·Basic Research·

Effects of corneal stromal cell- and bone marrow-derived endothelial progenitor cell-conditioned media on the proliferation of corneal endothelial cells

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Abstract

• **AIM: To explore the effects of conditioned media on the proliferation of corneal endothelial cells (CECs) and to compare the efficiency of different conditioned media (CM).**

• **METHODS: Rat CECs, corneal stromal cells (CSCs), bone marrow-derived endothelial progenitor cells (BEPCs), and bone marrow-derived mesenchymal stem cells (BMSCs) were isolated and cultured *in vitro*. CM was collected from CSCs, BEPCs, and BMSCs. CECs were cultivated in different culture media. Cell morphology was recorded, and gene and protein expression were analyzed.**

• **RESULTS: After grown in CM for 5d, CECs in each experimental group remained polygonal, in a cobblestone-like monolayer arrangement. Immunocytofluorescence revealed positive expression of Na^+/K^+-ATP, aquaporin 1 (AQP1), and zonula occludens 1 (ZO-1). Based on quantitative polymerase chain reaction (qPCR) analysis, Na^+/K^+-ATP expression in CSC-CM was notably upregulated by 1.3-fold (±0.036) (P<0.05, n=3). The expression levels of ZO-1, neuron specific enolase (NSE), Vimentin, paired homebox 6 (PAX6), and procollagen type Ⅷ (COL8A1) were notably upregulated in each experimental group. Each CM had a positive effect on CEC proliferation, and CSC-CM had the strongest effect on proliferation.**

• **CONCLUSION: CSC-CM, BEPC-CM, and BMSC-CM not only stimulated the proliferation of CECs, but also maintained the characteristic differentiated phenotypes necessary for endothelial functions. CSC-CM had the most notable effect on CEC proliferation.**

• **KEYWORDS:** conditioned medium; corneal endothelial cell; corneal stromal cell; bone marrow-derived endothelial progenitor cell; proliferation

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INTRODUCTION

The corneal endothelium is a physiologically important part of the cornea; it has an essential role in maintaining corneal clarity. To maintain the transparency of the cornea, the corneal endothelium needs to maintain the unique contact-inhibited monolayer, which has active pump and barrier functions. Additionally, the endothelial cell density (ECD) must remain above 400-500 cells/mm^2 [1]. However, the proliferation of corneal endothelial cells (CECs) *in vivo* is limited. Corneal endothelium decompensation resulting from the aging process or trauma ultimately leads to an inability to maintain its barrier and pump functions. This leads to a critical loss in ECD, corneal edema, bullous keratopathy, Fuchs' dystrophy, and reduced visual acuity. The current solution to restore vision is to replace dysfunctional endothelium with healthy donor corneal endothelium through a corneal transplant. With rapid advancements in endothelial keratoplasty, various methods for endothelial cell transplantation have been developed. These methods are aimed at providing a less invasive keyhole surgery option for the selective replacement of the corneal endothelial layer to minimize complications associated with penetrating keratoplasty [1]. They include Descemet's membrane endothelial keratoplasty, Descemet's stripping endothelial keratoplasty, deep anterior lamellar endothelial keratoplasty, and posterior lamellar keratoplasty[2-5]. The global shortage of donor corneal tissues for transplantation has become more severe, which greatly restricts the number of corneal transplantations that are performed. Accordingly, many researchers worldwide have sought to establish optimum methods for the *in vitro*

cultivation of CECs that can be used for transplantation, with the goal of developing a new clinical therapy for corneal endothelial dysfunction.

