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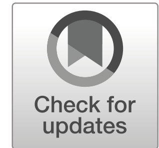

In Vivo Genome Engineering for the Treatment of Muscular Dystrophies

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Abstract

Purpose of Review Muscular dystrophies (MDs) are a heterogeneous collection of inherited disorders which cause progressive muscle loss and weakness/hypotonia. Owing to the genetic root of MDs, CRISPR/Cas9 genome editing has been investigated as a possible therapy, with significant advancements having been made. This review aims to provide an overview of recent progress on the in vivo utilization of CRISPR/Cas9 in MD animal models.

Recent Findings Three primary methods for correcting MD with CRISPR/Cas9 exist: restoration of the full-length protein, restoration of a truncated but partially functional protein, and modulation of gene expression. All these approaches have been (DMD) models with varying degrees of success. In congenital muscular dystrophy type 1A (MDC1A) mice, full-length protein restoration and disease modifier upregulation strategies significantly improved the phenotype. Lastly, efficient elimination of pathogenic CTG repeats via CRISPR/Cas9 was achieved in myotonic dystrophy type 1 (DM1) mice. Delivery of CRISPR machinery into MD animals was frequently accomplished with adeno-associated viruses (AAVs), which currently significantly outperform nanoparticle-based delivery. The targeting of satellite cells in vivo by AAVs has been evaluated by several groups in DMD mice, yielding conflicting results which require clarification.

Summary Partial or nearly complete phenotypic rescue has been achieved in DMD, MDC1A, and DM1 animals with numerous CRISPR/Cas9 strategies. While considerable work will be necessary to advance CRISPR/Cas9 genome editing past preclinical stages, its therapeutic potential for MD is extremely promising and warrants the investment.

Keywords Muscular dystrophy · Duchenne muscular dystrophy · Genome editing · CRISPR/Cas9 · Adeno-associated viruses · Nanoparticles

This article is part of Topical Collection on *Genome Editing*

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Introduction

Muscular Dystrophy

Muscular dystrophies (MDs) are a clinically and genetically heterogeneous group of diseases characterized by progressive weakness and loss of muscle mass [1]. The disorders differ by the affected muscles, age of onset, severity, and rate of progression [1, 2]. Medical interventions are currently restricted to symptom management or delaying disease progression [1, 3].

This review provides an overview of in vivo genome editing strategies in MD animal models that utilize clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) 9 technology. Published studies applying CRISPR/Cas9 as an in vivo therapeutic strategy have been limited to Duchenne muscular dystrophy (DMD), congenital muscular dystrophy type 1A (MDC1A), and myotonic dystrophy type 1 (DM1). However, the techniques and approaches discussed here are potentially applicable to a broad range of MDs. Furthermore, we examine which delivery systems have been tested in vivo, critical long-term considerations, what challenges lay ahead for the field, and key items to be addressed for genome editing to become a viable therapy.

DMD is the most common pediatric MD, affecting 1 in 5000 boys due to its recessive X-linked inheritance [4]. It is a life-limiting disorder resulting from mutations in the *DMD* gene encoding dystrophin [1, 5]. Dystrophin is a subsarcolemmal protein integral to the dystrophin glycoprotein complex which protects muscle from contraction-induced injury [6, 7]. *DMD* mutations generate out-of-frame transcripts, abolishing dystrophin expression and culminating in muscle atrophy [4]. In contrast, Becker muscular dystrophy (BMD) patients harbor *DMD* mutations which maintain the reading frame, producing a truncated yet partially functional dystrophin that results in a milder disease course [8, 9]. A major clinical goal is converting DMD mutations into BMD-like mutations to restore the dystrophin open reading frame (ORF) and improve the disease phenotype [10]. As the bulk of published in vivo MD genome editing studies are on DMD, this review will primarily summarize recent progress for DMD.

MDC1A is an autosomal, recessive neuromuscular disorder, characterized by neonatal onset of hypotonia, muscle weakness, and muscle wasting [1, 11]. Mutations in the *LAMA2* gene, which encodes laminin-α2, cause MDC1A [11, 12]. Laminins are extracellular matrix proteins which form complexes and are essential basement membrane components [13].

DM1 is an autosomal dominant condition affecting 1 in 8000 individuals and is the most common adult-onset MD [2, 14]. Apart from muscle weakness and stiffness, patients can develop intellectual impairment, respiratory insufficiency, and cardiac conduction abnormalities [2, 14]. A cytosine, thymine, guanine (CTG) repeat expansion in the 3′UTR of the dystrophia myotonica protein kinase (*DMPK)* gene causes DM1 [2]. These transcripts form nuclear foci which cause deleterious splicing defects in numerous pre-mRNAs [14].

Genome Editing Strategies

Various targeted MD therapies are currently approved or in clinical trials. These include gene therapy, antisense oligonucleotides (AONs), and stop codon read-through compounds [15–19]. However, relatively poor performance has hindered their widespread clinical use. An effective therapy must target and, ideally, permanently correct the genetic source of MDs. CRISPR/Cas9-based interventions hold extreme promise due to their unparalleled utility and precision in performing targeted genome editing [5, 20–22].

CRISPR/Cas9 Technology as a Genetic Engineering Tool

Genome editors include zinc finger nucleases, TALENs, and meganucleases, but their use has waned in favor of the more practical CRISPR/Cas9 [23]. The CRISPR/Cas9 system was first discovered in bacteria and archaea as an antiviral defense mechanism and has been repurposed as a programmable genome editor [24•, 25]. The most utilized type II CRISPR system has two components: a single guide RNA (sgRNA) with a region complementary to a target sequence and a Cas9 endonuclease [26]. Once guided to its DNA target by an sgRNA, Cas9 generates a double-strand break (DSB) upstream of its protospacer adjacent motif (PAM) [24•]. The most popular Cas9 from *Streptococcus pyogenes* (SpCas9) uses an NGG or NAG PAM [24•]. The smaller *Staphylococcus aureus* Cas9 (SaCas9) recognizes NNGRR(T), while *Campylobacter jejuni* Cas9 (CjCas9) uses an extended NNNNRYAC PAM [27]. Numerous other Cas9s have been discovered or developed, ensuring the availability of a suitable system for nearly any application [27].

