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MYOEDITING: TOWARD PREVENTION OF MUSCULAR DYSTROPHY BY THERAPEUTIC GENOME EDITING

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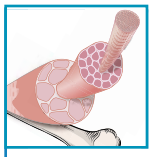

Zhang Y, Long C, Bassel-Duby R, Olson EN. Myoediting: Toward Prevention of Muscular Dystrophy by Therapeutic Genome Editing. *Physiol Rev* 98: 1205–1240, 2018. Published May 2, 2018; doi:10.1152/physrev.00046.2017.—Muscular dystrophies represent a large group of genetic disorders that significantly impair quality of life and often progress to premature death. There is no effective treatment for these debilitating diseases. Most therapies, developed to date, focus on alleviating the symptoms or targeting the secondary effects, while the underlying gene mutation is still present in the human genome. The discovery and application of programmable nucleases for site-specific DNA double-stranded breaks provides a powerful tool for precise genome engineering. In particular, the CRISPR/Cas system has revolutionized the genome editing field and is providing a new path for disease treatment by targeting the disease-causing genetic mutations. In this review, we provide a historical overview of genome-editing technologies, summarize the most recent advances, and discuss potential strategies and challenges for permanently correcting genetic mutations that cause muscular dystrophies.

I. INTRODUCTION

A. Skeletal Muscle Structure

From intense body movement in Greco-Roman wrestling to delicate vocal control in coloratura soprano, skeletal muscle supports a remarkably wide range of human activities. As one of the largest tissues, skeletal muscle accounts for ~40% of human body weight and is essential for physical support, locomotion, energy expenditure, and metabolism.

Skeletal muscle is a highly organized tissue and is composed of thousands of multinucleated myofibers, which are formed by fusion of mononucleated myoblasts during development and regeneration. Bundles of myofibers form a muscle fascicle, and groups of fascicles contribute to the structure of a skeletal muscle **(FIGURE 1*A*)**. The functional unit of a myofiber is the sarcomere, which comprises actin thin filaments and myosin thick filaments. The sliding of the thin and thick filaments past each other generates a muscle contraction.

B. Skeletal Muscle Regeneration and Satellite Cells

The adult musculature has a remarkable regenerative capacity, primarily due to the contribution of the skeletal muscle resident stem cells, known as satellite cells (48, 63, 454). Satellite cells reside between the sarcolemma and basal lamina of myofibers and are marked by expression of a paired-box transcription factor, Pax7 (350) **(FIGURE 1*B*)**. Upon muscle injury, quiescent satellite cells become activated and undergo proliferation and differentiation, and finally form multinucleated myofibers by fusion. Activated satellite cells can also undergo asymmetric division, in which one daughter cell maintains a satellite stem cell fate and the other one acquires a myogenic commitment, becoming a satellite cell committed myogenic progenitor (76, 202, 358). Pax7 is the canonical biomarker for quiescent and activated satellite cells and is downregulated during myogenic differentiation. Genetic ablation experiments demonstrated that $Pax7^+$ satellite cells are indispensable for adult skeletal muscle regeneration (215, 258, 283, 340).

Skeletal muscle regeneration is evolutionarily conserved among many bilaterians, requiring involvement of satellite cells or satellite-like cells (20). However, in certain bilateral species such as zebrafish and adult newt, myofiber dedifferentiation is also a unique mechanism for skeletal muscle regeneration. For example, extraocular muscle regeneration in adult zebrafish involves dedifferentiation of residual

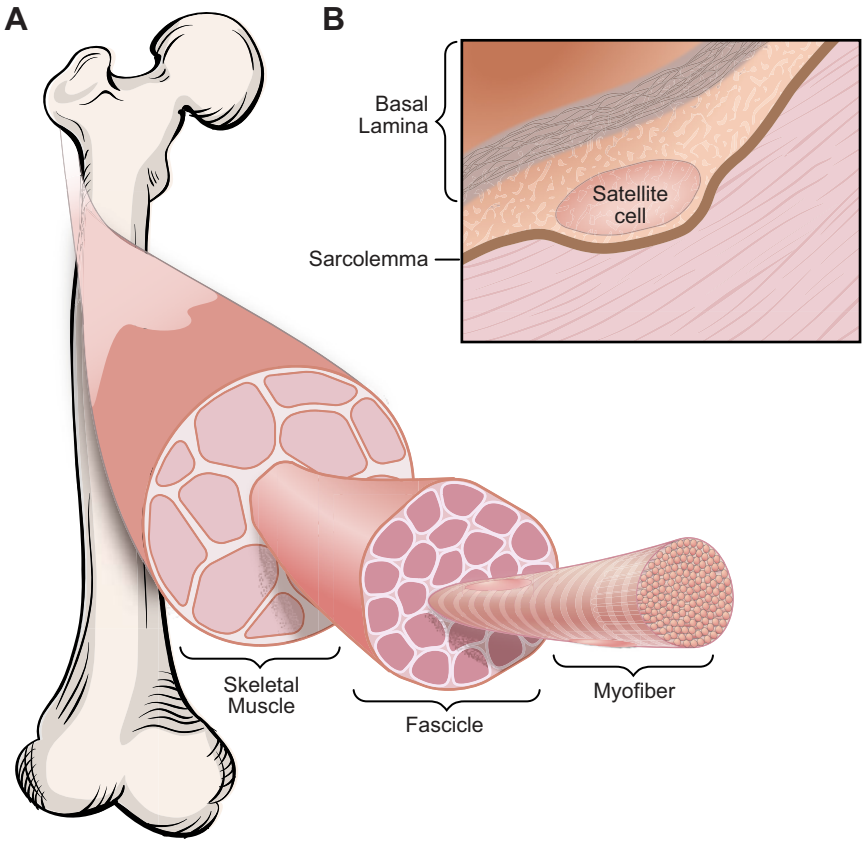

FIGURE 1. Skeletal muscle structure. *A*: skeletal muscle is composed of thousands of multinucleated myofibers. Bundles of myofibers form muscle fascicles, and groups of fascicles contribute to skeletal muscle structure. *B*: satellite cells are adult skeletal muscle stem cells, which reside between the sarcolemma and basal lamina of myofibers.

myocytes, which do not express Pax7 but express Mef2c (334). Similarly, limb muscle regeneration in the adult newt requires dedifferentiation of myocytes to Pax7-negative mononuclear cells (344).

C. Muscular Dystrophies

Despite the remarkable regenerative capacity of skeletal muscle, muscles are vulnerable to numerous disorders, including congenital myopathies, muscular dystrophies, and inflammatory myopathies. Muscular dystrophies are a large group of genetic disorders characterized by progressive weakness of multiple muscle groups. Owing to the advancement of genome research, the genetic causes of many muscular dystrophies have been identified, with many affecting sarcolemma-associated proteins, extracellular matrix proteins, glycosyltransferase enzymes, as well as nuclear proteins (174, 264). Depending on the mutation type and disease onset, muscular dystrophies can significantly impair the quality of life and cause premature death. There is no cure for these debilitating diseases. Initial efforts in gene therapy relied on gene replacement, but the source of the mutation remains present in the genome. Advancements in genome engineering technologies enable precise manipulation of the genetic mutations that cause muscular dystrophies and offer the prospect of a genetic therapy for the permanent correction of diverse genetic defects.

In this review, we provide a historical overview and consider the most recent advances in genome editing technologies. We also detail strategies of genome editing for the correction of genetic mutations that cause muscular dystrophies. Finally, we highlight current cell- and animal-based studies of muscular dystrophy and discuss current challenges and future perspectives of translating genome editing technologies to clinical applications.

II. GENOME AND EPIGENOME EDITING

A. History of Genome Editing: Meganuclease, ZFN, TALEN, and the CRISPR/Cas System

Three decades ago the laboratories of Mario Capecchi and Oliver Smithies independently developed methods for homologous recombination (HR)-mediated mammalian gene targeting technology by providing mammalian cells with exogenous plasmid DNA containing sequence homology to the endogenous genome (93, 250, 367, 393, 394). This HR-mediated technology allows precise gene knockout or correction of genetic mutations. HR-mediated embryonic stem (ES) cell gene targeting, together with mouse chimeras and germline transmission technologies developed by the laboratory of Martin Evans (49), paved the way for the

generation of "knockin" and "knockout" animal models, which significantly expanded our knowledge of gene function and advanced many fields of biological research. However, because DNA double-strand breaks (DSBs) occur randomly in the genome, the frequency of HR-mediated gene targeting is low (between 10^{-6} and 10^{-4}, depending on the length of sequence homology of the targeting vector) (86). Moreover, screening of correctly targeted clones requires positive-negative selection and/or Southern blot analysis, which is time consuming and labor intensive (57). Therefore, routine application of the conventional HR-mediated gene targeting technology for studying gene function was not feasible at that time.

In the early 1990s, it was discovered that HR-mediated gene targeting efficiency could be enhanced by more than 100-fold, when the DNA DSBs were initiated at the target region by providing mammalian cells with a rare-cutting meganuclease discovered in yeast (331). This discovery stimulated the development of programmable nucleases for creating site-specific DNA DSBs. Within the past two decades, four major classes of nucleases have been engineered, which are *1*) meganucleases (366), *2*) zinc-finger nucleases (ZFNs) (265, 406), *3*) transcription activator-like effector nucleases (TALENs) (38, 74, 266, 275), and *4*) CRISPR/Cas endonucleases (clustered regularly interspaced short palindromic repeats and CRISPR-associated proteins) (77, 168, 248, 466).

Permanent correction of genetic mutations that contribute to monogenic neuromuscular disorders offers the ultimate treatment for these diseases. Early attempts at genome editing for treatment of muscular dystrophies were challenged by low efficiency, cytotoxicity, and delivery issues (122, 217, 240, 303, 304, 316, 402, 443). The newly discovered CRISPR/Cas system has been effectively used in genome engineering and represents a new approach to therapeutic genome editing.

1. Meganucleases

Meganucleases are engineered homing endonucleases, which were initially discovered in archaea, bacteria, and unicellular eukaryotic genomes (375, 376). Unlike conventional type II restriction endonucleases that recognize short 4–8 base pairs (bp) of palindromic DNA sequences (315), meganucleases require extended DNA recognition sequences (typically 16–18 bp) to generate site-specific DNA DSBs (62). Meganucleases have been used to enhance HR-mediated gene targeting efficiency by introducing site-specific DNA DSBs in cultured mammalian cells and plants (72, 73, 94, 321, 331), but they have not been widely adopted for genome engineering because the DNA-recognition domain and the nuclease domain overlap (361, 366). This overlap may adversely affect the catalytic activity of the nuclease domain (14), making it very challenging to engineer the DNA recognition domain for specificity in new-sequence binding.

To address this issue, researchers began to focus on the type IIS restriction enzyme *Fok*I, which has two separate domains for DNA recognition and cleavage (188, 218–220). They engineered novel chimeric *Fok*I endonucleases with new DNA sequence specificities by swapping DNA-binding domains from other transcription factors, such as the *Drosophila* Ubx homeodomain (187), yeast Gal4 domain (189), zinc-finger protein (186), and TAL effector (74). The latter two chimeric *Fok*I endonucleases paved the way for the development of ZFNs and TALENs, respectively.

2. ZFNs

ZFNs are chimeric endonucleases containing multiple Cys_2-His_2 zinc-finger domains at the amino terminus (NH_2 terminus) for DNA-binding and a *Fok*I nuclease domain at the carboxyl terminus (COOH terminus) for DNA cleavage **(FIGURE 2*A*)** (186). Each individual zinc-finger domain contains ~30 amino acids folded in a $\beta\beta\alpha$ arrangement and contacts 3 bp of DNA sequence (310). Each ZFN monomer consists of 3–6 individual zinc-finger domains, and thus can bind to 9–18 bp of DNA sequence. Two approaches have been applied to improve genome targeting specificity and expand the targeting range of ZFNs. The first one is to engineer the wild-type (WT) *Fok*I nuclease to reduce the formation of cleavage-competent homodimers (265, 382). The engineered ZFNs require heterodimerization to form a functional nuclease, in which two monomers are separated by 5–7 bp of spacer (35). The second approach is to engineer zinc-finger domains for unique triplet DNA binding specificity by combinatorial library selection and/or oligomerized pool engineering (OPEN) (32, 95, 96, 128, 183, 237, 351).

These two approaches paved the way for modular assembly of customized ZFNs, which have been used for genome targeting in cultured cells, animals, and plants (116, 314, 352, 407). However, because of the context-dependent effects between adjacent zinc-finger domains, large-scale assembly of functional ZFNs remains challenging, and cytotoxicity caused by off-target effects is also a critical issue (115, 309, 343). Moreover, the genome targeting density of ZFNs is also limited because the engineered zinc-finger domains cannot target all 64 possible triplet DNA sequences, especially 5′-TNN-3′ sequences (N represents any nucleotide) (33). These obstacles prevent wide application of ZFNs for genome engineering.

