Endothelial Cell Whole Genome Expression Analysis in a Mouse Model of Early-Onset Fuchs' Endothelial Corneal Dystrophy

Mario Matthaei,^{1,2} Jianfei Hu,¹ Huan Meng,¹ Eva-Maria Lackner,^{1,3} Charles G. Eberhart,¹ Jiang Qian,¹ Haiping Hao,⁴ and Albert S. Jun¹

PURPOSE. To investigate the endothelial gene expression profile in a *Col8a2* Q455K mutant knock-in mouse model of early-onset Fuchs' endothelial corneal dystrophy (FECD) and identify potential targets that can be correlated to human late-onset FECD.

METHODS. Diseased or normal endothelial phenotypes were verified in 12-month-old homozygous *Col8a2*^{Q455K/Q455K} mutant and wild-type mice by clinical confocal microscopy. An endothelial whole genome expression profile was generated by microarray-based analysis. Result validation was performed by real-time PCR. Endothelial COX2 and JUN expression was further studied in human late-onset FECD compared to normal samples.

RESULTS. Microarray analysis demonstrated endothelial expression of 24,538 genes (162 up-regulated and 172 down-regulated targets) and identified affected gene ontology terms including *Response to Stress*, *Protein Metabolic Process*, *Protein Folding*, *Regulation of Apoptosis*, and *Transporter Activity*. Real-time PCR assessment confirmed increased *Cox2* ($P = 0.001$) and *Jun* mRNA ($P = 0.03$) levels in *Col8a2*^{Q455K/Q455K} mutant compared to wild-type mice. In human FECD samples, real-time PCR demonstrated a statistically significant increase in *COX2* mRNA ($P < 0.0001$) and *JUN* mRNA ($P = 0.002$) and tissue microarray analysis showed increased endothelial COX2 ($P = 0.02$) and JUN protein ($P = 0.04$).

CONCLUSIONS. The present study provides the first endothelial whole genome expression analysis in an animal model of FECD

and represents a useful resource for future studies of the disease. In particular endothelial COX2 up-regulation warrants further investigation of its role in FECD. (*Invest Ophthalmol Vis Sci.* 2013;54:1931-1940) DOI:10.1167/iops.12-10898

Fuchs endothelial corneal dystrophy (FECD) is a common disease of the corneal endothelium. It has been demonstrated to rank among the leading indications (9.3%–23.8%) for corneal transplant surgery in numerous Western countries but has a lower impact (1.7%–3.9%) in populations from the Asian region.^{1–9} An early-onset type and a more common late-onset type can be differentiated.¹⁰ The pathology of FECD is characterized by a progressive decrease in corneal endothelial cell (CEC) density over several decades and concomitant formation of posterior excrescences (guttae) and thickening of the Descemet membrane. The attenuated CEC monolayer is eventually unable to maintain corneal deturgescence, and stromal edema with sub- or intraepithelial bullae ensues. Recent investigations proposed that CEC oxidative stress and stress of the endoplasmic reticulum (ER) play critical pathogenetic roles and may result in endothelial apoptosis induction.^{11–14}

Recently, we have reported the development of the first transgenic knock-in mouse model of FECD harboring a point mutation in the *Col8a2* gene that has previously been associated with early-onset human disease.¹⁴ Using this mouse model, we have demonstrated that the *Col8a2* Q455K mutation is sufficient to elicit an FECD-like endothelial morphology; to activate the unfolded protein response (UPR), a cytoprotective signaling cascade; and to induce CEC apoptosis.¹⁴ These results were supported by our previous observation of ER stress, UPR activation, and apoptosis induction in human late-onset FECD.¹³

Studying FECD in an animal model offers important advantages, including the ability to obtain and investigate corneal tissues at earlier disease stages (compared to end-stage tissues retrieved after corneal transplant surgery in humans) and to use highly standardized testing conditions. In this respect, fresh tissues from an animal model can be processed with the exclusion of external factors like prolonged death-to-preservation time and exposure to other nonphysiological conditions like organ culturing that may bias gene expression, especially in normal control corneas.

The present study sought to obtain a deeper insight into the pathophysiology of early-onset FECD and, based on the results from the animal model, to detect potential parallels in the more common late-onset human disease. After verification of the diseased or normal corneal endothelial phenotype by clinical confocal microscopy, we performed endothelial whole genome expression profiling in midaged 12-month-old *Col8a2*^{Q455K/Q455K} mutant FECD mice and wild-type

From the ¹Wilmer Eye Institute, Johns Hopkins Medical Institutions, Baltimore, Maryland; the ²Department of Ophthalmology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany; the ³Department of Ophthalmology, Medical University of Graz, Graz, Austria; and the ⁴High Throughput Biology Center, Johns Hopkins Medical Institutions, Baltimore, Maryland.

Supported by Deutsche Forschungsgemeinschaft (DFG MA 5110/2-1 [MM]); Richard Lindstrom/Eye Bank Association of America Research Grant (MM); National Institutes of Health (NIH EY019874 [ASJ], EY001765 to Wilmer Microscopy Core Facility); Medical Illness Counseling Center (ASJ); grants from Stanley Friedler, MD, Diane Kemker, Jean Mattison, and Lee Silverman (ASJ); Research to Prevent Blindness (to Wilmer Eye Institute); and National Cancer Institute (NCI Cancer Center Support Grant P30CA068485 to Vanderbilt Translational Pathology Shared Resource).

Submitted for publication September 4, 2012; revised January 19, 2013; accepted February 18, 2013.

Disclosure: M. Matthaei, None; J. Hu, None; H. Meng, None; E.-M. Lackner, None; C.G. Eberhart, None; J. Qian, None; H. Hao, None; A.S. Jun, None

Corresponding author: Albert S. Jun, The Wilmer Eye Institute, 400 North Broadway, Baltimore, MD 21231; aljun@jhmi.edu.

