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Jiaxin Hu
UT Southwestern Medical Center

Et al.

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ORIGINAL ARTICLE

Oligonucleotides targeting TCF4 triplet repeat expansion inhibit RNA foci and mis-splicing in Fuchs' dystrophy

Jiaxin Hu^{1,2,3}, Ziyi Rong^{3,4}, Xin Gong³, Zhengyang Zhou^{4,5}, Vivek K. Sharma^{6,7}, Chao Xing^{4,8,9}, Jonathan K. Watts^{6,7}, David R. Corey^{1,2,†} and V. Vinod Mootha^{3,4,*,†}

¹Department of Pharmacology, ²Department of Biochemistry, ³Department of Ophthalmology and ⁴McDermott Center for Human Growth and Development, UT Southwestern Medical Center, Dallas, TX, USA, ⁵Department of Statistical Science, Southern Methodist University, Dallas, TX, USA, ⁶RNA Therapeutics Institute, ⁷Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, Worcester, MA, USA, ⁸Department of Bioinformatics and ⁹Department of Clinical Sciences, UT Southwestern Medical Center, Dallas, TX, USA

*To whom correspondence should be addressed at: Department of Ophthalmology, Eugene McDermott Center for Human Growth and Development, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd, Dallas, TX 75390, USA. Tel: +1 2146482367; Fax: +1 2146482469; Email: vinod.mootha@utsouthwestern.edu

Abstract

Fuchs' endothelial corneal dystrophy (FECD) is the most common repeat expansion disorder. FECD impacts 4% of U.S. population and is the leading indication for corneal transplantation. Most cases are caused by an expanded intronic CUG tract in the TCF4 gene that forms nuclear foci, sequesters splicing factors and impairs splicing. We investigated the sense and antisense RNA landscape at the FECD gene and find that the sense-expanded repeat transcript is the predominant species in patient corneas. In patient tissue, sense foci number were negatively correlated with age and showed no correlation with sex. Each endothelial cell has ~2 sense foci and each foci is single RNA molecule. We designed antisense oligonucleotides (ASOs) to target the mutant-repetitive RNA and demonstrated potent inhibition of foci in patient-derived cells. *Ex vivo* treatment of FECD human corneas effectively inhibits foci and reverses pathological changes in splicing. FECD has the potential to be a model for treating many trinucleotide repeat diseases and targeting the TCF4 expansion with ASOs represents a promising therapeutic strategy to prevent and treat FECD.

[†]These authors contributed equally to the work.

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Introduction

Corneal disorders impacting corneal clarity are a leading cause of vision loss and blindness globally. Fuchs' endothelial corneal dystrophy (FECD, MIM 136800) is an age-related degenerative disorder of the endothelium that affects 4% of individuals over the age of 40 in the United States and is the leading indication for corneal transplantation (1,2). The corneal endothelium is the inner hexagonal monolayer responsible for maintenance of stromal dehydration and corneal clarity. In FECD, the post-mitotic endothelium undergoes premature senescence and apoptosis (3–9). Descemet's membrane, the basement membrane of the endothelium, becomes markedly thickened and develops focal excrescences called guttae. These guttae are diagnostic of FECD and are detected by clinical slit-lamp biomicroscopy (10). Confluent central guttae and concomitant loss of endothelial cell density results in corneal edema, scarring and loss of vision.

Recently, the potential for treating FECD has been transformed by the discovery that expansions at the intronic CTG triplet repeat polymorphism of TCF4 (MIM 602272) account for 70% of FECD cases in the United States (11–14). TCF4 expansions of greater than 40 CTG repeats confer significant risk for developing FECD (12). Expanded CUG-repeat RNA transcripts (CUG^{exp}) accumulate as nuclear foci in FECD endothelial tissue that can be visualized by fluorescent *in-situ* hybridization (FISH) (15). The splicing factor muscleblind-like 1 (MBNL1) co-localizes with CUG^{exp} nuclear foci and its sequestration and functional depletion correlate with mis-splicing of MBNL1-sensitive exons (16). Myotonic dystrophy type 1 (MIM 160900) patients with CTG expansions within the 3'-untranslated region of DMPK (MIM 605377) are also at increased risk for FECD and form CUG^{exp}-MBNL1 foci in corneal endothelium (17).

Currently, the only effective therapy for FECD is corneal transplantation. Surgical outcomes including visual acuity have improved markedly over the last decade as endothelial keratoplasty (EK) techniques have overtaken penetrating keratoplasty for FECD (18). However, modern EK techniques are still associated with significant complications including a 28.8% detachment rate of allograft and 1.7% primary graft failure rate in the immediate post-operative period necessitating additional surgical interventions (19). Reported EK graft failure rates for FECD are 3.8–5% at 5 years in specialized, single-center studies (20,21). Even when the initial surgery is successful, nearly a quarter of patients develop glaucoma after EK that require further medical or surgical treatments (21). Access to tertiary-care corneal transplant surgeons and a suitable corneal donor pool with eye banking support to process tissue limits EK as a treatment option globally.

As a rule, visual disability must be substantial before surgery becomes an acceptable option to patients and many patients suffer loss in quality of vision for years prior to seeking surgical treatment. A less or non-invasive medical treatment option would benefit hundreds of thousands of individuals with early or moderate stage FECD by halting disease pathogenesis.

Over 20 diseases are caused by expansions of nucleotide repeats within genes (22). These expanded repeats can occur within coding regions, introns, or 3'-untranslated regions and the pathogenic mechanisms linking the molecular mutation to disease are diverse. For Huntington's disease, the disease appears to be caused by the mutant protein, while for Friedreich's ataxia the disease appears to be caused by repression of wild-type protein expression. Understanding mechanism and the molecular vulnerabilities of the mutant RNA is

critical to the design of gene specific drugs capable of treating disease.

FECD has the potential to be a model for studying the pathogenesis and treatment of diseases caused by expanded repeats. Unlike most other repeat diseases, where affected tissue is usually inaccessible and can be examined only by autopsy, the corneal endothelium is only a half-millimeter from the surface of the eye. Both disease progression and treatment outcomes can be followed by simple non-invasive measurements. For preclinical studies such as the one presented here, the ready availability of patient corneal tissue provides an *ex vivo* model for evaluating molecular pathology and drug action. Some lessons learned from FECD may be broadly applicable to other diseases that affect less accessible locations.

In this report, we qualitatively and quantitatively characterize RNA transcription at the FECD locus as a target for molecular therapy. We show that antisense oligonucleotides (ASOs) can block RNA foci in patient-derived cells. ASOs can enter corneal tissue and correct splicing changes in *ex vivo* human corneas with FECD. Targeting the TCF4 expansion with oligonucleotides is a promising therapeutic strategy to prevent or treat FECD, the most common human repeat expansion disorder.

