Endothelial Cell MicroRNA Expression in Human Late-Onset Fuchs' Dystrophy

Mario Matthaei,¹⁻³ Jianfei Hu,¹ Laura Kallay,¹ Charles G. Eberhart,¹ Claus Cursiefen,² Jiang Qian,¹ Eva-Maria Lackner,⁴ and Albert S. Jun¹

¹The Wilmer Eye Institute, Johns Hopkins Medical Institutions, Baltimore, Maryland

²Department of Ophthalmology, University of Cologne, Cologne, Germany

³Department of Ophthalmology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany

⁴Department of Ophthalmology, Medical University of Graz, Graz, Austria

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

Submitted: June 27, 2013

Accepted: November 29, 2013

Citation: Matthaei M, Hu J, Kallay L, et al. Endothelial cell microRNA expression in human late-onset Fuchs' dystrophy. *Invest Ophthalmol Vis Sci*. 2014;55:216-225. DOI:10.1167/iovs.13-12689

PURPOSE. MicroRNAs (miRNAs) are a class of endogenous noncoding RNA and post transcriptionally modulate gene expression during development and disease. Our study investigated the differential miRNA expression in human Fuchs' endothelial corneal dystrophy (FECD) compared with normal endothelium to identify miRNA sequences that are involved in the pathogenesis of FECD.

METHODS. Comparative miRNA expression profiles of endothelial samples obtained from FECD patients during lamellar corneal transplant surgery and from normal donor globes were generated using OpenArray plate technology. Differential expression of individual miRNAs was validated in the original and in independent samples using stem-loop RT qPCR assays. Expression of miRNA target genes was assessed using qPCR and tissue microarray (TMA) immunolabeling.

RESULTS. Our results demonstrate downregulation of 87 miRNAs in FECD compared with normal endothelium (>3-fold change; $P < 0.01$). Correspondingly, *DICER1*, (encoding an endoribonuclease critical to miRNA biogenesis) showed a moderate but significant decrease in FECD samples ($P < 0.05$). Significant repression of three miR-29 family members (miR-29a-3p, miR-29b-2-5p, and miR-29c-5p) was paralleled by upregulation of their extracellular matrix associated mRNA targets collagen I and collagen IV. Tissue microarray immunolabeling showed histologically verifiable subendothelial collagen I and collagen IV deposition and increased endothelial laminin protein expression in FECD samples.

CONCLUSIONS. The present study provides the first miRNA profile in FECD and normal endothelial cells and demonstrates widespread miRNA downregulation in FECD. Decreased endothelial expression of miR-29 family members may be associated with increased subendothelial extracellular matrix accumulation in FECD.

Keywords: Fuchs' endothelial corneal dystrophy, corneal endothelium, microRNA

Facing the aqueous humor of the anterior chamber, the endothelial monolayer regulates hydration of the corneal stroma and maintains corneal clarity through a pump-leak mechanism. Fuchs' endothelial corneal dystrophy (FECD) is a bilateral disorder of the corneal endothelium and among the most common reasons for corneal transplant surgery in the United States (US) and other Western countries.¹⁻⁶ Histopathologically, FECD is characterized by an accelerated loss of corneal endothelial cell (CEC) density. The endothelial basement membrane, Descemet membrane, thickens and forms focal excrescences called guttae. In advanced stages of the disease, the described morphologic characteristics are paralleled by functional loss of CECs, eventually resulting in corneal edema and opacification.⁷

Cellular stress of the endoplasmic reticulum activating a cytoprotective signaling cascade termed the unfolded protein response and oxidative stress have recently been implicated as important pathogenetic factors of FECD.⁷⁻⁹ Previous studies investigating the pathogenesis of FECD have analyzed endothelial differences in the most basic levels of gene expression.

These investigations include, but are not limited to, detailed genomic mutational analyses,¹⁰ RNA,^{11,12} and protein expression studies.^{13,14} However, to the best of our knowledge, a study of microRNA (miRNA) expression in both normal and in FECD endothelium has not yet been performed to date. MiRNA expression has attracted much attention recently and reports indicate that over 60% of protein-coding genes are subject to regulation by these small, noncoding 20 to 24 nucleotides (nt)-long RNAs.¹⁵ Their 5' seed region (nt 2-7) is preferably complementary to the highly conserved sites in 3' untranslated regions (UTRs) of mRNAs.¹⁶ Base pairing of at least five consecutive nucleotides is usually required for a corresponding miRNA-mRNA interaction and individual miRNAs may have hundreds of different mRNA targets.¹⁵ They cause mRNA destabilization and cleavage or direct translational repression and thereby regulate eukaryotic gene expression at the posttranscriptional level.¹⁶

Hypothesizing that miRNAs may be differentially regulated and involved in FECD pathogenesis, we generated a differential corneal endothelial miRNA expression profile of FECD speci-

mens and age-matched normal controls. Our results show widespread downregulation of corneal endothelial miRNA levels in FECD endothelium including significant downregulation of the miR-29 family. Follow-up experiments quantify increased mRNA and protein levels of miR-29 targets collagen I, collagen IV, and laminin in FECD samples and suggest involvement of miR-29 depletion in subendothelial extracellular matrix accumulation and thickening of Descemet membrane in FECD pathogenesis.

MATERIALS AND METHODS

Human Samples and Characteristics

Corneal endothelial samples (stripped Descemet membranes with adhering corneal endothelial cells) for immediate total RNA extraction were retrieved from patients undergoing endothelial keratoplasty due to clinically diagnosed end-stage FECD or from normal, whole autopsy globes without corneal pathologies or glaucoma. For immunohistochemical analyses of tissue microarray (TMA) sections, a TMA including triplicate 1-mm diameter cores from 50 FECD, five keratoconus (KC), 10 normal autopsy formalin-fixed paraffin-embedded corneas, and additional nonocular control tissues were used as previously described.¹⁷ Keratoconus cores on the TMA served as corneal controls in addition to cores from normal corneas since KC is not a primary disease of the corneal endothelium. Studies using human tissues were approved by The Johns Hopkins institutional review board and adhered to the tenets of the Declaration of Helsinki. Written informed consent was obtained from all patients.

