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The sustained expression of RNA-targeting Cas9 delivered systemically by adeno-associated viral vectors eliminates pathogenic foci of expanded-repeat transcripts and reverses muscle-disease phenotypes in mouse models of myotonic dystrophy type 1.

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The expansion of microsatellite repeats of 2–9 base-pair tracts leads to an increasing number of diseases[1]. In particular, myotonic dystrophy type 1 (DM1) — the most common muscular dystrophy in adults — is caused by the expression of expanded CTG repeats located within the 3' untranslated region of the *DMPK* gene[2]. Mutant transcripts containing expanded CUG repeats are retained in the nucleus as riboprotein complexes (foci) that sequester the muscleblind-like 1 (MBNL1) RNA-binding protein[3]. Because of its high binding affinity for CUG repeats, MBNL1 is titrated by the foci; the resulting functional loss of MBNL1 in skeletal muscles then leads to alterations in RNA metabolism, such as alternative-splicing dysregulations[4,5]. Some of such alterations are associated with clinical symptoms; for example, the aberrant splicing of the pre-RNA for skeletal muscle chloride channel 1 (CLCN1) is associated with myotonia, a hallmark of DM1 (ref. [6]).

Preclinical therapeutic strategies for DM1 (currently, there are no approved treatments for DM1) use antisense oligonucleotides or small molecules[7,8,9] to degrade *DMPK* transcripts or to interfere with CUG expansions. Gene therapy based on CRISPR-Cas9 or on RNA interference and delivered systemically by recombinant adeno-associated viruses (AAVs) is also being explored[10,11]. In particular, RNA-targeting Cas9 (a nuclease-dead Cas9 fused to the PIN RNA endonuclease domain; PIN-dCas9) can be used to target, via a designed guide RNA (gRNA), expanded RNA repeats[12]. By eliminating expanded CUG repeats in cultured DM1 muscle cells, RNA-targeting Cas9 reduced the number of nuclear RNA foci and reversed alternative splicing dysregulations. Reporting in *Nature Biomedical Engineering*, Gene Yeo and colleagues now show the performance of such RNA-targeting strategy in a mouse model of DM1 (ref. [13]). The researchers used a well-characterized transgenic mouse model of DM1 (the human skeletal actin long-repeat (HSA^{LR}) mouse model) harbouring 220 CTG repeats located within the 3' untranslated region of the human gene for actin[14]. HSA^{LR} mice express expanded CUG repeats exclusively in skeletal muscles, and display DM1-associated phenotypes, including nuclear RNA foci, alternative-splicing dysregulations, muscle defects and myotonia.

Yeo and co-authors packaged the RNA-targeting Cas9 (under the control of a short elongation factor-1 alpha promoter) and the CTG gRNA (driven by a U6 promoter) targeting CUG repeats in two different recombinant AAV9 vectors (the size of the DNA encoding for both RNAs is larger than the packaging capacity of a single AAV). HSA^{LR} mice co-injected intramuscularly with the AAVs for 4 weeks experienced a reduction in the number of foci with expanded CUG repeats, compared to mice injected with equivalent vectors bearing gRNAs that do not target CUG repeats. To eliminate the foci and to release sequestered MBNL1 into the nucleoplasm, it is necessary that the expression of PIN-dCas9 and CTG gRNA happens in the same nuclei. By using transcriptome-wide analysis by RNA sequencing, the authors confirmed the recovery of MBNL1 splicing activity in the muscles injected with the AAV-delivered PIN-dCas9, as well as a substantial correction of the altered splicing events that are regulated by MBNL1 and that are also normalized by the overexpression of MBNL1. For example, the correction of the aberrant MBNL1-dependent splicing of Clcn1 pre-mRNA (in HSA^{LR} mice, such aberrant splicing causes the nonsense-mediated decay of Clcn1 mRNA, the reduction of the levels of Clcn1 protein, and myotonia), restored the levels and localization of Clcn1 protein in the muscle. Concomitantly, muscle histology also improved. Analysis of molecular biomarkers (such as alternative-splicing defects) and of physiologic changes (myotonia) indicated a reversal of the DM1-associated phenotypes in the skeletal muscles.