Results

Sense and antisense transcription at the TCF4 locus

Because the CTG repeat expansion is outside the protein-encoding region of the TCF4 gene, defining the landscape of RNA synthesis is essential for understanding the molecular basis of FECD. The disease-associated expanded CUG repeat occurs within intron 2 of TCF4 pre-mRNA (Fig. 1A) (23). Many genes, including some with expanded repeats, express transcripts that are in an antisense orientation relative to mRNA and these antisense transcripts have the potential to contribute to disease (24,25). We used quantitative strand-specific PCR (qPCR) to evaluate antisense transcription at the TCF4 locus and detected an antisense transcript containing the expanded CAG repeat in the F35T (1500 CUG repeats) cell line derived from a patient with FECD (Fig. 1B; Supplementary Material, Fig. S1).

We used FISH to examine the presence of sense or antisense transcript foci within two different corneal endothelial cell lines derived from patients with FECD. For both F35T (1500 CUG repeats) and F45 (71 CUG repeats) cell lines, sense foci were detected (Fig. 1C). No obvious antisense foci were detected. We also used FISH to examine foci in corneal endothelial tissue from a FECD patient with the TCF4 repeat expansion and observed both sense and antisense foci (Fig. 1D). No foci were detected in endothelial cells of control donor cornea endothelial tissue lacking the expansion.

Analysis of sense and antisense foci in patient tissue

After establishing that both sense and antisense foci were detectable in FECD corneal endothelial tissue, we initiated a quantitative analysis of corneal surgery samples from 20 different FECD patients to assess the correlation of RNA expression with disease, age, expanded allele length and sex. Tissue samples were used for FISH to evaluate the percentage of endothelial cells with at least one detectable focus and the number of foci per 100 cells.

Both sense and antisense foci were detected in all 20 FECD tissues with the TCF4 triplet repeat expansion (Table 1). Sense foci were found in 62–95% of cells while the antisense foci

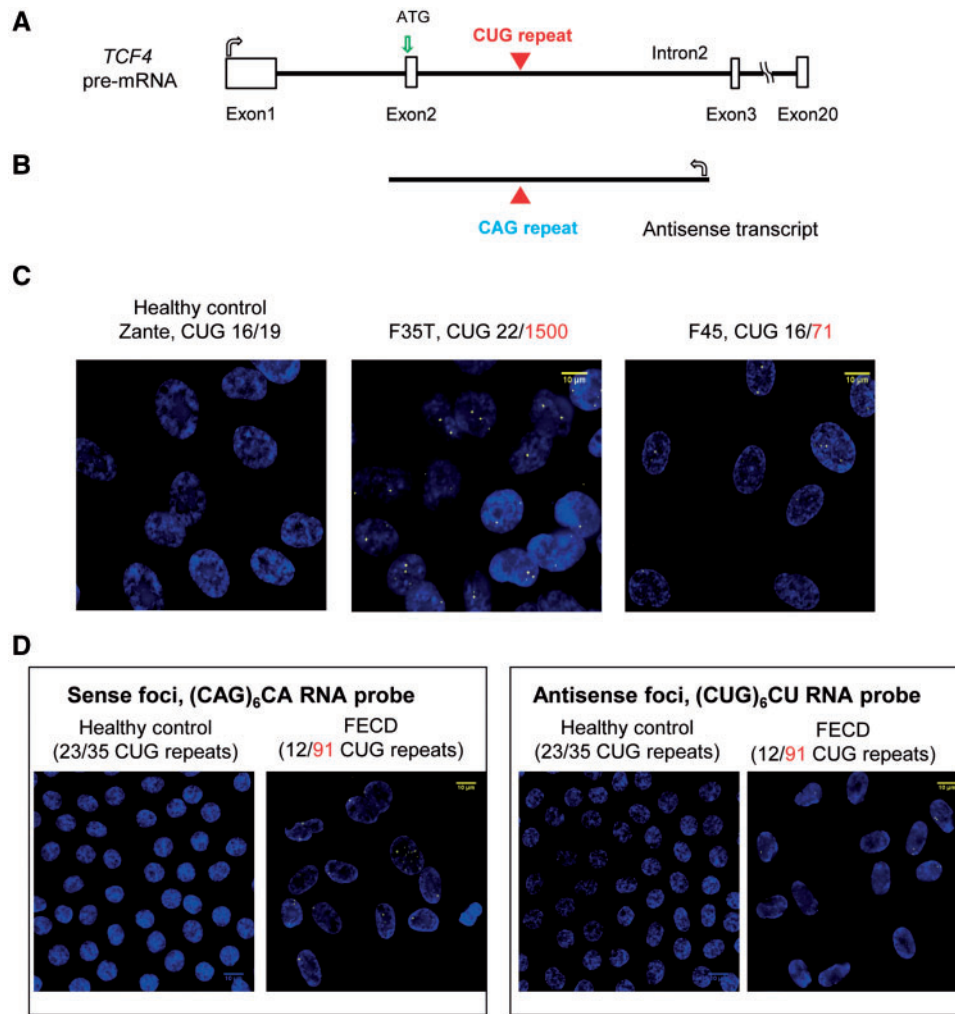


Figure 1. Sense and antisense RNA foci are detected in FECD corneal endothelial cells by fluorescent microscopy. (A, B) Scheme showing TCF4 pre-mRNA and antisense transcripts with the location of the expanded CUG and CAG repeat regions. (C) FISH images of CUG^{exp} RNA foci in two patient-derived FECD endothelial cell lines (F35T, F45) compared with a healthy control endothelial cell line (Zante). A (CAG)₆CA RNA probe was used for detecting the sense CUG^{exp} foci. (D) Representative images of sense CUG^{exp} or antisense CAG^{exp} RNA foci in one FECD patient corneal tissue compared with a healthy control endothelial tissue. A (CUG)₆CU RNA probe was used for detecting the antisense CAG^{exp} foci.

were found in only 3–27% of the cells. Foci are not numerous, with 1–2.7 sense foci and .07–0.6 antisense foci per cell. No foci were observed in a healthy control tissue or a FECD tissue without expansion. Genetic heterogeneity of FECD (17) would account for the lack of foci in endothelial tissue explants from patients without repeat expansions resulting in the same phenotype.

