Methodological study of directed differentiation of pluripotent stem cells into corneal endothelial cells

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Background: Corneal transplantation is the most effective clinical treatment for irreversible corneal endothelial decompensation. However, while visual rehabilitation can be achieved by corneal transplantation, transplant rejection, poor postoperative visual acuity, and lack of suitable donor tissue are currently the greatest obstacles to corneal transplantation. As a result, endothelial cell-based therapy has emerged as an alternative to corneal transplantation treatment. Human induced pluripotent stem cells (hiPSCs) were induced to differentiate into human corneal endothelial cell (hCEC)-like cells in our study, which aimed to provide an experimental basis for studying the clinical translation and application of induced PSC (iPSC) to corneal endothelial cell (CEC) differentiation.

Methods: A two-step method to convert hiPSCs into hCEC-like cells was applied in the study. First, the transforming growth factor beta (TGF-β) and Wnt signaling pathways were regulated by adding SB4315542 and CHIR99021, and hiPSCs were induced to differentiate into neural crest cells (NCCs) by a chemically defined and serum-free *in vitro* induction method. Subsequently, NCCs were induced to differentiate into hCEC-like cells by adding B27, platelet-derived growth factor (PDGF)-BB, and XAV939 (Wnt pathway inhibitor) to CEC culture medium.

Results: IPSCs were directionally differentiated into NCCs. During the process of differentiation, iPSCs gradually lost the monoclonal morphology of PSCs. Moreover, β-catenin and SRY-related HMG-box gene 10 (*SOX10*) proteins were immunohistochemically expressed on the 7th day of differentiation. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) results demonstrated that the cells expressed *SOX9*, *SOX10*, nerve growth factor receptor (*NGFR*), human natural killer-1 (*HNK-1*), and *β-catenin*, indicating that they were successfully directionally differentiated from iPSCs to NCCs. After 5–7 days of differentiation, the cells demonstrated a hexagonal morphology of monolayer tight junctions. Immunofluorescence results demonstrated that the cells expressed CEC indicator zonula occludens-1 (*ZO-1*) protein. QRT-PCR results demonstrated that the cells expressed collagen type IV alpha 1 (*COL4A1*), *COL8A2*, *COL8A1*, and *ZO-1*, which indicated that they were successfully directionally differentiated from NCC to CEC.

Conclusions: Our study successfully provided a simple and efficient method with clear chemical composition and serum-free media to directionally differentiate hiPSCs into hCEC-like cells, thereby advancing the results of previous studies on directional transformation into epithelial cells.

Keywords: Human induced pluripotent stem cells (hiPSCs); neural crest cell (NCC); human corneal endothelial cells (hCECs)

Introduction

The transparent outer surface of the eye, the cornea, is divided into five layers, which include, from outermost to innermost, the epithelial cell layer, Descemet's membrane, the stromal layer, Descemet's membrane, and the endothelial cell layer (1). The layers of the cornea are essential for the exertion of visual function, especially the corneal endothelial cell (CEC) layer. The CEC layer of the eye consists of a single layer of tightly connected hexagonal cells that can maintain the cellular matrix in a dehydrated state through the activity of Na$^+$/K$^+$-ATPase on the cell membrane and the presence of tight junctions, thereby maintaining corneal transparency (2,3). CECs do not have the capacity to regenerate *in vivo* and therefore cannot replace dead or damaged cells. Adult keratocyte density is about 2,500 cells/mm^2, and CEC dystrophy and surgical trauma can decrease the density of CECs. If cell density is below 500 cells/mm^2, corneal endothelial decompensation, progressive corneal edema, bullous keratopathy, and even blindness can result.

Corneal transplantation is the most effective clinical treatment for irreversible corneal endothelial decompensation. However, while visual rehabilitation can be achieved by corneal transplantation, corneal transplant rejection, poor postoperative visual acuity, and lack of suitable donor tissue are currently the greatest obstacles to corneal transplantation (4). Therefore, endothelial cell-based therapy has emerged as an alternative to corneal transplantation.

Because human CECs are difficult to culture and expand *in vitro* (5-8), pluripotent stem cell (PSC)-induced differentiated endothelial cells are an ideal source for cell-based endothelial cell transplantation. PSCs include embryonic stem cells (ESCs) and induced PSCs (iPSCs). ESCs are able to induce differentiation of CECs *in vitro*. However, this approach is ethically controversial and can result in rejection reactions, limiting its clinical application. The use of CECs obtained by induced differentiation of human iPSCs (hiPSCs) from autologous or other allogeneic donor sources reduces the risk of immune rejection and avoids socio-ethical issues. This method has become the main direction for clinical application of *in vitro* induced differentiation of CECs.

