The *TCF4* Trinucleotide Repeat Expansion of Fuchs' Endothelial Corneal Dystrophy: Implications for the Anterior Segment of the Eye

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Purpose. In the United States, 70% of Fuchs' endothelial corneal dystrophy (FECD) cases are caused by an intronic trinucleotide repeat expansion in the *TCF4* gene. CUG repeat RNA transcripts from this expansion accumulate as nuclear foci in the corneal endothelium. In this study, we sought to detect foci in other anterior segment cell types and assess their molecular impact.

Methods. We examined CUG repeat RNA foci appearance, expression of downstream affected genes, gene splicing, and *TCF4* RNA expression in corneal endothelium, corneal stromal keratocytes, corneal epithelium, trabecular meshwork cells, and lens epithelium.

Results. CUG repeat RNA foci, the hallmark of FECD in corneal endothelium (found in 84% of endothelial cells), are less detectable in trabecular meshwork cells (41%), much less prevalent in stromal keratocytes (11%) or corneal epithelium (4%), and absent in lens epithelium. With few exceptions including mis-splicing in the trabecular meshwork, differential gene expression and splicing changes associated with the expanded repeat in corneal endothelial cells are not observed in other cell types. Expression of the *TCF4* transcripts including full-length isoforms containing the repeat sequence at the 5′ end is much higher in the corneal endothelium or trabecular meshwork than in the corneal stroma or corneal epithelium.

Conclusions. Expression of the CUG repeat containing *TCF4* transcripts is higher in the corneal endothelium, likely contributing to foci formation and the large molecular and pathologic impact on those cells. Further studies are warranted to examine any glaucoma risk and impact of the observed foci in the trabecular meshwork of these patients.

Keywords: Fuchs' endothelial corneal dystrophy, trinucleotide repeat expansion disorder, corneal endothelium, *TCF4*, RNA nuclear foci

Corneal diseases represent one of the leading causes of vision loss and blindness globally.[1] Inherited corneal dystrophies can compromise the structure and transparency of the cornea. Fuchs' endothelial corneal dystrophy (FECD) occurs in 4% of the population in the United States over the age of 40 years.[2] FECD is the leading indication for corneal transplantation in the developed world.[3,4] The loss of vision is age related and is due to degeneration of the corneal endothelium. As the disease progresses, the post-mitotic corneal endothelium prematurely senesces, leading to a thickening of its underlying basement membrane, Descemet's membrane, and formation of hallmark focal excrescences called guttae.[2,5–11]

The endothelium is the innermost layer of the cornea that maintains the relative dehydration state of the corneal stroma required for the transparency of the cornea. Loss of the endothelium results in corneal edema and scarring. However, the more superficial layers of the cornea are also affected in FECD, including the loss of corneal nerves and development of corneal stromal haze.[12] Currently, there is a lack of consensus in the field as to whether patients with FECD may also be at risk for glaucoma, the leading cause of irreversible blindness worldwide.[13–17]

The cause of 70% of FECD cases in the United States is an intronic CTG repeat expansion within the *TCF4* gene.[18–21] Population-based studies are warranted to determine unbiased estimates of the prevalence and penetrance of the *TCF4* trinucleotide repeat expansion in different ethnic groups. CUG repeat RNA transcripts from this trinucleotide repeat expansion accumulate in corneal endothelial cells[22] and are

thought to bind muscleblind-like (MBNL) protein, an important splicing factor.[23–25] This binding reduces the cellular pool of MBNL protein and causes global splicing dysregulation. This splicing dysregulation and the differential expression of extracellular matrix genes likely account for the activation of fibrosis pathways and mitochondrial dysfunction leading to the observation of guttae and other physical manifestation of disease.[26]

Corneal transplantation is the only effective therapy for FECD.[3,4] Although endothelial keratoplasty has proven to be a transformative therapy for patients, it has limitations. There is a small but significant failure rate for the initial procedure, and the procedure requires considerable postoperative recovery time and care.[27] Even when modern endothelial keratoplasty techniques are used, there are complications, including detachment of the allograft, primary graft failure, and a substantial incidence of secondary glaucoma.[27–30] Many patients lack access to corneal transplant surgeons and a suitable donor cornea pool with eye banking support.[31] The development of drug therapies to treat FECD would complement surgical approaches and achieve better long-term benefits for patients. Drug development would benefit from a detailed understanding of the molecular mechanisms connecting the expanded CTG trinucleotide repeat polymorphism with findings in the corneal endothelium. One central unresolved question is whether the expanded CTG repeat mutation, which occurs in all cells, manifests its findings in cell types other than the corneal endothelium. Here, we examine *TCF4* RNA expression, expression of downstream affected genes, gene splicing, and foci appearance in cell types of the anterior segment of the eye, including the corneal endothelium, corneal stromal keratocytes, corneal epithelium, trabecular meshwork cells, and lens epithelium.

Methods

Anterior Segment Tissue Samples

The study was conducted in compliance with the tenets of the Declaration of Helsinki and with the approval of the institutional review board of the University of Texas Southwestern (UTSW) Medical Center. Subjects underwent a complete eye examination with a slit-lamp microscope by a cornea fellowship–trained ophthalmologist. Subjects underwent cataract surgery alone or combined cataract surgery with endothelial keratoplasty for FECD severity Krachmer grade 5 (≥5 mm central confluent guttae without stromal edema) or grade 6 (≥5 mm central confluent guttae with stromal edema) as assessed by slit-lamp microscopy.[32] Surgically explanted anterior lens capsule epithelium and 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 O.C.T. compound (Sakura Finetek, Torrance, CA, USA) for fluorescence in situ hybridization (FISH) studies as we have previously described.[22] Genomic DNA was extracted from peripheral blood leukocytes of each study subject using AutoGen FlexiGene (QIAGEN, Hilden, Germany).

Postmortem donor corneas were obtained from the eye bank of Transplant Services at UTSW. Certified eye bank technicians screened the donor corneal endothelium with slit-lamp biomicroscopy and Cellchek EB-10 specular microscopy (Konan Medical, Irvine, CA, USA). Donor corneal tissue with FECD was identified by the presence of confluent central guttae. The donor corneas procured by the eye bank were stored in Life4°C corneal preservation medium (Numedis, Isanti, MN, USA) at 4°C until dissection of the various cell types. Genomic DNA from subjects' peripheral leukocytes or donor tissue was used for genotyping the CTG18.1 trinucleotide repeat length in the *TCF4* gene as we have previously described.[18] For the repeat polymorphism, we dichotomized alleles such that those with ≥40 CTG repeats were considered an expanded allele.[18] The corneal endothelial surface of the donor cornea was stained with VisionBlue 0.06% trypan blue ophthalmic solution (Dutch Ophthalmic Research Center, Zuidland, The Netherlands). Trabecular meshwork tissue was dissected according to consensus recommendations for trabecular cell isolation and characterization as previously described.[33] Briefly, a #11 blade disposable scalpel (McKesson, Irving, TX, USA) was used to make vertical cuts along the anterior margin of the trabecular meshwork (immediately posterior to Schwalbe's line on the corneal side) and posterior margin of the trabecular meshwork (immediately anterior to the scleral spur). A Kahook dual blade (New World Medical, Rancho Cucamonga, CA, USA) was then used to remove the trabecular meshwork and inner wall of Schlemm's canal, which were collected in a 1.5-cc tube. An 8.0-mm Barron Donor Cornea Punch (Katena, Parsippany, NJ, USA) was used to make a partial thickness cut through the endothelium–Descemet's membrane. The central endothelium–Descemet's membrane monolayers from donor corneas were microdissected and stored as previously described.[22] Corneal epithelial cells were scraped off the underlying stroma using a #11 blade disposable scalpel and collected in a 1.5-cc tube. After the endothelium and epithelium were removed, the stroma was thoroughly rinsed in balanced salt solution. One-half to one-third thickness lamellar dissection of the cornea stroma tissue was performed using a #11 blade disposable scalpel for the purpose of obtaining thinner sections of the stroma. The dissected tissues were either stored at –80°C for RNA studies or further processed for FISH.

Fluorescence In Situ Hybridization

The dissected Descemet's membrane, trabecular meshwork, or stromal lamellar tissue was placed flat on a glass slide and then fixed in 4% formaldehyde in 1× PBS. The scraped corneal epithelial cells were resuspended in PBS and immobilized on glass slides with a Shandon Cytospin 4 cytocentrifuge (Thermo Fisher Scientific, Waltham, MA, USA) prior to fixation per the manufacturer's instructions. Cornea tissues were then permeabilized in 70% ethanol at 4°C overnight. After the permeabilization solution was removed, tissues were washed with the wash buffer (10% formamide in 2× SSC buffer) for 5 minutes and then incubated with the pre-hybridization buffer (40% formamide in 2× SSC) at 45°C for 20 minutes. Then, $(CAG)_6$CA-5′ Texas red-labeled 2′-*O*-methyl RNA probe in the hybridization buffer (100 mg/mL dextran sulfate and 40% formamide in 2× SSC) was added. The tissues were placed in a humidified chamber and incubated in the dark at 37°C overnight. The next day, tissues were washed twice with wash buffer at 37°C for 15 minutes and then stained with the mounting media with 4′,6-diamidino-2-phenylindole (DAPI, H-1500; Vector Laboratories, Newark, CA, USA).

