MicroRNA Profiles Qualify Phenotypic Features of Cultured Human Corneal Endothelial Cells

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PURPOSE. To elucidate a noninvasive method to qualify and identify cultured human corneal endothelial cells (cHCECs) devoid of cell-state transition and adaptable for cell-based therapy.

METHODS. The variations of cHCECs in their composition of heterogeneous subpopulations (SPs) were verified in relation to their surface cluster-of-differentiation (CD) markers and their morphology. The profiles of microRNA (miRNA) in cultured cells or supernatants were detected by 3D-Gene Human microRNA Chips (Toray Industries, Inc.). The profiles were also analyzed for fresh corneal tissues with distinct endothelial cell densities (ECD) with or without gutatata. To validate the 3D-Gene results, quantitative real-time polymerase chain reaction (PCR) was performed. RNAs were extracted from cHCECs transfected with selected miRNA, and target genes were presumed by PCR array (Qiagen).

RESULTS. Among a variety of morphologically different cHCECs, miRNA expression profiles were distinctively revealed. The one miRNA capable of discriminating CD44+ SP from SPs with CD44++~CD44+++ phenotypes was identified as miR34a. The downregulation of miRNAs in the 378 family paralleled the upregulation of surface CD44 on cHCECs. Interestingly, upregulated miRNAs in the 378 family in corneal endothelium dramatically decreased in the tissues with lower ECD with advanced gutatata, providing new insight on the pathogenesis of Fuchs’ endothelial corneal dystrophy.

CONCLUSIONS. The specified cultured SPs sharing the CD44+ surface phenotypes with matured HCECs showed the highest expression of miR-378. Conversely, SPs with upregulated CD44+++ showed a reduction of miR-378. Thus, miRNA in cultured cells may serve as an alternative method to qualify cHCECs.

Keywords: heterogeneity of cultured corneal endothelial cells, CD44, miR, gutatata, epithelium-mesenchymal transition (EMT)

Although human corneal endothelial cells (HCECs) have the ability to proliferate in vitro,1 culturing HCECs for an extended period of time is known to be extremely difficult.2 To date, most researchers have conceptualized cultured HCECs (cHCECs) only from the aspect that they are derived from corneal endothelium tissue, and disregarded details pertaining to the refinement of the biochemical features. In fact, cHCECs are known to be heterogeneous from culture to culture in their morphology and in their surface markers, such as cluster-of-differentiation (CD) antigens.3-5 Studies, either directly or indirectly, and including those from Joyce et al.,6-13 have shown that heterogeneity is present in HCEC cultures. Of particular and striking interest is the finding by Miyai et al.3 of the presence of frequent chromosomal aneuploidy in cHCECs, directly indicating the presence of heterogeneous subpopulations (SPs) with or without aneuploidy.

Cultured HCECs have an inclination toward cell-state transition (CST) into a senescence phenotype, epithelium-mesenchymal transition (EMT), and fibroblastic cell morphology. In addition, the plasticity of metabolic profiles of cHCECs might possibly interfere with the simple interpretation of heterogeneous SP composites in cHCECs.14

One specific cHCEC SP with surface markers CD166+, CD133+, CD105+, CD44+, CD24+, and CD26- was revealed to be the SP without CST among heterogeneous cHCEC SPs.15 This finding makes it possible to adapt this SP in the clinical setting, and the combination of CD markers defined in this study was found to be the most appropriate for quality control to ensure the functional characteristics of cHCECs. However, the flow cytometry application impedes cultured cells by scraping out cells for analysis, forcing us to seek a new noninvasive way to discriminate the quality of heterogeneous cHCEC SPs.

MicroRNAs (miRNAs or miRs) are noncoding RNAs that function as an endogenous regulator of gene expression. The dysregulation of miRNA has been implicated in the pathogenesis of diverse diseases. Moreover, a growing volume of evidence suggests that miRNAs play a critical role in various biologic processes, including cell proliferation, development, and differentiation.16,17 The expression of miRNAs is essential in the regulation of many cellular processes including the...
formation, maintenance, and remodeling of the extracellular matrix (ECM), which is linked to CST in cHCECs.

