

Title

Application of CRISPR/Cas9-mediated genome editing for the treatment of myotonic dystrophy type I

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Abstract

Myotonic dystrophy type 1 (DM1) is a debilitating multisystemic disorder, caused by expansion of a CTG microsatellite repeat in the 3' untranslated region of the *DMPK* gene. To date, novel therapeutic approaches have focused on transient suppression of the mutant, repeat-expanded RNA. However, recent developments in the field of genome editing have raised the exciting possibility of inducing permanent correction of the DM1 genetic defect. Specifically, repurposing of the prokaryotic CRISPR/Cas9 (clustered, regularly interspaced, short palindromic repeats/CRISPR-associated protein 9) system has enabled programmable, site-specific, and multiplex genome editing. CRISPR-based strategies for the treatment of DM1 can be applied either directly to patients, or indirectly through the *ex vivo* modification of patient-derived cells, and include excision of the repeat expansion, insertion of synthetic cleavage and polyadenylation signals upstream of the repeat, steric interference with RNA polymerase II procession through the repeat leading to transcriptional downregulation of *DMPK*, and direct RNA targeting of the mutant RNA species. Potential obstacles to such therapies are discussed, including the major challenge of Cas9 and guide RNA transgene/ribonuclear protein delivery, off-target gene editing, vector genome insertion at cut sites, on-target unintended mutagenesis (e.g. repeat inversion), pre-existing immunity to Cas9 or AAV antigens, immunogenicity, and Cas9 persistence.

Keywords

DM1, myotonic dystrophy, gene editing, CRISPR, Cas9, AAV

Introduction

Myotonic dystrophy type 1 (DM1, also known as Steinert's disease, OMIM #160900) is an autosomal dominant, multisystem disorder characterised by myotonia, skeletal muscle wasting, insulin resistance, cardiac conduction abnormalities, and ocular defects. DM1 is one of the most common muscular dystrophies in adults with an incidence of ~1 in 8,000 worldwide ¹⁻³. In severe cases, DM1 can be fatal, as a consequence of respiratory insufficiency or cardiac failure ⁴. The genetic cause of DM1 is expansion of a CTG microsatellite (CTGexp) in the 3' untranslated region (UTR) of the *DMPK* (*dystrophia myotonica* protein kinase) gene located at 19q13.32 ⁵ (**Figure 1A**). The number of CTG repeats is positively correlated with disease severity, and negatively correlated with age of disease onset ⁶. Healthy individuals typically have 5-37 repeats, whereas affected individuals carry >50 repeats ^{1,7} (**Figure 1B**). The CTG repeat is unstable in both somatic and germline cells, with a bias towards expansion as opposed to contraction. CTGexp length tends to increase with age, consistent with the progressive nature of disease pathology, and DM1 patient tissues exhibit somatic repeat length mosaicism ⁸⁻¹⁰. Anticipation is observed such that the disease tends to manifest earlier, and with more severe pathology, in successive generations ^{11,12}. Inheritance of very large repeat expansions (e.g. multiple thousands of repeats) is associated with congenital DM1, severe pathology, and intellectual disability ^{13,14}.

A related condition, myotonic dystrophy type II (DM2), is similarly caused by a microsatellite expansion, in this case a CCTG repeat region located in the first intron of the *ZNF9* gene ¹⁵, and is beyond the scope of the present review.

The prevailing model of DM1 pathogenesis is that of toxic RNA gain-of-function. Mutant CUG-repeat-expanded (CUGexp) *DMPK* transcripts adopt hairpin structures (**Figure 1C**) and

form nuclear foci that sequester RNA binding proteins (RBPs) involved in the alternative splicing, leading to spliceopathy (**Figure 1D**)^{16,17}. These include MBNL1 (muscleblind-like splicing regulator 1) which exhibits a loss of function^{18,19}, and CELF1 (also known as CUGBP1) which is upregulated in DM1 muscle^{20–22}. As such, abnormal splicing is observed at multiple genes associated with the various aspects of DM1 pathology including *CLCN1* (myotonia)²³, *TNNT2* (cardiac pathology)²⁰, *INSR* (insulin resistance)²¹, *PKM* (glucose metabolism perturbation)²⁴, *BINI* (T tubule alterations)²⁵, *DMD* (muscle fibre maintenance)²⁶, *MAPT* (brain pathology)²⁷, and *MBNL1* itself (which further exacerbates the spliceopathy)^{28,29}. However, there are multiple other factors that contribute to DM1 pathophysiology, including deficiency in DMPK protein expression^{30,31} (**Figure 1B**), toxic peptides generated by repeat-associated non-ATG (RAN) translation^{32,33}, and alterations in chromatin structure which lead to transcriptional silencing of neighbouring genes (e.g. *DMWD* and *SIX5*)^{34–36} (**Figure 1A**).

