Identification of Circulating Fibrocytes and Dendritic Derivatives in Corneal Endothelium of Patients With Fuchs’ Dystrophy

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Fuchs’ endothelial corneal dystrophy (FECD) is a degenerative eye disorder affecting 4% of Americans older than 40.1,4 The exact incidence is unknown because symptomatic FECD is preceded by an asymptomatic phase that does not always evolve into symptomatic disease.1,2

Although an early-onset form of FECD exists (manifesting in the 2nd to 3rd decade of life), this form is very rare.1 The current study included only late-onset cases of FECD (manifesting in the 5th to 6th decade of life).3

FECD usually affects both eyes and clinically progresses from blurred vision to painful erosions and even blindness.1,4 The blurred vision is caused by corneal swelling as a result of a defect in the inner corneal layer, that is, corneal endothelium (CE).1,4 CE is derived from the neural crest (not related to vascular endothelium).3 Normal CE is a monolayer of hexagonal cells in postmitotic arrest that rest on the DM.6 Normal CE acts as a selective barrier and active pumping system for water and nutrients across the avascular cornea, safeguarding corneal transparency.1,4 In FECD, CE function is impaired as a result of a critical loss of CE cells (CECs) and thickening of the DM with the appearance of guttae.1,4

FECD displays a high familial risk and can be inherited in an autosomal dominant-like way, but most cases are sporadic.4,7 Smoking is a risk factor and women are 2.5 to 3.5 times more frequently affected than men.1,4,8 Several mutations, sequence variants, and chromosomal loci have been linked to FECD,1,4,5,10 but each of those accounts for only a subpopulation of patients. Cellular functions that play a role in FECD include ECM deposition, oxidative stress, apoptosis, and epithelial-mesenchymal transition (EMT).1,4,9,11,12 but a unifying theory is lacking.

FECD is the leading indication for transplantation of the CE in the United States.13 Given the global shortage of donor corneas and the expected rise in prevalence of FECD with the aging of the population, this study aimed to gain more insight into the pathophysiology of FECD to identify targets for nonsurgical therapies.1,14

Keywords: Fuchs’ endothelial corneal dystrophy, circulating fibrocytes, dendritic cells, fibrosis, epithelial-mesenchymal transition
Materials and Methods

Acquisition of Human Samples and Study Approval

Fresh “CE-DM complexes” (CE monolayers with DM) were prospectively collected from patients with symptomatic late-onset FECD (clinical stages II to IV) during endothelial keratoplasty (CE transplantation). The diagnosis of FECD was made by corneal specialists, based on clinical history, slit-lamp biomicroscopic examination (bilateral presence of central guttae with or without edema), and disease progression and excluding other intraocular pathologies (such as uveitis or other inflammatory eye conditions). Patients with previous intraocular surgery other than cataract surgery were excluded from this study. Controls originated from donor corneas and enucleations for uveal melanoma (without corneal extension or prior radiotherapy). The retrieval of donor corneas was performed according to the European Eye Bank Association guidelines, within an average of 6 hours of warm ischemia or 14 hours of cold ischemia. Prior to transplantation and CE-DM collection, donor corneas were stored by organ culture in Stem Alpha medium (Stem Alpha, Saint-Genis-l’Argenti`ere, France), Eurobio medium (Eurobio, Les Ulis, France), or minimal essential medium supplemented with calf serum and antibiotics; dextran was added to reduce swelling prior to transplantation.

This material was either collected in RNAlater (Ambion by Life Technologies, Carlsbad, CA, USA or Qiagen) for RNA extraction or mounted on tissue slides, with CE facing up, for whole-mount staining. Some samples were partitioned and mounted on multiple slides to enable multiple stains for the same sample (these samples were counted only once to calculate the total number of samples for immunohistochemistry (IHC) and immunofluorescence (IF) and to calculate patient characteristics). Slides were air dried, fixed in acetone, and stored at −20°C.

This study included only residual human material and was approved by the local institutional ethics committee and biobank of UZ Leuven (Commissie Medische Ethiek UZ KU Leuven/Onderzoek; study number S55133) in adherence with the tenets of the Declaration of Helsinki.