The proliferative capacity of human CECs is limited; CECs *in vivo* do not exit the cell cycle, but are arrested in the G1 phase [6]. Furthermore, CECs are difficult to culture using standard tissue culture techniques [7]. Bone marrow mesenchymal stem cell (BMSC)-derived conditioned medium (CM) promotes CEC expansion, indicating that CEC proliferation can be stimulated *via* the regulation of G1 proteins of the cell cycle [8]. CM developed from human BMSCs can be partially attributed to the progenitor cell characteristics and secreted cytokines [9]. Our previous research has revealed that bone marrow-derived endothelial progenitor cells (BEPCs) co-cultured with CECs can differentiate into corneal endothelial-like cells [10-11]. Furthermore, corneal stromal cells (CSCs), which are components of the corneal endothelial microenvironment [12], can be induced into a functional tissue-engineered corneal endothelium [13]. These findings confirm that the proliferative potential of CECs can be stimulated and that such cells can be cultivated *in vitro*. To our knowledge, although a variety of methods to expand CECs *in vitro* have been explored, no studies have assessed the effect of CM obtained from CSCs and BEPCs on CECs proliferation, and different CMs have not been compared with respect to their efficiency in stimulating CECs proliferation.

In the present study, we provide evidence suggesting that CM obtained from CSCs and BEPCs stimulate CECs proliferation while maintaining the contact-inhibited monolayer with functional adherent junctions and pump functions. We also compare the proliferative effect of CSC-CM, BEPC-CM, and BMSC-CM on cultivated CECs. This study was aimed at finding more effective culture methodologies to expand proliferative, functional CECs, which may lead to the development of a novel clinical therapy for corneal endothelial dysfunction.

MATERIALS AND METHODS

Animals Sprague-Dawley (SD) rats aged 6wk were obtained from the Shanghai Tissue Engineering Animal Laboratory in Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine. All animals were treated with care, and all protocols complied with the institutional guidelines. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Shanghai Jiao Tong University.

Corneal Endothelial Cells Cultures CECs were obtained from the corneas of SD rats. The corneal endothelium was stripped from the cornea and incubated with 0.2% collagenase A (Roche Applied Science, Penzberg, Germany) at 37℃ overnight. Then, CECs were treated with 0.25% trypsin-EDTA (Gibco, Grand Island, NY, USA) for 6min at 37℃ and washed with OptiMEM-I (Life Technologies, Carlsbad, CA, USA). CECs obtained from the corneas of 24 SD rats were resuspended in basal growth medium [OptiMEM-I with 8% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA)] and plated into each well of a 6-well plate [14-15]. Cells were maintained at 37℃ in a 5% CO_2 humidified atmosphere, and the culture medium was replaced with fresh medium every 2d. When the cells reached confluence in 7d, they were treated with 0.25% trypsin-EDTA for subculturing, and seeded at a ratio of 1:2. CECs at the second passage were used for the experiment.

Corneal Stromal Cells Culture and Preparation of Corneal Stromal Cells-conditioned Media CSCs were obtained from 6-week-old SD rats, and cultured in dulbecco's modified eagle medium (DMEM; Life Technologies, Carlsbad, CA, USA) supplemented with 10% FBS. The culture medium was replaced with fresh medium every 2d to remove unattached cells. CSCs were then subcultured by treatment with 0.25% trypsin-EDTA after 4d, and seeded at ratio of 1:2 to 1:3. CSCs at the second passage were used to collect CM.

CSCs were treated with 0.25% trypsin-EDTA and subcultured; they were seeded at a ratio of 1:2 with DMEM. When CSCs reached 50% confluence, the medium was replaced with basal growth medium containing OptiMEM-I and 8% FBS. The CSCs were maintained for an additional 24h. The medium was collected and centrifuged at 1000 rpm for 10min, and the supernatant was filtered through a 0.22-μm filtration unit (EMD Millipore Corporation, Billerica, MA, USA) and used as CSC-CM.