We observed a significant negative correlation between age and both the percentage of cells with sense foci ($P = 0.006$) and the number of sense foci per cell ($P = 0.016$) (Fig. 2A). Tissues were harvested from patients with late stage disease and the correlation of decreased foci with age may be due to earlier disappearance of cells with relatively large numbers of foci. There was a trend towards a positive correlation between the number of sense foci and the length of the triplet repeat allele, but it did not reach the statistical significance (Fig. 2B). Although women are two to four times more likely to be affected by FECD (26,27), there was no significant correlation between sex of subjects and sense foci number (Fig. 2C). There was no significant correlation between foci number for the antisense transcript and age or sex (Supplementary Material, Fig. S2).

Copy number of mutant RNA molecules in patient cells

Defining the number of disease-causing molecules is fundamental to understanding of FECD pathology and successful drug development. Using qPCR (28), we measured a copy number of less than 3 TCF4 intronic transcripts per cell in patient tissue samples, control tissue, as well as in F35T cells (Fig. 3; Supplementary Material, Figs S3 and S4). The patient-derived cells used in this study were heterozygous for mutant TCF4 and our qPCR methods detect both the mutant and wild-type TCF4 intron 2 transcripts. Therefore, the number of mutant transcripts is a fraction of the total measured RNA molecules.

Using FISH, we determined that most cells have only one or two sense foci (Fig. 3A and B; Table 1). Thus, there is an approximately a one-to-one correspondence between mutant intronic RNA transcripts per cell and sense foci per cell, suggesting each focus is a single mutant TCF4 RNA molecule. Our laboratory recently reported that c9orf72 disease-related foci are also each composed of one mutant expanded repeat RNA (28). Similarly, Wansink and colleagues have reported that the mutant RNA expanded CUG transcript associated with myotonic dystrophy

Table 1. Summary table of patient corneal endothelial tissue samples

ID	Age	Sex	Ethnicity	CTG repeat	% of total cells with sense foci	Number of sense foci per 100 cells	% of total cells with antisense foci	Number of antisense foci per 100 cells
Control								
2014-1670	45	M	Black	23, 35	0.0	0.0	0.0	0.0
CA052	75	F	White	12, 17	0.0	0.0	0.0	0.0
FECD								
CA056	59	F	White	24, 150	92.0	211	6.0	7.2
VVM573	50	F	White	12, 130	91.2	217	7.7	9.0
VVM672	55	F	White	20, 130	93.0	171	2.8	5.0
CA105	58	M	White	12, 130	82.8	195	7.1	8.0
CA120	58	F	White	16, 130	84.9	168	5.8	7.0
AK009	61	F	White	18, 130	79.7	171	8.3	13.0
CA033	62	M	White	71, 130	95.0	277	10.0	12.0
CA063	64	F	White	12, 74	92.3	210	6.2	7.0
CA045	66	F	White	15, 120	91.7	210	8.0	9.0
CA026	67	M	White	12, 67	85.0	160	5.0	6.0
CA047	67	M	White	12, 91	69.0	129	41.0	62.0
CA054	68	M	White	12, 79	90.0	272	6.0	6.7
VVM513	70	M	White	12, 88	93.7	212	6.1	7.0
CA062	71	M	White	17, 87	89.0	173	17.0	20.0
CA027	71	F	White	12, 74	86.3	143	27.0	13.0
CA050	73	F	White	17, 74	90.0	191	13.0	16.0
CA048	74	F	White	12, 54	62.0	127	12.6	20.0
CA049	75	F	White	17, 1000	81.0	144	15.0	17.0
CA053	78	F	White	14, 84	70.0	134	4.0	4.0
VVM289	86	F	White	12, 87	61.9	96	3.8	4.0

Sample 2014-1670 is a healthy control. CA052 is a FECD tissue without CTG expansion (negative control). Twenty FECD samples are with CTG expansions. Sense foci were found in 84.0% of cells [standard deviation (SD) = 10.4] compared to the antisense foci found in 10.6% of the cells (SD = 9.1) ($P = 2.1 \times 10^{-24}$). Foci are not numerous, with 1.8 sense foci (SD = 4.7) and 0.13 antisense foci per cell (SD = 0.13) ($P = 2.9 \times 10^{-13}$). $n = 300$ – 500 cells evaluated per patient sample.

is also low abundance (29). These data cumulatively suggest that small numbers of RNA molecules have a big impact on repeat expansion disease pathogenesis making them ideal therapeutic targets.

Antisense oligonucleotides inhibit foci *in vitro*

ASOs have several advantages as starting points for FECD drug discovery. ASOs are synthetic nucleic acids that can recognize complementary RNA sequences inside cells and sequence-specifically modulate gene expression *in vivo* (30). Several ASOs have shown encouraging results in late stage clinical trials, including two recent FDA approvals to alleviate spinal muscular atrophy (31) and muscular dystrophy (32).

We chose to test the ability of anti-CUG ASOs to block foci and serve as a starting point for therapeutic development. ASOs targeting the DMPK CUG repeat RNA can reverse cellular phenotypes of myotonic dystrophy (33). Our laboratory and others have shown that synthetic nucleic acids can target expanded CAG repeats within genes causing Huntington's disease, Machado Joseph Disease and dentatorubral-pallidoluysian atrophy (34–36).

FECD sense foci are much more prevalent than antisense foci, making the expanded repeat within the sense transcript the logical target of a molecular therapy. We synthesized locked nucleic acid (LNA) ASOs complementary to the CUG repeat in all three registers relative to the target sequence (Table 2). LNA substitutions were spread through the ASO, creating designs that would not be expected to recruit RNase H to cause degradation of target RNA. LNA is an advantageous chemical modification for drug development because it contains a conformationally restricted

ribose that enables high-affinity binding to cellular RNA targets (37,38).

All three 19-mers (LNA 1–3) were potent inhibitors of CUG foci in F35T or F45 endothelial cell lines (Fig. 4A and B). The 16-mer LNA 4 is less effective. LNAs 1, 2 and 3 had higher affinities for complementary targets, as measured by melting temperature, than LNA 4 (Table 2), probably explaining their greater efficacy. We were unable to assess the impact of the ASO-LNAs complementary to the sense strand on the antisense foci because the lack of any detectable antisense foci in the FECD cell lines utilized in this study. We also tested a 21-mer ASO that was entirely substituted with 2'-methyl RNA and found that it was less effective (Supplementary Material, Fig. S5). Transfection of LNAs did not reduce levels of TCF4 RNA (Supplementary Material, Fig. S6).