To prepare the paraffin-embedded tissue cross-sections, each donor cornea was bisected and fixed in 4% formaldehyde for 24 hours. Then, the fixed tissue was washed in PBS

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twice and stored in 70% ethanol for the paraffin embedding protocol of the UTSW histology core facility. Vertical sections were made from the center that included all layers of the cornea and trabecular meshwork. The cross-sections were deparaffinized by xylene (three times for 10 minutes each time), rehydrated by 100% ethanol (three times for 3 minutes each time), and then rehydrated by 95%, 70%, and 50% ethanol sequentially for 3 minutes each. After the slides were rinsed with water, they were permeabilized in 0.2% Triton X-100 in 2× SSC for 15 minutes on ice. The slides were rinsed with 2× SSC twice, incubated with the prehybridization buffer, and then treated with the hybridization buffer with FISH probe overnight at 37°C.

The tissues were imaged using a 60× lens for RNA foci with a DeltaVision widevision microscope (GE HealthCare, Chicago, IL, USA). Images were processed by blind deconvolution with AutoQuant X3 software. Visualizations of RNA foci were made using ImageJ (National Institutes of Health, Bethesda, MD, USA) as previously described.[34] We used signal intensity (by adjusting the brightness and contrast of the image) to detect RNA foci, which appear as distinct, bright, and round-shaped dots in the nucleus. The same brightness and contrast parameters were then used to process and analyze the entire batch of FISH images. For quantification, at least 20 pictures were taken from randomly chosen microscopic fields containing 100 to 400 cells for each tissue.

RNA Extraction

Total RNA from the corneal endothelium and trabecular meshwork was directly isolated using the NucleoSpin RNA XS kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. Total RNA from corneal epithelial cells was extracted with TRIzol Reagent (Sigma-Aldrich, St. Louis, MO, USA) and purified with the NucleoSpin RNA XS kit. The stromal lamellar tissues were cut into smaller pieces by scissors and placed into 1 mL of the TRIzol Reagent for 30 minutes on ice. After centrifuge, the liquid supernatant was carefully removed and collected so as not to disturb the stromal tissue. Then, 1 mL of fresh TRIzol Reagent was added into the tube with stromal tissue prior to centrifuging and collection of the supernatant, and this process was repeated once more. After combining the collected TRIzol solution, the stromal RNA was isolated by chloroform and isopropanol. The crude RNA was then purified using the NucleoSpin RNA XS kit.

Validation of Differential Gene Expression and Alternative Splicing Patterns

Total RNA concentration was analyzed using a NanoDrop spectrophotometer (Thermo Fisher Scientific). The cDNAs were prepared by reverse transcription of equal amounts of RNAs from tissue samples using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). The quantitative PCR (qPCR) experiments were performed on a 7500 Real-Time PCR System (Applied Biosystems, Waltham, MA, USA) using iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). Data were normalized relative to the levels of the housekeeping gene *RPL19* mRNA. Reverse transcription PCR (RT-PCR) was performed using ChoiceTaq Blue Mastermix (Thomas Scientific, Swedesboro, NJ, USA). PCR amplification was performed as follows: 94°C for 3 minutes (one cycle), 94°C denaturation for 30 seconds, 60°C annealing for 30 seconds, 72°C extension for 1 minute (38 cycles), and a 7-minute 72°C extension. The amplification products were separated by 1.5% agarose gel electrophoresis. The qPCR or PCR primers were used as previously reported.[26]

Statistics

Unpaired Student's *t*-tests were applied to reveal statistical differences between different tissue groups for the molecular studies. P values less than 0.05 ($^{*}P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$) were considered significant.

Results

Experimental Design to Examine Molecular Consequences of Repeat Expansion in Anterior Segment Cell Types

Corneal endothelial tissue samples and anterior lens capsule specimens were obtained after surgery from FECD patients with the trinucleotide repeat expansion in the *TCF4* gene who had undergone endothelial keratoplasty and/or cataract surgery (Fig. 1A, Table 1). Donor corneoscleral rims were obtained from the eye bank and dissected to obtain corneal epithelium, corneal stroma, corneal endothelium, and trabecular meshwork cells (Fig. 1B; Tables 1, 2). Donor corneas were obtained from individuals who (1) had no signs of FECD and no CTG expansion (CTRL), (2) had FECD findings (guttae-positive upon specular microscopy) with CTG expansion (FECD_REP), or (3) had no signs of FECD noted upon specular microscopy (guttae-negative) but were positive for the CTG repeat expansion after genotyping (non-FECD_REP). Note that the non-FECD_REP samples were rare, present in only a few percent of all donor corneas made available for analysis.

When viewed using FISH, the expanded CUG repeat RNA (CUG^{exp}) can be detected as nuclear foci (Fig. 2A). Each foci is a single mutant RNA molecule.[34] RNA was extracted from dissected tissues and qPCR was used to evaluate the expression of *TCF4* and genes known to be affected by the presence of the trinucleotide repeat mutation in corneal endothelial cells. The splicing of genes that are sensitive to MBNL is altered by expression of the trinucleotide repeat expansion.[23,26] Therefore, to examine the molecular consequences of mutant RNA expression, we examined splicing of genes that are known to change during the progression of FECD using RT-PCR followed by agarose gel electrophoresis. These techniques (foci detection, qPCR, and splicing of sensitive genes) allowed us to gain insights into the consequences and potential impact of expression of CUG repeat RNA.

Foci Are Absent in Lens Epithelium

We had previously reported that RNA foci were observed in most corneal endothelial cells and that cells had a median of one or two foci per cell in endothelial keratoplasty surgical samples of patients with late-stage FECD with the *TCF4* trinucleotide repeat expansion.[22,34] For some patients, endothelial keratoplasty is combined with cataract surgery, providing access to the anterior lens capsule tissue. In this current study, we examined anterior lens capsule epithelium from FECD patients with the expansion.

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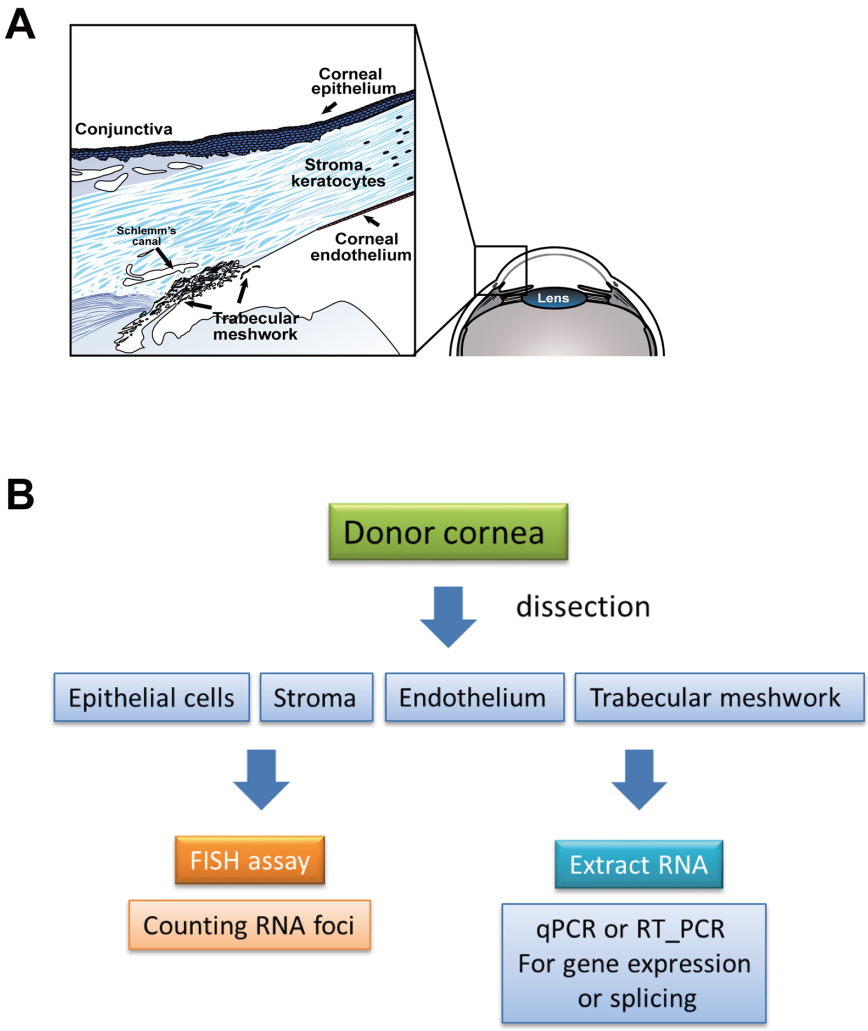

Figure 1. Experimental design. (**A**) Anterior segment structures of the eye. (**B**) Scheme outlining the experiments utilizing human donor corneoscleral rims. Pairs of corneas from donors with FECD findings and healthy donors were obtained from the eye bank and were dissected for detecting RNA foci or for assessing differential gene expression and mis-splicing.

