Long-term observation and sequencing analysis of SKPs-derived corneal endothelial cell-like cells for treating corneal endothelial dysfunction

Lin Shen

Qilu Hospital of Shandong University <https://orcid.org/0000-0002-4741-9584>

Peng Sun

Department of Ophthalmology, the affiliated Yantai Yuhuangding Hospital of Qingdao University

Liqun Du

Department of Ophthalmology, Qilu Hospital of Shandong University

Jing Zhu

Department of Ophthalmology, Qilu Hospital of Shandong University

Chengqun Ju

Department of Ophthalmology, Qilu Hospital of Shandong University

Hui Guo

Department of Ophthalmology, Qilu Hospital of Shandong University

Kunpeng Pang

Department of Ophthalmology, Qilu Hospital of Shandong University

Xinyi Wu (✉ xywu8868@163.com)

Research

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Abstract

Background

Corneal endothelial dysfunction is a principal cause of visual deficiency. Corneal transplantation is the most effective treatment for corneal endothelial dysfunction. However, a severe shortage of available donor corneas remains a global challenge. Previously, we acquired corneal endothelial cell-like cells (CEC-like cells) derived from skin-derived precursors (SKPs). CEC-like cells exerted excellent therapeutic effect in rabbit and monkey corneal endothelial dysfunction models.

Method:

We prolonged the observation in the monkey experiment for 2 years. Further transcriptome sequencing was also carried out on HCEC, CEC-like cells before transplantation and after transplantation.

Results

We confirmed that the monkey cornea remained transparent and the CEC-like cells could adapt to the microenvironment after transplantation. The gene expression pattern of CEC-like cells was similar to HCEC and changed slightly after transplantation.

Conclusions

This study presented a brand-new insight into CEC-like cells and further provided a promising prospect of cell-based therapy for corneal endothelial dysfunction. The renewable cell source, novel derivation method and simple treatment strategy may be clinically applied in regenerative medicine in the future.

Background

Corneal blindness, the 4th cause of blindness globally (5.1%), is one of the principal causes of visual deficiency[1]. Corneal endothelial dysfunction which is characterized by excessive endothelial loss is a major reason for corneal blindness. Severe endothelial cell loss may occur as a result of previous ocular surgeries, infections, trauma of some corneal disease such as Fuchs' corneal endothelial dystrophy (FCED), posterior polymorphous corneal dystrophy (PPCD), and iridocorneal endothelial syndrome (ICE) [2]. Corneal transplantation, such as penetrating keratoplasty (PKP) and corneal endothelial keratoplasty, is an effective way for treating corneal blindness [3, 4]. Corneal endothelial dysfunction is the leading indication for endothelial keratoplasty which account for about 39% of all corneal transplantation[5, 6]. However there is a worldwide shortage of cornea donors which is an obstacle for their application[7].

A new source of functional CECs combine with cell-based therapy is an extremely good choice to overcome the shortage of cornea donors. Human corneal endothelial cells (HCEC) have limited proliferative capacity *in vivo* and cannot be sub-cultured for more than a few passages *in vitro*[8, 9]. However, we previously invented a new method that could acquire corneal endothelial cells (CEC-like cells). We co-cultured skin-derived precursors (SKPs) with B4G12 cells using Transwell inserts in human endothelial serum free medium (SFM). The acquired CEC-like cells had similar morphology and characteristic with human CECs. After they were injected into the anterior chamber of rabbit and monkey corneal endothelial dysfunction models, they had exerted excellent therapeutic effect[10]. The invention provided a new treatment strategy for corneal endothelial dysfunction and also provided efficient source of seed cells to construct tissue engineered corneas[11].

In this study, the observation after CEC-like cell transplantation into the monkey endothelial dysfunction model was prolonged for 2 years. In addition, further transcriptome sequencing was carried out on HCEC, CEC-like cells before transplantation and after transplantation. Results showed that CEC-like cells could adapt to the microenvironment and have a wonderful effect after being transplanted into the monkey endothelial dysfunction model in long-term observation. The gene expression pattern of CEC-like cells was similar to HCEC and changed slightly after transplantation. Our studies have provided a promising prospect of cell-based therapy with CEC-like cells for corneal endothelial dysfunction in future clinical applications.