TABLE 1. Assays Used for Quantitative Real-Time PCR Assessment of Mouse (m) and Human (h) Samples

Taqman Assay	Species	Gene Symbol	RefSeq	Gene Name
Mm00478374_m1	m	<i>Ptgs2</i>	NM_011198.3	Prostaglandin-endoperoxide synthase 2
Mm01213380_s1	m	<i>Sod3</i>	NM_011435.3	Superoxide dismutase 3, extracellular
Mm00495062_s1	m	<i>Jun</i>	NM_010591.2	Jun oncogene
Mm00472715_m1	m	<i>Serp1</i>	NM_030685.3	Stress-associated endoplasmic reticulum protein 1
Mm00459056_m1	m	<i>Slc38a4</i>	NM_027052.3	Solute carrier family 38, member 4
Mm00444330_s1	m	<i>Slc5a3</i>	NM_017391.3	Solute carrier family 5 (inositol transporters), member 3
Mm00442612_m1	m	<i>Atp1b2</i>	NM_013415.5	ATPase, Na ⁺ /K ⁺ transporting, beta 2 polypeptide
Mm01329588_m1	m	<i>Slc4a11</i>	NM_001081162.1	Solute carrier family 4, sodium bicarbonate transport
Mm00607939_s1	m	<i>Actb</i>	NM_007393.3	Actin, beta
Hs00153133_m1	h	<i>PTGS2</i>	NM_000963.2	Prostaglandin-endoperoxide synthase 2
Hs01103582_s1	h	<i>JUN</i>	NM_002228.3	JUN oncogene
Hs99999903_m1	h	<i>ACTB</i>	NM_001101.3	Actin, beta

controls by high resolution gene microarray analysis. The results from this study were validated by quantitative real-time PCR. The expression of two individual targets, cyclooxygenase 2 and jun-proto-oncogene, was further studied in our mouse model and in human samples of late-onset FECD.

MATERIALS AND METHODS

Animals

Homozygous mutant knock-in mice (MUT) harboring the *Col8a2* Q455K point mutation and *Col8a2* wild-type (WT) mice were generated as previously described.¹⁴ Animals were maintained and treated under specific pathogen-free conditions. All experiments were performed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and adhering to protocols approved and monitored by the Animal Care and Use Committee of the Johns Hopkins University School of Medicine.

Human Samples

Studies using human tissues were approved by the Johns Hopkins Institutional Review Board and adhered to the tenets of the Declaration of Helsinki. Tissue microarray (TMA) studies were performed using triplicate 1-mm-diameter cores of formalin-fixed paraffin-embedded tissue samples from 50 FECD corneas, 5 keratoconus corneas, 10 normal corneas, and nonocular control tissue specimens as previously described.¹⁵

Unfixed corneal endothelial samples (Descemet membrane and adhering CECs) for immediate endothelial mRNA extraction were retrieved from corneal transplant surgeries in FECD patients ($n = 13$ eyes from 13 patients, mean age $[\pm \text{SEM}]$ 68.6 ± 2.3 , 7:6 male to female ratio) or retrieved directly from whole donor eyes without corneal pathologies or glaucoma ($n = 11$ eyes from 9 donors, mean age $[\pm \text{SEM}]$ 70.4 ± 4.6 , 3:6 male to female ratio, mean death to preservation time $[\pm \text{SEM}]$ 12.2 ± 2.1 hours). Written informed consent was obtained from all patients.

Clinical Confocal Microscopy

Clinical confocal microscopy of the right eye was performed in all animals to confirm the diseased or normal endothelial phenotype, respectively. A ConfoScan3 microscope (Nidek, Fremont, CA) with a $\times 40$ surface-contact objective was used as previously described.¹⁴ Mice were anesthetized using isoflurane (Vedco, St. Joseph, MO) and euthanized by cervical dislocation. Whiskers were trimmed. Mice were placed on a customized platform and the head was fixed with the right eye pointing towards the objective. Lubricant eye gel (Gentel; Novartis, East Hanover, NJ) was used as an immersion fluid, and approximately 100 images of the central corneal endothelium were acquired. Mean CEC density was calculated from randomly selected microscopic images of the corneal endothelium using the ConfoScan software (Nidek).

RNA Isolation and Gene Array Analysis

RNA was isolated from corneal endothelium of both eyes from three groups of MUT and three groups of WT mice. Each group consisted of

FIGURE 1. Clinical confocal microscopy of the central corneal endothelium of 12-month-old *Col8a2*^{Q455K/Q455K} MUT ($n = 12$) and WT ($n = 12$) mice. MUT mice show loss of hexagonal shape (pleomorphism), irregularity of size (polymegathism), hyperreflective nuclei (open triangles), and guttae (closed triangles), whereas WT mice exhibit a normal endothelial morphology. The bar graph depicts loss of CEC density in MUT compared to WT animals as previously described.¹⁴ Data are mean $\pm$ SEM; * $P < 0.05$.

two 12-month-old male animals (four corneas) of the respective strain (MUT or WT). Mice were euthanized, and clinical confocal microscopy was performed as already described. Corneal buttons from excised eyes were dissected for each group. Descemet membranes and adhering CECs were peeled off the corneal buttons using jewelers forceps under a dissecting microscope, pooled, and immediately disrupted by gentle pipetting in TRIzol Reagent (Invitrogen, Carlsbad, CA) at 4°C. RNA was extracted by combined TRIzol and RNeasy spin column (Qiagen, Valencia, CA) purification. Quantity and quality of the extracted RNA was measured by spectrophotometry (NanoDrop 2000; Thermo Scientific, Waltham, MA) and by bioanalyzer assessment (Bioanalyzer Series II Pico Chip; Agilent, Palo Alto, CA).

Gene array analysis in endothelial RNA samples was performed on Mouse Exon 1.0 ST microarrays (Affymetrix, Santa Clara, CA). The Ovation Pico WTA system (NuGEN Technologies, San Carlos, CA) was used to generate SPIA-amplified cDNA from 10 ng of RNA. Sense transcript cDNA (ST-cDNA) was created from 3 µg of purified cDNA using the WT-Ovation Exon module (NuGEN Technologies). Five micrograms of ST-cDNA were subsequently enzymatically fragmented, biotin labeled using the Encore Biotin module (NuGEN Technologies), and hybridized onto Mouse Exon 1.0 ST arrays (Affymetrix) for 18 hours at 45°C with constant rotation at 60 rpm. Affymetrix Fluidics Station 450 was used to wash and stain the arrays, removing the nonhybridized target and incubating with a streptavidin-phycoerythrin conjugate to stain the biotinylated cDNA. Fluorescence was detected using the Affymetrix G3000 GeneArray Scanner and image analysis of each GeneChip was performed through the Affymetrix GeneChip Command Console version 2.0 (AGCC v2.0) software.

For gene expression analysis, data sets were processed using PARTEK Genomics Suite (Partek, St. Louis, MO). Raw intensity levels of probe sets were normalized using the Robust Multichip Average (RMA) method. Two-way analysis of variance (ANOVA) was applied for statistical analysis of differential expression of individual genes in MUT compared to WT mice. Selection of significant genes was performed using a fold-change of >1.5 or <-1.5 and $P < 0.05$ as the cutoff criteria.

