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Trinucleotide Repeat-Targeting dCas9 as a Therapeutic Strategy for Fuchs' Endothelial Corneal Dystrophy

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Purpose: Fuchs' endothelial corneal dystrophy (FECD) is the leading indication for corneal transplantation. Seventy percent of cases are caused by an intronic CTG triplet repeat expansion in the *TCF4* gene that results in accumulation of pathogenic expanded CUG repeat RNA (CUG^{exp}) as nuclear foci in corneal endothelium. A catalytically dead Cas9 (dCas9) can serve as an effective guide to target genomic DNA or RNA transcripts. Here, we examined the utility of the clustered regularly interspaced short palindromic repeats (CRISPR)-dCas9 system to effectively target and reduce CUG^{exp}.

Methods: We delivered dCas9 and repeat-targeting single guide RNA (sgRNA) expression plasmids to patient-derived endothelial cells using lipofection or lentiviral transduction. We used fluorescence in situ hybridization (FISH) and RNA dot-blot hybridization to quantify CUG^{exp} foci and repeat RNA levels, respectively. *TCF4* expression levels were assessed using quantitative PCR (qPCR).

Results: Using FISH, we found that expression of both dCas9 and a $(CAG)_n$ sgRNA complementary to CUG^{exp} are necessary to reduce foci. We observed a reduction in percentage of cells with foci from 59% to 5.6% and number of foci per 100 cells from 73.4 to 7.45 ($P < 0.001$) in cells stably expressing dCas9-$(CAG)_n$ sgRNA but saw no decrease in cells expressing dCas9-$(CUG)_n$ sgRNA or nontargeting control sgRNA. In cells with dCas9-$(CAG)_n$ sgRNA, we detected a reduction in CUG^{exp} RNA by dot-blot without any reduction in *TCF4* mRNA levels using qPCR.

Conclusions: Using CRISPR-dCas9 to target the trinucleotide repeat is a promising treatment for FECD contingent on effective in vivo delivery.

Translational Relevance: This work advances a gene therapy for a common age-related degenerative disorder.

Introduction

Fuchs' endothelial corneal dystrophy (FECD) is an age-related degenerative disorder that impacts 5% of white people over the age of 40 years in the United States.[1] In FECD, the post-mitotic endothelium undergoes premature senescence and apoptosis resulting in corneal edema and vision loss.[2–9] FECD is the leading indication for corneal transplantation in the developed world.[1,10]

An intronic CTG triplet repeat expansion in the *TCF4* gene (CTG18.1 locus) accounts for 70% of FECD cases in white people,[11–14] making FECD the most common DNA repeat expansion disorder. We found that expansions of > 40 CTG repeats in DNA of peripheral leukocytes confer a > 30-fold increased risk for FECD,[13] and the

repeat expansion co-segregates with the trait in 52% of families with complete penetrance and 10% with incomplete penetrance.[13] Our laboratory and others have shown that expanded CUG repeat RNA transcripts (CUG^{exp}) accumulate as nuclear foci in FECD endothelial cells that can be visualized by fluorescent in situ hybridization (FISH).[15,16] The splicing factors MBNL1 and 2 colocalize with CUG^{exp} foci and their functional depletion correlates with mis-splicing of MBNL-sensitive exons in FECD tissue contributing to disease pathogenesis.[16–18]

FECD is 1 of over 40 degenerative diseases caused by DNA repeat expansions.[19] Myotonic dystrophy type 1 (DM1) is a multisystem neurodegenerative disorder caused by a CTG repeat expansion within the 3′ UTR of the *DMPK* gene.[20,21] Of note, this mutation has also been associated with FECD with the accumulation of CUG^{exp} nuclear foci in affected corneal endothelial cells.[22,23] This remarkable observation that FECD can be caused by the same expanded repeat within noncoding regions of RNAs associated with two different genes reinforces the conclusion that the mutant expanded CUG repeat RNA is a root cause of FECD.

By the examination of endothelial tissue of presymptomatic individuals harboring the *TCF4* triplet repeat expansion mutation, we revealed that the presence of expanded CUG repeat RNA triggers mis-splicing of MBNL-sensitive exons and upregulation of fibrosis genes early in the disease course.[24] Therefore, we feel that the mutant CUG repeat RNA species has advantages as a target for drug development to halt FECD disease pathogenesis at its molecular origins.

Although advances in corneal transplantation in the form of endothelial keratoplasty have improved patient outcomes over the last decade, medical methods for prevention and treatment are needed. Our laboratory's previous efforts to develop a molecular therapy for FECD have focused on synthetic nucleic acids targeting the CUG repeat RNA with antisense oligonucleotides, duplex RNAs, and single-stranded silencing RNAs.[25,26] Here, we turn our efforts to the use of clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) system methodology as a potential therapeutic approach for FECD.

