Directed Differentiation of Human Corneal Endothelial Cells From Human Embryonic Stem Cells by Using Cell-Conditioned Culture Media

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Purpose. A shortage of human corneal endothelial cells (HCEC) for transplant and current methods of differentiation induction require chemical compounds, which might cast further influences after differentiation induction. Therefore, we developed a simple and straightforward approach to endothelial cell differentiation from human embryonic stem cells (hESC).

Methods. hESC are used to differentiate into HCEC by employing a two-stage method, which involves the application of two different types of conditioned culture medium, human corneal stromal cell–conditioned medium (HCSC-CM) and lens epithelial cell (LEC) plus HCSC-CM in the presence of the second-conditioned culture medium. The differentiation of POMPs into CEC-like cells is regulated by a TGFβ/FOXC1 signaling pathway that is activated by the factors present in the conditioned culture medium.

Results. In the presence of conditioned culture medium, embryonic stem cells differentiate first under the control of periocular mesenchymal precursors (POMPs). Consequently, the expression of several POMP markers was observed. Following this first stage differentiation, POMPs were further directed to differentiate into corneal endothelial cell (CEC)-like cells in the presence of the second-conditioned culture medium. The differentiation of POMPs into CEC-like cells is regulated by a TGFβ/FOXC1 signaling pathway that is activated by the factors present in the conditioned culture medium.

Conclusions. HCEC-like cells could be differentiated from hESC by simply using a two-step, preconditioned, medium-mediated approach, which could significantly minimize the workload to generate HCEC for potential clinical use. This research may provide an ideal cell source for corneal regenerative medicine and clinical treatment for corneal diseases in the future.

Keywords: human embryonic stem cells, human corneal endothelial cells, differentiation, TGFβ-2, conditioned medium, neural crest

The cornea is the outermost structure of the eyeball; thus, it is highly prone to damage caused by infection, trauma, and other possibilities. Historically, corneal opacity is one of the most frequent causes of blindness. The proportion of blindness caused by various corneal diseases in all kinds of blinding eye diseases is 4%, after cataract, glaucoma, and AMD. Functional impairments of corneal endothelial cells (CECs) may manifest clinically as corneal edema, haze, and eventually corneal scarring. The transparency of the cornea is vital for light refraction. Corneal endothelium, a monolayer of cells with a hexagonal structure beneath the Descemet membrane, maintains corneal transparency through the regulation of aqueous humor flow to the corneal stroma. The Na⁺- and K⁺-dependent ATPase (Na⁺/K⁺-ATPase) expressed in the basolateral membrane of CECs is primarily responsible for the pumping function of the corneal endothelium. Human CECs are arrested in the G1 phase of the cell cycle; therefore, CECs have a poor proliferative capability in vivo. Corneal pathologies can dampen the barrier function of CECs, and damaged CECs cannot be easily self-renewed. Therefore, endothelial transplant is always performed to correct CEC dysfunction. Due to the lack of human cornea donors in many regions of the world, an alternative source of CECs is urgently required for clinical practices. Human embryonic stem cells (hESCs), which usually form as embryoid bodies (EBs) in a cell culture, have an extensive capability of self-renewal and multipluripotent to differentiate into all lineages under appropriate conditions. Stem cell–based therapy for corneal diseases is a cutting edge field in regenerative medicine, evidenced by the treatment of ocular surface disorders using stem cell autografts, but it is only common to treat epithelial defects by inducing differentiation from stem cells from...
patients’ own tissue.11–14 CECs regenerative treatments are still unclear so far.15 It has been documented that hESCs can be induced into keratocytes (corneal stromal cells)16; however, the directed differentiation of hESCs into human CECs is still controversial.