DSB Resolution by HDR and NHEJ

The power of CRISPR/Cas9 for genome editing comes from site-specific DSB generation. This DSB induces DNA repair pathways which can be utilized to yield desired genomic modifications. Depending on the proliferative status of the cell and the presence of an exogenous DNA template, the DSB will be repaired by homology directed repair (HDR) or non-homologous end joining (NHEJ) [24•, 28, 29].

HDR results in faithful resolution of the DSB but is typically restricted to S/G2 phases of proliferating cells [30]. HDR proceeds by homologous recombination, enabling knock-in of complete or partial wild-type genes [30]. Therefore, for

CRISPR to initiate HDR, a DNA template with homology to the regions flanking the DSB must be provided alongside the Cas9 and sgRNA [24•]. Insertion of exogenous DNA is a powerful therapeutic strategy for MD. Unfortunately, an enormous barrier to applying HDR is its poor efficiency in post-mitotic muscle cells [29, 31].

In the absence of a DNA template, Cas9-induced DSBs are typically repaired via NHEJ. NHEJ is the primary DSB repair pathway, with the free ends being directly ligated. Imprecise repair can introduce random insertions and/or deletions (indels) [24•]. NHEJ-based strategies have been used to correct splicing and excise mutated sequences like duplications and out-of-frame exons [32•, 33–36].

Applications of a Nuclease Deficient Cas9

A groundbreaking application of CRISPR/Cas9 has been the manipulation of gene expression with a catalytically inactive or "dead" Cas9 (dCas9) [24•, 35]. While DNA binding is retained, dCas9 cannot generate DSBs. Gene repression is achievable by targeting dCas9 to regulatory elements, sterically hindering transcription machinery [37]. Expression can alternatively be activated or enhanced by fusing transcriptional activators to dCas9 and localizing them to promoters [29].

Single nucleotide mutations can be corrected without DSBs through base editing [38]. A Cas9 capable of generating single-stranded DNA (ssDNA) breaks, called a nickase, is fused to a nucleobase deaminase which can chemically alter a base [38]. Current deaminase enzymes are restricted to C-to-T and A-to-G transitions, limiting which point mutations can be corrected [38, 39]. Unfortunately, base editors risk off-target editing when adjacent bases, identical to the target, are present [39]. Nevertheless, when correctly applied, base editing can restore full-length protein expression.

NHEJ-Mediated Restoration of a Truncated Dystrophin Protein

As previously mentioned, one promising strategy is converting DMD mutations into BMD-like variants, with the expectation that disease phenotype will improve. The most straightforward method to restore the reading frame is by Cas9-mediated deletion, skipping, or reframing of exons via NHEJ (Fig. 1a). It is important to note that while the following approaches are applicable to a wide range of DMD patients, converting DMD into BMD can only reduce disease severity, not cure it. Table 1 provides a comprehensive summary of the Cas9 variants, delivery vectors, routes of administration, and treatment regimens from these studies.

Exon Deletion

The deletion of one or more exons with a pair of flanking sgRNAs has been demonstrated to effectively restore the *DMD* ORF. Single exon deletion has been accomplished in DMD mice [40–42, 52, 60, 61, 62•, 64] and, more recently, pigs [57••]. Moretti et al. induced robust dystrophin expression in a Δ52 DMD pig model following systemic administration of SpCas9 and a pair of sgRNAs to achieve exon 51 deletion [57••]. Only two other studies using CRISPR/Cas9 in large DMD animals have been conducted [54•, 58]. Data from these studies have provided compelling support for the clinical utility of gene editing in DMD patients. The deletion of multiple exons has also been demonstrated in DMD mice, a strategy which could be applied across a greater range of mutations [36, 43, 44, 45•, 47, 48, 63]. For additional information on this topic, we invite the reader to consult additional reviews [5, 68–70].

Exon Skipping and Reframing with a Single sgRNA

Simultaneous DSBs produced by sgRNA pairs increase the risk of unintended insertions, deletions, and other complex rearrangements [71, 72]. A single sgRNA targeting the splice acceptor or donor site of a frameshifted exon can restore the ORF while minimizing undesirable mutations. NHEJ indels will disrupt the splice site, causing omission of the frameshifted exon from the mature mRNA [73]. Another option is to target an sgRNA near a premature stop codon. Indels could remove the stop codon and reframe the transcript. Exon skipping and reframing using CRISPR/Cas9 has been accomplished in DMD mice [50, 52, 55, 56, 59, 74] and dogs [54•].

CRISPR-Mediated Restoration of a Full-Length Protein

To truly correct MD, restoration of the full-length protein is required. To achieve this, CRISPR/Cas9 editing typically requires HDR (Fig. 1b) or base editing (Fig. 1d) or, in unique circumstances, NHEJ. Most strategies are mutation specific and are therefore not broadly applicable, which may provide barriers for rapid regulatory approval.

Gene Correction Via HDR

Unfortunately, HDR is highly inefficient in muscle, and as such only three studies have applied CRISPR/Cas9-mediated HDR in vivo [45•, 49, 58]. Bengtsson et al. achieved an HDR rate of 0.18% following intramuscular injection of SpCas9 and a repair template into the *mdx*^{4c} DMD mouse model [45•]. Interestingly, HDR occurred in