The CRISPR-Cas system is part of the immune system of prokaryotic organisms that uses RNA-guided nucleases to target and cleave foreign DNA elements in a sequence-specific manner.[27–29] The CRISPR-Cas system has been exploited widely for gene editing and regulation purposes.[30] The

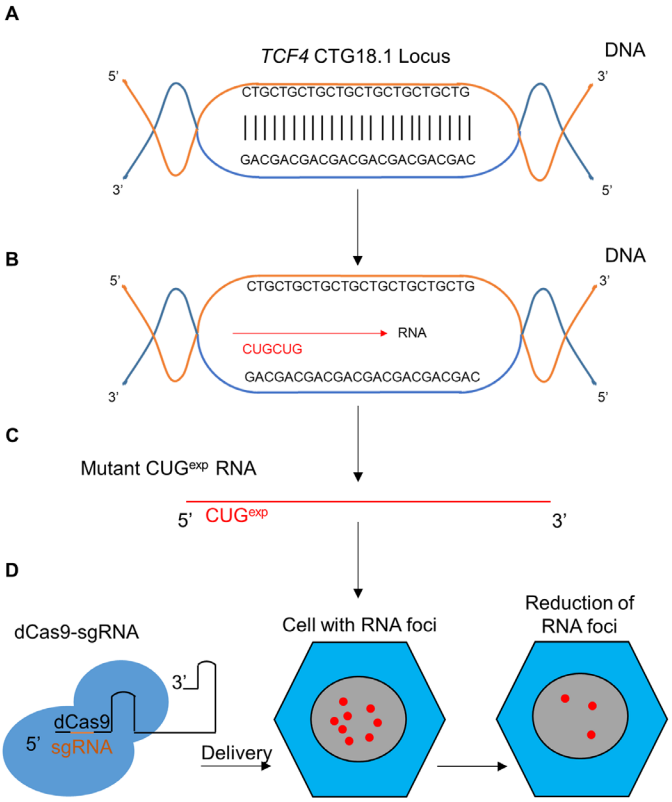


Figure 1. Application of repeat-targeting dCas9-sgRNA for treatment of FECD. (**A**) Schematic description of the *TCF4* CTG18.1 trinucleotide repeat locus. (**B**, **C**) Mutant expanded CUG repeat RNA (CUG^{exp}) is generated through transcription of DNA. (**D**) Delivery of a catalytically dead Cas9 (dCas9) with repeat-targeting sgRNAs to assess impact on mutant CUG repeat RNA in FECD corneal endothelial cells.

wild-type Cas9 endonuclease binds to a single guide RNA (sgRNA) and forms a protein-RNA complex. The sgRNA directs the complex to a specific DNA target that is complementary to the sequence of the sgRNA and is also adjacent the proto-spacer sequence motif (PAM) to generate a double-stranded break (DSB) in the target DNA strands.[31]

Mutations in both endonuclease domains of Cas9 result in a catalytically dead Cas9 (dCas9), which can still be used as an effective guiding vehicle to both genomic DNA and RNA transcripts.[32,33] Recently, dCas9 in conjunction with an sgRNA complementary to the mutant repeat RNA species has been reported to target and eliminate microsatellite repeat expansion RNAs that can result in DM1 and other rare neurodegenerative disorders.[34,35]

In this report, we introduce the use of the CRISPR-Cas system as a potential therapeutic strategy for CUG repeat RNA-mediated FECD (Fig. 1). Specifically, we examine the utility of dCas9 with a repeat-targeting sgRNA to reduce CUG^{exp} nuclear foci and mutant

intronic RNA levels in FECD patient-derived corneal endothelial cells.

Materials and Methods

Constructs and Transient Plasmid Transfection

For transient transfections, a pair of vectors encoding Cas9 and sgRNA were co-transfected with Lipofectamine LTX (Invitrogen, 15338030). The pCDNA3.1-dCas9-2xNLS-EGFP was purchased from Addgene (Addgene plasmid #74710). Different sequences of sgRNAs were inserted in pBluescriptSKII+ U6-sgRNA(F+E) empty (Addgene plasmid #74707) with BbsI sites.

LentiCRISPRv2-dCas9-hygro was created via replacement of wtCas9 in lentiCRISPRv2 hygro (Addgene plasmid #98291) with dCas9 from lenti-CRISPRv2 dCas9 (Addgene plasmid #112233) via AfeI and BamHI restriction enzymes sites. Different sequences of sgRNAs were inserted in lentiCRISPRv2-dCas9-hygro with BsmBI sites (Supplementary Table S1). All constructs were verified by Sanger sequencing.