There are currently no effective treatments for DM1, although a number of experimental therapies are in clinical and pre-clinical development³⁷. Oligonucleotide approaches targeting the expanded repeat RNA are among the most advanced therapeutic strategies^{38–40}, having reached the stage of clinical trials (NCT02312011). RNase H-competent antisense oligonucleotides (e.g. gapmers) and small interfering RNAs promote cleavage of the repeat expanded *DMPK* mRNA^{39,40}, whereas steric block oligonucleotides (e.g. peptide-morpholino conjugates) mask the repeat sequence and prevent sequestration of splicing factors such as MBNL1^{38,41}. Importantly, such approaches necessitate a lifetime of treatment in order to achieve persistent silencing of mutant CUGexp RNA expression, and thereby suppress disease pathology. An alternative therapeutic strategy is the use of gene editing technologies to induce permanent correction of the DM1 genetic defect. To this end, zinc finger nucleases (ZFNs) and

transcription activator-like effector nucleases (TALENs) have been utilised to induce contraction of CTG/CAG repeat expansions via targeted introduction of a DNA double stranded break (DSB) within the repeat expansion, excision of the CTGexp from the patient's genome, or otherwise interfering with the generation of toxic repeat-expanded RNA^{42–46}. With the recent development and repurposing of the prokaryotic CRISPR (clustered, regularly interspaced, short palindromic repeats)/Cas9 (CRISPR-associated protein 9) system there has been renewed interest in the utility of gene editing therapies for a plethora of disease indications. Here we discuss the therapeutic potential of CRISPR/Cas9-based technologies for the treatment of DM1.

CRISPR/Cas9-mediated gene editing

The CRISPR/Cas9 system was discovered as an adaptive immune system present in bacteria and archaea that protects against infection by invading foreign nucleic acid sequences^{47,48}. For example, bacteria that survive bacteriophage infection incorporate short phage-derived DNA sequences into the CRISPR locus of their genome, thereby serving as a form of 'immunological' memory^{49–51}. Transcription of these sequences produces CRISPR RNA (crRNA), that directs the Cas9 DNA endonuclease to the complementary bacteriophage DNA sequences during subsequent bacteriophage reinfection. Once bound, the Cas9 protein induces the formation of a DSB, thereby inactivating the invading parasitic DNA⁵².

The Cas9 endonuclease is comprised of recognition (REC) and nuclease (NUC) lobes connected via a linker loop region and an arginine-rich bridge helix^{53–55}. The two catalytic domains, HNH and RuvC, are contained within the NUC lobe and each cleave one of the strands of the targeted DNA, which together results in a DSB (**Figure 2A**).

Although several CRISPR/Cas systems have been identified across different prokaryotes, the bacterial CRISPR/Cas9 system has received the most attention for its utility as a programmable, site-specific DNA endonuclease. This is largely due to its simplicity, being comprised of a single effector protein (Cas9) and two small non-coding RNAs: the Cas9-guiding crRNA, and a transactivating RNA (tracrRNA) that is necessary for crRNA maturation and priming of Cas9 cleavage activity ⁵⁶.

Subsequently, it was demonstrated that the CRISPR/Cas9 system could be repurposed for use in eukaryotic cells in culture ⁵⁷ and *in vivo* ⁵⁸, leading to a revolution in gene editing for research, biotechnology, and gene therapy applications. The major advantage of CRISPR/Cas9 technology is that the protein component is invariant, whereas a large number of guide RNAs (gRNAs) against many different targets can be screened rapidly, and cheaply. This is in contrast to ZFNs and TALENs, which are cumbersome to design and expensive to produce, requiring that a unique synthetic protein be engineered for every target ⁵⁹. The flexibility and ease of use of CRISPR/Cas9 also means that multiplex gene editing is possible via the co-administration of Cas9 with multiple gRNAs ⁵⁷.

A key advance was the demonstration that the two non-coding RNA components could be combined into a ~100 nt single guide RNA (sgRNA) ^{60,61}, and this simplified configuration has rapidly become the most widely-used approach. Exogenously designed sgRNAs typically contain ~20 nucleotides of sequence at the 5' terminus which are complementary to a target genomic sequence, while the remainder of the sgRNA forms a scaffold structure that is recognised by the Cas9 protein.

A requirement of the Cas9 system that limits what sequences can be targeted is that the complementary region of the gRNA must be immediately upstream of, and adjacent to, an orthologue-specific protospacer adjacent motif (PAM) which is required for target recognition and catalysis of DSB formation^{48,56,57,61–63}. The PAM site is a crucial evolutionary feature of the natural bacterial system which enables the CRISPR/Cas9 system to avoid self-recognition⁶⁴.

After binding to a cognate target site, a DSB is induced at a position three nucleotides upstream of the PAM site, in the case of the most commonly used *Streptococcus pyogenes* and *Staphylococcus aureus* Cas9 variants (SpCas9 and SaCas9 respectively)^{57,58,63}. The PAM sequence for SpCas9 is 5'-NGG-3' (**Figure 2B**)⁶⁵ and for SaCas9 is 5'-NNGRRT-3' (where R = A or G) (**Figure 2C**)⁵⁸. Given the short length and relatively low complexity of these PAM sequences, potential Cas9 target sites are highly abundant in the human genome⁶⁶. Notably, the PAM requirements of SpCas9 and SaCas9 variants mean that direct targeting within the expanded CTG repeat is not favourable. However, for SpCas9, a 5'-NAG-3' is also recognised, although with reduced efficiency⁶⁷, thereby enabling the CTGexp DNA to be targeted on the reverse strand (where the sequence is 5'-CAG-3'). Similarly, SaCas9 has been shown to tolerate a less stringent 5'-NNGRRN-3' PAM sequence to a limited extent, thereby expanding the target space of this alternative analogue⁶⁸. Notably, direct targeting of the DNA repeat has been demonstrated with both SpCas9 and SaCas9 variants, presumably based on sub-optimal PAM site recognition^{69,70}.