Bone Marrow-derived Endothelial Progenitor Cell Culture and Preparation of Bone Marrow-derived Endothelial Progenitor Cells-conditioned Media Primary BEPCs were prepared according to previously published methods [10-11]. Briefly, limb bone marrow samples from 6-week-old SD rats were separated, washed, and dispersed with phosphate-buffered saline (PBS; Gibco, Grand Island, NY, USA). Next, mononuclear cells were isolated from the tissue samples by Histopaque density gradient centrifugation (1.083 g/mL, Sigma-Aldrich, St. Louis, MO, USA) [11]. The cells were suspended in EGM-2 culture medium (Clonetics, Lonza, Walkersville, MD, USA) enriched with 10% FBS (HyClone, Logan, UT, USA), hydrocortisone, human fibroblast growth factor-basic (hFGF-B), vascular endothelial growth factor (VEGF), long R3 insulin-like growth factor-1 (R3-IGF-1), ascorbic acid, human epidermal growth factor (hEGF), and gentamycin and amphotericin (GA-1000) on 6-well plates precoated with 0.2 mg/mL human plasma fibronectin (EMD Millipore Corporation, Billerica, MA, USA) and maintained at 37℃ in a 5% CO_2 humidified

atmosphere. The culture medium was replaced with fresh medium every 4d to remove unattached cells. When the cells reached 70%-80% confluence, they were treated with 0.25% trypsin-EDTA for subculturing, and seeded at a ratio of 1:2.

BEPCs after 1 passage were used for the experiment. BEPCs were treated with 0.25% trypsin-EDTA for subculturing and seeded at a ratio of 1:2 with EGM-2. When BEPCs reached 50% confluence, the medium was replaced with basal growth medium (OptiMEM-I with 8% FBS). The BEPCs were maintained for an additional 24h. The cultured medium was collected and centrifuged at 1000 rpm for 10min, and the supernatant was filtered through a 0.22-μm filtration unit and used as BEPC-CM.

Bone Marrow –derived Mesenchymal Stem Cells Culture and Preparation of Bone Marrow –derived Mesenchymal Stem Cells –conditioned Media Primary BMSCs were prepared according to previously published methods [16]. Briefly, BMSCs were obtained from limb bone marrow of SD rats aged 6wk, and cultured in modified eagle medium (MEM) Alpha (Life Technologies, Carlsbad, CA, USA) supplemented with 10% FBS. The culture medium was replaced with fresh medium every 4d to remove unattached cells. When the cells reached 70% -80% confluence, they were treated with 0.25% trypsin-EDTA for subculturing, and seeded at a ratio of 1:2. Cells at the first passage were used for the experiment.

BMSCs were treated with 0.25% trypsin-EDTA for subculturing and seeded at a ratio of 1:2 with MEM Alpha. When BMSCs reached 50% confluence, the medium was replaced with basal growth medium. The BEPCs were maintained for an additional 24h. The medium was collected and centrifuged at 1000 rpm for 10min, and the supernatant was filtered through a 0.22-μm filtration unit and used as BMSC-CM.

Experimental Group Based on the medium used to culture CECs, a control group and three experimental groups were established. For the control group, corneal basal growth medium OptiMEM-I was used. The experimental groups used CSC-CM, BEPC-CM, or BMSC-CM respectively. CECs were seeded on a single well of a 6-well plate at a density of 2×10^4 cells/cm^2 with 5 mL of CM and maintained for the same time period in each experiment.

Immunocytochemistry CECs after 2 passages were seeded on 18-mm glass coverslips (VWR, West Chester, PA, USA) coated with laminin (Sigma-Aldrich, St. Louis, MO, USA) in 12-well plates and maintained for 24h. The medium was replaced with CEC basal growth medium, CSC-CM, BEPC-CM, or BMSC-CM respectively. Culture medium was changed every 2d. After 5d of culture, when the cells were 70%-80% confluent, they were fixed with 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA), permeabilized with 0.3% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) in PBS, and blocked with 10% normal goat serum (Invitrogen, CA, USA) [17]. Next, the cells were incubated with rabbit polyclonal anti-ZO-1 (1:50; Santa Cruz Biotechnology, CA, USA), mouse monoclonal anti-aquaporin 1 (AQP1) (1:100; Abcam, Cambridge, MA, USA), and mouse polyclonal anti-alpha 1 Sodium Potassium ATPase (ATP1A1; 1:100; Abcam, Cambridge, MA, USA) at 4℃ overnight. The next day, cells were incubated with fluorescently labeled secondary antibodies (1:800; Alexa Fluor 546-goat anti-mouse/rabbit, BD, San Jose, CA, USA). The cells were then rinsed 3 times in PBS, and cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen). Negative controls were performed in parallel using the same protocol but without the primary antibody. Immunoreactive cells were visualized and imaged using a fluorescence microscope (Olympus BX51, Tokyo, Japan). Additionally, the percentage of positive cells was estimated using Image-Pro Plus 6.0 (Media Cybernetics, Bethesda, MD, USA), which automates cell counting after merging images of immunopositive cells with DAPI-stained nuclei and immunopositive cells treated with primary antibodies.