Total RNA Extraction, Quantification, and Evaluation of Integrity

Total RNA (including miRNA fraction) isolation was performed by combined TRIzol/spin-column RNA extraction using the mirVana miRNA isolation kit (Ambion, Austin, TX). The present study was based on reverse transcription quantitative pcr (RT-qPCR) analysis of mature miRNA expression. MicroRNAs can be accurately detected using RT-qPCR even in RNA preparations with reduced RNA integrity.¹⁸ However, RNA integrity number (RIN) values obtained by our standard protocol were confirmed in preliminary RNA extraction experiments using bioanalyzer assessment (Bioanalyzer Series II Pico chip; Agilent, Palo Alto, CA) and showed exclusively RIN values greater than 7.0. RNA quantity and purity/quality was tested in subsequent experiments using NanoDrop 2000 spectrophotometry (Thermo Scientific, Waltham, MA). All samples included had a 260/280 ratio greater than or equal to 1.6.

Human MicroRNA Array Procedure

Total RNA extraction from individual FECD ($n = 6$) and normal ($n = 6$) samples was performed as described above. The experiment was performed according to the standard protocol provided by the manufacturer for small sample sizes ("Optimized protocol with low sample input"; Applied Biosystems [ABI], Foster City, CA). For each sample, 10 ng of total RNA was converted to cDNA using the Taqman MicroRNA Reverse Transcription Kit (ABI) and corresponding Megaplex RT Primers (Human Pool A+B; ABI). Seven and one-half microliters of the reverse transcription product were mixed with 20 μ L preamplification master mix (2 $\times$ concentrate ABI), 8.5 μ L nuclease free water and 4 μ L preamplification primer mix (10 $\times$ concentrate Pool A or B; ABI). Thermal cycling for 16 cycles

followed the standard protocol. The preamplification product was diluted 1:20 in Tris-EDTA (TE) buffer. The diluted preamplification product (Pool A and Pool B) was 1:1 mixed with 22.5 μ L Taqman Open Array Real Time PCR Master Mix (ABI), and the expression of 754 miRNA sequences was analyzed using the TaqMan OpenArray Human MicroRNA Panel, QuantStudio 12K Flex (ABI) according to manufacturer's instructions.

MicroRNA Array Data Analysis

The array Pool A and B datasets were analyzed using the ExpressionSuite v1.0 software package (ABI). The experimental data were grouped into files from diseased (FECD) and normal biological replicates using the respective normal group as reference. A threshold cycle value of 35 was set as a threshold for detectable miRNAs expression. Quantitative PCR reactions presenting uneven amplification were manually omitted. MiRNA expression values were normalized using reference RNAs, RNU48 and U6. The relative threshold cycle values of the reference RNAs showed good reproducibility with low intra- and intergroup variability using constant input quantities of RNA for cDNA synthesis and their mean expression was used in the following as reference values. Differentially expressed miRNAs were identified using a fold-change greater than 3 with P less than 0.01, respectively, as cut-off criteria. Unsupervised hierarchical clustering was performed using Cluster 3.0 ([in the public domain] <http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm>) and the average linkage method.¹⁹ The statistics package of R language ([in the public domain] <http://www.r-project.org>) was used for principal component analysis (PCA).

Assessment of Individual MicroRNA Expression

Endothelial total RNA was extracted from an additional set of human FECD ($n = 3$) and normal ($n = 3$) samples as described above. These new samples were added to the two original sets of n equals 6 samples per group and subsequent stem-loop RT-qPCR assessment was performed in n equals 9 samples per FECD and normal group, respectively, according to manufacturer's instructions (Publication Part No. 4465407; ABI). The individual assays (all ABI) used were hsa-let-7g_002282, hsa-miR-26b_000407, hsa-miR-29b-2#_002166, hsa-miR-30d_000420, hsa-miR-125b_000449, hsa-miR-181c_000482, hsa-miR-184_000485, and RNU48_001006. Briefly, total RNA was reverse transcribed to cDNA using the Taqman MicroRNA Reverse Transcription Kit (ABI) and a custom RT Primer Pool prepared from Taqman MicroRNA Assay 5xRT primers (ABI). Complimentary DNA samples were preamplified for 12 cycles using the Taqman PreAmp Master Mix (ABI) and a custom PreAmp primer pool prepared from individual 20x Taqman MicroRNA assays (ABI). Preamplified cDNA samples were diluted 1:8 in 0.1x TE buffer and used for preparation of the PCR reaction mix. Samples were run in technical triplicates on a 96-well plate using individual 20x Taqman MicroRNA assays (ABI) and Universal Master Mix, no UNG (ABI) on a Step-One Plus Cyclor (ABI) under standard conditions. Data evaluation was performed using ExpressionSuite v1.0 (ABI) and normalizing expression of individual miRNAs to RNU48.

Assessment of Individual mRNA Expression

Ten microliters of RNA from FECD ($n = 20$) and control ($n = 12$) samples were reverse transcribed to cDNA using the High-Capacity cDNA Reverse Transcription kit (ABI) according to manufacturer's instructions. The RT-product was preampli-

FIGURE 1. Array analysis of endothelial microRNA expression in FECD ($n = 6$) and normal corneas ($n = 6$). **(A)** Heat map demonstrates differential expression of 87 targets (cut-off criteria: >3 -fold change and $P < 0.01$), the *dendrogram* shows clear segregation of the individual samples in the FECD and normal groups. **(B)** Principal component analysis mapping shows clustering of the samples in conformity with their origin from the FECD (blue) and the normal (red) groups. **(C)** Volcano plot demonstrates fold-change and P value of each miRNA with detectable endothelial expression level in FECD compared with normal corneas. *Horizontal threshold line:* $P = 0.01$, *vertical threshold lines:* fold-change $= -3.0$ (left) and fold-change $= 3.0$ (right). Significantly altered miRNAs are marked in red. Arrows mark respective members of the miR-29 family with significantly altered expression levels.