However, only three reports of miRNA expression concerning corneal endothelium have been published, and no information on cHCECs is available to date. Matthaei et al. reported the widespread downregulation of miRNA levels in the corneal endothelium of patients with late-onset Fuchs’ endothelial corneal dystrophy (FECD), and indicated significant downregulation of the miR-29 family. Zhao et al. identified miRNAs differentially expressed in the corneal endothelium during aging. In addition, Iliff et al. described the single-base-pair substitution in miR-184 in regard to the disease phenotype of EDIC, a syndrome characterized by endothelial dystrophy, iris hypoplasia, congenital cataract, and stromal thinning.

In the present study, the 3D-Gene miRNA Microarray Platform (Toray Industries, Inc., Tokyo, Japan) and hierarchical clustering revealed a distinct expression pattern of miRNAs in a comparative analysis of cHCECs with distinct phenotypes. Moreover, a unique miRNA expression pattern including up- and downregulated miRNA clusters was revealed in cultured cells. In addition, our findings revealed that some isoforms of miR were also greatly upregulated in corneal endothelium tissues, while they were dramatically decreased in the tissue with pathogenically progressed gutatta. This observation may provide new insights on the pathogenesis of bullous keratopathy (BK), including FECD.

Materials and Methods

HCEC Donors

The human tissue used in this study was handled in accordance with the tenets set forth in the Declaration of Helsinki. Human CECs were obtained from more than 20 human donor corneas and were cultured before performance of karyotyping analysis. Human donor corneas were obtained from SightLife, Inc. (Seattle, WA, USA). Informed written consent for eye donation for research was obtained from the next of kin of all deceased donors. All tissues were recovered under the tenets of the Uniform Anatomical Gift Act (UAGA) of the particular state in which the donor consent was obtained and the tissue was recovered.

The donor age ranged from 2 to 75 years (mean age: 43.7 ± 26.4 years). All donor corneas were preserved in Optisol GS (Chiron Vision, Inc., Irvine, CA, USA) and imported via international air transport for research purposes. Donor information accompanying the donor corneas showed that they were all considered healthy and free of any corneal abnormality. For the analysis of corneal endothelium tissues with gutatta, the tissues were stripped from donor corneas and then stored in Optisol GS until use.

Cell Culture of HCECs

Unless otherwise stated, the HCECs were cultured according to the published protocols, with some modifications. Human donor corneas at the distinct ages were used for the experiments. Briefly, the Descemet’s membranes with the CECs were stripped from donor corneas and digested at 37°C with 1 mg/mL collagenase A (Roche Applied Science, Penzberg, Germany) for 2 hours. The HCECs obtained from a single donor cornea were seeded in one well of a type I collagen-coated six-well plate (Corning, Inc., Corning, NY, USA). The culture medium was prepared according to published protocols. Briefly, basal medium was prepared with OptiMEM I (Life Technologies Corporation, Carlsbad, CA, USA), 8% fetal bovine serum (FBS), 5 mg/mL epidermal growth factor (EGF; Life Technologies Corporation), 20 μg/mL ascorbic acid (Sigma-Aldrich Corp., St. Louis, MO, USA), 200 mg/L calcium chloride (Sigma-Aldrich Corp.), 0.08% chondroitin sulfate (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and 50 μg/mL gentamicin. The HCECs were cultured using mesenchymal stem cell (MSC)-conditioned medium at 37°C in a humidified atmosphere containing 5% CO2, and the culture medium was changed twice per week. When they reached confluence, the HCECs were passaged at ratios of 1:3 after treatment with 10X TrypLE Select (Life Technologies Corporation) for 12 minutes at 37°C. The HCECs at passages 2 through 5 were used for all experiments. More than 70 cultures were set up for the analysis.

Phase-Contrast Microscopy

Phase-contrast microscopy images were taken by use of an inverted microscope system (CKX41; Olympus Corporation, Tokyo, Japan).