RNA Extraction for Microarray Expression Analysis and Reverse Transcription Quantitative Real-Time PCR

CE-DM complexes were manually homogenized with a disposable pestle. RNA extraction was performed with an RNeasy Micro Kit (Qiagen) without DNase treatment (to maximize RNA yield).

Microarray Expression Analysis

Eight RNA samples were selected for microarray expression analysis: four FECD (age 75.5 [4.0]; mean [standard deviation] in years; female:male [F:M] ratio 3:1) and four normal control samples (age 52.8 [18.8]; F:M ratio 0:4). RNA concentration and quality was measured using spectrophotometry (NanoDrop ND-1000, Thermo Scientific, Waltham, MA, USA). RNA integrity ranged between 6.70 and 7.90, as measured with a Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA). For each sample, 100 ng of total RNA was spiked with bacterial poly-A RNA positive controls (Affymetrix, Santa Clara, CA, USA) and used to generate second-cycle cDNA with the Ambion WT Expression Kit. Samples were fragmented and biotin-labelled using the Affymetrix WT Terminal Labeling Kit. A mixture of fragmented biotinylated cDNA and hybridization controls (Affymetrix) was hybridized on Affymetrix GeneChip Human Gene 1.0 ST Arrays, followed by staining and washing in a GeneChip fluidics station 450 (Affymetrix). Chips were scanned using a GeneChip scanner 3000 (Affymetrix) to assess the raw probe signal intensities.

MEA was completed in the R programming environment with Bioconductor packages (http://www.bioconductor.org, in the public domain). The analysis was based on the robust multiarray average expression levels of the probe sets with the package xps (version 1.7.2). Probe sets with ≥1 present detection call above background were taken into account for further analysis (27,818 probe sets). The average number of present calls per sample was 26,342 (91.2%), with a standard deviation of 416 (1.4%), and comparable average background and scaling factors across the slides. Differential expression was assessed via the moderated t statistic. To control the false discovery rate (FDR), multiple testing correction was performed.

Pathway and function analysis was completed using Ingenuity Pathway Analysis version 8.5-2803 (IPA; Qiagen, Redwood City, CA, USA), with all 27,818 probe sets as input. Of the entries, 19,254 could be mapped to molecules in the Ingenuity Knowledge Base. Probes with log2 (FECD/normal) ≥ 1 and FDR-corrected P value < 0.05 were selected. Probe sets referring to the same molecule were resolved by taking the average of the log-ratio values. This resulted in 617 eligible molecules for gene networks and 628 for functions, pathways, and lists.

Reverse Transcription Quantitative Real-Time PCR (RT-qPCR)

Two Custom RT2 Profiler PCR Arrays (CAPH10409 and CAPH10410; Qiagen) were designed, comprising 197 genes of interest based on MEA data: 85 fulfilled the cut-off criteria ([log2 (FECD/normal)] ≥ 1 and FDR-corrected P value < 0.05; 94 genes were added to complement the pathways of interest, as identified by IPA. RNA concentration and quality was measured using spectrophotometry (NanoDrop ND-1000). Nine unpooled FECD (age 67.8 [8.0]; F:M ratio 5:4) and eight unpooled normal control samples (age 59.6 [12.3]; F:M ratio 2:6), different from those used for MEA, were analyzed with RT-qPCR. Five of 9 FECD samples and seven of eight normal samples contained enough mRNA to be used on both arrays; the other samples were used on one array each. Per array, 100 ng of total RNA was reverse transcribed to cDNA with the RT2 First Strand Kit (Qiagen). Arrays were run on the 7900 HT Fast Real-Time PCR System (Applied Biosystems) using RT2 SYBR Green ROX qPCR Mastermix (Qiagen).

Data analysis was performed through the web portal of Qiagen (http://qiagen.com/geneglobe, in the public domain). The software performed comparative threshold cycle calculations on the uploaded raw threshold cycle (Ct) data. Normalization was done using the average arithmetic mean of Ct values from three reference genes (RPL13A, RPL19, and RP55) that were stably expressed in FECD and normal control samples. Ct cut-off was set to 35. Higher Ct values and missing values were imputed with a Ct value of 35. A 2-sided t statistic was used (t ≥ 0.05).