We compared detection of foci between lens epithelium and corneal endothelium of two consecutive FECD patients at the time of endothelial keratoplasty and cataract surgery (Figs. 2A, 2B; Table 1). We did not detect any foci in the anterior lens capsule epithelium. To put the lack of foci in the lens epithelium into perspective, more than 80% of the corneal endothelial cells collected at the same time from the same patients possessed RNA foci (Fig. 2C). We subsequently confirmed the absence of foci in the lens epithelium of five additional FECD patients with the expansion obtained at the time of their cataract surgery (data not shown).

Table 1. Patient Surgical Specimens Including Cornea Endothelium and Anterior Lens Capsule Tissues for RNA FISH Assay

ID	Diagnosis*	CTG18.1†	Sex	Age (y)
VVM868	Ctrl	18, 24	M	73
VVM876	Ctrl	16, 36	F	53
VVM877	Ctrl	24, 27	F	71
VVM873	FECD_REP	15, 73	F	86
VVM716	FECD_REP	15, 83	F	71
VVM795	FECD_REP	25, 83	F	62
VVM38	FECD_REP	26, 130	F	66
VVM609	FECD_REP	18, >100	F	73
VVM932	FECD_REP	18, >100	F	61

* Ctrl indicates control tissue without CTG expansion; FECD_REP, tissue with FECD or guttae-positive with CTG expansion in the *TCF4* gene.

† CTG18.1 is the CTG repeat length in the *TCF4* gene.

Foci Are Detected More Frequently in Corneal Endothelium Relative to Corneal Stroma

We next examined postmortem donor corneas to allow us to examine a wider range of anterior segment cell types for analysis. Because the expanded CUG repeat is such a common inherited disease mutation, it is possible to obtain mutant tissue from postmortem donors. Tissue from the corneal endothelium from donor corneas with FECD findings (detection of guttae by specular microscopy) and repeat expansion (Fig. 3A) yielded a high percentage of cells with foci (84%) similar to that observed in endothelial tissue

Table 2. Donor Corneoscleral Tissues for FISH Assay and RNA Extraction

ID	Diagnosis*	CTG18.1†	Age (y)	Sex	Usage
5564	Ctrl	16, 17	72	M	FISH
2463	Ctrl	12, 15	70	F	FISH
5243	Ctrl	14, 15	53	F	RNA
5245	Ctrl	25, 33	69	F	RNA
5564	Ctrl	16, 17	72	M	RNA
5600	Ctrl	12, 15	74	M	RNA
5677	Ctrl	12, 18	71	F	RNA
5704	Ctrl	15, 15	67	M	RNA
5706	Ctrl	12, 25	63	F	RNA
5702	Ctrl	12, 18	71	M	RNA
5763	Ctrl	12, 18	60	M	RNA
5794	Ctrl	17, 24	74	M	RNA
5370	Non-FECD_REP	24, >100	56	M	FISH
4463	Non-FECD_REP	18, 73	73	M	FISH
2234	FECD_REP	18, 67	62	M	FISH
2535	FECD_REP	18, 83	49	M	FISH
4000	FECD_REP	22, >100	73	F	FISH
5286	FECD_REP	12, 85	68	F	FISH
5477	FECD_REP	17, 73	63	F	FISH
887	FECD_REP	12, 70	63	M	RNA, FISH
8102	FECD_REP	12, >100	70	M	RNA, FISH
8162	FECD_REP	12, 70	73	M	RNA, FISH
8394	FECD_REP	12, >100	59	F	RNA, FISH
74452	FECD_REP	12, >100	65	F	RNA
74646	FECD_REP	15, 73	66	F	RNA

* Ctrl indicates control tissue without CTG expansion; FECD_REP, tissue with FECD or guttae-positive with CTG expansion in the *TCF4* gene; and Non-FECD_REP, guttae negative tissue but with CTG expansion.

† CTG18.1 is the CTG repeat length in the *TCF4* gene.

samples of patients with late-stage FECD (83% or 89%) (Fig. 2) undergoing corneal transplantation.

We then examined the corresponding corneal epithelium and stroma (Figs. 3B–3D). Unlike the endothelial monolayer, the epithelium is composed of multiple layers of cells. To prepare a single layer for FISH, we dissected epithelial cells from tissue and spread the cells on a coverslip on a glass plate. Corneal stromal keratocytes are dispersed between densely packed collagen lamellae. To visualize stromal foci, we split the stroma in half in the horizontal plane with a lamellar dissection. This reduced the tissue thickness. Because the tissue is transparent, we were able to detect RNA foci in stromal keratocytes.

Visual inspection of the stroma and epithelium revealed detectable, but much lower, numbers of foci compared with endothelial cells (Figs. 3B, 3C). Relative to corneal endothelial cells, quantitation of foci across over 100 imaged cells revealed an 8- to 20-fold decrease in the total number of cells with foci. We also evaluated the number of foci per 100 cells to account for the possibility that some cells may have more foci than others, and we observed a 10- to 25-fold decrease in the number of foci per cell (Fig. 3D).

Foci Are Also Detected in the Trabecular Meshwork Cells

We investigated the presence of foci in the trabecular meshwork, because the trabecular meshwork is a key tissue involved in glaucoma, but we encountered technical challenges. Analysis of foci using FISH in the trabecular meshwork and Schlemm's canal is demanding because, unlike the corneal endothelium, epithelium, or corneal stroma, this tissue is not transparent. In addition, it is composed of several distinct tissue layers, including the uveal meshwork, which is comprised of a three-dimensional network of connective tissue beams covered by trabecular meshwork cells; corneoscleral meshwork, consisting of collagen and elastin lamellae (sheets) lined by trabecular meshwork cell monolayers; juxtacanalicular connective tissue with sparsely imbedded trabecular meshwork cells; and the Schlemm's canal with an inner wall covered by an endothelial monolayer.[33]

We dissected the trabecular meshwork from donor corneas and performed FISH. RNA foci were clearly detected only in trabecular meshwork cells located at the surface of the trabecular beams and lamellae from FECD tissue (41% of cells) and non-FECD_REP sample (37 % of cells) using whole-mount microscopy (Fig. 4A). Our analysis was limited, however, by a high background due to the trabecular beams trapping probe and preventing clear visualization of cells imbedded inside of the trabecular meshwork.

Because we were concerned that this background might affect our conclusions, we prepared cross-sections of paraffin-embedded FECD corneoscleral rims and visualized cells within the trabecular meshwork and surrounding area. Because the paraffin treatment may damage RNA detection, we also analyzed the corneal endothelial cells at the same time as a positive control. A picture of a typical trabecular meshwork is shown in Figure 4B (left) using a low-magnification 20× lens. We then used a higher magnification 60× lens to visualize individual cells and foci (Fig. 4B, right).

Using whole-mount microscopy, we observed foci in approximately 40% of cells from FECD-REP or non-FECD_REP trabecular meshworks (Figs. 4A, 4C) compared to 84% of cells in flatmount corneal endothelium monolayers (Fig. 3A, 3D). When we quantitated images from the analysis of cross-sections of six different FECD_REP and non-FECD_REP tissue samples from separate donors, we observed a substantial amount of foci in the corneal endothelium; from 47% to ~67% of the cells had foci, which was lower compared to the 84% detection by whole flatmount microscopy of endothelial tissue monolayers (Figs. 3A, 3D, 4B, 4D). For the trabecular meshwork, we calculated that about 16% to ~43% of trabecular meshwork cells had foci in our six donor samples on cross-sections (Fig. 4D). Judging by our comparison of flatmount versus cross-sectional results of corneal endothelium, our quantitation of trabecular meshwork foci may represent a lower estimate of the percentage of detected foci. These data from two imaging strategies suggest that the number of foci per cell in trabecular meshwork is intermediate between the number of foci in corneal endothelium and the number of foci in stroma. A summary of the RNA foci findings in all the tissues examined is shown in Supplemental Table S1.

Effect of CTG Expansion on Gene Splicing

Altered splicing of MBNL-sensitive exons in the corneal endothelium is a genetic hallmark that unambiguously distinguishes FECD caused by an expanded CTG repeat from cases of FECD that cannot be traced to a CTG expansion. We extracted RNA from the corneal epithelium, stroma, endothelium, and trabecular meshwork from FECD_REP and control donor corneal samples (Table 2).

