MOLECULAR PATHOGENESIS OF GENETIC AND INHERITED DISEASES

Function-Related Protein Expression in Fuchs Endothelial Corneal Dystrophy Cells and Tissue Models

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Fuchs endothelial corneal dystrophy (FECD) is a corneal pathology that affects the endothelial cell's ability to maintain deturgescence, resulting in a progressive loss of corneal transparency. In this study, we investigated the expression of function-related proteins in corneal endothelial cells using FECD or healthy corneal endothelial cells, either in a cell culture two-dimensional model or in an engineered corneal endothelium three-dimensional tissue model. No statistically significant difference in gene regulation was observed for the function-related families *ATP1*, *SLC4*, *SLC16*, *AQP*, *TJP*, and *CDH* between the FECD and the healthy cell models. Similarly, no difference in barrier integrity (transendothelial electrical resistance measurements and permeability assays) was observed *in vitro* between FECD and healthy cultured cells. Protein expression of the key function-related families was decreased for Na⁺/K⁺-ATPase α 1 subunit, monocarboxylate transporters 1 and 4 in native *ex vivo* end-stage FECD specimens, whereas it returned to levels comparable to that of healthy tissues in the engineered FECD model. These results indicate that cell expansion and tissue engineering culture conditions can generate a corneal endothelium from pathologic FECD cells, with levels of function-related proteins similar to that of healthy tissues. Overall, these results explain why it is possible to reform a functional endothelium using corneal endothelial cells isolated from nonfunctional FECD pathologic specimens. (*Am J Pathol* 2018, 188: 1703–1712; <https://doi.org/10.1016/j.ajpath.2018.03.014>)

The corneal endothelium forms a monolayer of cells on the posterior surface of the cornea. It is responsible for corneal deturgescence, keeping the stroma partially dehydrated, which is a prerequisite for corneal transparency.¹ The barrier and pump functions of the endothelium are responsible for maintaining corneal transparency by regulating stromal hydration. The barrier integrity (structural and functional integrity of the intercellular junctions) is made possible by the tethering and sealing capacity of intercellular multi-protein complexes, such as adherens and tight junctions.² In the corneal endothelium, these junctions are respectively composed, among others, of N-cadherin and zonula occludens protein 1.^{2,3} The corneal endothelium has a low transendothelial electrical resistance (TEER; approximately 15 to 30 Ω cm²) and is permeable to macromolecules up to

60 to 90 kDa.^{1,4–8} Transcellular and paracellular passage of solutes and fluid is counterbalanced by an active fluid pump

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Disclosures: None declared.

Figure 1 Characterization of the sodium-potassium pump family. **A:** Heat map representation and mean linear signal of all Na⁺/K⁺-ATPase subunit genes expressed by healthy and Fuchs endothelial corneal dystrophy (FECD) cell populations. The number on top of each column corresponds to the age of the patients/donors. **B:** The expression of *ATP1A1* and *ATP1B1* was also evaluated by real-time quantitative PCR. **C:** Immunofluorescence (red) directed against Na⁺/K⁺-ATPase α1 and β1 subunits on native and tissue-engineered (TE) healthy and FECD endothelium (E). Nucleus staining = Hoechst (blue). The specimens are presented stromal side up and endothelial side down. Dashed white lines delineate Descemet membranes (DMs). Data are expressed as means ± SD (A and B). Scale bar = 10 μm (C).

mechanism powered exclusively by the Na⁺/K⁺-ATPase sodium generated gradient. It involves many membrane water and ion facilitating proteins, including bicarbonate transporters, anion exchangers (AEs) AE2, sodium (Na) bicarbonate cotransporter 1, monocarboxylate transporters MCT1, MCT2, MCT4, and aquaporin 1.^{9,10} Altered expression of these proteins by pharmacologic or genetic means dampens to various degrees the endothelium capacity to maintain deturgescence.^{9,10} For example, ouabain, a potent Na⁺/K⁺-ATPase pump inhibitor, is known to cause reversible endothelial dysfunction.¹¹