Method

Animals

Five rhesus monkeys weighting 2.5–3.5 kg (Hongli Medical Animal Experimental Research Center, Jinan, Shandong Province, China) were used for corneal endothelial transplantation. All animals were treated in accordance to the ARVO (Association for Research in Vision and Ophthalmology) Statement for the Use of Animals in Ophthalmic and Vision Research. All animal experiments were approved by the Medical Ethics Committee of Qilu Hospital of Shandong University, China (approval number 12028).

Cell Culture

Human SKPs were isolated and cultured as previously described[10, 12, 13]. Human skin was taken from 10 patients (age: 32.9 ± 15.82 years, mean $\pm$ S.D.) that underwent a double-eyelid operation. Samples were collected following written informed consents, and the study was performed in adherence to the Declaration of Helsinki. All experiments were approved by the Ethical Committee of Qilu Hospital of Shandong University and were conducted following the institutional guidelines (approval number 12028). Briefly, SKPs was cultured to a cell density of 25,000 or more cells/ml. The growth medium was DMEM/F12 Nutrient Mixture (3:1; Thermo Fisher) supplemented with 1% penicillin/streptomycin (Solarbio), 50 μ g/mL fungizone, 2% B27 supplement (Thermo Fisher), 40 ng/mL human recombinant

bFGF, and 20 ng/mL epidermal growth factor (both from Peprotech). SKPs spheres were passaged every 1–2 weeks. SKPs between passages 2 and 4 were used for further experiments.

The immortalized HCEC population B4G12 cells were generously provided by Monika Valtink and were cultured according to the previous protocol[14, 15]. Briefly, B4G12 cells were cultured in human endothelial SFM (Thermo Fisher) supplemented with 10 ng/ml human recombinant bFGF in T25 culture flasks.

CEC-like cells were derived from human SKPs as our previously protocol[10]. Briefly, human SKPs spheres were separated into single cells by 0.05% trypsin-0.02% EDTA, and then they were plated into 6-well plates coated with 10 mg/ml chondroitin sulphate and 10 µg/ml laminin (both from Sigma). The B4G12 cells were cultured in six-well Transwell inserts (Corning) and the Transwell inserts were added to the 6-well plates and co-cultured with the human SKPs in B4G12 culture medium for 8 days to acquire CEC-like cells. CEC-like cells were passaged and cultured in CEC-like cells culture medium consisting of human endothelial SFM supplemented with 10 ng/ml bFGF.

CEC-like cells transplantation into the monkey corneal endothelial dysfunction model

CEC-like cells were transplanted according to our previous method[10]. Briefly, the corneal endothelium was mechanically scraped with a lacrimal passage irrigator (Shandong Weigao) from the Descemet's membrane to create monkey corneal endothelial dysfunction models. 50 µl aqueous humor was first extracted from the models' anterior chamber. Then, 4.0×10^5 CEC-like cells suspended in 50 µl culture medium supplemented with 1.6 µg of Y-27632 were injected into the anterior chamber. Four monkeys that had cells injected were the experimental group and the other one monkey that had no cells injected was the control group. Peribulbar and subconjunctival injection of triamcinolone and dexamethasone were given after surgery. The eyes of all monkeys were kept in a face-down position for 6 hours under general anesthesia. Tobramycin and Dexamethasone were given 3–4 times a day. The corneas were examined by a slit-lamp microscope (Topcon), Visante OCT (Carl Zeiss), non-contact specular microscopy (Topcon), tenonometer (Suzhou liuliu), gonioscope (Volk), B-ultrasonography (Suoer), and fundus camera (Carl Zeiss) at certain times. The contralateral normal eyes of the monkeys were observed as the normal group.