Lentivirus Production

LentiCRISPRv2-hygromycin derived constructs were co-transfected with psPAX2 (packaging plasmid, Addgene plasmid #12260) and VSV-G (viral envelope, Addgene plasmid #11259) into HEK293T cells using Lipofectamine 3000 (Invitrogen, L3000008). Viral supernatants were collected, filtered (0.45 µm, GE Healthcare, 6780-2504), and aliquoted at -80°C for storage.

Cell Culture and Lentiviral Transduction

FECD patient-derived F45SV corneal endothelial cells (SV40-mediated immortalization by ALSTEM, Richmond, CA) and F35T corneal endothelial cells (a generous gift of Dr. Albert Jun, Johns Hopkins, Baltimore, MD, USA) were cultured as described before.[25] The 0.5 mL of unconcentrated lentivirus was placed into cells 1 day after plating in growth media. Polybrene (MilliporeSigma, TR1003G) was added to a final concentration of 5 µg/mL. After 3 days, hygromycin B was added (50 µg/mL, Invitrogen, 10687-010) to select cells stably expressing lentiCRISPRv2-hygromycin derived constructs. The surviving cells were collected and used for later experiments.

DNA Extraction and Hygromycin Resistant Gene Confirmation

Cells stably expressing lentiCRISPRv2-hygromycin derived constructs were collected and DNA was extracted by QuickExtract DNA extraction solution (Epicentre, QE09050). DNA concentrations were determined by Nanodrop spectrophotometer. Hygromycin B gene was amplified by PCR with GoTaq G2 Flexi DNA Polymerase (Promega, M7808). PCR products were used to run a 1% agarose gel. DNA fragments were visualized with Gel Doc XR+ imaging system (Bio-Rad).

Fluorescence In Situ Hybridization and Immunofluorescence

FISH of cultured cells was performed as previously described.[25] Briefly, cells were fixed and immersed in 70% ethanol at 4°C overnight. After treatment with wash buffer and prehybridization buffer, slides were incubated with $(CAG)_6CA$-5′ Texas red-labeled 2-O-methyl RNA 20-mers probe (Integrated DNA Technologies). On the next day, slides were washed twice and then stained with mounting media with DAPI (Vector Labs, H-1500).

The slides were sealed and imaged at 60 × magnification using a Widefield Deltavision microscope. Images were processed by deconvolution with AutoQuant X3 (Media Cybernetics) software. Visualization of RNA foci and GFP signals were made using ImageJ. For quantification, data were analyzed from at least 50 cells for each sample in 2 independent experiments. Results are shown as the mean ± SD.

RNA Dot-Blot Hybridization

After harvesting cells, RNA isolations were carried out with Trizol (Sigma, St. Louis, MO) according to the manufacturer's protocol. RNA concentrations were determined by the Nanodrop spectrophotometer.

The 2 µg of total RNA was denatured as mentioned before.[34] Then, the RNA was directly loaded onto the positively charged nylon membrane (Roche, 11417240001). The membrane was placed in the XL-1000 UV crosslinker (Spectronics Corporation) with the setting of 120 mJ. Hybridization oven (Hybaid) was used to pretreat the membrane in DIG Easy Hybridization solution (Roche, 11796895001) for 60 minutes at 55 °C. The probes were purchased from IDT and end-labeled with DIG by DIG Oligonucleotide Tailing Kit (Roche, 03353583910). The DIG-labeled probe was added to the DIG Easy Hybridization

solution at a ratio of 1 µl/mL. Hybridization was conducted overnight at 55 °C.

The next day, the membrane was washed twice with 2X SSC, 0.1% SDS, and twice with 0.5X SSC, 0.1% SDS at 55 °C. The membrane was then rinsed with B1 washing buffer (Roche, 11585762001) and incubated in B2 blocking solution (Roche, 11585762001). After incubation with anti-Digoxigenin-AP (Roche, 11093274910), the membrane was washed twice with B1 washing buffer. Then, the membrane was treated with CSPD (Roche, 11655884001) in detection buffer (Roche, 11585762001). DIG-AP signals were exposed to autoradiography film (Thermo Fisher) in the dark.