Flow Cytometry Analysis of the cHCECs

Human CECs were collected from the culture dish by TrypLE Select treatment as described above and suspended at a concentration of 4 × 10⁶ cells/mL in flow cytometry (FACS) buffer (phosphate-buffered saline containing 1% bovine serum albumin and 0.05% NaN3). Next, an equal volume of antibody solution was added and incubated at 4°C for 2 hours. The antibody solutions were as follows: FITC-conjugated anti-human CD26 mAb, PE-conjugated anti-human CD166 mAb, PerCP-Cy 5.5-conjugated anti-human CD24 mAb, PE-Cy 7-conjugated anti-human CD44 (all from BD Biosciences, San Jose, CA, USA), and APC-conjugated anti-human CD105 (eBioscience, Inc., San Diego, CA, USA). After washing with FACS buffer, the HCECs were analyzed by use of the BD FACSCanto II Flow Cytometry System (BD Biosciences).

3D-Gene Microarray Analysis

miRNA Extraction. Both corneal endothelial and epithelial tissues were stripped from donor corneas and then stored in QIAzol Lysis Reagent (Qiagen) until total RNA extraction. Cultured CECs were lysed by QIAzol Lysis Reagent and then stored at −80°C until total RNA extraction. Total RNA was extracted by use of the miRNeasy Mini kit (Qiagen). The quality of the purified total RNA was analyzed by use of an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

miRNA Expression Profiling. Most of the experiments for the analysis of miRNA were carried out at least five times for each culture. For miRNA expression profiling, 3D-Gene Human microRNA Chips (miRBase versions 17-19; Toray Industries, Inc.) were used, and 250 to 500 ng total RNA derived from both tissue and cell samples was labeled with Hy5 by use of the miRCURY LNA microRNA Power Labeling Kits (Exiqon A/S, Vedbaek, Denmark). The labeled miRNAs were individually hybridized onto the surface of the miRNA chips and then incubated at 32°C for 16 hours. The washed and dried miRNA chips in an oxygen-free environment were scanned using 3D-Gene Scanner 3000 (Toray Industries, Inc.) and analyzed using 3D-Gene Extraction Software (Toray Industries, Inc.).

Normalized Data Processing. The digitalized fluorescent signals provided by the above-described software were regarded as the raw data. All of the normalized data were globally normalized per microarray, such that the median of the
The signal intensity was adjusted to 25. In a case where a bar graph of tissue or cHCECs, normalized levels were adjusted to the 100th value from the high rank.

**Polymerase Chain Reaction (PCR) Array.** Total RNA was extracted from the cHCECs by use of the miRNeasy Mini kit (Qiagen). Complementary DNA synthesis was performed with 100 ng total RNA for a 96-well plate format by use of an RT² First Strand kit (Qiagen). Expression of endothelial mRNAs was investigated using the RT² Profiler PCR-Array Human Extracellular Matrix and Adhesion Molecules (EMA), Human p53 Signaling Pathway, Human Fibrosis, Human Cellular Senescence, and Human EMT (Qiagen), and then analyzed using RT² Profiler PCR Array Data Analysis Tool version 3.5 (Qiagen).

**Statistical Analysis**

Student’s t-test was used to determine the statistical significance (P value) of the mean values for two-sample comparisons, and Dunnett’s multiple-comparisons test was used to determine the statistical significance for the comparison of multiple sample sets. Values shown on the graphs represent the mean ± SE.

**RESULTS**

**Distinct miR Expression Profiles Among cHCEC Cultures**

In this study, we dealt with improved cultures of cHCECs mostly without conspicuous EMT. However, it is difficult to discriminate the presence of a trace amount of EMT among heterogeneous SPs. At first, we compared the profiles of miRs detected by 3D-Gene between a CD44-SP (effector cell, lot 66P5 in Fig. 1A) and several cultured cells not clear regarding their composition of SPs (lots 29, 34, and 35, all at passage 1).
slight upregulation in miR29c expression and decrease in miR-378 expression were found in the latter SPs compared with the CD44+/C0 effector cells. Next, the effector CD44+/C0 SP was compared with cultured cell lot 67 evident in CST (Fig. 1B), whose composition of SPs is shown in Figure 2. That comparison also revealed a downregulation in the miR-378 family. However, the comparison with cultured cell lot C11, also evident in CST but different from that of lot 67, did not reveal a downregulation of the miR-378 family (Fig. 1C). These findings clearly indicate the presence of at least two different types of CST in cHCECs, even in the absence of conspicuous EMT. The expression profiles of the miR-378 family are summarized in Tables 1 and 2. The striking reduction of miR-378 expression levels from cHCEC lot 66 was confirmed in morphologically distinguishable cultures of poor quality, and the expression levels of the miR-378 family were comparable between cell cultures 66 and C11.