Total RNA Extraction and Reverse Transcription Polymerase Chain Reaction Human CECs after 2 passages were cultured in basal growth medium and maintained for 1d, and the medium was replaced with CEC basal growth medium, CSC-CM, BEPC-CM, or BMSC-CM respectively. The cultures were maintained for 5d. Total RNA from each sample was extracted by Trizol reagent (Invitrogen). The concentration and purity of the total extracted RNA was assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) at ODs of 260 nm and 280 nm. Samples with OD 260/280 nm ratios between 1.9 and 2.1 were used for cDNA synthesis.

One microgram of total RNA extracted from CECs was reverse transcribed using the PrimeScript™ RT Reagent Kit (Perfect Real Time; TaKaRa, Dalian, Liaoning Province, China)[18]. After reverse transcription, 1 μL of cDNA diluted 10-fold in nuclease-free water (Invitrogen) was used as a template for quantitative polymerase chain reaction (qPCR), which was performed using the 7500 Real-Time PCR System (Applied Biosystems, Foster, CA, USA) in a total volume of 20 μL containing 10 μL of 2×Power SYBR Green PCR Master Mix (Applied Biosystems), 10 μL of diluted cDNA, and 300 nmol/L gene-specific primers. The primer sequences are shown in Table 1. The genes encoding Na^+/K^+-ATP and AQP1 were used to detect pump function. ZO-1 was used to detect barrier function. Procollagen type Ⅷ (COL8A1) was used to examine the secretion function of Descemet's membrane in endothelial cells. Neuron specific enolase (NSE) was used to identify the cell type. Vimentin and paired homebox 6 (PAX6) were used to detect

Table 1 Primers used for qPCR

Genes	Accession No.	Forward (5'-3')/reverse (5'-3')	Product size (bp)
Na^+/ K^+-ATP	NM_012504.1	TACATGGCAGTGACTTGAAGGACA CTTCTGTTGAGGAGAGGTCCTAGC	101
AQP1	NM_012778.1	ACCTGCTGGCCATTGACTAC AGGGCACTCCCAATGAATGG	127
ZO-1	NM_001106266.1	ATGACCGAGTCGCAATGGTT TCTATCCCTTGCCCAGCTCT	263
NSE	NM_139325.3	CCCGATGCATCACTGGGGAC GGGTTGGTCACCGTCAGGTC	172
COL8A1	NM_001107100.1	TTGCTTACCATGTTCACTGCAAGG AAAGCCCTTCTTGTACTCGTCGTA	101
Vimentin	NM_031140.1	GAGCTGAATGACCGCTTCGC ACGGGCCTTGTCATTGGTGA	186
PAX6	NM_0130012.2	CCGAATTCTGCAGGTGTCCA AGTCGCCACTCTTGGCTTAC	112
Ki67	NM_001271366.1	GGGCAGCTTCTACCAAGAGG GCATCAAACTTGGGGCTTGG	214
GAPDH	NM_017008.4	CATGTTTGTGATGGGTGTGAACCA AAAGTTGTCATGGATGACCTTGGC	115

the self-renewal ability. PCR efficiency was measured with primers using serial dilutions of cDNA (1:1, 1:5, 1:25, 1:125, 1:625, and 1:3125). Each sample was tested in triplicate. The relative mRNA or microRNA expression levels were analyzed using the Pfaffl method [19]. The relative mRNA or microRNA levels are expressed as fold-changes relative to the untreated controls after normalization to the expression of β-actin or 5S rRNA[17], respectively.