Real-Time Quantitative PCR Analysis of *TCF4* Transcripts

Cells were harvested and RNA was extracted with Trizol reagent. Quantitative PCR (qPCR) was performed to analyze *TCF4* transcripts level with iTaq SYBR Green Supermix. Sequences of primers were described previously.[25]

Results

Transient Expression of dCas9 with Repeat-targeting sgRNA Reduces Pathogenic CUG^{exp} RNA Foci in FECD Cells

For our initial experiments to examine the therapeutic utility of the CRISPR-Cas system in FECD, we chose to determine the effects of transient expression of DNA plasmids encoding for dCas9 and repeat-targeting sgRNAs in F45SV patient-derived corneal endothelial cells with 1500 CTG repeats in the *TCF4* gene (Fig. 2). Using cationic lipid, we delivered a plasmid encoding for a GFP-tagged dCas9 and CUG repeat-targeting sgRNA with a CAG repeat in spacer sequence $(CAG)_n$ or alternatively a plasmid encoding GFP-tagged dCas9 and CAG repeat-targeting sgRNA with a CUG repeat in spacer sequence $(CUG)_n$ (see Supplementary Table S1).

Expression of GFP was used to identify the subset of successfully transfected cells and was the focus of our analysis (see Fig. 2A; Supplementary Figure S1). We found that corneal endothelial cells were difficult to transfect with liposomes and calculated an efficiency of approximately 12% in F45SV cells (Fig. 2B). FISH was used to count the number of RNA foci in each experimental group. At least 100 cells were analyzed in each sample and cells with the GFP signal were compared to cells without the GFP signal for each experimental cohort. For cells that were successfully transfected with dCas9 and $(CAG)_n$ sgRNA, we observed a significant reduction of RNA foci (both the percentage of cells with foci and number of foci per 100 cells) compared with untreated cells (Fig. 2C). We saw no decrease in foci in cells transfected with dCas9 and $(CUG)_n$ sgRNA.

A similar transient expression experiment was performed in a different FECD patient-derived corneal endothelial cell line, F35T cells with an expanded *TCF4* allele of 1500 repeats. We observed a reduction of RNA foci in transfected F35T cells that expressed dCas9 and $(CAG)_n$ sgRNA relative to untransfected cells suggesting that the effect is not cell line specific and generalizable to FECD cells harboring the triplet repeat expansion (Supplementary Figure S2).

Generation of FECD Corneal Endothelial Cell Lines with Stable Expression of dCas9-sgRNA Constructs or sgRNA Constructs Alone

Encouraged by the reduction of foci by the transient expression of dCas9-$(CAG)_n$ sgRNA, we turned to lentiviral infection followed by stable cell selection to identify FECD cell clones containing genomic integration of dCas9-sgRNAs. Lentiviruses containing different dCas9-sgRNA constructs along with a hygromycin B resistance cassette were used to infect FECD F45SV cells followed by selection in hygromycin B. This approach allowed us to establish FECD F45SV cell lines that uniformly express various dCas9-sgRNA constructs including $(CAG)_n$, $(CUG)_n$, or non-targeting control (NTC) sgRNAs for further quantitative and mechanistic studies. Similar methods were used to generate FECD F45SV cell lines that uniformly express sgRNA constructs alone (Supplementary Figure S3).

Stable Expression of Repeat-Targeting dCas9 Effectively Reduces Mutant Repeat RNA

To study the impact of stable, long-term expression of dCas9 and various sgRNAs on mutant repeat RNA, we quantified the CUG^{exp} RNA nuclear foci using FISH in these newly generated FECD F45SV cell lines (Fig. 3A). We found a significant reduction in foci in cells expressing the dCas9 and $(CAG)_n$ sgRNA plasmid relative to untreated cells ($P < 0.001$; (Figs. 3B, 3C). The percentage of cells with RNA foci decreased from 59% to 5.6%. The number of foci per 100 cells decreased from 73.4 to 7.5. Expression of dCas9 with an NTC sgRNA or $(CUG)_n$ sgRNA had no effect on foci (see Figs. 3B, 3C).