At present, many corneal endothelial differentiation methods and practices simulate the process of corneal endothelial development and differentiation *in vitro*. Neural crest cells (NCCs) can differentiate into CECs during embryonic development (9), and this process could provide a strategy for inducing multifunctional stem cells into NCCs, after which, NCCs are induced into CECs. In a previous study, epithelial cell-conditioned medium and/or corneal stroma cell-conditioned medium was included with the induction medium (10) (the conditioned medium). However, because the chemical composition of the culture medium was not clear, the components that play a role in inducing differentiation could not be clearly obtained by these methods. With clear and simple components without serum or immunogenicity problems, the feeder-free induction method, using recombinant protein or small-molecule substances, is more suitable for future clinical applications.

In this study, the differentiation of hiPSCs into NCCs was induced by regulating transforming growth factor beta (TGF-β) and Wnt signaling pathways by adding SB4315542 and CHIR99021. The differentiation of NCCs into human CECs (hCECs) was then induced by adding B27, platelet-derived growth factor (PDGF)-BB, and XAV939 to CEC culture medium. This approach provided a simple and efficient method for directed differentiation of iPSC into CEC with clear chemical composition and serum-free media. We present the following article in accordance with the MDAR reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-22-1586/rc).

Methods

Cell culture and differentiation

Differentiation from hiPSC to NCC

(I)   HiPSCs and ncEpic hPSC medium were obtained from Nuwacell Biotechnologies Co., Ltd. (Anhui, China). HiPSCs were seeded in Matrigel-coated 6-well plates and digested with ethylenediamine tetraacetic acid (0.5 mM EDTA; Nuwacell Biotechnologies Co., Ltd.) for 7.5 minutes. Once the

degree of fusion reached 80%, 2.5 µM non-muscle myosin II inhibitor (Sigma-Aldrich, St. Louis, MO, USA) was added to the medium.

(II)   The ncEpic medium (2 mL) was changed daily. After 48 hours, the cells reached 30% confluence and were replaced with NCC cell culture medium. The ingredients were: 50% Iscove's modified Dulbecco's medium (IMDM; 13390, Sigma-Aldrich), 50% F12 (N-6658, Sigma-Aldrich), 1× chemically defined lipid concentrate (11905-031, Thermo Fisher Scientific, Waltham, MA, USA), 5 µg/mL transferrin (T8158, Sigma-Aldrich), 5 µg/mL insulin (Sigma-Aldrich), 5 µg/mL fibroblast growth factor 2 (FGF2; PeproTech, Windsor, NJ, USA), 5 mg/mL bovine serum albumin (BSA; Sigma-Aldrich), 0.5× ncB27 Serum-Free Supplement (ncB27; Nuwacell Biotechnologies Co., Ltd.), 10 µM SB431542 (A8249, ApexBio, Houston, TX, USA), and 0.5 µM CHIR99021 (ApexBio). The medium was changed daily until day 7.

Differentiation from NCC to CEC

(I)   NCCs were inoculated with laminin-511 (5 µg/well) coated in 6-plate wells at 0.1 mL/well. Once cell fusion reached 80%, cells were digested with 0.125% trypsin (Sigma-Aldrich) for 2–3 minutes, and an equal volume of trypsin inhibitor (Sigma-Aldrich) was added. NCCs were collected, and 2.5 µM non-muscle myosin II inhibitor (Sigma-Aldrich) was added to the CEC01 medium. The CEC01 medium was composed of: 50% IMDM (13390, Sigma-Aldrich), 50% F12 (Sigma-Aldrich), 1× chemically defined lipid concentrate (Thermo Fisher Scientific), 5 µg/mL transferrin (Sigma-Aldrich), 5 µg/mL insulin (Sigma-Aldrich), 5 mg/mL BSA (Sigma-Aldrich), 10 ng/mL FGF1 (PeproTech), 10 ng/mL PDGF-BB (PeproTech), 1 µM XAV939 (Sigma-Aldrich), and 0.5× ncB27 (Nuwacell Biotechnologies Co., Ltd.).