Immunostaining on Whole-Mount Specimens

Staining was performed manually or in an automated way with a BOND-MAX stainer (Leica Microsystems, Wetzlar, Germany) or a Link 48 (Dako, Glostrup, Denmark) using diaminobenzidine (brown), alkaline phosphatase (red), or amino-ethylcarbazole (red) as chromogens. Prior to staining with the peroxidase substrates (diaminobenzidine and amino-ethylcarbazole), endogenous peroxidase activity was blocked with
Peroxidase Blocking Solution (Dako) for 10 minutes at room temperature. Pretreatment, dilution, and incubation conditions (Supplementary Table S1) were optimized on fresh-frozen positive control specimens that also served as batch controls. Horseradish peroxidase-labelled EnVision FLEX (Dako) or Bond Polymer Refine Red Detection (Leica) were used as a secondary antibody for 30 minutes. Sections were counterstained with hematoxylin and mounted.

If double staining consisted of combinations of antisera from different hosts (mouse and rabbit) and goat-anti-mouse or goat-anti-rabbit antibodies labelled with Alexa Fluor 488 or Alexa Fluor 568 (Invitrogen by Life Technologies) as secondary antibodies. Dye swaps ensured the specificity of staining. Slides were counterstained with DAPI (Invitrogen) and mounted with glycerol gelatin or ProLong Gold antifade reagent (Life Technologies).

In total, 55 CE-DM samples from patients with FECD (age 70.1 [15.8]; F:M ratio 10:5) and 15 normal control samples (age 60.1 [15.8]; F:M ratio 10:5) were tested with IHC and IF.

**Image Acquisition and Processing**

Brightfield images were acquired with a NanoZoomer 2.0-RS (Hamamatsu, Hamamatsu City, Japan), Ultra Fast Scanner 1.6 IVD (Philips, Amsterdam, Netherlands), or DM LB microscope with DC400 camera and IM50 software (Leica). Sample surfaces were calculated with an NDP viewer 2.2.10 (Hamamatsu), IntelliSite Pathology Solution IMS 2.3 (Philips), or Fiji17 (ImageJ 1.48c; ImageJ, Madison, WI, USA), respectively. Images acquired with the Leica microscope were white balanced, matched for brightness, and corrected with a flatfield background image using EZ Image Prep from ChromaCal (Supplementary Methods) confirmed the significant upregulation of genes related to dendritic cell maturation (Fig. 1d, Supplementary Fig. S1, Supplementary Table S2). Note that hepatitis stellate cells are a subset of myofibroblasts that play a role in fibrotic liver disorders.9 Gene set enrichment analysis (Supplementary Methods) confirmed the significant upregulation of genes related to dendritic cell maturation (Fig. 1d, Supplementary Fig. S1).20

In addition, MEA revealed biological functions (P < 0.001) that are consistent with previously reported processes in FECD (Supplementary Table S2), supporting the external validity of this experiment. These functions included connective tissue development and function (FECD is characterized by ECM deposition),1 posttranslational modification and free radical scavenging (unfolded protein stress and oxidative stress play a role in FECD),1,9 cell cycle and cell death (senescence and aberrant apoptosis of CECs have been reported in FECD),1,4 and nervous system development and function (CE originates from the neural crest).5

**RT-qPCR provides technical and biological validation of the MEA results**

We used RTqPCR to validate the MEA results on a new and larger set of unpooled samples (n = 9 FECD, n = 8 normal). For this, we selected 179 genes of interest based on their MEA expression values and role in MHC class II type antigen presentation, dendritic cell maturation, inflammation, chemotaxis, growth factor activity, EMT, and ECM production.

The RT-qPCR results correlated strongly with the MEA results (R² = 0.82, P < 0.0001, Fig. 1c), and unsupervised clustering could again segregate FECD from normal samples (Fig. 1b). MEA and RT-qPCR data have been deposited in NCBI’s Gene Expression Omnibus21 and are accessible through the Gene Expression Omnibus Series accession number GSE75676 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE75676, in the public domain).