3. TALENs

TALENs are chimeric endonucleases that contain multiple DNA-binding domains, known as transcription activator-like effectors (TALEs), at the NH_2 terminus, and a *Fok*I

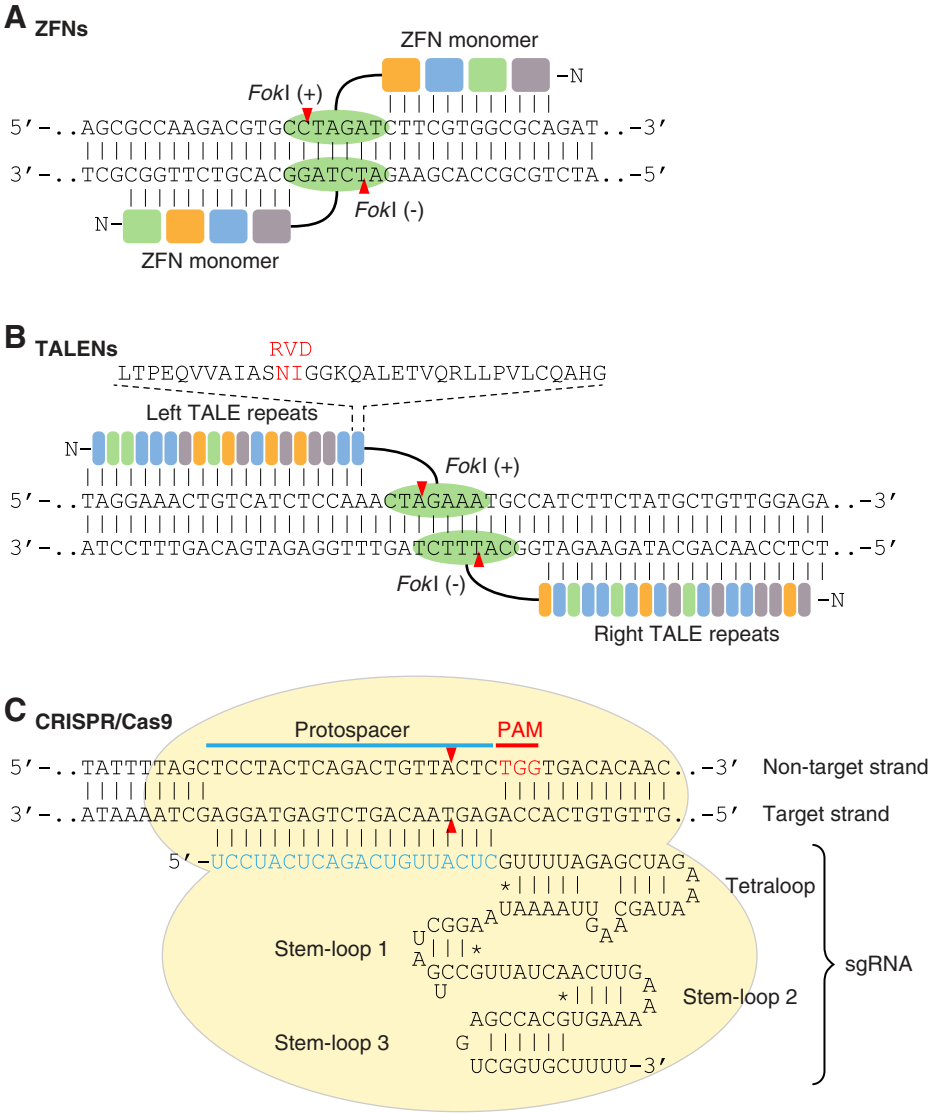

FIGURE 2. Programmable nucleases used for genome editing. *A*: a schematic illustration of a pair of zinc-finger nuclease (ZFN) monomers bound to DNA. ZFN is a chimeric endonuclease composed of multiple zinc finger domains (colored boxes) at the NH_2 terminus for DNA binding and a *Fok*1 nuclease domain (green oval) at the COOH terminus for DNA cleavage. Dimerization of two *Fok*1 nucleases induces a DNA double-strand break (DSB) with 4 bp of 5′ overhang. *B*: a schematic illustration of a pair of transcription activator-like effector nucleases (TALENs) bound to DNA. TALEN is a chimeric endonuclease composed of multiple TALE repeats (colored rectangles) at the NH_2 terminus and a *Fok*1 nuclease domain (green oval) at the COOH terminus. Each TALE repeat recognizes 1 bp of DNA, and the sequence specificity is determined by repeat-variable diresidues (RVD; shown in red). TALEN-mediated DNA DSBs are induced by dimerization of two *Fok*1 nucleases. *C*: a schematic illustration of the engineered CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats-CRISPR associated protein 9) system from *Streptococcus pyogenes*. In the CRISPR/Cas9 system, target recognition is mediated by DNA hybridization with a single guide RNA (sgRNA), which is an engineered RNA chimera composed of CRISPR RNA (crRNA) and *trans*-activating crRNA (tracrRNA). CRISPR/Cas9-mediated DNA DSB requires a protospacer adjacent motif (PAM; shown in red), and cleavage is induced at the nucleotide 3 bp proximal to the PAM. Red arrowheads indicate cleavage site.

nuclease domain at the COOH terminus for DNA cleavage (**FIGURE 2*B***) (74). Unlike the zinc-finger domain in ZFNs, which binds to a triplet DNA sequence, the TALE domain, consisting of 33–35 amino acids in tandem arrays, recognizes a single base pair (87, 242). The sequence specificity of each TALE repeat is determined by the 12th and 13th amino acids at the TALE domain, known as repeat variable diresidues (RVDs) (38, 275). Similar to ZFNs, functional TALENs require dimerization of the *Fok*I nuclease domain with each TALE arm targeting 15–20 bp of DNA sequence separated by 12–21 bp of spacer. TALENs have been widely used to target genomes of various species including cultured cells, animals, and plants (116, 170, 352, 380). Although many cloning methods have been developed for the construction of functional TALENs, such as type II restriction enzyme-based Golden Gate assembly (58), solid-phase assembly (50, 327), and ligation-independent cloning (347), modular assembly of customized TALENs is still challenging and time consuming because each TALEN arm consists of up to 20 highly repetitive TALE arrays.

Despite the difficulty of assembling TALE arrays, TALENs still offer many advantages over other programmable nucleases. First, TALENs have the highest genome-targeting density compared with ZFNs and CRISPR/Cas because each TALE array recognizes DNA sequence at single nucleotide resolution (38, 275). Second, TALENs have minimal off-target effects because a functional TALEN requires dimerization of two TALEN pairs, which can bind 30–40 bp of DNA sequence (87, 182, 242). Therefore, TALENs offer benefits for genome engineering.

4. CRISPR/Cas system

The discovery of CRISPR can be dated back to 1987, when a Japanese research group identified a series of directed repeats interspaced with short spacer sequences in the genome of *Escherichia coli*, although the function of these repeats was unknown at that time (161). It was not until the mid 2000s that researchers discovered that these directed repeats are widely present in over 40% of sequenced bacte-

ria and 90% of archaea genomes (270) and found that the short spacer sequences between the directed repeats are of plasmid and viral origin (41, 269, 319). After realizing that the CRISPR locus is actively transcribed and the protein product has potential nuclease and helicase activities, scientists proposed that the CRISPR/Cas system functions as an adaptive immune system in bacteria and archaea to defend against viral infection (23, 41, 52, 139, 163, 244, 253, 319). The CRISPR/Cas system can be grouped into two classes and six subtypes: the class 1 system encodes multiple effector proteins forming a Cascade complex (CRISPR-associated complex for antiviral defense) with their corresponding signature proteins, such as Cas3, Cas10, and Csf1 from type I, III, and IV CRISPR systems, respectively (243, 245, 246, 359). The class 2 system encodes a single Cas protein with multiple functions, including Cas9, Cpf1, and Cas13a/C2c2 from type II, V, and VI CRISPR systems (5, 102, 359, 466).

The mechanism of CRISPR immunity in bacteria and archaea varies between different CRISPR types, but generally can be divided into three stages, which are protospacer acquisition, precursor CRISPR RNA (pre-crRNA) processing, and crRNA-guided cleavage of exogenous nucleic acids (252, 440). Most CRISPR immunity requires a protospacer adjacent motif (PAM) located next to the crRNA target region in the exogenous invading genome (151, 440).

Owing to the simplicity of the class 2 CRISPR system in which only one RNA-guided endonuclease is required for nucleic acid cleavage, scientists engineered Cas9 endonuclease in conjunction with a hybrid crRNA-tracrRNA duplex, known as single guide RNA (sgRNA), for efficient site-specific genome cleavage in eukaryotic cells **(FIGURE 2C)** (77, 168, 248). Currently, the most widely used Cas9 endonuclease is from *Streptococcus pyogenes* with 5′-NGG-3′ or 5′-NAG-3′ PAM preference. Other Cas9 orthologs are also available for genome targeting, including Cas9 endonucleases from *Staphylococcus aureus* (322), *Neisseria meningitides* (150), and *Streptococcus thermophilus* (238, 279), although these Cas9 orthologs recognize longer and more complicated PAM sequences. Besides the type II CRISPR/Cas9 system, the most recently discovered type V and VI CRISPR effectors including Cpf1 (466) and Cas13a/C2c2 (5, 102) further expand the range of genome editing and nucleic acid detection. We will further discuss these two CRISPR effectors in section II*D*.

B. CRISPR/Cas-Mediated Genome Editing: C-NHEJ, HDR, and MMEJ

On average, each human cell undergoes ~50 spontaneous DNA DSBs during each cell cycle (416). DNA DSBs occur randomly, so the efficiency of HR-mediated gene targeting in the absence of programmable nucleases is extremely low (86). The RNA-guided CRISPR/Cas system significantly enhances and simplifies genome editing, in which the Cas9-sgRNA ribonucleoprotein complex binds to DNA by base-pairing with sgRNA, generating a site-specific DNA DSB adjacent to the PAM sequence. Depending on the cell cycle stage and repair machinery, the DNA DSBs can be repaired by error-prone nonhomologous end joining (NHEJ) or by accurate homology-directed repair (HDR). Additionally, there is a third DNA DSB repair pathway known as microhomology-mediated end joining (MMEJ), which is a subtype of alternative NHEJ (alt-NHEJ).

1. Classical NHEJ

Classical NHEJ (C-NHEJ) DNA repair machinery is triggered when a CRISPR/Cas-induced DNA DSB occurs in the absence of a repair template **(FIGURE 3A)**. Although C-NHEJ is active in all stages of the cell cycle, it occurs preferentially during the G_1 phase when the DNA-end resection activity is low (160). The end of a DNA DSB is recognized by Ku70/Ku80 heterodimers, which recruit and activate the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs). Depending on the nature of the break, the ends of DNA DSBs can be directly ligated by the DNA ligase IV-XRCC4 complex, or requires additional processing steps, such as end resection by Artmis, WRN, or APLF nucleases and nucleotide synthesis by DNA polymerases μ and λ (75, 83).

DNA DSBs repaired by C-NHEJ usually generate insertions or deletions (INDELs). Depending on the location of the site-specific cleavage, C-NHEJ has been used for different purposes of genome editing. The most widely used application of C-NHEJ is gene disruption, because INDELs often cause a frameshift of an exon and subsequently disrupt gene function, resulting in a gene knockout. However, C-NHEJ can also cause exon skipping if the INDELs disrupt the splice acceptor site (217), or an exonic splice enhancer/silencer sequence (277), although the outcome of the latter scenario is less predictable. Depending on the reading frame of the skipped exon and adjacent exons, exon skipping can cause gene knockout when the newly spliced adjacent exons are out of frame. Conversely, exon skipping can also produce a truncated protein if the newly spliced adjacent exons are perfectly in frame with each other.

C-NHEJ was generally considered as an error-prone DSB repair pathway. However, some recent studies also demonstrated the precision of C-NHEJ (18, 251) and used this repair pathway for homology-independent targeted integration (HITI) of DNA fragments into postmitotic cells and animals, further expanding the application of CRISPR/Cas-mediated C-NHEJ in genome editing (381).

2. HDR

DNA DSBs can also be repaired by HDR during S and G_2 phases of the cell cycle, when sister chromatids can be used

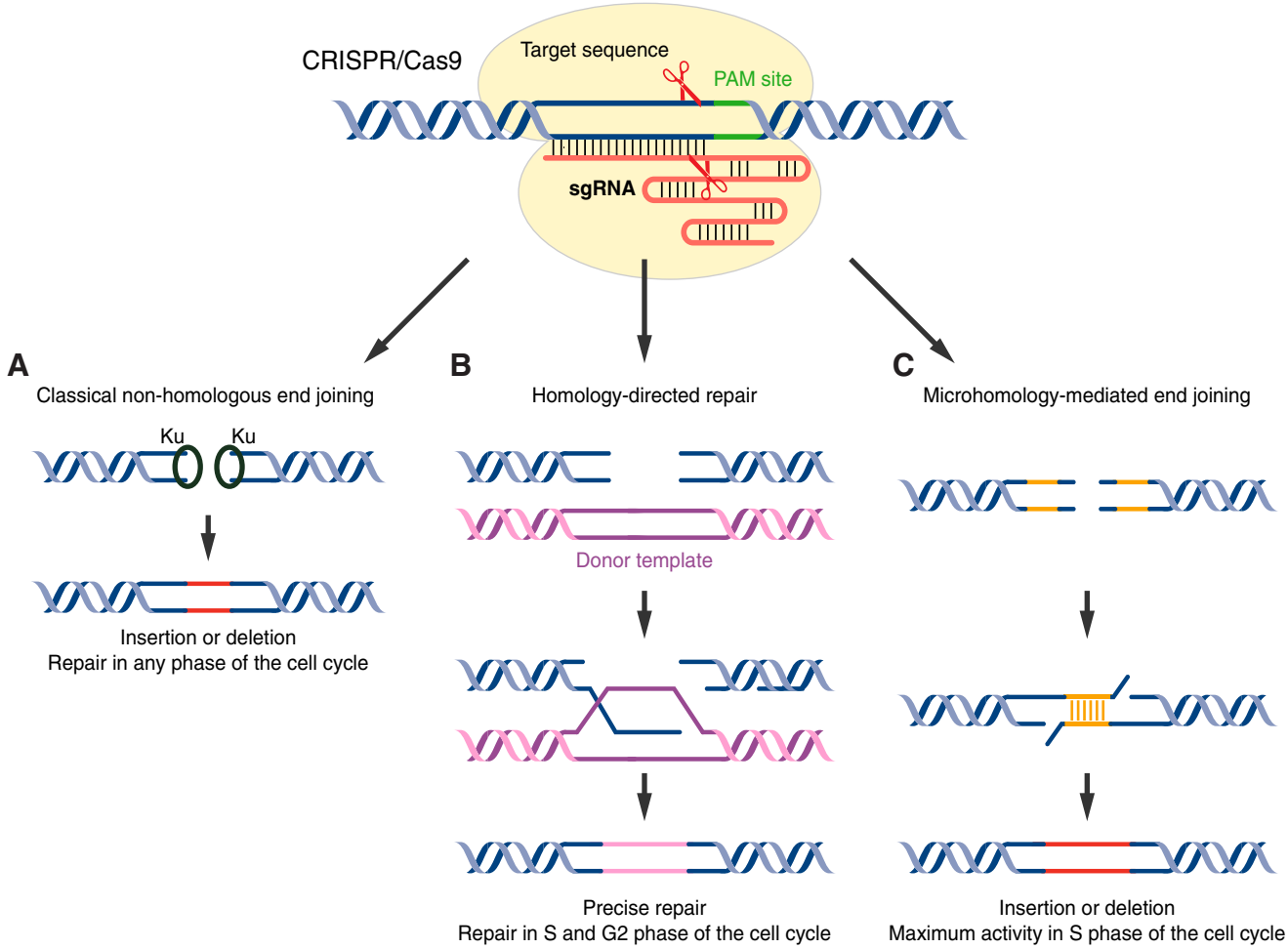

FIGURE 3. DNA repair pathways involved in CRISPR/Cas-induced DNA double-strand break repair. *A*: classical nonhomologous end joining (c-NHEJ) is a Ku-dependent DNA repair pathway that is active throughout the cell cycle. In the absence of a donor template, c-NHEJ generates insertions or deletions (INDELs; shown in red) in the genome. *B*: when a DNA double-strand break (DSB) is induced in the S or G_2 phase of the cell cycle, homology-directed repair (HDR) can be triggered if a donor template is present (magenta), leading to precise repair of the genome. *C*: a Ku-independent microhomology-mediated end joining (MMEJ) pathway can be used for DNA DSB repair if the DNA breakage site shares sequence homology. MMEJ-mediated repair generates INDELs in the genome (red).

as a template for HR (**FIGURE 3*B***) (456). During HDR, the end of the DNA DSB is recognized by the MRE11-RAD50-NBS1 (MRN) complex, which undergoes initial DNA end resection induced by MRE11 (434), followed by extensive end resection induced by the EXO1-BLM complex (40), producing single-stranded DNA (ssDNA). The exposed ssDNA is coated by RPA until RAD51 detects the homology sequence, leading to strand invasion and Holliday junction formation (431). HDR is completed when the Holliday junction is either dissolved by the BLM/TOPOIII complex or resolved by GEN1 or SLX1/SLX4 nucleases (442).