Studies in developmental biology have revealed that the neural crest (NC), which is a transient structure migrated from the neural tube in vertebrate embryos, is the origin of corneal stromal cells (HCSC) and human lens epithelial cells (HLECs).17,18 In related research, mouse PA6 fibroblasts were co-cultured with hESCs to induce differentiation toward stromal keratocytes19; however, the mechanisms governing human NC cell differentiation to CECs remains unclear. During cornea development in the embryonic stage, the microenvironment surrounding the migrated NC cells determines the ultimate fate of CECs, including human corneal stromal cells (HCSC) and human lens epithelial cells (HLECs).17,20

In this in vitro study, we tried to mimic the in vivo microenvironment of corneal development by using conditioned medium containing HLECs, HCSC, and human corneal endothelial cells (HCEC). To validate the effectiveness of the co-culture system, a two-step approach of induction from hESCs to HCEC was established. By using this novel co-culture system, we successfully generated CEC-like cells, which share both phenotypic and genetic characteristics of normal human CECs from hESCs. The result of this study could provide a better understanding of the embryonic development of the cornea; meanwhile, it could serve as a potential replacement of cadaveric cornea in the context of regenerative medicine.

MATERIALS AND METHODS

Cell Culture

The hESC cell lines (including H9 and H1) were generously provided by the Stem Cell and Regenerative Medicine Lab, Beijing Institute of Transfusion Medicine (Beijing, China), and cultured on irradiated mouse embryonic fibroblast (MEFs) feeder layers. The hESC culture medium was composed of 78% Dulbecco’s modified Eagle’s medium (DMEM)/F12 (1:1), 20% knockout serum replacement (KSR), 1 mM l-glutamine, 5 ng/ml basic fibroblast growth factor (bFGF; all obtained from Gibco, Waltham, MA, USA), 1% nonessential amino acids (NEAA), and 0.1 mM β-mercaptoethanol (Invitrogen, Carlsbad, CA, USA). Cell cultures were incubated at 37°C in a humidified atmosphere containing 5% CO2 with medium changes every 2 to 3 days.

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HCSC for conditioned medium were obtained from the Eye Tissue Bank of Beijing (Beijing, China). Corneas were collected after the central buttons were used for corneal transplantation, 7 to 8 days postmortem. The age of donors was from 30- to 65-years old. The remaining conjunctiva, uveal, and scleral tissues were removed but the corneal limbus was preserved. (DMEM)/F12 (1:1) supplemented with 10% FBS and 10 ng/mL penicillin, and then centrifuged at 300g for 4 minutes. Initial culture was performed in 10-cm dishes in a humidified atmosphere of 5% CO2 at 37°C. The medium was changed every 2 to 3 days.

Preparation of Conditioned Medium and Two-Stage Differentiation of CEC-Like Cells

HCSC was harvested after culturing for 15 to 20 days by the application of 0.25% trypsin. HCSC-conditioned medium (HCSC-CM) was prepared by collecting the supernatant from these cultured cells at 70% to 90% confluence every 12 hours. The collected supernatant was filtered (0.22 mm) to remove dead cells and stored at –80°C to preserve its biological activity. HCSC-CM was used in the first stage of differentiation (EBs to periocular mesenchymal precursors [POMPs]).

SV-40-transformed HLEC were obtained from the Shanghai Yansheng Biological Technology Co., Ltd. (Shanghai, China). LEC was cultured in (DMEM)/F12 (1:1) supplemented with 10% FBS. HCEC (HCEC-B4G12 cell lines) were purchased from Creative Bioarray (Creative Dynamics, Inc., NY, USA) and cultured in Human-Endothelial-SFM (Creative Dynamics, Inc.). The supernatant of LEC and HCEC were collected and preserved as described in the HCSC-CM, and labeled human LEC-CM and HCEC-CM. LEC-CM was mixed with HCEC-CM at the ratios of 3:1, 2:1, and 1:1 to induce the second stage of differentiation (POMPs to CEC-like cells).

In Vitro Differentiation

EBs were cultured in low-attachment culture dishes for 14 days, then 0.1 mM retinoic acid (RA) was added (MilliporeSigma) into the EBs culture medium and cultured for 4 days.