**RESULTS**

**Genes related to inflammation, antigen presentation, and dendritic cell maturation are significantly differentially expressed in FECD**

MEA was used to compare the corneal endothelial transcriptome of four FECD and four normal control samples. For this, RNA was extracted from CE-DM complexes, which contain only CECs, allowing a reliable comparison of the gene expression profiles. Unsupervised computational analysis clearly segregated FECD samples from normal donor samples (Fig. 1a), indicating a marked difference in the gene expression between both groups. This experiment revealed 954 significantly differentially expressed probe sets (487 probe sets were ≥ twofold upregulated and 467 were ≥ twofold downregulated in FECD with an FDR-corrected P < 0.05). IPA analysis of the MEA data indicated a role for functions that are currently not linked to the pathophysiology of FECD, such as inflammatory response, inflammatory disease, and antigen presentation (Supplementary Fig. S1, Supplementary Table S2). Notably, one of the top gene networks was centered on upregulation of major histocompatibility complex (MHC) class II molecules related to antigen presentation (Fig. 2), with major histocompatibility complex, class II, DR alpha (HLA-DRA) being the second most upregulated gene in FECD (fold regulation = 73.95, P < 0.001). The most significant canonical pathways included dendritic cell maturation; role of macrophages, fibroblasts, and endothelial cells in rheumatoid arthritis; and hepatic fibrosis/hepatic stellate cell activation (Supplementary Fig. S1, Supplementary Table S2). Note that hepatic stellate cells are a subset of myofibroblasts that play a role in fibrotic liver disorders.9 Gene set enrichment analysis (Supplementary Methods) confirmed the significant upregulation of genes related to dendritic cell maturation (Fig. 1d, Supplementary Fig. S1).20

As HLA-DRA was one of the top upregulated genes in FECD, according to MEA (fold regulation = 73.95, P < 0.001), which was confirmed by RT-qPCR (fold regulation = 169.07, P = 0.009), IHC was performed for the HLA-DRα chain (HLA-DRA) on CE-DM whole mounts. Surprisingly, this revealed the presence of HLA-DRA+ cells with dendritic morphology in all of the examined FECD specimens (Fig. 3b), but in none of the controls (Fig. 3a). Another marker related to antigen presentation, namely CD45 (marker of hematopoietic cells),22 unveiled two subsets of cells, that is, cells with dendritic morphology and moderate CD45 expression, and a smaller subset of monocyte-like cells with bean-shaped nuclei, strong CD45 expression, and lacking dendritic processes (Fig. 3c). If double staining for CD45 and α smooth muscle actin (αSMA; mesenchymal marker, and marker of EMT; Fig. 4c), HLA-DRA
and αSMA (Fig. 4d), as well as HLA-DRA and CD45 (Fig. 5a) showed coexpression of each of these marker pairs within cells with dendritic morphology. In addition, αSMA diffusely stained the CE layer in FECD (Fig. 3f).

Notably, IHC staining for HLA-DRA and CD45 on transversal sections of full-thickness corneas was not suited to demonstrate the presence of cells with dendritic or monocyte-like morphology in FECD. This was a result of sampling bias (for the monocyte-like subset) and the long and slender dendritic processes with scant cytoplasm that only occasionally presented as small dots and were easily overlooked (for the dendritic subset). Transversal staining for αSMA, on the other hand, confirmed a general upregulation of this protein in the CE layer of patients with FECD.

**Immunofluorescent Double Staining of CE-DM Complexes Identifies Circulating Fibrocytes in FECD**

Next, we aimed to identify the origin and nature of the dendritic and monocyte-like subsets of cells in FECD: either they are derived from CECs (through the process of EMT) or they are a different cell type that has thus far not been described in FECD. Although the expression of αSMA is compatible with EMT, the expression of CD45 (a specific marker for cells of hematopoietic origin that cannot be obtained by cells of another lineage, not even during the process of EMT)22–24 pleads strongly against CE origin. Furthermore, these observed bean-shaped nuclei are typical for cells from the monocyte-lineage.

IF double staining for CD34 and collagen I (Fig. 4a) and for CD45 and collagen I (Fig. 4b) revealed coexpression of both marker pairs within cells with monocyte-like morphology. This coexpression profile is the specific signature of circulating fibrocytes. Circulating fibrocytes are bone marrow–derived cells that coexpress hematopoietic markers (such as CD34 and CD45) and mesenchymal markers (such as collagen I)25 and that are derived from the monocyte/macrophage lineage (with characteristic bean-shaped nuclei). They can differentiate into a variety of phenotypes related to their mixed hematopoietic character (cytokine producing or antigen presenting [HLA-DRA⁺]) and mesenchymal character (ECM producing [αSMA⁺]).25 During this differentiation they can lose surface markers such as CD34 and CD45.26,27 In our study, cells with dendritic morphology indeed displayed less CD45 and CD34
and more HLA-DRA and αSMA (myofibroblast marker; Figs. 3c, 4c, 5a–e, 6a), suggesting a more differentiated stage.