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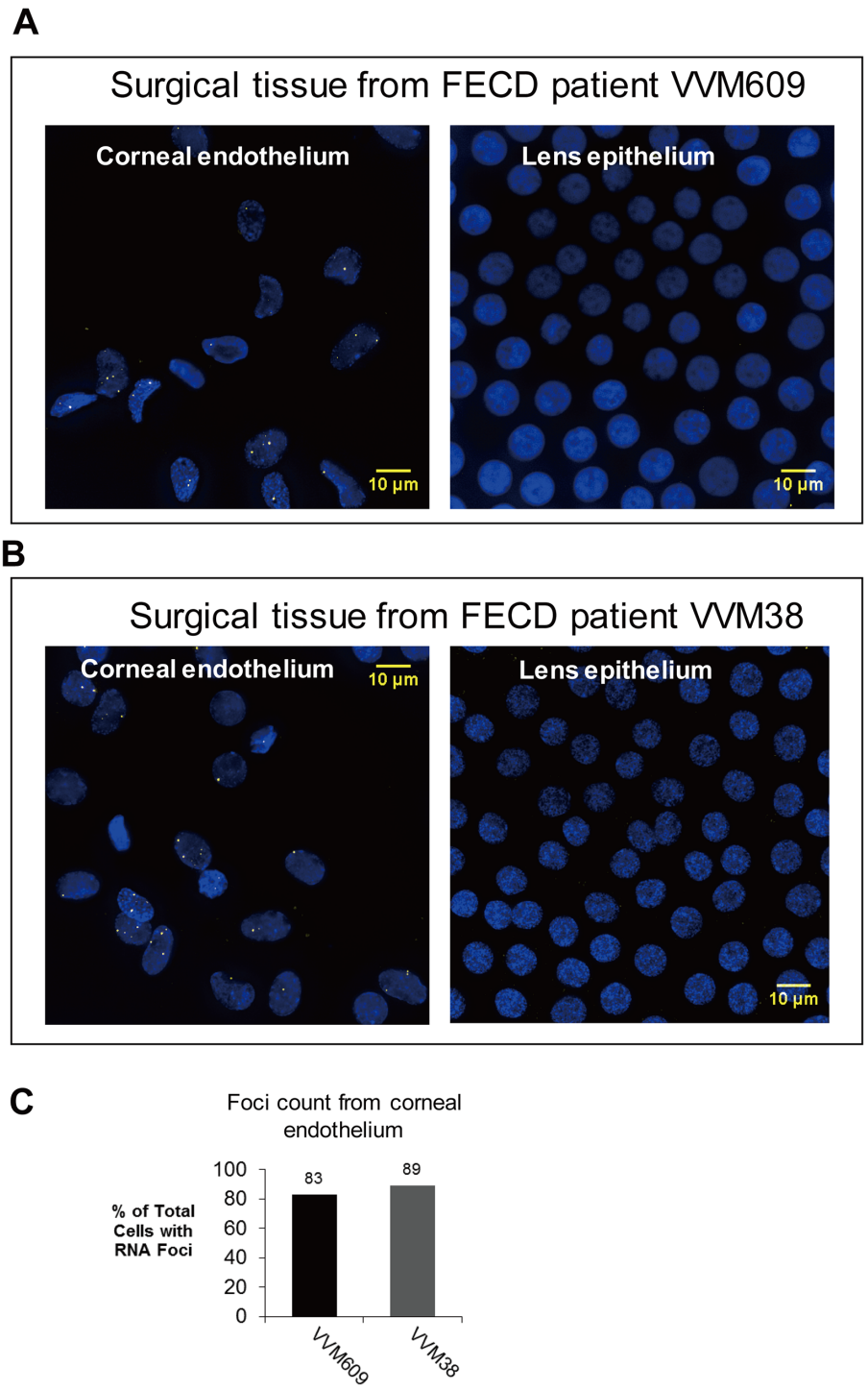

Figure 2. *TCF4* expanded CUG RNA nuclear foci were abundant in the corneal endothelium but absent in the lens epithelium of FECD patients. Representative FISH images of the corneal endothelium and lens epithelium from the same patient. The cell nuclei were stained with DAPI (*blue*). A $(CAG)_6CA$-5′ Texas red-labeled 2-*O*-methyl RNA probe was used for RNA foci (*white*). A total of seven lens epithelium samples were examined.

The splicing changes were examined for three benchmark genes—inverted formin gene (*INF2*), muscleblind-like splicing regulator 1 gene (*MBNL1*), and muscleblind-like splicing regulator 2 gene (*MBNL2*)—which have been previously shown to have altered splicing in corneal endothelium of patients with late-stage disease.[20,26] We confirmed these previous results in corneal endothelium from donor corneas with FECD findings and repeat expansion (Fig. 5A, Supplementary Table S2). In contrast to the substantial changes in corneal endothelium, we observed no significant change in stromal keratocytes or corneal epithelium (Figs. 5B, 5C; Supplementary Fig. S1; Supplementary Table S2). Samples from the trabecular meshwork showed splicing changes in *INF2* but not *MBNL1* or *MBNL2* (Fig. 5D, Supple-

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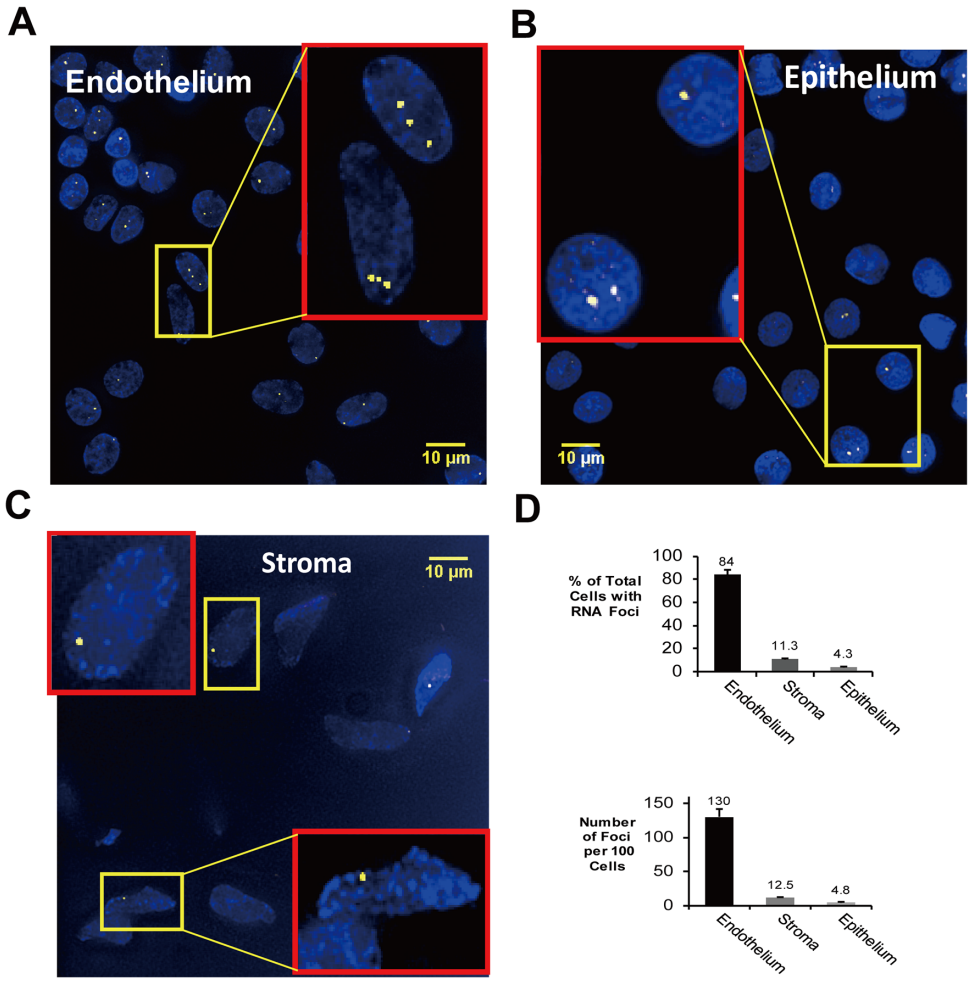

Figure 3. *TCF4* expanded CUG repeat RNA foci were detected in the corneal epithelium, stroma, and endothelium of FECD donor corneal tissue. (**A–C**) Representative FISH images for *TCF4* CUG foci in FECD corneal endothelium (**A**), corneal epithelium (**B**), and corneal stroma (**C**). (**D**) Percentage of cells with foci or number of foci per 100 cells averaged from the three layers of FECD corneal tissues.

mentary Table S2). These data are consistent with the relative percentage of foci detected in these tissue (corneal endothelium > trabecular meshwork > stroma > epithelium).

Effect of CTG Expansion on Gene Expression

Our lab had previously observed that expression of the expanded CUG RNA in corneal endothelial tissue results in activation of genes in the fibrosis pathway.[26] Activation of fibrosis genes including those encoding for the extracellular matrix is consistent with the clinical observation of thickening of Descemet's membrane and guttae formation. We used RNA from donor corneal epithelium, corneal stroma, corneal endothelium, and trabecular meshwork tissues and examined the expression of fibrosis genes and other genes known to be affected by the repeat expansion in the corneal endothelium using qPCR (Table 2).

As we had observed previously in the FECD corneal endothelium, expression of *FN1*, *COL1A1*, *COL4A2*, *COCH*, *CTGF*, *MSI1*, and *BCL2* significantly increased, whereas the expression of *SOD3*, *KDR*, and *LUM* significantly decreased (Fig. 6). No significant expression changes were observed in the corneal epithelium, stroma, or trabecular meshwork for these genes, with one exception: *KDR* showed a significant decrease in FECD corneal epithelium. These data are consistent with the observation that the same transcriptional signature in corneal endothelium triggered by the CUG repeat RNA is not observed in other cell types of the anterior segment.