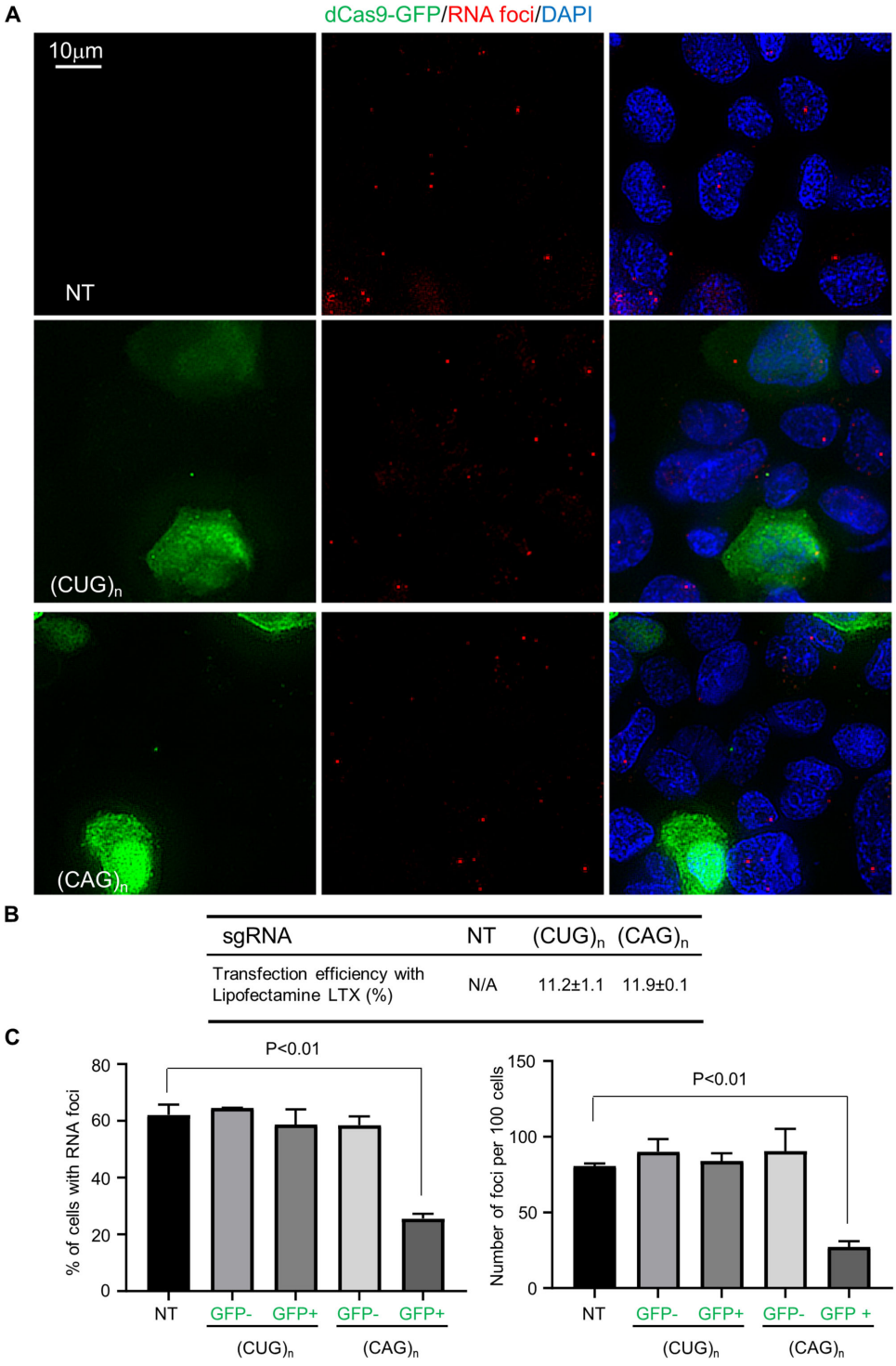
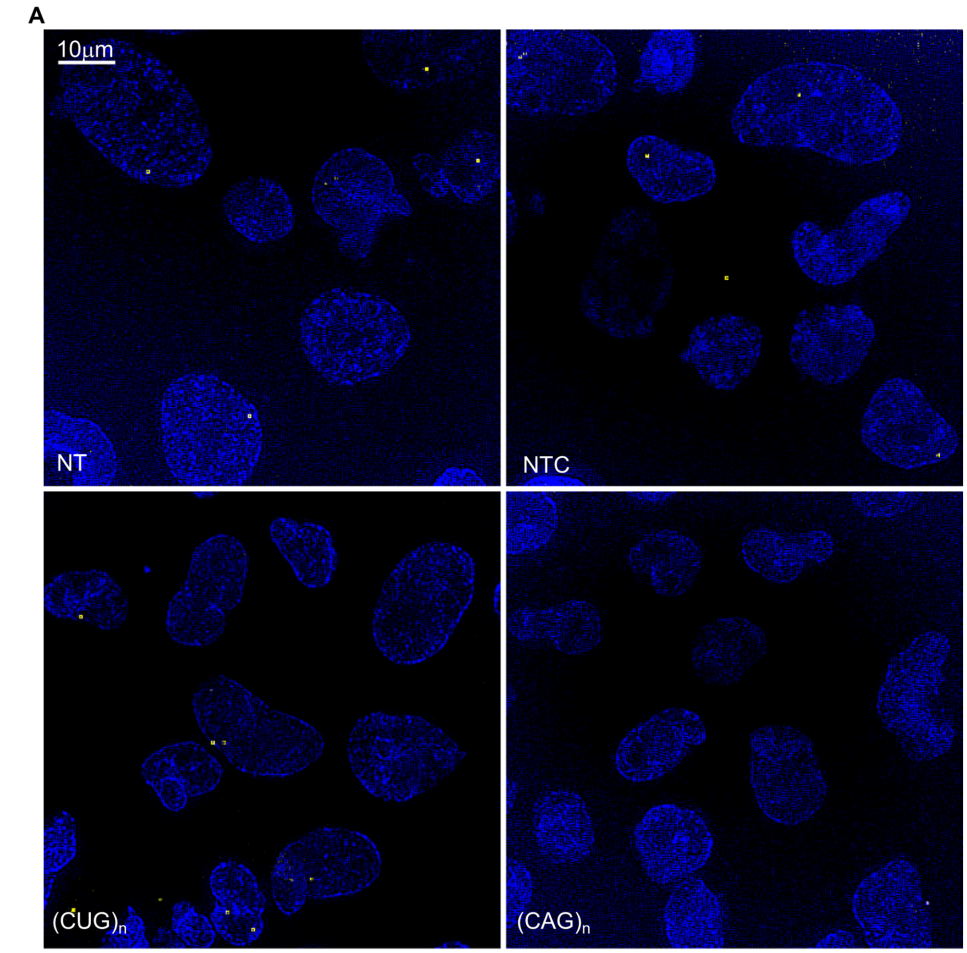