Fuchs endothelial corneal dystrophy (FECD) is a disorder of the corneal endothelium that causes progressive corneal edema and vision loss.¹² Mutations within genes coding for *COL8A2*, *TCF4*, *TCF8*, *SLC4A11*, and *LOXHD1* have been characterized in family subtypes, but most cases are considered sporadic or of unknown origin.^{13–23} Pathophysiology of the disease is associated with an irregular thickening of the endothelium's basement membrane (Descemet membrane),^{24–26} oxidative stress imbalance,^{27–30} mitochondrial dysfunction,^{31–33} endoplasmic reticulum stress,^{34–36} increased level of apoptosis,^{37–39} and a reduced cell density.⁴⁰ Cell density is important for the corneal endothelium. In healthy adults, endothelial cell density

ranges between 2500 and 2700 cells/mm².^{41,42} However, when endothelial cell density decreases to 400 to 700 cells/mm², the remaining cells can no longer fulfill their barrier function, which results in corneal swelling.⁴³

Na⁺/K⁺-ATPase pump sites have been shown to increase in the early stages of FECD and gradually decline over time, reaching values considerably lower than in healthy controls.^{44–47} Expression of the pump-related protein genes *ATP1B1*, MCT1 (*SLC16A1*), and MCT4 (*SLC16A3*) has been shown to be significantly lowered in end-stage FECD specimens.³⁵ A decrease in aquaporin 1, a water channel protein, has also been shown in 40% of the studied FECD surgical specimens.⁴⁸ The endothelium becomes progressively unable to maintain deturgescence, leading to clinically significant corneal edema, corneal opacification, and vision loss.

Our laboratory has developed two cell and tissue models in which endothelial corneal cells from FECD specimens are expanded *in vitro* (two-dimensional cell model) or used to recreate an endothelium on a healthy devitalized cornea (three-dimensional tissue model).^{25,49} *In vitro* FECD models showed that FECD cells rapidly deposit high amounts of fibronectin on the surface of their basement membrane.

Figure 2 Characterization of the bicarbonate transporter family. **A:** Heat map representation and mean linear signal of all *SLC4* genes expressed by healthy and Fuchs endothelial corneal dystrophy (FECD) cell populations. The number on top of each column corresponds to the age of the patients/donors. **B:** The expression of *SLC4A4* was also evaluated by real-time quantitative PCR. **C:** Immunofluorescence (red) directed against $\text{Na}^+/\text{HCO}_3^-$ cotransporter $\alpha 4$ on native and tissue-engineered (TE) healthy and FECD endothelium (E). Nucleus staining = Hoechst (blue). The specimens are presented stromal side up and endothelial side down. **Dashed white lines** delineate Descemet membranes (DMs). Data are expressed as means $\pm$ SD (A and B). Scale bar = 10 μm (C).

Deposition of extracellular matrix was also observed when the engineered FECD corneas were transplanted in living felines.⁴⁹ Interestingly, the engineered FECD corneas showed partial functional rehabilitation, because they were able to maintain corneal deturgescence for up to 7 days after transplantation in these animals. These tissue-engineered models offer consequently a unique opportunity to study the behavior of FECD cells in a healthy environment and to analyze and understand the initial events of this disease.

The goal of this study was to examine the functionality of the cultured FECD endothelium by assessing the presence of key proteins important for ion transport and barrier integrity. Specifically, we analyzed the gene and protein expression of the *ATP1*, *SLC4*, *SLC16*, *AQP*, *CDH*, and *TJP* families. To our knowledge, this is the first article that extensively studies the expression of function-related proteins in FECD cells using *in vitro* models of this disease. Knowledge gained from this research will help to develop future therapies aimed at maintaining corneal transparency in FECD patients.

Materials and Methods

This study was conducted in accordance with the institutional guidelines [Centre de Recherche du CHU de Québec—Université Laval (Québec, QC, Canada), Maisonneuve-Rosemont Hospital—University of Montreal (Montréal, QC, Canada), and Centre Hospitalier de l'Université de Montréal (Montréal, QC, Canada)] and the Declaration of Helsinki.

FECD and Healthy Specimen Distribution

Specimens from 24 late-stage FECD patients were collected after graft surgery, as described previously.²⁵ These

specimens were randomly assigned and used for gene profiling and real-time quantitative PCR (qPCR) analysis ($n = 6$; age range, 58 to 82 years; mean $\pm$ SD, 69 ± 10 years), for TEER and 10-kDa fluorescein isothiocyanate—dextran permeability analysis ($n = 4$; age range, 57 to 71 years; mean $\pm$ SD, 66 ± 5 years), to engineer FECD endothelia ($n = 7$; age range, 58 to 77 years; mean $\pm$ SD, 67 ± 8 years), and for immunostaining analysis (native *ex vivo* FECD specimens; $n = 7$; age range, 57 to 82 years; mean $\pm$ SD, 70 ± 6 years).