Cell Proliferation The effect of CM on CEC proliferation was assessed using the cell counting kit (CCK-8; Dojindo, Kumamoto, Japan). Human CECs were cultured at a density of 5000 cells/well in a 96-well plate in the presence or absence of CM derived from CEC basal growth medium, CSC-CM, BEPC-CM, or BMSC-CM. After treatment with 4 different CM types, the CCK-8 solution was added to each well at days 0, 1, 2, and 3 of the culture period. Then, the cells were incubated for another 4h at 37℃ according to the reagent instructions and absorbance at 450 nm was measured using an enzyme-linked immuno sorbent assay (ELISA) microplate reader (ELX800, BioTeK, Winooski, VT, USA). The cell viability was directly proportional to the absorbance at 450 nm; therefore, the viability was expressed as the A450 value.

Statistical Analysis The results are expressed as the mean± standard derivation (SD). Each experiment was repeated at least three times, unless otherwise specified. Statistical significance of the differences in CEC expression between the experimental and control groups was analyzed using the Student's *t* -test (P <0.05 and P <0.01 were deemed to indicate statistical significance).

RESULTS

Shapes of Corneal Endothelial Cells Cultured *in Vitro* CECs were collected from the corneal endothelia of 24 rats and cultured in 6-well plates. Three days after the initial plating, CECs grew as isolated, oval-shaped colonies. The cells were passaged after 7d in culture. After 3d, the CECs of the first passage that reached confluence were polygonal in appearance. These results indicated that the CECs cultured *in vitro* maintain their morphology and viability.

BEPC-CM, BMSC-CM, and CSC-CM Maintain the Corneal Endothelial Phenotype During *in Vitro* Expansion CECs were maintained in basal growth medium, BEPC-CM, BMSC-CM, or CSC-CM for 5d. Inverted phase-contrast microscopy revealed that a portion of CECs in the control group (Figure 1A) exhibited a loss of the characteristic polygonal cell morphology and had irregular cell shapes, whereas the morphology of CECs maintained in CSC-CM, BEPC-CM, and BMSC-CM assumed a contact-inhibited monolayer of hexagonal cells, similar to corneal endothelial cells *in vivo* (Figure 1B-D). These results indicated that CECs cultivated in CM maintain the characteristic polygonal cell morphology.

Protein and mRNA Expression Levels in Corneal Endothelial Cells After Culturing In order to examine the pump function and intercellular adherent junctions of CECs after cultivation in CMs, we detected Na^+/K^+-ATP, AQP1, and ZO-1 expression. Immunocytofluorescence revealed that CECs cultured with different CMs all express Na^+/K^+-ATP (Figure 2A-D), AQP1 (Figure 2E-H), and ZO-1 (Figure 2I-L). The qPCR showed that Na^+/K^+-ATP expression in CSC-CM

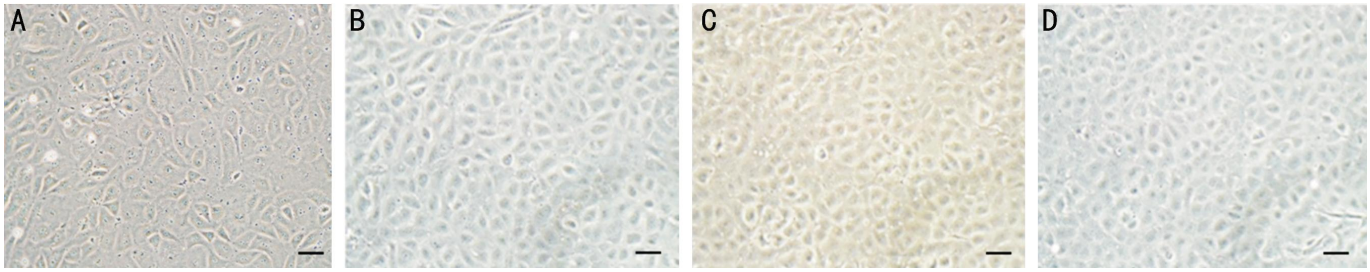

Figure 1 BEPC−CM, BMSC−CM and CSC−CM maintain corneal endothelial phenotype *in vitro* expansion After CECs maintained in the four culture media for 5d, inverted phase-contrast microscopy was used to compared phenotype of CECs. A: CECs cultured in control; B: CECs cultured in CSC-CM; C: CECs cultured in BEPC-CM; D: CECs cultured in BMSC-CM. Scale bars: 50 μm.

