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

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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.
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\textsuperscript{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 (M BN1L1) co-localizes with CUG\textsuperscript{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\textsuperscript{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 treatment rate of allograft and 1.7% primary graft failure 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 treatment.

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 TCF4 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...
were found in only 3–27% of the cells. Foci are not numerous, with 1–2.7 sense foci and 0.07–0.6 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...
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 complementarity 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'-O-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

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**Table 1. Summary table of patient corneal endothelial tissue samples**

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

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 × 10^{-10}]). 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 × 10^{-12}]). n = 300–500 cells evaluated per patient sample.
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<sup>exp</sup> foci formation and pathologic splicing in ex vivo human FECD corneas.

Discussion

ASOs as treatment for FECD

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.

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. Vitrawene, 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

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence 5’-3’</th>
<th>Mass (observed/calculated)</th>
<th>$T_m$ (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LNA 1</td>
<td>CAGCAGCAGCAGCAGCAG</td>
<td>6273.56/6273.05</td>
<td>78.1</td>
</tr>
<tr>
<td>LNA 2</td>
<td>AGCAGCAGCAGCAGCAG</td>
<td>6325.52/6325.08</td>
<td>77.4</td>
</tr>
<tr>
<td>LNA 3</td>
<td>GCAGCAGCAGCAGCAGCAG</td>
<td>6313.56/6313.07</td>
<td>77.9</td>
</tr>
<tr>
<td>LNA 4</td>
<td>AGCAGCAGCAGCAGCAG</td>
<td>5317.50/5317.27</td>
<td>74.1</td>
</tr>
</tbody>
</table>

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 CUGexp 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 CUGexp 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 CUGexp 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 PK 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 cryoprotection, 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’-TGAGATGAGACCCTGACAC-3’; R 5’-GGCAATGTCCATTTCCATCT-3’. Primers for TCF4 intron2: F 5’-GAGAGAGGGAGTAAAGAGAG-3’; R 5’-GGCAATGTCCATTTCCATCT-3’.

To detect antisense transcript, cDNA was generated from 0.25 μg 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 µg/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 transcription with corresponding purified PCR fragment in vitro, the diameter of trephine used for dissection; endothelial cell transcript with approximately 71 CUG repeats. The dissected Descemet’s membrane monolayer was incubated 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)6-C5’ Texas red-labeled 2-O-methyl 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 (CUG)6CU 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 anti-sense 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 CUGexp 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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