Free *ex vivo* uptake and activity of ASOs in FECD human corneas

To evaluate whether delivery to corneal tissue was feasible, we first tested 'gymnotic' or 'naked' (transfection reagent free) uptake (39) of a fluorophore-labeled oligonucleotide into the endothelium of *ex vivo* human corneas (Supplementary Material, Fig. S7). Uptake of ASO was observed within both the cytoplasm and nuclei.

After establishing that *ex vivo* uptake was possible, we were fortunate to be able to extend our investigation by accessing human corneas deemed unsuitable for transplantation because of findings of FECD and subsequent confirmation of the mutation by genotyping. We treated the corneas of human FECD-patient donors using a non-complementary control (LNA LC) or

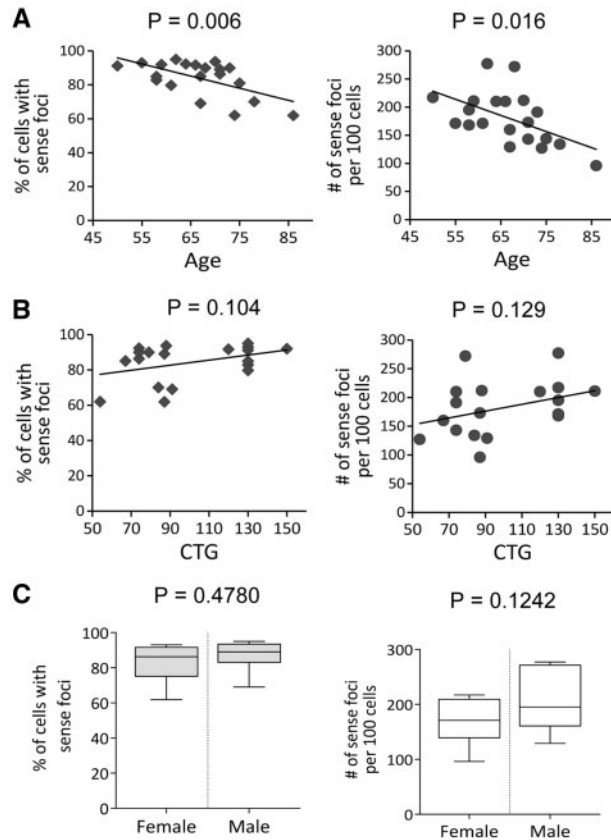


Figure 2. Effect of age, repeat length, and sex on foci. (A) Graphs show significant negative correlation between % of cells with sense RNA foci or number of sense foci per 100 cells with age of subject. (B) There is a trend toward a positive correlation between sense foci and triplet repeat allele length. (C) Graphs show no correlation between sense foci and patient gender.

ASO LNA 1 (Fig. 5A; Supplementary Material, Fig. S8). After dissection, the Descemet's membrane with endothelium was analyzed by FISH. We found that LNA 1, but not the non-complementary LNA LC, blocked foci formation (Fig. 5B and C).

The expanded repeat within TCF4 has been shown to affect splicing of MBNL1-sensitive exons in FECD corneal tissue (16). Binding of the LNAs would block the mutant repeat, preventing the RNA from interacting with MBNL1 or other factors that influence splicing. To determine whether LNA 1 was affecting a cellular phenotype, we examined splicing of *INF2*, *MBNL1* and *ADD3*, all genes previously identified as being influenced by mutant TCF4. We observed alteration of splicing in all three genes upon administration of LNA 1, partially restoring alternative splicing to that observed in normal corneal tissues (Fig. 5D and E).

Discussion

ASOs as treatment for FECD

FECD is a leading cause of vision loss and by far the most common disease caused by mutant expanded trinucleotide repeats. We introduce ASOs as a potential molecular therapy to correct a genetic disorder in human corneas. ASOs targeting the mutant TCF4 repeat effectively reduced CUG^{exp} foci formation and pathologic splicing in *ex vivo* human FECD corneas.

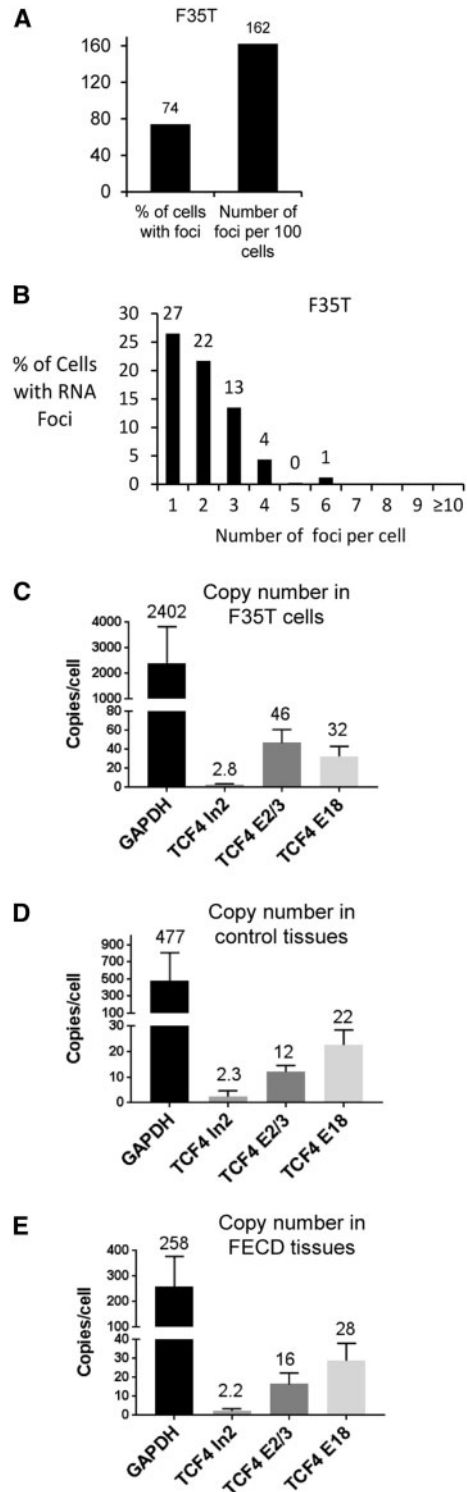


Figure 3. One-to-one correspondence between sense foci and mutant TCF4 RNA molecules in FECD. (A) Analysis of sense foci in F35T cells. (B) Sense foci distribution in F35T cell line. Majority of F35T cells have one or two sense foci in nuclei. (C) Copy number of GAPDH, TCF4 intron 2, TCF4 exon 2/3, TCF4 exon 18 transcripts measured with qPCR in F35T cells. F35T cells have less than three TCF4 intron 2 molecules. (D) Copy number of TCF4 transcripts in healthy control corneal endothelial tissues ($n = 3$). (E) Copy number of TCF4 transcripts in FECD corneal endothelial tissues ($n = 3$). TCF4 intron 2 molecules are rare RNA species in endothelial tissue. One-to-one correspondence between intronic RNA transcripts per cell and sense foci per cell suggests that each focus is a single mutant TCF4 RNA molecule.