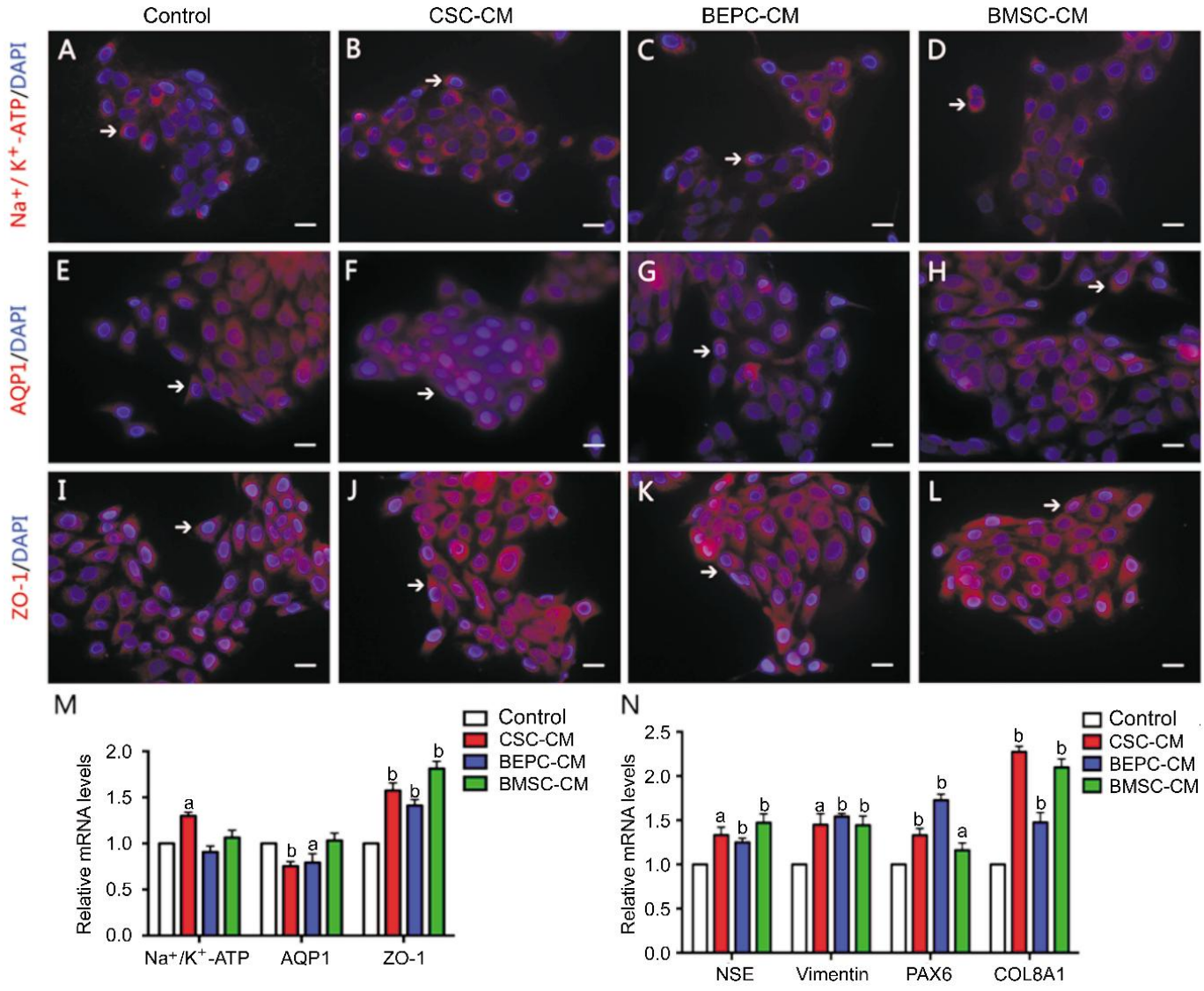

Figure 2 Expression of proteins and mRNA levels in CECs after cultured in four CMs A-L: Five days after CECs were grown in CMs, the markers Na^+/ K^+-ATP, AQP1 and ZO-1 were evaluated by immunostaining analysis. The cells were immunostained with antibodies against Na^+/ K^+-ATP, AQP1 and ZO-1 with red fluorescence. Cell nuclei were counterstained with DAPI showing blue fluorescence. Scale bars: 50 μm. M: The qPCR results revealed that the expression level of Na^+/ K^+-ATP and AQP1 changed slightly in CM-cultured compared to control group. The expression level of ZO-1 increased approximately 1-fold in the four CMs, especially in BMSC-CM. N: The expression levels of NSE, COL8A1, vimentin and PAX6 increased in the four CMs. Among them, COL8A1 expression level increased approximately 2-fold in CSC-CM and BMSC-CM. Error bars indicate the standard deviation of the mean. $^aP<0.05$, $^bP<0.01$ *vs* control by Student's *t*-test.

was upregulated. AQP1 expression was downregulated in CSC-CM and BEPC-CM. ZO-1 expression was upregulated in each experimental group (Figure 2M). These results indicated that the pump function of CECs changed after cultivation in CMs and intercellular adherent junctions were enhanced. To further characterize the CEC changes, we examined the expression of NSE, vimentin, PAX6, and COL8A1, which are corneal endothelial-related markers, in