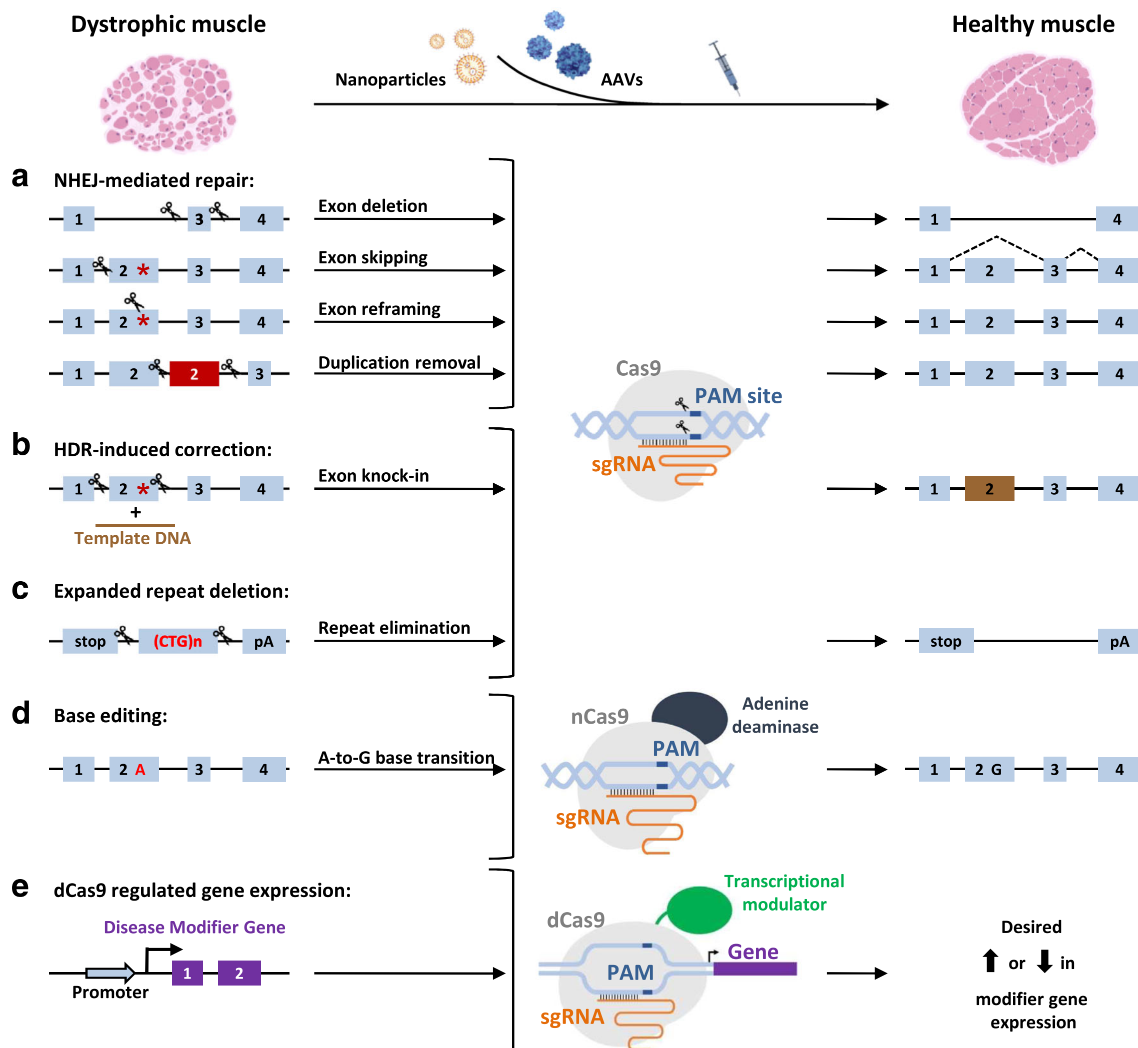

Fig. 1 CRISPR/Cas9-mediated genome editing strategy for the treatment of MDs. On the left side are gene stretches shown with an exon 2 deletion mutation, a nonsense mutation (*), an exon 2 duplication or expanded CTG repeats in the 3′UTR region. **a** Non-homologous end joining (NHEJ)-meditated exon deletion, exon skipping, exon reframing, or duplication removal. **b** CRISPR/Cas9-induced gene correction via homology-directed repair (HDR)-mediated exon knock-in with an additional DNA template. **c** CRISPR/Cas9-mediated targeted elimination of expanded repeats. These (CTG)n repeats are located in 3′UTR of a gene between the stop codon (stop) and the polyadenylation signal (pA). **d** A-to-G base transition carried out by CRISPR/nCas9 attached with an adenine deaminase. **e** CRISPR/dCas9-mediated gene regulation. Gene expression of a disease modifier gene can be modulated by CRISPR/dCas9 fused with a transcriptional modulator

CRISPR, clustered regularly interspaced short palindromic repeats; AAV, adeno-associated virus; PAM, protospacer adjacent motif; nCas9, Cas9 nickase; dCas9, catalytically deficient Cas9

a fraction of myogenic cells, but not satellite cells due to promoter choice. Lee et al. achieved a 0.8% HDR frequency in *mdx* mice, demonstrating HDR worked, albeit inefficiently, in muscle [49]. In the third study, a splice site mutation was corrected in GRMD dogs using HDR, which yielded dystrophin recoveries between 2 and 16% [58].

To date, HDR-based strategies have yet to restore clinically relevant levels of dystrophin, which precludes their consideration as an MD therapy. These studies highlight the limitations of HDR and that novel approaches to enhance its efficiency in muscle, such as by upregulation of HDR factors, are necessary.

NHEJ-Mediated Intronic Deletion to Restore Correct Splicing

The dy^{2J}/dy^{2J} MDC1A mouse model has a *LAMA2* donor splice site mutation in intron 2 which results in aberrant exon 2 skipping, yielding a truncated and unstable protein [75]. Kemaladewi et al. used two sgRNAs to excise a genomic region that included the defective splice site and harnessed NHEJ repair to restore a functional donor site [32•]. Systemic administration of SaCas9 and sgRNAs into neonatal pups resulted in robust restoration of full-length laminin-α2 in muscle and sciatic nerve, improving motility and paralysis. Given the diversity of Cas9 enzymes with different PAM specificities, NHEJ splice site

Table 1 Summary of studies that have used CRISPR/Cas9 approaches for the in vivo treatment of MDs