(II)   After 24 hours, the medium was changed into CEC02, composing of: 50% IMDM (13390, Sigma-Aldrich), 50% F12 (Sigma-Aldrich), 1× chemically defined lipid concentrate (Thermo Fisher Scientific), 1 µg/mL transferrin (Sigma-Aldrich), 5 µg/mL insulin (Sigma-Aldrich), 5 mg/mL BSA (Sigma-Aldrich), 5 ng/mL FGF1 (PeproTech), 10 ng/mL PDGF-BB (PeproTech), 0.5 µM XAV939 (Sigma-Aldrich), and 0.5× ncB27 (Nuwacell Biotechnologies Co., Ltd.). The medium was changed every 2 days.

(III)   When the fusion reached 80%, the cells were passaged at a ratio of 1:6 by trypsin (Sigma-Aldrich).

Immunofluorescence staining

On the 7th and 14th days of differentiation, the culture medium was discarded. The cells were then washed once with Dulbecco's phosphate buffer saline (DPBS; Sigma-Aldrich) and fixed with 4% paraformaldehyde (Sigma-Aldrich) at room temperature for 15 minutes, washed 3 times with DPBS for 5 minutes each time, permeabilized with 0.3% TritonX-100 (Sigma-Aldrich) at room temperature for 20 minutes, and blocked with 10% goat serum at room temperature for 1 hour. Primary antibody incubation was first performed at room temperature for 30 minutes, kept overnight at 4 ℃, and then rewarmed at room temperature for 30 minutes. The cells were washed 3 times with DPBS for 5 minutes each time, and secondary antibody was added. Cells were then incubated at room temperature in the dark for 1 hour, washed 3 times for 5 minutes each time with DPBS, and 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) staining solution was applied at room temperature for 10 minutes. The cells were then washed a further 3 times with DPBS for 5 minutes, and double distilled water was added once. Finally, the film was sealed with antifluorescent dampening agent. The primary antibodies used for photography with the laser confocal microscope (Olympus, Tokyo, Japan) were: rabbit anti-SRY-related HMG-box gene 10 (*SOX10*; 1:500; ab155279, Abcam, Cambridge, England), rabbit anti-*β-catenin* (1:200; 8480, Cell Signaling, Boston, MA, USA), and rabbit anti-zonula occludens-1 (*ZO-1*; 1:800; 18-7430, Invitrogen, Carlsbad, CA, USA), and the secondary antibody was goat anti-rabbit immunoglobulin G (IgG) antibody (1:1,000; ab150077, Abcam).

Flow cytometry detection

Differentiated cells from day 7 were digested with 0.25% Trypsin-0.5 mM EDTA (Sigma-Aldrich) into individual cells, and 100,000 single cells were removed, washed with DPBS, and centrifuged in a palm centrifuge. The supernatant was absorbed and discarded, fluorescence-activated single cell sorting (FACS) buffer was added to resuspend the cell pellet, and FITC-p75 (345104, BioLegend, San Diego, CA, USA) and PE-human natural killer-1 (*HNK-1*) flow antibody (359612, BioLegend) were added. The cells were incubated in the dark at 4 ℃ for

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Table 1 Primers identified by qRT-PCR

Cell type	Primer	Forward sequences	Reverse sequences
NCC	SOX9	GCCAGGTGCTCAAAGGCTA	TCTCGTTCAGAAGTCTCCAGAG
	SOX10	CACAAGAAAGACCACCCGGA	AAGTGGGCGCTCTTGTAGTG
	NGFR	AAGAAAAGTGGGCCAGTGTG	AACAGTCCTTTGCAGGGTTG
	HNK-1	AATGGGTGGGTTGTGAGTGC	GCCAGACAGTGATGAGCAGA
	β-catenin	GACGGAGGAAGGTCTGAGGA	TGGCCATGTCCAACTCCATC
CEC	COL4A1	GGCAGATTCGGACCACTAGG	GGGCCATTTCCGTGGTTTCT
	COL8A1	CCTGGGTCAGCAAGTACCTC	TTGTTCCCCTCGTAAACTGG
	COL8A2	ATCGCTGGGGACTCTGACAC	GGTAGAGGCATTTCCAGGTACT
	ZO-1	ACCAGTAAGTCGTCCTGATCC	TCGGCCAAATCTTCTCACTCC
RPE	RPE65	GCATCCTGCTGGTGGTTACA	TTCAAAGAGTCCTGGCCCAC

qRT-PCR, quantitative reverse transcription polymerase chain reaction; NCC, neural crest cell; CEC, corneal endothelial cell; RPE, retinal pigment epithelium; SOX10, SRY-related HMG-box gene 10; NGFR, nerve growth factor receptor; HNK-1, human natural killer-1; COL4A1, collagen type IV alpha 1; ZO-1, zonula occludens-1.