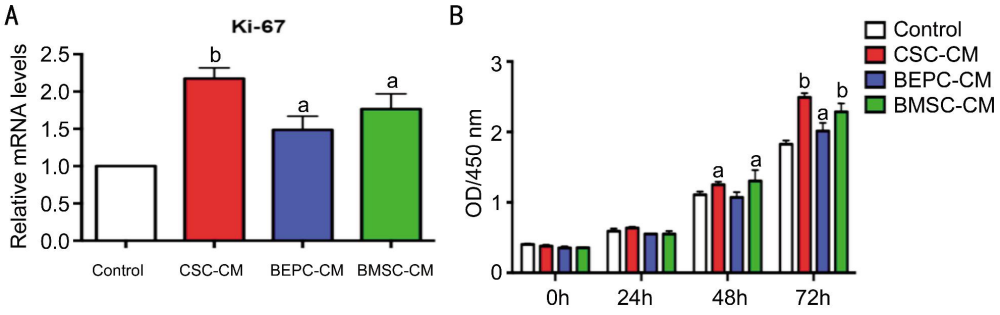

Figure 3 Effect of the four CMs on the proliferation of CECs Proliferative potential in the four CMs were assessed by Ki67 gene expression and CCK-8 analysis A: The qPCR results revealed that the expression level of Ki67 increased approximately 2-fold in CSC-CM, 1.5-fold in BMSC-CM and 0.5-fold in BEPC-CM; B: The proliferation ability of CECs cultivated in the four CMs were assessed using CCK-8 analysis. The proliferation ability of CECs was obviously increased in CSC-CM and following by BMSC-CM and BEPC-CM in 48 and 72h cultures under proliferation conditions. Error bars indicate the standard deviation of the mean. $^{a}P<0.05$ and $^{b}P<0.01$ *vs*control by Student's *t*-test.

CECs cultivated in CMs by qPCR. The results of the qPCR analysis (Figure 2N) showed that in CECs cultured in CSC-CM, BEPC-CM, and BMSC-CM, the expression levels of NSE (1.33±0.064, 1.248±0.054, and 1.471±0.078, respectively; $P<0.05$, $n=3$), vimentin (1.449±0.139, 1.541±0.039, 1.444±0.114, $P<0.05$, $n=3$), and PAX6 (1.329±0.084, 1.726±0.07, 1.16±0.094, $P<0.05$, $n=3$) were significantly higher in the experimental groups than in the control group. The expression levels of COL8A1 were notably upregulated by more than 2-fold (2.273±0.063, 2.098±0.039, respectively; $P<0.05$, $n=3$) in cells cultured in CSC-CM and BMSC-CM.