Currently, the most widely used repair templates for HDR are double-stranded DNA (circular or linearized plasmid) and single-stranded oligodeoxynucleotides (ssODNs). Before programmable nucleases were employed in HR-mediated gene targeting, the length of sequence homology on the targeting vector for HDR could be up to 14 kb for efficient gene targeting (86). The development of programmable nucleases, especially the CRISPR/Cas system, significantly enhanced site-specific DNA DSBs and further reduced the length of sequence homology on the targeting vector to several hundred base pairs (255, 468).

A ssODN can also serve as a repair template for HDR, especially for introducing small DNA modifications (231, 329, 441, 469). Interestingly, asymmetrical ssODN complementary to the nontarget strand (the DNA strand that does not base pair with the CRISPR sgRNA) can drive the efficiency of HDR up to 60%, because the Cas9 endonuclease first releases the PAM-distal nontarget DNA strand, which is more available for ssODN binding (329). Due to the accuracy of HDR, it is possible that the Cas9 endonuclease will continuously generate DSBs at the target site as long as the PAM and the sgRNA target sequence remain intact, even when HDR is completed. Because of codon

degeneracy, introducing a silent mutation at the third nucleotide of the triplet codon for the disruption of the PAM and/or sgRNA target sequence can effectively overcome the recleavage event (231, 469).

3. MMEJ

MMEJ is a Ku-independent alt-NHEJ repair pathway for DNA DSBs, which displays maximal activity in S phase **(FIGURE 3C)** (398). Similar to HDR, MMEJ undergoes initial DNA end resection induced by MRE11 but does not require extensive end resection induced by the EXO1-BLM complex (324, 398, 444). If microhomology is present, the exposed ssDNA ends generated by initial DNA end resection will anneal with each other and the gap between the newly annealed ssDNA will be filled by DNA polymerases θ (61, 460) and finally ligated by the LIG3-XRCC1 complex (17, 362, 421).

In the absence of template DNA, MMEJ is an error-prone DNA repair pathway because of INDEL formation (356, 398). However, several studies have adopted MMEJ for precise integration of exogenous reporter genes into the genome after TALEN or CRISPR/Cas9-mediated DNA DSBs (146, 285, 335). This method, known as Precise Integration into Target Chromosome (PITCh), requires three DNA DSBs, with one DSB located at the target locus in the genome, and the other two DSBs located at the 5′- and 3′-ends of a reporter cassette (e.g., GFP). The reporter cassette is cloned into a plasmid with 5–25 bp of sequence homology to the target locus, serving as the MMEJ repair template. Therefore, MMEJ provides an alternative method for precise genome editing similar to HDR.

C. Engineered Cas9 With Mutant Nuclease Domains

CRISPR/Cas9-mediated DNA DSBs are induced by two separate nuclease domains, in which the HNH nuclease domain cuts the target strand that hybridizes with the sgRNA and the RuvC nuclease domain cuts the nontarget strand (295). A single amino acid mutation at the RuvC-I domain (D10A) generates a Cas9 nickase that is only active for target strand cleavage **(FIGURE 4A)** (323). Double mutations at both RuvC-I and HNH nuclease domains (D10A, H840A) abolish the Cas9 nuclease activity, generating a deactivated Cas9 (dCas9) **(FIGURE 4B)** (168). A pair of Cas9 nickases can be used to induce DNA DSBs with high specificity, since only two adjacent DNA single-strand breaks can generate a DSB (323). Although dCas9 lacks its nuclease activity, it still has RNA-guided DNA binding activ-

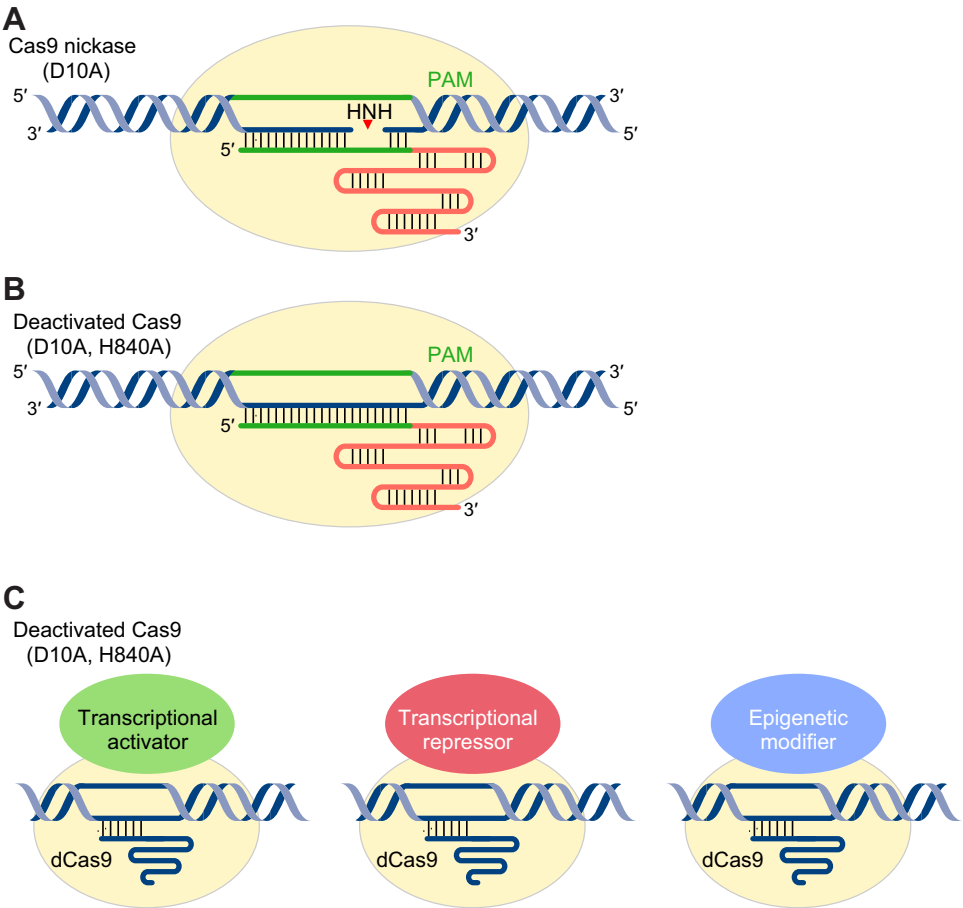

FIGURE 4. CRISPR/Cas9 with mutant nuclease domain. CRISPR/Cas9-mediated DNA double-strand break (DSB) is induced by two separate nuclease domains, in which the HNH domain cleaves the target strand and the RuvC domain cleaves the nontarget strand. *A*: D10A Cas9 nickase having a mutation in the RuvC domain is only active for target strand cleavage by the HNH nuclease domain. *B*: mutations at both RuvC and HNH nuclease domains (D10A, H840A) abolish the Cas9 nuclease activity, generating a deactivated Cas9 (dCas9). *C*: CRISPR/dCas9-mediated gene regulation is achieved by fusing dCas9 to transcriptional activation domains, transcriptional repression domains, or epigenetic modifiers.

ity and can be fused with different effectors, including cytidine deaminase, transcriptional activators/repressors, and epigenetic modifiers for different purposes, such as nucleotide conversion as well as genome and epigenome regulation (66, 126, 142, 179, 194, 196, 228, 274, 294). Unlike CRISPR/Cas9-mediated permanent genome alternation, CRISPR/dCas9-mediated genome and epigenome regulation does not modify the genome. Therefore, the CRISPR/dCas9 system provides a powerful tool for inducible and reversible control of gene expression and changing the epigenetic landscape without modifying endogenous genomic sequence.

1. Base editing: Cas9 nickase fused to cytidine deaminase

Base editing is a CRISPR/Cas-mediated genome editing technology in which the dCas9 or Cas9 nickase is fused to a cytidine deaminase for site-specific C-G to T-A conversion (194, 294) or fused to an engineered adenine deaminase for site-specific A-T to G-C conversion (123). Classically, CRISPR/Cas-mediated gene disruption introduces INDELs into the genome, which is imprecise and unpredictable, potentially leading to cytotoxicity by unintended alterations in the genome due to off-target effects. In contrast, gene knockout by Cas9 nickase-mediated base editing does not generate DNA DSBs. Several studies have applied this technology for precise gene knockout through site-specific introduction of premature stop codons (34, 203).

Based on the NCBI ClinVar database, more than 900 human genetic diseases are caused by T-to-C or A-to-G mutations, and these can be corrected by base editing (194, 206). Therefore, CRISPR/Cas9 nickase-mediated base editing represents a promising technology for therapeutic genome editing because its efficiency is higher than HDR, while INDEL formation is minimized since a DNA DSB is not required.

2. Transcriptional regulation: dCas9 fused to transcriptional activator/repressor

CRISPR/Cas9-mediated genome editing was developed to alter DNA sequences. In contrast, CRISPR/dCas9-based technology was developed to regulate gene expression at the transcriptional level without altering genome integrity. The general mechanism of CRISPR/dCas9-mediated transcriptional regulation is achieved by direct fusion or recruitment of transcriptional activators or repressors to the dCas9-sgRNA complex, forming a CRISPR activation or interference complex (CRISPRa or CRISPRi). In the presence of sequence-specific sgRNA, CRISPRa or CRISPRi is targeted to the transcription start site of a gene, thereby inducing or repressing gene expression, respectively (193, 391, 420).

Gene repression in eukaryotes requires that dCas9 be fused with a transcriptional repression domain, such as KRAB (Krüppel-associated box), MXI1 (MAX-interacting protein 1), or SID4X (four copies of mSin3 interaction domain) **(FIGURE 4*C*)** (126, 195, 392). Conversely, CRISPR/dCas9-mediated gene activation is achieved by fusion of transcriptional activation domain(s), such as VP64 (four copies of the Herpes Simplex Virus VP16 transcriptional activation domain), p65 (NF-Kb activation domain), and Rta (Epstein-Barr Virus-derived R transactivator) to the NH_2 terminus and/or COOH terminus of dCas9, leading to gene activation in a variety of cell types (59, 66, 70, 236, 247, 313).

Several strategies have been exploited to further improve the potency of the CRISPRa system. One strategy is to use antibody/epitope-based recruitment of transcriptional activators, known as the SunTag system (387). Another strategy is to engineer the sgRNA scaffold for recruitment of the RNA binding protein fused with multiple transcriptional activators (196, 465). CRISPR/dCas9-based transcriptional activation can also be used for cell lineage reprogramming. As an example, dCas9 fused with two copies of the VP64 transcriptional activation domain can be directed to the *Myod1* promoter, leading to reprogramming of fibroblasts to the myogenic lineage (59).

One of the applications of dCas9-based transcriptional regulation for the treatment of muscular dystrophies is to upregulate compensatory or paralogous proteins. Utrophin is an autosomal paralogue of dystrophin, and upregulation of utrophin can partially alleviate the dystrophic phenotype seen in the mouse model of Duchenne muscular dystrophy (DMD) (145, 396). Therefore, it would be interesting to evaluate the efficacy and efficiency of dCas9-based transcriptional upregulation of the endogenous utrophin gene for the treatment of DMD.

3. Epigenetic regulation: dCas9 fused to epigenetic modifiers

The CRISPR/dCas9 system can also be used for site-specific epigenome editing when fused with epigenetic modifiers, leading to histone code modification or changes in DNA methylation **(FIGURE 4*C*)**. In an example of histone modification, dCas9 engineered from *Neisseria meningitides* was fused to histone demethylase LSD1 and used to target the enhancer regions of the *Oct4*, *Sox2*, and *Tbx3* genes (179). This decreased the levels of H3K4me2 and H3K27ac and suppressed gene expression. Conversely, fusing dCas9 with the catalytic core of the histone acetyltransferase p300 induced robust transcriptional activation of the targeted genes (142). In addition to histone code modification, the DNA methylation pattern can also be altered. Fusing dCas9 with either Tet1 (Ten-eleven translocation methylcytosine dioxygenase 1) or Dnmt3a (DNA methyltransferase 3A) produced dCas9-Tet1 or dCas9-Dnmt3a, which can be used to target methylated or unmethylated promoter re-

gions, leading to promoter activation or silencing (228, 274).

D. Novel CRISPR/Cas Systems: CRISPR/Cpf1 and CRISPR/Cas13a/C2c2

Currently, the most widely used forms of Cas9 and its orthologs are from the class 2 type II CRISPR/Cas system. Recent studies in class 2 type V and VI CRISPR/Cas systems revealed several new RNA-guided CRISPR effectors capable of nucleic acid cleavage and detection, such as Cpf1 (CRISPR from *Prevotella* and *Francisella 1*) and Cas13a (formerly C2c2). These new CRISPR/Cas systems further expand the genome editing range of the CRISPR system (5, 103, 111, 466).