Groups And Corneal Endothelium Acquisition After Sacrifice

The five monkeys were sacrificed by an intravenous overdose of pentobarbital sodium. The postoperative and normal eyes were removed. The two monkeys in the experimental group were sacrificed at 1 year and labeled as the EXP1 group. The other two monkeys in the experimental group were sacrificed at 2 years and labeled as the EXP2 group. The monkey in the control group was sacrificed at 2 years. The normal monkey eyes in normal groups were labeled as the MON group. Human eyes were obtained from discarded eyes after penetrating keratoplasty and from the Eye Tissue Bank of Shandong Province, China (approval number 12028). The normal human eyes group was labeled as the HCEC group. The Descemet

membranes with corneal endothelial cells were torn off monkey and human eyes of different groups with microscopic tweezers.

Polymerase Chain Reaction And DNA-sequencing

DNA was extracted from CEC-like cells and corneal endothelial cells of EXP1, EXP2, MON, and HCEC groups using a DNA extraction kit (Tiangen) according to the manufacturer's protocol. Human and monkey specific Cytb gene fragments were amplified by polymerase chain reaction (PCR) with the Ultra HiFidelity PCR Kit (Tiangen) according to the manufacturer's protocol. Primers designed by BioSune Biotech (Shanghai, China) are listed in Table 1. Agarose gel electrophoresis was carried out with the PCR product according to the standard method. Amplified DNA was purified using the TIANGel Midi Purification Kit (Tiangen). The sequencing of DNA was performed in BioSune Biotech (Shanghai, China) with the ABI3730XL sequenator. Data was analyzed with Chramas.

Histological Examination

Part of the corneas was frozen and embedded in OCT compound. Part of the corneas were fixed in 4% formaldehyde and embedded in paraffin. Sections were cut at 5 μ m slices, and subjected to immunofluorescent staining and HE staining according to standard methods. For immunofluorescent staining, the primary antibody was mouse anti- Na^+/K^+ ATPase (1:100, Millipore), secondary antibodies (1:100, Beijing Zhongshan) was coupled to TRITC, and the nuclear stain was DAPI.

RNA-sequencing And Data Analysis

The total RNA of CEC-like cells and corneal endothelial cells of EXP1 and EXP2 groups were isolated using TRIzol reagent (Life Technologies) according to the manufacturer's protocol. RNA-seq transcriptome library was prepared following the TruSeq™ RNA sample preparation Kit from Illumina (San Diego, CA) using 1 μ g of total RNA. Sequencing was performed in Majorbio Biotech (Shanghai, China) using the Illumina HiSeq 4000 150 bp Paired-End Platform. The raw paired end reads were trimmed and quality controlled by SeqPrep and Sickle with default parameters. Then clean reads were separately aligned to human reference genome with orientation mode using HISAT software. The mapped reads of each sample were assembled by StringTie. The HCEC RNA-sequencing raw paired end reads data of was downloaded from GEO datasets[16].

To identify DEGs (differential expression genes) between two different samples, the expression level was calculated according to the TPM method. RSEM was used to quantify gene abundances. R statistical package software EdgeR was utilized for differential expression analysis. Genes with $\text{FDR} < 0.05$ and $|\log_2\text{FC}| \geq 1$ were considered as significant. Venn analysis ($\text{TPM} \geq 1$) was used to demonstrate common and uniquely expressed genes transcripts between samples. The correlation analysis provides basic

reference for the analysis of differential genes. GO functional enrichment analysis were performed by Goatools to identify which DEGs were significantly enriched at Bonferroni-corrected P-value ≤ 0.05 compared with the whole-transcriptome background.

Statistical Analyses

All data are presented as mean $\pm$ S.D. The Student's t test was used to examine differences between the two groups. Comparisons among three or more groups were made using one-way ANOVA and post hoc analysis with the Bonferroni test. $P < 0.05$ was considered to be statistically significant. All data analyses were calculated by GraphPad Prism version 6 (GraphPad Software, Inc.).