miR Expression Profiles Among SPs With Distinct CD Markers

In an aim to elucidate the dependency of expression profiles of miRs on the compositions of SPs distinct in their CD marker expression levels such as CD44, RNA extracted from FACS-defined cHCEC SPs (Fig. 2) was used for 3D-Gene analysis. To compare the miR profiles among cHCECs distinct in their CD44 expression status, we selected distinct culture methods to skew the composition of SPs in cHCECs. In the presence of SB431542 in our culture condition, we got different dominancy of CD44++ cHCECs with distinct morphologic phenotypes shown in Figure 2C, a2. These three cHCECs, that is, a5, a1, and a2, contain SPs mainly composed of CD44−/C0, CD24+/C0, CD26+/C0, CD44++/C0, CD24+/C0, CD26++/C0 SPs, respectively. As above, the expression levels of the miR378 family were comparable between a5 and a1, but a dramatic decrease was confirmed in a2 along the line with the elevated CD44 expression.

Table 1. Expression Profiles of the miR378 Family Among 17 Distinct Lots of cHCECs (Culture Lot 66 as a Reference Culture With CD44+ SP)

<table>
<thead>
<tr>
<th>Name</th>
<th>C1</th>
<th>C2</th>
<th>C3</th>
<th>C4</th>
<th>C5</th>
<th>C6</th>
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<td>153.3</td>
<td>60.7</td>
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<td>656.2</td>
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<td>9.1</td>
<td>0.1</td>
<td>12.3</td>
<td>10.2</td>
<td>12.0</td>
<td>15.6</td>
<td>30.0</td>
<td>17.4</td>
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<td>0.1</td>
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<td>13.9</td>
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<td>154.2</td>
<td>105.2</td>
<td>38.4</td>
<td>8.2</td>
<td>552.5</td>
<td>460.0</td>
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<td>104.8</td>
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66-1, 66-2, 66-3: same culture lot, but differences in flasks.

expression. The typical changes of miR-3778a-3p, -378a-5p, and -378b are illustrated in Figure 3. In addition to the miR378 family, the 23, 27, 30, 130, and 181 families inversely paralleled CD44 expression, whereas the 29, 31, 193, and 199 families positively paralleled CD44 expression (Fig. 3). To clarify that situation, the changes were divided into five classes. As shown in Figure 3B, the inverse decrease of miR-378 family expression was gradual along the line with the increase of CD44. Thus, the changes did not discriminate the CST between a5 and a1 but did between a5 and a2. The most relevant finding is that the changes in expression level of miR34 between a5 and a1 is gradual along the line with the increase of CD44. Thus, the purity of cHCECs should be carefully monitored from the aspect of heterogeneous SP composites with a view toward clinical applications, where it is vital that safety be ensured.

In the present study, not only the morphologic variations of cultured cells but also the composites of cHCEC SPs classified by CD markers varied greatly, even under seemingly similar morphologic figures, from culture to culture. Combined analysis of CD markers clearly specified the SPs (effector cells) adaptable for cell-based therapy among diverse SPs, and we successfully elucidated a method to quantitate the proportion of effector SPs in cultures (E-ratio).15 The E-ratio is the ratio of the effector cells contained in cHCECs and suitable for cell-injection therapy. We defined the SP with CD133, CD105, CD90, CD44, CD26, CD24, and HLA-DR negative and CD166, HLA-ABC, and PD-L1 positive as effector cells, with complete absence of karyotype aneuploidy and with a CD marker profile consistent with that of HCECs in fresh corneal tissues.