20 minutes, washed with DPBS once, and FACS buffer was added. Flow cytometry was then performed (FACSCalibur, BD Biosciences, Franklin Lakes, NJ, USA).

Identification by quantitative reverse transcription polymerase chain reaction (qRT-PCR)

RNA was extracted from cells differentiating on days 7 and 14 using TRIzol reagent (Invitrogen) in accordance with the manufacturer's protocol. Reverse transcription with the TaqMan Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) was performed using 1 µg of template RNA. QRT-PCR analysis was performed according to the manufacturer's protocol using SYBR Green Mastermix and the 7500 real-time PCR System (Applied Biosystems, Carlsbad, CA, USA). Primers identified by qRT-PCR were NCC-specific primers [SOX9, SOX10, nerve growth factor receptor (NGFR), HNK-1, and β-catenin], CEC-specific primers [collagen type IV alpha 1 (COL4A1), COL8A1, COL8A2, and ZO-1] with retinal pigment epithelial cell-specific primers (RPE65) (Table 1).

Statistical analysis

QRT-PCR was performed for three parallel assays. The obtained data were statistically analyzed by SPSS 17.0, and the results are expressed as mean ± standard deviation (SD). The differences between the two groups were examined by the Student's t-test. P<0.05 was considered statistically significant.

Results

Differentiation of hiPSC into NCC

During development, CECs originate from NCCs. NCCs are a pluripotent progenitor cell population that are transiently present during development and can differentiate into many cell types, including neuronal cells, colloid cells, connective tissue, bone, CECs, corneal stromal cells, and other anterior segment cells. Therefore, our strategy involved first inducing the differentiation of hiPSCs into NCCs using two small-molecule compounds, CHIR99021 [glycogen synthase kinase 3 (GSK-3) inhibitor, Wnt pathway activator] and SB431542 (TGF-β/SMAD pathway inhibitor), based on a previously published induction protocol by Menendez et al. (11).