To analyze the data quality among individual samples and between both biological groups, principal component analysis (PCA) was performed using the statistics package of R language (<http://www.r-project.org>). Unsupervised hierarchical clustering was performed using Cluster 3.0 (<http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm>) and the average linkage method.¹⁶ For gene ontology (GO) analysis, a Perl program was coded to identify significantly affected functional groups among the differentially expressed genes that showed enrichment of their respective GO term. The GO of all transcripts with a P value of <0.05 and a fold-change of >1.5 or <-1.5 was analyzed.

Quantitative Real-Time PCR

RNA was extracted from three additional and independent groups of 12-month-old MUT and WT mice, respectively, and from human samples using TRIzol and RNeasy spin column (Qiagen) purification. RNA was reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The cDNA samples were preamplified using the Preamp Master Mix (Applied Biosystems) and Taqman inventoried gene expression assays (Table 1; Applied Biosystems). Preamplified cDNA was subjected to real-time PCR on a StepOne Plus Cycler (Applied Biosystems). Universal Master Mix, no UNG (Applied Biosystems), and the Taqman inventoried gene expression assays listed in Table 1 were used. Cycling conditions were 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles at 95°C for 15 seconds, and 60°C for 1 minute. Target gene expression was normalized to expression of actin-beta (*ActB/ACTB*) in mouse and human samples. Fold changes between diseased tissues and controls were calculated using the comparative Ct Method and ExpressionSuite Software 1.0 (Applied Biosystems).

FIGURE 2. Microarray analysis. (A) The first three principal components (PCs) of the PCA mapping account for 91.4% variance (PC1, 79.8%; PC2, 6.4%; PC3, 5.2%) of the data set. The endothelial samples are clearly arranged in clusters in conformity with their origin from *Col8a2*^{Q455K/Q455K} MUT (red) and WT (blue) mice. (B) The heat map and the dendrogram summarizing the unsupervised hierarchical clustering of all differentially expressed transcripts (fold change of >1.5 [red] or <-1.5 [green] with $P < 0.05$ from ANOVA) demonstrate the clear distinction between biological replicates from MUT and WT animals. (C) Volcano plot of significantly changed transcripts in MUT compared to WT animals with the x-axis representing the mean fold change (−10-fold to 10-fold change) and the y-axis representing the negative log10 of the P values. Horizontal threshold line, $P = 0.05$; vertical threshold lines, fold-change = −1.5 and 1.5, respectively.

TABLE 2. Top 35 Differentially Regulated Genes in MUT Compared to WT Animals*

	Gene Symbol	RefSeq	Fold Change, MUT versus WT	P Value, MUT versus
1	<i>Bard1</i>	NM_007525	23.71	0.0012
2	<i>Rnps1</i>	NM_009070	8.78	0.0113
3	<i>Stoml1</i>	NM_026942	7.65	0.0249
4	<i>Magix</i>	NM_018832	−6.34	0.0015
5	<i>Tbl3</i>	NM_145396	5.37	0.0258
6	<i>Cdv3</i>	NM_175565	−5.25	0.0075
7	<i>Gabrb2</i>	NM_008070	−5.16	0.0042
8	<i>Lman1</i>	NM_001172062	5.16	0.0474
9	<i>1700029I01Rik</i>	NM_027285	−5.13	0.0085
10	<i>3110048L19Rik</i>	NR_003549	−5.09	0.0398
11	<i>Tmtc4</i>	NM_028651	−5.04	0.0010
12	<i>B230220N19Rik</i>	AK053483	−4.89	0.0215
13	<i>Sbisa7</i>	NM_172737	−4.45	0.0353
14	<i>LOC100046744</i>	XM_001475765	4.27	0.0006
15	<i>Ccnd2</i>	NM_009829	4.26	0.0011
16	<i>D730045A05Rik</i>	NR_045390	−4.22	0.0468
17	<i>Timp1</i>	NM_001044384	4.12	0.0011
18	<i>Ptgs2</i>	NM_011198	4.00	0.0029
19	<i>Med28</i>	NM_025895	3.97	0.0361
20	<i>Osmr</i>	NM_011019	3.83	0.0038
21	<i>Tmem175</i>	NM_001163531	−3.70	0.0289
22	<i>Wars</i>	NM_001164314	3.61	0.0252
23	<i>Gm10590</i>	XR_001565	−3.54	0.0299
24	<i>Slc38a4</i>	NM_027052	−3.48	0.0000
25	<i>Sorcs3</i>	NM_025696	−3.43	0.0202
26	<i>1700021N20Rik</i>	AK006223	−3.40	0.0282
27	<i>Lect1</i>	NM_010701	−3.35	0.0025
28	<i>Ebf1</i>	NM_007897	−3.30	0.0042
29	<i>Bptf</i>	NM_176850	−3.30	0.0434
30	<i>Cnn3</i>	NM_028044	−3.30	0.0497
31	<i>Gm6455</i>	NR_003596	−3.30	0.0220
32	<i>Hmox1</i>	NM_010442	3.22	0.0001
33	<i>Rragc</i>	NM_017475	3.20	0.0246
34	<i>Gnpat1</i>	NM_019425	3.18	0.0035
35	<i>Tulp4</i>	NM_054040	−3.11	0.0143

* Fold-change values of >1.00 indicate up-regulation and values of <−1.00 indicate down-regulation.

FIGURE 3. Enriched GO terms: bars in the diagram indicate false discovery rates (FDRs) of GO terms showing enrichment in the groups of the most up-regulated (*white*) and the most down-regulated (*black*) transcripts in *Col8a2*^{Q455K/Q455K} mutant compared to wild-type mice.

TMA Immunohistochemical Staining and Analysis

Four-micrometer sections were taken from the TMA paraffin block (for human tissue samples included on TMA see Methods subsection “Human Samples”).¹⁵ Staining was performed through the Translational Pathology Core Laboratory, Department of Pathology and Laboratory Medicine, David Geffen School of Medicine at UCLA (COX2) and Translational Pathology Shared Resource at Vanderbilt University Medical Center (JUN).