Donor human corneas, without endothelial disease (Healthy) and unsuitable for transplantation, were obtained from the Centre Universitaire d'Ophtalmologie Eye Bank (Québec, QC, Canada). Six were used for gene expression profiling and qPCR (age range, 58 to 82 years; mean $\pm$ SD, 71 ± 10 years), four for TEER and permeability analysis (age range, 56 to 72 years; mean $\pm$ SD, 66 ± 7 years), two to engineer healthy endothelia (aged 47 and 68 years), nine as carriers for the engineered corneal endothelia (age range, 54 to 84 years; mean $\pm$ SD, 69 ± 10 years), and eight for immunostaining experiments (native *ex vivo* healthy tissue; age range, 68 to 84 years; mean $\pm$ SD, 72 ± 12 years).

Isolation and Culture of Corneal Endothelial Cells from Healthy and FECD Corneas

Isolation and culture of healthy corneal endothelial cells (CEC) was performed, as described previously.⁵⁰ Briefly, Descemet membranes were peeled off from healthy donor corneas and incubated overnight in culture medium at 37°C. Descemet membranes were then incubated in EDTA 0.02% buffered solution (Sigma-Aldrich, St. Louis, MO) and incubated for 1 hour. Physical force (up and down through a flamed-polished pipette) was used to detach cells from the Descemet

Figure 3 Characterization of the monocarboxylate cotransporter (MCT) family. **A:** Heat map representation and mean linear signal of all *SLC16* genes expressed by healthy and Fuchs endothelial corneal dystrophy (FECD) cell populations. The number on top of each column corresponds to the age of the patients/donors. **B:** The expression of *SLC16A1* and *SLC16A3* was also evaluated by real-time quantitative PCR. **C:** Immunofluorescence (red) directed against MCT1 and MCT4 on native and tissue-engineered (TE) healthy and FECD endothelium (E). Nucleus staining = Hoechst (blue). The specimens are presented stromal side up and endothelial side down. Dashed white lines delineate Descemet membranes (DMs). Data are expressed as means $\pm$ SD (A and B). Scale bar = 10 μ m (C).

membrane. After centrifugation, cells were seeded in culture dishes covered with FNC coating mix (Athena Enzyme Systems, Baltimore, MD), in culture medium made of OptiMem-I (Invitrogen, Burlington, ON, Canada), 8% fetal bovine serum (HyClone, Logan, UT), 5 ng/mL human epidermal growth factor (Austral Biologicals, San Ramon, CA), 20 ng/mL nerve growth factor (Biomedical Technologies, Stoughton, MA), 100 μ g/mL bovine pituitary extract (Biomedical Technologies), 20 μ g/mL ascorbic acid (Sigma-Aldrich), 0.08% chondroitin sulfate (Sigma-Aldrich), 25 μ g/mL gentamicin sulfate (Scheringm Pointe Claire, QC, Canada), and 100 IU/mL penicillin G (Sigma-Aldrich). The same procedure was used for the isolation and culture of FECD endothelial cells, with the exception that the peeling was performed by corneal surgeons during the Descemet's stripping automated endothelial keratoplasty procedure.²⁵ Cells were used at passage 2 or 3 for all experiments. Cultures were maintained at confluency for at least 2 weeks before being used for the experiments.

Gene Expression Profiling

Cells were maintained at confluency for 25 to 40 days. Hydrocortisone (0.4 μ g/mL; Calbiochem, La Jolla, CA) was added to the growth medium in the last 7 days. Hydrocortisone was added because adding glucocorticoids, such as dexamethasone⁵¹ or hydrocortisone (M.T. and S.P., unpublished data), during post-confluency improves the