HiPSCs were seeded in Matrigel-coated 6-well plates, and the fusion degree reached about 30% to induce differentiation. The cells gradually lost the morphology of monoclonal hiPSCs, switching from cells with a high nucleocytoplasmic ratio to cells with a shuttle shape. Differentiation on day 7 was induced, cell confluence reached 100%, there were a large number of bulging cells between the cell pellets, and cell morphology was significantly changed (*Figure 1A*). Immunofluorescence staining for β-catenin, a positive correlation protein of the
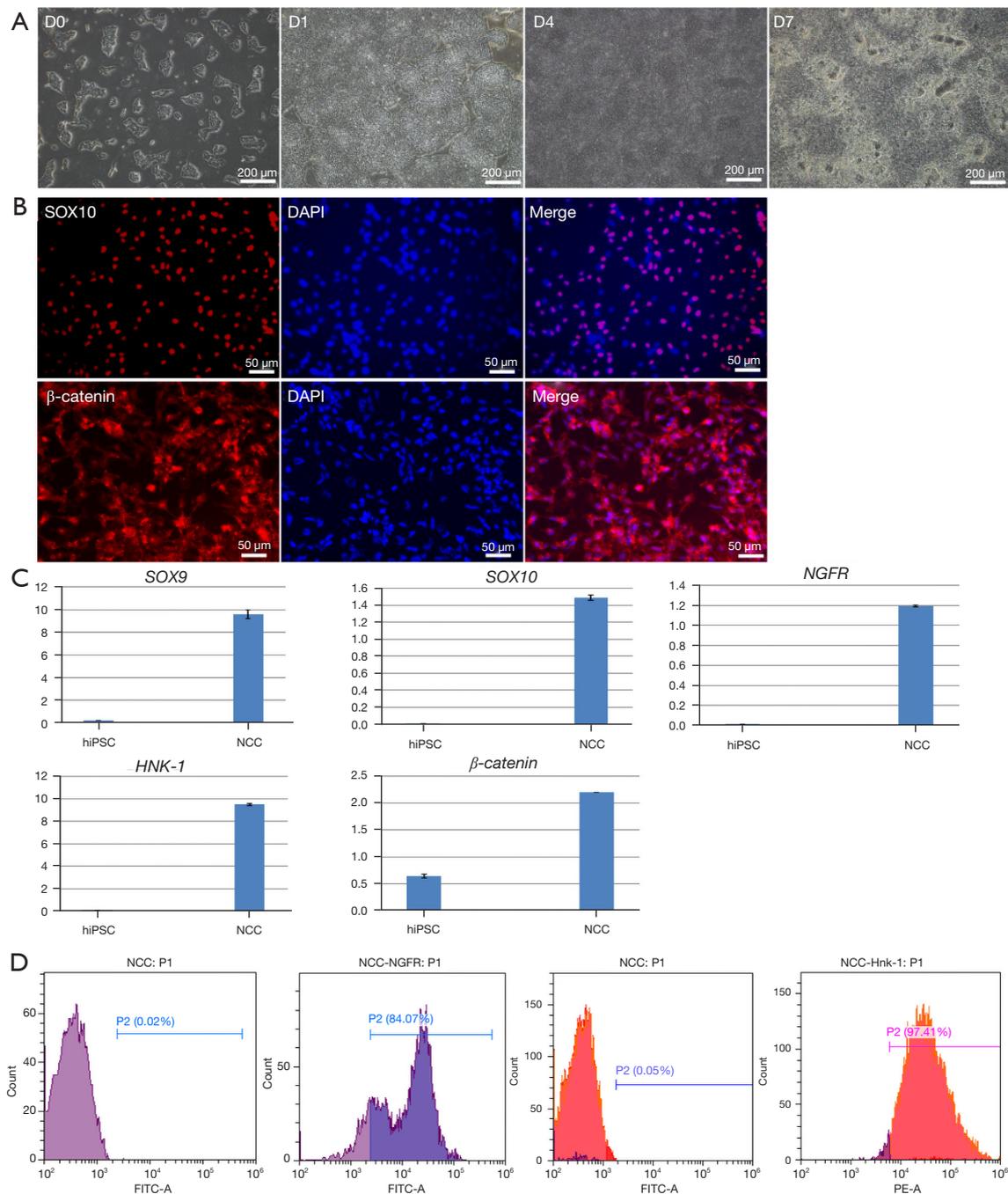
Figure 1 Deformation of hiPSC-induced differentiated NCCs and detection and identification of NCC-specific indicators. (A) Picture of cells before (D0) and after (D1, D4, D7) differentiation were observed by light microscope. (B) Immunofluorescence staining showing the expression of *SOX10* with *β-catenin* in induced differentiated day 7 cells. (C) The expression of neural crest specific indicators detected by qRT-PCR (*SOX9*, *SOX10*, *NGFR*, *HNK-1*, *β-catenin*) in hiPSCs and NCCs. The Y-axis represents normalization to relative fold. (D) The expression of *p75* and *HNK-1* protein on the cell membrane of induced differentiation on day 7, detected by flow cytometry. *SOX10*, SRY-related HMG-box gene 10; DAPI, 4',6-diamidino-2-phenylindole; *NGFR*, nerve growth factor receptor; *HNK-1*, human natural killer-1; hiPSC, human induced pluripotent stem cell; NCC, neural crest cell; FITC-A, fluorescein isothiocyanate antibody; qRT-PCR, quantitative reverse transcription polymerase chain reaction.

Wnt pathway, along with *SOX10* staining, an indicator of NCC specificity, demonstrated that about 60% of the cells were positive for *SOX10* expression (*Figure 1B*). QRT-PCR results demonstrated that cells harvested from induced differentiation on day 7 significantly expressed neural crest specific parameters (*SOX9*, *SOX10*, *NGFR*, *HNK-1*, and *β-catenin*) compared with undifferentiated hiPSCs (*Figure 1C*). The results of flow cytometry demonstrated that more than 80% of cells expressed *p75/NGFR* with *HNK-1* protein (*Figure 1D*). These results indicated that cells harvested from day 7 of hiPSC-induced differentiation were NCCs in response to neural crest inducing differentiation medium.

Differentiation into CEC induced by NCC

As described in a number of previous induction protocols (12-14), neural growth supplement (B27), endothelial mitogen (PDGF-BB), and FGF were added to the CEC culture medium. In addition, several studies (12-14) have reported that dickkopf-related protein 2 (DKK 2), a Wnt pathway inhibitor, is involved in NCC-induced differentiation to CEC. Our experiments confirmed that inhibition of the Wnt pathway was involved in NCC-induced differentiation to CEC, and the Wnt pathway inhibitor XAV939 was added to the medium, rather than DKK 2. XAV939 is a small-molecule Wnt signaling pathway inhibitor that selectively inhibits β-catenin-mediated transcription and inhibits the Wnt signaling pathway by stabilizing axin (15).