Reference	Animal model/s	Strategy	Age	Application	Delivery	Cas enzyme and sgRNA	Highlights
DMD: short-term analysis (≤ 6 months after treatment)							
Long et al. [40]	mdx mouse; stop codon in exon 23	Exon 23 skipping; NHEJ	P1, P12, P18	IP, IM in TA, RO	AAV9	SpCas9 + 2 sgRNAs (exon 23 and intron 23); 2 vectors	All injection methods led to dystrophin expression in myofibers (1.4–7.7%) and CMs (1.8–25%), and RO is the best. Dystrophin expression increased over time. IP injection improved grip strength
Nelson et al. [41]	mdx mouse; stop codon in exon 23	Exon 23 skipping; NHEJ	P2, 6–8 we	IM in TA, IV, IP	AAV8	SaCas9 + 2 sgRNAs (intron 22 and 23); 2 vectors	All injection techniques led to dystrophin expression in myofibers; intramuscular injection restored localization of DGC proteins and nNOS activity at the sarcolemma and increased muscle function (specific twitch and tetanic force)
Tabebordbar et al. [42]	mdx (stop codon in exon 23);Ai9 mouse, Pax7-Zsgreen ± mdx; Ai9 mouse	Exon 23 skipping; NHEJ	P3, 6 we	IM in TA, IP, IV (tail vein)	AAV9	SaCas9 + 2 sgRNAs (intron 22 and 23); 2 vectors	Dystrophin expression in myofibers was restored following all injection methods; DGC proteins and nNOS were relocated to the sarcolemma. This study was able to edit satellite cells
Iyombe-Engembe et al. [43]	hDMD/mdx mouse (stop codon in exon 23)	Deletion of exon 50–54; hybrid exon; NHEJ	–	IM in TA	Electroporation	SpCas9 + 2 sgRNAs (exons 50 and 54); 2 vectors	First study editing the human *DMD* gene in vivo. Hybrid exon formation thought to preserve dystrophin rod domain structure better
Xu et al. [44]	mdx mouse; stop codon in exon 23	Skipping of exon 21–23; NHEJ	P1–3, 2 mo	IM in GA, Qua and FDL	Electroporation/AdV transduction	SpCas9 + 2 sgRNA (intron 20 and 23)	Electroporation experiments normalized osmotic shock-induced Ca^{2+} sparks. AdV transduction led to 50% of Wt dystrophin expression and reduced the EBD uptake (with or without treadmill exercise)
Bengtsson et al. [45•]	mdx 4cv mouse; stop codon in exon 53	Strategy 1, deletion of exon 52 + 53, or strategy 2, deletion of a part of exon 53; NHEJ	2, 10–12 we	IM in TA, RO	AAV6	SaCas9, SpCas9 + 2 sgRNAs (strategy 1, introns 51 and 53, and strategy 2, exon 53: 5′ and 3′); 2 vectors	Multiple muscle-specific AAV-CRISPR/Cas9-driven gene editing strategies by using dual- or single-vector approaches. Systemic delivery yielded widespread dystrophin expression in the heart (34% of CMs). Injection of the high virus dose generated dystrophin expression in all analyzed muscle tissues
		Strategy 2: HDR, mutation correction	2, 10–12 we	IM in TA	AAV6	SpCas9 + 2 sgRNAs (exon 53: 5′and 3′); 2 vectors	Successful HDR was detected in 0.18% of total genomes; 8% of the edited genomes resulted from HDR. HDR occurred in a fraction of myogenic cells, but not in satellite cells
Perrin et al. [46]	rag/mdx mouse (stop codon in exon 23)	Upregulation of Lama1	4 mo	IM in TA	Electroporation	dSpCas9-VP160 + 1 sgRNA	Overexpression experiments caused heterogeneous distribution of the Lama 1 protein. Expression of alpha7 and beta1 integrins expression were not increased
Young et al. [47]	hDMD/mdx and hDMD/mdxD2 mice with hDMD exon 45 deletion	Deletion of exons 45–55; NHEJ	12 we/18.5 we	IM in FDB	Electroporation	SpCas9 + 2 sgRNA (introns 44 and 55)	First study targeting the human DMD/*mdx* mice models (with exon 45 del.) with the CRISPR system. Successful deletion of multiple exons (exon 45–55)
Refaey et al. [48]	mdx mouse and mdx/Utrn ± mouse; stop codon in exon 23	Skipping of exon 21–23; NHEJ	P1–3, 16 we	TV, RO, IP, IV (tail vein)	Adenovirus/AAVrh74	SpCas9 + 2 sgRNA (intron 20 and 23); 2 vectors	Adenovirus-CRISPR-delivery induced limited cardiac dystrophin expression shown by IF, PCR, and WB. After AAVrh74 treatment, 40% of CMs were dystrophin+, cardiac fibrosis was reduced, and contractility of papillary muscles was improved
Lee et al. [49]	mdx mouse; stop codon in exon 23	HDR correction	4 we	IM in TA and GA	Gold nanoparticles	SpCas9 RNP + 1 sgRNA (exon23)	CRISPR-Gold treatment led to a 5.4% HDR correction with cardiotoxin co-injection and to 0.8% HDR

Table 1 (continued)

Reference	Animal model/s	Strategy	Age	Application	Delivery	Cas enzyme and sgRNA	Highlights
							correction without CTX co-injection, which increased the hanging time
Amoasii et al. [50]	deltaEx50 mouse	Reframing and skipping of exon 51; NHEJ	P4, P12	IP, IM in TA	AAV9	SpCas9 + 1 sgRNA (exon 51 splice acceptor site); 2 vectors	Single-cut genome editing by targeting the splice acceptor site led to exon reframing mainly by insertion of a single adenosine. The first study which used muscle-specific Cas9 expression and 3 different RNA poly III promoters for guide expression. Systemic application reduced the serum CK concentrations and relocated the DGC proteins to the sarcolemma. Cas9-treated mice exhibited improved grip strength 4 weeks after injection
Liao et al. [51]	Cas9/mdx mouse (stop codon in exon 23)	Upregulation of utrophin	P2.5, 3 we	IM in (fore+)hind limbs	AAV9	SpCas9 + 1 sgRNA; 2 vectors	Intramuscular injection increased utrophin expression and this improved hind-limb grip strength
Koo et al. [52]	DMD knockout mouse with deletion in exon 23	Reframing by insertion of indels; NHEJ	8 we	IM in TA	AAV2/9	CjCas9 + 1 sgRNA (exon 23)	CjCas9 nuclease targeted the *Dmd* gene in skeletal muscles in a highly specific manner, without any detectable off-target effects in vivo. CRISPR-treated mice demonstrated robust dystrophin expression (IF: 26–39%) and an increased specific maximal force
Ryu et al. [53]	DMD mouse with a nonsense mutation in exon 20	Base editing	7 we	IM in TA	AAV2/9	nSpCas9-ABE7.10 + 1 sgRNA (exon 20); 2 vectors	Treatment of a dual trans-splicing adeno-associated virus (tsAAV) vector system restored dystrophin expression in 17% of myofibers and re-localized nNOS to the sarcolemma
Amoasii et al. [54•]	deltaE50-MD dog	Exon 51: reframing (insertion of A) and skipping; NHEJ	4 we	IM in cranial tibialis muscle; intravenous	AAV9	SpCas9 + 1 sgRNA (exon 51 splice acceptor site); 2 vectors	First study applying the CRISPR/Cas9 system to treat a large DMD animal. IM injection did not change the amount of infiltrating immune cell, but it relocated ß-dystroglycan to the sarcolemma. IV injection of a high viral dose increased dystrophin expression (WB: 3–92%) and slightly decreased serum CK activity
Duchêne et al. [36]	del52hDMD/mdx mouse (stop codon in exon 23)	Deletion of exon 47–58; hybrid exon; NHEJ	4–5 we	IV (tail vein)	AAV9	SaCas9 + 2 sgRNAs (exons 47 and 58); 2 vectors	The genomic deletion to restore ORF produced a truncated dystrophin with normally phased spectrin-like repeats. Dystrophin is expressed in CM, but not in TA or diaphragm
Min et al. [55]	deltaEx44 DMD	A insertion; exon 45 skipping; NHEJ	P12	IM in TA	AAV9	SpCas9 + 1 sgRNA (exon 45); 2 vectors	First study highlighting the importance of the dosages of the gene editing components for optimal gene correction. Cas9 expression is affected by the amount of sgRNA present, and thus, sgRNA is the limiting factor for successful in vivo editing. Systemic application of a 1:10 ratio of Cas9:sgRNA led to 90–95% of WT dystrophin expression in CMs and myofibers
Amoasii et al. [56]	deltaEx50-Dmd-Luc	Exon 51 reframing (insertion of a single nucleotide); NHEJ	P4, 12	IP, IM in TA	AAV9	SpCas9 + 1 sgRNA (exon 51 splice acceptor site); 2 vectors	Novel in vivo noninvasive bioluminescence monitoring of dystrophin correction post-treatment. Gene editing occurs within the first week after injection, and 10 weeks after injection, the