The plasticity by morphologic and phenotypic conversions, such as expression of mesenchymal markers and loss of epithelial markers, is collectively referred to as EMT. Cultured HCECs have an inclination toward CST into EMT. Numerous miRNAs have been implicated in EMT (i.e., the miR-200, miR-203 families). In the comparison of the distinct cultures shown in Figure 1, no evident changes in these EMT-related miRs were detected, which is consistent with our judgment that our culture conditions exclude such conspicuous EMT. Conversely, and as summarized in Table 1, many of the miR378 family were evidently downregulated in cultures heterogeneous in their SPs or with low E-ratios. In fresh human corneal endothelium (HCE) tissues, we observed contrasting features of miR signatures. MiRs relating to EMT were upregulated in those cells (data not shown).

Candidate Target of miR-378

Finally, we performed a preliminary transfection of miR-378a-3p or 5f mimics into CD44++ cHCEC SP, in which the expression of these two was not detected. As shown in Supplementary Figure S1A, the gene signature of ECM and the adhesion molecules class was analyzed. Moreover, the cells showed the gene signature upregulated in many of the collagen, integrin (ITG), and matrix metalloproteinase (MMP) families as well as CD44. Supplementary Figure S1B shows the heat map of the gene signatures after transfection of two miR mimics as assayed by PCR arrays of senescence, EMT, fibrosis, p53, and EMA. We plan to present a more detailed analysis in the future. Some of the genes, such as CCNE, TWIST, and GADD45, were upregulated in the senescence array. Many genes showed distinct signatures in the p53 PCR array after transduction by miR-378 mimic, as well as the downregulation of TCF4, TGF3, TGFβ2, TGFβ3, and SOX10 in the EMT array.

DISCUSSION

Expansion of cHCECs from donor corneal endothelium could provide a pragmatic tool for the clinical application of cHCEC-based therapy. Cultured HCECs expanded in an in vitro culture system can be a mixture of SPs with distinct CST, and the cultured cells tend to be inclined toward karyotype changes.3 Thus, the purity of cHCECs should be carefully monitored from the aspect of heterogeneous SP composites with a view toward clinical applications, where it is vital that safety be ensured.

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**FIGURE 3.** The profiles of miR expression in cHCECs. The values were the average of global normalization values (n = 3). Cultured HCECs, a5, a1, and a2, were the same as in Figure 2. (A) The expression value of miR378 family in these three cHCECs. (B) The expression value of miR378 family, the 23, 27, 30, 130, and 181 families inversely paralleled CD44 expression, whereas the 29, 31, 193, and 199 families positively paralleled CD44 expression (Fig. 3). To clarify that situation, the changes were divided into five classes. As shown in Figure 3B, the inverse decrease of miR-378 family expression was gradual along the line with the increase of CD44. Thus, the changes did not discriminate the CST between a5 and a1 but did between a5 and a2. The most relevant finding is that the changes in expression level of miR34 between a5 and a1 is gradual along the line with the increase of CD44. Thus, the purity of cHCECs should be carefully monitored from the aspect of heterogeneous SP composites with a view toward clinical applications, where it is vital that safety be ensured.

In the present study, not only the morphologic variations of cultured cells but also the composites of cHCEC SPs classified by CD markers varied greatly, even under seemingly similar morphologic figures, from culture to culture. Combined analysis of CD markers clearly specified the SPs (effector cells) adaptable for cell-based therapy among diverse SPs, and we successfully elucidated a method to quantitate the proportion of effector SPs in cultures (E-ratio).15 The E-ratio is the ratio of the effector cells contained in cHCECs and suitable for cell-injection therapy. We defined the SP with CD133, CD105, CD90, CD44, CD26, CD24, and HLA-DR negative and CD166, HLA-ABC, and PD-L1 positive as effector cells, with complete absence of karyotype aneuploidy and with a CD marker profile consistent with that of HCECs in fresh corneal tissues.