NCCs were revived and seeded in Matrigel-coated well plates and changed to CEC02 medium after 24 hours of culture in CEC01 medium. On differentiation day 11, the cells no longer demonstrated an elongated morphology of NCC (the cell morphology became flat), and on differentiation day 14, irregular hexagonal cells with tight junction structures could be observed (*Figure 2A*). The hexagonal cells of tight junctions were identified by immunofluorescence staining with qRT-PCR, and the cells were *ZO-1*-positive cells, with positive cells accounting for about 90% (*Figure 2B*). QRT-PCR analysis of specific genes (*COL4A1*, *COL8A1*, *COL8A2*, and *ZO-1*) expressed early in the corneal endothelium of the eye demonstrated that *COL8A1* and *COL8A2* expression increased nearly 10- to 100-fold, respectively, compared with undifferentiated hiPSCs. Furthermore, *RPE65*, a specific indicator of retinal pigment epithelial cells, was not expressed (*Figure 2C*). The results of immunofluorescence and qRT-PCR

demonstrated that the CECs were harvested from hiPSC-induced differentiation on day 14 in the presence of corneal endothelium-inducing differentiation medium.

Discussion

During eye development, when the surface ectoderm and lens vesicle are separated, there are periocular cells that migrate and differentiate from NCCs, with the interocular cells differentiating into a variety of cells, including CECs, which remain in the G1 phase of the cell cycle (16). This process may occur in response to the TGF-β signaling system and/or the Wnt signaling system (17,18). Since the corneal endothelium is located between the corneal stroma and the lens and develops after the lens, CECs may be produced under the influence of lens epithelial cells and corneal stromal cells. Zhang *et al.* (10) exposed human ESCs to corneal stromal cells to stimulate the differentiation of corneal stromal cells into ocular cells. NCCs were placed in lens epithelial cell-conditioned medium for co-culture to derive corneal endothelial-like cells from human ESC. Na$^+$/K$^+$-ATPase alpha-1 and beta-1 subunits were upregulated in these corneal endothelial-like cells. Similarly, in a study by Chen *et al.* (19), mouse ESC and iPSC differentiation into CEC was induced using an embryonic development process, in which all-trans retinoic acid (RA) was added to the culture medium in the first step, followed by a second induction in conditioned culture of lens epithelial cells, leading to the generation of CEC-like cells. RA is an active product of fat-soluble vitamin A, which plays an important role in embryonic development. Selection of effective all-trans RA concentrations could upregulate interepithelial transition-related transcription factors and NNC marker gene expression (20,21). It has also been shown that (22) RA and GSK 3β inhibitors (Wnt/β-catenin signaling activators) can upregulate the expression of paired-like homeodomain 2 (*Pitx2*), a homeobox gene involved in anterior segment development. In addition to lens epithelial conditioned medium, Song *et al.* (23) used bovine corneal endothelial conditioned medium to induce differentiation of NCC induced by embryonic stem cells and obtained human corneal endothelial-like cells after 40 days of induced differentiation culture. These studies verify that the corneal endothelium is differentiated from NCC (10,16-23). Moreover, the components in the conditioned medium of lens epithelial cells or corneal stromal cells are capable of inducing differentiated CEC (9).
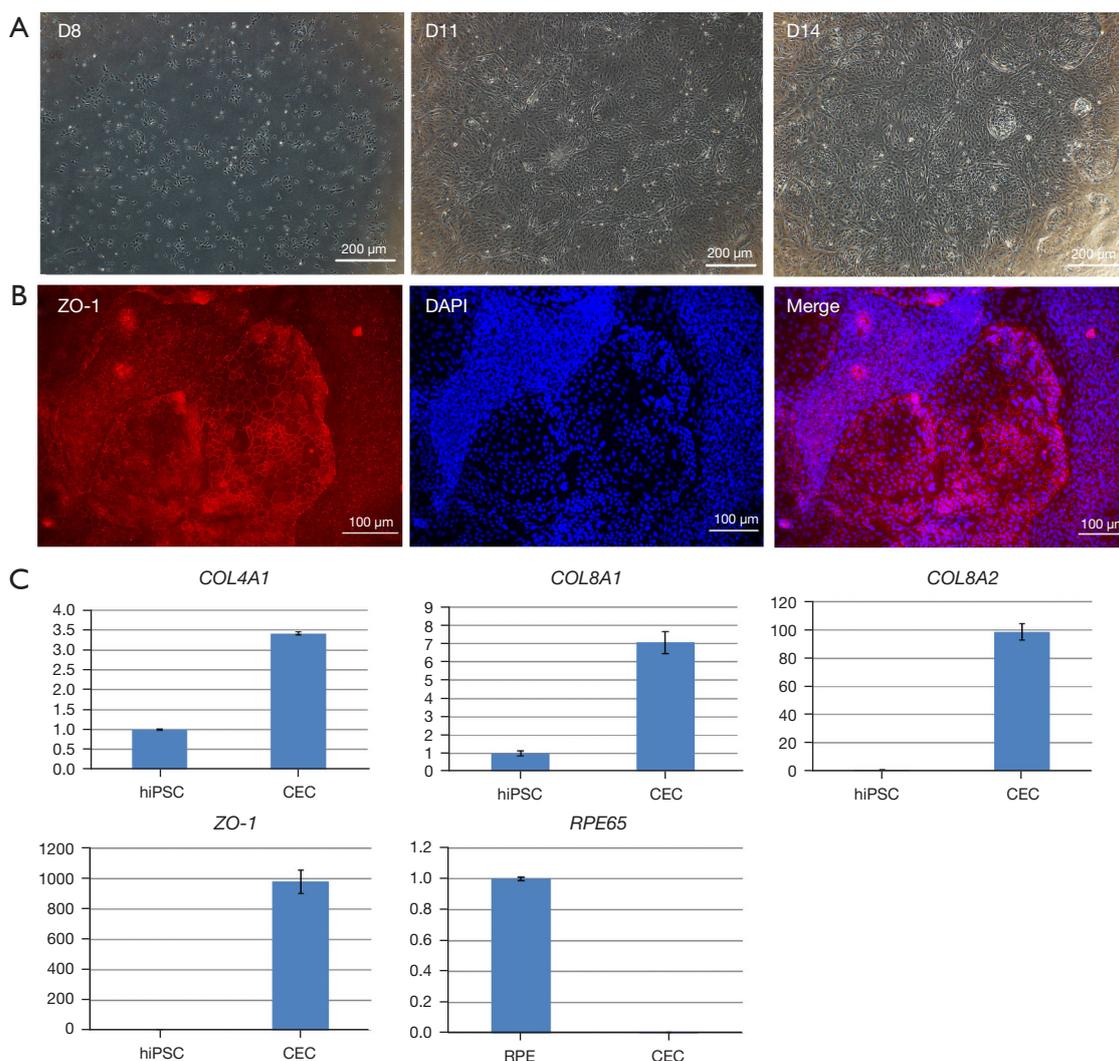
There are some drawbacks to the methods mentioned