endothelial phenotype of cultured human CECs. Total RNA was isolated using RNeasy Mini Kit (Qiagen, Toronto, ON, Canada) or TRIzol (Ambion; Life Technologies, Grand Island, NY). All microarray analyses were conducted by the gene profiling service of the molecular genetic platform from the Centre Universitaire d'Ophthalmologie—Recherche (Québec, QC, Canada). RNA was labeled with cyanine 3-CTP using Agilent One-Color Microarray-Based Gene Expression Analysis kit (Agilent Technologies Canada Inc., Mississauga, ON, Canada). Obtained cRNAs were incubated on a G4851A SurePrint G3 Human GE 8 $\times$ 60K array slide (60,000 probes; Agilent Technologies Canada Inc.). After being washed and stained, the slides were digitized with an Agilent SureScan Scanner. The results were analyzed by robust multiarray analysis using the ArrayStar software version 12 (DNASTAR, Madison, WI), as described previously.^{52–54} The color scale used to display the log₂ expression level values is determined by the hierarchical clustering algorithm of the euclidian metric distance between genes. Genes indicated in dark blue correspond to those whose expression is low, whereas highly expressed genes are shown in orange/red. The microarray data presented in this study complied with the Minimum Information About a Microarray Experiment requirements⁵⁵ and have been deposited in the National Center for Biotechnology Information's Gene Expression Omnibus repository (<https://www.ncbi.nlm.nih.gov/geo>; accession number GSE112039).

Quantitative PCR

The RNA was reverse transcribed to cDNA with SuperScript II Reverse Transcriptase (Invitrogen), according to the manufacturer's instructions. Primers used to amplify were as follows: *ATP1A1*, 5'-TGTCAGAATTGCAGGTCTTTG-3' (forward) and 5'-TGCCCGCTTAAGAATAGGTAGGT-3'⁵⁶ (reverse); *ATP1B1*, 5'-TGATCCCAAGAGCTATGAGGC-3' (forward) and 5'-GCACATCG-CCACAATCTTCAAAA-3' (reverse); *SLC4A4*, 5'-CGGCATCAAA-CCAAGAAATCC-3' (forward) and 5'-CATGGCTGGGCTACCATTAT-3' (reverse); *SLC16A1*, 5'-TTGGAGTCATTGGAGGTCTTG-3' (forward) and 5'-TGGTCGCCTCTTGTAAGAAATAC-3' (reverse); and *SLC16A3*, 5'-CCATGCTCTACGGGACAGG-3' (forward) and 5'-GCTTGCTGAAGTAGCGGT-3' (reverse). *B2M* was used as housekeeping gene, and primers were 5'-GTGCTCGCGCTAC-TCTCTC-3' and 5'-GTCAACTTCAATGTCCGAT-3'.⁵⁷ Quantitative PCR was performed using a Rotor Gene Q Real-Time PCR Cycler (Qiagen). Synthesis of double-stranded DNA during PCR was monitored using Brilliant III UltraFast SYBR Green (Agilent Technologies). A standard calibration curve was prepared for each gene using serial dilutions of the calibrator sample. Crossing point values were plotted to the log of the relative concentration of each dilution. This standard curve was used to correct for differences in efficiencies of the PCRs. Relative gene expressions were then calculated by the $\Delta\Delta C_T$ method and normalized to *B2M* expression.⁵⁸

Transendothelial Resistance and 10-kDa Fluorescein Isothiocyanate—Dextran Permeability Analysis

Cells were seeded in quadruplet on FNC-coated 12-mm 0.4- μ m polycarbonate isopore membrane filters (PIHP01250; Millipore, Billerica, MA) inserted in 24-plate wells (Millipore). TEER was measured using a Millicell ERS-2 voltohmmeter (EMD Millipore, Burlington, MA), according to the manufacturer's instructions. TEER was measured after each medium change ($n = 5$ measures per insert). For the permeability analysis, 10-kDa dextran—fluorescein isothiocyanate (40 μ g/mL) was added into the insert. The plates were then incubated for 2.5 hours before collecting the medium from the bottom of the well. Fluorescence was measured using a CytoFluor series 4000 (PerSeptive Biosystems, Framingham, MA). Inserts without cells were used as baseline control for both tests. Fibroblasts, which do not generate a polarized cell barrier, were used as a negative control.

Tissue Engineering of the Corneal Endothelium

Corneas were devitalized, as described previously, using three freeze-thaw cycles and stored at -20°C until use.⁵⁹ Before cell seeding, they were rinsed to remove dead cells. Corneas with detached Descemet membrane were discarded. On seeding, devitalized corneas were laid down, posterior side facing up, in

a well of a 6-well plate. Cultured cells were seeded in 200 μ L of culture medium on the Descemet membrane and allowed to adhere for 4 hours. The corneas were then immersed in culture medium and further cultured for 2 weeks.