The large size of the SpCas9 protein (4.1 kb) is at the limit of what can be effectively packaged in an adeno associated virus (AAV) genome (capacity ~4.7 kb), especially when considering the additional non-coding regulatory sequences that are required for transgene expression. In

contrast, SaCas9 is much more compact (3.2 kb) and has consequently become the Cas9 variant of choice for *in vivo* gene editing studies⁵⁸. Even with the development of SaCas9, the use of multiple, co-administered AAV vectors has typically been required to deliver both the Cas9 and sgRNA components^{58,71–73}. Further engineering of truncated versions of Cas9 and AAVs with improved packaging capacities may address the issue of AAV packaging limitations^{74,75}.

Importantly, the utility of the CRISPR/Cas9 gene editing system is predicated on the activity of host cell DNA damage repair pathways to resolve the Cas9-induced lesions, of which the key pathways are non-homologous end joining (NHEJ) and homology directed repair (HDR)^{56,65}. NHEJ is operative in all cells, and is the more active of these two pathways. DSB repair via NHEJ typically leaves an indel (insertion/deletion) ‘scar’ at the cut site⁷⁶. Conversely, HDR requires the presence of a single-stranded DNA template, but results in high-fidelity, ‘scarless’ lesion repair⁷⁷.

Therapeutic gene editing strategies for DM1

nCas9-mediated repeat contraction

One of the first studies to demonstrate the potential of CRISPR/Cas9 for the treatment of microsatellite expansion disorders utilised a reporter model system consisting of an intronic CAG (CTG in the reverse strand) repeat tract located upstream of a green fluorescent protein (GFP) mini-gene⁶⁹. In this model, the length of the repeat is negatively correlated with GFP expression. While this system is not a model of DM1 *per se*, it does provide important insights into potential gene editing approaches for targeting CTGexp regions.

The CRISPR/Cas9-mediated induction of DSBs at the repeat region resulted in repeat instability, with both repeat contraction and expansion observed⁶⁹. While repeat contraction

would be expected to alleviate DM1 pathology, repeat expansion is likely to worsen the disease phenotype. In contrast, when the repeat was targeted using a ‘nickase’ Cas9 variant (nCas9, in which a D10A mutation in the RuvC-I domain of SpCas9 inactivates one of its two catalytic domains⁵⁷) the resulting single-strand breaks (SSBs) also resulted in repeat instability, but with a strong bias towards contraction⁶⁹ (**Figure 3A**). Notably, this effect was limited to reporters with large (≥ 101 repeats) expansions, and not observed with shorter repeats (≤ 42), suggesting that repeats with lengths in the normal range are not subject to nCas9-induced repeat instability⁶⁹. The authors proposed a model in which multiple single SSBs on the same strand of the CAG repeat resulted in the formation of large regions of single-stranded DNA. They further demonstrated the involvement of the serine/threonine kinase ATM (ataxia–telangiectasia mutated) in the repeat contraction process⁶⁹.

Notably, there have been subsequent conflicting reports as to whether DSBs at the *DMPK* CTG expansion induce repeat instability^{78,79}.

Excision of the CTGexp repeat expansion

The most thoroughly investigated DM1 gene editing approach aims to excise the CTGexp DNA sequence in order to achieve permanent correction of the disease phenotype. This is achieved using a pair of sgRNAs targeting sequences which flank the CTGexp, leading to the formation of two DSB. Therapeutic correction is dependent upon the correct joining of the flanking regions via the NHEJ pathway in a manner that excludes the intervening, repeat-containing DNA (**Figure 3B**). The resulting indel at the repair site is expected to be well-tolerated as it occurs in the noncoding 3' UTR and therefore does not disrupt the translation reading frame of *DMPK*. However, it is possible that the deletion of regulatory sequence in the CTGexp-flanking

regions may mean that binding sites for *trans* regulators like microRNAs or RNA binding proteins are lost.

This repeat excision approach has primarily been investigated in myoblasts derived from the DM500 DM1 mouse model ⁷⁸, and in multiple DM1 patient-derived cell models including myoblasts ^{78,80,81}, human embryonic stem cells (hESCs) ⁸⁰, induced pluripotent stem cells (iPSCs) ⁸², iPSC-derived myogenic cells ⁸², iPSC-derived neural stem cells ⁸³, and fibroblasts transdifferentiated into myogenic cells via the forced expression of MYOD1 ⁷⁹. In all cases, excision of the CTGexp was accompanied by evidence of correction of the DM1 phenotype, including the reductions in nuclear foci, cytoplasmic localisation of MBNL proteins, and reversal of aberrant splicing patterns ^{78–82}. This was even true in a cell model with ~2,600 CTG repeats ⁸¹.

Interestingly, CRISPR/Cas9-mediated CTGexp excision was shown to reverse aberrant silent state epigenetic chromatin marks at the *DMPK* locus (i.e. DNA methylation, H3K9me3 enrichment) and to rescue expression of the neighbouring *SIX5* gene in DM1 patient-derived hESCs ⁸⁰. However, such effects were not observed in DM1 myoblasts, suggesting that the DM1-associated changes to the epigenetic landscape surrounding the *DMPK* locus are less malleable in differentiated cells ⁸⁰.