Figure 2 Deformation of hiPSC-induced differentiated NCCs and identification of specific indicators of NCCs. (A) Picture of cells before (D8) and after (D11, D14) differentiation were observed by light microscope. (B) The expression of *ZO-1* in induced differentiated cells on day 14, shown by immunofluorescence staining. (C) The expression of corneal endothelial cell-specific indicators (*COL4A1*, *COL8A1*, *COL8A2*, *ZO-1*, and *RPE65*) in hiPSCs and CECs identified by qRT-PCR. The Y-axis represents normalization to relative fold. *ZO-1*, zonula occludens-1; DAPI, 4',6-diamidino-2-phenylindole; *COL4A1*, collagen type IV alpha 1; RPE, retinal pigment epithelium; hiPSC, human induced pluripotent stem cell; CEC, corneal endothelial cell; NCC, neural crest cell; qRT-PCR, quantitative reverse transcription polymerase chain reaction.

above, including unclear medium composition, long differentiation time, and low differentiation rate (24). Therefore, the use of culture methods with clear components has become an important research direction for CEC differentiation *in vitro*. This method is beneficial for revealing the regulatory mechanisms of embryonic development, and the induction of differentiation protocols through chemically well-defined media is simpler, more

time-efficient, and does not require additional human corneal donors. Studies have shown that TGF-β, bone morphogenetic protein, and Wnt signaling pathways play important roles in NCC and corneal endothelial development (9,24-27). Thus, current methods of inducing differentiation mostly focus on the above signaling pathways. The corneal endothelial induction method proposed by McCabe *et al.* (13) is a two-step generation