Our laboratory and others have reported that a greater than 30-fold increased risk for FECD is correlated with an expanded CUG repeat within the *TCF4* gene (11–13). There are at least three possible explanations linking the mutation to disease: 1). The expansion with the chromosomal DNA affects gene expression directly, either at the *TCF4* locus or at nearby genes; 2). the expansion within the RNA affects gene expression or gene splicing or 3). the expansion within the RNA acts as a template for synthesis of RAN peptides, a phenomenon that has been noted for other expanded repeat disease genes (40).

Our study was not intended to definitively distinguish between these options but does provide some insights into potential roles for mutant RNA. There are an average of 1–2 foci or mutant RNA molecules per cell suggesting that, if the mutant RNA is involved directly, small numbers of RNA molecules must have an outsized impact on disease.

Evidence suggesting that these small numbers can impact disease includes our previous report that FECD is observed in myotonic dystrophy (DM1) patients that have a CUG expansion within the *DMPK* gene (17). DM1 cells are also characterized by 1–2 foci per cell (29) and the association of FECD with CUG expanded repeats in two different genes implicates the mutant RNA as causative rather than the host gene. The repeat DM1 CUG RNA transcripts within the *DMPK* gene have been demonstrated to serve as templates for repeat-associated non-ATG

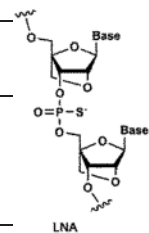
(RAN) translation resulting in toxic peptides (40). We did not examine the expression of RAN peptides in this study and such experiments are warranted to understand how repeat CUG RNA transcripts result in dysfunction of the corneal endothelium.

In DM1, experimental evidence suggests that the mutant expanded repeat CUG RNA transcripts sequester the splicing factor MBNL1 and contribute to cellular dysfunction by triggering mis-splicing (41) and splice defects have previously been noted in FECD pathogenesis (16). We provide experimental data suggesting that ASOs targeting the sense RNA transcript reverses foci formation and pathologic splicing. The ability of ASOs to target the mutant expanded RNA and at least partially correct a splicing defect is further evidence supporting the hypothesis that small numbers of mutant RNA can contribute to disease.

ASOs are experiencing a wave of success in the clinic and the eye may be a particularly advantageous organ for targeting with synthetic oligonucleotides. Vitravene, the first ASO drug approved by the FDA, was administered by intraocular (intravitreal) injection for cytomegalovirus retinitis (42). Intracameral injection would place an ASO into the anterior chamber of the eye in direct contact with the corneal endothelium. The small volume of the eye will greatly reduce the cost of treatment relative to systemic administration. Other trials with ASOs have shown long duration of effects (31), suggesting that infrequent

Table 2. List of CUG repeat-targeting LNA-ASOs

Name	Sequence 5'-3'	Mass (observed/calculated)	T _m (°C)
LNA 1	CAGCAGCAGCAGCAGCAGC	6273.56/6273.05	78.1
LNA 2	AGCAGCAGCAGCAGCAGCA	6325.52/6325.08	77.4
LNA 3	GCAGCAGCAGCAGCAGCAG	6313.56/6313.07	77.9
LNA 4	AGCAGCAGCAGCAGCA	5317.50/5317.27	74.1



LNA base: bold; DNA base: normal caps; all oligomers are fully phosphorothioate backbones. T_m was detected by equal amount (1 μM each) of LNAs with complementary DNAs.

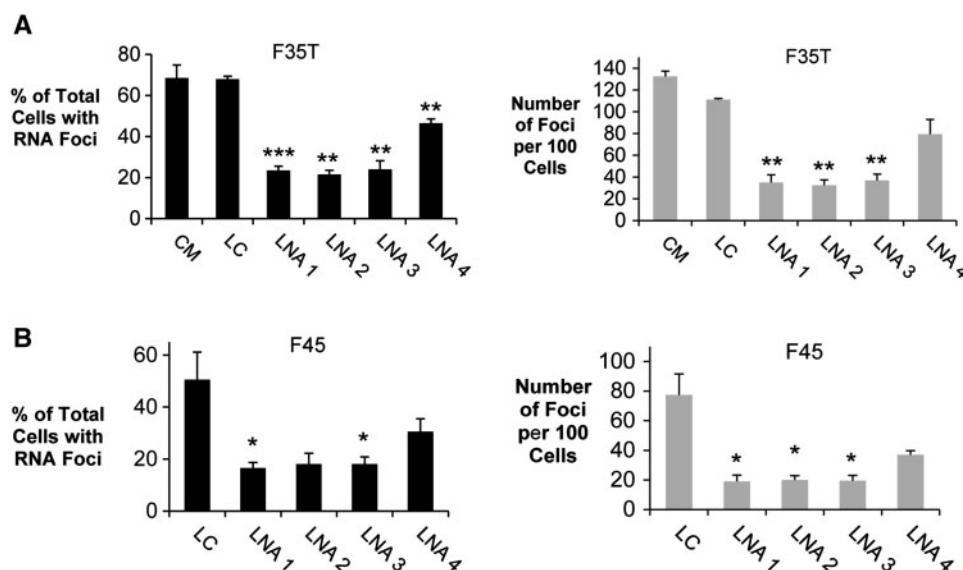


Figure 4. LNA-ASOs inhibit CUG^{exp} foci in F35T (CUG 22/1500) and F45 (CUG 16/71) endothelial cell lines. (A) Effect of LNAs on inhibition of CUG^{exp} RNA foci in F35T cells. CM: non-complementary negative control duplex RNA; LC: LNA analog of CM. (B) Effect of LNAs on inhibition of CUG^{exp} foci in F45 cells. Error bars represent SEM. *P < 0.05; **P < 0.01; ***P < 0.001 compared with control LC. At least one hundred cells were analyzed for each experiment.