For COX2 staining, sections were deparaffinized and rehydrated through graded ethanol. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 10 minutes. Heat-induced antigen retrieval was carried out in 0.01 M citrate buffer, pH 6.00 at 95°C for 25 minutes. Rabbit antihuman COX2 antibody (Neomarkers, Clone SP21, concentration 1:100; Thermo Scientific) was applied for 45 minutes followed by incubation in secondary antibody (Dakocytomation Envision, System Labelled Polymer HRP antirabbit; Dako, Carpinteria, CA) for 30 minutes and 3,3'-diaminobenzidine (DAB) substrate-chromogen (Dako) for 10 minutes. Nuclei were counterstained with Mayer's hematoxylin.

For JUN staining, slides were placed on the Bond Max IHC stainer (Leica, Deerfield, IL) and deparaffinized. Heat-induced antigen retrieval was performed using Epitope Retrieval 2 solution (Leica) for 20 minutes. Slides were incubated with anti-c-Jun (Clone 60A8; Cell Signaling Technology, Boston, MA) for 1 hour at a 1:400 dilution. The

TABLE 3. Genes Associated with Top Three Up-Regulated GO Terms in MUT Compared to WT Animals*

Gene Symbol	RefSeq	Response to Stress	Catalytic Activity	Protein Metabolic Process	Fold Change, MUT versus WT	P Value, MUT versus WT
<i>Bard1</i>	NM_007525	✓	✓		23.71	0.0012
<i>Ptgs2</i>	NM_011198	✓	✓		4.00	0.0029
<i>Wars</i>	NM_001164314		✓	✓	3.61	0.0252
<i>Hmox1</i>	NM_010442	✓	✓		3.22	0.0001
<i>Rragc</i>	NM_017475		✓		3.20	0.0246
<i>Gnpnat1</i>	NM_019425		✓		3.18	0.0035
<i>Pde3a</i>	NM_018779		✓		3.04	0.0076
<i>Tyrobp</i>	NM_011662		✓		2.86	0.0084
<i>Serpine1</i>	NM_008871	✓	✓		2.80	0.0074
<i>Exosc6</i>	NM_028274		✓		2.63	0.0299
<i>Itcb</i>	NM_008395		✓	✓	2.60	0.0210
<i>Ero1lb</i>	NM_026184		✓	✓	2.45	0.0109
<i>Sod3</i>	NM_011435	✓	✓		2.30	0.0002
<i>Manba</i>	NM_026968		✓		2.25	0.0058
<i>Ptptra</i>	NM_008980		✓	✓	2.24	0.0488
<i>Dusp6</i>	NM_026268		✓	✓	2.23	0.0026
<i>Iyz2</i>	NM_017372	✓	✓		2.20	0.0235
<i>Fanca</i>	NM_016925	✓			2.17	0.0465
<i>Dnajb13</i>	NM_153527			✓	2.15	0.0465
<i>Ddb1</i>	NM_015735	✓			2.03	0.0131
<i>Gls</i>	NM_001081081		✓		2.03	0.0070
<i>Pcsk7</i>	NM_008794		✓	✓	2.02	0.0020
<i>Jun</i>	NM_010591	✓			1.92	0.0001
<i>B4galt6</i>	NM_019737		✓		1.89	0.0435
<i>Cpe</i>	NM_013494		✓	✓	1.89	0.0089
<i>Abr</i>	NM_013464	✓			1.85	0.0375
<i>Ugcg</i>	NM_011673		✓		1.82	0.0045
<i>Epbx3</i>	NM_001033163		✓		1.78	0.0008
<i>Plat</i>	NM_008872	✓	✓	✓	1.77	0.0328
<i>Mtif2</i>	NM_133767		✓	✓	1.75	0.0349
<i>Fut10</i>	NM_134161		✓	✓	1.74	0.0250
<i>Pdia4</i>	NM_009787		✓		1.73	0.0051
<i>Ctsc</i>	NM_009982		✓	✓	1.72	0.0088
<i>Ppil3</i>	NM_027351		✓	✓	1.69	0.0071
<i>Ccl2</i>	NM_011333	✓			1.69	0.0036
<i>Ddit3</i>	NM_007837	✓			1.66	0.0163
<i>Clec2d</i>	NM_053109	✓			1.66	0.0378
<i>Hdac5</i>	NM_001077696	✓	✓		1.65	0.0012
<i>Uba5</i>	NM_025692		✓	✓	1.64	0.0024
<i>Stk39</i>	NM_016866		✓	✓	1.63	0.0041
<i>Cd74</i>	NM_001042605	✓		✓	1.62	0.0057
<i>Prss23</i>	NM_029614		✓	✓	1.62	0.0065
<i>Jak1</i>	NM_146145		✓	✓	1.60	0.0339
<i>Rbobb3</i>	NM_028493		✓		1.60	0.0223
<i>Iars</i>	NM_172015		✓	✓	1.59	0.0012
<i>Acsl4</i>	NM_207625		✓		1.59	0.0197
<i>Scd1</i>	NM_009127	✓	✓		1.58	0.0190
<i>Smok4a</i>	NR_030763		✓	✓	1.58	0.0183
<i>Myof</i>	NM_001099634	✓			1.56	0.0103
<i>Cct6a</i>	NM_009838			✓	1.55	0.0288
<i>Pgm3</i>	NM_028352		✓		1.54	0.0015
<i>Steap1</i>	NM_027399		✓		1.54	0.0248
<i>Ece2</i>	NM_025462		✓	✓	1.53	0.0212
<i>Entpd7</i>	NM_053103		✓		1.53	0.0321
<i>Serp1</i>	NM_030685	✓		✓	1.52	0.0401

* Differential expression of bold genes was validated by real-time PCR.

Bond Polymer Refine Detection system (Leica) was used for antibody detection.

Immunolabeled TMA sections were analyzed by light microscopy. Evaluation was performed manually by one observer (MM) masked for all data (including diagnosis) from the donors' records. Antecedent-

masked parallel scorings of multiple sections with a board-certified pathologist (CGE) ensured agreement in the grading procedure.