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process; the first step is to induce differentiation from ESC to NCC with a TGF-β signaling blocker (SB431542) and Noggin (BMP inhibitor). As the TGF-β-SMAD-2/3 signaling pathway and the BMP SMAD-1/5/8 signaling pathway are blocked, the process is called "double SMAD inhibition". Menendez et al. (11) induced NCCs positive for the NCC markers *p75/NGFR* and *SOX10* using two small-molecule compounds, CHIR99021 (GSK-3 inhibitor) and SB431542 (TGF-β/SMAD pathway inhibitor). The cell morphology altered from high nucleocytoplasmic ratios to elongated structures with neuronal morphology, with 80–99% differentiation. In the first step of the study, NCCs were induced within 7 days and expressed NCC-specific markers from Menendez et al.'s induction method. Among them, *β-catenin* protein is an indicator of Wnt canonical pathway activation during neural crest formation (28). *SOX10* protein was also produced by Wnt pathway regulation during NCC formation.

In the second step of differentiation from NCC to CEC, McCabe et al. (13) induced differentiation of corneal endothelial-like cells with a hexagonal structure from NCCs by inducers PDGF-B, DKK 2, and basic FGF. Previous studies have varied from 14–25 days for differentiation from hiPSC-NCC to CEC (12,14). DKK 2 is an antagonist of Wnt/β-catenin signaling, and it could play an important role in normal ocular development (29). The role of Wnt/β-catenin signaling in differentiation from NCC to CEC was confirmed in our study by replacing DKK 2 with another antagonist of Wnt/β-catenin signaling, XAV939. XAV939, described in a study in Nature in 2009 (15), is a novel small-molecule Wnt signaling pathway inhibitor that selectively inhibits β-catenin-mediated transcription and inhibits the Wnt signaling pathway by stabilizing axin. HiPSC-NCCs were differentiated for 7 days to produce CECs and showed high expression of CEC-associated markers. *ZO-1* is a tight junction protein on the endothelial cell membrane, and collagen type 4 and type 8 are components of Descemet's (30) and are secreted by endothelial cells. The assay results demonstrated that the use of XAV939 instead of *DKK 2* induced differentiation of NCC into CEC, with an induced differentiation time of 7 days, which was shorter than that in previous studies (12,14).

In terms of *in vivo* tests, Japanese scholars (31) successfully injected allogeneic CECs expanded *in vitro* into the anterior chamber of patients and observed them for 24 weeks, finding that the endothelial cell count increased, the corneal thickness decreased, and visual acuity improved. This was the first demonstration that anterior chamber injection with primary cultured endothelial cells could maintain corneal transparency. In terms of an *in vivo* test of CECs induced by PSCs, in 2017 Shen *et al.* (32) cultured skin-derived precursors and B4G12 cells (an immortalized human endothelial cell) together to obtain CEC-like cells and then injected them into the anterior chamber of experimental monkeys with corneal endothelial decompensation. The cornea gradually became transparent 7 days after surgery, and the cornea remained transparent during 3 months of follow-up. Gong *et al.* (33) published a study in 2021 in which differentiation of hiPSC into NCC was induced by CHIR99021 and SB431542. NCCs were injected into the anterior chamber of model rabbits with corneal endothelial decompensation, with the results showing that the rabbit cornea gradually became transparent 7 days after injection. However, the effect disappeared 14 days after injection. The reappearance of corneal edema may be associated with improper maturation, senescence, and interepithelial transition of hiPSC-CEC (33). Therefore, on the basis that the mechanism of differentiation from NCC into CEC has not been fully clarified, there is still no optimal method for differentiation induced by small molecules or recombinant proteins *in vitro* (25). Further study of the function and stability of endothelial cells induced by stem cells with clear chemical composition *in vitro*, as well as the medium composition and steps of *in vitro* culture, is needed.

In our study, we used a differentiation method with clear chemical composition and no serum or serum substitutes to successfully induce hiPSCs to differentiate into NCCs (day 7) with high expression of *β-catenin*, *p75*, and *HNK-1* protein. CECs expressing CEC-specific indicators *COL4A1*, *COL8A1*, *COL8A2*, and *ZO-1* could be obtained by continuing differentiation for 5 to 7 days. In the future, further optimization of protocols for the differentiation of stem cells into CECs *in vitro* is required to keep the induced endothelial-like cells more stable in morphology and function. Studies on endothelial cell function *in vitro*, as well as *in vivo* animal experiments, are required to evaluate safety and efficacy.

Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://atm.amegroups.com/article/view/10.21037/atm-22-1586/rc

Data Sharing Statement: Available at https://atm.amegroups.com/article/view/10.21037/atm-22-1586/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://atm.amegroups.com/article/view/10.21037/atm-22-1586/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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