Tissue Fixation and Immunofluorescence Analysis

Corneas were embedded in OCT compound (Somagen, Edmonton, AB, Canada) and frozen in liquid nitrogen. They were then stored at -80°C . Immunofluorescence analysis was performed, as previously described.⁵⁹ Briefly, 10- μ m cryosections were fixed for 10 minutes at -20°C in acetone (100%; EMD, Mississauga, ON, Canada) and immunostained with a mouse anti- Na^+/K^+ -ATPase $\alpha 1$ (Millipore), a mouse anti- Na^+/K^+ -ATPase $\beta 1$ (Thermo Scientific, Waltham, MA), a rabbit anti- $\text{Na}^+/\text{HCO}_3^-$ cotransporter (Chemicon, Temecula, CA), a mouse anti-aquaporin 1 (Novus Biologicals, Oakville, ON, Canada), a mouse anti-monocarboxylate cotransporter 1 (Santa Cruz, Dallas, TX), and a rabbit anti-monocarboxylate cotransporter 4 (Santa Cruz) as primary antibodies. Goat anti-mouse (Invitrogen) and chicken anti-rabbit (Invitrogen) antibodies conjugated with Alexa 594 were used as secondary antibodies. The antibodies were diluted in phosphate buffer saline with 1% bovine serum albumin (Sigma-Aldrich) at room temperature for 1 hour with primary antibodies and for 45 minutes with secondary antibodies. Nuclei were stained with Hoechst reagent 33258 (Sigma-Aldrich). Fluorescence was observed using a Zeiss Axio Image.Z2 microscope (Carl Zeiss, Toronto, ON, Canada). Immunofluorescence analysis using second antibodies only was used as negative control. They showed negligible background staining.

Statistical Analysis

Statistical analyses consisted in Student's t -distribution test for differences in means. Mean values are reported with their SDs. $P < 0.05$ was considered statistically significant. All statistical tests were two sided. The analyses were conducted using Microsoft Excel 2016 software (Microsoft Corp., Redmond, WA).

Results

Sodium-Potassium Pump Family

Gene profiling of cultured cells revealed a predominant and ubiquitous expression of *ATP1A1* compared with the other α subunits. *ATP1A1*'s gene expression was, on average, 56 times higher than the second most expressed α subunit, *ATP1A4* (Figure 1A). For the regulatory β subunits, *ATP1B3* and *ATP1B1* were the only subunits expressed, the former covering an average of $76.5\% \pm 10.9\%$ of the total family expression (Figure 1A). Because *ATP1B1* was shown to be down-regulated in FECD,⁶⁰ and because the Na^+/K^+ -ATPase $\alpha 1$ protein has been shown to be decreased in

FECD^{44–47,61} and in an experimental model of FECD,⁴⁹ the gene profiling results of *ATP1A1* and *ATP1B1* were verified by qPCR. No statistically significant difference was observed in both the gene profiling and the qPCR analysis between FECD and healthy populations (Figure 1, A and B). Protein expression measured by immunofluorescence revealed a lower staining of Na⁺/K⁺-ATPase $\alpha 1$ in native FECD specimens compared with healthy equivalents. Engineering an endothelium on a devitalized carrier using FECD cells restored expression to a level similar to that of an endothelium engineered using healthy cells (Figure 1C). No difference was observed in the protein expression of Na⁺/K⁺-ATPase $\beta 1$ between FECD and healthy native specimens (Figure 1C), and no expression was observed in the engineered endothelia, both healthy and FECD.

Bicarbonate Transporter SLC4 Family

The first three members of the *SLC4A* family are anion exchangers (Cl[−]/HCO₃[−]; AEs 1 to 3). Cultivated endothelial cells predominantly expressed *SLC4A2* (AE2) and *SLC4A3* (AE3), the former covering 62.7% $\pm$ 11.0% of the total expression. Cultivated endothelial cells also expressed two membrane Na⁺/HCO₃[−] cotransporters: *SLC4A4* (67.3% $\pm$ 15.8% of total NBCs) and *SLC4A7* (Figure 2A). No differences in expression were observed between FECD and healthy cultivated cells for those two proteins subfamilies. The borate cotransporter and FECD marker *SLC4A11* appeared to have a lower expression in FECD (less than half); however, this difference was not statistically significant ($P = 0.41$), probably because of the high variation of expression in the samples (Figure 2A). It has previously been shown that the sodium bicarbonate cotransporter-1 protein (*SLC4A4* gene) was decreased in a short-term experimental model of FECD.⁴⁹ We thus wanted to confirm the absence of difference in gene expression of

SLC4A4 between cultured healthy and FECD cells by qPCR (Figure 2B). The sodium bicarbonate cotransporter-1 protein (*SLC4A4*) was present in both native and engineered tissues of healthy and FECD cells (Figure 2C).