Results

Long-term observation of CEC-like cells transplantation into the monkey corneal endothelial dysfunction model

In the experimental cell group, the cornea remained transparent, and no new keratic precipitates or corneal neovascularization appeared (Fig. 1A). In addition, the pupil was round with a positive light reflex, the iris texture was clear, the anterior chamber depth was normal, the aqueous fluid was clear, and the lens was transparent. In the control group, the cornea remained opaque with edema and as a consequence the iris could not be seen (Fig. 1B).

The corneal thickness in the experimental group remained steady within the range of 0.5 mm – 0.55 mm from 1 month to 2 years after transplantation. The corneal thickness in the control group continued to be above 1.0 mm 2 years after transplantation, and neovascularization appeared (Fig. 2A, C). The cells after transplantation were in the form of a polygonal monolayer (Fig. 2B). The cell density continued to increase in the first three months, peaking at an average of 3022 cells/mm², 84% of pre-operation. But after three months it gradually fell. The cell density rapidly decreased in the first year and slowly in the second year. It decreased by about 20% during the first year and by 5% during the second year compared to the third month. However, the cell density was still more than 2000 cells/mm² within 2 years, 63% of pre-operation (Fig. 2D). Other ophthalmic examinations including intraocular pressure, chamber angle, B-ultrasonography, and fundus imaging showed no apparent pathological abnormality (Fig. 2E, F, G).

Polymerase Chain Reaction And Dna-sequencing After Transplantation

PCR was carried out to show the existence of human or monkey DNA in the samples. We designed primers for human and monkey specific Cytb gene fragments to identify human or monkey DNA. With the human Cytb gene fragments primer, a 459 bp gene fragment in HCEC group was obtained (Fig. 3A HCEC).

With the monkey Cytb gene fragments primer, a 130 bp gene fragment in MON group was obtained (Fig. 3A MON). But, with the two primers, only a 459 bp but not a 130 bp gene fragment in CEC-like cells was obtained (Fig. 3A CEC-like). However, with the two primers, both a 459 bp and a 130 bp gene fragment in the EXP1 group were obtained (Fig. 3A EXP1), the same as the EXP2 group (Fig. 3A EXP2).

DNA-sequencing of the 459 bp gene fragment products in EXP2 group was carried out. It showed that the base pairs sequence highly corresponded to that of the designed human Cytb DNA fragment (Fig. 3B). This indicated that the amplified DNA products acquired in the experimental group had a high homology with human DNA, which proved the long-term existence of injected CEC-like cells in the 1-year and 2-year experimental groups (EXP1 and EXP2).

Histological Examination After Transplantation

Immunofluorescent staining of frozen section showed Na^+/K^+ ATPase expression in EXP2 group, indicating the persistent pump function (Fig. 3C). Through HE staining it could be seen that the corneal thickness in the EXP2 group was similar to the normal group. Under magnified observation, the CEC-like cells tightly adhered to the posterior surface of the cornea in a monolayer. The morphology of the corneal epithelium, stromal cells, and endothelium in the EXP2 group were all similar to the normal group (Fig. 3D, E). The EXP1 data was same as EXP2, and the EXP1 data are not shown. However, in the control group, the cornea was very thick and edematous. Under magnified observation, the Descemet's membranes were bare and almost no cells were detected. The corneal stromal layer had severe edema and a lot of inflammatory cells. Collagen fibers were irregularly arranged, layered, and broken. Stromal cells had an abnormal morphology. The corneal epithelial layer was also vacuolate (Fig. 3F).

Functional pattern of gene expression in HCEC, CEC-like cells, EXP1 cells, and EXP2 cells

Venn analysis was carried out to show co-expressed and specially expressed genes between samples or groups. The GO annotation analysis showed the top 20 GO sets of abundance in specially expressed genes, including classification of the biological process, cellular component, and molecular function.