Moreover, Yeo and co-authors' transcriptome analyses and immunostaining revealed the upregulation of genes linked to immune responses as well as high levels of T-cell infiltration in the muscles of HSA^{LR} mice that were injected with PIN-dCas9. The levels of PIN-Cas9 protein progressively decreased 8 weeks and 12 weeks after injection, which suggests the existence of an adaptive immune response to the injected non-self Cas9 protein. Intriguingly, intramuscular injection of AAVs encoding for PIN-dCas9 in wild-type mice at the same dose induced low cytotoxic immune responses and the absence of clearance of the

injected Cas9 for over 12 weeks. Hence, a pre-existing inflammation environment induced by the expression of pathogenic CUG RNA repeats must potentiate the adaptive immune response to PIN-dCas9 protein in the muscles of HSA^{LR} mice. However, a 2-weeks co-treatment with the immunosuppressors tacrolimus and abatacept prevented T-cell activation after PIN-dCas9 administration, limited the cytotoxic immune response, and sustained the expression of PIN-dCas9 for 12 weeks. In HSA^{LR} neonates, the therapy without immunosuppression (because of the lack of immune response at this developmental stage in mice), led to sustained levels of PIN-dCas9 protein in the muscles of systemically injected mice for at least 16 weeks, and the continuous expression of the protein correlated with a drastic reduction of expanded CUG repeats. The authors also show, via gene-ontology analyses, that the therapy changed the expression of genes associated with muscle function and muscle maturation and with the production of muscle extracellular matrix. They also tested whether PIN-dCas9 induced off-target effects, especially on short CTG repeats. Gene-expression analysis performed on muscles of treated wild-type mice did not show any changes in the mRNA levels of hundreds of genes containing short CTG repeats, thus supporting the therapy's targeting specificity for large CUG repeats.

Yeo and colleagues also show that, despite being systemic delivered by distinct AAV9 vectors alongside transient immunosuppression in adult HSA^{LR} mice (Fig. 1), the therapy led to the simultaneous expression of PIN-dCas9 and CTG gRNA at different levels in various muscle tissues after 4 and 8 weeks. Four weeks of sustained expression of CTG led to a significant reduction of the levels of expanded CUG repeats and of nuclear RNA foci in the quadriceps muscles, and alternative-splicing dysregulations were significantly corrected in these muscles. Yet myotonia was reduced in the tibialis anterior muscles.

Although gene therapies using AAVs to deliver a functional protein to compensate for a loss of gene product in muscle diseases such as X-linked myotubular myopathy or Duchenne muscular dystrophy are in clinical trials, AAV-mediated gene therapies for genetic disease dominated by expanded RNA repeats are in preclinical development. To improve disease phenotypes, AAV-delivered transgenes have to neutralize the production of toxic RNAs or reverse their toxicity. Yeo and colleagues' work provides proof-of-principle evidence that this may be possible. In contrast to approaches relying on the Cas9-mediated excision of expanded repeats at the genomic level, for which Cas9 is preferably expressed transiently, PIN-dCas9 needs to be continuously expressed to reduce the transcription of the expanded repeats. Despite the immune responses induced by non-self Cas9 protein in HSA^{LR} mice (except in neonate mice), PIN-dCas9 administration combined with transient immunosuppression appears to be sufficient to sustain therapeutic levels of PIN-dCas9 protein for up to 8 weeks. The pre-existing inflammation environment observed in HSA^{LR} mice should be examined in other mouse models of DM1 and will eventually need to be assessed in human patients. Also, DM1 patients will require long-lasting therapeutic levels. In this respect, packaging a smaller Cas9 protein and the guide RNA within a single AAV vector should improve the therapeutic efficacy, as it would facilitate their co-expression within nuclei (especially in large muscle fibres containing hundreds of nuclei). Furthermore, the regulation of PIN-dCas9 expression using either tissue-specific promoters or any other transgene-regulatory system should also be considered, as DM1 is a multisystemic disease affecting skeletal, cardiac and smooth muscles as well as tissues of the central nervous system. Targeted tissue expression could also modulate the immune response after systemic administration. And RNA-targeted Cas9 may also be applicable to other microsatellite repeats in other RNA contexts and expressed in different cell types and tissues.