The First Stage of Differentiation (Stage 1). After the RA treatment, EBs were transferred into 6-well culture plates precoated with 10 mg/ml fibronectin, 10 mg/ml laminin, and 10 mg/ml chondroitin sulfate, and then the culture medium was replaced with HCSC-CM to acquire POMPs phenotype.

The Second Stage of Differentiation (Stage 2). Eight days after Stage 1, POMPs were induced to the CEC-like cells with the mixed CM (LEC-CM and HCEC-CM). Medium was changed every 2 days, and the cell morphology was monitored as well.

Immunofluorescent Staining

EBs were cultured in a confocal dish coated with chondroitin sulfate, laminin, and fibronectin. After 1 day of culture, the EBs were fixed with paraformaldehyde, permeabilized with 0.2% Triton X-100, and then antibodies were added. Primary antibodies (and dilutions) used were mouse anti-CD73 (1:500; Becton Dickinson Biosciences), rabbit anti-p75 NGF (1:200; Abcam), rabbit anti-zonula occludens-1 (ZO-1; 1:100; Zymed Laboratory, Invitrogen), and mouse anti-N-cadherin (1:500; Becton Dickinson Biosciences), rabbit anti-Vimentin (1:200; Abcam), rabbit anti-AP2 alpha (1:50; Santa Cruz Biotechnologies, Dallas, TX, USA), rabbit anti-CD73 (1:50; Calbiochem), and mouse anti-AP2 alpha (1:200; Abcam). All images were acquired by laser confocal microscope (Olympus, Tokyo, Japan).

Western Blotting

Total protein of CEC-like cells was extracted using 1% radioimmunoprecipitation assay lysis buffer (Becton Dickinson Biosciences) and then quantified with a bicinchoninic acid

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protein assay kit (Becton Dickinson Biosciences). Cells were lysed in RIPA buffer that was composed of 50 mM Tris-Cl (P0013B; Beyotime Biotechnology, Shanghai, China) and protein concentration was measured by the Bradford assay (Thermo Fisher Scientific, Waltham, MA, USA). Fifty micrograms of total protein was loaded in the 6% to 15% SDS-PAGE and then transferred to a polyvinylidene fluoride (PVDF) membrane (MilliporeSigma), which was blocked by 5% non-fat milk, probed with a primary antibody overnight at 4°C, and then washed three times with triethanolamine buffered saline with 0.05% tween (TBS-T). It was then incubated with a horseradish 4 peroxidase–conjugated secondary antibody. Primary antibodies used were against Na\(^+\)/K\(^+\)-ATPase \(\alpha_1\) (1:2000; Santa Cruz Biotechnologies) and \(\beta_1\) (1:1000; Santa Cruz Biotechnologies) and \(\beta\)-actin (1:500; obtained from Beijing Zhongshan Company, Beijing, China) overnight at 4°C. After immunoblotting with secondary antibodies (1:5000; MilliporeSigma) at room temperature for 1 hour, a chemiluminescence image was acquired and analyzed by using a charged couple device (CCD) camera system (ChemiDoc-IIt 600, UVP, LLC, Upland, CA, USA).

Quantitative PCR (qPCR)

Total RNA was isolated using TRIzol (Invitrogen). The TaqMan Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and a Gene Amp PCR System 9700 (Applied Biosystems) were used to generate complimentary (c)DNA. Gene expression analysis was determined by quantitative real-time PCR using the SYBR Green Mastermix and a 7500 real-time PCR System (Applied Biosystems). The results were analyzed using the 2\(^{-\Delta\Delta CT}\) method that was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression (\(n = 4\) for each group). The primers were listed in the Table.

RESULTS

Induced Differentiation of hESCs to Human CEC-Like Cells

To confirm the pluripotency of hESCs, the common markers of undifferentiated ESCs, including Nanog, Oct-4, and Sox2,\(^{21}\) were stained positively by immunofluorescent staining (Fig. 1A).