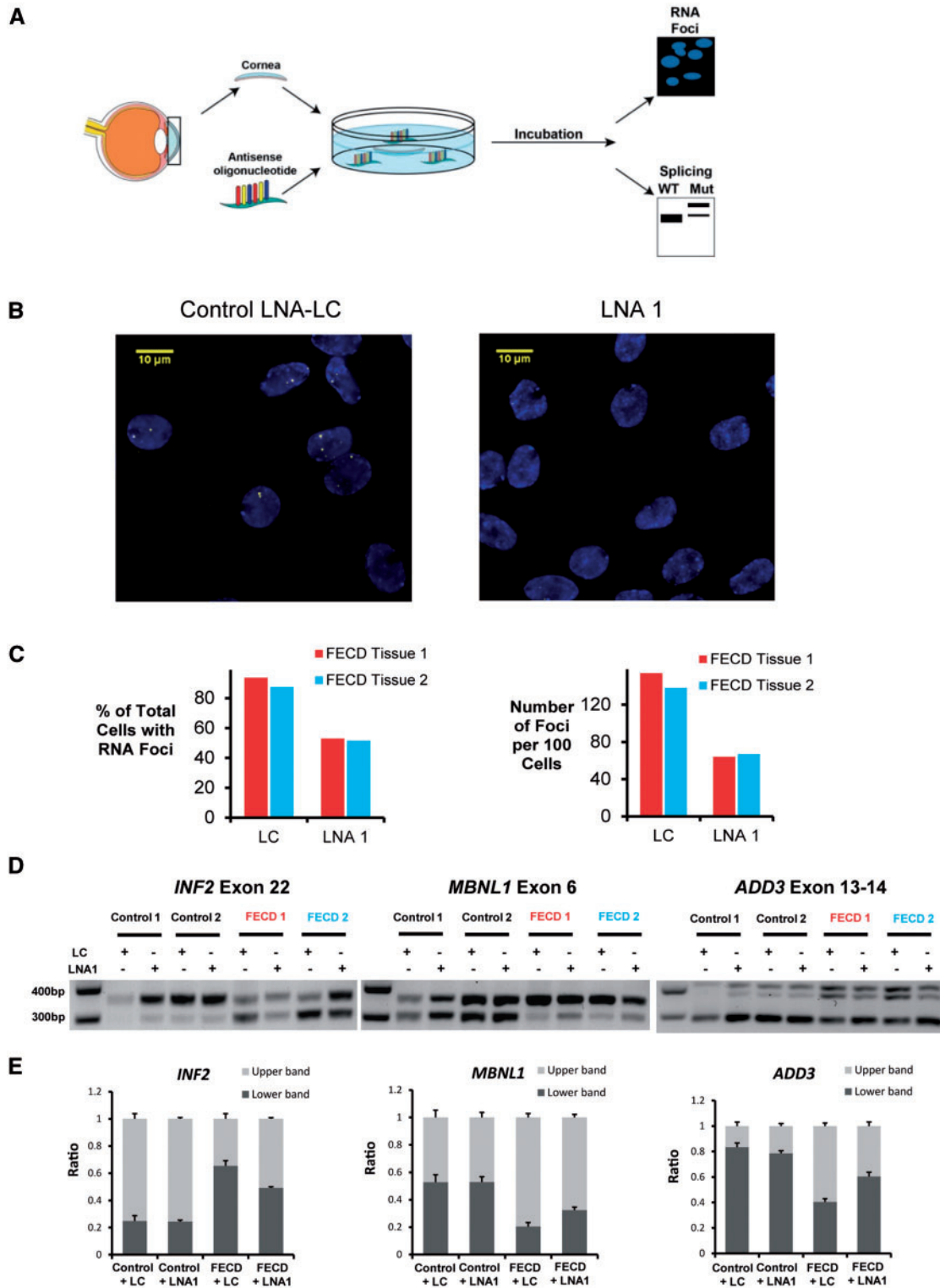


Figure 5. An LNA-ASO reduces CUG^{exp} foci and reverses mis-splicing in ex vivo human FECD corneas. (A) Scheme outlining the experiment in ex vivo human corneas. Pairs of corneas from FECD-patient donors and control donors were obtained from eye bank and were treated with ASOs. Corneas from right eyes were used to assess CUG^{exp} foci. Corneas from corresponding left eyes were used to assess splicing events. (FECD Tissue 1 from 54-year-old FECD-patient donor, 17/110 CTG repeat; FECD Tissue 2 from 51-year-old FECD-patient donor, 13/130 CTG repeat). (B) FISH images of FECD corneal endothelial tissue 1 treated with control LNA-LC and LNA 1. (C) Effect of LNA 1 on inhibition of CUG^{exp} foci in two FECD corneas. About 200 cells or more were analyzed for each treatment. (D) RT-PCR gel images showing effect of LNA 1 on the splicing of *INF2*, *MBNL1* and *ADD3* in FECD corneal endothelial tissues. Flanking RT-PCR primers were used to assess exon inclusion or exon exclusion. (E) Averaged quantitative bar graphs of splicing events shown in (D). ImageJ was used to compare density of bands on gel. Error bars represent SEM.

dosing may be possible, further lowering cost and increasing patient convenience.

The affected tissue in FECD is only a half-millimeter from the surface of the eye and topical administration by eye drops may be possible. Topical administration of ASO eye drops have already been found to be effective, safe and well tolerated in phase II FDA trials of aganirsen for viral keratitis-induced corneal neovascularization (43). Because systemic administration is unnecessary, the likelihood of systemic off-target effects that might cause toxicity is low.

Early detection of FECD in patients with the TCF4 trinucleotide repeat expansion is possible by genetic testing and routine examination of the cornea. In parallel with further testing of compounds that modulate mutant repeat RNA, it will be essential to rigorously explore the molecular natural history of FECD to guide clinical trials. Our group has documented incomplete penetrance of the FECD trait in the setting of the triplet repeat expansion in the TCF4 gene (12). Additional studies are necessary to develop a FECD risk propensity scoring system based on parameters such as TCF4 triplet repeat allele length, sex, age, ethnicity, family history and corneal examination findings to identify individuals who would benefit from preventative molecular therapies (14).

Any drug treatments for FECD would likely need to start early in the disease process and continue for an extended period. Identification of biomarkers will provide critical guides for convenient and timely monitoring of disease progression so that the length of clinical trials can be minimized. Treatments will therefore need to be minimally invasive and convenient for patients. A safe, non-toxic and convenient preventative therapy may be indicated even in subjects where physical manifestation of early stage FECD is not yet apparent but would certainly be warranted after early stage disease is detected but before significant loss of vision occurs.