Table 1 (continued)

Reference	Animal model/s	Strategy	Age	Application	Delivery	Cas enzyme and sgRNA	Highlights
							bioluminescence level was at 70% of WT
Moretti et al. [57••]	DMD delta52 pig	Deletion of exon 51; NHEJ	10–14 d, 4 we	IM in fore- and hindlimbs, IV in ear vein	AAV9, G2-AAV9	SpCas9 (Cas9-intein half) + 2 sgRNAs (introns 50 and 51); 2 vectors	First published study on dystrophin gene correction in a pig model. Increased myotropism by coating AAV9 with G2-PAMAM nanoparticles. Treated pigs showed increased muscle function, reduced CK levels, and prolonged survival
Mata López et al. [58]	GRMD dog; mutation in intron 6 acceptor splice site	HDR correction	3 mo–8 y	IM (together in CT, LDE and PL)	–	SpCas9 + 2 sgRNAs (intron 6 and exon 7)	Although intramuscular injection led to inclusion of exon 7 for all CRISPR/Cas9 treatments, dystrophin protein restoration (WB: 2–16%) was minimal. TALEN did not restore any dystrophin expression
Zhang et al. [59••]	deltaEx44 DMD mouse	Insertion of 1 nt or deletion of 2 nt to reframe exon 45; exon 45 skipping; NHEJ	P4	IP	Single-stranded AAV9, self--complementary AAV9	SpCas9 + 1 sgRNA (exon 45); 2 vectors	Efficient genome editing by using a self-complementary AAV delivery system requires at least a 20-fold lower dose than with single-stranded AAV. Dystrophin restoration is viral vector dose dependent. More than 60% of the NHEJ events contained a + 1-nt insertion
Gee et al. [60]	NOG-mdx (NOD.Cg-Prkdc^{scid} Il2rg^{tm1Sug}Dmd^{mdx}/-Jic) mouse (stop codon in exon 23)	Exon 23 skipping; NHEJ	–	IM in TA	NanoMEDIC	SpCas9 + 2 sgRNAs (exon 23 near the splice acceptor and donor site); 2 vectors	Usage of all-in-one EV delivery system termed NanoMEDIC (nanomembrane-derived extracellular vesicles for the delivery of macromolecular cargo). IM treatment caused 1.1% large genomic DNA deletion (194 bp) between the two sgRNA target sites
DMD: long-term analysis (≥ 12 months after treatment)							
Hakim et al. [61]	mdx mouse; stop codon in exon 23	Exon 23 skipping; NHEJ	6 we	IV, IM in TA	AAV9	SaCas9 + 2 sgRNAs (intron 22 and 23); 2 vectors	First study evaluating the long-term systemic AAV-CRISPR therapy in *mdx* mice. They observed disproportional sgRNA vector depletion after systemic application. 18 month-old, Cas9-treated mice exhibited 20% and 2% of WT dystrophin levels in the heart and skeletal muscle, respectively
Nelson et al. [62•]	mdx mouse; stop codon in exon 23	Exon 23 skipping; NHEJ	P2, 8 we	FVI, IP, IM in TA, IV	AAV8, AAV9	SaCas9 + 2sgRNAs (intron 22 and 23); 2 vectors	AAV-CRISPR is immunogenic when administered to adult mice, but this can be avoided by treating neonatal mice. Quantifiable and heterogeneous genome-editing events (deletions, inversions, indels, AAV integrations) at the on-target *Dmd* locus occurred in all treated mice
Xu et al. [63]	mdx mouse; stop codon in exon 23	Exons 21–23 skipping; NHEJ	P3	IP	AAVrh74	SaCas9 + 2 sgRNA (intron 20 and 23); 2 vectors	CRISPR-Cas9-induced DSBs were mainly repaired by the precise ligation of the two cut sites. No signs of tumor development or other deleterious defects. All injected mice developed substantial humoral immune response against AAV viral particles, but not against SaCas9. The analysis was restricted to the heart and only 11% of CMs were dystrophin^{+} by IF
Nance et al. [64]	mdx mouse (stop codon in exon 23) or NGS.mdx4cv mouse	Exon 23 skipping; NHEJ	6 we	IV (tail vein)	AAV9	SaCas9 + 2 sgRNAs (introns 22 and 23); 2 vectors	Only study using a free muscle graft model to investigate muscle stem cell editing efficiency. 22.4% of the TA muscle fibers were dystrophin^{+} 17 months post-treatment