fied using the Taqman PreAmp Master Mix (ABI) and a target specific primer pool (individual Taqman assays [ABI] diluted 1:100 in TE-buffer). Taqman assays (2 $\times$ concentrate, ABI) used for preamplification and qPCR were Hs00229023_m1 (*DICER1*), Hs00203008_m1 (*DROSHA*), Hs00256062_m1 (*DGCR8*), Hs00164004_m1 (*COL1A1*), Hs00266237_m1 (*COL4A1*), Hs00267056_m1 (*LAMC1*), and Hs99999903_m1 (*ACTB*). Quantitative PCR was performed in technical

triplicates. Per reaction, 5 μ L of preamplification product were mixed with 1 μ L Taqman assay, 10 μ L Taqman Universal Master Mix II, no UNG (ABI), and 4 μ L nuclease free water, loaded onto a 96-well plate and run on a Step-One Plus Cycler (ABI) under standard conditions. Data evaluation was performed with the ExpressionSuite v1.0 software (ABI) normalizing the expression of individual targets to the housekeeping gene beta-actin (*ACTB*). *Beta-actin* was chosen

TABLE. Differentially Expressed MicroRNAs as Detected by Array Analysis with a 3-Fold Change and $P < 0.01$ Comparing Endothelium Obtained During Posterior Lamellar Keratoplasty in Patients With Fuchs' Dystrophy and Healthy Human Donor Endothelium

Assay Name ID	miRBase Names	Fold-Change	P Value
hsa-miR-199b_000500	hsa-miR-199b-5p	-1000.00	0.003
hsa-miR-616_002414	hsa-miR-616-3p	-250.00	0.006
hsa-miR-29a_002112	hsa-miR-29a-3p	-166.67	0.005
hsa-miR-140-3p_002234	hsa-miR-140-3p	-50.00	0.004
hsa-miR-184_000485	hsa-miR-184	-50.00	0.000
hsa-miR-30c_000419	hsa-miR-30c-5p	-45.45	0.000
hsa-miR-30b_000602	hsa-miR-30b-5p	-34.48	0.000
hsa-miR-101_002253	hsa-miR-101-3p	-32.26	0.004
hsa-miR-502_001109	hsa-miR-502-5p	-18.18	0.004
hsa-miR-181a_000480	hsa-miR-181a-5p	-17.86	0.001
hsa-miR-455_001280	hsa-miR-455-5p	-17.24	0.000
hsa-miR-340_002258	hsa-miR-340-5p	-16.95	0.002
hsa-miR-130b_000456	hsa-miR-130b-3p	-12.82	0.001
hsa-miR-95_000433	hsa-miR-95	-11.76	0.000
hsa-miR-484_001821	hsa-miR-484	-11.36	0.006
hsa-miR-532-3p_002355	hsa-miR-532-3p	-9.80	0.003
hsa-miR-15b_000390	hsa-miR-15b-5p	-9.62	0.003
hsa-miR-195_000494	hsa-miR-195-5p	-9.52	0.000
hsa-miR-34a#_002316	hsa-miR-34a-3p	-9.09	0.004
hsa-miR-628-5p_002433	hsa-miR-628-5p	-8.62	0.003
hsa-miR-330_000544	hsa-miR-330-3p	-8.13	0.007
mmu-miR-374-5p_001319	hsa-miR-374b-5p	-8.00	0.001
hsa-miR-502-3p_002083	hsa-miR-502-3p	-7.81	0.000
hsa-miR-148b_000471	hsa-miR-148b-3p	-6.80	0.000
hsa-miR-185_002271	hsa-miR-185-5p	-6.62	0.009
hsa-miR-107_000443	hsa-miR-107	-6.58	0.000
hsa-miR-191_002299	hsa-miR-191-5p	-6.58	0.000
hsa-miR-1244_002791	hsa-miR-1244	-6.54	0.002
hsa-miR-374_000563	hsa-miR-374a-5p	-6.49	0.001
hsa-miR-135a_000460	hsa-miR-135a-5p	-6.45	0.010
hsa-miR-186_002285	hsa-miR-186-5p	-6.45	0.001
hsa-miR-125b_000449	hsa-miR-125b-5p	-6.41	0.000
hsa-miR-181c_000482	hsa-miR-181c-5p	-6.37	0.000
hsa-miR-660_001515	hsa-miR-660-5p	-6.25	0.004
hsa-miR-125a-5p_002198	hsa-miR-125a-5p	-6.13	0.003
hsa-miR-30d_000420	hsa-miR-30d-5p	-6.10	0.006
rno-miR-7#_001338	hsa-miR-7-1-3p	-5.95	0.001
hsa-miR-378_000567	hsa-miR-378a-5p	-5.88	0.000
hsa-miR-29b-2#_002166	hsa-miR-29b-2-5p	-5.75	0.000
hsa-miR-671-3p_002322	hsa-miR-671-3p	-5.65	0.008
hsa-miR-26a_000405	hsa-miR-26a-5p	-5.62	0.001
hsa-let-7d_002283	hsa-let-7d-5p	-5.46	0.000
hsa-miR-30a-5p_000417	hsa-miR-30a-5p	-5.41	0.008
hsa-miR-26b_000407	hsa-miR-26b-5p	-5.38	0.000
hsa-miR-340#_002259	hsa-miR-340-3p	-5.38	0.000
hsa-miR-93#_002139	hsa-miR-93-3p	-5.15	0.004
hsa-miR-148a_000470	hsa-miR-148a-3p	-5.08	0.001
hsa-miR-151-3p_002254	hsa-miR-151a-3p	-4.88	0.008
hsa-miR-151-5p_002642	hsa-miR-151a-5p	-4.88	0.001
hsa-miR-99a_000435	hsa-miR-99a-5p	-4.83	0.001
hsa-let-7g_002282	hsa-let-7g-5p	-4.76	0.000
hsa-miR-532_001518	hsa-miR-532-5p	-4.72	0.003
hsa-miR-23b_000400	hsa-miR-23b-3p	-4.67	0.000
hsa-miR-19a_000395	hsa-miR-19a-3p	-4.61	0.003
hsa-miR-362_001273	hsa-miR-362-5p	-4.61	0.008
hsa-miR-30e-3p_000422	hsa-miR-30e-3p	-4.59	0.000
hsa-miR-361_000554	hsa-miR-361-5p	-4.59	0.000
hsa-miR-149_002255	hsa-miR-149-5p	-4.46	0.002
hsa-miR-100_000437	hsa-miR-100-5p	-4.42	0.001
hsa-miR-99b_000436	hsa-miR-99b-5p	-4.39	0.001
hsa-miR-483-3p_002339	hsa-miR-483-3p	-4.33	0.003

