene therapy vectors. The Phase I/II clinical study of recombinant AAV gene therapy for hemophilia conducted by the University of Pennsylvania revealed a serious cellular immune response. The patients’ transaminase level rose sharply, liver function was seriously impaired, and clinical studies were forced to terminate. Furthermore, a 2018 study found severe liver and neurotoxicity at a high dose of $2 \times 10^{14}$ vg/kg in rhesus monkeys and piglets using the vector AAV9. Studies have shown that systemic and sensory neuronal toxicity may be a common property of high-dose i.v. delivery of AAV vectors, independent of capsid serotypes or gene transfer. Elucidating the causes and mechanisms of AAV toxicity is thus of great significance for future clinical research for treatment of DMD. The use of alternative methods, including nanoparticles and other nonviral delivery systems, are currently being studied.

CRISPR components immunogenicity

CRISPR-Cas9 is a gene editing system derived from microorganisms, and the most widely used Cas9 proteins are SaCas9 from Staphylococcus aureus and SpCas9 from Streptococcus pyogenes. In the CRISPR-Cas9-mediated gene therapy process, when AAV or other vectors carry Cas9 protein into humans, these vectors mediate the sustained expression of Cas9 protein. If the patient has preexisting specific memory T cells against Cas9 antigen, then their memory T cells are rapidly activated by cytotoxic lymphocyte to kill cells directly with Cas9 protein expression in the cell, resulting in failure of gene therapy, and this approach may trigger a serious immune response, leading to organ dysfunction. In 2018, the Porteus Lab at Stanford University found that 70% of healthy humans had Cas9 protein homologs and approximately 50% had preexisting antigen-specific T cells specific for these homologs. These results show that humoral and cellular immunity against Cas9 protein is present in most humans, which would limit the use of gene therapy using CRISPR-Cas9 for most DMD patients. Studies have been conducted to control the immune response by engineering Cas9 nucleases such as CasX to reduce their immunogenicity in vivo or by using Cas9 reactive regulatory T cells. However, more work needs to be done to determine if these methods are feasible and safe.

Wagner et al. found that while cellular immunity against SaCas9 and SpCas9 was detected in adults, no cellular immunity was found in fetal cord blood. This suggests a new gene therapy approach for treating the fetus affected by DMD in utero that could potentially prevent the fetus from generating an immune response to the viral vector and to the foreign transgenic product. The team successfully performed intrauterine gene editing therapy on mouse embryos using an AAV-mediated single base editor without eliciting an immune response. Due to the presence of novel mutations in about 30% of patients with DMD, when prenatal diagnosis fails to detect these, a live birth cannot be avoided. Genetic editing of the fetus in the uterus during pregnancy could significantly improve the efficiency of DMD gene repair and possibly at a higher AAV concentration and with reduced AAV toxicity.

Off-target genome editing

This is the foremost challenge to be overcome, as it could result in DNA insertions and deletions in
non-target genes, leading to unexpected genomic instabilities, such as off-target mutations and chromosomal translocations. These off-target effects could create larger risks than the editing technique aims to correct, such as cancer and other diseases. Several approaches have been developed to reduce Cas9-mediated off-target editing: off-target monitoring and improved target specificity. Off-target gene editing effects are efficiently detected using assays based on whole-genome DNA sequencing (WGS), for example using next-generation and nanopore sequencing technologies, together with the analysis tool Basic Local Alignment Search Tool (BLAST; blast.ncbi.nlm.nih.gov/Blast.cgi). To improve target specificity or to minimize off-target editing, several new strategies have been developed, such as truncated guide RNA, titration of dosage for Cas9-sgRNA, high-fidelity SpCas9(SpCas9-HF1), and “enhanced specificity” SpCas9 (eSpCas9). Above all, the off-target toxicity and the immunity of CRISPR components are related to the genetic makeup of each individual and persistent nuclease expression in their genome after gene editing treatment. It may be much safer for each DMD patient for ex vivo cell gene editing to be performed or for individualized gene therapy to be developed. For in vivo gene editing, it may be necessary to enhance tissue targeting specificity while restricting nuclease expression to a certain time window. Methods to do this include inducible expression, controlled dosage, induced degradation, or imposing spatiotemporal restrictions within the cell that can restrict the exposure window to CRISPR components, such as self-restricted CRISPR system or the CRISPR inhibitors AcrIIC1 or AcrIIC3. Toxic effects associated with the gene editing components need to be addressed in appropriate preclinical studies prior to human clinical trials.

OTHER USES OF THE CRISPR STRATEGY TO TREAT DMD

Myostatin downregulation

A catalytically dCas9 can be repurposed as a platform for RNA-guided transcription regulation when co-expressed with a guide RNA. This directly blocks transcription in a manner that can be easily programmed by designing sgRNAs. It can also modulate transcription by targeting key cis-acting motifs and blocking the association of their cognate trans-acting transcription factors. Myostatin (Myostatin; MSTN) is a cytokine released specifically by skeletal muscles cells that causes muscle atrophy by activating the transcriptional program of SMAD2/SMAD3 signaling. AAV8-mediated delivery of SaCas9 driven by a tissue-specific double muscle creatine kinase (dMCK) promoter can be used to disrupt MSTN and prevent muscle wasting in mouse muscle in vivo, which might alleviate muscle-wasting syndrome caused by DMD. In addition, the fusion of dCas9 with KRAB (a transcriptional repressor domain) can effectively suppress the transcription of endogenous genes through the recruitment of heterochromatin, which can silence about 80% of genes. This novel approach could potentially be used for DMD treatment.