Examples for individual TMA cores stained for COX2 and JUN from the FECD, KC, autopsy, and noncorneal control groups are shown in Figures 5A and 6A. For COX2, the endothelial staining intensity of each

TABLE 4. Genes Associated with Top Three Down-Regulated GO Terms in MUT Compared to WT Animals*

Gene Symbol	RefSeq	Transporter Activity	Carbohydrate Metabolic Process	Establishment of Localization	Fold Change, MUT versus WT	P Value, MUT versus WT
<i>Gabrb2</i>	NM_008070	✓		✓	−5.16	0.0042
<i>Slc38a4</i>	NM_027052	✓		✓	−3.48	0.0000
<i>Cacng7</i>	NM_133189	✓		✓	−2.63	0.0023
<i>Mgat5</i>	NM_145128		✓		−2.48	0.0427
<i>Pglyrp4</i>	NM_001165968		✓		−2.45	0.0181
<i>Slc5a3</i>	NM_017391	✓	✓	✓	−2.39	0.0085
<i>Txnip</i>	NM_023719			✓	−2.36	0.0088
<i>Gpm6a</i>	NM_153581	✓			−1.97	0.0022
<i>Atp1b2</i>	NM_013415	✓		✓	−1.91	0.0069
<i>Trpm3</i>	NM_001035240	✓		✓	−1.86	0.0004
<i>Gdpd2</i>	NM_023608		✓		−1.82	0.0132
<i>Ogdb</i>	NM_010956		✓		−1.82	0.0289
<i>Man2a1</i>	NM_008549		✓		−1.80	0.0237
<i>Ttpal</i>	NM_181734	✓		✓	−1.78	0.0419
<i>Igf2</i>	NM_010514		✓		−1.73	0.0040
<i>Lrp1b</i>	NM_053011			✓	−1.68	0.0293
<i>Syt9</i>	NM_021889	✓		✓	−1.67	0.0142
<i>Hyal1</i>	NM_008317		✓		−1.66	0.0316
<i>Vps4b</i>	NM_009190			✓	−1.61	0.0047
<i>Slc4a11</i>	NM_001081162	✓		✓	−1.61	0.0245
<i>Cox7c</i>	NM_007749	✓			−1.60	0.0412
<i>B3galt2</i>	NM_020025		✓		−1.60	0.0230
<i>Atp2a2</i>	NR_027838	✓		✓	−1.60	0.0007
<i>Fras1</i>	NM_175473			✓	−1.58	0.0139
<i>Grik5</i>	NM_008168	✓		✓	−1.53	0.0038
<i>Atp8a1</i>	NM_001038999	✓		✓	−1.53	0.0137
<i>Cacna1g</i>	NM_009783	✓		✓	−1.53	0.0050
<i>Crabp2</i>	NM_007759	✓		✓	−1.52	0.0066
<i>Slc6a1</i>	NM_178703	✓		✓	−1.52	0.0041
<i>Expb5</i>	NM_176846			✓	−1.51	0.0341
<i>Pitpnc1</i>	NM_145823	✓		✓	−1.50	0.0145

* Differential expression of bold genes was validated by real-time PCR.

corneal core was evaluated using a $\times 100$ oil immersion objective. Scoring was standardized on the basis of a four grade scoring system: 3, intense; 2, moderate; 1, weak; 0, negative (examples shown in Fig. 5B) applied to the most intense endothelial staining of each core section. For JUN, all endothelial nuclei per section were analyzed using a $\times 40$ objective to determine the JUN-positive percentage of nuclei (example shown in Fig. 6B). Mean staining intensity scores (COX2) and mean percentages of positive nuclei (JUN) were calculated for each specimen and for the three respective corneal entities (FECD, KC, and autopsy/normal cornea) present on the TMA. Core sections with fewer than four evaluable CECs were excluded.

Immunofluorescent Double-Labeling of Corneal Wholemounts

A modified protocol from Blitz et al.¹⁷ was used. Nine-month-old MUT and WT animals ($n = 3$ each) were euthanized, and clinical confocal microscopy was performed. Eyes were dissected and excised corneoscleral buttons were fixed in 0.5% paraformaldehyde for 30 minutes. Tissue samples were permeabilized in 0.5% Triton-X (Sigma-Aldrich, St. Louis, MO) in PBS for 15 minutes, and nonspecific binding sites were blocked by incubation in 5% goat serum for 30 minutes. Mouse anti-ZO-1 antibody (concentration 1:200; Invitrogen) and rabbit anti-COX2 antibody (concentration 1:500; Cayman Chemical, Ann Arbor, MI) diluted in 2% BSA served as primary antibodies. Alexa Fluor 555 goat antimouse and Alexa Fluor 488 goat antirabbit (both concentrations 1:500; Invitrogen) in 2% BSA served as secondary antibodies. Tissue samples were incubated at 37°C for 60 minutes in primary antibody solution and for 45 minutes in secondary antibody

solution. Corneoscleral buttons were flatmounted in Prolong Gold Antifade Reagent with DAPI (Invitrogen) after making four radial incisions. Images were acquired with a LSM510 laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany) using a $\times 40$ oil-immersion objective. COX2-positive CECs within three randomly selected microscopic visual fields were counted and used to calculate the proportions of COX2-positive CECs per sample.

Statistical Analysis

Unless stated otherwise, PRISM4 software (Graphpad Software, La Jolla, CA) was used for statistical analyses applying the unpaired, two-tailed *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

Endothelial Phenotype of MUT and WT Animals

The endothelium of 12-month-old MUT mice exhibited a FECD-like phenotype with loss of hexagonal shape (pleomorphism) and irregularity of size (polymegethism) of the CECs as previously described and quantified.¹⁴ None of these morphologic abnormalities were observed in WT endothelium. The CEC densities in representative cohorts of 12-month-old mice from each strain (measured in right corneas from $n = 12$ mice per strain) were $1050 \pm 39/\text{mm}^2$ in MUT animals (mean $\pm$ SEM) and $2086 \pm 27/\text{mm}^2$ in WT animals ($P < 0.0001$; Fig. 1).

FIGURE 4. Validation of microarray data. (A) Four up-regulated and four down-regulated genes were selected for quantitative real-time PCR validation of Exon 1.0 ST microarray data in endothelial samples from *Col8a2*^{Q455K/Q455K} MUT ($n = 6$) and WT ($n = 6$) mice. Bar diagram shows fold change (MUT compared to WT) of individual transcripts as detected by the two different methods ($^*P < 0.05$). (B) Whole corneas from MUT and WT animals ($n = 3$ each) were immunofluorescence double-labeled for cyclooxygenase 2 protein (COX2, green), which is encoded by *Ptgs2*, and for zonula-1 (ZO-1, red). Nuclei were counterstained with DAPI (blue). COX2-positive CECs within three microscopic fields at the given magnification were counted, and proportions of COX2-positive CECs per sample were calculated. Original magnification $\times 400$. Data are mean $\pm$ SEM.

Comparative Analysis of Expression Profiles

Pooled endothelial mRNA samples of MUT and WT mice with an RNA integrity number (RIN) value of at least 7.5 were used for hybridization onto EXON 1.0 ST microarrays. One strong point of the EXON 1.0 ST microarray platform is the high-resolution analysis of the whole transcript by approximately 40 probes per gene. In total, we could detect transcriptional expression of 24,538 genes.