**Morphology, Expression Profile, Number, and Localization of Circulating Fibrocytes/Dendritic Derivatives in FECD**

To characterize the circulating fibrocyte phenotype in FECD, we applied a set of 18 markers on CE-DM whole mounts from 55 patients with FECD (clinical stages II to IV) and 15 normal controls using either IHC (one marker per slide) or IF double staining (two markers per slide; Figs. 3–6). From the 55 FECD samples, 48 were used to test markers of circulating fibrocytes. Four of these 48 samples originated from phakic eyes (two male and two female patients).

These markers included antibodies to CD11b (marker for precursors of circulating fibrocytes); CCR5, CXCR1, CXCR2, and CXCR4 (chemokine C-C and C-X-C motif receptors involved in recruitment of circulating fibrocytes) and matrix metallopeptidase 9 (MMP9; which is used by circulating fibrocytes to enter the target tissue). Staining for fibronectin ($n = 1$) and vimentin ($n = 1$), two other markers of circulating fibrocytes, proved not useful as both markers also stained CECs (Supplementary Fig. S2). Indeed, fibronectin is overexpressed in CECs with FECD, and vimentin is a marker of normal CECs. Furthermore, glial fibrillary acidic protein (GFAP; a marker of hepatic myofibroblasts or stellate cells) was included because of the canonical pathway hepatic stellate cell activation that was identified by MEA. In addition, lymphatic vessel endothelial hyaluronan receptor 1 (LYVE1) was included because it was one of the top 10 significantly upregulated genes in FECD when compared with controls according to MEA (fold regulation $= 51.68, P = 0.003$) and confirmed by RT-qPCR (fold regulation $= 39.87, P = 0.046$). Because LYVE1 can be expressed by macrophages, we wanted to check whether it was also expressed by circulating fibrocytes (or derivatives) in FECD. Finally, we included antibodies directed to CD1a (marker for dendritic Langerhans cells in the corneal epithelium, $n = 3$) and T cell markers CD4 ($n = 2$) and CD8 ($n = 1$), for which no immunoreactivity was observed in FECD specimens.

Circulating fibrocytes/dendritic derivatives were present in all of the 48 tested FECD samples. No difference in number of circulating fibrocytes/dendritic derivatives was observed in phakic versus pseudophakic eyes with FECD. None of the normal controls contained such cells. Sample sizes per antiserum are given in Figure 6b for FECD. For normal controls, the sample sizes were CD45, $n = 10$; αSMA, $n = 9$; HLA-DRA, $n = 7$; collagen I, $n = 2$; CD34, $n = 1$; CD11b, $n = 1$; CCR5, $n = 1$; and LYVE1, $n = 1$.

Figure 6 summarizes the protein expression profile per cell morphology and per localization within the CE layer and gives the average number of immunoreactive cells per mm² of CE for each of the markers. On pure morphology, we observed a subset of cells with dendritic morphology and elongated nuclei and a smaller subset of monocyte-like cells with bean-shaped nuclei lacking dendritic processes. There were also cells of intermediate phenotype, with bean-shaped nuclei and wider cytoplasm, but without clear dendritic processes, suggesting a continuum between both subsets of cells. With respect to protein expression profile, the markers CD45, CD34, CD11b, collagen I, CCR5, CXCR2, and MMP9 were most prominent in
the monocyte-like subset, whereas HLA-DRA, GFAP, CXCR4, and CXCR1 were most prominent in the dendritic subset of cells. LYVE1 was expressed by both subsets, again suggesting a common origin between the cells with monocyte-like and dendritic morphology. With respect to localization, the monocyte-like subset was mostly observed in the peripheral zone of the CE layer, whereas the dendritic subset was mostly observed in the center, and cells of intermediate phenotype were concentrated at the interface between the central confluency of guttae and the peripheral remaining CECs. HLA-DRA was the most prevalent marker, with a weighted average of 17.60 cells per mm$^2$ of CE (Fig. 6b). Thus, on an average whole mount of 42 mm$^2$ (7–8 mm in diameter), more than 700 HLA-DRA$^+$ cells were present. Not a single FECD sample ($n=11$) lacked HLA-DRA$^+$ cells, and not a single control sample ($n=7$) contained HLA-DRA$^+$ cells.