Figure 2. Effects of transient expression of dCas9 and repeat targeting sgRNA on CUG^{exp} nuclear foci in patient-derived FECD F45SV corneal cells. (**A**) Immunofluorescence images of dCas9-GFP and RNA foci in F45SV corneal endothelial cells. DAPI staining (*blue*) defines cell nuclei. (**B**) Transfection efficiencies of specific dCas9-sgRNA constructs were calculated. Results of two independent experiments are shown. NT, no plasmid treatment. (**C**) Percentage of cells containing foci and number of foci per 100 cells are shown. In cells transfected with GFP-tagged dCas9 and $(CUG)_n$ or $(CAG)_n$ sgRNA by Lipofectamine LTX, GFP negative and positive cells were analyzed separately. Results are shown as the mean ± SD, $n = 2$ independent experiments. *P* value was obtained by *t*-tests analysis of NT compared with dCas9-$(CAG)_n$ sgRNA GFP+ cells. At least 100 cells were analyzed for each sample (GFP- and GFP+ cells).



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sgRNA	NT	NTC	$(CUG)_n$	$(CAG)_n$
% of cells with RNA foci	59.0±0.8	54.9±2.3	54.5±4.4	5.6±1.7
Number of foci per 100 cells	73.4±0.8	67.5±5.4	68.5±4.9	7.5±0.1

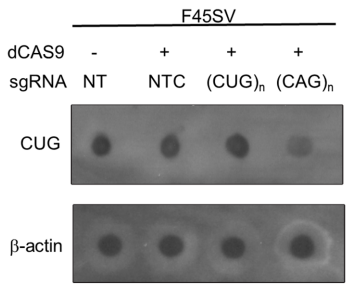

Figure 3. **Decrease of mutant CUG repeat RNA in FECD cell lines with stable expression of dCas9-$(CAG)_n$ sgRNA plasmid.** (**A**) FISH images of CUG^{exp} RNA foci in F45SV corneal endothelial cells transduced with the indicated dCas9-sgRNAs lentivirus. (**B, C**) Percentage of cells containing foci and number of foci per 100 cells were analyzed. Results are shown as the mean ± SD, $n = 2$ independent experiments. *P* value was obtained by *t*-tests analysis of NT compared with dCas9-$(CAG)_n$ sgRNA. At least 100 cells were analyzed for each sample. NT, no treatment. NTC, non-targeting control. (**D**) RNA dot-blot hybridization assay of CUG^{exp} levels in F45SV cells transfected with dCas9-NTC/$(CUG)_n$/$(CAG)_n$ sgRNA. β-actin was used as a loading control.