1. CRISPR/Cpf1: more than an alternative to Cas9

CRISPR/Cpf1, from the class 2 type V CRISPR system, is a RNA-guided endonuclease capable of DNA cleavage **(FIGURE 5*A*)** (111, 466). Two Cpf1 orthologs, LbCpf1 (from *Lachnospiraceae bacterium ND2006*) and AsCpf1 (from *Acidaminococcus sp. BV3L6*), have been engineered for genome editing in a variety of systems, including mammalian cells, animals, and plants (106, 156, 166, 184, 185, 235, 281, 317, 397, 405, 464, 469). The CRISPR/Cpf1 system has many unique features compared with CRISPR/Cas9: *1*) Cas9-mediated genome cleavage requires two RNA components consisting of a crRNA and a tracrRNA (which can be engineered as a single sgRNA hybrid), whereas Cpf1-mediated genome cleavage is tracrRNA-independent so it only requires a short crRNA. *2*) The PAM

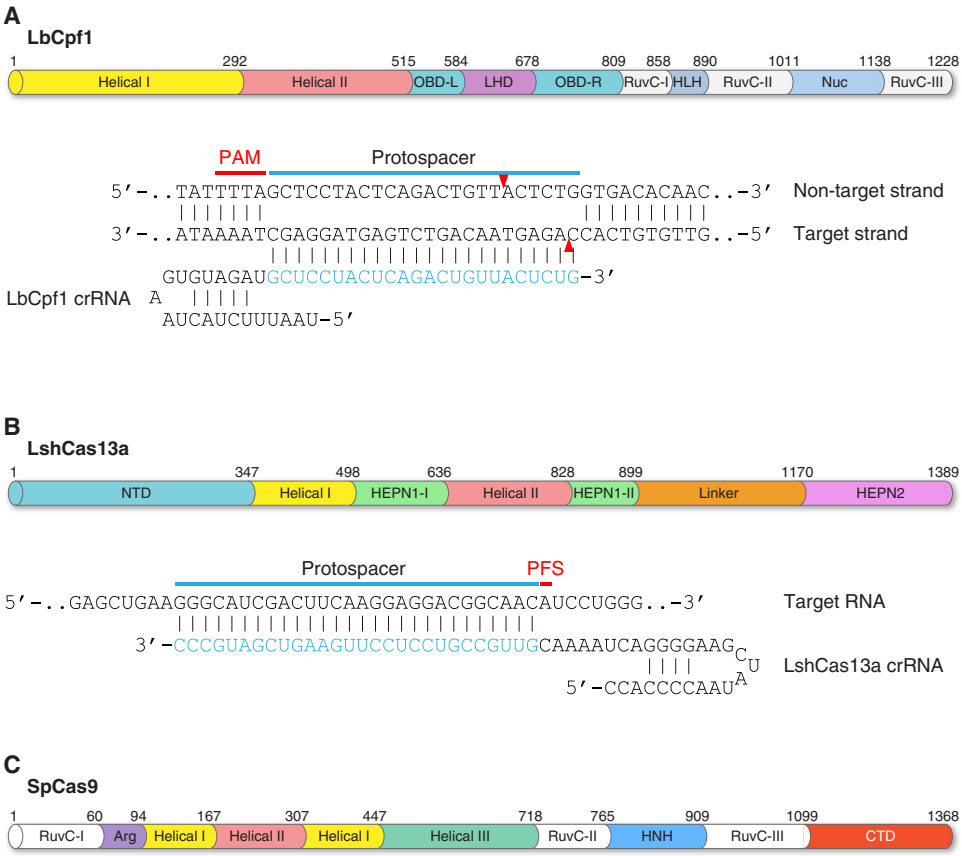

FIGURE 5. Novel CRISPR/Cas systems. Two novel class 2 CRISPR/Cas systems have been engineered for nucleic acid recognition and cleavage, such as Cpf1 (CRISPR from *Prevotella* and *Francisella 1*) and Cas13a (formerly C2c2). *A*: domain organization of the LbCpf1 protein discovered in *Lachnospiraceae bacterium ND2006*. All Cpf1 orthologs have two nuclease domains: *1*) the RuvC domain which cleaves the nontarget DNA strand and *2*) the Nuc domain which cleaves the target DNA strand. The LbCpf1 crRNA is shown hybridizing with its DNA target. The PAM is highlighted in red. Red arrowheads indicate cleavage site. *B*: domain organization of the LshCas13a protein discovered in *Leptotrichia shahii*. Cas13a has dual RNase activities, one specific for pre-crRNA processing and maturation, which is catalyzed by the helical-I domain, and the other one for RNA-guided single-stranded RNA (ssRNA) degradation, which is catalyzed by the HEPN1 and HEPN2 domains. The LshCas13a crRNA is shown hybridizing with its RNA target. CRISPR/Cas13a-mediated ssRNA cleavage is independent of a PAM; instead, it requires a 3′-protospacer flanking site (PFS; shown in red). *C*: domain organization of the SpCas9 protein discovered in *Streptococcus pyogenes*. The RuvC nuclease domain cuts the nontarget strand. The HNH nuclease domain cuts the target strand that hybridizes with the sgRNA. CTD, COOH-terminal domain.

sequence of Cpf1 is 5′-TTTN-3′, located at the 5′ end of a protospacer; in contrast, the 5′-NGG-3′ or 5′-NAG-3′ PAM for SpCas9 is located at the 3′ end of a protospacer. *3*) Cas9-mediated DNA DSB is blunt-ended and proximal to the PAM site, whereas Cpf1-mediated DNA DSBs are cleaved as a staggered cut distal to the PAM site. *4*) The pre-crRNA processing in the CRISPR/Cas9 system is catalyzed by an additional RNase III, whereas Cpf1 has intrinsic RNase activity and can directly process pre-crRNA by itself (111, 466).

Because of its T-rich PAM preference, Cpf1 represents an alternative to Cas9 for genome editing at AT-rich loci. In addition to the canonical 5′-TTTN-3′ PAM sequence, Cpf1 also recognizes 5′-CTTV-3′, 5′-TCTV-3′, 5′-TTCV-3′ (V represents A, G, or C) as noncanonical PAMs, because the PAM-binding channel of Cpf1 has conformational flexibility (449) that further expands the targeting range of the CRISPR/Cpf1 system. Another advantage of Cpf1 compared with Cas9 is the convenience of multiplex genome editing. CRISPR/Cas9-mediated multiplex genome editing requires multiple sgRNAs transcribed from separate promoters or additional RNA sequences for recognition and cleavage by other nucleases if multiple sgRNAs are transcribed from a single promoter (171, 336, 400, 445). However, CRISPR/Cpf1-mediated multiplex genome editing only requires a single promoter for the transcription of multiple crRNAs, because Cpf1 can process polycistronic crRNAs into individual ones using its own RNase activity, which significantly simplifies multiplex genome editing (467). Therefore, Cpf1 is more than an alternative to Cas9 in terms of genome and epigenome editing because it offers a broader range of editing options.

2. CRISPR/Cas13a/C2c2: programmable RNA-guided RNA-targeting CRISPR effector

Most CRISPR effectors discovered so far are RNA-guided deoxyribonucleases. To date, RNA-guided RNA-targeting activity has been shown in only three types of CRISPR systems: the type III-A, III-B systems and type VI CRISPR/Cas13a system (formerly C2c2) (5, 103, 140, 165, 178, 339, 372, 373, 386). Type III-A, III-B systems belong to the class 1 CRISPR family and require a multicomponent effector complex for RNA degradation, and so have limited application in RNA biology. In contrast, the type VI CRISPR/Cas13a system belongs to the class II CRISPR family and is a single-effector system for crRNA processing and RNA targeting (5, 102) **(FIGURE 5*B*)**. These engineered Cas proteins further extend the CRISPR/Cas system from DNA editing to RNA editing **(FIGURE 5)**.

Several Cas13 orthologs have been engineered for RNA recognition and cleavage both in vitro and in vivo (4, 80, 102, 129). For example, the Cas13a ortholog from *Leptotrichia wadei* (LwaCas13a) has been engineered for pathogenic virus and bacteria detection, mutation genotyping, and identification, which further expands the clinical application of the CRISPR/Cas13a system (129). In addition, LwaCas13a can be used in mammalian cells and plants for targeted RNA knockdown with high specificity, and its catalytically inactive form can also be applied for tracking RNA transcripts in vivo (4). Most recently, the Cas13b ortholog from *Prevotella sp. P5–125* (PspCas13b) has been engineered for mammalian RNA targeting, and its catalytically inactive form fused with ADAR2 deaminase domain can also be applied for RNA base editing (80). Therefore, the recently characterized CRISPR/Cas13 system is becoming a powerful tool for studying RNA biology.

III. MYOEDITNG: PREVENTION OF MUSCULAR DYSTROPHIES

A. DMD: Dystrophin Gene Structure and Mutations

DMD is an X-linked recessive monogenic disease caused by mutations in the *DMD* gene, which encodes dystrophin (149). DMD is the most common type of monogenic muscular dystrophy, affecting ~1 in every 5,000 boys (135). DMD patients seem normal at birth, but within a few years they begin having trouble walking and lose ambulation between 7 and 12 yr of age. Cardiac and respiratory failure causes premature death, often by the early 30s.

Dystrophin is a key component of the dystrophin glycoprotein complex, which is a large multicomponent protein complex essential for sarcolemma integrity and stability **(FIGURE 6)** (120, 135). The structure of the full-length dystrophin protein can be organized into four major domains: *1*) the NH_2-terminal region containing an actin-binding domain; *2*) the central region containing a stretch of 24 spectrin-like repeats, forming the rod domain, which is interrupted by 4 hinge regions; *3*) the cysteine-rich domain which contains several subdomains, including a WW domain, two EF-hand-like domains, and a ZZ domain, which are important for interacting with β-dystroglycan, calmodulin, and ankyrin-B; and *4*) the COOH-terminal domain which interacts with dystrobrevin and syntrophins (6, 120, 299). The DMD gene, comprised of 79 exons **(FIGURE 7)** (184, 264), gives rise to different isoforms of the dystrophin protein which are expressed in various tissues by tissue-specific promoters and/or alternative splicing (280). The large 427-kDa cytoskeletal protein that is primarily expressed in skeletal muscle and heart is transcribed from the Dp427m promoter.

More than 7,000 mutations have been identified in the *DMD* gene (36). These mutations can be categorized as deletion (68%), duplication (11%) of single or multiple exons, or small point mutations (20%), such as missense and nonsense substitutions (3, 36, 271). Mutations in the

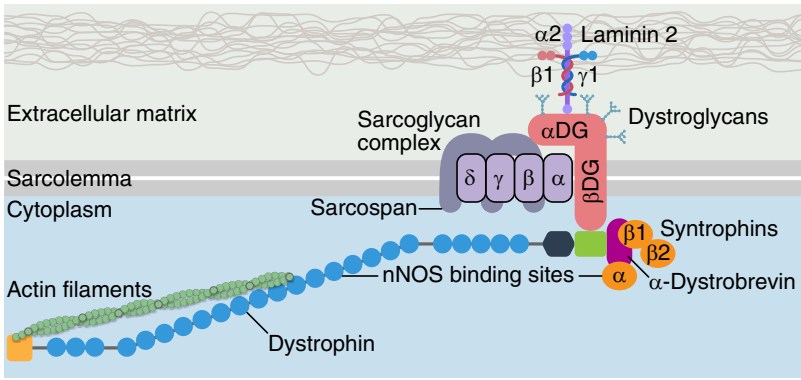

FIGURE 6. Structure of the dystrophin-glycoprotein complex (DGC). The main components of the DGC are the dystroglycan complex, sarcoglycan complex, and dystrophin. The DGC provides sarcolemma stability and integrity through interaction with laminin in the basement membrane on the extracellular matrix and actin in the cytoplasm. Other dystrophin-associated proteins include neuronal nitric oxide synthase (nNOS), dystrobrevins, syntrophins, and sarcospan. Mutations of the main components of the DGC cause muscular dystrophies, such as Duchenne or Becker muscular dystrophy (dystrophin mutation), and limb-girdle muscular dystrophy types 2C, 2D, 2E, and 2F (sarcoglycan mutations).

DMD gene are not uniformly distributed but cluster into hot spots, which are clustered within exons 2–20 and exons 45–55 (36). Approximately 15% of all exon deletion events and 50% of all exon duplication events are observed within exons 2–20, whereas 70% of all exon deletion events and 15% of all exon duplication events are observed within exons 45–55 (36, 451). In-frame deletion or duplication of exon(s) within the central region of the *DMD* gene retains the protein reading frame and generates either a truncated or extended dystrophin protein. These mutant dystrophin proteins retain their NH_2 and COOH termini, which are essential for actin cytoskeleton and dystrophin glycoprotein complex interaction, leading to a milder form of muscular dystrophy, known as Becker muscular dystrophy (BMD) (2, 135). In contrast, out-of-frame deletion or duplication of exon(s) either disrupts the protein reading frame or generates a premature termination codon (PTC) and leads to DMD.

B. Animal Models of DMD

The most commonly used animal model for DMD is the *mdx* mouse, in which a C-to-T transition in exon 23 creates a nonsense mutation, leading to loss of full-length dystrophin expression (54, 360). The *mdx* mice do not develop severe DMD phenotypes, such as muscle wasting, scoliosis, and cardiomyopathy until reaching 15 mo of age. In contrast to DMD patients whose lifespan is significantly reduced, the lifespan of *mdx* mice is reduced by only 25% (60). Four chemically induced *mdx* strains have also been developed, known as mdx^{2cv}, mdx^{3cv}, mdx^{4cv}, and mdx^{5cv}, with a point mutation in intron 42, intron 65, exon 53, or exon 10, respectively (65). In addition to the *mdx* strains with point mutations, four additional DMD mouse models have been established with either exon 2 duplication, exon 45 deletion, exon 50 deletion, or exon 52 deletion (12, 417, 458).

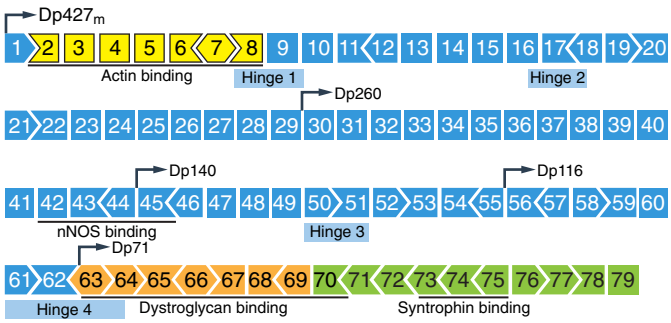

FIGURE 7. Structure of the dystrophin gene. The dystrophin gene has 79 exons. Different dystrophin isoforms can be transcribed from various promoters (demarcated as Dp, followed by a numeric number indicating isoform molecular weight in kilodaltons). The dystrophin protein expressed in skeletal muscle and heart is transcribed from the Dp427m promoter. Domains essential for binding with other DGC components or cytoskeletal proteins are underlined. Exons are color-coded according to the domain they encode: NH_2 terminus (yellow), central rod domain (blue), cysteine-rich domain (orange), and COOH terminus (green).

Dystrophin-deficient mouse models generally do not develop severe pathological phenotypes as seen in DMD patients. Several double knockout (dKO) mouse models were generated, in which the *Dmd* gene was knocked out, along with additional genes required for sarcolemma integrity, stem cell maintenance, and muscle homeostasis (85, 130, 259, 278, 332). Genome editing technology also played a role in expanding the rodent models of DMD. For example, two DMD rat models were created by TALEN- or CRISPR/Cas-mediated targeting of the *Dmd* exon 23 or exons 3–6, leading to an exon 23 frame shifting or exon 3–6 deletion (208, 286). Most recently, CRISPR/Cas9 was used to create a mouse model lacks exon 50, representing the most common mutational "hot spot" in humans (7).

In addition to small rodent models, large animal models of DMD have been developed, including dogs (16, 197, 346, 365, 409, 418, 435), pigs (192, 353, 461), and non-human primates (69). Monkey models of DMD are still at F_0 with mosaicism, which requires additional breeding to generate a pure background (69). Disease progression in some porcine models of DMD is so severe that the majority of the affected pigs die within the first week of life, which limits its application in therapeutic translation (353). In contrast, canine DMD models share more similar clinical phenotypes as seen in human patients, including limb muscle fibrosis, joint contracture, hypersalivation, and an early cardiac defect (259). Moreover, canine DMD models have fewer re-

generated myofibers than *mdx* mice as indicated by central nucleation, which is histologically similar to human patients (81, 365). In addition, canine DMD models develop limb muscle weakness at 2–3 mo of age and have ~75% reduction of lifespan, showing similar disease progression as human patients (410). Therefore, the canine model of DMD seems superior to the other large animal models in regard to current availability, genetic background, and speed of disease progression.