Competing interests

The authors declare no competing interests.

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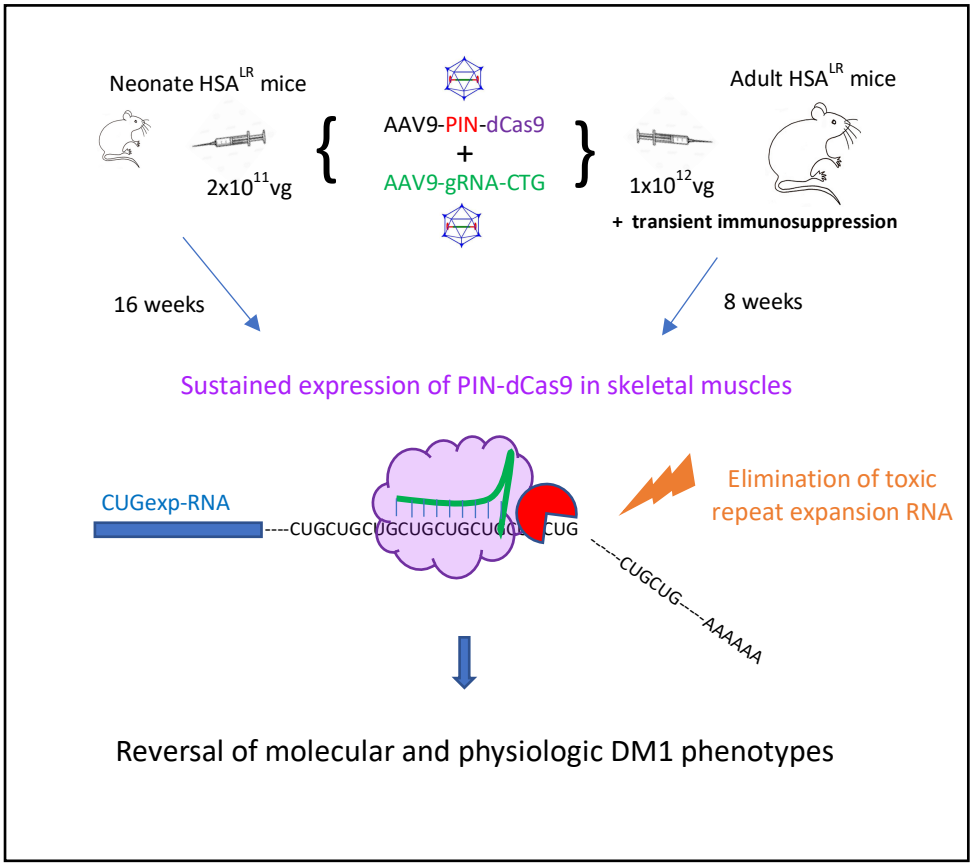

Fig. 1 | Systemic administration of Cas9-targeting toxic expanded RNA repeats in mouse models of myotonic dystrophy type 1 (DM1). HSA^{LR} mice (an animal model of DM1 in which the expression of expanded CUG repeats is restricted to skeletal muscle) display nuclear foci of toxic CUG RNA repeats that sequester MBNL1, resulting in alternative-splicing dysregulation as well as in muscle abnormalities and myotonia. The treatment of both neonate and adult HSA^{LR} mice (the latter co-treated with the immunosuppressors tacrolimus and abatacept for 2 weeks) with two adeno-associated virus vectors encoding for a nuclease-dead Cas9 fused to the PIN RNA endonuclease domain and for a guide RNA (gRNA) targeting expanded CUG repeats results in the reversion of muscle-disease phenotypes for as long as the PIN-dCas9 protein is expressed (approximately 16 weeks for neonate mice and 4 weeks for adult mice, after single injections of the indicated number of vector genomes; vg).