TABLE. Continued

Assay Name ID	miRBase Names	Fold-Change	P Value
hsa-miR-574-3p_002349	hsa-miR-574-3p	-4.02	0.003
hsa-miR-455-3p_002244	hsa-miR-455-3p	-4.00	0.000
rno-miR-29c#_001818	hsa-miR-29c-5p	-3.97	0.009
mmu-miR-140_001187	hsa-miR-140-5p	-3.95	0.001
hsa-miR-301_000528	hsa-miR-301a-3p	-3.95	0.005
hsa-miR-152_000475	hsa-miR-152	-3.85	0.000
hsa-miR-328_000543	hsa-miR-328	-3.68	0.000
hsa-miR-197_000497	hsa-miR-197-3p	-3.64	0.000
hsa-let-7f_000382	hsa-let-7f-5p	-3.62	0.001
hsa-miR-181a-2#_002317	hsa-miR-181a-2-3p	-3.51	0.000
hsa-miR-27b_000409	hsa-miR-27b-3p	-3.51	0.008
hsa-miR-324-3p_002161	hsa-miR-324-3p	-3.51	0.005
hsa-miR-103_000439	hsa-miR-103a-3p	-3.46	0.001
hsa-miR-128a_002216	hsa-miR-128	-3.40	0.005
hsa-miR-17_002308	hsa-miR-17-5p	-3.36	0.000
hsa-miR-320_002277	hsa-miR-320a	-3.32	0.000
hsa-miR-106a_002169	hsa-miR-106a-5p	-3.31	0.000
hsa-miR-20a_000580	hsa-miR-20a-5p	-3.24	0.005
hsa-miR-27b#_002174	hsa-miR-27b-5p	-3.16	0.002
hsa-miR-423-5p_002340	hsa-miR-423-5p	-3.15	0.000
hsa-miR-34a_000426	hsa-miR-34a-5p	-3.08	0.008
hsa-miR-590-3p_002677	hsa-miR-590-3p	-3.08	0.006
hsa-miR-19b_000396	hsa-miR-19b-3p	-3.06	0.001
hsa-miR-638_001582	hsa-miR-638	-3.06	0.001
hsa-miR-708_002341	hsa-miR-708-5p	-3.06	0.005
hsa-miR-28_000411	hsa-miR-28-5p	-3.00	0.002

as housekeeping gene since previous studies indicate its stable expression in human corneal endothelial cells under certain conditions and also in mouse corneal endothelium.^{20–22}

Tissue Microarray Immunohistochemical Analysis

Sections measuring 4 to 5 μm in thickness were taken from the TMA paraffin block (for information on human tissue samples included on TMA, see Methods, Human Samples section).¹⁷ The staining was performed through HistoTox Labs, Inc. (Boulder, CO). Sections were deparaffinized and rehydrated. Immunohistochemical stainings were performed using an autostainer (Dako Cytomation, Carpinteria, CA). Primary antibodies/antigen retrieval used were anticollagen I (ab34710; Abcam, Cambridge, MA)/no retrieval, anticollagen IV (ab6586; Abcam)/enzyme retrieval, antilaminin (ab11575; Abcam)/enzyme retrieval. Nuclei were counterstained with Mayer's hematoxylin. Slides were dehydrated and permanently cover-slipped.

Evaluation of immunolabeled TMA sections was performed under a light microscope by one observer (MM) masked for all data from the donors' records. Antecedent masked parallel scorings with a board certified pathologist (CGE) established consistency in the grading procedure.

Corneal subendothelial collagen I or collagen IV deposition was quantified as follows: a positive case was defined as at least one out of the triplicate cores per case showing an immunohistochemically-labeled subendothelial layer, which could clearly be distinguished from the underlying Descemet membrane. Positive cases were identified and the percentage of positive cases per group (FECD, KC, and Normal) was calculated. A previously described four grade scoring system was used to quantify corneal endothelial laminin staining

FIGURE 2. Stem-loop RT-qPCR validation of individual miRNA expression levels. Differential endothelial expression of seven miRNAs was validated using individual stem-loop RT-qPCR assays in the original FECD (FECD₁, $n = 6$, blue) and normal (Control₁, $n = 6$, red) samples and in a second set of independent FECD (FECD₂, $n = 3$, red) and normal (Control₂, $n = 3$) samples. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

(grades: 3 intense, 2 moderate, 1 weak, 0 negative).²³ Core sections with fewer than four evaluable CECs were excluded.

RESULTS

MiRNA Expression Profile in Normal and FECD Endothelium

Endothelial total RNA from n equals 6 (age $\pm$ SEM: 59.5 ± 3.1 ; male to female ratio: 1:5) FECD and n equals 6 (age $\pm$ SEM: 63.0 ± 1.9 ; male to female ratio: 3:3; mean death to preservation time $\pm$ SEM 11.9 ± 2.7 hours) normal autopsy globes was subjected to miRNA expression analysis using OpenArray plate technology on the QuantStudio 12K Flex system (ABI) for high-throughput real-time quantification. This method is based on stem-loop RT-based Taqman real-time PCR, which is considered as the gold standard for miRNA detection.²⁴ A total of 311 miRNAs showed detectable levels in CECs from FECD patients and normal donors (for a complete list of all expressed miRNAs see Supplementary Table S1). This represents 41.2% of the total number of 754 well-characterized miRNA sequences analyzed in our study. The expression values of each miRNA were normalized to the mean expression value of the two

commonly used reference RNAs RNU48 and U6. These two RNAs showed stable expression among the samples when loading an equal amount of total RNA (see Supplementary Fig. S1). Unsupervised hierarchical clustering (Fig. 1A) demonstrates that the individual samples clearly segregate into a FECD and a normal group, and emphasize good reproducibility of the data. In a similar manner, the PCA map in Figure 1B explains 40% of the variance and demonstrates the distinct grouping of the respective FECD and normal samples.