C. Introduction of Myoediting

To date, more than 800 monogenic neuromuscular disorders with mutations in over 400 different genes have been recorded (177). The discovery and application of programmable nucleases for genome editing paves the way for permanent correction of these genetic diseases (79, 318, 320). Meganucleases, ZFNs, and TALENs have been reported to correct mutations responsible for certain muscular dystrophies including DMD, limb-girdle muscular dystrophy (LGMD), and myotonic dystrophy (DM) (122, 217, 240, 303, 304, 316, 402, 443). However, these early versions of programmable nucleases were not widely adopted for correcting mutations in various muscular dystrophies because of the low genome targeting density, difficulty of assembly of the functional nuclease domains, and cytotoxicity caused by off-target effects. The CRISPR/Cas system revolutionized the genome editing field and significantly simplified the process of permanent correction of monogenic neuromuscular disorders.

CRISPR/Cas-mediated genome editing in skeletal muscle and heart, which we termed myoediting (230, 231), can permanently correct various *DMD* mutations and restore dystrophin function. Initially, myoediting was performed in the germline of *mdx* mice, a mouse model of DMD with a nonsense mutation in exon 23. By injecting Cas9 mRNA, a sgRNA targeting the mutated exon 23, and a ssODN repair template into the zygotes of *mdx* mice, it was demonstrated that CRISPR/Cas9-mediated myoediting can successfully correct the *Dmd* mutation by HDR or NHEJ and restore dystrophin expression (231). However, germline editing in humans is currently not feasible, necessitating alternative strategies for therapeutic genome editing. Therefore, we and other groups used recombinant adeno-associated virus (rAAV) to deliver the CRISPR/Cas9 genome editing components to postnatal *mdx* mice for skipping or deleting the mutated exon in vivo (29, 104, 230, 289, 383). The rAAV-delivered CRISPR/Cas9-mediated postnatal genome editing successfully restored dystrophin expression and improved muscle function in *mdx* mice. These studies underscore the therapeutic potential of the CRISPR/Cas9 system for treating devastating muscle diseases.

The CRISPR/Cpf1 system was also used to correct *DMD* mutations in human induced pluripotent stem cells (iPSCs) and in *mdx* mice either by exon skipping or HDR (469), which further expands the range of CRISPR/Cas-mediated genome editing in AT-rich loci. Due to postmitotic and multinucleation features, skeletal muscle is ideal for therapeutic CRISPR/Cas9 genome editing because genomic correction of a subpopulation of nuclei leads to steady improvement of muscle function (29, 104, 230, 231, 289, 383, 469). Therefore, CRISPR/Cas-mediated myoediting represents a novel method for DMD treatment. In the following sections, different strategies of applying the CRISPR/Cas system for correcting *DMD* mutations will be discussed in detail. In addition, the potential of applying CRISPR/Cas-mediated genome editing for the correction of other muscular dystrophies will also be explored.

D. Strategies of CRISPR/Cas-Mediated DMD Correction

Initial efforts to apply programmable nucleases such as meganuclease, ZFN, and TALEN for precise genome editing provided many insights into the permanent correction of *DMD* mutations (217, 240, 303, 304, 316). The CRISPR/Cas system significantly simplified the genome editing process. To date, four strategies have been developed for CRISPR/Cas-mediated correction of *DMD* mutations, which are exon deletion, exon skipping, exon reframing, and exon knock-in.

1. Exon deletion

Approximately 80% of mutations found in the *DMD* gene are out-of-frame exon deletions or duplications, leading to reading frame incompatibility between adjacent exons **(FIGURE 8*A*)** (3, 36). The most traditional strategy to permanently restore the *DMD* open reading frame (ORF) is in-frame exon deletion, in which a pair of sgRNAs is used to generate two simultaneous DNA DSBs within the intron regions flanking the out-of-frame exon, leading to complete removal of a single exon or multiple exons to generate a compatible reading frame outcome with the adjacent exon **(FIGURE 8*B*)**. CRISPR/Cas-mediated exon deletion is best suited for correcting *DMD* mutations caused by exon duplication and has been reported with high efficiency in human DMD myoblasts with exon 2 or exon 18–30 duplications (209, 437). Removal of the duplicated exons restores the *DMD* ORF and produces full-length dystrophin protein that is indistinguishable from wild-type or normal dystrophin, although small INDELs can be observed at the genomic level.

DMD mutations caused by an out-of-frame exon deletion can be corrected by an in-frame exon deletion, producing a truncated dystrophin protein with internal deletions. For example, cultured myoblasts from DMD patients with an out-of-frame deletion of exons 48–50 have an incompatible reading frame when exon 47 is spliced with exon 51. A pair

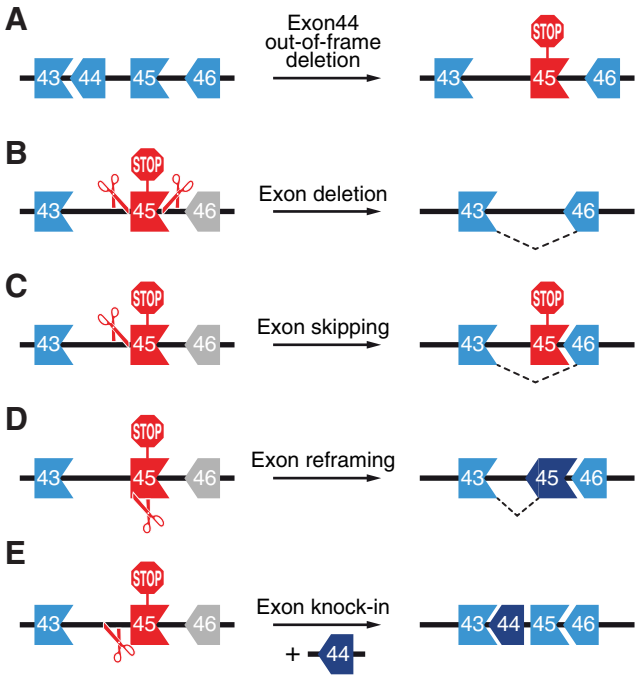

FIGURE 8. Strategies for CRISPR/Cas-mediated correction of *DMD* mutations. *A*: a schematic illustration showing arrangement of exons 43–46 of the *DMD* gene in terms of their reading frame compatibility. This genomic region is used here as an example to highlight the strategies for CRISPR/Cas9 correction of *DMD* mutations. An out-of-frame deletion of *DMD* exon 44 results in splicing of exon 43 to exon 45. This creates a premature stop codon in exon 45 (red STOP sign). *B*: exon deletion is used to restore the *DMD* reading frame. Two sgRNAs targeting introns 44 and 45 will generate two DNA DSBs flanking exon 45. This leads to excision of exon 45 and subsequent splicing of exon 43 to exon 46. *C*: exon skipping is mediated by a single sgRNA which targets the splice acceptor site of exon 45. The INDELs generated by NHEJ-mediated repair disrupt the splice acceptor site of exon 45, leading to splicing of exon 43 to exon 46. *D*: exon reframing is mediated by a single sgRNA targeting exon 45. The INDELs in exon 45 generated by NHEJ-mediated repair may restore the reading frame compatibility of exon 45 with exons 43 and 46. *E*: exon knock-in relies on HDR DNA repair pathway in the presence of a donor template. A single sgRNA targeting intron 44 will generate a DNA DSB and be repaired by HDR when exon 44 is used as a donor template, leading to exon 44 knock-in between exons 43 and 45.

of sgRNAs targeting intron 50 and 51 was used to delete exon 51, restoring the reading frame between exon 47 and 52 (302). Similarly, the dystrophin reading frame incompatibility caused by an out-of-frame deletion of exons 45–52 has been corrected by exon 53 deletion, leading to splicing of exon 44 to exon 54 and subsequently restoring the dystrophin reading frame (239, 240). Multiple exons can also be deleted to restore the dystrophin reading frame. By using a pair of sgRNAs targeting intron 44 and 55, a large deletion extending from exon 45 to 55 was generated, leading to reading frame restoration of exon 44 to exon 56 (302, 457). Similarly, a large deletion extending from exons 44 to 54 was generated, by using a pair of sgRNAs targeting intron 43 and 54, leading to reading frame restoration between exon 43 and 55 (239, 240). Several in vivo studies in postnatal *mdx* or *mdx*^{4cv} mice used exon deletion strategies to remove a single or multiple exons with a point mutation and thereby restored dystrophin expression and muscle function (29, 104, 230, 289, 383, 446).

Exon deletion is a promising strategy to correct mutations clustered in the second hot spot region (exons 45–55) because the spectrin-like repeats within the central rod domain are tolerant of large in-frame deletions (120, 135). However, special consideration should be given to mutations at the NH_2 and COOH termini of dystrophin because these regions encode many essential domains known to interact with the actin cytoskeleton and dystrophin glycoprotein complex. For example, three different exon deletion strategies were applied to correct the *DMD* mutation caused by an out-of-frame deletion of exons 8–9, and different outcomes were observed in regard to dystrophin protein stability and function (205). Specifically, an in-frame deletion of exons 7–11 retained all three actin binding sites, but this truncated dystrophin was structurally unstable and showed minimal recovery of cardiomyocyte function in vitro. In contrast, in-frame deletion of exons 3–9 only retained actin binding site 1 but was the most effective strategy to restore functionality of human iPSC-derived cardiomyocytes. Reading frame restoration does not guarantee functional recovery, and hence, additional empirical analysis should be performed to further evaluate different correction strategies.

2. Exon skipping

Exon skipping has been achieved using anti-sense oligonucleotide (AON)-based therapy (1). However, AON-based exon skipping corrects at the mRNA level, while retaining the mutant *DMD* in the genome. Thus this approach requires life-long treatment. In contrast, CRISPR/Cas-based exon skipping is achieved by NHEJ-mediated disruption of the splice acceptor or donor sequence at the genomic level, leading to permanent exon skipping and completely eliminating the source of the mutation. For example, human iPSCs derived from DMD patients with exon 44 deletion have an incompatible reading frame between exon 43 and 45 **(FIGURE 8*A*)**. A single sgRNA was designed to specifically target the intron 44 and exon 45 boundary, thereby inducing a DNA DSB at the splice acceptor site of exon 45 **(FIGURE 8*C*)**. The INDELs generated by NHEJ-based DSB repair disrupted the splice acceptor sequence of exon 45, leading to exon 45 skipping during mRNA splicing (217). Similarly, *DMD* mutations caused by an out-of-frame deletion of exons 48–50 or exons 45–52 have been corrected by skipping exon 51 or exon 53, respectively (239, 240, 469). Recently, exon skipping has also been used to correct *Dmd* in a mouse model representing the most commonly deleted hot spot mutation in humans (7). This mouse model has an out-of-frame deletion of exon 50, which generates a premature stop codon in exon 51. A single sgRNA was designed to target the exon 51 splice acceptor site, leading to exon 51

skipping. Therefore, using a single sgRNA-mediated exon skipping strategy, which abolishes either the splice acceptor site or splice donor site or allows for reframing, overcomes the necessity of double sgRNA-based exon deletion.

Usually, the single sgRNA-mediated exon skipping strategy generates a relatively small INDEL at the intron/exon boundary, destroying the exon splice acceptor or donor site but retaining the residual part of the exon sequence in the genome. If "AG" nucleotides are present in the residual part of the exon, they can serve as a pseudo-splice acceptor sequence, rendering the single sgRNA-mediated exon skipping ineffective. Therefore, additional experimental studies, such as reverse transcription polymerase chain reaction (RT-PCR) or Western blot analysis, should be performed to confirm exon skipping at the RNA and protein level.

3. Exon reframing

A NHEJ-based reframing strategy can also be applied to restore the dystrophin ORF, in which a single or a pair of sgRNAs are used to generate DNA DSBs within the exon region, leading to a targeted frameshift, since in theory, one-third of INDELs created by NHEJ should be in-frame **(FIGURE 8*D*)**. Several studies have applied this strategy to restore the dystrophin reading frame by inducing targeted frameshifts in exons with an incompatible reading frame in regard to the adjacent exon, including exons 23, 45, 50, 51, 53, and 54 (29, 162, 217, 231, 239, 240, 302, 469). Unlike the exon deletion strategy, which excises a single or multiple exons, exon reframing only creates small INDELs, and hence, minimizes the length of the genomic deletion.

Both exon skipping and exon reframing strategies require using one sgRNA-mediated single cut in the genome. These two strategies are considered more efficient than using two sgRNA-mediated double cuts in the genome. This is because exon deletion by excision using two sgRNAs requires two cooperative DNA DSBs. However, two DNA DSBs do not always occur simultaneously since there is a possibility that a single DNA DSB can be rapidly rejoined by NHEJ-mediated DNA repair, leaving the second intronic DSB ineffective. In this situation, exon deletion cannot be achieved because of the latency between the two DNA DSBs. In contrast, one sgRNA-mediated single cut near the splice acceptor site can be sufficient to restore the *DMD* ORF. For example, if the INDEL disrupts the splice acceptor sequence, this could lead to exon skipping. Alternatively, if the INDEL does not disrupt the splice acceptor sequence, there is still a possibility that one-third of the INDELs within the exon could be in-frame, leading to exon reframing.

4. Exon knock-in

In general, *DMD* mutations corrected by exon deletion, skipping, or reframing strategies will generate truncated dystrophin proteins with internal deletions. In principle, *DMD* mutations can also be corrected by exon knock-in, leading to expression of full-length dystrophin protein **(FIGURE 8*E*)**. Exon knock-in requires a DNA donor template and active cell cycle to induce HDR-mediated precise editing in the S and G_2 phases. This repair strategy has been used in DMD patient-derived iPSCs to correct a mutation caused by an out-of-frame deletion of exon 45 (217). In addition, point mutations in mouse *Dmd* exon 23 and 53 have also been corrected by HDR-mediated precise editing (29, 231, 469, 471).

Mutations at specific regions in the NH_2 and COOH termini of dystrophin generally are not feasible for exon deletion or skipping-based correction because essential domains known to interact with cytoskeletal actin or the sarcoglycan complex are encoded within these regions. Therefore, exon knock-in is required to correct these types of mutations. However, due to the postmitotic nature of mature skeletal muscle and cardiomyocytes, HDR efficiency remains low in CRISPR/Cas-mediated postnatal genome editing (29). Recently, precise genome editing in postmitotic cells and animals with high efficiency was reported (381). This technology, which was termed homology-independent targeted integration (HITI), only relies on the NHEJ pathway and can be used to precisely integrate DNA fragments into the mammalian genome, regardless of the cell cycle state, which may provide opportunities to correct certain *DMD* mutations by exon knock-in.