Table 1 (continued)

Reference	Animal model/s	Strategy	Age	Application	Delivery	Cas enzyme and sgRNA	Highlights
	(stop codon in exon 53)						
MDC1A							
Kemaladewi et al. [32•]	dy^{2J}/dy^{2J} mouse of MDC1A; point mutation in intron 2 of *LAMA2*	Correction of slice donor site; NHEJ	P2, 3 we	IM in TA, IP, IV	AAV9	SaCas9 + 2 sgRNAs (exon 2 and intron 29); 2 vectors	First study using the CRISPR/Cas9 system to correct a *Lama2* splice site mutation. Cas9-treated mice exhibited reduced of fibrosis and increased muscle fiber size. The systemic application successfully edited muscle and sciatic nerve
Kemaladewi et al. [65••]	dy^{2J}/dy^{2J} mouse of MDC1A; point mutation in intron 2 of *LAMA2*	Upregulation of Lama1	P2, 3 we	IM in TA, TV, IV	AAV9	dSaCas9-VP64 + 1 sgRNA; 1 vector	Lama1 overexpression to compensate for the Lama2 deficiency. Treatment of older, symptomatic mice improved and effectively reversed disease progression
DM1							
Pinto et al. [66]	HSA^{LR} mouse; human skeletal actin transgene containing 250 CTG repeats	Decreased transcription of the expanded microsatellite repeats	P2	TV	AAV2/6, AAV2/9	dSpCas9 + 1 sgRNA; 1 vector	First published study on treating a DM1 mouse model with dCas9 to inhibit transcription. 5–15% of fibers showed complete loss of CUG RNA foci
Lo Scrudato et al. [67]	DMSXL mouse; human DMPK gene with 1200 CTG repeats (45 kb)	Deletion of expanded CTG repeats; NHEJ	3, 5–9 we	IM in TA	rAAV9	SaCas9 + 2 sgRNAs; 2 vectors	IM injection of homozygous mice caused expression of SaCas9 and GFP in 18% of myonuclei. These mice did not show any sign of damaged muscle tissue

DMD, Duchenne muscular dystrophy; MDC1A, muscular dystrophy type 1A; DM1, myotonic dystrophy type 1; CRISPR, clustered regularly interspaced short palindromic repeats; dCas9, catalytically inactive Cas9; NHEJ, non-homologous end joining; HDR, homology-directed repair; AAV, adeno-associated virus; AdV, adenovirus; IM, intramuscular; IP, intraperitoneal; IV, intravenous; RO, retroorbital; TV, temporal vein; TA, tibialis anterior; FDB, flexor digitorum brevis; GA, gastrognemius; Qua, quadriceps, FDL, flexor digitorum longus; CT, cranial tibialis; LDE, long digital extensor, PL, peroneus longus; WB, western blot; IF, immunofluorescence staining; TALEN, transcription activator-like effector nuclease; nNOS, neuronal nitric oxide synthase; sgRNA, single guide RNA; Utrn, utrophin; P, postnatal day; d, days; we, weeks; mo, months; y, years; DSB, double-strand break; del, deletion; CTX, cardiotoxin

restoration may be applicable to a variety of pathogenic splice site mutations [27].

Therapeutic Base Editing to Correct Point Mutations

The correction of a specific mutation via the CRISPR/Cas9 system can also be accomplished with base editing. In the context of DMD, Ryu et al. utilized this method to correct a nonsense mutation and restore full-length dystrophin [53]. With an adenine base editor (ABE), the nonsense mutation was converted into glutamine via an A-to-G transition. Intramuscular injection of the Cas9-ABE led to dystrophin restoration in 17% of myofibers.

While base editing can currently correct only a limited group of mutations, this study represents a milestone in demonstrating its feasibility in treating DMD without truncating dystrophin.

Modulation of Gene Expression

Gene editing strategies are applicable to MD patients harboring deletion, insertion, duplication, or point mutations. However, those lacking large genomic regions or possessing complex mutations require alternative therapeutic strategies. The application of CRISPR/Cas9 to modulate gene expression holds great promise for MD. It is primarily mutation independent, allowing for broad applicability. Typically, dCas9 is used, bypassing concerns for undesirable on- and off-target mutations resulting from DSBs [76]. Both the mutated gene and disease modulators can be targeted for modulation and multiplex regulation is possible (Fig. 1e) [76].

Activation and Upregulation of Disease Modifiers

In *mdx* mice, expression of utrophin, a homologue of dystrophin, can partially compensate for dystrophin deficiency [77]. Building on pioneering work by Wojtal et al., who achieved utrophin upregulation in DMD patient derived myoblasts [33], Liao et al. used a Cas9 activation system in *mdx* mice to upregulate utrophin [51]. For the first time in vivo, Cas9-mediated upregulation of the disease modifier utrophin was shown to improve the dystrophic phenotype.

Laminin-α1 protein is structurally similar to laminin-α2 and can compensate for its loss in MDC1A [78]. However, *LAMA1*, which encodes laminin-α1, is only expressed during embryogenesis [11]. Perrin et al. demonstrated that laminin-α1 expression can be induced by intramuscular delivery of dCas9-VP160 in *mdx* mice [46]. Recently, Kemaladewi et al. showed that systemic administration of dCas9-2xVP64 in neonatal dy^{2J}/dy^{2J} pups prevented muscle fibrosis and hindlimb paralysis [65••]. Furthermore, treatment of older, symptomatic mice resulted in drastic clinical improvements and effectively reversed disease progression.

Results from these studies demonstrate that upregulation of disease modifiers is a valuable therapeutic approach which could improve and possibly partially reverse some MDs. Additional studies are required to provide more insight into the long-term persistence and efficacy of transcriptional modulation.

Cas9 Interference (Deletion and Transcriptional Repression)

To treat DM1, the formation of nuclear foci from expanded DMPK transcripts must be prevented. This can be achieved by deletion or transcriptional repression of the expanded CTG repeats (Fig. 1c).

The DMSXL DM1 mouse model carries the human *DMPK* gene with > 1000 CTG repeats [79]. Lo Scrudato et al. decreased the number of pathological RNA foci within myonuclei by deleting the CTG repeats by intramuscular injection of SaCas9 and two sgRNAs. This provided compelling evidence that genome editing to remove a large trinucleotide expansion was a feasible strategy for treating DM1 afflicted muscle.