TCF4 Gene Expression

To help evaluate reasons for the difference in foci detection in the corneal endothelium versus corneal epithelium, corneal stroma, and trabecular meshwork, we used qPCR to evaluate the expression of *TCF4*. There are 46 different *TCF4* transcripts documented, and 25 of these isoforms have sequences that span the CTG repeat on the 5′ end of the gene.[35] We analyzed *TCF4* mRNA expression using primer sets targeting either exons 2 and 3 for transcripts containing CUG repeats or constitutive exon 18 to evaluate all of the transcripts (Fig. 7A). We used primer sets complementary to sequences up- or downstream of the expanded CUG repeat to evaluate the expression of intron 2.

Expression of the constitutive exon 18 of *TCF4* in corneal epithelium and stroma was low in all samples, regardless of the presence of the CUG repeat. Expression of *TCF4* exon 18 in trabecular meshwork was much higher than in epithelium

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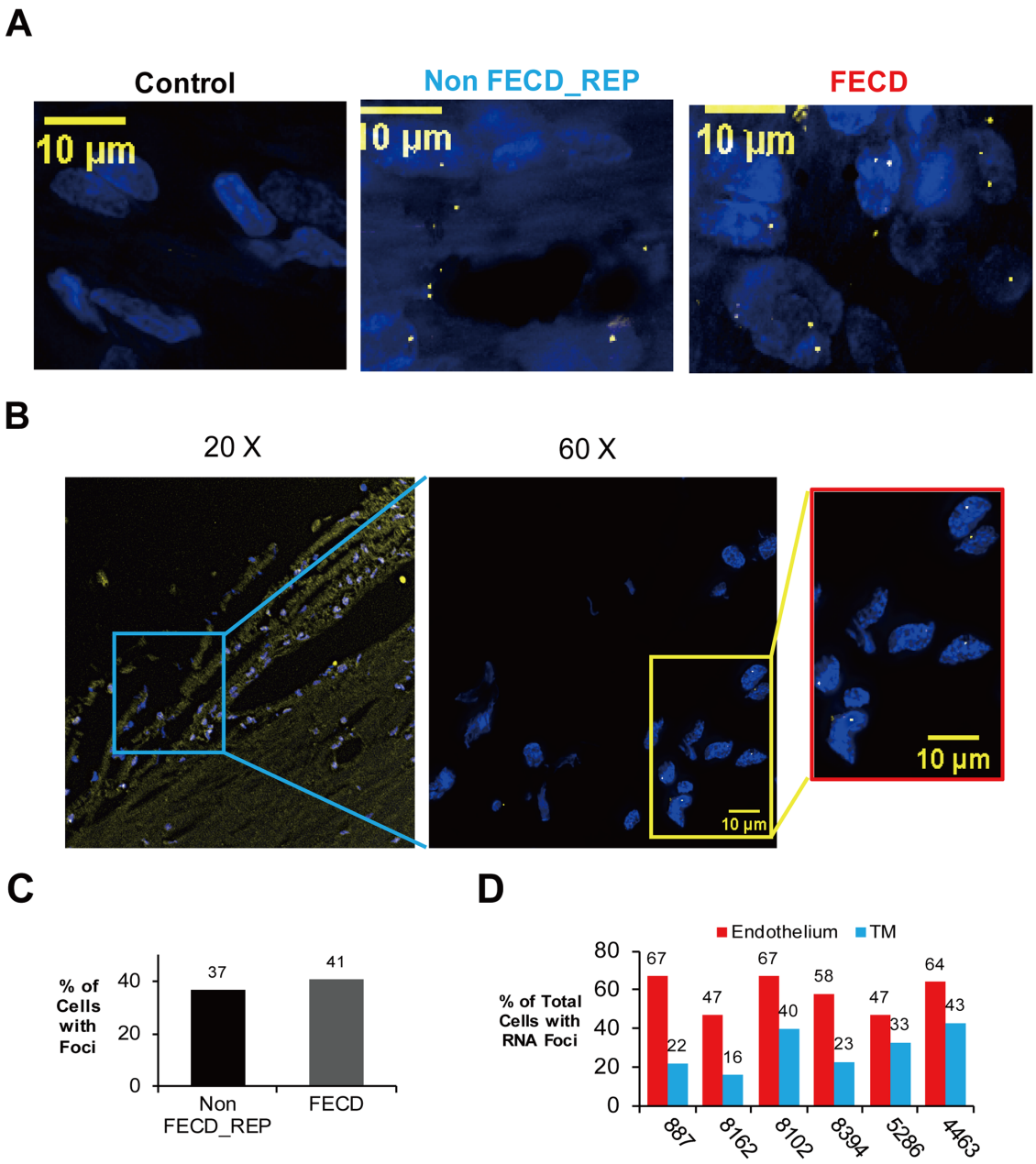

Figure 4. *TCF4* expanded CUG repeat RNA foci were found in the trabecular meshwork (TM) cells of FECD donors. (**A**) FISH images of trabecular meshwork tissues from control, non-FECD_REP, and FECD subjects. The pictures were taken from whole-mount microscopy of the TM, which was dissected from donor corneoscleral rim. The images were processed by Z-stack and show multiple layers of cells in a single picture. (**B**) Images from cross-sections of FECD corneoscleral rim. The *left picture* was captured by a 20× lens. The *middle picture* was captured by a 60× lens and shows the area in the *blue box* from the left. The *red-outlined image* on the right is the enlarged area from the *yellow box*. (**C**) Summary of from whole-mount TM samples. (**D**) Foci counting in endothelium and TM of donor corneoscleral rim cross-sections.

or stroma (Fig. 7B), reaching levels that were comparable to those of the corneal endothelium. When the expression of exons 2 and 3 was analyzed, expression in the trabecular meshwork remained much higher than expression levels of the stroma or epithelium, but they were also four fold lower than that observed in endothelial tissue (Fig. 7B). These data suggest that expression of the *TCF4* mRNA transcripts including those isoforms with exons 2 and 3 is higher in the corneal endothelium or trabecular meshwork than in the corneal stroma or corneal epithelium.

The expression of intron 2 presents a more complex picture (Fig. 7C). Primer sets that are upstream of the trinucleotide repeat expansion detect much more intronic RNA expression in FECD donor corneal endothelium than control samples, consistent with results previously observed from FECD_REP tissue of patients with late-stage disease.[26] For intron 2 downstream sequences, expression levels are similar among all of the tissues, with or without repeat expansion. The difference in the detection of the sequence upstream of the repeat (high) and downstream of the

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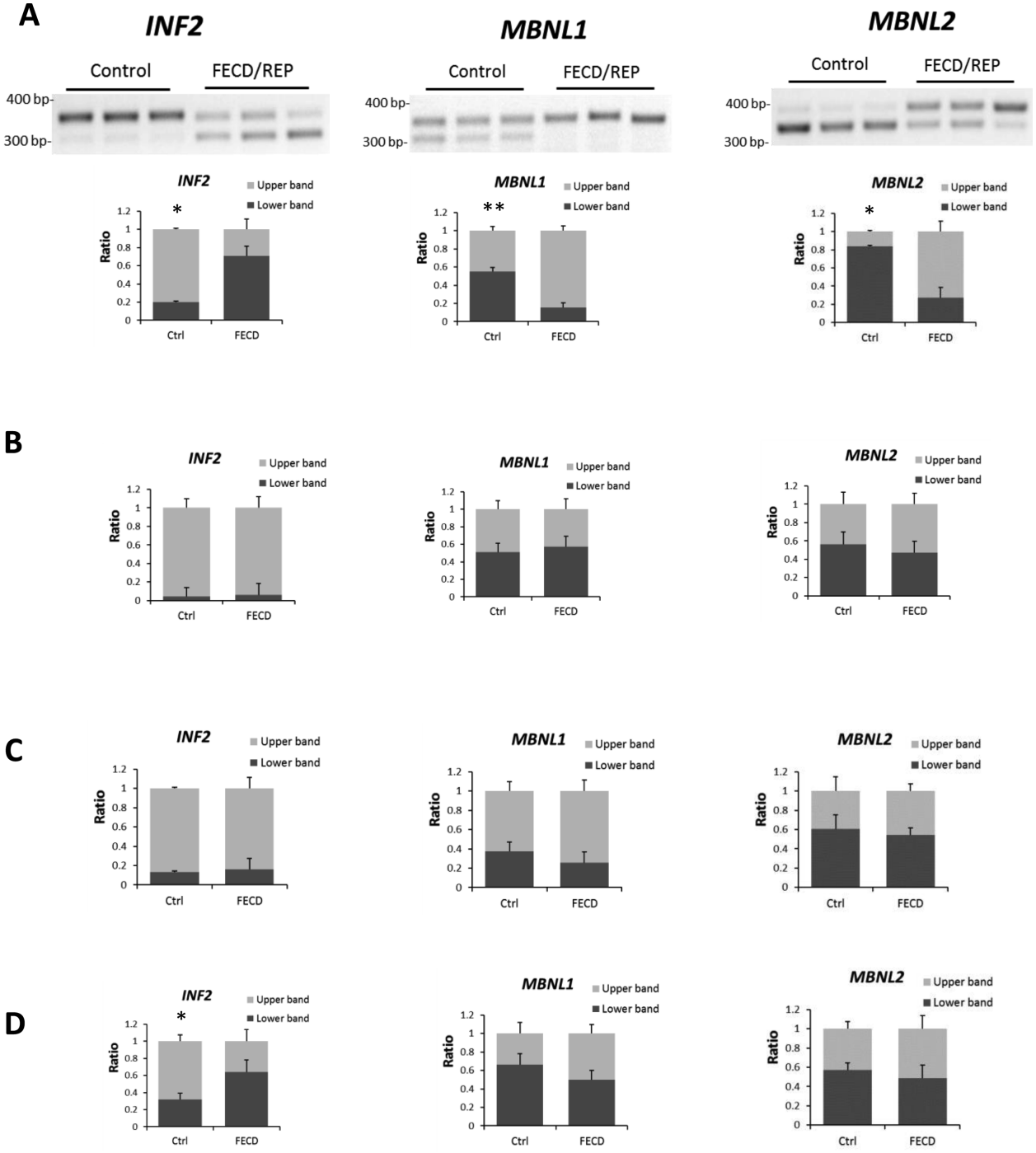