Corneal Endothelial MiRNA Expression in FECD Compared With Normal Samples

Two hundred seventy-nine miRNAs showed a mean relative quantity (RQ) of less than 1 and 32 miRNAs showed a mean relative quantity of greater than 1 (Fig. 1C) in FECD compared with normal endothelium. However, none of the increased expression levels proved to be statistically significant, whereas among the downregulated miRNAs significantly changed expression levels were detected for 87 miRNAs (listed in the Table) applying a 3-fold change with P less than 0.01, respectively, as cut-off criteria.

FIGURE 3. Taqman qPCR analysis of genes involved in miRNA biogenesis. Relative endothelial expression of *DICER1*, *DROSHA*, and *DGCR8* in FECD ($n = 20$) compared with normal ($n = 12$) samples. Data are mean $\pm$ SEM. * $P < 0.05$.

Validation of Individual MiRNA Expression Levels

Seven of the significantly ($P < 0.01$) downregulated miRNAs were validated by stem-loop RT-qPCR using individual Taqman assays. This validation was performed in two cohorts of samples: the first cohort consisted of the original 12 samples from the array analysis ($n = 6$ FECD; $n = 6$ normal) and the second cohort consisted of three additional independent samples per biological group ($n = 3$ FECD: mean age $\pm$ SEM: 67.7 ± 4.7 ; male to female ratio 0:3; $n = 3$ normal: mean age $\pm$ SEM: 79.3 ± 3.5 , male to female ratio: 2:1, mean death to preservation time $\pm$ SEM: 14.2 ± 8.0), respectively. RNU48 was used as reference RNA. We found good consistency of the data from both experiments (Fig. 2). The mean fold-change (significance levels) values for each individual miRNA in original array analysis/cohort 1/cohort 2 were for let-7g: -4.76 ($P < 0.001$)/ -2.64 ($P < 0.0001$)/ -3.63 ($P = 0.037$); miR-26b: -5.38 ($P < 0.001$)/ -3.44 ($P < 0.0001$)/ -5.68 ($P = 0.006$); miR-29b-2: -5.75 ($P < 0.001$)/ -2.55 ($P = 0.002$)/ -3.83 ($P = 0.008$); miR-30d: -6.1 ($P = 0.006$)/ -8.05 ($P = 0.001$)/ -19.23 ($P = 0.026$); miR-125b: -6.41 ($P < 0.001$)/ -4.64 ($P < 0.0001$)/ -8.40 ($P = 0.109$); miR-181c: -6.37 ($P < 0.001$)/ -3.25 ($P = 0.007$)/ -5.56 ($P = 0.075$); miR-184: -50.00 ($P < 0.001$)/ -2.61 ($P < 0.0001$)/ -4.53 ($P = 0.196$).

Expression of *DICER1*, *DROSHA*, and *DGCR8* in FECD and Normal Endothelium

Our results demonstrate downregulation of global miRNA expression in FECD compared with normal endothelium. In order to analyze potential underlying reasons for this deregulation, we examined mRNA expression of individual genes, which are crucial for miRNA processing including, *DICER1*, *DROSHA*, and *DGCR8* in FECD ($n = 20$, mean age: 65.6 ± 2.0 , male to female ratio: 6:14) compared with normal ($n = 12$, mean age: 66.8 ± 4.7 , male to female ratio: 5:7, mean death to preservation time $\pm$ SEM: 14.2 ± 2.4) endothelial samples. Our results demonstrate -1.38 -fold ($P = 0.039$) downregulation of *DICER1* in FECD endothelium, whereas the transcriptional expression of *DROSHA* (1.03-fold, $P = 0.82$) and *DGCR8* (1.11-fold, $P = 0.60$) showed no significant alterations (Fig. 3).

Differential Expression of MiR-29 Family-Targets in FECD and Normal Endothelium

Three members of the miR-29 family (miR-29a-3p, miR-29b-2-5p, and miR-29c-5p) were significantly downregulated and miR-29a ranked among the three most deregulated miRNAs in FECD compared with normal samples. The miR-29 family plays

an important role in the regulation of extracellular matrix (ECM) turnover and fibrotic conditions of different organs and tissues including the eye.^{25–35} To identify parallels between the significantly reduced expression of the miR-29 family and the transcriptional expression of corresponding target genes, we performed qPCR analyses for the expression of miR-29 targets *COL1A1*, *COL4A1*, and *LAMC1* using the same 20 patient and 12 normal samples described in the preceding section. *COL1A1*, *COL4A1*, and *LAMC1* were chosen for further studies because they belong to the group of experimentally validated miR-29 targets (mirWALK database, [in the public domain] <http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/>) and in particular have also been shown to be suppressed by the miR-29 family in cells derived from the trabecular meshwork neighboring the corneal endothelium.³⁵ We found transcriptional overexpression of two of these three genes in FECD compared with normal samples: *COL1A1* 42.65-fold ($P = 0.026$; Fig. 4A) and *COL4A1* 4.21-fold ($P = 0.004$; Fig. 5A). *LAMC1* showed a trend for transcriptional upregulation (1.35-fold expression; $P = 0.061$; Fig. 6A).

The evaluation of immunohistochemically-stained TMA section showed in 59.2% of FECD cases an additional subendothelial collagen I-positive layer (Fig. 4B) and in 60.4% of FECD cases an additional subendothelial collagen IV-positive layer (Fig. 5B). Corresponding subendothelial deposits were found in none of the normal or KC cases for collagen I (Fig. 4B) and in none of the KC and in 12.5% of normal corneas for collagen IV (Fig. 5B). According to the applied scoring system, we found significantly increased endothelial cytoplasmic laminin expression in FECD compared with KC (2.26-fold; $P < 0.0001$) and normal corneas (1.42-fold; $P = 0.0034$) (Fig. 6B).

DISCUSSION

The present study investigated the differential endothelial miRNA expression in FECD compared with normal corneas. Analysis of 754 well-characterized miRNA sequences demonstrated widespread downregulation of miRNA levels in FECD endothelial cells. Follow-up experiments validating these results in the original and in independent endothelial samples showed good reproducibility of the data. Three members of the miR-29 family were among the most downregulated mature miRNA transcripts. This was paralleled by increased transcriptional and translational expression of extracellular matrix associated miR-29 targets (collagen I, collagen IV, and laminin) suggesting impact of altered miR-29 regulation on subendothelial ECM accumulation in FECD.