Monocarboxylate Cotransporter Family SLC16

The SLC16 gene family has 14 members, four of which (*SLC16A1*, *SLC16A3*, *SLC16A7*, and *SLC16A8*) encode monocarboxylate cotransporters (MCT1, MCT4, MCT2, and MCT3, respectively).⁶² Gene profiling revealed an expression of three MCTs: MCT1 (*SLC4A1*), MCT2 (*SLC4A7*), and MCT4 (*SLC4A3*), the latter corresponding to 98.1% $\pm$ 0.5% of these genes' expression (Figure 3A). No difference of expression between FECD and healthy cultivated cells was observed using gene profiling. Because *SLC16A1* and *SLC16A3* genes were previously shown to be deregulated in FECD,³⁵ qPCR analysis was also performed for these two genes (Figure 3B). Results show no statistical difference in expression between the FECD and the healthy cultivated cells. Immunofluorescence protein analysis showed an absence of expression for MCT1 and MCT4 in native FECD specimens, whereas they were both present in the healthy specimens. The engineered endothelium using healthy and FECD cells did not express the MCT1 protein. However, MCT4 protein was present in the endothelium engineered using healthy and FECD cells (Figure 3C).

Assessment of Aquaporin Expression

Figure 4 shows the results of the aquaporin protein and transcriptional expressions. Gene profiling revealed a disparate expression of four aquaporins: *AQP1* (24.8% $\pm$ 22.6% of total expression), *AQP11* (24.1% $\pm$ 13.6% of total expression), *AQP9* (14.7% $\pm$ 9.1% of total expression), and *AQP5*

Figure 4 Characterization of the aquaporin (AQP) family. **A:** Heat map representation and mean linear signal of all AQP genes expressed by healthy and Fuchs endothelial corneal dystrophy (FECD) cell populations. The number on top of each column corresponds to the age of the patients/donors. **B:** Immunofluorescence directed against aquaporin 1 on native and tissue-engineered (TE) healthy and FECD endothelium (E). Nucleus staining = Hoechst (blue). The specimens are presented stromal side up and endothelial side down. Dashed white lines delineate Descemet membranes (DMs). Data are expressed as means $\pm$ SD (A). Scale bar = 10 μ m (B).

(11.2% $\pm$ 4.8% of total expression). Other aquaporins were expressed in a scarce and elusive way (Figure 4A). Protein analysis of aquaporin 1 by immunofluorescence showed a lot of variability, which corroborated gene expression results (Figure 4B). FECD cells showed no significant differences in gene and protein expression of aquaporins.

Assessment of the Barrier Function

CDH2 (N-cadherin) was the most abundantly expressed classic cadherin in CEC populations, as was *TJP1* (zonula occludens protein 1) for the zonula occludens protein 1 family (Figure 5A). However, their gene expression in FECD and healthy cultured cells did not differ. No significant difference in TEER was found between FECD (16.7 $\pm$ 7.0 Ω cm²) and healthy (23.0 $\pm$ 4.1 Ω cm²; P = 0.30) CEC populations. The fibroblast population (9.0 $\pm$ 1.4 Ω cm²) showed a reduction in TEER attributed to cell contraction within the first week of culture (Figure 5B). No difference in permeability was observed between FECD (34.6% $\pm$ 2.3% relative to controls) and healthy (22.2% $\pm$ 3.1%; P = 0.43) CEC, or between FECD CEC and fibroblast (34.9% $\pm$ 10.4%) populations (Figure 5C).

Discussion

FECD is a multifactorial disease that progresses over decades and results in a vision-impairing endothelial dysfunctionality. The only efficient treatment available consists of replacing the diseased endothelium through a transplantation surgery.⁶³ Most reported studies use small damaged surgery specimens or mutation-based mice models. Although both bring enlightening information for the understanding of FECD, they remain limited in their ability to characterize more specific pathogenic cues. For

example, the exact role of the endothelial cells in the pathogenesis of the disease is unclear. The ability to cultivate and reengineer an FECD endothelium on a nonpathologic cornea therefore offers a unique opportunity to discriminate between what could be innate cell alterations and other pathologic markers, such as, among others, altered Descemet membrane^{40,64–66} and altered aqueous humor composition.^{67–71}

In this article, we used a cell model and a tissue model to evaluate the expression of function-related proteins and the endothelial barrier integrity of cultured FECD endothelial cells. After cell cultivation, no difference in the expression of *ATP1*, *SLC4*, *SLC16*, *AQP*, *CDH*, and *TJP* families and in the endothelial barrier integrity between FECD and healthy cell populations was found. FECD-cultivated cells thus became similar to healthy cultivated cells, suggesting some degree of rehabilitation from their initial dysfunctional phenotype.