CTGexp excision has the potential to cure patients of DM1 through a one-time treatment, but carries the risk of unintended off-target genome cleavage that may cause pathological mutagenesis. The aforementioned cell culture studies reported minimal off-target genome editing, as assessed by sequencing *in silico*-predicted potential sites of unintended editing ^{78–83}, which exhibit complementarity to the sgRNA. Unbiased genome-wide Cas9 off-target

detection tools, such as GUIDE-seq⁸⁴, have shown that these *in silico* approaches miss cleavage at unexpected sites, and thus may overestimate Cas9 specificity^{84,85}. Improvements in methods for detecting genome-wide off-target editing, and the development of engineered Cas9 variants with enhanced specificity will likely help to reduce the risks of detrimental off-target mutagenesis^{86–89}. However, it is likely that there will always be some risk of off-target gene editing despite these technological improvements. As such, the potential for the introduction of unintended deleterious mutations must necessarily be weighed against the benefits of the therapy.

An arguably greater problem for the CTGexp excision approach is the relatively common reports of on-target unintended editing^{78,79,82,83}. These included the formation of large indels at the cut sites⁷⁹, microdeletions in which the PAM site was deleted but the repeat remained intact⁸¹, repeat instability^{69,78}, partial repeat deletions⁸², and most notably, inversion of the CTG repeat^{78,79} (including at both mutant and wild-type alleles⁸³). Notably, Wang *et al.* detected CAGexp ribonuclear foci in Cas9-treated iPSC-derived neural stem cells in which CTGexp inversion had occurred⁸³. Further research is needed to determine the frequency and consequences of such sequence inversion, alongside the other potential unintended DNA repair consequences such as translocation and duplication. The *DMPK* locus is known to be transcribed in the antisense orientation, and CAGexp foci have been reported in the nuclei of DM1 tissues⁹⁰. There are conflicting reports as to whether such foci could sequester splicing factors (e.g. MBNL1)^{90,91}, but toxicity may result from alternative mechanisms, such as a consequence of RAN translation³³. Notably, a CAG repeat expansion in the 5' UTR of the *PPP2R2B* gene causes spinocerebellar ataxia 12⁹², suggesting that unintended CTGexp inversion after gene editing DM1 cells may itself be pathogenic. Furthermore, there is growing awareness of CAGexp RNA-mediated toxicity in CAGexp disorders whose pathogenesis has

been traditionally associated with the production of mutant polyglutamine proteins, such as in the case of Huntington's disease^{93,94}. If found to be harmful, an inability to prevent these large on-target mutations would represent a major obstacle to the clinical translation of the CTGexp excision approach.

Expanding on the promising cell culture studies described above, Lo Scrudato *et al.* demonstrated CTGexp excision after a single intramuscular injection of AAV vector-mediated sgRNA/SaCas9 delivery in the tibialis anterior (TA) of adult (5-9 week old) DMSXL mice⁹⁵ (homozygous for a 45 kb fragment of human genomic DNA consisting of *DMPK* with >1,000 CTG repeats, and the neighbouring genes *DMWD* and *SIX5*)⁸¹. The components of the CRISPR system were encoded on separate vectors with an SaCas9 expression cassette driven by the muscle-specific SPc5-12 promoter, and the two sgRNA cassettes each driven by U6 small nuclear RNA promoters. These two AAV (serotype 9) vectors were co-administered in equal amounts, with a total dose of 1×10^{11} vector genomes per muscle⁸¹. A ~24% reduction in the number of TA myonuclei containing CUGexp ribonuclear foci was observed four weeks post treatment, when compared to the contralateral, PBS-treated control muscles⁸¹. The authors did not observe changes in the weight or strength of the treated muscle, and analysis of the correction of DM1-associated alternative splicing patterns was not possible due to the mild spliceopathy observed in the DMSXL model⁸¹. It is currently unclear whether this degree of gene correction crosses the threshold of what would be clinically beneficial, although patient responses to CTGexp excision will likely vary due to differences in factors such as disease severity, repeat mosaicism, and time since disease onset.

Polyadenylation signal insertion

An alternative gene editing strategy to ameliorate DM1 pathology is the insertion of an exogenous polyadenylation signal (PAS) upstream of the CTGexp (consisting of an array of both simian virus 40 and bovine growth hormone polyA signals). As such, transcription of the *DMPK* mRNA is terminated before RNA polymerase II reaches the microsatellite repeat, and so toxic RNA molecules are no longer generated (**Figure 3C**). This PAS-insertion strategy utilises the homology dependent DNA repair (HDR) pathway, which requires that a single-stranded DNA template (encoding the PAS together with flanking homology arms) is co-administered with the Cas9 and gRNA components^{44,83}. Using this approach, Wang *et al.* were able to insert a PAS sequence into the *DMPK* 3' UTR in DM1 patient-derived iPSCs, leading to stable expression of PAS-containing *DMPK* transcripts and a reduction in CUGexp ribonuclear foci⁸³. The repeat length was unaffected in the edited iPSC lines, which maintained their potential to differentiate into skeletal muscle, cardiomyocytes, and neural stem cells⁸³. The nCas9 (nickase) variant was used together with a pair of sgRNAs in order to induce a pair of adjacent SSBs on opposite strands which together form a DSB. This strategy is more specific than with the standard Cas9 as two nicks must occur in relative close proximity, meaning that the possibility of off-target gene editing is greatly reduced^{96,97}.