A total of 87 miRNAs showed significantly decreased abundance in FECD endothelium, whereas none of the increased miRNA levels proved to be statistically significant (see volcano plot, Fig. 1C). Such global unidirectional alterations in miRNA expression have been previously demonstrated in other pathologic conditions such as cancer tissue, human and rat cigarette smoke exposed lung tissue, and cortical tissue affected by neuropsychiatric disorders; the underlying reasons for the global changes in miRNA expression seem to be diverse and not yet fully understood.^{36–41} It is anticipated that global changes in miRNA expression might be explained by alterations in transcription or epigenetic regulation of primary miRNA transcripts, by changes in miRNA biogenesis, by altered expression of Argonaute protein homologue, or by changed miRNA turnover.³⁶ The present study analyzed the transcriptional levels of genes involved in miRNA biogenesis including *DICER1*, *DROSHA*, and *DGCR8*. The results raise the possibility that altered miRNA biogenesis caused by a decrease in *DICER1* may contribute to reduction

FIGURE 4. Endothelial collagen I expression in FECD. (A) Taqman qPCR analysis of relative endothelial *COL1A1* mRNA levels in FECD ($n = 20$, blue) compared with normal ($n = 12$, red) corneas. Data are mean $\pm$ SEM. $*P < 0.05$. (B) Immunohistochemical analysis of collagen I protein in FECD ($n = 50$), KC ($n = 5$), and normal ($n = 10$) corneas included in respective triplicate cores from the corneal center on a TMA as reported previously.¹⁷ Fuchs' endothelial corneal dystrophy samples that showed a collagen I-positive subendothelial layer (closed triangle, example sections FECD 1 and FECD 2) were at 59.2%. No such layer was observed in any of the KC or normal samples. Vertical arrow, corneal endothelium; open triangle, Descemet membrane; open horizontal arrow, corneal stroma. Original magnification 800 $\times$.

of miRNA abundance. Further studies will have to investigate this aspect in more detail. It is of interest, however, that recent studies demonstrated cytotoxicity of DICER1 depletion in various tissues like the retina and the heart, and that this downregulation may be triggered by oxidative stress.^{42–44} This tempts us to speculate that a similar oxidative stress (known to

occur in FECD)–DICER1 depletion cascade may be involved in FECD pathogenesis.⁴⁵

MiR-29a-3p was among the three most depressed miRNAs and two more members from the miR-29 family, miR-29b-2-5p, and miR-29c-5p, ranked among the significantly downregulated miRNAs in the analyzed FECD specimens. It is believed that all

FIGURE 5. Endothelial collagen IV expression in FECD. (A) Taqman qPCR analysis of relative endothelial *COL4A1* mRNA levels in FECD ($n = 20$, blue) compared with normal ($n = 12$, red) corneas. Data are mean $\pm$ SEM. $**P < 0.01$. (B) Immunohistochemical analysis of collagen IV protein in FECD ($n = 50$), KC ($n = 5$), and normal ($n = 10$) corneas included in respective triplicate cores from the corneal center on a TMA as reported previously.¹⁷ Fuchs' endothelial corneal dystrophy samples at 60.4% (example sections FECD 1 and FECD 2) and 12.5% of the normal samples showed a collagen IV-positive subendothelial layer (closed triangle), which could clearly be differentiated from the underlying part of Descemet membrane. No such layer was observed in any of the KC samples. Vertical arrow, corneal endothelium; open triangle, Descemet membrane; open horizontal arrow, corneal stroma. Original magnification: 800 $\times$.

FIGURE 6. Endothelial laminin expression in FECD. **(A)** Taqman qPCR analysis of relative endothelial *LAMC1* mRNA levels in FECD ($n = 20$, blue) compared with normal ($n = 12$, red) corneas. Data are mean $\pm$ SEM. **(B)** Immunohistochemical analysis of laminin protein in FECD ($n = 50$), KC ($n = 5$), and normal ($n = 10$) corneas included in respective triplicate cores from the corneal center on a TMA as reported previously¹⁷ shows increased cytoplasmic laminin expression in FECD compared with KC or normal endothelium. Vertical arrow, corneal endothelium; open triangle, Descemet membrane; open horizontal arrow, corneal stroma. Original magnification: 800 $\times$.

members of the miR-29 family have a largely overlapping spectrum of target genes due to the similarities in their sequences and their identical seed sequences.²⁵ The miR-29 family is a putative key modulator of ECM homeostasis. Members of the miR-29 family inhibit a variety of ECM-associated proteins and show aberrant, generally reduced expression in fibrotic conditions of different organs such as liver, kidney, lung, heart, as well as systemic sclerosis.^{25–30,32} In ocular tissues, an antifibrotic effect of the miR-29 family has also been reported. It was demonstrated that miR-29b suppresses type I collagen in human tenon fibroblasts and that the miR-29 family induces suppression of ECM proteins such as SPARC, collagen I, and IV in human trabecular meshwork (HTM) cells.^{31,34,35} Furthermore, it was suggested that chronic exposure of HTM cells to oxidative stress may result in miR-29b downregulation.³³ Activation of the TGF- β pathway is frequently discussed as the inciting event that triggers reduction in miR-29 levels in ocular- and nonocular-derived cell types.^{31,35} Transforming growth factor- β stimulates ECM production in human corneal endothelial cells, which can at least in part be avoided by TGF- β receptor inhibition.^{46,47} However, the effect of TGF- β on miRNA expression in corneal endothelial cells and its role in FECD have not yet been studied in detail.