PCA and hierarchical clustering were employed to visualize and assess the differences in gene expression profiles between the tested endothelial MUT and WT samples. Figure 2A shows a PCA map explaining 91.4% of the variance and demonstrating a distinct grouping of the respective MUT and WT samples. Also, the data analysis by unsupervised hierarchical clustering demonstrated a clear distribution of all biological replicates in the respective WT and MUT group as presented in the dendrogram in Figure 2B. A volcano plot of significantly changed transcripts is shown in Figure 2C.

Using a positive or negative fold change of 1.5 and a P value of 0.05 as the cutoff, 162 (0.66%) genes were up-regulated, and 172 (0.7%) genes were down-regulated. A complete list of differentially expressed genes is presented as Supplementary Material (see Supplementary Tables S1 [up-regulated genes] and S2 [down-regulated genes], <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-10898/-/DCSupplemental>). The 35 genes with the highest differential expression in either direction in MUT compared to WT mice are presented in Table 2.

Gene Ontology of Differentially Expressed Genes

Statistically significant enrichment of at least 15 functional groups was detected (false discovery rate < 0.05 , Fig. 3).

Real-Time PCR and Immunofluorescence Validation of Microarray Data

Real-time PCR analysis investigating examples of four overrepresented and four underrepresented targets (Tables 1, 3, 4) in the six original ($n = 3$, MUT and WT samples, respectively) and in six independent endothelial samples ($n = 3$, MUT and WT samples, respectively) from 12-month-old mice was performed to validate the microarray data. Real-time PCR analysis confirmed relative mRNA expression levels compared with the microarrays and demonstrated a consistency of the data retrieved by both methods (Fig. 4A).

PTGS2 (*COX2*), which encodes the enzyme cyclooxygenase 2 (*COX2*), was chosen for further evaluation in mouse and human tissues due to its comparably high transcriptional overexpression in MUT mice (4.3-fold, $P = 0.001$) and the previously established role of cyclooxygenases in injured corneal endothelium.¹⁸ By immunofluorescence labeling, $4.5 \pm 1.6\%$ COX2-positive CECs were detected in the endothelium of 9-month-old MUT mice, whereas no COX2 expression was found in the endothelium of WT mice (Fig. 4B).

COX2 and JUN in Human Samples

Two TMA sections, each including triplicate cores from 50 FECD, 10 autopsy, and 5 KC corneas, respectively, were investigated per protein (data presented as COX2/JUN, mean $\pm$ SEM): adequate tissue quality (at least one evaluable core) on the immunolabeled TMA sections was present for 42/45 FECD corneas (mean value of $2.8 \pm 0.3/3.6 \pm 0.3$ evaluable cores per cornea), 5/5 KC corneas (mean value of $3.0 \pm 0.5/4.6 \pm 0.7$ evaluable cores per cornea), and 8/8 autopsy corneas (mean value of $3.3 \pm 0.7/3.0 \pm 0.7$ evaluable cores per cornea). The mean donor ages (and male to female ratios) of these evaluable corneal specimens were $69.2 \pm 1.3/69.6 \pm 1.3$ (14:28/15:30) for the FECD group, $58.6 \pm 8.6/58.6 \pm 8.6$ (2:3/2:3) for the KC group, and $62.1 \pm 3.0/62.1 \pm 3.0$ (5:3/5:3) for the autopsy group. The mean scores according to the COX2 four grade scoring system (Fig. 5B)/the mean percentages of JUN positive cells were $1.5 \pm 0.1/35.3 \pm 0.1$ for the FECD group, $0.6 \pm 0.2/4.8 \pm 1.0$ for the KC group, and $0.7 \pm 0.3/15.2 \pm 4.3$ for the autopsy group (Figs. 5C, 6C). The difference between the FECD and autopsy groups (for COX2, $P = 0.02$ and for JUN, $P = 0.04$) and the difference between the FECD and KC groups (for COX2, $P = 0.03$ and for JUN, $P = 0.01$) were statistically significant for both targets.

Real-time PCR showed statistically significant transcriptional up-regulation of *PTGS2* and *JUN* (4.00- and 1.92-fold up-regulated, respectively, in MUT mouse endothelium) in human endothelial FECD samples ($n = 13$ eyes from 13 patients) compared to normal controls ($n = 11$ eyes from nine donors; in the case of two available eyes from the same donor, the expression values from both eyes were averaged). Up-regulation for *COX2* was 3.1-fold ($P < 0.0001$; Fig. 5D) and for *JUN* was 3.1-fold ($P = 0.002$; Fig. 6D).

DISCUSSION

In the present study we used high-resolution microarrays to detect endothelial gene expression changes in a *Col8a2*^{Q455K} mutant mouse model of early-onset FECD. Our results demonstrate distinct changes in the endothelial transcriptome of mutant animals, providing further insight into the pathophysiology of early-onset FECD. *Cox2* and *jun* were selected from the subset of up-regulated genes for further evaluation. In human late-onset FECD endothelium, elevated *COX2* and *JUN*

FIGURE 5. COX2 expression in late-onset human FECD specimens. Sections of a TMA including triplicate cores from 50 FECD, 5 KC, and 10 normal autopsy corneas and additional noncorneal control cores as previously described were immunolabeled for COX2 protein.¹⁵ (A) A representative core from each group (arrows indicate endothelial side, original magnification $\times 100$). Staining intensities of core sections were evaluated using a four-grade scoring system (0 = negative; 1 = weak; 2 = moderate; 3 = intense; examples shown in [B]), original magnification $\times 1000$). Placenta tissue, also included on the TMA, served as positive control for the specificity of the staining (shown in [A, B]). Mean grades were calculated for each of the three groups (shown in [C], error bars indicate SEM). (D) Real-time PCR was used to assess relative *PTGS2* transcriptional expression in FECD specimens ($n = 13$) compared to normal controls ($n = 9$). Horizontal bars indicate the mean value for each group. * $P < 0.05$.

expression was demonstrated by TMA analysis and real-time PCR.