E. Facioscapulohumeral Muscular Dystrophy

1. Facioscapulohumeral muscular dystrophy type 1 and type 2

Facioscapulohumeral muscular dystrophy (FSHD) is an autosomal dominant neuromuscular disease with an estimated prevalence ranging from 1:14,000 to 1:20,000 (109, 276, 298, 305, 390). Clinically, FSHD shows asymmetric regional muscle weakness in the face, shoulders, and upper arms. Symptoms progress to the trunk and leg muscles while the extraocular, pharyngeal, and cardiac muscles remain unaffected (389). In contrast to other muscular dystrophies that show a phenotype of severe myofiber degeneration, FSHD shows a minimal myopathic phenotype at the histopathological level, with evidence of endomysial inflammation specific to the perivascular region (10, 390). The mechanism of FSHD can be either genetic or epigenetic, leading to the classification of type 1 FSHD (FSHD1) and type 2 FSHD (FSHD2) (211, 333, 390).

FSHD1 represents 95% of the FSHD cases and is caused by contraction of D4Z4 macrosatellite repeats located in the subtelomeric region of chromosome 4, leading to DUX4-induced cytotoxicity in skeletal muscles (213, 390, 413, 433). Normal individuals carry 11–100 D4Z4 repeats on

chromosome 4q35, while FSHD patients are limited to 1–10 copies of D4Z4 repeats (413). The reduced number of D4Z4 repeats alters the heterochromatic DNA structure at chromosome 4q35 and induces the expression of the *DUX4* gene within the last D4Z4 repeat when a polyadenylation signal is present on the FSHD permissive 4qA haplotype. The FSHD nonpermissive 4qB haplotype lacks the exon distal to the D4Z4 repeat and its *DUX4* transcript is destabilized (92, 213, 368). *DUX4* encodes a double-homeobox transcription factor, which is normally only expressed in the testis (369, 459). When DUX4 is ectopically expressed in skeletal muscle, it activates various genes that are normally expressed only in the germline, stem cells, and the immune system, leading to oxidative stress, apoptosis, and inhibition of muscle regeneration (44, 124, 390). Unlike FSHD1 with D4Z4 repeats contraction, FSHD2 patients generally retain normal-sized D4Z4 repeat arrays but show strong reduction of D4Z4 methylation because of a mutation of the *SMCHD1* gene (212). *SMCHD1* is essential for hypermethylation of CpG islands, and its mutated form fails to methylate D4Z4 repeats, thereby causing DUX4 expression (212). Similar to FSHD1, *DUX4* expression in FSHD2 requires the acquisition of a polyadenylation signal present on the FSHD permissive 4qA haplotype.

2. Animal models of FSHD

FSHD1 is caused by contraction of subtelomeric D4Z4 macrosatellite repeats, leading to DUX4-induced cytotoxicity in skeletal muscles (213, 390, 413, 433). Currently four DUX4 transgenic mouse models have been developed, which are D4Z4–2.5, D4Z4–12.5, iDUX4–2.7, and iDUX4-pA mice (43, 82, 201). Both D4Z4–2.5 and D4Z4–12.5 mouse models display abundant expression of DUX4 transcripts in the testis, consistent with germline DUX4 expression in humans. In addition to germline expression, DUX4 transcripts can also be detected in multiple tissues in D4Z4–2.5 mouse model, indicating that contraction of D4Z4 repeats leads to inefficient DUX4 repression in somatic tissues.

In contrast to D4Z4–2.5 and D4Z4–12.5 mouse models which uses an endogenous DUX4 promoter, two inducible DUX4 transgenic mouse models (iDUX4–2.7 and iDUX4-pA) were also generated in which the DUX4 expression is induced by doxycycline (43, 82). Low-level DUX4 expression in the iDUX4-pA mouse model without doxycycline induction results in progressive degenerative myopathy and other muscle phenotypes, such as inflammatory infiltration and fibrosis. In contrast, muscle-specific DUX4 induction in the iDUX4-pA mouse model leads to dystrophic phenotypes and impaired muscle regeneration after injury.

The FSHD pathogenic gene *DUX4* is specific to primates, and retrotransposon-mediated expansion of its binding sites in the primate genome is not conserved in the murine system (124, 210, 459). Therefore, studies using both FSHD mouse models and human cell models, such as FSHD primary myoblasts and iPSCs, may provide more thorough information about disease progression and translational application.

3. CRISPR/Cas-mediated correction of FSHD1 and FSHD2

A CRISPR/dCas9-based gene editing strategy has been reported to reduce *DUX4* expression in primary FSHD myocytes, in which dCas9 was fused to a KRAB transcriptional repressor and targeted to the *DUX4* promoter or the first exon region, leading to transcriptional repression of *DUX4* (144). However, CRISPR/dCas9-based transcriptional repression is transient while the 4q35 chromatin landscape and D4Z4 repeat number remain pathogenic, so any therapeutic benefit would be only temporary.

Two potential genome editing strategies might provide longer term benefit. The first strategy is CRISPR/dCas9-based DNA methylation. It has been reported that fusing dCas9 with a DNA methyltransferase (Dnmt3a) or with a hybrid form of two DNA methyltransferases (Dnmt3a-Dnmt3L) can create site-specific DNA methylation (228, 274, 374). It might be effective to use the dCas9-DNA methyltransferase system to revert the hypomethylated 4q35 chromatin landscape, leading to *DUX4* silencing, since both FSHD1 and FSHD2 have reduced DNA methylation levels at the D4Z4 repeat arrays. The second strategy entails CRISPR/Cas9-based genome editing of the FSHD permissive 4qA haplotype **(FIGURE 9*A*)**. The Cas9 nuclease can be directed to the 4qA haplotype by specific sgRNA(s) that target the polyadenylation signal region and induce DNA DSBs to either disrupt the polyadenylation sequence or excise the polyadenylation signal, thereby converting the permissive 4qA haplotype to the nonpermissive 4qB haplotype.

F. LGMD

LGMD is a general term for a highly heterogeneous group of autosomal neuromuscular diseases with variable disease phenotypes, ranging from progressive muscle weakness in proximal limbs with a normal life span to rapid disease progression in early childhood. LGMD can also affect distal limbs and the heart and cause life-threatening symptoms such as respiratory compromise and cardiac abnormalities (282, 292). To date, mutations in more than 30 loci have been reported to cause LGMD, including eight autosomal dominant forms (LGMD1A-1H) and 25 autosomal recessive forms (LGMD2A-2Y) (177). Owing to the scope of this review, only two subtypes of LGMD2 will be covered, which are dysferlinopathy and sarcoglycanopathies. The potential of applying CRISPR/Cas-mediated genome editing for the correction of LGMD2 subtypes will also be discussed.

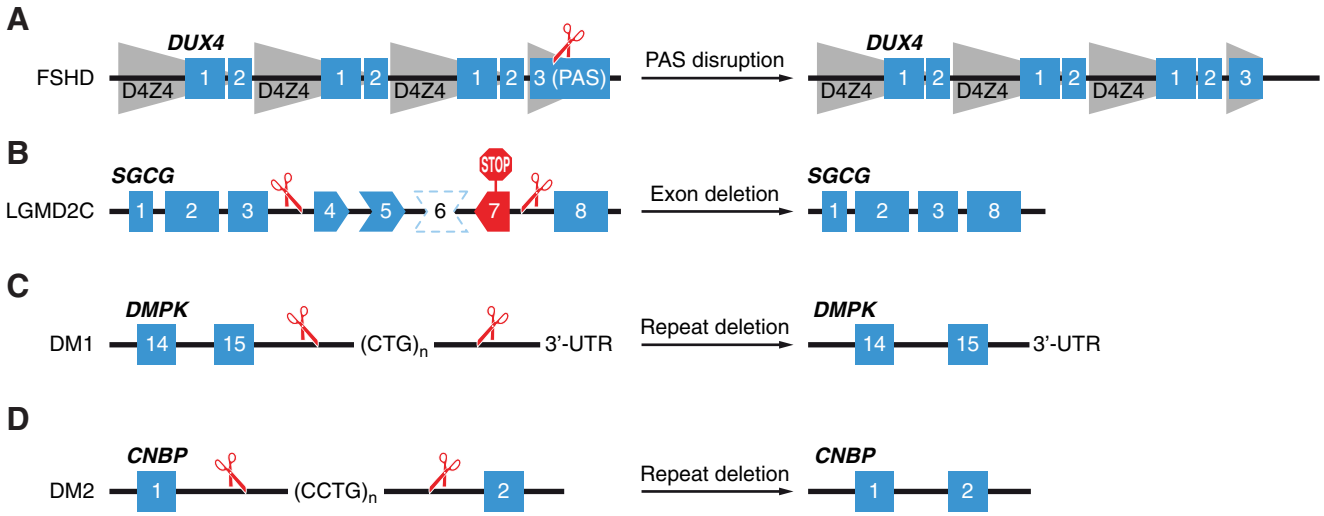

FIGURE 9. Strategies for CRISPR/Cas-mediated correction of other muscular dystrophies. *A*: FSHD type I and type II are caused by acquisition of a poly-adenylation signal on the permissive 4qA haplotype, leading to *DUX4* transcript stabilization. The Cas9 nuclease is directed to the 4qA haplotype by specific gRNA(s) targeting the polyadenylation signal and converts the permissive 4qA haplotype to the nonpermissive 4qB haplotype. The gray shaded area depicts D4Z4 macrosatellite repeats. PAS indicates poly-adenylation signal on the permissive 4qA haplotype. *B*: LGMD2C is caused by loss-of-function mutation of the *SGCG* gene. An out-of-frame deletion of exon 6 (shown by a dotted line around exon 6) results in splicing of exon 5 to exon 7, which creates a premature stop codon in exon 7 (red STOP sign). Two sgRNAs that target introns 3 and 7 will generate two DNA DSBs flanking exons 4–7. This leads to excision of exons 4–7 and subsequent splicing of exon 3 to exon 8 and permanent restoration of the *SGCG* reading frame, since deletion of exons 4–7 of the *SGCG* gene was shown to partially restore function of γ-sarcoglycan (121). *C*: myotonic dystrophy type I (DM1) is caused by trinucleotide CTG repeat expansion in the 3′ untranslated region (3′-UTR) of the *DMPK* gene. Two sgRNAs can be designed to generate two DNA DSBs that flank the CTG repeats, leading to deletion of the CTG repeats. *D*: myotonic dystrophy type 2 (DM2) is caused by a tetranucleotide CCTG repeat expansion in intron 1 of the *CNBP* gene. Two sgRNAs can be designed to generate two DNA DSBs that flank the CCTG repeats, leading to deletion of the CCTG repeats.

1. Dysferlinopathy: LGMD2B and Miyoshi myopathy

Dysferlinopathy is caused by autosomal recessive mutation of the *DYSE* gene on chromosome 2p13 (25, 26, 226). Clinically, dysferlinopathy can be classified into LGMD2B and Miyoshi myopathy, depending on the muscle groups being affected. LGMD2B causes proximal muscle weakness in the pelvic and shoulder girdle muscle regions but spares other muscle groups (282, 293). In contrast, Miyoshi myopathy shows distal muscle weakness specifically affects the gastrocnemius and soleus muscles, without affecting other muscle groups (9).

The *DYSE* gene encodes a 230-kDa single-pass transmembrane protein, known as dysferlin, which is ubiquitously expressed in many tissues but enriched in striated muscles (25). Dysferlin binds to phospholipids in the presence of Ca^{2+} and is important for repairing membranes of skeletal muscle (22, 214). In addition, dysferlin also interacts with the dihydropyridine receptor (DHPR), caveolin-3, annexin A1, and desmoyokin (AHNAK) in t-tubule membranes, indicating its role in t-tubule maintenance (8, 153, 214, 256). Mutations in the *DYSE* gene are diverse, including missense and nonsense mutations, splice site and 3′-UTR mutations, and small insertions or deletions, leading to nonsense-mediated mRNA decay, protein misfolding, or mislocalization (158, 200, 430).

Dysferlin-deficient mice maintain an intact dystrophin glycoprotein complex but are defective in Ca^{2+}-dependent membrane repair and display progressive dystrophic phenotypes, including sarcolemma lesions, muscle necrosis, inflammatory infiltration, and fatty tissue deposition (22, 147). AAV-based gene replacement therapy has been applied to treat dysferlinopathy. However, the size of the dysferlin cDNA (6.5 kb) exceeds the packaging limit of AAV. To address this issue, dysferlin cDNA was split into two cDNA fragments cloned into a dual-AAV system. After administration of the dual recombinant AAV vectors, full-length dysferlin transcripts were shown to be produced by either trans-splicing or homologous recombination (133, 232, 370). Although the AAV-based dysferlin replacement can restore muscle function, the endogenous *DYSE* mutations are still present in the genome. A recent study using TALENs and CRISPR/Cas9-genome editing successfully corrected the endogenous *DYSE* mutations in iPSCs derived from LGMD2B patients (402). After differentiation of corrected iPSCs into skeletal muscles, dysferlin expression was restored and correct protein localization was observed. This proof-of-concept study demonstrates the efficiency and ac-

curacy of the CRISPR/Cas-genome editing system in permanent correction of monogenic neuromuscular disorders and represents an alternative method for the treatment of dysferlinopathy.

2. Sarcoglycanopathies: LGMD2C, 2D, 2E, and 2F

Sarcoglycanopathies can be classified as LGMD2C, 2D, 2E, or 2F, which are caused by loss-of-function mutations of the *SGCG*, *SGCA*, *SGCB,* or *SGCD* genes, respectively (293, 311). These genes encode γ-, α-, β-, and δ-sarcoglycans, which are single pass transmembrane proteins and together with ε- and ζ-sarcoglycans can form the sarcoglycan complex that resides in the sarcolemma (107, 260, 432). The sarcoglycan complex is a key component of the dystrophin glycoprotein complex, and its stability requires lateral association with the dystroglycan complex linked with dystrophin. Therefore, mutations in dystrophin can cause loss of the sarcolemma distribution of the sarcoglycan subunits, and patients affected with sarcoglycanopathies have similar phenotypes seen in DMD and BMD, displaying both muscular dystrophy and cardiomyopathy (24, 207, 261). Interestingly, mutations in any single sarcoglycan gene can lead to a significant reduction in or complete absence of the entire sarcoglycan complex (408). Many mouse models of sarcoglycanopathies have been developed (11, 78, 100, 101, 138, 428). These mouse models recapitulate many of the pathophysiological phenotypes seen in human patients, and hence serve as a reliable animal model of LGMD.

CRISPR/Cas9-mediated genome editing has successfully corrected *SGCG* and *SGCA* mutations in iPSCs derived from LGMD2C and LGMD2D patients (181, 402). However, these CRISPR/Cas9-mediated LGMD corrections rely on HDR, which is less efficient in postmitotic mature skeletal muscles. Interestingly, a truncated γ-sarcoglycan was engineered with in-frame deletion of exons 4–7 and shown to reduce pathophysiological phenotypes associated with LGMD2C with improvement in both skeletal muscle and heart function (121). Moreover, AON-mediated exon skipping was used to correct *SGCG* mutations in human cells. This proof-of-concept study challenges the conventional knowledge that exon skipping is restricted to correct *DMD* mutations and paves the way for the application of the CRISPR/Cas system to permanently correct *SGCG* mutations in LGMD2C by NHEJ-mediated exon skipping **(FIGURE 9*B*)**.