Normal Descemet membrane consists of an anterior banded layer (ABL) subjacent to the corneal stroma and a posterior nonbanded layer (PNBL) located anterior to the corneal endothelium.⁴⁸ The thickness of the ABL remains stable after birth (approximately 3 μ m), whereas the thickness of the PNBL continuously increases (from 3 μ m at the age of 20 years to 10 μ m at the age of 80 years).^{48,49} The PNBL in FECD specimens may be thinner compared with normal corneas, even though the overall thickness of the Descemet's membrane from FECD patients usually exceeds the one from normal tissues: an essential characteristic of FECD is the formation of subendothelial excrescences of Descemet membrane (guttae) and accelerated thickening of Descemet membrane by subendothelial deposition of ECM in the form of an additional subendothelial posterior collagenous layer.⁵⁰

We hypothesized that repression of the miR-29 family may be associated with uncontrolled and increased expression of their ECM-related target genes and accumulation of their proteins in FECD. Indeed, our results demonstrate in addition to significantly reduced expression of miR-29a-3p, miR-29b-2-5p, and miR-29c-5p reciprocally increased mRNA levels of their targets collagen I and collagen IV in FECD endothelium and show in the majority of cases distinct subendothelial collagen I or collagen IV deposition. Laminin protein showed increased endothelial cytoplasmic expression in FECD specimens, whereas no clear subendothelial deposits were observed. These quantitative results confirm results from a previous descriptive study, which showed a similar expression pattern for collagen IV and laminin in normal and late-onset FECD corneas.⁵¹

Future studies need to clarify the influence of miRNA expression changes in FECD pathogenesis in more detail. Apart from changes in miR-29 expression this also extends to other differentially expressed miRNAs in FECD. One of them, miR-184, has been shown to be affected by a mutation in its seed region in Endothelial Dystrophy, Iris hypoplasia, congenital Cataract, and stromal Thinning (EDICT) syndrome, and mutation or downregulation may contribute to the endothelial abnormalities found in both EDICT syndrome and FECD.⁵² Pharmacological modulation of miRNA expression could provide a conservative approach to treat FECD. A recent study showed that the Rho-associated kinase inhibitor fasudil prevents renal fibrosis and restores the expression of miR-29.⁵³ It would be interesting to investigate if a similar miRNA-associated mechanism also plays a role in the known therapeutic effect of this drug class in FECD.⁵⁴

In summary, the present study provides the first analysis of differential endothelial miRNA expression in FECD and normal corneas and may serve as a valuable base for future studies of the disease. The observed global suppression of miRNA expression levels in FECD endothelial cells might be influenced by the diminished endothelial expression of DICER1. Reduced expression of miR-29 family members paralleled by reciprocal overexpression of their ECM related targets suggest

a role of the miR-29 family in FECD pathogenesis and as a potential target of future conservative therapeutic approaches.

Acknowledgments

The authors thank Roxann Ashworth, MHS, Gerald L. Vandergrift, and Andrea De Biase, MS, for technical support. They also thank Shannath Merbs, MD, PhD, Ray Enke, PhD, Verity Oliver, PhD, and Jin Song, MD, PhD, for providing human corneal control samples.

Supported by grants from National Institutes of Health (NIH EY019874 [ASJ], EY001765 [Wilmer Microscopy Core Facility]), Medical Illness Counseling Center (ASJ), Research to Prevent Blindness (Wilmer Eye Institute), Deutsche Forschungsgemeinschaft (DFG MA 5110/2-1 [MM]), Fuchs' Dystrophy Research Grant (Wilmer Eye Institute [MM]), Richard Lindstrom/Eye Bank Association of America Research Grant (MM), and grants from the J. Willard and Alice S. Marriott Foundation, Edward Colburn, Lorraine Collins, Richard Dianich, Barbara Freeman, Mary Finegan, Stanley Friedler, MD, Diane Kemker, Jean Mattison, Lee Silverman, and Norman Tunkel, PhD.

Disclosure: **M. Matthaei**, None; **J. Hu**, None; **L. Kallay**, None; **C.G. Eberhart**, None; **C. Cursiefen**, None; **J. Qian**, None; **E.-M. Lackner**, None; **A.S. Jun**, None

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, Klitworth 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.
- Cursiefen C, Kuchle M, Naumann GO. Changing indications for penetrating keratoplasty: histopathology of 1,250 corneal buttons. *Cornea*. 1998;17:468-470.
- Elhalis H, Azizi B, Jurkunas UV. Fuchs endothelial corneal dystrophy. *Ocul Surf*. 2010;8:173-184.
- 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.
- 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.
- Aldave AJ, Han J, Frausto RE. Genetics of the corneal endothelial dystrophies: an evidence-based review. *Clin Genet*. 2013;84:109-119.
- Gottsch JD, Seitzman GD, Margulies EH, et al. Gene expression in donor corneal endothelium. *Arch Ophthalmol*. 2003;121:252-258.
- 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.
- Jurkunas UV, Rawe I, Bitar MS, et al. Decreased expression of peroxiredoxins in Fuchs' endothelial dystrophy. *Invest Ophthalmol Vis Sci*. 2008;49:2956-2963.
- Jurkunas UV, Bitar MS, Rawe I, Harris DL, Colby K, Joyce NC. Increased clusterin expression in Fuchs' endothelial dystrophy. *Invest Ophthalmol Vis Sci*. 2008;49:2946-2955.
- Friedman RC, Farh KKH, Burge CB, Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res*. 2009;19:92-105.
- Bartel DP. MicroRNAs: target recognition and regulatory functions. *Cell*. 2009;136:215-233.
- Matthaei 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.
- Jung M, Schaefer A, Steiner I, et al. Robust microRNA stability in degraded RNA preparations from human tissue and cell samples. *Clin Chem*. 2010;56:998-1006.
- 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.
- Wilson SE, Lloyd SA. Epidermal growth factor and its receptor, basic fibroblast growth factor, transforming growth factor beta-1, and interleukin-1 alpha messenger RNA production in human corneal endothelial cells. *Invest Ophthalmol Vis Sci*. 1991;32:2747-2756.
- 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.
- Shei W, Liu J, Htoon HM, Aung T, Vithana EN. Differential expression of the Slc4 bicarbonate transporter family in murine corneal endothelium and cell culture. *Mol Vis*. 2013;19:1096-1106.
- Matthaei M, Hu J, Meng H, et al. Endothelial cell whole genome expression analysis in a mouse model of early-onset Fuchs endothelial corneal dystrophy. *Invest Ophthalmol Vis Sci*. 2013;54:1931-1940.
- Hurley J, Roberts D, Bond A, Keys D, Chen C. Stem-loop RT-qPCR for microRNA expression profiling. *Methods Mol Biol*. 2012;822:33-52.
- Kriegel AJ, Liu Y, Fang Y, Ding X, Liang M. The miR-29 family: genomics, cell biology, and relevance to renal and cardiovascular injury. *Physiol Genomics*. 2012;44:237-244.
- Qin W, Chung AC, Huang XR, et al. TGF-beta/Smad3 signaling promotes renal fibrosis by inhibiting miR-29. *J Am Soc Nephrol*. 2011;22:1462-1474.
- He Y, Huang C, Lin X, Li J. MicroRNA-29 family, a crucial therapeutic target for fibrosis diseases. *Biochimie*. 2013;95:1355-1359.
- Maurer B, Stanczyk J, Jungel A, et al. MicroRNA-29, a key regulator of collagen expression in systemic sclerosis. *Arthritis Rheum*. 2010;62:1733-1743.
- Pandit KV, Milosevic J, Kaminski N. MicroRNAs in idiopathic pulmonary fibrosis. *Transl Res*. 2011;157:191-199.
- Roderburg C, Urban GW, Bettermann K, et al. Micro-RNA profiling reveals a role for miR-29 in human and murine liver fibrosis. *Hepatology*. 2011;53:209-218.
- Li N, Cui J, Duan X, Chen H, Fan F. Suppression of type I collagen expression by miR-29b via PI3K, Akt, and Sp1 pathway in human Tenon's fibroblasts. *Invest Ophthalmol Vis Sci*. 2012;53:1670-1678.
- van Rooij E, Sutherland LB, Thatcher JE, et al. Dysregulation of microRNAs after myocardial infarction reveals a role of miR-29 in cardiac fibrosis. *Proc Natl Acad Sci U S A*. 2008;105:13027-13032.
- Luna C, Li G, Qiu J, Epstein DL, Gonzalez P. Role of miR-29b on the regulation of the extracellular matrix in human trabecular meshwork cells under chronic oxidative stress. *Mol Vis*. 2009;15:2488-2497.