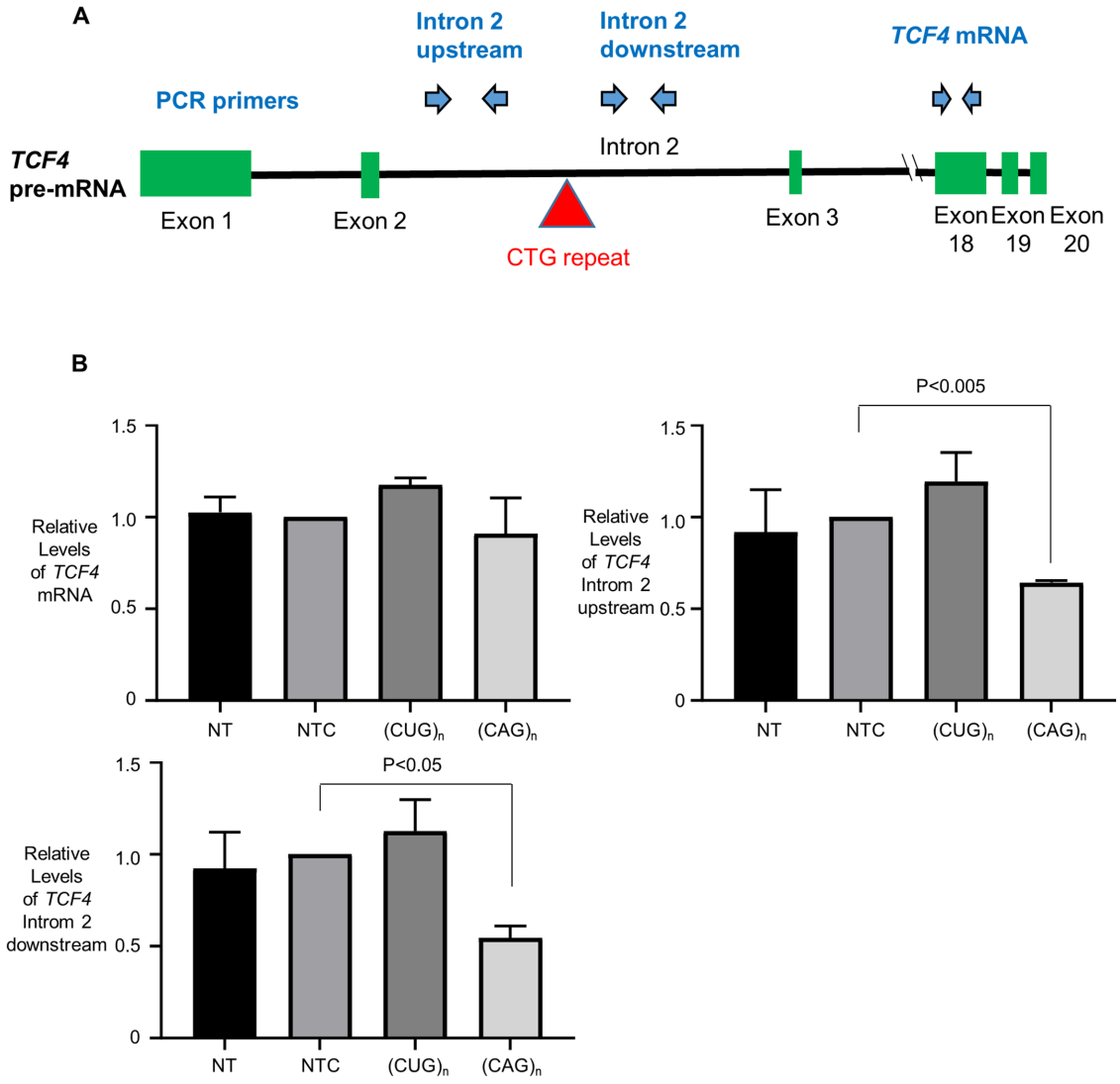

Figure 4. **Effect of dCas9-sgRNAs on *TCF4* expression levels.** (**A**) Schematic with quantitative PCR (qPCR) primers for *TCF4* mRNA and intronic RNA transcripts. (**B**) The qPCR results showing that dCas9-$(CAG)_n$ sgRNA reduces *TCF4* intron two RNA levels and has no effects on *TCF4* mRNA. Levels of *TCF4* mRNA were assessed by measuring expression levels of constitutive exon 18 found in all isoforms. Results are shown as the mean ± SD, $n = 2$ independent experiments. *P* value was obtained by *t*-test analysis of NTC compared with dCas9-$(CAG)_n$ sgRNA.

FISH assay was also performed in F45SV cells stably express sgRNA alone. No changes in RNA foci were found in F45SV cells transduced with $(CAG)_n$ sgRNA alone (Supplementary Fig. S4), indicating that expression of the 20-mer CAG repeat in the sgRNA spacer sequence alone is not sufficient to reduce the CUG^{exp} foci in the absence of dCas9. Expression of NTC sgRNA or $(CUG)_n$ sgRNA alone also had no effect on the foci (see Supplementary Fig. S4).

To quantify the levels of the CUG repeat RNA molecular species, we used the RNA dot-blot hybridization assay. We detected a reduction of the mutant repeat RNA levels in FECD cells that stably express dCas9-$(CAG)_n$ sgRNA and not in those transduced with dCas9-NTC sgRNA or dCas9-$(CUG)_n$ sgRNA (Fig. 3D).