The Venn analysis of HCEC and CEC-like cells showed that 71.6% of all expressed genes were co-expressed. The number of specific genes of CEC-like cells and HCEC was 1671 and 3186, accounting for 9.8% and 18.6% (Fig. 4A). The GO annotation analysis of specific genes in the two groups showed that the abundance ranking in the two groups was almost the same with only few differences. The percent of the signaling and developmental process ranked higher in CEC-like cells than that of HCEC. The percent of the extracellular region ranked higher than the catalytic activity in CEC-like cells, whereas in HCEC they were the opposite (Fig. 4B).

The Venn analysis of CEC-like/EXP1/ EXP2 cells showed that 79.3% of all expressed genes were co-expressed. The number of specific genes of CEC-like/EXP1/ EXP2 cells was 356 /508/519, accounting for 2.1%/3.0%/3.1%, respectively (Fig. 4C). The GO annotation analysis of specific genes in the three

groups showed that the abundance ranking was similar with only a few differences. The percent of signaling and positive regulation among biological process ranked higher in CEC-like cells. However, the macromolecular complex and extracellular region among cellular component ranked higher in EXP1 and EXP2 (Fig. 4D).

The Venn analysis of HCEC/EXP1/ EXP2 cells showed that 68.4% of all expressed genes were co-expressed. The number of specific genes of HCEC/EXP1/ EXP2 cells was 2119/392/414, accounting for 19.1%/2.1%/2.2%, respectively (Fig. 4E). Through GO annotation analysis it was found that the abundance ranking was similar to that in CEC-like/EXP1/ EXP2 cells GO annotation analysis (Fig. 4F).

The correlation analysis was carried out to detect the relativity between samples or groups. The r value between each biological repeat sample of HCEC and CEC-like cells was more than 0.98 (data not shown), demonstrating good biological repeatability. The mean r value between CEC-like cells and EXP1 or EXP2 cells was both about 0.954. The r value between EXP1 and EXP2 cells was 0.991. The mean r value between CEC-like cells and HCEC was about 0.87, but after 1–2 years transplantation the r value between EXP1 or EXP2 cells and HCEC was 0.873 and 0.874, respectively (Fig. 4G).

Differential expression gene analysis of CEC-like cells, EXP1 cells, and EXP2 cells

Expression variance analysis was used to detect differential expression genes (DEGs). Genes with $FDR < 0.05$ and $|\log_2FC| \geq 1$ were considered as significant. GO enrichment analysis was carried out to obtain the GO Term of significant DEGs enrichment.

4566 significant DEGs were detected between CEC-like cells and EXP1 cells, among which 3385 (74.1%) genes were up-regulated and 1181 (25.9%) were down-regulated. The GO enrichment analysis showed that the significant DEGs in EXP1 cells vs CEC-like cells mainly took part in regulation of water loss, mitotic recombination, sensory organ morphogenesis, and kidney development (Fig. 5B C).

4524 significant DEGs were detected between CEC-like cells and EXP2 cells, among which 3360 (74.3%) genes were up-regulated and 1164 (25.7%) were down-regulated. Significant DEGs in EXP2 cells vs CEC-like cells mainly took part in cell-cell junction organization/assembly, cell adhesion, chromosome segregation, pattern specification, and DNA replication (Fig. 5D E).

19 significant DEGs were detected between EXP1 cells and EXP2 cells, among which 12 genes were up-regulated and 7 were down-regulated. The genes could not be GO enriched.

Discussion

In our previous study, we invented a creative method to acquire CEC-like cells. We co-cultured skin-derived precursors (SKPs) with B4G12 cells using Transwell inserts in human endothelial SFM. B4G12 cells were for the first time applied in cell induction and the serum free medium were for the first time was used for corneal endothelial cells culture. The CEC-like cells we acquired had similar morphology and characteristics to normal human CECs. CEC-like cells could be passaged in vitro. CEC-like cells could

survive after being injected into anterior chamber of the monkey corneal endothelial dysfunction model and had an excellent therapeutic effect within 3 months[10].