We have recently reported the development of the first transgenic mouse model of FECD.¹⁴ The point mutation harbored by this animal model causes a glutamine to lysine substitution at amino acid position 455 (Q455K) of the *Col8a2* gene, and the equivalent mutation in the human *COL8A2* gene has been associated with early-onset FECD.^{14,19} Homozygous *Col8a2*^{Q455K/Q455K} mutant mice exhibit an endothelial phenotype comprising important characteristics of the human disease. These include progressive loss of CEC density, alterations in endothelial cell morphology, and basement membrane guttae.¹⁴

Our microarray analysis revealed 334 differentially regulated genes (162 up-regulated, 172 down-regulated) in the corneal endothelium of 12-month-old mutant mice. Real-time PCR experiments reinvestigating four respective up- or down-regulated transcripts in *Col8a2* mutant and wild-type animals validated our results and demonstrated good accordance with the microarray data. *Response to Stress*, *Protein Metabolic Process*, *Protein Folding*, *Regulation of Apoptosis*, and *Transporter Activity* were among the functional groups that showed enrichment in the GO analysis. A complete list of all affected GO terms is presented in Figure 3.

Cellular stress, particularly oxidative stress and ER stress, have been recently assigned central roles in the pathogenesis of FECD.^{12,13} Our microarray data also support a pathogenesis involving cellular stress-associated mechanisms in our mouse model based on the detection of a significant number of up-regulated stress response-related targets in the endothelium of *Col8a2*^{Q455K/Q455K} mutant mice. It is noteworthy that this finding is paralleled by increased transcriptional expression of genes related to the functional groups of *Protein Metabolic Process* and *Protein Folding* like the chaperone-encoding genes *Cct6a* and *Ppil3* or the stress associated endoplasmic reticulum protein 1 (*Serp1*). We consider this as potential further evidence for endothelial ER stress resulting in activation of the UPR, supporting results from our previous studies in the same mouse model and in a population of genetically heterogeneous late-onset FECD patients.^{13,14} The hypothesis that the misfolding or aggregation of proteins in FECD contributes to cellular stress and apoptosis induction in CECs has also been proposed in studies investigating other genes affected in late-onset FECD like *SLC4A11* and *LOXHD1*.^{20,21}

Increased transcriptional regulation of other important stress-associated genes such as *Ptgs2* (*Cox2*), *Hmox1*, *Serpine1*, and *Sod3* (complete list in Table 3) was found in the corneal endothelium of mutant animals. Although these

FIGURE 6. JUN expression in late-onset human FECD specimens. TMA sections were immunolabeled for JUN protein. (A) A representative core from each group (arrows indicate endothelial side, original magnification $\times 100$). (B) Representative endothelial sections (original magnification $\times 1000$). Myometrium tissue, also included on the TMA, served as positive control for the specificity of the staining (shown in [A, B]). (C) The percentage of JUN-positive endothelial nuclei was determined in each core section. Mean percentages of positive nuclei were calculated for each of the three groups (shown in [C], error bars indicate SEM). (D) Real-time PCR was used to assess relative *JUN* transcriptional expression in FECD specimens ($n = 13$) compared to normal controls ($n = 9$). Horizontal bars indicate the mean value for each group. $*P < 0.05$.

markers point at least in part to the presence of endothelial oxidative stress, we observed no down-regulation of the antioxidant defense system, which has been demonstrated by previous studies in human late-onset end-stage FECD specimens.¹² The underlying reasons for this inequality may be among other possibilities differences in the species (mouse versus human), the entities (early-onset versus late-onset FECD) or the stages of the disease (midstaged samples from 12-month-old mice versus end-staged tissues from human patients).

The corneal endothelial monolayer maintains corneal deturgescence through a “pump-leak” mechanism. Previous studies have demonstrated decreased transcriptional expression of pump function-related genes in human late-onset FECD endothelium²² and reduced ATPase pump site density in the mid- and end-stage phase of the disease.^{23,24} Our study revealed the down-regulation of *Slc4a11* and of numerous other transport-related genes like the Na/K ATPase encoding gene *Atp1b2* in *Col8a2* mutant animals. Mutations and down-regulation of *Slc4a11* have been associated with human late-onset FECD before.^{20,22} Moreover, it was recently shown that the knock-down of *SLC4A11* causes a reduction of cellular growth and proliferation in HeLa cells and that a depletion of the *SLC4A11* gene alone may already lead to apoptosis induction in CECs.^{25,26}

Prostaglandin-endoperoxide synthase 2 (*PTGS2*, *COX2*) was further evaluated in the early-onset FECD mouse model and in human late-onset FECD. *PTGS2* was chosen due to its 5-fold endothelial transcriptional up-regulation in mutant animals, the previously established corneal endothelial expression of cyclooxygenases and its modulation capability with readily available drugs like COX2 inhibitors. Increased expression of cyclooxygenases was previously detected in CECs involved in endothelial wound closure.²⁷ Cyclooxygenases influence changes of CECs in morphology, mitosis, and migration through their most important product, the eicosanoid prostaglandin E2 (PGE2).^{18,27–29} Jumblatt et al.¹⁸ have shown that injury-induced PGE2 synthesis exhibits increased sensitivity for diclofenac and indomethacin but not for aspirin, suggesting a central role of COX2. To our knowledge, studies on the expression of COX2 in FECD endothelium have not yet been performed. The present study demonstrates transcriptional and translational overexpression of COX2 in both the *Col8a2* mutant mouse model and human late-onset FECD samples. Whether COX2 dysregulation has a protective or detrimental impact in FECD will be the subject of future studies.

The expression of another stress-responsive target, jun proto-oncogene (*JUN*), which had shown moderate but significant up-regulation in the mouse model, was investigated in human FECD tissue to further pursue the transferability of the mouse model data to the human disease. Jun is a protein of

the AP-1 complex with a complex spectrum of functional properties being involved in cellular processes such as proliferation, cellular survival or death, and differentiation.^{30,31}

We demonstrate that up-regulation of *JUN* mRNA and protein occur in the human disease and further corroborate the parallels between the two systems. However, comprehensive expression studies are needed to identify in more detail the extent of shared genes related to endothelial cell stress and death in the early-onset FECD mouse model and the late-onset human disease.

In conclusion, the present study provides the first endothelial whole genome expression analysis in an animal model of early-onset FECD. It delineates important parallels and differences to previous findings in human late-onset FECD endothelium. *Response to Stress*, *Protein Metabolic Process*, *Protein Folding*, *Regulation of Apoptosis*, and *Transporter Activity* are among the differentially expressed functional groups of genes. The results may serve as an important guideline for the future application of the *Col8a2* mutant mouse as a model of endothelial cell death and warrant further investigation of the role of COX2 up-regulation in FECD.