Effect of Repeat-Targeting dCas9 on *TCF4* RNA Levels

Finally, we used qPCR to examine the impact of the various dCas9-$(CAG)_n$-sgRNAs on the transcript levels of the mRNA of the parent gene *TCF4* as well as intronic RNA immediately upstream and downstream from the repeat (Fig. 4A). We found no differences in expression of *TCF4* mRNA in any of the transduced cell lines relative to untreated cells (Fig. 4B).

However, we noted that expression levels of intronic RNA upstream and downstream of the triplet repeat were decreased only in the cells transduced with dCas9-$(CAG)_n$ sgRNA.

Discussion

CRISPR-Cas9 approaches are an effective strategy for recognizing and regulating mutant DNA or RNA responsible for human disease. Our results suggest that use of dCas9 programmed with an anti-CUG repeat guide RNA strand can target and reduce the mutant RNA in FECD patient-derived corneal endothelial cells (see Figs. 2, 3, 4, Supplementary Figs. S1, S2). The expanded repeat is located within the *TCF4* gene that encodes a ubiquitously expressed transcription factor. By targeting the intronic repeat, our approach affects the disease-causing mutant RNA without affecting the parent gene, increasing the potential of the strategy as a therapy for FECD.

With a $(CAG)_n$ sgRNA, dCas9 reduces nuclear foci and mutant CUG^{exp} RNA levels. This reduction of detectable RNA may be due to a competition for association with the expanded CUG repeat between dCas9-$(CAG)_n$-sgRNA protein complex and the RNA binding proteins that normally associate with the repeat. The RNA binding proteins MBNL1 and MBNL2 co-localize with the CUG^{exp} RNA foci[16,18] and stabilize the foci.[17] Blocking these RNA binding proteins with dCas9-$(CAG)_n$-sgRNA complex may destabilize the expanded repeat, causing a decrease in the foci and the pathogenic RNA levels in FECD patient cells. Use of a dCas9 fused to an RNAase (PIN-endonuclease) domain may further enhance the elimination of repeat RNA.[34] An alternative explanation of our results may be that dCas9-$(CAG)_n$ sgRNA impedes the transcription of the *TCF4* CTG repeat harboring DNA strand to result in reduction of mutant repeat RNA levels and CUG^{exp} foci.[35]

Excision of the CTG18.1 repeat expansion from the genomic DNA of FECD endothelial cells using dual CRISPR-Cas9 cleavage has also been proposed as a potential therapy for FECD.[36] Previous studies have demonstrated the feasibility of this approach in excising the causal CTG DNA repeat expansion in DM1 cell lines with CRISPR-Cas9 and a pair of flanking gRNAs followed by repair of the two DSBs with nonhomologous end joining albeit with variable cutting and repair efficiencies.[37] It remains to be seen whether this approach of excision of a pathogenic genomic DNA repeat is feasible in terminally differentiated cells such as corneal endothelium. Additionally, there is a risk of permanent and off-target genome editing with this approach.

Our data suggest that use of dCas9 and repeat targeting sgRNA can effectively reduce CUG repeat RNA nuclear foci in patient-derived corneal endothelial cells. However, we acknowledge the relatively low transfection efficiency with either cationic lipid (12%) or lentivirus (36%) in our in vitro model systems. Further advances in plasmid delivery to corneal endothelial cells are required to achieve the levels of CUG^{exp} foci reduction what we have previously reported for steric-blocking antisense oligonucleotides and repeat-targeting duplex RNAs.[25,26] We have already shown that oligonucleotides can be effectively delivered in vivo to corneal endothelium by intravitreal injections[38] but such treatments would need to be administered repeatedly over the lifetime of a patient with FECD.

Additional challenges must be addressed before CRISPR-dCas9 technology could be considered for clinical use. Because the transformed cells used in this study do not faithfully recapitulate the mis-splicing phenotype seen in FECD endothelial tissue, generation of a suitable mouse model of FECD would be required to test the feasibility of this gene therapy approach to restore normal MBNL localization and/or splicing in endothelial tissue in vivo. Limitations of the dCas9-$(CAG)_n$ sgRNA approach include the potential to bind other CTG repeats in the genome warranting additional studies of unanticipated off-target effects.

A gene therapy approach may be an attractive therapeutic strategy for FECD that may avoid the need for repeated treatments. Adeno-associated virus (AAV) has been identified as a useful tool for gene delivery, especially in terminally differentiated cells. High transduction efficiency of corneal endothelial cells following a single inoculation of AAV has been reported previously.[39–41] AAV delivery to FECD corneal endothelial tissue could enable long-term production of encoded therapeutic materials for repeat-targeting dCas9 for FECD. Our results open the possibility to harness the CRISPR-Cas system as a gene therapy for one of the most common inherited age-related degenerative ocular diseases.

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