**DISCUSSION**

MEA with IPA analysis ($n=4$ FECD, $n=4$ normal) was validated on a larger independent set of samples with RT-qPCR ($n=9$ FECD, $n=8$ normal) and led to the immunohistological identification of circulating fibrocytes and dendritic derivatives in the CE layer patients with FECD ($n=48$ FECD, $n=15$ normal). The coexpression of CD34 and CD45 respectively with collagen I proved their circulating fibrocyte identity,25 a cell type that causes fibrosis in a variety of organs throughout the body,25,33 but that has not previously been described in FECD. Circulating fibrocytes/dendritic derivatives were present in all examined FECD samples (ranging from clinical stages II to IV),2 irrespective of prior cataract surgery (which is usually performed prior to endothelial keratoplasty).

Circulating fibrocytes are considered to be derived from the monocyte/macrophage lineage (with characteristic bean-shaped nuclei) because they express monocyte surface markers such as CD11b.26 They should not be confused with bone marrow-derived mesenchymal stem cells (which have been reported to improve corneal recovery via anti-inflammatory mechanisms), which by definition do not express hematopoietic markers such as CD45, CD34, and CD11b or HLA-DR.34–37 Circulating fibrocytes are recruited from the blood to peripheral sites of injury via chemokine ligand/chemokine receptor axes, such as chemokine C-X-C motif ligand 12 and receptor 4 (CXCL12/CXCR4).25,33 Upon arrival, they penetrate the site of injury and can differentiate into a variety of phenotypes related to their mixed hematopoietic character (cytokine producing or antigen presenting) and mesenchymal character (ECM producing).25 They can enhance physiologic wound healing or cause pathologic fibrosis.27

It is tempting to think that the observed cells with dendritic morphology are merely CECs with degenerative changes or CECs that have become myofibroblast-like through the process of EMT.38 However, the expression of CD45 (a cell-lineage marker of bone marrow-derived hematopoietic cells that cannot be acquired by nonhematopoietic cells, not even during EMT),22–24 strongly pleads against this hypothesis. Furthermore, the bean-shaped nuclei are characteristic of cells from the monocyte lineage, as are circulating fibrocytes. Nevertheless, our data also support the process of EMT in FECD, consistent with current literature (Supplementary Table S2). We did not observe an upregulation of the EMT-inducing

![Image](https://arvojournals.org/12/12/2018)
genes ZEB1 and SNAI1, as reported by Okumura et al., who studied the EMT profile in immortalized CECs from late-onset FECD, but we did observe a significant upregulation of ZEB2 in FECD versus normal as reported earlier in a conference poster of the same group (Ho L, et al. IOVS 2013;54:ARVO E-Abstract 1680). Differences in these results might be a result of the difference between in vivo and in vitro (without circulating fibrocytes) gene expression and to the use of GAPDH as a reference gene for normalization (as discussed later). In contrast to Hidayat and Cockerham, we observed diffuse αSMA expression in the CE layer of patients with FECD (Fig. 4d) using two different antisera (Supplementary Table S1) with proper internal and external controls and consistent with the upregulation of ACTA2 at the gene expression level (fold regulation = 3.69, P = 0.025, according to RT-qPCR).

Rather than being mutually exclusive, our data suggest that both processes (EMT and circulating fibrocytes) play a role in FECD. Notably, EMT and circulating fibrocytes are frequently reported to contribute simultaneously to tissue fibrosis, whereby EMT is suggested to occur in later stages (contributing to the irreversible progression of fibrosis) and circulating fibrocytes would be present in earlier stages. Further study of the interplay of both processes would certainly be of interest to elucidate the pathogenesis of FECD.