Acknowledgments

The authors thank Clara M. Magyar, PhD, Anthony Frazier, Rhonda Grebe, BS, and Mary E. Pease, MS, for technical support and Shannath Merbs, MD, PhD, and Ray Enke, PhD, for providing human corneal control samples.

References

- Al-Yousuf N, Mavrikakis I, Mavrikakis E, Daya SM. Penetrating keratoplasty: indications over a 10 year period. *Br J Ophthalmol*. 2004;88:998-1001.
- Dorrepal SJ, Cao KY, Slomovic AR. Indications for penetrating keratoplasty in a tertiary referral centre in Canada, 1996-2004. *Can J Ophthalmol*. 2007;42:244-250.
- Ghosheh FR, Cremona FA, Rapuano CJ, et al. Trends in penetrating keratoplasty in the United States 1980-2005. *Int Ophthalmol*. 2008;28:147-153.
- Kang PC, Klintworth GK, Kim T, et al. Trends in the indications for penetrating keratoplasty, 1980-2001. *Cornea*. 2005;24:801-803.
- Lois N, Kowal VO, Cohen EJ, et al. Indications for penetrating keratoplasty and associated procedures, 1989-1995. *Cornea*. 1997;16:623-629.
- Higa A, Sakai H, Sawaguchi S, et al. Prevalence of and risk factors for cornea guttata in a population-based study in a southwestern island of Japan: the Kumejima study. *Arch Ophthalmol*. 2011;129:332-336.
- Kanavi MR, Javadi MA, Sanagoo M. Indications for penetrating keratoplasty in Iran. *Cornea*. 2007;26:561-563.
- Shimazaki J, Amano S, Uno T, Maeda N, Yokoi N. National survey on bullous keratopathy in Japan. *Cornea*. 2007;26:274-278.
- Sony P, Sharma N, Sen S, Vajpayee RB. Indications of penetrating keratoplasty in northern India. *Cornea*. 2005;24:989-991.
- Schmedt T, Silva MM, Ziaei A, Jurkunas U. Molecular bases of corneal endothelial dystrophies. *Exp Eye Res*. 2012;95:24-34.
- Wang Z, Handa JT, Green WR, Stark WJ, Weinberg RS, Jun AS. Advanced glycation end products and receptors in Fuchs' dystrophy corneas undergoing Descemet's stripping with endothelial keratoplasty. *Ophthalmology*. 2007;114:1453-1460.
- 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.
- Engler C, Kelliher C, Spitze AR, Speck CL, Eberhart CG, Jun AS. Unfolded protein response in fuchs endothelial corneal dystrophy: a unifying pathogenic pathway? *Am J Ophthalmol*. 2010;149:194-202. e192.
- Jun AS, Meng H, Ramanan N, et al. An alpha 2 collagen VIII transgenic knock-in mouse model of Fuchs endothelial corneal dystrophy shows early endothelial cell unfolded protein response and apoptosis. *Hum Mol Genet*. 2012;21:384-393.
- Matthaeci M, Lackner EM, Meng H, et al. Tissue microarray analysis of cyclin-dependent kinase inhibitors p21 and p16 in Fuchs dystrophy. *Cornea*. 2013;32:473-478.
- Eisen MB, Spellman PT, Brown PO, Botstein D. Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci U S A*. 1998;95:14863-14868.
- Blitzer AL, Panagis L, Gusella GL, Danias J, Mlodzik M, Iomini C. Primary cilia dynamics instruct tissue patterning and repair of corneal endothelium. *Proc Natl Acad Sci U S A*. 2011;108:2819-2824.
- Jumblatt MM, Willer SS. Corneal endothelial repair. Regulation of prostaglandin E2 synthesis. *Invest Ophthalmol Vis Sci*. 1996;37:1294-1301.
- Biswas S, Munier FL, Yardley J, et al. Missense mutations in COL8A2, the gene encoding the alpha2 chain of type VIII collagen, cause two forms of corneal endothelial dystrophy. *Hum Mol Genet*. 2001;10:2415-2423.
- Vithana EN, Morgan PE, Ramprasad V, et al. SLC4A11 mutations in Fuchs endothelial corneal dystrophy. *Hum Mol Genet*. 2008;17:656-666.
- Riazuddin SA, Parker DS, McGlumphy EJ, et al. Mutations in LOXHD1, a recessive-deafness locus, cause dominant late-onset Fuchs corneal dystrophy. *Am J Hum Genet*. 2012;90:533-539.
- Gottsch JD, Bowers AL, Margulies EH, et al. Serial analysis of gene expression in the corneal endothelium of Fuchs' dystrophy. *Invest Ophthalmol Vis Sci*. 2003;44:594-599.
- McCartney MD, Robertson DP, Wood TO, McLaughlin BJ. ATPase pump site density in human dysfunctional corneal endothelium. *Invest Ophthalmol Vis Sci*. 1987;28:1955-1962.
- McCartney MD, Wood TO, McLaughlin BJ. Moderate Fuchs' endothelial dystrophy ATPase pump site density. *Invest Ophthalmol Vis Sci*. 1989;30:1560-1564.
- Park M, Li Q, Shcheynikov N, Zeng WZ, Muallem S. NaBC1 is a ubiquitous electrogenic Na⁺-coupled borate transporter essential for cellular boron homeostasis and cell growth and proliferation. *Mol Cell*. 2004;16:331-341.
- Liu J, Seet LF, Koh LW, et al. Depletion of SLC4A11 causes cell death by apoptosis in an immortalized human corneal endothelial cell line. *Invest Ophthalmol Vis Sci*. 2012;53:3270-3279.
- Jumblatt MM. Autocrine regulation of corneal endothelium by prostaglandin E2. *Invest Ophthalmol Vis Sci*. 1994;35:2783-2790.
- Joyce NC, Matkin ED, Neufeld AH. Corneal endothelial wound closure in vitro. Effects of EGF and/or indomethacin. *Invest Ophthalmol Vis Sci*. 1989;30:1548-1559.
- Jumblatt MM, Paterson CA. Prostaglandin E2 effects on corneal endothelial cyclic adenosine monophosphate synthesis and cell shape are mediated by a receptor of the EP2 subtype. *Invest Ophthalmol Vis Sci*. 1991;32:360-365.
- Shaulian E, Karin M. AP-1 as a regulator of cell life and death. *Nat Cell Biol*. 2002;4:E131-E136.
- Meng QH, Xia Y. c-Jun, at the crossroad of the signaling network. *Protein Cell*. 2011;2:889-898.