An advantage of the PAS-insertion strategy is that it avoids the problem of inversions of the repeat, as the CTGexp itself is not targeted, although truncation of the 3' UTR may also result in the loss of some *DMPK* regulatory sequence. Furthermore, as the CTGexp DNA is unchanged by this approach, pathogenic transcription regulation of neighbouring genes in *cis* is unlikely to be corrected.

Importantly, HDR activity has been reported to be very low, or negligible, in post-mitotic tissues, such as skeletal muscle^{98–100}, and so this strategy is not expected to be efficacious if

applied to DM1 patient muscle *in vivo*. As such, the major therapeutic application of this strategy is for the *ex vivo* modification of patient-derived cells and cell transplantation. Cell therapy applications for disease of muscle are reviewed elsewhere ¹⁰¹.

CTGexp silencing using dCas9

A further development of CRISPR/Cas9 technology is the use of nuclease deficient or ‘dead’ Cas9 variants (dCas9) through mutations which inactivate the nuclease activities of both the RuvC-I and HNH domains ⁵⁶. Such variants are guided to specific genomic loci based on complementarity with the sgRNA but do not cleave the target DNA. In some cases, the direction of dCas9 to bind at a promoter or terminator sequence of a gene can result in a transient steric block of transcription initiation or termination respectively ^{102,103}. Furthermore, fusions of dCas9 with additional protein moieties allows for transcriptional repression through CRISPR interference (CRISPRi) ^{104,105}, or transcriptional activation (CRISPRa) ¹⁰⁶ using the dCas9-KRA or dCas9-VP64-p65-Rta (dCas9-VPR) fusion constructs respectively. The versatility of the dCas9 variant has also enabled the development of improved chromatin immunoprecipitation ¹⁰⁷ and live cell genomic imaging ¹⁰⁸ techniques.

dCas9 has been used to interfere with *DMPK* transcription utilising sgRNAs targeting the CTGexp region. In this manner, the dCas9 protein itself acts to sterically block RNA polymerase II procession through the CTG repeat DNA ⁷⁰ (**Figure 3D**). Treatment of primary DM1 patient myoblasts with dSaCas9 and a repeat-targeting (CAG)₆ sgRNA was shown to reduce the number of CUGexp nuclear foci and restore wild-type splicing patterns ⁷⁰.

Similar results were obtained in a DM1 mouse model (*HSA*^{LR}, which carries 250 CTG repeats inserted into the 5' UTR of a human skeletal α -actin transgene ¹⁷). AAV-mediated delivery of

the same repeat-targeting approach dSaCas9 approach resulted in a reduction in CUGexp nuclear foci in *HSA*^{LR} extensor digitorum longus isolated myofibres *ex vivo* ⁷⁰. *HSA*^{LR} mice were injected at P2 with AAV-dSaCas9-(CAG)₆-gRNA (1×10¹⁰ viral genomes per mouse) via the temporal vein and harvested 5 weeks later. Reductions in myotonia were observed in some animals as determined by electromyography, and a complete loss of CUGexp ribonuclear foci was observed in 5-15% of extensor digitorum longus (EDL) fibres. Importantly, immunostaining for dSaCas9 revealed a mosaic pattern of expression, and restoration of CLCN1 expression (indicative of local amelioration of spliceopathy) was observed in a subset of fibres. However, RNA analysis in bulk muscle revealed no correction in splicing patterns ⁷⁰.

The targeting of the repeat DNA sequence itself is advantageous as the stoichiometry of dCas9 to target sequence is expected to confer some degree of allele specificity, as a greater number of dCas9 complexes are likely to be recruited to the expanded repeat, thereby inducing a greater degree of transcriptional repression. In contrast, the dCas9-KRAB fusion protein could theoretically be utilised to silence the *DMPK* promoter at the transcriptional level, but would be unable to distinguish between the wild-type and repeat expanded alleles. To date, the potential for the dCas9 CTGexp-targeting approach to affect other genes containing CTG-repeats has not been investigated.

Given that therapeutic strategies based on dCas9 do not induce breaks in genomic DNA they are inherently safer than cutting approaches. However, as the effects of dCas9 binding are transient, these approaches necessarily require long-term retention of the episomal AAV genome and persistent expression of the dCas9 and sgRNA components. AAV genomic DNA may be lost or epigenetically silenced over time, which may limit the utility of such dCas9-based therapies.