When comparing our gene expression results with existing literature (Supplementary Table S2), our data confirm the significant upregulation of extracellular matrix genes agrin, collagens (COL1A1, COL4A3, COL4A4, COL4A5, COL4A6,
**Figure 5.** Immunofluorescent coexpression of circulating fibrocyte markers in CE of patients with FECD. Expression of CD45 is observed in monocyte-like cells, coexpressing CCR5 (c), CD34 (d); CD11b (e), and collagen I (Fig. 4b) in cells with dendritic morphology; coexpressing HLA-DRA (a) and αSMA (Fig. 4c). Coexpression of CD45 and LYVE1 (b) was seen in cells of both morphologies. This suggests that both morphological subsets represent different differentiation stages of circulating fibrocytes. Sample (c) originates from a phakic eye with FECD, indicating that the presence of (CD45+/CCR5+) cells with monocyte-like morphology in FECD is not secondary to cataract surgery. (c, f, g) The expression of chemokine C-C motif 5 (CCR5) suggests that this receptor might play a role in recruitment of circulating fibrocytes to the CE layer in FECD. (h) Coexpression of HLA-DRA and GFAP was observed in cells with dendritic and intermediate morphology.

**COL5A1, COL6A2,** and fibronectin, and the significant downregulation of ribonuclease **DICER1** (involved in miRNA biogenesis) in FECD versus normal, as reported in literature.39,41-43 In contrast with existing literature,41,44,45 we found a significant downregulation of clusterin (**CLU**), integrins **ITGA3** and **ITGB3**, **MMPs** (**MMP10** and **MMP14**), transforming growth factor beta induced and tissue inhibitor of metalloproteinase **TIMP1** in FECD versus normal. The upregulation of extracellular matrix molecules (agrin, collagen, and fibronectin) and the downregulation of matrix-degrading molecules (MMPs, which notably degrade agrin) are consistent with the thickening of the DM in FECD. Furthermore, agrin is a critical signal for hematopoietic niches,46 and its upregulation might be related to the presence of circulating fibrocytes in FECD. The downregulation of integrins (cell adhesion molecules) and **CLU** (prosurvival factor) is compatible, respectively, with dysfunctions and apoptosis of CE in FECD. With respect to Du et al.,47 who studied differential splicing events in FECD versus normal, we cannot directly compare our results because we studied gene expression irrespective of splicing variants. However, our data show a significant upregulation of EMT-related genes **CSNK1G3** and **PPP1BPF1**, and no differential expression of splicing regulators **MBNL1** and **MBNL2**. With respect to oxidative stress genes, our data confirm the significant downregulation of superoxide dismutase **SOD2** and heme oxygenase **HMOX1**, as reported by Jurkunas et al.48 In addition, we observed a significant downregulation of **SOD3** and a significant upregulation of **NADPH** oxidase activator **NCE2** in FECD versus normal. With respect to senescence genes, our data confirm the significant upregulation of **ARHGAP18, CCND1, CDK6, CDKN2A, CDKN2B, IGFI, IGFBP5, IGFBP7, IGF5, NOX3, and PIK3CG** in FECD versus normal, as reported by Matthaei et al.42 However, aldehyde dehydrogenase **ALDH1A3**, which was found to be significantly upregulated in their study,42 was significantly downregulated in our data, together with dehydrogenases **AKR1C2** and **ALDH3A1**, which were also reported to be downregulated in a serial analysis of gene expression experiment by Gottsch et al.49 Several other genes that were upregulated according to a serial analysis of gene expression (apolipoprotein D, metallothionein **MT2A**, and serum amyloid genes **SA41** and **SA42**) were significantly downregulated in our data; and the reverse was true for activator protein 1 transcription factor subunit **FOSB** and ion transporter **SLC4A11**. These differences in results could be a result of differences in FECD stages, that is, the patients examined with a serial analysis of gene expression were derived from penetrating keratoplasty surgery, whereas our samples were derived from endothelial keratoplasty (penetrating keratoplasty is usually performed in later stages than endothelial keratoplasty). Furthermore, others did not find a significant difference in gene expression for **SLC4A11** between FECD and normal,50 and they argued that expression levels of ion transporters could be increased in the early stages of FECD. Apart from **FOSB**, another activator protein 1 transcription factor subunit, namely **JUN**, was also significantly upregulated in our data, consistent with the current literature.50,51 With respect to unfolded protein response, our data confirm a significant downregulation of prefoldin subunit 5, as reported by Jalimarada et al.50 As a last remark, differences in gene expression levels between studies can also be a result of the use of different reference genes for normalization. **GAPDH**, a frequently used reference gene,53,54 is unstably expressed in FECD versus normal (significantly upregulated in our study and significantly downregulated in other studies48,49) and is therefore not the best reference gene to study gene expression in FECD.52 We have selected **RPL13A, RPL19** and **RP55** as reference genes (over **GAPDH, ACTB** and **HPRT1**) because of their stable expression in FECD and normal. **RPL13A** was also used by others48,50 because of its small difference in expression between FECD and control groups.