The plasticity by morphologic and phenotypic conversions, such as expression of mesenchymal markers and loss of epithelial markers, is collectively referred to as EMT. Cultured HCECs have an inclination toward CST into EMT. Numerous miRNAs have been implicated in EMT (i.e., the miR-200, miR-203, and miR-203 families). In the comparison of the distinct cultures shown in Figure 1, no evident changes in these EMT-related miRs were detected, which is consistent with our judgment that our culture conditions exclude such conspicuous EMT. Conversely, and as summarized in Table 1, many of the miR378 family were evidently downregulated in cultures heterogeneous in their SPs or with low E-ratios. In fresh human corneal endothelium (HCE) tissues, we observed contrasting features of miR signatures. MiRs relating to EMT were upregulated in HCE tissues with low ECD, as was the case for miR146 (also upregulated in tissues with advanced gutatta). The miR378 family was downregulated in cultures with low E-ratios (Fig. 5). In the context of the recently reported buffering effect of miRs against cell stresses,25 the observed changes can be considered reasonable. Extensive regulatory loops have
been described between promesenchymal transcription factors and epithelial-enforcing miRs. These operate not only to buffer the effects of stress on gene expression programs, but also to function as robust developmental reprogramming switches.23 In the same context, the upregulation of miR146 in stressed HCE tissues may also be understandable.24 Reportedly, miR-146a affects structural ECM proteins important in the assembly, composition, and organization of the ECM.18 CD44 is known to be the key to distinguish differentiated cHCECs from either undifferentiated cells or cHCECs with CST by E-ratios. CD44 ablation increased metabolic flux to mitochondrial respiration and concomitantly inhibited entry into glycolysis. Such metabolic reprogramming, induced by CD44 ablation, reportedly results in a marked depletion of cellular reduced glutathione.25 It is noteworthy that the only miR capable of discriminating CD44+ effector cells from CD44+++/CD44+++ SPs was identified as miR34a (Fig. 3B). The miR34a downregulates the expression of CD44, and the miR-34a/CD44 axis activates the downstream factors of CD44, including Ras homolog gene family, member A (RhoA) and MMP-2.26 In 2007, several groups identified the members of the miR-34 family as the most prevalent p53-induced miRNAs, and the members of the miR-34 family are now recognized as important mediators of tumor suppression.27 Coincidentally, this is in accordance with our previous observation that the CD44+ effector SP only showed the absence of karyotype abnormality (Hamuro et al., unpublished data).

The miR378 family showed a gradual decrease in parallel with decreases of CD44 expression (Fig. 3D). MicroRNA-378 performs a metabolic shift leading to a reduction in tricarboxylic acid (TCA) cycle gene expression, as well as an increase in lactate production.28 The miR-378 family is known to play roles in mitochondrial energy homeostasis.28,29 This indicates that the shift from aerobic oxidative metabolism to glycolytic metabolism is a characteristic feature of some CST in cHCECs. Pyruvate is processed by the TCA cycle through oxidative phosphorylation. Citrate/lactate ratios discriminated CD44+ effector cells not only from CD44+++, but also from CD44+++ SPs with a minuscule amount of CST.30 Several miRs, including miR-378a-5p, have also been shown to be involved in senescence targeting the p53 pathway. Senescence induction could provide miR-378a-5p with an additional mechanism in regard to how it is involved in CST regulation in cHCECs.31,32