Conclusions

Our data provides a rationale of the use of ASOs targeting the small number of mutant TCF4 repeat RNA molecules within cells as a therapeutic strategy to prevent and treat vision loss caused by FECD. Reversal of the molecular phenotype in an *ex vivo* human FECD cornea model may serve as relevant pre-clinical development data to justify use of a lead ASO therapeutic compound in human trials once dosing and delivery protocols are optimized in animal and *ex vivo* human corneal models (44,45). An oligonucleotide treatment for FECD would advance clinical practice by realizing the promise of precision medicine for a common human disease. Beyond FECD, the ability to introduce oligonucleotides into corneal tissue suggests the potential to control gene expression associated with many other disorders, such as the group of TGFBI-associated corneal dystrophies and broaden the options for using ASOs in the eye.

Materials and Methods

Subjects

Subjects underwent a complete eye examination including slit lamp biomicroscopy by a cornea fellowship-trained ophthalmologist. Subjects underwent EK for FECD severity Krachmer grade 5 (≥ 5 mm central confluent guttae without stromal edema) or 6 (≥ 5 mm central confluent guttae with stromal edema) assessed by slit lamp microscopy (46). Surgically explanted endothelium-Descemet's membrane monolayers

were fixed in a 4% phosphate-buffered formaldehyde, equilibrated in a 30% sucrose solution for cytoprotection, and frozen in Tissue-Tek Optimal Cutting Tissue (OCT) compound (Sakura, Torrance, CA) for FISH studies (15). Genomic DNA was extracted from peripheral blood leukocytes of each study subject using Autogen Flexigene (Qiagen, Valencia, CA).

Corneal endothelial samples from post-mortem donor corneas preserved in Optisol GS corneal storage media (Bausch & Lomb, Rochester, NY) were obtained from the eye bank of Transplant Services at UT Southwestern. Certified eye bank technicians screened the donor corneal endothelium with slit lamp biomicroscopy and Cellchek EB-10 specular microscopy (Konan Medical). Donor corneal tissue with FECD was identified by the presence of confluent central guttae. Endothelium-Descemet's membrane monolayers from donor corneas were micro-dissected and stored as previously described (15). DNA from the remaining corneal tissue of each sample was extracted with TRIzol reagent (ThermoScientific).

TCF4 CTG18.1 polymorphism genotyping

Genomic DNA from subjects' peripheral leukocytes or corneal tissue was used for genotyping. The CTG18.1 trinucleotide repeat polymorphism in the TCF4 gene was genotyped using a combination of short tandem repeat (STR) and triplet repeat primed polymerase chain reaction (TP-PCR) assays as we have previously described (12). For the STR assay, a pair of primers flanking the CTG18.1 locus was utilized for PCR amplification with one primer labeled with FAM on 5' end. The TP-PCR assay was performed using the 5' FAM-labeled primer specific for the repeat locus paired with repeat sequence targeted primers for PCR amplification. PCR amplicons were loaded on an ABI 3730XL DNA analyzer (Applied Biosystems, Foster City, CA) and the results analyzed with ABI GeneMapper 4.0 (Applied Biosystems). Large triplet repeat expansions were sized by southern blot analysis using digoxigenin-labeled probes (14).

Detection of TCF4 repeat transcript

TCF4 expression was analyzed by qPCR on a 7500 real-time PCR system (Applied Biosystems) using iTaq SYBR Green Supermix (Bio-rad). Data was normalized relative to levels of HPRT1 mRNA. Primers specific for TCF4 exon18 are as follows: F 5'-TG ACGATGAGGACCTGACAC-3'; R 5'-GTCTGGGGCTTGTCACCTT-3'. Primers for TCF4 intron2: F 5'-GAGAGAGGGAGTGAAGAGAG A-3'; R 5'-GGCAATGTCCATTTCATCT-3'.

To detect antisense transcript, cDNA was generated from 0.25 μ g of total RNA using the SuperScript III system (Invitrogen) with strand-specific reverse primers attaching a linker sequence LK. Next, cDNA was amplified by PCR with strand-specific forward and LK primers (Supplementary Material, Fig. S1). The PCR products were cloned and Sanger sequenced to verify their specificity.

Human corneal endothelial cell culture

The 'Zante' human corneal endothelial cell line derived from a healthy control subject expressing TCF4 transcript with approximately 16–19 CUG repeats was a generous gift of Dr Danielle Robertson (UT Southwestern) (47). The F35T corneal endothelial cell line derived from FECD patient expressing TCF4 transcript with approximately 1500 CUG repeats was a generous gift of Dr Albert Jun (Johns Hopkins). The F45 corneal endothelial cell line

was a primary culture from a donor cornea from FECD patient expressing TCF4 transcript with approximately 71 CUG repeats. The dissected Descemet's membrane monolayer was incubated with 2 mg/ml collagenase A (Roche) in culture media at 37°C for 4 h to dissociate the cells, spun down at 800 g for 5 min, and plated on culture dish pre-coated with fibronectin (FNC) (Athena Environmental Sciences). Cells were grown in modified Eagle's minimal essential media (OptiMEM) (ThermoFisher) supplemented with 8% fetal bovine serum, 5 ng/mL human epidermal growth factor (ThermoFisher), 20 ng/mL nerve growth factor (Fisher Scientific), 100 µg/mL bovine pituitary extract (ThermoFisher), 20 µg/mL ascorbic acid (Sigma-Aldrich), 200 mg/L calcium chloride (Sigma-Aldrich), 0.08% chondroitin sulfate (Sigma-Aldrich), 50 µg/mL gentamicin (ThermoFisher) and antibiotic/antimycotic solution (diluted 1/100) (Sigma-Aldrich) (48). Cultures were incubated at 37°C in 5% CO₂ and passed when confluent.