Figure 5. (**A–D**) Comparison of splicing of *INF2*, *MBNL1*, and *MBNL2* in corneal endothelium (**A**), corneal stroma (**B**), corneal epithelium (**C**), and trabecular meshwork (**D**) samples from donor corneas with FECD findings and repeat expansion.

repeat (low) is consistent with the hypothesis that the mutant CUG trinucleotide repeat stabilizes the upstream RNA. These data in human tissue are consistent with our previous observation of much longer half-lives for sequences upstream relative to downstream of the expanded trinucleotide repeat in patient-derived cells.[26] The upstream intron 2 level may also be increased in FECD corneal stroma and trabecular meshwork cells compared with controls, but the differences that we have detected are not statistically significant.

Discussion

CUG Trinucleotide Repeat Foci and Corneal Endothelial Disease

A central question for the study of all disease genes containing trinucleotide repeats is why disease findings occur in only a subset of tissues. We examined the molecular burden of CUG repeat RNA foci in cell types of the anterior segment of the eye of FECD subjects with

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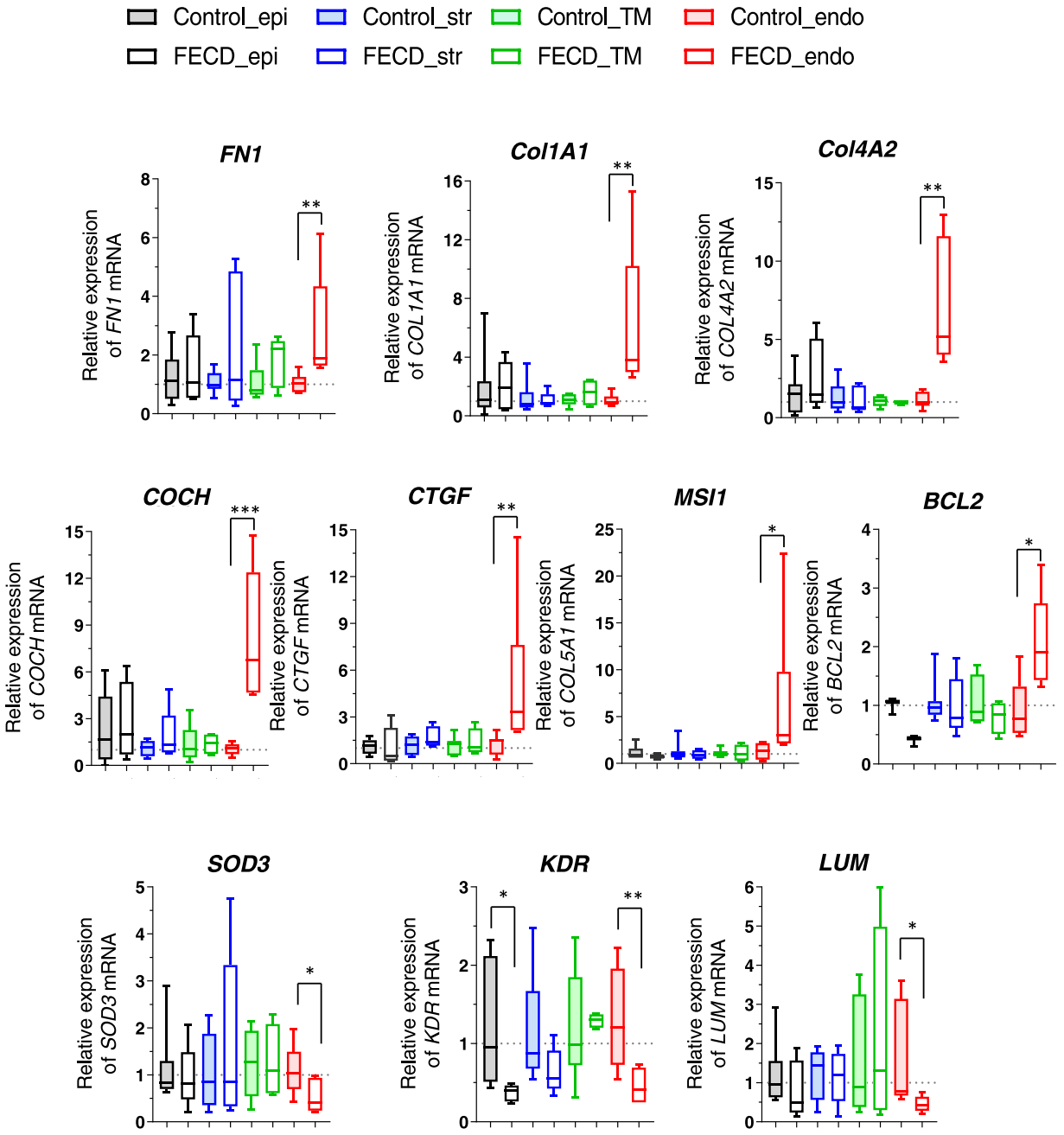

Figure 6. Differential gene expression in the corneal epithelium, corneal stroma, trabecular meshwork, and corneal endothelium from donor corneas with FECD findings and repeat expansion.

the intronic *TCF4* trinucleotide repeat expansion. The cell type specificity for foci is pertinent to studying disease pathogenesis triggered by mutant repeat RNA, understanding the FECD clinical phenotype, and therapeutic development.

Our molecular data agree with clinical observations indicating that the corneal endothelium is the primary tissue affected by FECD. We observed that expanded CUG repeat RNA nuclear foci, the hallmark of FECD in corneal endothelium and a visual indication of the presence of disease-causing repeat RNA, are less detectable in trabecular meshwork cells, much less prevalent in corneal stroma or epithelium, and absent in lens epithelium (Figs. 2–4). The corneal endothelium may be more prone to foci formation, given its high expression levels of all *TCF4* transcripts and especially those full-length isoforms containing the repeat sequence at the 5′ end (Fig. 7). The detection of intronic RNA upstream of the repeat was most profound in the corneal endothelium. Similarly, differential gene expression changes, including the upregulation of extracellular matrix genes (Fig. 6) and splicing changes (Fig. 5), are most obvious in corneal endothelium. All findings reported here are consistent with clinical observations that FECD primarily affects the corneal endothelium.