FIGURE 4. The typical expression levels of representative miRs among distinct cHCECs. The subjected cHCECs were 66 (passage 4/passage 5, donor age: 23 years, donor ECD: 3504 cells/mm²), C11 (passage 2, donor age: 26 years, donor ECD: 3322 cells/mm²), C09 (passage 2, donor age: 16 years, donor ECD: 3590 cells/mm²), 55 (passage 5, donor age: 49 years, donor ECD: 2699 cells/mm²), and 73 (passage 2, donor age: 59 years, donor ECD: 2696 cells/mm²). Expression levels of miRs were evaluated by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR).
FIGURE 5. miR profiles in fresh corneal endothelial tissues. The tissues were a gift from SightLife, Inc. It should be noted that no diagnosis was performed to determine whether or not the donors of the endothelium tissues with guttata were afflicted with Fuchs’ endothelial corneal dystrophy (FECD). (A) Images of fresh corneal endothelial tissues with guttata (donor age: 73 years, donor ECD: 378 cells/mm²; donor age: 73 years, donor ECD: 1552 cells/mm²; and donor age: 51 years, donor ECD: 2457 cells/mm²). (B) Scatter plots of cHCEC miR expression profiles. The Toray 3D-Gene forms of Ver. 19/20 were converted to Ver. 17, and the values and the upper/lower of 2-fold lines are as explained for Figure 1. The upper plot shows the comparison of tissues with normal-level ECD (donor age: 3 days, donor ECD: 7470 cells/mm²; donor age: 7 months, donor ECD: 5070 cells/mm²; donor age: 12 months, donor ECD: 4551 cells/mm²; donor age: 12 years, donor ECD: 3987 cells/mm²; donor age: 20 years, donor ECD: 3812 cells/mm²; donor age: 63 years, donor ECD: 2598 cells/mm², Ver. 17) (donor age: 66 years, donor ECD: 2918 cells/mm², Ver. 20) with tissues with lowered ECD of 378 cells/mm² (donor ECD: 378, donor age: 73 years, Ver. 19). The lower plot shows the comparison among tissues with guttata but different in ECD. The tissues were those with an ECD of 378 cells/mm² and averaged 2318 cells/mm² (donor age: 50 years, donor ECD: 2187 cells/mm², donor age: 51 years, donor ECD: 2379 cells/mm², Ver. 17). (C) The normalized value of miR378a-5p and 378f in corneal endothelial and epithelial tissues is depicted. The miR378f expression level was dramatically decreased in tissues with lowered ECD. (D) The normalized value of miR146a-5p and 146b-5p in corneal endothelial/epithelial tissues, cHCECs, and supernatants of cHCECs are shown. CST+ means cHCECs with morphologically abnormal cells differing from CD44+/CD9+. The miR146b-5p expression levels were uniformly upregulated during cultures. (E) Expression levels of miR-378a-5p, -378e, and -378f were evaluated by qRT-PCR. The miRs were extracted from fresh HCE tissues distinct in ages and ECDs (donor age: 54 years, donor ECD: 3107 cells/mm²; donor age: 72 years, donor ECD: 1411 cells/mm² [guttata+], donor age: 60 years, donor ECD: 795 cells/mm² [guttata+]).
What conclusions can be drawn from our study with respect to therapeutic options for BK or FECD? Remodeling of the tissue under chronic stress appears to result, albeit in part, from downregulation of miR-378 and upregulation of EMT regulating the miR-200 family. Consequently, insufficient adaptation to tissue stress may be the progression of pathogenesis controlled by these miRs.

The findings of this study demonstrate that cHCECs are composed of a dysregulated expression of a hierarchy of miR clusters, probably due to the presence of CST and senescence. Isoforms of miR-378 were downregulated and miR146 isoforms, in contrast, were upregulated in fresh HCE tissues with gutatta. The cell state–transitioned CD44<sup>++</sup> cHCEC was decisively distinguished from CD44<sup>/--</sup> effector cells by miR-34a. In addition to this practical purpose, the new findings presented here warrant further investigation to develop a better understanding of the role of the dysregulated expression of miRs in pathogenesis of BK and FECD.

The upregulated miR29 in cHCEC SPs with CST is also remarkable in regard to the recent report describing its role in the EMT-promoting effect through Wnt/β-catenin signaling. The details of the downregulation of TCF4 and others, by the miR-378 family mimic transfection into the CD44<sup>++</sup> cHCEC SP with EMT, will hopefully be elucidated in future investigations.

It should be noted that the primary purpose of this study was to elucidate and describe the usefulness of intracellular miRs to distinguish cHCECs with or without CST. In order to more definitively establish the precise roles of miRs in CST in HCECs, further thorough experiments will be critical.

In closing, global single-cell analysis will provide precise mapping of the lineage relationship among the individual cells described here. At the same time, it may help clarify the relative relevance of the factors dictating the different cell states, that is, maturation, differentiation, transformation, and senescence. We are mostly interested in factors such as miRNA, exosomes, and the metabolites, either intracellular or secreted.

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**References**