From our understanding, this loss of the FECD phenotype on cultivation could be caused by two mechanisms: selection of the healthiest cells and/or absence of a pathologic environment. The first mechanism would be explained by the fact that FECD usually begins in the central cornea, expanding progressively toward the periphery. The large FECD surgical specimens received in the laboratory thus contain cells at different stages of the disease, with healthier, smaller, and more hexagonal cells in the periphery of the specimen. Cell amplification may favor the selection of these peripheral-healthier cells with retained endothelial functionality. The second mechanism would be the absence in culture of a pathologic environment, such as, potentially, an abnormal basement membrane or aqueous humor composition, thus allowing cells to recover some functionality. Another option to consider is that *in vitro* the culture medium contains factors that may help to reassert functionality, factors that are lacking

Figure 5 Characterization of Fuchs endothelial corneal dystrophy (FECD) endothelial barrier function *in vitro*. **A:** Heat map representation and mean linear signal of zonula occludens protein 1 and classic cadherin gene families expressed by healthy and FECD cell populations. The number on top of each column corresponds to the age of the patients/donors. **B** and **C:** Mean transendothelial electrical resistance (TEER; **B**) and 10-kDa fluorescein isothiocyanate–dextran permeability (**C**) were measured in FECD and healthy corneal endothelial cell populations and a healthy population of fibroblasts cultured 23 days on 12-mm 0.4- μ m polycarbonate isopore membranes. Data are expressed as means $\pm$ SD.

in the normal *in vivo* environment. In any case, a better understanding of these rehabilitating mechanisms will allow us to define pharmacologic therapeutic strategies, either by promoting rehabilitation of the healthiest cells, by minimizing toxicity of the cell environment, or by adding factors that are missing *in vivo*. These strategies would likely slow down progression of the disease and delay the need for corneal transplantation.

These results are in accordance with our previous work showing that an engineered FECD endothelium can maintain corneal deturgescence and transparency in a short-term *in vivo* animal model.⁴⁹ Of interest, this study also evidenced an excess of subendothelial loose fibrillar material deposition, which prompted us to conclude that the cultured FECD cells still carried the disease. This conclusion is also supported by the recent demonstration of a rapid accumulation of fibronectin under the cells of our engineered FECD corneas,⁷² suggesting that fibronectin is implicated from the early stages of the disease. It also suggests that the reported decrease of Na⁺/K⁺-ATPase pump sites^{44–47} and the disruption of the barrier function⁷³ observed in late-stage FECD may be secondary to the extracellular matrix deposition.

Our results confirm the previously described protein and mRNA decrease of Na⁺/K⁺-ATPase α 1, MCT1, and MCT4 in native FECD.^{35,44–47} We confirm that change in mRNA expression of *SLC4A4* (alias sodium bicarbonate cotransporter-1) was not associated with FECD.³⁵ Reduced expression levels of Na⁺/K⁺-ATPase β 1 and MCT1 were noted between the native and the engineered corneas. Because the reduced protein expression was shared by both the diseased and the healthy engineered corneas, we believe it was not related to FECD and would instead be caused by the cultivation of the cell.

Of interest, aquaporin family showed high variability in gene expression between its members compared with other proteins studied in this article. Trying to understand why cells would privilege one type of aquaporin over another is beyond the scope of this research. Because other aquaporins are expressed in the corneal endothelium, these findings may explain why *AQP1* knockout fails to cause corneal edema in the mouse model.⁷⁴ Kenney et al⁴⁸ also detected aquaporins other than *AQP1* in the endothelium. Unlike them, our *in vitro* models did not address any relation with FECD.

To conclude, this study illustrates the hidden potential of FECD corneal endothelium to regain its ability to function once cultivated. This highly suggests that dysfunction of the FECD endothelium is secondary to other factors, which ought to be investigated in further studies.

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Supplemental Data

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