34. Luna C, Li GR, Qiu JM, Epstein DL, Gonzalez P. Cross-talk between miR-29 and transforming growth factor-betas in trabecular meshwork cells. *Invest Ophthalmol Vis Sci*. 2011;52:3567–3572.
35. Villarreal G Jr, Oh DJ, Kang MH, Rhee DJ. Coordinated regulation of extracellular matrix synthesis by the microRNA-29 family in the trabecular meshwork. *Invest Ophthalmol Vis Sci*. 2011;52:3391–3397.
36. Smalheiser NR, Lugli G, Rizavi HS, Torvik VI, Turecki G, Dwivedi Y. MicroRNA expression is down-regulated and reorganized in prefrontal cortex of depressed suicide subjects. *PLoS One*. 2012;7:e33201.
37. Beveridge NJ, Gardiner E, Carroll AP, Tooney PA, Cairns MJ. Schizophrenia is associated with an increase in cortical microRNA biogenesis. *Mol Psychiatry*. 2010;15:1176–1189.
38. Graff JW, Powers LS, Dickson AM, et al. Cigarette smoking decreases global microRNA expression in human alveolar macrophages. *PLoS One*. 2012;7:e44066.
39. Schembri F, Sridhar S, Perdomo C, et al. MicroRNAs as modulators of smoking-induced gene expression changes in human airway epithelium. *Proc Natl Acad Sci U S A*. 2009;106:2319–2324.
40. Izzotti A, Calin GA, Arrigo P, Steele VE, Croce CM, De Flora S. Downregulation of microRNA expression in the lungs of rats exposed to cigarette smoke. *FASEB J*. 2009;23:806–812.
41. Lu J, Getz G, Miska EA, et al. MicroRNA expression profiles classify human cancers. *Nature*. 2005;435:834–838.
42. Kaneko H, Dridi S, Tarallo V, et al. DICER1 deficit induces Alu RNA toxicity in age-related macular degeneration. *Nature*. 2011;471:325–330.
43. Damiani D, Alexander JJ, O'Rourke JR, et al. Dicer inactivation leads to progressive functional and structural degeneration of the mouse retina. *J Neurosci*. 2008;28:4878–4887.
44. Chen JF, Murchison EP, Tang R, et al. Targeted deletion of Dicer in the heart leads to dilated cardiomyopathy and heart failure. *Proc Natl Acad Sci U S A*. 2008;105:2111–2116.
45. 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.
46. Okumura N, Kay EP, Nakahara M, Hamuro J, Kinoshita S, Koizumi N. Inhibition of TGF-beta signaling enables human corneal endothelial cell expansion in vitro for use in regenerative medicine. *PLoS One*. 2013;8:e58000.
47. Usui T, Takase M, Kaji Y, et al. Extracellular matrix production regulation by TGF-beta in corneal endothelial cells. *Invest Ophthalmol Vis Sci*. 1998;39:1981–1989.
48. Wilson SE, Bourne WM. Fuchs' dystrophy. *Cornea*. 1988;7:2–18.
49. Cogan DG, Kuwabara T. Growth and regenerative potential of Descemet's membrane. *Trans Ophthalmol Soc U K*. 1971;91:875–894.
50. Waring GO III, Bourne WM, Edelhauser HF, Kenyon KR. The corneal endothelium. Normal and pathologic structure and function. *Ophthalmology*. 1982;89:531–590.
51. Gottsch JD, Zhang C, Sundin OH, Bell WR, Stark WJ, Green WR. Fuchs corneal dystrophy: aberrant collagen distribution in an L450W mutant of the COL8A2 gene. *Invest Ophthalmol Vis Sci*. 2005;46:4504–4511.
52. Iliff BW, Riazuddin SA, Gottsch JD. A single-base substitution in the seed region of miR-184 causes EDICT syndrome. *Invest Ophthalmol Vis Sci*. 2012;53:348–353.
53. Wang B, Komers R, Carew R, et al. Suppression of microRNA-29 expression by TGF-beta1 promotes collagen expression and renal fibrosis. *J Am Soc Nephrol*. 2012;23:252–265.
54. Koizumi N, Okumura N, Ueno M, Nakagawa H, Hamuro J, Kinoshita S. Rho-associated kinase inhibitor eye drop treatment as a possible medical treatment for Fuchs corneal dystrophy. *Cornea*. 2013;32:1167–1170.