In this study, we prolonged the observation to 2 years and discovered that the CEC-like cells still worked. The cornea remained transparent and its thickness was constant. This investigation indicates that the cells were stable and could adapt to the microenvironment after transplantation. In addition, there was no apparent immune rejection during observation period. Few inflammatory keratic or anterior chamber precipitates appeared during observation period. This demonstrates the low immunogenicity of CEC-like cells. This may be due to that SKPs' ability to suppress T-lymphocytes activation[17] had been passed to CEC-like cells. On the other hand, the anterior-chamber-associated immune deviation (ACAID) mechanism, the less irritating surgical procedure, and the serum free medium culture of CEC-like cells were also factors that could reduce cell immunogenicity[18–20].

The count of corneal endothelial cells gradually increased within 3 months, indicating that the injected CEC-like cells survived well in the microenvironment and still proliferated early after transplantation. During 3 months to 1 year, the cell density significantly decreased, possibly because CEC-like cells stopped proliferating and some CEC-like cells that could not adapt to the microenvironment gradually died. After 1 year, the rest of the CEC-like cells had adapted and the cell density tended to be stable remaining above 2000 cells/mm² within 2 years. A few cells died possibly due to the physiological aging process[21]. The cell density of corneal endothelial cells in normal adults is above 2000 cells/mm². Therefore, the cell density in our experiment conforms to the normal physiological state.

To verify that the origin of endothelial cells in the long-term experimental group, we extracted the endothelial cell DNA for PCR and sequencing. Nucleotide sequences of human and animal mitochondrial cytochrome b genes are known to be species-specific and have been used for species identification in forensic investigations[22, 23]. In order to distinguish human and monkey species, we designed specific Cytb primers. The results showed that the corneal endothelial cells in the experimental group contained both CEC-like cells and monkey cells. This suggests that CEC-like cells can survive and persist in the monkey corneal dysfunction model for a long time, despite a few residual monkey cells. Because of the lack of tracking technology, we were unable to determine the percentage of CEC-like cells and monkey cells. Perhaps the relatively stable cell density after 2 years is a result of the peaceful coexistence of CEC-like cells and monkey cells.

Transcriptome sequencing was also used in this study to analyze the gene expression pattern of HCEC, CEC-like cells, and cells after transplantation (EXP1, 2). The three Venn analyses showed a high proportion of co-expressed genes in these cells. This indicates that the gene expression pattern of the SKP-derived CEC-like cells are similar to HCEC and after transplantation CEC-like cells still retain their characteristics. The three GO annotation analyses of specially expressed genes showed the top 20 GO sets of abundance, including classification of biological process, cellular component, and molecular function. The abundance ranks in the three analyses were almost same with only a few differences. This also indicates the similarities between these four kinds of cells. The gene transcriptional sequencing

analysis reveals the reason why the CEC-like cells can effectively work on the primate models and work so long.

Correlation analysis provides basic reference for the analysis of differential genes. The correlation analysis showed a high correlation between HCEC, CEC-like cells, and cells after transplantation (EXP1, 2), suggesting a high similarity of their gene expression pattern. The r value between EXP1 and EXP2 cells reached 0.991, indicating that cells tended to be stable after one year of transplantation. The r value between EXP cells and HCEC was higher than that between CEC-like cells and HCEC, suggesting that long after transplantation these CEC-like cells become more and more similar to human corneal endothelial cells. This might be due to the influence of the monkey anterior chamber microenvironment *in vivo*[24]. CEC-like cells retained their characteristics after transplantation and only slightly changed. Thus, we carried out gene differential analysis of CEC-like cells and EXP cells. Differential analysis showed that most (EXP1:74.1%, EXP2:74.3%) genes were up-regulated and a few were down-regulated. The up-regulated genes function focused on mitotic recombination, DNA replication, organ development and regulation, cell junction, and adhesion. Therefore, CEC-like cells still proliferated and cell attachment was enhanced after transplantation.