The transgenic HSA^{LR} mouse carries a fragment of the human skeletal actin (HSA) gene with 250 CTG repeats in the 3′UTR [79]. Pinto et al. treated these mice systemically with dCas9 targeted to CTG repeats, blocking their inclusion in HAS transcripts. They observed a notable decrease in repeat transcription and an improved phenotype, validating the therapeutic potential of dCas9 repression [66].

In Vivo CRISPR Delivery Systems

CRISPR/Cas9 gene editing strategies are composed of two elements: the editing machinery and the delivery system. Efficient treatment of MDs will require robust expression of CRISPR/Cas9 components throughout skeletal and cardiac muscle. As such, we will now cover the two predominant in vivo vectors for MD genome editing.

AAVs

The ssDNA adeno-associated viruses (AAVs) are strong candidates for use in CRISPR/Cas9 MD therapies. While lentiviruses and adenoviruses have been used as delivery vehicles of therapeutic components in the past, AAVs are becoming the frontrunners for efficient and safe systemic delivery to muscle. Unfortunately, even AAVs cannot be re-administered without significant intervention due to the production of neutralizing antibodies after treatment. For detailed information on AAV-based therapies, we refer to these excellent reviews [80••, 81–83].

At present, high AAV titers are required for MD therapies; thus, efforts have been made to reduce the effective dose, with some success. Moretti et al. coated AAV9s in polyamidoamine dendrimers which significantly increased skeletal and cardiac muscle transduction in DMD pigs [57••]. These dendrimers are suspected to enhance cellular uptake through electrostatic interactions with cell surfaces. Increasing the sgRNA-to-Cas9 ratio by encoding multiple sgRNA copies on a second AAV has enhanced corrective DMD exon skipping. Unexpectedly, Hakim et al. observed disproportionate depletion of sgRNA encoding AAVs following systemic administration [61]. Min et al. studied this phenomena in detail and concluded that optimization of the AAV ratio for dual-AAV strategies was necessary to minimize the impact of sgRNA loss on editing efficiency [55]. Zhang et al. circumvented this problem by packaging sgRNAs in self-complementary AAV (scAAVs), enabling efficient editing without sgRNA depletion [59••].

It is possible that encoding multiple, identical sgRNA sequences as ssDNA can cause vector loss. Upon reaching the nucleus, ssDNA of the AAV stabilizes by synthesizing its complementary strand, a step the dsDNA scAAVs skip [84]. AAVs also package plus and minus strands equally, allowing direct annealing and skipping second strands synthesis. We speculate that repetitive sgRNA sequences may result in mis-annealing between plus and minus strands, yielding unstable DNA species which are degraded. While this hypothesis is untested, it could explain why scAAVs were not disproportionally lost. Assessing if specific depletion of sgRNA encoding vectors occurs when only a single sgRNA cassette is present would shed light on this issue.

Nanoparticles

Nonviral nanoparticles are an attractive alternative to AAVs for CRISPR/Cas9 delivery. They can deliver DNA, RNA, or proteins for transient expression, are extremely diverse, and allow for re-treatment, giving them enormous potential [85, 86].

Gold nanoparticles (GNPs) are easily taken up by cells and bind both DNA and protein [49]. CRISPR-Gold consists of a GNP conjugated with DNA to allow hybridization with the HDR repair template [49]. A Cas9/sgRNA ribonucleoprotein then associates with the DNA. Lee et al. used CRISPR-Gold to restore full-length dystrophin via HDR in *mdx* mice [49]. Intramuscular injection of CRISPR-Gold corrected the mutated dystrophin gene to the wild-type sequence with an HDR frequency of 0.8%, which increased to 5.4% when cardiotoxin was used to induce muscle stem cell proliferation.

NanoMEDIC is a novel nanoparticle formulation consisting of vesicles purified from genetically modified packaging cells [60]. They contain SpCas9 protein and transcribed sgRNAs which are also produced by the packaging cells. NanoMEDIC was utilized by Gee et al. to correct NOG-*mdx* mice through exon skipping [60]. Analysis following intramuscular injection revealed an exon skipping efficiency of 1.6%.

Nanoparticles are extremely promising AAV alternatives. Unfortunately, the necessary editing efficiencies to restore fully protective dystrophin levels (estimated at ~ 20% of normal) have not been realized with these systems [87, 88]. Additionally, all MD studies assessing nanoparticles have been limited to local administration. The inability to perform systemic delivery to muscle is a major barrier to moving past the preclinical stage.

Satellite Cell Genome Editing

Satellite cells are quiescent muscle stem cells which are activated by signals triggered by muscle growth, turnover, or damage. Activated satellite cells generate myoblasts via asymmetric division which fuse together to yield new myotubes or repair existing ones.

Satellite cells are a critical target of MD genome editing strategies. If they remain uncorrected, fusion of their progenitor cells may render treatments ineffective due to dilution and loss of corrected nuclei within the myofiber [80••, 89]. While this may not be problematic for several years due to the slow rate of muscle turnover, it will be over the course of a patient's life [80••]. Additionally, as already revealed with the role of dystrophin in satellite cells, other MD genes may also be essential for stem cell function and will require correction in these cells [90]. Therefore, future CRISPR/Cas9 MD strategies will likely need to target satellite cells to achieve long-lasting clinical benefits.

While in vitro correction of dystrophin in *mdx* satellite cells has been successful, in vivo results have been ambiguous. Arnett et al. reported rare transduction of satellite cells by AAV8 but none with AAV6 or AAV9 [91]. In contrast, Tabebordbar et al. achieved modest genome editing (~ 35%) of the satellite cell population with AAV9, whereas both Nance et al. and Goldstein et al. observed maximal genome modification rates of ~ 60% [42, 64, 92]. Due to significant differences in study design, the reasons behind these conflicting results are difficult to ascertain. Treatment age or injection routes might be factors as Goldstein et al. injected the AAVs systemically instead of intramuscularly. Transduction levels with Cre systems were notably higher than with CRISPR/Cas9, likely due to the superior editing efficiency of Cre. Despite the myriad of differentiating factors between these four studies, the most recent data is quite strong and suggests that AAVs, particularly AAV9, are capable of transducing satellite cells better than previously thought. While promising for AAV-based MD therapies, further validation is necessary.