Screening of DMD-related functional genes

The pathogenesis of DMD is not clear. The CRISPR-Cas9 system can be used to screen for related molecular pathway genes by targeting genes for gene knockdown or disease suppression after transcriptional repression. Thousands of genes are simultaneously targeted to screen for candidate genes by co-infecting a disease model with mixtures of thousands of gRNA-containing viruses and viruses that express Cas9 or dCas9. This method has been used for genome-wide recessive gene and high-throughput genetic screening in mammalian cells. For example, Liu et al. successfully identified 326 functional sites of long non-coding RNA in human iPSCs using lentivirus-mediated CRISPRi. Another study used CRISPR-Cas9 technology to construct acute myeloid leukemia (AML) gene mutations successfully in mouse cells, identified 46 potential candidate genes, and found that METTL3 is one of the most strongly affected genes for AML cell survival but is not required for healthy blood cells. The entire set of proteins expressed that are required by AML can be blocked by inhibiting METTL3 activity, which identified METTL3 as a potential drug target. This approach is expected to reveal new functional genes and specific signaling pathways, guiding the establishment of new drug screening and treatment programs.

Early studies have found that dystrophin-associated glycoprotein complex structures interact with ligands in the Notch signaling pathway in Drosophila, suggesting that dystrophin protein abnormalities may lead to dysregulation of Notch signaling pathways in muscle tissue. In 2014, Jiang et al. demonstrated in vivo that in the DMD mouse model (mdx mice), the activity of the Notch signaling pathway is decreased in mdx mouse muscle satellite cells, and the proliferation of muscle satellite cells is corrected after activation by transgenic approaches. A study at the end of 2015
found that two Golden Retriever dogs with DMD had mutations in the DMD gene but did not exhibit a disease phenotype, and their JAG1 gene expression was higher than normal dogs. Screening for Notch pathway-associated genes in individuals affected by DMD and regulating these genes could potentially improve the activity of their muscle satellite cells and provide a new treatment for DMD.

**DMD genome image**

A deficiency of muscle satellite cells plays a key role in muscular dysfunction in DMD patients. Studies have confirmed that dystrophin is highly expressed in muscle satellite cells and regulates the polar and asymmetric division of these cells, suggesting that DMD muscle regeneration and repair disorders may be related to the failure to regulate this process properly after dystrophin protein deficiency. The mechanisms for this deficiency are still being researched. Wang et al. created a temporal recording in arrays by CRISPR expansion system and converted CRISPR-Cas into a microdisplay. Researchers combine a modified trigger plasmid that responds to external signals with a recording plasmid that can express CRISPR-Cas system components. The time and signal sequence of cellular processes are recorded by the different CRISPR sites with or without external signals. The study showed that the system can handle at least three synchronization signals and store data for a few days. CRISPR-Cas9 can also be used to monitor the status of the genome visually during the cell cycle. Researchers targeted the genomic DNA in combination with dCas9-GFP and modified gRNA that dynamically monitors the status of the genome during the cell cycle. The method can be used to monitor the genomic activity of stem cells and has great significance for studying the pathological changes and pathogenesis of DMD, especially for monitoring satellite cells during disease progression. In addition, the method can also be used to assess the efficacy of drugs by monitoring changes in cell-cycle DMD-related pathway genes.

**RNA editing**

Due to the presence of off-target effects, direct DNA modification in the treatment of genetic diseases is influenced by the duration of expression and can cause serious damage. Genome editing techniques are much safer for gene therapy when used at the RNA level to modifying disease-associated sequences to block production of disease-causing proteins. The newly discovered CRISPR-Cas13a can target knockdown of endogenous transcripts to interfere with gene expression and improve specificity. RNA sequencing indicated that in human cell lines, Cas13a only targets the specified RNA under the guide RNA and keeps other RNA sequences intact. In addition, CRISPR-Cas13a and Cas13b, another member of Cas13 family, can target and edit the required specific RNA fragments for therapeutic purposes. Moreover, dCas13, another variant, lacking the ability to cleave RNA, can track the trajectory of targeted RNA by binding a bright fluorescent dye to the RNA, a technology that may play an important role in the study of DMD pathogenesis.

**CONCLUSIONS**

Though facing many challenges, currently the CRISPR-mediated genomic editing is the most advanced technology able to rectify DMD mutations permanently. Prior to the implementation of CRISPR-mediated DMD clinical gene therapy, the safety and efficacy of this strategy in animal models, both in vivo and in vitro, should be fully evaluated under ethical constraints. Progress in this field is advancing at a great pace and is expected to provide more individualized and effective gene editing procedures, safer and more efficient transfer methods, and cheaper therapy options for DMD patients.

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**AUTHOR DISCLOSURE**

The authors declare that there are no competing financial interests associated with the data presented in this manuscript.
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