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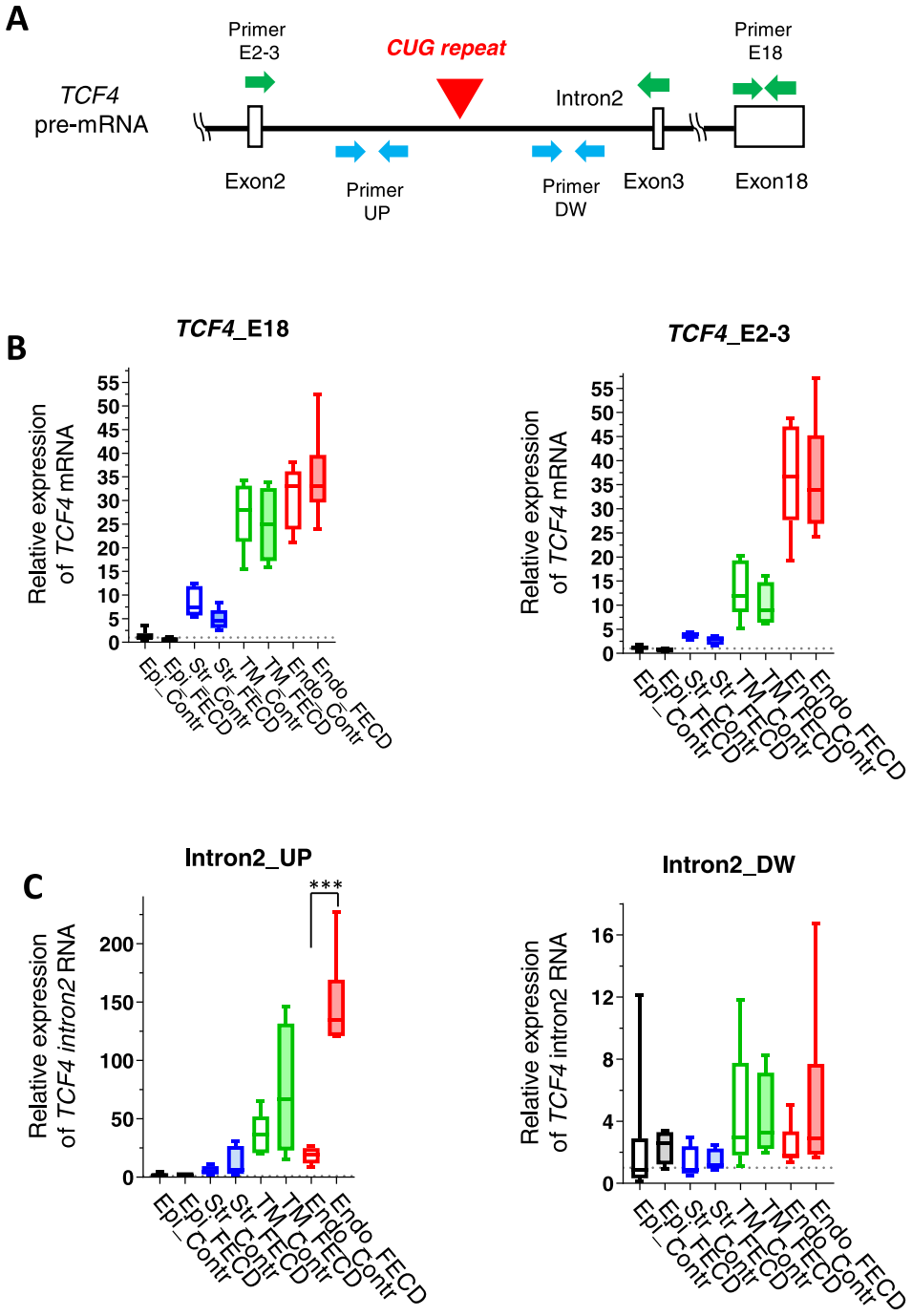

Figure 7. *TCF4* expression in corneal epithelium, corneal stroma, trabecular meshwork, and corneal endothelium. (**A**) Primer design for *TCF4* mRNA and intronic RNA. (**B**, **C**) Comparison of expression level of *TCF4* coding transcripts (**B**) and intronic RNA (**C**) upstream or downstream of CUG repeat.

CUG Foci Occur in the Trabecular Meshwork

Although our observations support the assumption that the corneal endothelium is the primary tissue impacted by the trinucleotide repeat mutation, we also observed significant numbers of foci in the trabecular meshwork (Fig. 4). It is important to consider how the presence of the toxic expanded CUG repeat RNA in trabecular meshwork might impact FECD disease findings. The cells of the trabecular meshwork are anatomic neighbors of the corneal endothelium. Both cell types originate from neural crest tissue embryonically, exhibit limited proliferative capacity, and may have a common stem cell niche in the transition zone between the termination of endothelium on Descemet's membrane (Schwalbe's line) and endothelium of the trabecular meshwork (Fig. 2).[36]

Trabecular meshwork cells and the corneal endothelium had higher levels of *TCF4* expression (Fig. 7) and CUG

repeat RNA foci (Fig. 4) compared to the other cell types examined in this study. The foci appear in non-FECD_REP tissue, suggesting the need to consider potential impacts early in disease (Figs. 4A, 4C). We observed mis-splicing of the *INF2* gene in the cells of the trabecular meshwork, but not the *MBNL1* or *MBNL2* genes prone to changes in splicing in FECD corneal endothelial tissue. Myotonic dystrophy type 1 (DM1), a multisystem disease that is also caused by expanded CTG repeats, may offer some clues as to why the splicing changes are different in the trabecular meshwork versus the corneal endothelium and altogether absent in the other corneal layers in FECD. In DM1, a major pathogenic event is the functional sequestration of MBNL proteins by CUG repeat RNA molecules transcribed from the expanded CTG repeats. Differences in the extent of MBNL sequestration dependent on both the triplet repeat length and expression levels of the repeat RNA may account for tissue specificity for disease and the variability of disease findings across different affected tissues in DM1.[37] Interestingly, the alternative splicing of some genes is specific to the affected heart tissue or skeletal muscle in DM1.[37] Relative to the corneal endothelium, expression levels of *TCF4* transcripts and especially those full-length isoforms containing the repeat sequence at the 5′ end (Fig. 7) are lower in trabecular meshwork and may account for why the splicing pattern is different in this tissue.

Our data show that foci can be detected at relatively high levels in trabecular meshwork cells, that detection occurs early in non-FECD_REP cells, and that splicing changes diagnostic for FECD occur. Expression levels of the transcripts containing the expanded CUG and other tissue specific factors likely impact the functional sequestration of the MBNL protein and observed splicing patterns. However, despite the presence of foci in trabecular meshwork cells, the changes in gene expression and splicing are less than those observed in corneal endothelial cells. These mixed results are consistent with the conclusion that corneal endothelial cells are the primary tissue impacted by FECD, but the trabecular meshwork is also affected by the disease. Additional studies are warranted to examine if patients with the *TCF4* trinucleotide repeat expansion are at increased risk for glaucoma and any contributory role of the CUG repeat foci observed in the trabecular meshwork cells of these patients.

CUG Foci Are Rare in Corneal Stroma and Absent in Lens Epithelium

We also observed rare foci in the corneal stroma. The corneal stroma is the thickest layer of the cornea located between the outer corneal epithelium and inner corneal endothelium. The keratocytes in the corneal stroma are also of neural crest origin and play an important role in the assembly of collagen fibrils and the maintenance of corneal transparency.[38] The corneal subbasal nerve plexus is located in the superficial layers of the corneal stroma. Loss of the subbasal nerve plexus and corneal stromal haze secondary to activation of keratocytes and production of aberrant extracellular matrix are observable findings early in the disease course of patients with FECD with the trinucleotide repeat expansion.[12] It is unknown at this time if the foci observed in the stromal keratocytes and other corneal layers could contribute to the loss of corneal nerves and stromal haze disease findings observed in FECD. Additional studies are warranted to determine if there is any difference in foci formation in the anterior stroma compared to the posterior stroma. Further studies are also required to determine if storage of donor corneas in the corneal preservation medium affects the cells of the corneal epithelium or stroma differently from the cells of the corneal endothelium and trabecular meshwork to affect the FISH results.

The almost complete absence of foci in lens epithelium stands out from other tissues examined. To our knowledge, patients with FECD with the *TCF4* repeat expansion are not prone to early-onset or peculiar forms of cataract such as the early-onset posterior subcapsular "snowflake" or iridescent "Christmas-tree" cataracts common in patients with DM1 caused by expanded CTG repeats in the *DMPK* gene.[39] The lens epithelium derives embryonically from neural ectoderm, and the corneal endothelium, stromal keratocytes, and trabecular meshwork cells originate from neural crest tissue. Anterior segment cells of neural crest origin may be more prone to CUG repeat RNA foci formation in FECD patients with the *TCF4* repeat expansion. Gene expression patterns have been shown to be specific to lineage development.[40]

The lens epithelium and corneal epithelium proliferate prolifically. In contrast, the corneal endothelium and trabecular meshwork endothelium have limited proliferative capacity, perhaps making these cell types more vulnerable to age-related somatic mutations that result in larger trinucleotide repeat expansions and subsequent disease as are observed in other repeat expansion disorders.[41] The lack of adequate amounts of DNA from surgical FECD endothelial tissue samples has hindered direct measurements of the repeat length using current technologies. However, results from one recent study that used long read sequencing of the RNA isolated from FECD corneal endothelium suggest that the *TCF4* repeat expansion in corneal endothelium is much larger than in peripheral leukocytes of patients.[42] We hypothesize that cells with larger repeat lengths may be more prone to foci formation.

Conclusions

The presence of detectable CUG repeat RNA foci is a hallmark of age-related FECD mediated by the *TCF4* repeat expansion. We observed abundant foci in the corneal endothelium. In comparison, foci were less detectable in trabecular meshwork cells, much less prevalent in stromal keratocytes, and absent in lens epithelium. Expression of the *TCF4* mRNA transcripts including full-length isoforms containing the repeat sequence was much higher in the corneal endothelium and trabecular meshwork, which may predispose these cell types to foci formation. Our molecular data are consistent with the conclusion that the pathologic consequences of FECD are primarily due to the impact of the expanded CTG repeat mutation on corneal endothelial cells. Additional studies are warranted to examine any impact of the observed foci and splicing changes in the trabecular meshwork cells on FECD disease findings.