It is generally known that CECs originate from NC cells; therefore, we first cultured hESCs for 3 days without a feeder layer to directionally induce hESC differentiation into the neural ectoderm progenitor of NC cells. RA was added to EB culture on day 4. RA plays a key role in cell growth and differentiation by activating RA receptors (\(\alpha\), \(\beta\), and \(\gamma\)), which are nuclear receptors.\(^{22}\) Early animal studies showed that RA regulates ventral eye development. RA is a widely used factor in both mouse and hESCs. It suppresses differentiation to mesoderm and enhances differentiation to ectoderm.\(^{23-25}\) RA-treated EBs were then transferred to the chondroitin sulfate, laminin, and fibronectin precoated plate, following which the culture medium was changed to the two types of conditioned
co-culture medium (CM) for the induction of differentiation (Fig. 1B). 1 day after the RA treatment, a large number of spindle-like cells had migrated out from EBs (Fig. 1C; III). They are not like the spherical morphology of EBs (Fig. 1C; II) or the colony form of hESCs (Fig. 1C; I).

**Retinoic Acid Treatment and Corneal Stroma Conditioned Medium Induced Embryonic Stem Cells-Derived EBs to Directly Differentiate Into NC Cells**

Quantitative PCR showed that compared with hESCs, the expression of NC cell marker P75 increased after the differentiation to EBs (Fig. 2A; P < 0.05). We also found that compared with EBs, the expression of P75 increased to the greatest extend at the fourth day after 1-μM RA treatment, and then the expression gradually declined (Fig. 2B; P < 0.05). Thus, after RA treatment for 4 days, we transferred EBs to 6-wells plates coated with chondroitin sulfate, laminin, and fibronectin. It was observed under a light microscope that after 1 day of settling down in the coated plate, a large number of spindle-shaped cells migrated from EBs to the periphery (Fig. 1C; III). The NC cell markers AP2α, P75/NGF receptor, and CD57, were evaluated and the results showed the cells, which had migrated from EBs, co-expressed these markers (Fig. 2C). These results indicated that EBs, which had been treated for 4 days were able to differentiate into NC cells.

**Corneal Matrix Conditioned Medium Induced NC Cells to Differentiate Into POMPs**

Through the observation of the anatomic structure of the eye, we find that the corneal stroma is located in the front of the CEC layer. At the same time, HCSC and HCEC are both developed from NC-derived POMPs. During eye development, NC-derived POMPs migrate to form the lens bubbles, which originate from epidermal ectoderm. They fill the gap between the two types of cells and eventually differentiate into HCEC and HCSC. HCEC and HCSC interact with each other

**TABLE. Primers Used for qPCR**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tbody>
<tr>
<td>P75</td>
<td>CTATTCGACGAGCCACC</td>
<td>CAGGAATCTTGCAGCACTC</td>
</tr>
<tr>
<td>FOXC1</td>
<td>GCTGTCAAATGGCCTTCCCT</td>
<td>TCCTGCTTTGGGCATT</td>
</tr>
<tr>
<td>PITX2</td>
<td>GGACCAACCTTACGGAAGCC</td>
<td>CTACTTGGCGCTTCACTCAC</td>
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<td>TGF-β2</td>
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<td>TTACAGGAGGAGGAGGAG</td>
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<td>TGF-βR1</td>
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</tr>
<tr>
<td>GAPDH</td>
<td>GCCATGACATGCGCTTCACT</td>
<td>TGCACCACCAGCAGACACGC</td>
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during embryonic development. Therefore, we selected HCS-CM as the differentiation induction medium for the first stage to simulate the development of HCEC in vivo and to provide an in vitro microenvironment that is similar to in vivo development. From qPCR results we were able to determine that EBs treated with RA showed the highest expression of P75 in the first day of adherent culture, and then the expression decreased gradually. *P < 0.05 compared with NCs group. (B) The optimal time for HCS-CM-induced NC differentiation into POMPs. The expression of POMPs markers FOXC1 and PITX2 peaked at the third day co-culture in HSC-CM, and then decreased gradually. *P < 0.05 compared with NCs group. (C) Immunofluorescent staining of day 3 HSC-CM cultured cells. After culturing in the HSC-CM for 3 days, most cells expressed periplasmic mesenchymal cell markers FOXC1 and PITX2, thus showing that the NC cells have differentiated into mesenchymal cells. Scale bar: 20 μm. Data were representative of four independent experiments.