CUGexp RNA elimination (RCas9)

CRISPR/Cas9 technology has been repurposed to directly target single-stranded RNA (RCas9)¹⁰⁹. This was achieved by providing a synthetic DNA oligonucleotide containing the PAM sequence (PAMmer) in *trans*¹⁰⁹. The RCas9 approach has been utilised to target the CUGexp for the purposes of treating DM1 using dCas9 variants fused to either GFP (dCas9-GFP), or to the RNA endonuclease domain, PIN (dCas9-PIN)¹¹⁰ (**Figure 3E**). These strategies were designed to displace MBNL proteins from CUGexp, or to degrade the mutant expanded *DMPK* mRNA respectively. However, both approaches were found to induce a reduction in CUGexp foci in primary DM1 patient-derived myotubes¹¹⁰, consistent with reports using steric block ASOs which lack RNase H activity⁴¹, suggesting that the displacement of RBPs from the repeat RNA is sufficient to destabilise the mutant *DMPK* mRNA. Notably, the PAMmer was found to be dispensable when targeting the CUG repeat, which simplifies this therapeutic approach as this oligonucleotide cannot be co-delivered using viral vectors. Elimination of CUGexp nuclear foci in DM1 myotubes treated with dCas9-PIN was associated with redistribution of MBNL1 to a diffuse pattern of nuclear staining, global reversal of DM1-associated splicing defects, and improved myogenic differentiation¹¹⁰. dCas9-PIN treatment had no effect on the CTGexp repeat size, and did not affect the expression of a gene with a short CTG expansion (*TCF4*), suggestive of minimal off-target activity¹¹⁰.

As with dCas9-mediated CTGexp transcriptional silencing, the RCas9 approach avoids the possibility of permanent, undesirable genome editing, but may still induce cleavage of non-target RNA transcripts. However, direct targeting of mutant RNA does not correct CTGexp DNA-mediated transcriptional silencing of neighbouring genes^{34–36}, and would require long-

term expression of the dCas9 and sgRNA elements in order to maintain suppression of CUGexp RNA synthesis.

Truncated versions of the dCas9-PIN fusion protein were generated which retained nuclear foci elimination activity ¹¹⁰. These include variants in which the HNH domain or HNH and REC2 domain were deleted from the dCas9 component. These variants are ~4.3 kb (PIN-dCas9 Δ HNH) and ~3.9 kb (PIN-dCas9 Δ HNH, Δ REC2) respectively, thereby enabling their packaging into AAV vectors. To this end, the same group recently reported successful utility of the RCas9 approach in an *in vivo* DM1 model ¹¹¹. *HSA*^{LR} mice were treated with a mixture of two AAV9 vectors (carrying the dCas9-PIN construct and the U6-sgRNA transgene respectively) via both intramuscular and systemic routes. For the intramuscular study, tibialis anterior muscles from adult mice were injected with $2.5\text{-}5\times 10^{10}$ vector genomes, which resulted in elimination of CUGexp foci, promoted a diffuse pattern of cellular MBNL1 distribution, and reversed splicing defects ¹¹¹. For the systemic treatments, *HSA*^{LR} mice were treated at both the neonatal (2×10^{11} vector genomes via the temporal vein) and adult (1×10^{12} vector genomes via the lateral tail vein) stages ¹¹¹. Again, a reversal of DM1 molecular pathological features were ameliorated, in addition to a reduction in myotonia ¹¹¹. Importantly, the AAV vectors were coadministered with a combination of immunosuppressive agents (tacrolimus and CTLA4-Ig) in order prevent an immune response towards the dCas9 protein, thereby facilitating sustained therapeutic benefit ¹¹¹.

Other simple and compact CRISPR systems with an inherent preference for RNA targeting, such as Cas12g ¹¹² and the Cas13 family ¹¹³, that do not require provision of a PAMmer oligo, could be employed to target repeat-expanded *DMPK* mRNA, either for transcript downregulation, or for steric hindrance of MBNL protein binding.

Challenges for the clinical development of DM1 CRISPR/Cas9 therapy

The major obstacle to the translation of gene therapies is effective delivery to target tissues and cells. In the case of DM1, delivery to skeletal and cardiac muscle is particularly challenging due to poor drug penetrance, in part due to the tight endothelial barrier surrounding blood vessels in these tissues ¹¹⁴. Importantly, the multisystemic nature of DM1 pathology may require body-wide correction (e.g. in tissues beyond muscle) to treat all aspects of the disease.

AAVs are currently the leading strategy for muscle gene therapy, due to the availability of skeletal/cardiac tropic serotypes (such as AAV1, AAV6, AAV8, and AAV9) ^{73,115,116}. The therapeutic potential of AAV vectors to treat neuromuscular disorders is exemplified by the recent approval of zolgensma, an intravenous AAV-based gene therapy for spinal muscular atrophy ¹¹⁷. Similarly, AAV-based products are also in late stage development for Duchenne muscular dystrophy ¹¹⁸ and X-linked myotubular myopathy (NCT03199469), for the delivery of micro-dystrophin or *MTM1* transgenes respectively.

While generally considered safe, there is the risk of pathogenic immune responses to AAV proteins, or to the Cas9 transgene product itself. Recently, two clinical trial patients treated with high dose AAV therapy died as a result of liver toxicity and sepsis ¹¹⁹, suggesting a re-appraisal of the risks associated with AAV-based therapy is warranted. This is a particular concern for the delivery of CRISPR/Cas9-based therapies, where high doses of AAV may be required for effective treatment. Another potential limitation of AAV is the risk of vector-sequence integration into the host genome at DSBs ^{120–123}.