G. Myotonic Dystrophy

1. Myotonic dystrophy type 1 and type 2

Myotonic dystrophy (DM) is an autosomal dominant neuromuscular disorder affecting ~1 in every 8,000 individuals (263, 404). Clinically, patients with DM suffer muscle degeneration leading to weakness and myotonia with other symptoms including cardiac conduction defects, cataracts, brain abnormalities, as well as gastrointestinal and endocrine disorders (132, 229, 268, 330, 436).

DM is classified into myotonic dystrophy type 1 (DM1) and type 2 (DM2). DM1, also known as Steinert's disease, is caused by trinucleotide CTG repeat expansion in the 3′ untranslated region (3′-UTR) of the *DMPK* gene located on chromosome 19 (51, 114, 241). Healthy individuals carry 5–37 CTG repeats while repeat numbers greater than 37 are unstable and may further expand in length during cell division (15, 403, 404). *DMPK* mRNA transcripts with increased CUG expansion can form stable secondary structures, leading to increased steady-state expression of CELF1 (CUGBP Elav-like family member 1) by hyperphosphorylation, downregulation of DMPK itself, and aberrant splicing of other genes by sequestering splicing factors such as MBNL1 in the ribonuclear foci (97, 154, 167, 172, 204, 337). Dysregulation of CELF1 and MBNL1 splicing factors causes not only RNA toxicity and aberrant splicing, but also transcriptional dysregulation, mRNA instability, and microRNA dysregulation (45, 312, 325, 328, 415).

In contrast to DM1 with trinucleotide CTG repeat expansion in the 3′-UTR of the *DMPK* gene, DM2 is caused by a tetranucleotide CCTG repeat expansion in intron 1 of the *CNBP* gene in chromosome 3 (84, 225). Similar to DM1, the expansion of the CCUG repeats in *CNBP* mRNA transcripts also causes MBNL1 sequestration, leading to CNBP downregulation, RNA toxicity, and abnormal splicing of other genes (404). Because CNPB is required for cap-independent translation, other pathogenic effects such as abnormal protein translation or turnover may also contribute to DM2 (155, 341).

2. Animal models of DM

Approximately 20 mouse models of DM1 and DM2 have been established and are categorized into two groups: *1*) mouse models that recapitulate the toxic RNA gain-of-function and *2*) mouse models with abnormal splicing regulators (127). One of the most informative DM1 models is the HSA^{LR} transgenic line, in which 250 copies of the CTG repeats are placed in the 3′-UTR of the *HSA* gene that encodes the human α-skeletal actin. This mouse model develops severe myotonia, nuclear sequestration MBNL1, and aberrant splicing of multiple target transcripts (249).

Another transgenic mouse model, known as DM300–328, carries a large fragment of the human *DMPK* locus with expanded CTG repeats. This mouse model displays ribonuclear foci accumulation in multiple tissues, progressive muscle weakness, myotonia, tau protein distribution abnormalities, as well as defects in splicing and glucose metabolism, recapitulating many pathophysiological phenotypes seen in DM1 patients (136, 354, 355, 414). An inducible mouse model was also developed in which a floxed concatemer of

three polyadenylation signals is inserted upstream of 960 copies of CTG repeats in exon 15 of the *DMPK* gene. This allows tissue-specific expression of the transgene by the Cre-lox system (300, 419).

In addition to mouse models that recapitulate toxic RNA gain-of-function, mouse models with abnormal splicing regulators were also developed, including *Mbnl1* and *Mbnl2* knockout mice and *CELF1* overexpression mice (141, 148, 173, 198, 223, 233, 257, 395, 429). As with HSA^{LR}, the mouse model of DM1, a CCTG transgenic mouse model was developed to model DM2, in which the expanded $(CCTG)_{121}$ repeats are introduced in intron 1 of the *ZNF9* gene, displaying CELF1 upregulation, altered protein translation and degradation, and recapitulating muscle pathology, as seen in DM2 (338). DM mouse models with repeat expansion are more suitable for therapeutic application especially for CRISPR/Cas-mediated genome editing because the microsatellite repeats present in both DM1 and DM2 can conceivably be excised by NHEJ-mediated DNA repair.

3. TALENs and CRISPR/Cas-mediated correction of DM1 and DM2

In several studies, researchers have employed programmable nucleases for genome editing of DM1 and DM2. A TALEN-based system was used to generate site-specific DNA DSBs in the 3′-UTR of the *DMPK* gene and introduced multiple polyadenylation signals upstream of the trinucleotide CTG repeats by HDR, leading to early transcriptional termination and subsequently preventing the production of the toxic *DMPK* transcripts (122, 443). However, HDR-mediated knockin of polyadenylation signals requires a repair template and an active cell cycle, which may not be efficient for genome editing in postmitotic tissues such as skeletal muscle.

The CRISPR/Cas system and its derivatives have also been used to treat both DM1 and DM2 as well. For example, an engineered dCas9 fused to a PIN RNA endonuclease domain was shown to be active for RNA cleavage and could specifically eliminate microsatellite repeat expansions in *DMPK* and *CNBP* mRNAs, reducing pathophysiological phenotypes seen in DM1 and DM2 (27). Although bypassing the potential off-target genetic lesions caused by conventional CRISPR/Cas9-mediated DNA cleavage, this CRISPR/dCas9-based RNA-targeting system cannot permanently reduce the microsatellite repeats in *DMPK* and *CNBP* loci. Another study used CRISPR/Cas9-mediated DNA DSBs at the *DMPK* trinucleotide repeat region and successfully excised the entire expanded CTG/CAG repeats in human and mouse myoblasts. This approach successfully reverted the pathogenic hallmarks of DM1, including the *cis* epigenetic effects and the *trans* effects on the transcriptome and proteome **(FIGURE 9*C*)** (411). Therefore, CRISPR/Cas-mediated excision of expanded microsatellite repeats represents a promising strategy for permanent correction of DM1 and DM2 mutations **(FIGURE 9, *C* AND *D*)** because it requires only NHEJ-mediated DNA repair, which is active throughout the cell cycle and hence can be used in many cell types.

IV. MYOEDITING IN "DISEASE-IN-A-DISH" AND ANIMAL MODELS

A. Human Induced Pluripotent Stem Cell Models of Muscular Dystrophies

The development of iPSCs has opened up new opportunities for disease modeling (307, 384, 385, 462). In principle, human iPSCs can self-renew indefinitely and can be differentiated into a variety of cell types, which become an inexhaustible and scalable source for stem cell research. Moreover, human iPSCs offer a similar genetic background to model human genetic diseases. For example, spinal muscular atrophy (SMA), caused by a mutation of the survival motor neuron 1 (*SMN1*) gene (53), cannot easily be modeled in mice. This is because humans have multiple copies of the paralogous *SMN2* gene while mice do not carry the *SMN2* gene (28). Therefore, additional knockout and transgenic strategies are required in animal models to model human SMA, which is time-consuming and expensive. In contrast, iPSCs derived from human SMA patients can be directly used for disease modeling and large-scale screening for drug discovery. Therefore, human iPSCs represent a complementary cellular source to the widely used animal models. Many human iPS cell lines have been established to model muscular dystrophies, including DMD, FSHD, LGMD, and DM. CRISPR/Cas has been utilized to correct a variety of *DMD* mutations found in human myoblasts and patient-derived iPS cell lines by different correction strategies **(TABLE 1)**.

Because of different genetic backgrounds and reprogramming methods, different human iPS cell lines can be variable in terms of their differentiation properties and phenotypic output (39, 46). To address this issue, isogenic control cell lines can be generated by introducing mutations in WT iPSCs, mimicking mutational genotypes found in patients. Conversely, mutations in patient-derived iPSCs can be corrected by programmable nucleases. The direct comparison between genome-edited iPSCs with unedited isogenic control cell lines, in principle, can reduce genetic background variation and improve genotype-phenotype correlation. Moreover, long-term in vitro iPSC culturing may cause genomic abnormalities, and hence routine karyotyping is recommended to monitor genome stability (254).

B. In Vivo Genome Editing of Animal Models

The ultimate goal of therapeutic application of genome editing is to permanently correct mutations that contribute to

Table 1. *CRISPR/Cas-mediated correction of DMD mutations in human cells*

Strategy	Cell Type	CRISPR/Cas Type	*DMD* Mutations	sgRNA Targeting Site	Genome Editing Outcome	Reference Nos.
Exon deletion	Myoblast	SpCas9	Δ Ex45–52	i52, i53	Δ Ex53	239, 240
	Myoblast	SpCas9	Δ Ex45–52	i43, i54	Δ Ex44–54	239, 240
	Myoblast	SpCas9	Δ Ex48–50	i43, i54	Δ Ex44–54	239, 240
	Myoblast	SpCas9	Δ Ex48–50	i50, i51	Δ Ex51	302
	Myoblast	SpCas9	Δ Ex48–50	i44, i55	Δ Ex45–55	302
	Myoblast	SpCas9	Dup. Ex2	i2	Δ Dup. Ex2	209
	Myoblast	SpCas9	Dup. Ex18–30	i27	Δ Ex28–30, Δ Dup. Ex18–27	437
	iPSC	SpCas9	Δ Ex46–47	i44, i55	Δ Ex45–55	457
	iPSC	SpCas9	Δ Ex46–51	i44, i55	Δ Ex45–55	457
	iPSC	SpCas9	Δ Ex8–9	i2, i7	Δ Ex3–7	205
	iPSC	SpCas9	Δ Ex8–9	i5, i7	Δ Ex6–7	205
	iPSC	SpCas9	Δ Ex8–9	i6, i11	Δ Ex7–11	205
Exon skipping	Myoblast	SpCas9	Δ Ex48–50	e51	Skp. Ex51	239, 240
	Myoblast	SpCas9	Δ Ex45–52	e53	Skp. Ex53	239, 240
	iPSC	SpCas9	Δ Ex44	e45 SA	Skp. Ex45	217
	iPSC	LbCpf1	Δ Ex48–50	i50, e51	Skp. Ex51	469
Exon reframing	Myoblast	SpCas9	Δ Ex45–52	e53	Ex53 reframing	239, 240
	Myoblast	SpCas9	Δ Ex48–50	e51	Ex51 reframing	239, 240, 302
	Myoblast	SpCas9	Δ Ex51–53	e50, e54	Ex50, Ex54 reframing	162
	iPSC	SpCas9	Δ Ex44	e45 SA	Ex45 reframing	217
	iPSC	LbCpf1	Δ Ex48–50	e51	Ex51 reframing	469
Exon knock-in	iPSC	SpCas9	Δ Ex44	e45 SA	Ex44 knock-in	217

i, intron; e, exon; Δ, deletion; Dup., duplication; SA, splice acceptor; Skp., skipping.

human monogenic diseases. Programmable nucleases such as the CRISPR/Cas system have been demonstrated to be effective in precise correction of pathogenic mutations found in the human embryo (176, 221, 234, 388). Several proof-of-concept studies have shown the efficacy and efficiency of the CRISPR/Cas system in correcting *Dmd* mutations in different mouse models by germline editing and postnatal editing **(TABLE 2)**. However, ethical issues, as well as public policies, restrict therapeutic application of human germline editing, leaving postnatal genome editing as the means to achieve the same goal.

Therapeutic genome editing requires delivering programmable nucleases and other genome editing components to target cells, which can be achieved ex vivo or in vivo. Ex vivo editing requires in vitro editing of the cellular genome,

Table 2. *CRISPR/Cas-mediated correction of Dmd mutations in mice*

Strategy	Mouse Model	CRISPR/Cas Type	*DMD* Mutations	sgRNA Targeting Site	Genome Editing Outcome	Reference Nos.
Exon deletion	*mdx*	SpCas9 and SaCas9	Ex23 point mutation	i22, i23	Δ Ex23	230, 289, 383
	mdx/Utr$^{+/-}$	SpCas9 and SaCas9	Ex23 point mutation	i20, i23	Δ Ex21–23	104
	mdx	SpCas9	Ex23 point mutation	i20, i23	Δ Ex21–23	446
	mdx^{4cv}	SpCas9 and SaCas9	Ex53 point mutation	i51, i53	Δ Ex52–53	29
Exon skipping	Δ Ex50	SpCas9	Δ Ex50	e51 SA	Skp. Ex51	7
Exon reframing	*mdx*	SpCas9	Ex23 point mutation	e23	Ex23 reframing	231
	Δ Ex50	SpCas9	Δ Ex50	e51 SA	Ex51 reframing	7
	mdx^{4cv}	SpCas9	Ex53 point mutation	e53	Ex53 reframing	29
Exon HDR	*mdx*	SpCas9	Ex23 point mutation	e23	Ex23 HDR	231
	mdx	LbCpf1	Ex23 point mutation	e23	Ex23 HDR	469
	mdx^{4cv}	SpCas9	Ex53 point mutation	e53	Ex53 HDR	29

i, intron; e, exon; Δ, deletion; SA, splice acceptor; Skp., skipping.

followed by transplantation of the targeted cell population to the original host. CRISPR/Cas-mediated ex vivo editing has been shown to be effective in the hematopoietic system (88, 125, 137, 345, 423). In theory, ex vivo genome editing can be applied to the skeletal muscle system because satellite cells serve as adult stem cells of skeletal muscle and are capable of surviving manipulation in vitro. However, skeletal muscle is the largest tissue in the human body, comprising ~40% of body weight. Transplantation of edited satellite cells or progenitor cells may improve local muscle function, but systemic functional recovery remains questionable (471). In addition to skeletal muscle, the heart is also affected in a variety of muscular dystrophies, but it is still under debate whether the heart contains stem cells or stem cell-like cell populations capable of transplantation and regeneration (21, 105, 284, 301, 378, 412). Taken together, ex vivo transplantation-based approaches are generally not feasible to treat muscular dystrophies, especially for those affecting both skeletal muscle and the heart. Therefore, in vivo postnatal genome editing turns out to be a more practical approach to permanently correct genetic mutations causing muscular dystrophies.

1. Delivery of genome editing components by nonviral vectors

Achieving in vivo postnatal genome editing requires an efficient and effective delivery system. Genome editing components can be physically or chemically delivered to target cells by nonviral vectors **(FIGURE 10)**. For example, microinjection and electroporation have been demonstrated as effective methods to deliver CRISPR/Cas genome editing components to target cells (68, 190, 262, 296, 450). However, these physical approaches are widely used for germline editing or embryo manipulation but are not feasible for systemic delivery in the postnatal host. In contrast, hydrodynamic intravenous injection can achieve systemic delivery in multiple tissues, including liver, kidney, lung, skeletal muscle, and heart (42, 377). Some studies have applied this technology to deliver CRISPR/Cas genome editing components to postnatal mice for mutating cancer genes in the mouse liver, correcting a tyrosinemia mutation and disrupting hepatitis B virus (222, 447, 455, 470). However, hydrodynamic injection requires a large injection volume and high pressure, which may damage tissues or organs.

