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 pericellular 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.1 The proportion of blindness caused by various corneal diseases in all kinds of blindness is 4%, after cataract, glaucoma, and AMD.2 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.3,4 The Na+/K+-dependent ATPase (Na+/K+-ATPase) expressed in the basolateral membrane of CECs is primarily responsible for the pumping function of the corneal endothelium.5-6 Human CECs are arrested in the G1 phase of the cell cycle; therefore, CECs have a poor proliferative capability in vivo.7 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 multipluripotency to differentiate into all lineages under appropriate conditions.8-10 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 endothelium and stroma.17,18 In related research, mouse PA6 derived from the neural tube in vertebrate embryos, is the origin of corneal endothelium and stroma.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 (HLEC).17,20

In this in vitro study, we tried to mimic the in vivo microenvironment of corneal development by using conditioned medium containing HLEC, 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 day. Cells were passaged once per week. For further cell differentiation, hESCs were digested with Accutase solution (MilliporeSigma, St. Louis, MO, USA), and then transferred into AggreWell (STEMCELL Technologies, Vancouver, Canada) plates for 24 hours to obtain EBs. The EBs culture medium was composed of 78% (DMEM)/F12 (1:1), 20% KSR, 1 mM l-glutamine (all obtained from Gibco), 1% NEAA, and 0.1 mM β-mercaptoethanol (Invitrogen).

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]).

SV40-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 (1:200; Abcam), rabbit anti-zonula occludens-1 (ZO-1; 1:100; Zymed Laboratory, Invitrogen), and mouse anti-Na+/K+ ATPase alpha (1:50; Santa Cruz Biotechnologies, Dallas, TX, USA), rabbit anti-p75 NGF (1:200; Abcam), and rabbit 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...
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 α1 (1:2000; Santa Cruz Biotechnologies) and β1 (1:1000; Santa Cruz Biotechnologies) and β-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-Ilt 600, UVR 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^-ΔΔCT 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 Sox-2,^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 (α, β, and γ), which are nuclear receptors. 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. 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 media (Fig. 1B).
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-\( \mu \)M 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-well 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 AP2, P75, 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

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**Table.** Primers Used for qPCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5'-3')</th>
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<tbody>
<tr>
<td>P75</td>
<td>Forward primer: GTATTCCGACGAGGCCAACC</td>
</tr>
<tr>
<td></td>
<td>Reverse primer: CAGGGATCTCTCPCACACTC</td>
</tr>
<tr>
<td>FOXC1</td>
<td>Forward primer: GTCTGGCATTGGCGGTGATT</td>
</tr>
<tr>
<td></td>
<td>Reverse primer: TCCTGGGTTGCGGTGATT</td>
</tr>
<tr>
<td>PITX2</td>
<td>Forward primer: GACCAACCTTTACGAGACCTC</td>
</tr>
<tr>
<td></td>
<td>Reverse primer: TTAACCTCCTTGACAGGCCC</td>
</tr>
<tr>
<td>TGF-β2</td>
<td>Forward primer: GCCAGCAAGATGCTACCCGAC</td>
</tr>
<tr>
<td></td>
<td>Reverse primer: CGGAGGAGGAGGAGGAGGAGG</td>
</tr>
<tr>
<td>TGF-βR1</td>
<td>Forward primer: TCCAACTACTGTAAGTCCTCACA</td>
</tr>
<tr>
<td></td>
<td>Reverse primer: TGAGATGCAGACGAGACCACA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward primer: GCC ATG GAC TGT GGT CAT GAG</td>
</tr>
<tr>
<td></td>
<td>Reverse primer: TGC ACC ACC AAC TGC TTA GC</td>
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PITX2 is involved in the regulation of CEC development. QPCR results showed that the expression of PITX2 increased significantly at the first day after HCSC-CM induced first stage differentiation, the expression of 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 PITX2 increased significantly (Fig. 5A; P < 0.05). Meanwhile, the expression of TGFβ-2 and its receptor, TGFβ-R1, 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, TGFβ-R1, 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/TGFβ-R1/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 HCSC, 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.

**Figure 3.** The differentiation of NCs into POMPs. (A) The optimal time for NCs culture. EBs treated with RA showed the highest expression of PITX2 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 HCSC-CM-induced NC differentiation into POMPs. The expression of POMPs markers FOXC1 and PITX2 peaked at the third day of co-culture in HCSC-CM, and then decreased gradually. *P* < 0.05 compared with NCs group. (C) Immunofluorescent staining of day 3 HCSC-CM cultured cells. After culturing in the HCSC-CM for 5 days, most cells expressed perilaminar 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.
The hESC-CEC–like cells were eventually derived from POMPs by activating the TGF-β-2 and TGF-β-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...
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.59
Meanwhile, POMPs begin to receive signals related to lens and
prepare to complete the MET process. POMPs respond to the
differentiation signals of corneal endothelium generated by the
MET process. The CECs migrate away from the anterior part of
lens in the first 13.5 and 14.5 days of the embryonic
development. The anterior chamber is formed by the gap
between the corneal endothelium and the lens, ultimately. At
present, the mechanism of how CECs, which appeared to be a
regular single-layer structure, is formed is not yet clear.
However, experiments performed to remove lenses in both
chicken and mouse embryos show that lens removal inhibits
the normal formation of corneal endothelium and corneal
stromal cells.39

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.10,11 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.12 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.53 Based on these
findings, to simulate the microenvironment of internal corneal
endothelial development in vitro, our study selected HCSC-
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 Afshar14 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 karyotyp-
ically normal cell lines.45,46 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.17 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 differentiation induction and gradually decreased after differentiation. TGF-

**References**


41. Sanford LP, Ormsby I, Gittenberger-de Groot AC, et al. TGFβ2 knockout mice have multiple developmental defects that are non-overlapping with other TGFβ knockout phenotypes. *Development*. 1997;124:2659–2670.