The recent discovery of pre-existing immunity to Cas9 within the human population, in the form of both Cas9-specific antibodies and CD8⁺ cytotoxic T cells, presents an additional challenge to effective therapy ^{124–126}. This is thought to arise from common exposure to *S. aureus* and *S. pyogenes*. Pre-existing immunity to AAVs is also widespread as a result of natural infection in early life ¹²⁷. This will foreseeably inhibit cell transduction or drive a cytotoxic response to those successfully transduced. Moreover, viral delivery of gene editing transgenes is expected to immunise the treated individuals against AAV-derived antigens, and possibly also against Cas9 itself, thereby ruling out the possibility of repeat administration. Screening will be necessary to determine pre-existing immunity in prospective patients. Plasmapheresis to remove AAV/Cas9-specific antibodies from the blood, and/or transient immunosuppression during treatment could be utilised to enable therapy in these patients ¹²⁸. There is already work on engineering AAV and Cas9 variants that can evade immune detection ^{115,129,130}. Importantly, if successful for initial treatment, these methods may enable re-administration.

Considering these safety concerns, the ability to readily halt *in vivo* Cas9 activity is desirable. Possible strategies to achieve some degree of temporal control of Cas9 include self-deleting Cas9s, anti-CRISPR molecules, and small molecule-dependent Cas9 variants, but may themselves have side effects or issues of delivery and immunogenicity ^{131–133}.

The future development of AAV-based delivery system for DM1 CRISPR/Cas9 therapies will undoubtedly benefit from progress towards similar therapies for Duchenne muscular dystrophy (DMD) which are at a more advanced stage. Systemic AAV injection has been utilised to deliver therapeutic levels of Cas9 and sgRNA(s) to the heart, diaphragm, and other muscles, in mouse, canine, and porcine models of Duchenne muscular dystrophy (DMD) ^{71,73,134–138}. In the

porcine model of DMD, AAV myotropism was further enhanced by coating AAVs with generation 2 polyamidoamine (G2-PAMAM) dendrimers ^{139,140}. An alternative approach for improving muscle tropism is the use of AAVs engineered to express cell-penetrating peptides on the vector surface, as such peptides recently enhanced skeletal muscle uptake of CUGexp RNA-targeting antisense oligonucleotides in mice ³⁸.

Notably, the highly promising results observed in the DMD context have typically been achieved with much higher doses of AAV (in the range of 10^{13} - 10^{14} vector genomes per kilogram) than were used in similar DM1 studies ^{81,110}. The requirements for very high viral titres is problematic for safety reasons (as described above), but also for the purposes of virus manufacture. However, work from the Olson group has shown several strategies which have enabled effective DMD gene editing treatments with lower doses, including the use of self-complementary AAV vectors (which express their transgenes rapidly as there is not requirement for second-strand DNA synthesis) ¹⁴¹, and using a Cas9:sgRNA vector ratio of 1:10 ¹⁴².

A relatively under-appreciated facet of CRISPR/Cas9 is the consequences of sub-optimal gene correction. Given that muscle fibres are long, syncytial structures containing hundreds of nuclei, gene editing therapy is likely to result in a situation whereby chimeric fibres are generated containing both edited and non-edited myonuclei. As muscles undergo normal growth or repair over time, additional un-edited nuclei will fuse with the fibre, further exacerbating this situation. In the case of DMD, we have demonstrated that patchy dystrophin expression in myofibres is insufficient to correct dystrophic pathology using a genetic model ¹⁴³. These findings suggest that there is likely a threshold number of nuclei which must be edited for therapeutic benefit. Consistent with this notion, CRISPR/Cas9-mediated CTGexp

excision in the DMSXL mouse resulted in mosaic expression of Cas9, with Cas9 expressed in some nuclei, but not others, within the same fibre⁷⁰. Whether incomplete CTGexp excision will be therapeutic for DM1 remains to be determined.

Non-viral delivery vehicles are also in development for gene editing therapies¹⁴⁴. In this manner, complexes of recombinant Cas9 proteins and synthetic sgRNAs can be delivered with the aid of lipid nanoparticles, cationic lipids, gold nanoparticles, or cell-penetrating peptides^{145–148}. Such non-viral ribonuclear protein (RNP) complex delivery technologies offer several advantages: (i) there is the potential for repeat administration, (ii) there is no possibility of vector integration at edit sites, (iii) transient Cas9 expression reduces the risk of off-target gene editing^{145,149,150}, (iv) they are generally less immunogenic than viral vectors with minimal risk of pre-existing immunity¹⁵¹. Conversely, non-viral delivery approaches present a number of additional challenges, such as poor tissue uptake, and preferential liver delivery following systemic administration¹⁵². Similarly, ensuring that the Cas9 and gRNA components are not denatured or degraded during formulation, or in the circulation, are further challenges. A recent study by Wei *et al.* demonstrated efficient delivery of Cas9:sgRNA RNPs via a modified lipid nanoparticle strategy after systemic delivery¹⁵³. The addition of a permanently cationic charged lipid (DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane) to existing lipid, lipid-like, or dendrimer nanoparticles enabled gene editing in the liver and lungs after intravenous injection¹⁵³. Furthermore, intramuscular injection of RNP-carrying modified lipid nanoparticles in a DMD mouse model resulted in ~4% dystrophin protein recovery¹⁵³. While, these results are highly promising for the development of non-viral delivery of gene editing technologies for myopathies such as DM1, additional development will be needed to improve their efficacy.