In addition to physical vectors, chemical vectors, such as polymeric carriers and lipid-based carriers, are also widely used for in vivo delivery. Polymeric carriers are cationic polymers that can condense negatively charged DNA or RNA, whereas lipid-based carriers can spontaneously assemble into liposomes consisting of nucleic acids and cationic or neutral lipids (453). The CRISPR/Cas system and other genome editing components can be chemically delivered into animals in the form of plasmid DNA, mRNA, or ribonucleoprotein complexes. Chemical vectors can protect nucleic acids or ribonucleoproteins from degradation by

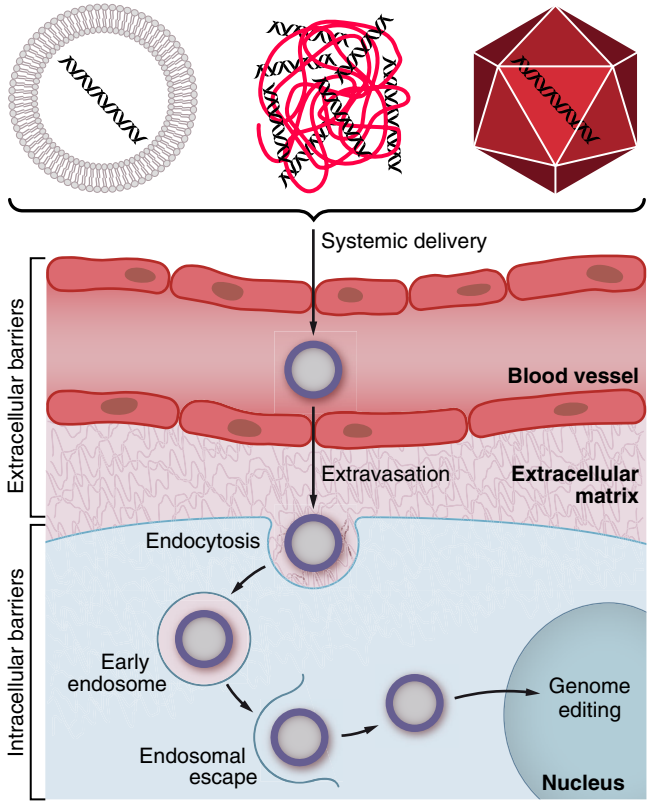

FIGURE 10. Systemic delivery of genome editing components. Nonviral (lipid-based carriers or polymeric carriers) and viral (rAAV) vector-based delivery of genome editing components into target tissue. Following administration, the delivery vectors pass through the blood vessel by extravasation to reach their target tissues. In the tissue, they undergo cytoplasmic trafficking, endosomal escape, and nuclear entry to perform genome editing in the nucleus.

endonucleases or proteases in physiological fluids and extracellular space, improving stability and half-life. For example, chemically modified CRISPR/Cpf1 mRNA and crRNA have been reported to enhance genome editing efficiency (216). However, many challenges still remain, including efficient delivery to the tissue of interest, cellular internalization, and protection from the lysosomal degradation pathway (422, 453).

It has been reported that plasmid DNA can be retained in skeletal muscle for over a year after intramuscular injection, suggesting the likelihood of long-term gene expression and retention in postmitotic tissue (439). In contrast, localized nonviral delivery such as intramuscular injection cannot generate a systemic effect, which is not ideal for treating muscular dystrophies since skeletal muscle is one of the largest tissues in the human body and multiple muscle groups can be affected. Systemic nonviral delivery, such as intravenous or intraperitoneal injection, have extended the targeting range but may cause gene expression in nontargeted tissues. Therefore, delivery of genome editing components to animals by nonviral vectors still requires improvement and optimization before clinical translation.

2. Delivery of genome editing components by viral vectors

Many viral vectors have been used for gene therapy, including lentivirus, retrovirus, herpes simplex virus, poxvirus, adenovirus, adeno-associated virus, baculovirus, and Epstein-Barr virus (288, 426). Among these, adeno-associated virus (AAV) is the most promising viral vector for the delivery of genome editing components to specific tissues, such as muscle and heart. AAV is a nonenveloped DNA virus with an ~5 kb linear ssDNA genome (371). The genome of wild-type AAV has two major ORFs flanked by two inverted terminal repeats (ITRs), while in the recombinant AAV (rAAV), the viral ORFs encoding the replication and capsid proteins are replaced by the customized gene expression cassette (110). Both wild-type and rAAVs are nonpathogenic in humans or animals, and their propagation requires a helper virus, making them a safe delivery system for therapeutic genome editing (199, 342, 379).

The rAAVs have many appealing features, including broad spectrum tissue tropism with minimal integration risk and long-term transgene expression from the episomal genome after viral transduction (98, 297, 348). Currently, 13 AAV serotypes are widely available for gene delivery, and each of them shows different tissue tropism. AAV serotypes 1, 6, 8, and 9 have high tropism in skeletal muscle and heart (37, 64, 119, 131, 159, 306, 427, 463, 472). In addition, tissue tropism and transduction efficiency can also be improved by pseudo-typing. For example, AAV2 genomes pseudo-packaged into AAV5 capsids can enhance gene delivery to skeletal muscle, whereas improved cardiomyocyte transduction has been observed by pseudo-packaging AAV2 genomes into AAV6 capsids (99, 363). rAAV has been successfully used to as a delivery system to administer CRISPR/Cas9 and other genome editing components to postnatal *mdx* or *mdx*^{4cv} mice and correct *Dmd* mutations by exon deletion or reframing strategies (29, 104, 230, 289, 383) **(FIGURE 10)**. In addition, several other studies reported rAAV-delivered CRISPR/Cas9-mediated in vivo genome editing in mouse models of human Huntington disease and congenital muscular dystrophy (180, 273, 452). These studies demonstrated that the combination of a rAAV-based delivery system with CRISPR/Cas9-mediated postnatal genome editing is a compelling strategy to permanently correct mutations responsible for monogenic neuromuscular disorders. However, long-term benefits and effects in animal models still need to be examined to prepare for future clinical trials.

C. Challenges of Therapeutic Genome Editing

Despite many appealing features, the rAAV-based delivery system entails major challenges. rAAV has a limited packaging capacity which limits the packaging size of gene therapy components. Currently, in the CRISPR/Cas-based system, the size limitation has been addressed by various groups, whereby SpCas9 and SaCas9 have been efficiently packaged into different rAAV serotypes for correcting *Dmd* mutations in mice (29, 104, 230, 289, 383). To effectively deliver the rAAV-based genome editing components, tissue and cell type specificity is another parameter to be considered. Selection of the appropriate rAAV serotype is necessary for tissue tropism. Additionally, implementation of tissue-specific promoters can be used to regulate expression of the editing components. Recent administration of rAAV serotypes with muscle tropism and gene expression regulated under a muscle-specific promoter successfully delivered genome editing components to skeletal muscle and heart (7, 29, 143).

1. Immunogenicity

One of the greatest challenges of using rAAV as a delivery system is the immune response to the vector. Potential immunogenicity elicited by rAAV-based delivery of the CRISPR/Cas system can be evoked by *1*) the restored protein product, *2*) the CRISPR/Cas system, and *3*) capsid proteins on the surface of rAAV virus.

Mutated genes in monogenic disorders encode abnormal proteins or cause a complete loss of protein. After CRISPR/Cas-mediated correction, epitopes derived from the newly restored protein may elicit immunogenicity. However, in the case of DMD, due to somatic mutation or alternative splicing, more than 50% of DMD patients display low level of dystrophin-positive revertant fibers (0.2–4%), which may mitigate a potential immune response (55, 290, 291). Indeed, in a gene transfer study, expression of murine full-length or mini-dystrophin in *mdx* mice did not evoke humoral or cytotoxic immune responses (108). Therefore, immunogenicity elicited by the rescued protein may not be a significant concern, at least in the case of DMD.

In regard to immunogenicity elicited by the CRISPR/Cas system, it was demonstrated that Cas9 endonucleases delivered by rAAV did evoke a humoral immune response in mice (71). However, they did not observe significant muscle cell damage or a repair response at 2 wk after rAAV administration. Another concern is if the rAAV needs to be readministered since once the SpCas9-mediated humoral immunity is established in the host, further application of SpCas9 for therapeutic genome editing may no longer be effective. Several potential strategies could be applied to address this issue, including *1*) large-scale functional variant profiling of SpCas9 for epitope mutation; *2*) replacing SpCas9 with other Cas endonucleases such as SaCas9, LbCpf1, and AsCpf1 after initial SpCas9 administration; and *3*) performing plasmapheresis or transient immunosuppression to reduce the circulating antibody titer.

In addition to the immunogenicity response elicited by the CRISPR/Cas system, the humoral immune response evoked by rAAV is another challenge for in vivo therapeutic genome editing. Several studies in non-human primates have shown that high incidence of neutralizing antibodies (NAbs) after initial exposure to AAV can block AAV transduction, rendering gene delivery ineffective (164, 425). Moreover, the prevalence of AAV NAbs in human populations is also relatively high, ranging from 30 to 60% in AAV2, to 15–30% in AAV7, AAV8, and AAV9 serotypes (47, 56). Several strategies have been developed to overcome AAV-induced humoral immune responses, including *1*) AAV capsid mutagenesis to alter NAb binding epitopes (287), 2) plasmapheresis to reduce AAV NAb titer (157, 272, 424), and *3*) transient immunosuppression (267).

2. Off-target effects

Another concern around using programmable nucleases for therapeutic genome editing is the potential for mutagenesis caused by off-target effects. Initial programmable nuclease-like TALENs generally show minimal off-target effects because TALEN-mediated DNA DSBs require dimerization of two TALEN monomers and the dimerized TALEN pairs can recognize 30–40 bp of DNA sequence (182). In contrast, DNA DSBs induced by SpCas9, currently the most prevalently used Cas endonuclease, only requires a 20-nt sgRNA forming DNA-RNA duplex with the target DNA strand, which may increase mismatching frequency. Indeed, several early studies about SpCas9 specificity have shown that high-frequency mutagenesis caused by off-target effects is possible at mismatched sites (112, 152, 224). For example, INDELs caused by SpCas9 off-target cleavage can be detected at certain sites with up to five mismatches relative to the on-target site (112, 152, 308). Several approaches can be used to evaluate potential CRISPR/Cas-induced off-target effects. Computational prediction of off-target sites followed by DNA mismatch cleavage assay serves as a rapid and convenient method to evaluate sgRNA specificity. More recently, computational prediction of off-target sites followed by deep sequencing or unbiased whole-genome sequencing represents a more reliable method for systematic evaluation of CRISPR/Cas specificity (399).

Many strategies have been developed to reduce off-target effects caused by CRISPR/Cas9 nonspecific cleavage. Using truncated sgRNA with 2–3 nt deletion at the 5′-end can reduce the off-target-induced mutagenesis by 5,000-fold or more, and reduce the genome-wide off-target sites by 2- to 5-fold (113, 401). Another strategy is to use a pair of Cas9 nickases to generate two DNA nicks in close proximity, and this strategy has been shown to reduce off-target mutations by 50- to 1,500-fold (247, 323, 357). Other strategies such as titration of dosage for Cas9/sgRNA (152), fusion dCas9 to *Fok*I nuclease (134, 400) have also been developed to reduce off-target effects.

Resolving the crystal structure of SpCas9 in complex with sgRNA and target DNA provided many insights into the potential improvement of Cas9 specificity through logistic engineering (295). The high-fidelity SpCas9 (SpCas9-HF1) was generated by alanine substitution to disrupt nonspecific interaction with the target DNA strand (191). Recent structural analysis demonstrated that binding SpCas9-HF1 to a substrate with even a single base pair mismatch at the PAM distal end completely abolishes stable docking of the HNH nuclease domain (67). In addition, enhanced SpCas9 (eSpCas9 1.1) was developed by neutralizing positively charged residues to weaken interactions with the non-target DNA strand (364). Additionally, a hyper-accurate Cas9 variant (HypaCas9) was developed in which the HNH nuclease activation is allosterically regulated by REC3, leading to high specificity of on-target cleavage (67). All of these engineered SpCas9 variants have been shown to significantly reduce off-target mutagenesis and have the potential to push therapeutic genome editing toward high specificity.

3. Long-term effects and benefits of postnatal genome editing

Postnatal genome editing can correct the genetic mutation in muscular dystrophies and, in the short term, leads to improved muscle function. At this time, little is known about the longevity of restored dystrophin protein in vivo. It is uncertain, for example, whether genome-edited, dystrophin-positive myofibers will be diluted out by dystrophin-negative myofibers generated from satellite cells carrying the *DMD* mutation. In contrast, cardiomyocytes in the adult human heart have a very low turnover rate (1% at the age of 25 to 0.45% at the age of 75) (30, 31). Therefore, therapeutic genome editing in the human heart should provide long-term clinical benefit. Indeed, several studies have demonstrated that CRISPR/Cas-mediated genome editing can restore cardiac function in dystrophic mice (104, 230).

V. CONCLUSIONS AND FUTURE PERSPECTIVES

In addition to mutations in the nuclear genome, mutations in the mitochondrial genome also cause primary mitochondrial DNA (mtDNA)-related diseases. Mutations in mitochondrial tRNAs or protein-coding genes can lead to MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes) or MERRF (myoclonus epilepsy and ragged red fibers) (89–91, 349). These genetic disorders not only affect muscle function but also result in pathogenesis in other systems, including brain, blood vessels, and the endocrine system. Currently, mitochondrial replacement therapy (MRT) is used to treat genetic disorders caused by mtDNA mutations, in which the meiotic spindle apparatus with chromosomes from an unfertilized maternal oocyte is transferred into a donor oocyte cytoplasm containing healthy mtDNA (438). However, healthy

mtDNA replacement is not absolute, and <1% carryover of mutant mtDNA can be present after MRT. This carryover may lead to a gradual loss of healthy donor mtDNA and reversal to the maternal haplotype by genetic drift (175, 448). This brings up an interesting question of whether programmable nucleases can be used to eliminate mutant mtDNA. Indeed, ZFNs and TALENs have been applied to selectively degrade pathogenic mitochondrial genomes (19, 118, 326). Currently, efficient mitochondrial genome editing by the CRISPR/Cas system remains controversial (117, 169). Perhaps, efficient delivery of sgRNA into the mitochondrial matrix is an impediment for this application. We anticipate that in the near future, CRISPR/Cas-mediated genome editing can be further expanded to the mitochondrial genome.

To date, there are 840 neuromuscular diseases known to be caused by mutations in 465 different genes, with 72 mapped loci awaiting gene identification (177). These debilitating diseases cause early death or significantly impair the quality of life. Currently, there is no effective treatment for these diseases since most therapies developed to date focus on alleviating the symptoms or targeting the secondary effects, while the source of mutations is still present in the human genome. The discovery and application of programmable nucleases for site-specific DNA DSBs provide a powerful tool for precise genome engineering. In particular, the CRISPR/Cas system has revolutionized the genome editing field and provides a new path for disease treatment by removing the genetic mutations that cause disease rather than simply treating the symptoms.

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