Long-Term Considerations for the Use of In Vivo CRISPR Gene Editing

As the MD genome editing field embarks into new, uncharted territory, understanding the long-term impacts and effects is a necessity. A primary concern is the longevity of phenotypic rescue. Further research will be needed to answer this, though it is likely that without satellite cell correction, life-long benefits from a single treatment will be limited. This question is particularly relevant for MDs, as the optimal therapeutic window is early childhood, prior to severe disease progression. Rapid muscle growth at this age may swiftly render non-satellite cell targeting CRISPR therapies ineffective.

Important consideration must be given to the host's immune system, owing to the introduction of several antigens by CRISPR/Cas9 treatments: the restored therapeutic protein, Cas9, and AAV capsid in the case of viral delivery. Restoring a protein which was absent during elimination of self-reactive lymphocytes can trigger immune responses. Anti-dystrophin antibodies and immune-system rejections have been noted in DMD animals and patients given corrective therapies [93–97]. Pre-existing Cas9 immunity has also been extensively reported; thus, caution should be taken towards Cas9 immunogenicity since AAV episomes can remain for at least a decade in human muscle, enabling prolonged expression of CRISPR machinery [98]. Xu et al. however demonstrated that at 19 months post-treatment, *mdx* mice developed a humoral response to the AAVs, not Cas9 [63]. Perhaps Cas9 immune responses are only transient in nature. Nevertheless, transient expression by nanoparticles is ideal, but they are far from ready for clinical use. Thus, rigorous safety monitoring will be essential during clinical trials to mitigate immune reactions in MD patients. Checking for Cas9 neutralization by the immune system should also be conducted to ascertain therapy effectiveness.

Recently, Nelson et al. revealed severe underestimations of AAV integration following Cas9 DNA cleavage [62•]. AAVs can integrate into the AAVS1 site of mammalian genomes at low frequencies [99, 100]. However, Nelson and colleagues demonstrated significant integration at on-target DSBs generated by Cas9 [62•]. This finding suggested that AAVs as delivery vehicles for genome editing applications are less benign than previously thought, though the authors still observed substantial dystrophin recovery in their *mdx* mice with no noted toxicity [62•]. On the other hand, 19 months post-treatment, Xu et al. found that the CRISPR-Cas9-induced DSBs were mainly repaired by the precise ligation of the two cut sites [63]. Further investigation into the impact of Cas9 on AAV integration is imperative to understanding the consequences of genomic incorporation of CRISPR systems.

Conclusions and Future Directions for the MD Gene Editing Field

Recently, pioneering advancements in CRISPR/Cas9 utilization have opened numerous new therapeutic opportunities for MD. There is palpable optimism in the field, but new challenges lie ahead, and the future of MD gene editing will be contingent on surmounting them. The ambiguity surrounding AAV transduction of satellite cells in vivo needs to be resolved, so focus can be appropriately directed towards optimizing vector targeting if necessary. It is straightforward to see the need for satellite cells correction, but it must be experimentally established if extensive muscle turnover will negatively impact CRISPR strategies.

The poor editing efficiency of HDR in muscle is another problematic area. Efficient integration of exogenous DNA would open a myriad of new CRISPR applications, even beyond MD. Either canonical HDR must be improved or alternative mechanisms will need to be developed. The post-mitotic nature of myotubes and dormant satellite cells suggests that the latter may be most successful. Three promising novel approaches are homology-independent targeted integration (HITI), homology-mediated end joining (HMEJ), and prime editing. HITI leverages NHEJ, achieving absolute knock-in rates of 3.4% and 10% in murine heart and quadriceps respectively after systemic AAV9 administration [101]. Unfortunately, relative knock-in rates for whole muscles were not reported. HMEJ is speculated to proceed through single-strand annealing by employing a modified HDR DNA template which incorporates sgRNA sites for excision from its delivery vector [102]. AAV9 administration into the cortex of mice yielded a ~50% knock-in rate within the transduced neuron population [102]. The post-mitotic state of neurons suggests that such a knock-in rate may be achievable in muscle. Prime editing is a recent development which fuses Cas9 nickase to reverse transcriptase [103]. Using a 3′-extended pegRNA encoding the desired template, knock-in rates similar to HDR have been achieved in vitro [103]. However, validation in muscle, miniaturization of the system, and greater editing efficiencies are necessary for in vivo MD applications.

An alternative method to restore full-length proteins is through duplication removal. Duplications are the second most common DMD mutation, and an elegant, single sgRNA approach has been demonstrated to restore dystrophin [33, 34]. At this time, CRISPR/Cas9-mediated duplication removal has only been performed in DMD patient cells and must now be evaluated in vivo [33, 34].

The first generations of MD CRISPR therapies will likely utilize AAVs due to their proven track record in in vivo studies and clinical trials [17, 80••]. AAVs may be the best available option currently, but future therapies will likely require an alternate delivery system. It may come in the form of nanoparticles as they can deliver a variety of transiently expressed

cargo and are high modifiable. Their potential to avoid triggering immune responses will be particularly important if satellite cells cannot be corrected [104, 105]. Life-long rescue of MD would be possible through nanoparticle re-administration to mitigate the effects of muscle turnover. But, efficient in vivo editing with CRISPR via nanoparticle delivery has not been achieved in muscle. As this has not been a problem with AAVs, focus must be directed towards novel nanoparticle formulations rather than optimization of CRISPR/Cas9 systems. Systemic muscle delivery by nanoparticles remains a significant barrier as revealed by the lack of published studies. Local intramuscular injections are not feasible due to the amount of muscle in the human body and the need to target muscles within the thoracic cavity [106]. Development of an efficient, muscle-specific nanoparticle which can be delivered through circulation is paramount for future research.

The challenges ahead are arduous, but with the appropriate focus and investment of resources, solutions will arise, bringing therapeutic genome editing ever closer to the clinic. While this review was heavily centered on DMD, the discussed approaches and techniques can and undoubtedly will be applied to treating the plethora of other MDs.

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Compliance with Ethical Standards

Conflict of interest Monika Kustermann, Matthew J. Rok, Ronald D. Cohn, and Evgueni A. Ivakine declare that they have no conflict of interest.

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- • Of importance
- •• Of major importance

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