Although Descemet's stripping automated endothelial keratoplasty (DSAEK) and Descemet membrane endothelial keratoplasties (DMEK) are now the most commonly used surgical method of endothelial keratoplasty[25–27], the anterior chamber injection method may be a very promising method to be clinically used. Recently, Okumura reported serial studies that confirmed injection of cultivated corneal endothelial cells supplemented with the Rock inhibitor (Y-27632) into rabbit or monkey anterior chambers could exert good therapeutic effect for corneal endothelial dysfunction[28–31]. Their clinical trials also succeeded in 11 corneal endothelial dysfunction patients[32]. The anterior chamber injection method is simple and easy to do. In our study, we not only applied the anterior chamber injection method but also used our novel CEC-like cells. CEC-like cells could be produced abundantly and avoid using cultivated human endothelial cells. What is more, the monkey is the species most similar to humans[33]. Thus, our research is preclinical research that is significantly meaningful for cell-based therapy of corneal endothelial dysfunction. We are ready do clinical trials in the near future.

Conclusions

CEC-like cells could adapt to the microenvironment and had a good therapeutic effect after being transplanted into the monkey endothelial dysfunction model during long-term observation. The cornea remained transparent and cell density remained in a normal range. The gene expression pattern of CEC-like cells was similar to HCEC and slightly changed after transplantation. We have acquired a deep understanding of CEC-like cells in terms of gene, protein, and animal experimentation. Our researches provided a promising prospect of cell-based therapy with CEC-like cells for corneal endothelial dysfunction. The renewable cell source, novel derivation method and simple treatment strategy may be clinically applied in cellular replacement therapy or regenerative medicine in the future.

Abbreviations

ACAID: anterior-chamber-associated immune deviation; CEC-like cells: endothelial cell-like cells; DNA: deoxyribonucleic acid; DSAEK: Descemet's stripping automated endothelial keratoplasty; DMEK: Descemet membrane endothelial keratoplasties; SKPs: skin-derived precursors; FITC: Fluorescein isothiocyanate; HCECs: human endothelial cells; H&E: Hematoxylin and eosin; OCT: Optical coherent tomography; PBS: Phosphate-buffered saline; PCR: Polymerase Chain Reaction; RNA: Ribose Nucleic Acid.

Declarations

Ethics approval and consent to participate

All animals were treated in accordance with the ARVO (Association for Research in Vision and Ophthalmology) Statement for the Use of Animals in Ophthalmic and Vision Research. All animal experiments were approved by the Medical Ethics Committee of Qilu Hospital of Shandong University, China. Samples were collected following written informed consents, and the study was performed in adherence to the Declaration of Helsinki. All the experimental protocols were approved by the Ethical Committee of Qilu Hospital of Shandong University and were conducted following the institutional guidelines.

Consent for publication

Not applicable.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

LS and XW were responsible for conception and design. PS, LS cultured CEC-like cells SKPs. LS, PS, and LD performed the animal experiment. LS, JZ, HG and KP were responsible for assembly of data, and data analysis and interpretation. PS and LS were responsible for manuscript writing. LS and XW were responsible for revision of the manuscript. XW was responsible for provision of study material. All authors read and approved the final manuscript.

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Table

Table.1 Primers used for PCR

	Forward	Reverse
Homo-Cytb	5'-GGCATTATCCTCCTGCTTGC-3'	5'-GATGTGGGGAGGGGTGTTTA-3'
Macaca-Cytb	5'-ACCCAGACAACTACATCCCG-3	5'-AAGAGTGCTAGTACGCCTCC-3'

Figures

Figure 1

Long-term observation of CEC-like cells transplantation into the monkey corneal endothelial dysfunction model

Figure 2

Long-term observation of CEC-like cells transplantation into the monkey corneal endothelial dysfunction model

Figure 3

Polymerase chain reaction and DNA-sequencing after transplantation

Figure 4

Functional pattern of gene expression in HCEC, CEC-like cells, EXP1 cells, and EXP2 cells

Figure 5

Differential expression gene analysis of CEC-like cells, EXP1 cells, and EXP2 cells