It has been shown that FOXC1 affects the embryonic development of the anterior segment significantly.28–30 Also, the PITX2 gene plays an important role in the embryonic development of the NC into ocular tissue.29,31 Studies have shown that PITX2 could be detected in POMPs progenitor for CECs, and that POMPs has a regulatory role in the development of corneal stroma, sclera, and ocular vessels.17 Taken together, FOXC1 and PITX2 can serve as markers for POMPs. The results showed that expression of FOXC1 and PITX2 increased significantly at the first day after HCS-CM induction, peaked at the third day, and then decreased gradually (Fig. 3B; *P < 0.05). The POMP markers were subjected to immunofluorescent staining after 3 days of HCS-CM culture, and the majority of cells showed a positive expression of FOXC1 and PITX2 (Fig. 3C). These results indicated that NCs were induced to differentiate into POMPs by conditioned medium.

The Isolation and Characterization of CEC-Like Cells

According to the anatomic structure of the eye, LECs are beneath the CEC layer. It has been reported that human umbilical cord blood mesenchymal stem cells, which have been cultured by conditioned medium (LEC-CM) of HLEC, can be induced to differentiate into CECs by simulating the development of the eye.32 Meanwhile, mature CECs can also provide some inducing factors for undifferentiated CECs by autocrine or some regulatory factors.33–34 At this stage, we induced the differentiation of POMPs using a mixed-conditioned medium of both LEC-CM and HCEC-CM. The morphologic changes of cells were documented by light microscopy (Fig. 4A). The cells gradually changed into spindle shapes after the culture medium was switched to LEC-CM plus HCEC-CM. Eight days after the induction of second stage differentiation, some cells were observed to show a hexagonal morphology (Fig. 4A). The number of cells significantly increased at day 12, after culture medium exchange, and the morphology of the cells was not significantly changed compared with those cells on day 8. After day 8, the hexagonal cells do not significantly change their morphology in the presence of LEC-CM plus HCEC-CM. Therefore, we selected cells on day 8 to evaluate their HCEC-related markers, including Na+/K+-ATPase, ZO-1, N-cadherin, and vimentin. The results of immunofluorescent microscopy showed that selected HCEC markers were expressed in the CEC-like cells (Fig. 4B). We further evaluated mRNA and protein expression of Na+/K+-ATPase, a hallmark of CEC, in cells at different stage of differentiation (Figs. 4C–4E).

These results indicated that we acquired the human endothelial cell-like cells by using our co-culture system with related cell types in the anterior chamber.

Induction of POMPs Into CEC-Like Cells is Regulated by the TGFβ-2/FOXC1/PITX2 Signaling Pathway

In the development of mouse corneal endothelial cells (mCEC), mouse lens epithelial cells (mLEC) provide the internal environment for the differentiation and maturation of CECs by paracrine factors.35,36 It has been reported that TGFβ-2 treatment regulates the FOXC1 expression in POMPs, which are derived from the anterior region of the eyes in E12.5 mouse embryos. The LEC-CM could mimic the E12.5 environment in CEC differentiation by regulating FOXC1 through TGFβ-2.37 Therefore, in this part of the study, we examined the FOXC1 signaling pathway components involved in the regulation of CEC development. qPCR results showed that the expression of FOXC1 and PITX2 began to increase in the EB stage of differentiation after adding RA (Fig. 5A). After HSC-CM induced first stage differentiation, the expression of FOXC1 and PITX2 increased significantly (Fig. 5A; *P < 0.05). Meanwhile, the expression of TGFβ-2 and its receptor, TGFβR1, began to increase. With the addition of mixed LEC-CM plus HCEC-CM, the expression of FOXC1 and PITX2 was downregulated, TGFβ-2 and its receptor, TGFβR1, increased first, and gradually decreased with the maturation of CECs (Fig. 5B; *P < 0.05). These results indicated that the mixed LEC-CM plus HCEC-CM induced POMPs to differentiate into corneal endothelial-like cells by regulating the TGFβ-2/TGFβR1/FOXC1/PITX2 signaling pathway.