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References

1. Barrientez B, Nicholas SE, Whelchel A, Sharif R, Hjortdal J, Karamichos D. Corneal injury: clinical and molecular aspects. *Exp Eye Res*. 2019;186:107709.
2. Lorenzetti DW, Uotila MH, Parikh N, Kaufman HE. Central cornea guttata. Incidence in the general population. *Am J Ophthalmol*. 1967;64:1155–1158.
3. Sarnicola C, Farooq AV, Colby K. Fuchs endothelial corneal dystrophy: update on pathogenesis and future directions. *Eye Contact Lens*. 2019;45:1–10.
4. Gain P, Jullienne R, He Z, et al. Global survey of corneal transplantation and eye banking. *JAMA Ophthalmol*. 2016;134:167–173.
5. Bigar F. Specular microscopy of the corneal endothelium. Optical solutions and clinical results. *Dev Ophthalmol*. 1982;6:1–94.
6. Borderie VM, Baudrimont M, Vallee A, Ereau TL, Gray F, Laroche L. Corneal endothelial cell apoptosis in patients with Fuchs' dystrophy. *Invest Ophthalmol Vis Sci*. 2000;41:2501–2505.
7. Chi HH, Teng CC, Katzin HM. Histopathology of primary endothelial-epithelial dystrophy of the cornea. *Am J Ophthalmol*. 1958;45:518–535.
8. Jurkunas UV, Bitar MS, Funaki T, Azizi B. Evidence of oxidative stress in the pathogenesis of Fuchs endothelial corneal dystrophy. *Am J Pathol*. 2010;177:2278–2289.
9. Laing RA, Leibowitz HM, Oak SS, Chang R, Berrospi AR, Theodore J. Endothelial mosaic in Fuchs' dystrophy. A qualitative evaluation with the specular microscope. *Arch Ophthalmol*. 1981;99:80–83.
10. Li QJ, Ashraf MF, Shen DF, et al. The role of apoptosis in the pathogenesis of Fuchs endothelial dystrophy of the cornea. *Arch Ophthalmol*. 2001;119:1597–1604.
11. Matthaei M, Zhu AY, Kallay L, Eberhart CG, Cursiefen C, Jun AS. Transcript profile of cellular senescence-related genes in Fuchs endothelial corneal dystrophy. *Exp Eye Res*. 2014;129:13–17.
12. Gillings M, Mastro A, Zhang X, et al. Loss of corneal nerves and corneal haze in Fuchs' endothelial corneal dystrophy patients with the *TCF4* trinucleotide repeat expansion. *Ophthalmol Sci*. 2022;3:100214.
13. Loewenstein A, Hourvitz D, Goldstein M, Ashkenazi I, Avni I, Lazar M. Association of Fuchs' corneal endothelial dystrophy with angle-closure glaucoma. *J Glaucoma*. 1994;3:201–205.
14. Nagarsheth M, Singh A, Schmotzer B, et al. Relationship between Fuchs endothelial corneal dystrophy severity and glaucoma and/or ocular hypertension. *Arch Ophthalmol*. 2012;130:1384–1388.
15. Ali ZK, Whitson JT, Mootha VV, et al. Glaucoma in patients with corneal endothelial dystrophy. *Eye Contact Lens*. 2011;37:332–336.
16. Buxton JN, Preston RW, Riechers R, Guilbault N. Tonography in cornea guttata. A preliminary report. *Arch Ophthalmol*. 1967;77:602–603.
17. Rice GD, Wright K, Silverstein SM. A retrospective study of the association between Fuchs' endothelial dystrophy and glaucoma. *Clin Ophthalmol*. 2014;8:2155–2159.
18. Mootha VV, Gong X, Ku HC, Xing C. Association and familial segregation of CTG18.1 trinucleotide repeat expansion of *TCF4* gene in Fuchs' endothelial corneal dystrophy. *Invest Ophthalmol Vis Sci*. 2014;55:33–42.
19. Soliman AZ, Xing C, Radwan SH, Gong X, Mootha VV. Correlation of severity of Fuchs endothelial corneal dystrophy with triplet repeat expansion in *TCF4*. *JAMA Ophthalmol*. 2015;133:1386–1391.
20. Wieben ED, Aleff RA, Tosakulwong N, et al. A common trinucleotide repeat expansion within the transcription factor 4 (*TCF4*, E2-2) gene predicts Fuchs corneal dystrophy. *PLoS One*. 2012;7:e49083.
21. Xing C, Gong X, Hussain I, et al. Transethnic replication of association of CTG18.1 repeat expansion of *TCF4* gene with Fuchs' corneal dystrophy in Chinese implies common causal variant. *Invest Ophthalmol Vis Sci*. 2014;55:7073–7078.
22. Mootha VV, Hussain I, Cunnusamy K, et al. *TCF4* triplet repeat expansion and nuclear RNA foci in Fuchs' endothelial corneal dystrophy. *Invest Ophthalmol Vis Sci*. 2015;56:2003–2011.
23. Du J, Aleff RA, Soragni E, et al. RNA toxicity and missplicing in the common eye disease Fuchs endothelial corneal dystrophy. *J Biol Chem*. 2015;290:5979–5990.
24. Rong Z, Hu J, Corey DR, Mootha VV. Quantitative studies of muscleblind proteins and their interaction with *TCF4* RNA foci support involvement in the mechanism of Fuchs' dystrophy. *Invest Ophthalmol Vis Sci*. 2019;60:3980–3991.
25. Zarouchlioti C, Sanchez-Pintado B, Hafford Tear NJ, et al. Antisense therapy for a common corneal dystrophy ameliorates *TCF4* repeat expansion-mediated toxicity. *Am J Hum Genet*. 2018;102:528–539.
26. Chu Y, Hu J, Liang H, et al. Analyzing pre-symptomatic tissue to gain insights into the molecular and mechanistic origins of late-onset degenerative trinucleotide repeat disease. *Nucleic Acids Res*. 2020;48:6740–6758.
27. Deng SX, Lee WB, Hammersmith KM, et al. Descemet membrane endothelial keratoplasty: safety and outcomes: a report by the American Academy of Ophthalmology. *Ophthalmology*. 2018;125:295–310.
28. Ang M, Soh Y, Htoon HM, Mehta JS, Tan D. Five-year graft survival comparing Descemet stripping automated endothelial keratoplasty and penetrating keratoplasty. *Ophthalmology*. 2016;123:1646–1652.
29. Lee WB, Jacobs DS, Musch DC, Kaufman SC, Reinhart WJ, Shtein RM. Descemet's stripping endothelial keratoplasty: safety and outcomes: a report by the American Academy of Ophthalmology. *Ophthalmology*. 2009;116:1818–1830.
30. Price MO, Fairchild KM, Price DA, Price FW. Descemet's stripping endothelial keratoplasty five-year graft survival and endothelial cell loss. *Ophthalmology*. 2011;118:725–729.
31. Oliva MS, Schottman T, Gulati M. Turning the tide of corneal blindness. *Indian J Ophthalmol*. 2012;60:423–427.
32. Krachmer JH, Purcell JJ, Young CW, Bucher KD. Corneal endothelial dystrophy. A study of 64 families. *Arch Ophthalmol*. 1978;96:2036–2039.
33. Keller KE, Bhattacharya SK, Borras T, et al. Consensus recommendations for trabecular meshwork cell isolation, characterization and culture. *Exp Eye Res*. 2018;171:164–173.
34. Hu J, Rong Z, Gong X, et al. Oligonucleotides targeting TCF4 triplet repeat expansion inhibit RNA foci and mis-splicing in Fuchs' dystrophy. *Hum Mol Genet*. 2018;27:1015–1026.
35. Sepp M, Kannike K, Eesmaa A, Urb M, Timmusk T. Functional diversity of human basic helix-loop-helix transcription factor TCF4 isoforms generated by alternative 5′ exon usage and splicing. *PLoS One*. 2011;6:e22138.

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36. Sie NM, Yam GH, Soh YQ, et al. Regenerative capacity of the corneal transition zone for endothelial cell therapy. *Stem Cell Res Ther*. 2020;11:523.
37. Wang ET, Treacy D, Eichinger K, et al. Transcriptome alterations in myotonic dystrophy skeletal muscle and heart. *Hum Mol Genet*. 2019;28:1312–1321.
38. Zhang L, Anderson MC, Liu CY. The role of corneal stroma: a potential nutritional source for the cornea. *J Nat Sci*. 2017;3:e428.
39. Burian HM, Burns CA. Ocular changes in myotonic dystrophy. *Am J Ophthalmol*. 1967;63:22–34.
40. Kluger Y, Tuck DP, Chang JT, et al. Lineage specificity of gene expression patterns. *Proc Natl Acad Sci USA*. 2004;101:6508–6513.
41. Kacher R, Lejeune FX, Noel S, et al. Propensity for somatic expansion increases over the course of life in Huntington disease. *eLife*. 2021;10:e64674.
42. Wieben ED, Aleff RA, Rinkoski TA, et al. Comparison of *TCF4* repeat expansion length in corneal endothelium and leukocytes of patients with Fuchs endothelial corneal dystrophy. *PLoS One*. 2021;16:e0260837.

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