A limitation to this study is that the samples used for MEA and RT-qPCR experiments were not matched for age and sex. However, despite this limitation, the MEA and RT-qPCR results led to the identification of circulating fibrocytes and dendritic derivatives in FECD using immunohistology on whole-mount samples that were matched for age and sex within the constraints of sample availability (FECD exhibits a female predominance and late onset,1,3 whereas donor corneas...
This finding was novel and consistent. The fact that these cells have not been described previously despite advanced techniques such as confocal laser scanning microscopy can be explained by their relatively low numbers and morphology. Circulating fibrocytes are typically described as spindle shaped, compatible with the morphology of cells in our samples with intermediate and dendritic morphology, when these would be cross-sectioned. Full-thickness cross-sections were not suited to detect cells with dendritic morphology. Sampling problems also arise because only an estimated <700 HLA-DRAþ cells with dendritic morphology (1.4% of cells in a layer containing 50,400 remaining CECs at 1200 cells/mm2) and <300 collagen Iþ cells with monocyte-like morphology.
like morphology (<0.6% of the total cell number) are present in an average CE-DM specimen of 42 mm². Furthermore, these cells cover a broad spectrum of morphologies and marker combinations, depending on their differentiation state (from cells with monocyte-like morphology to myofibroblast-like cells with dendritic morphology), similar to what has been reported in other fibrosing disorders that involve circulating fibrocytes.40 These small numbers and diverse phenotypes would also make it difficult to isolate them from the CE layer for in vitro studies on their recruitment and exact function in FECD. Alternatively, circulating fibrocytes could be isolated from the peripheral blood, similar to what has been done for other fibrosing disorders.53

With respect to the recruitment of circulating fibrocytes toward the CE layer in FECD, our mRNA data suggest a role for chemokine receptors CCR5 (RT-qPCR fold regulation = 10.84, P = 0.019; Figs. 3i, 3c, 5i, 5g, 6), CCR1 (MEF fold regulation = 3.62, P = 0.056; RT-qPCR fold regulation = 10.05, P = 0.042), and CX3CR1 (MEF fold regulation = 10.32, P = 0.003; RT-qPCR fold regulation = 4.92, P = 0.017). The differential localization of cells with different morphology in the CE layer of patients with FECD (Fig. 6c) suggests that circulating fibrocytes enter the CE layer peripherally, where we observed the monocyte-like subset of cells (with rounded contours, consistent with a recent exit from the circulation), and differentiate into larger dendritic myofibroblast-like cells toward the center. Therefore, we speculate that circulating fibrocytes might enter the anterior eye chamber by extravasation from blood vessels in the iris-ciliary body, as is common during intraocular inflammation,54 or via the trabecular meshwork, where rare CD45+ dendritic cells coexpressing monocyte markers have been reported.55 Although FECD is not accompanied by overt ocular inflammation, a process of para-inflammation might be active, similar to other age-associated degenerative diseases of the immunoprivileged eye (such as adult macular degeneration, glaucoma, and diabetic retinopathy), which might allow circulating fibrocytes to enter the anterior chamber.56 Further research is needed to study the recruitment of circulating fibrocytes to the CE layer in FECD.

Based on this observational study, we cannot conclude on the exact function of circulating fibrocytes/dendritic derivatives in FECD. However, these cells are known to cause organ fibrosis,57,58 such as CCR1 antagonists (inhibit fibrocyte differentiation),59 and other risk factors with fuchs’ endothelial corneal dystrophy.

In conclusion, circulating fibrocytes and their dendritic derivatives are a novel and consistent aspect of symptomatic FECD. Their presence should be taken into account in all future studies that investigate the CE layer of patients with FECD. Their recruitment, differentiation, and function in FECD deserve further investigation because these insights might present new therapeutic avenues for patients with FECD.

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