We illustrated the schematic diagram of the differentiation process (Fig. 5C). Targeted cells expressed specific markers during different stages of cellular state. In the origin of HCEC and HSC, NCs were derived from hESCs successfully by RA treatment. Also, the co-culture approach following the pattern of embryonic development was considered to be more efficient.
The hESC-CEC–like cells were eventually derived from POMPs by activating the TGFβ-2 and TGFβ-R1 signaling cascade.

**DISCUSSION**

The mammalian eye is composed of tissues from three embryonic layers, including epidermal ectoderm, neuroectoderm, and POMPs that were derived from NCs. The development of the cornea is closely related to NCs; however, there are still controversies about the specific process of NC differentiation and its related signaling pathways on the development of mammalian ocular tissue during embryogenesis. The primary role of POMPs is to develop various types of cells with visual function, including CECs, corneal stromal cells, Schlemm’s canal, trabecular meshwork, ciliary muscle, sclera, extraocular muscle, iris stroma, ocular vascular tissue, and so on.

Another essential function of POMPs is to provide basic signal pathways for the development of ectoderm ocular primordia, such as the development of epidermal ectoderm into lacrimal gland, retinal cup, RPE, and so on. Because research on human embryos has significant ethical concerns, we mostly learn the pattern of human embryonic development from avian and mammalian animals. In the past, it was generally believed that POMPs originated from mesoderm, but by observing and studying the development process of the avian eye, it has been suggested that POMPs was formed under a combined action of both NC and mesoderm. Some studies have shown that the development of the corneal endothelium in both birds and mice is through the POMPs stage. However, more and more evidence indicates that there are some differences in the developmental processes of birds as opposed to mammals, such as migration time and path of NCs. In mammals, most CECs are derived from the NC while a small population of CECs may originate from mesoderm. Therefore, we speculated that the development of HCEC is similar to development in mice due to many factors. Thus, we chose to determine if the induction method of hESCs was used to induce cells to differentiate into NC and POMPs.

As the initial signal pathway involved in POMPs differentiation, FOXC1 and PITX2 can be detected in early embryogenesis. PITX2 has a significant regulatory effect in a variety of tissue originated from POMPs, such as corneal endothelium, corneal stroma, and extraocular muscles. Many studies have shown that PITX2 knockout mice lack normal function of the...
expression of transcription factors PITX2 and FOXC1 it can be stated that the lens is considered to be the center for expression in cell cultures. Based on the above findings, PITX2 expression in the EBs + RA-treated group began to increase. The expression of FOXC1 and PITX2 began to decrease after replacing the HSC-CM with mixed-conditioned medium. The expression of TGF-β2 and its receptor TGF-βR1, did not change significantly. (B) The expression of these four markers as described above at different days after POMPs differentiation. All genes began to decrease at day 8. *P < 0.05 compared with hESCs group, and #P < 0.05 compared with day 3 after HSC-CM culture. Data are representative of four experiments. (C) Schematic diagram of the differentiation stages and the relevant markers of each stage of the differentiation.

corneal endothelium and the corneal stroma, which suggests that the PITX2 gene itself is also one of the targets for early signal transduction during the development of the cornea. Therefore, we evaluated both PITX2 and FOXC1 expression, as they were corresponding markers of POMPs to verify the direction the first stage of differentiation.