Transcript copy number measurement by qPCR

Transcript copy number measurement was performed as we described previously (28). Briefly, standard RNA was made by *in vitro* transcription with corresponding purified PCR fragment containing SP6 promoter sequence. After checking RNA with Bioanalyzer (Agilent), a serial dilution of purified standard RNA ranging from 10 to 10⁵ copies was used to construct standard curve for qPCR efficiency following reverse transcription. The qPCR was performed on a CFX Connect Real-Time System (Bio-Rad). Ct values in each dilution were measured in triplicate. To determine transcript copy number per cell, total RNA was extracted by TRIzol (ThermoScientific) for F35T cell line or by NucleoSpin RNA XS kit (Fisher Scientific) for endothelial tissue samples and used to do reverse transcription. Cell number of F35T was counted by two methods: hemocytometer and Z1 Particle Counter (Beckman Coulter). Cell number of control endothelial tissue was calculated as $\pi \times (d/2)^2 \times \text{cell density}$ (d is the diameter of trephine used for dissection; endothelial cell density measured by specular microscopy was provided by eye bank). Endothelial cell density of FECD tissue was estimated as 500 cells/mm². For each transcript, a serial dilution of cDNA was used. Ct values in each dilution were measured in triplicate. Copy number per cell was calculated by using the standard curve for qPCR efficiency of each transcript. For cells, data were analyzed from two batches of F35T cells. For tissues, data were analyzed from three control tissues and three FECD tissues. Results are showed as the mean \pm SD. Primers used to make PCR product are: GAPDH E1F1 SP6 (5'-ATTTAGGTGACACTATAGAAGCTCTGCTCCTCTGTTGAC-3'), GAPDH E5R1 (5'-TTGATTTTGGAGGGATCTCG-3'), TCF4 E2F1 SP6 (5'-ATTTAGGTGACACTATAGAATGCTAAAATGCATCACCAACA-3'), TCF4 E3R1 (5'-GAGCAGTAAAATGTCCACTTG-3'), TCF4 intron2dwF1 SP6 (5'-ATTTAGGTGACACTATAGAATGTTGCACTTTCTCCATTTCG-3'), TCF4 intron2dwR1 (5'-CAGGAGAGCAATTTGAAGCA-3'), TCF4 E18F1 SP6 (5'-ATTTAGGTGACACTATAGAATGACGATGAGGACCTGACAC-3') and TCF4 E18R1 (5'-AGGATCAGGAGCTTGGTCTG-3'). Primers and probes used for qPCR are: Human GAPD (GAPDH) Endogenous Control (Thermo Fisher 4310884E), TCF4 E2F2 (5'-TAGGGACGGACAAAGAGCTG-3'), TCF4 E3R2 (5'-CCACTTGCCAAAGAAGTTGG-3'), TCF4 E2/3 probe (5'-TTCACCTCTGTGAGCAGTG-3'), TCF4 intron2dwF2 (5'-TTCTCCATTGCTTCTTGC-3'), TCF4 intron2dwR2 (5'-CACTCCCTCTCTCCAGCA-3'), TCF4 intron2dw probe (5'-GCTCTGACTCAGGGAAGGTG-3'), TCF4 E18F2 (5'-ACGATGAGGACCTGACACCA-3'), TCF4 E18R2 (5'-GGGCTTGTAC

TCTTGAGGT-3') and TCF4 E18 probe (5'-AGAAGGCAGACGGTGA GAAG-3').

Synthesis and transfection of oligonucleotides

LNA phosphoramidites were synthesized from the 3'-hydroxyl precursors (Rasayan) and assembled into oligonucleotides as described previously (49). ASOs were deprotected using concentrated aqueous ammonia for 16 h at 55°C and were characterized by LCMS (Table 2). LNA ASOs were transfected into cells with Lipofectamine RNAiMAX (Life Technologies) as described previously (34). Cells were plated at a density of 300 000 per well of a 6-well plate and transfected at the same time using an oligonucleotide concentration of 25 nM. After 72 h, the cells were transfected again as mentioned above and harvested 4 days later to assess the effects for reduction of CUG^{exp} foci using RNA FISH/immunofluorescence.

Fluorescence in-situ hybridization

Cornea endothelial cells post oligonucleotide transfection were harvested by trypsin and replated on glass slides. Cells were fixed with 4% formaldehyde in 1X phosphate-buffered saline (PBS) and permeabilized in 70% ethanol at 4°C overnight. After removing the permeabilization solution, cells were washed with buffer (10% formamide in 2X saline sodium citrate buffer [SSC]) for 5 min, and then incubated with prehybridization buffer (40% formamide in 2XSSC) at 45°C for 20 min. (CAG)₆CA-5' Texas red-labeled 2-Omethyl RNA 20-mers probe in hybridization buffer (100 mg/mL dextran sulfate and 40% formamide in 2XSSC) was added. The slides were placed in a humidified chamber and incubated in the dark at 37°C overnight. On the next day, cells were washed twice with wash buffer at 37°C for 15 min, and then stained with mounting media with DAPI (H-1500; Vector Labs).

Cells were imaged at 60× magnification using a Widefield Deltavision microscope. Images were processed by blind deconvolution with AutoQuant X3. Visualization of RNA foci were made using ImageJ. For quantification, at least 20 pictures were taken from randomly chosen microscopic fields, containing 100–300 cells for each treatment. Counting of foci was performed by different investigators.

FISH of corneal endothelial tissue monolayers were performed using previously described methods (15). Twenty random archived EK samples from patients with the CTG18.1 expansion were studied. Genotyping of CTG18.1 locus was performed after the time of EK using genomic DNA from subject leukocytes. Each endothelial tissue sample was bisected with one half used to detect sense foci using (CAG)₆CA RNA probe and other half used to detect antisense foci using (CUG)₆CU RNA probe (Integrated DNA Technologies).

Ex vivo oligonucleotide treatment of human corneas

Post-mortem human donor corneas were obtained from UT Transplant Services. The donor corneas were incubated in Optisol GS corneal storage media with a single stranded antisense oligonucleotide LC-Cy5 (final concentration 10 µM) at 37°C. The Descemet's membrane-endothelium monolayers were dissected as reported previously (15) either after 1 day or 7 days of treatment, stained with DAPI (Vector Labs), and imaged at 60× magnification using a Widefield Deltavision microscope.

FECD donor corneas with confluent corneal endothelial guttae on specular microscopy and the TCF4 expansion and control donor corneas were bisected and incubated in Optisol GS corneal storage media with control LNA (LC) or LNA1 (final concentration 10 μM) at 37°C. After treatment for 7 days, RNA FISH analysis was performed on the dissected Descemet's membrane-endothelium monolayers from the right eye from each donor to assess CUG^{exp} sense foci as described above. After treatment for 9 days, the total RNA was extracted from the dissected Descemet's membrane-endothelium monolayers using TRIzol (ThermoScientific) per manufacturer's instructions. Alternative splicing of MBNL1-sensitive exons were assessed by RT-PCR as described previously (16) using endothelial tissue from corresponding left eyes of donors.

Statistics

Correlation between four foci parameters (percentage of total cells with sense foci, number of sense foci per 100 cells, percentage of total cells with antisense foci and number of antisense foci per 100 cells) and age, sex and CTG repeat number were examined on the 20 FECD subjects by linear regression models.

Study approval

The study was approved by the institutional review board of the University of Texas Southwestern Medical Center and conducted in adherence with the tenets of the Declaration of Helsinki. Written informed consent was obtained from participants prior to inclusion in the study.

Supplementary Material

Supplementary Material is available at HMG online.

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Conflict of Interest statement: Authors are inventors on a planned patent related to findings reported in the study.

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