Conclusions

The impressive flexibility of the CRISPR/Cas9 system has been utilised to develop multiple distinct gene editing strategies for the treatment of DM1. Each approach has shown promise in proof-of-concept studies. The delivery of the gene editing machinery is a major obstacle for the effective translation of gene editing therapy to the clinic. Whether experimental CRISPR/Cas9 therapies can reverse the pathological features of DM1 remains to be convincingly demonstrated. The reliance on AAV vectors also presents challenges, such as limited packaging capacity, requirement for very high viral doses, inability to repeat dose, pre-existing immunity to AAV antigens or Cas9, issues associated with persistent Cas9 expression, and unintended insertion of AAV genomic DNA at DSBs. In this regard, gene editing therapies for DM1 will undoubtedly benefit from developments in therapies for other indications, and for DMD in particular. Non-viral alternatives are currently at early stages of development relative to AAV systems.

Targeting of the CTGexp in DM1 is associated with its own unique challenges. Due to their repetitive nature, such sequences present an inherent challenge for DNA replication and repair which is further exacerbated when DNA lesions are introduced in the vicinity of the CTGexp. As such, unintended mutagenesis at the repeat has been reported by multiple groups. The inherent instability of the repeat, means that there is a risk of promoting expansion and exacerbating disease pathology. Furthermore, inversions of the repeat sequence may result in pathogenic CAGexp sequences.

Despite the multiple challenges, CRISPR/Cas9-based technologies present exciting opportunities for the treatment of DM1.

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Conflict of Interest Statement

The authors declare no conflicts of interest.

Author Contributions

The first draft was written by S.M. and T.C.R. Additional content was added by B.H., M.A.V. and M.J.A.W. All authors contributed to the editing of the final draft.

Figure Legends

Figure 1

Pathogenesis of myotonic dystrophy type 1.

(A) DM1 is caused by an expansion of a CTG repeat sequence in the 3' UTR of the *DMPK* gene. Repeat expansion results in epigenetic silencing of the adjacent *SIX5* and *DMWD* genes. An antisense transcript (DM1-AS1) overlaps with the repeat region. (B) Wild-type (WT) *DMPK* transcripts contain 5-37 CUG repeats, whereas mutant *DMPK* transcripts typically contain more than 50 CUG repeats. Repeat expansion results in reduced DMPK protein output from the mutant allele. (C) The CUGexp forms a hairpin structure. (D) Toxic CUGexp *DMPK* transcripts sequester muscleblind proteins such as MBNL1. CELF1 is also upregulated in DM1 muscle. The altered balance between MBNL1 and CELF1 results in a global shift in splicing patterns. Altered splicing of specific genes leads to the various tissue pathologies of DM1.

Figure 2

The CRISPR/Cas9 system.

(A) Schematic of primary protein domain structures for SpCas9 (1,368 amino acids) and SaCas9 (1,053 amino acids). Both Cas9 proteins are bilobed, consisting of the nuclease (NUC) lobe with three RuvC motifs (RuvC-I, -II and -III) and the HNH domain, as well as the recognition (REC) lobe which, in the case of SpCas9, is divided further into four subdomains. (BH, bridge helix; L1 and L2, linker 1 and 2 regions; PLL, phosphate lock loop; WED, wedge domain; PI, PAM interacting domain; TOPO, topoisomerase-homology domain; CTD, C-terminal domain). Amino acid start positions of each domain are indicated. Schematics of (B) SpCas9 and (C) SaCas9 in complex with their cognate single guide RNAs (sgRNA), targeted to a genomic DNA (gDNA) target site. The target is defined by a variable guide sequence within the sgRNA which is typically ~17nt for SpCas9 or ~21nt for SaCas9. Protospacer adjacent motif (PAM) sequences are located on the non-target strand and have the motif 5'-NGG-3' for SpCas9 and 5'-NNGRRT-3' for SaCas9 are highlighted in yellow. (N indicates any nucleotide, R is guanine or adenine, and Y is cytosine or thymidine). RuvC and HNH cleavage domain cut sites are indicated by arrowheads on the non-target and target strands respectively (three nucleotides upstream of the PAM sequence). The invariant sgRNA scaffold sequences (highlighted in white) are comprised of a tetraloop region and either three or two stem loops for SpCas9 and SaCas9 respectively.

Figure 3

CRISPR/Cas9 therapeutic approaches for myotonic dystrophy type 1.

(A) Targeting the expanded CTG repeat (CTGexp) with the nickase nCas9 results in repeat contraction and expansion, with a bias towards contraction. (B) Excision of the CTGexp DNA using a double-cut strategy with guide RNAs targeting the regions flanking the repeat. Non-homologous end-joining (NHEJ) results in the formation of an indel at the edit site (productive repeat excision), or non-productive inversion to generate a CAGexp. (C) Insertion of a polyadenylation signal (PAS) upstream of the CTGexp using a single-cut strategy and homology dependent repair (HDR) and a single-stranded DNA repair template. Transcription of the edited *DMPK* locus results in the generation of a truncated mRNA that lacks the toxic CUGexp repeat. (D) Targeting the CTG repeat with nuclease-deficient dCas9 results in steric interference of RNA polymerase II (RNAPII) processions through the repeat sequence, meaning that generation of toxic *DMPK* transcripts is reduced. (E) Direct targeting of mutant *DMPK* CTGexp mRNA with dCas9 displaces MBNL proteins and results in a reduction in *DMPK* transcript levels.