The development of CECs begins with the migration of POMPs to the gap between the lens and corneal epithelium. Meanwhile, POMPs begin to receive signals related to lens and corneal epithelium. PITX2 and FOXC1 expression, as they were corresponding markers of POMPs to verify the direction the first stage of differentiation.

Studies have shown that when the TGFβ-1- and TGFβ-2-signaling cascades went abnormally, the differentiation and development of corneal endothelium would also be abnormal. Several studies showed that mouse embryos lacking TGFβ-2 have multiple defects in ocular structures, including having a thin cornea without corneal endothelium, malformation of the anterior chamber, an immature retina, and persistent vitreous vessels. TGFβ-2 released from the lens is required for the expression of transcription factors PITX2 and FOXC1 in the NC-derived cornea and in the chamber-angle structures of the eye that controls IOP. TGFβ-2 enhances FOXC1 and induces PITX2 expression in cell cultures. Based on the above findings, it can be stated that the lens is considered to be the center for the development of corneal endothelium and stroma, and the signal molecules generated by them reach the targeted part possibly through the anterior chamber. Based on these findings, to simulate the microenvironment of internal corneal endothelial development in vitro, our study selected HSC-conditioned medium as the first stage differentiation co-culture medium, then the conditioned medium of LEC and HCEC were collected as the second stage differentiation conditioned medium. After repeated tests of the ratio of LEC-CM and HCEC-CM, the ratio of 3:1, 2:1, and 1:1 to POMPs was used to induce and to differentiate respectively. The role of TGFβ in ocular development is still vague and different systems yield different perspectives. One study done by Zhao and Afshari shows that using TGFβ inhibitor together with ROCK inhibitor promotes HCEC induction from induced pluripotent stem cells (iPSCs). The difference in findings could be explained in several ways. For example, although iPSC and ESC are similar in many aspects, there are numerous reports showing dissimilarities. Considering the difference between iPSCs and ESCs, the role of TGFβ might be slightly different for in vitro ocular development. For the purposes of the study, the reason we chose ESC rather than iPSC was that experimentally, ESCs are more robust than iPSC with established protocols to expand and maintain karyotypically normal cell lines. Meanwhile, the conditioned medium from ocular cells we used in our study to induce ESC differentiation may be more physiologically reasonable because it contains a variety of cytokines, macromolecules, and micromolecules. However, the small molecule chemical compound might possess some off-target effect that would harm the normal physiology of cells. In our understanding, the simulated microenvironment would provide a better guide for the partially differentiated NC cells to further differentiate into HCEC and this is the major reason we favor conditioned
medium rather than chemical compounds. Further, the reason we postulated the role of TGFβ in HCEC differentiation came directly from our observation that the components of the TGFβ signaling pathway were highly upregulated, without any pharmacologic interference. According to the observation of HCEC culture in vitro, endothelial-mesenchymal transformation causes the loss of characteristic endothelial phenotypes, such as loss of the apical junctional proteins at the plasma membrane, and loss of the contact-inhibited monolayer. The use of the inhibitor to TGFβ receptor (SB431542) and/or anti-EMT molecules (BMP-7) enables HCECs to grow while maintaining normal physiological function (i.e., barrier and endothelial function). These studies support knowledge that TGFβ plays a critical role in both function and morphology of HCEC, which indicated that the activity of the related signaling pathway might also be crucial to corneal development in the embryonic stage. Again, our data indicated that TGFβ-2 and TGFβ-β1 are highly expressed in the early stage of differentiation induction and gradually decreased after differentiation. This partially falls into the notion that inhibition of TGFβ promotes endothelial differentiation, but the exact role of TGFβ signaling in different time points of HCEC differentiation is still controversial.

In subsequent parts of this study, we plan to isolate HCEC-like cells by using different approaches such as fluorescent-activated cell sorting, and to select the appropriate scaffold for tissue engineering. By doing so, target cells would be seeded on the scaffold for animal studies for a later functional test. We believe that our work will hopefully provide an optimal method to obtain adequate CECs in vitro, and that it could be directed to clinical studies in future.

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