Effect of Conditioned Medias on Corneal Endothelial Cells Proliferation CECs were cultured in basal growth medium, CSC-CM, BEPC-CM, or BMSC-CM. Compared to CECs maintained in basal growth medium, qPCR analyses (Figure 3A) showed that the expression of Ki67 was most highly upregulated (> 2-fold) in CSC-CM, followed by in BMSC-CM and BEPC-CM. The CCK8 analysis also demonstrated a positive effect of CMs with respect to CEC proliferation (Figure 3B), especially for CSC-CM. These results indicated that, among the four experimental groups, CSC-CM has the most positive effect on proliferation.

DISCUSSION

The cornea is mainly composed of three tissue layers: the outer stratified squamous epithelium, the intermediate stroma, and the inner endothelium[20]. The corneal endothelial monolayer helps to maintain corneal transparency *via* its barrier and ionic pump functions [21]. Due to limited CEC proliferation, cell's enlargement and migration are the major means of endothelial monolayer repairation [22]. CEC loss during the aging process or trauma results in a critical reduction in ECD, corneal edema, bullous keratopathy, Fuchs' dystrophy, and a loss of visual acuity[23-25]. Endothelial function is eventually compromised. The current solution for the restoration of vision is to replace the dysfunctional endothelium with a healthy donor corneal endothelium through a corneal transplant [26]. However, a global shortage of donor corneas, corneal graft rejection, and continual cell damage that occurs after transplantation greatly restrict the number of corneal transplantations that are performed [27]. Therefore, there is great clinical interest in the development of an effective method to improve CEC proliferation *in vitro* to solve the shortage of corneal transplant material[15].

CECs are arrested at the G1 phase of the cell cycle, and this characteristic property indicates their potential of proliferate in response to growth factor stimulation. A wide variety of culture media as well as various cell factors affect the growth and proliferation of CECs [20]. Corneal stroma is localized in the anterior region near the endothelium of the cornea and is a component of the corneal endothelial microenvironment[12]. A small population of stem cells in the stroma displays properties of mesenchymal stem cells. Additionally, both CECs and CSCs originate from neural crest-derived mesenchymal cells [20], but have distinct phenotypes and functions in the cornea [28]. The functional corneal endothelium can be derived from mouse and human corneal stroma stem cells [13]. Therefore, we inferred that some cytokines secreted by CSCs affect the proliferation of CECs. The use of pluripotent stem cells is also a popular direction in CEC regeneration research. BMSC-CM has a positive effect on CEC expansion. Another subgroup of pluripotent stem cells is BEPCs, which have similar morphologies and functionality as CECs; both function as carriers in nutrition exchange and as liquid barriers. In our recent study, we co-cultured BEPCs with CECs [10-11]. After 10d of induction, BEPCs resembled CECs, they were polygonal and expressed characteristic CEC genes, indicating the differentiation potential of BEPCs into corneal endothelial-like cells. However, CM obtained from BEPCs has not been studied. In the present study, we used fresh isolated BEPCs, BMSCs, and CSCs from SD rats to obtain CM. Then, we cultivated fresh CECs obtained from SD rats in the CM for 5d.

Various CEC properties have been described, such as their polygonal cell shape, pump function, barrier function,

components of Descemet's membrane secreted by endothelial cells, and NSE expression[11]. In order to observe the effect of CSC-CM and BEPC-CM on CEC proliferative ability and determine the relative efficiency of each CM, several tests were performed to compare CEC characteristics.

In our study, the CECs reached contact inhibition in each CM 3-4d after seeding. In this period of time, the CECs appeared hexagonal in CSC-CM, BEPC-CM, and BMSC-CM. The adherent CECs that proliferated in each CM showed similar growth dynamics, despite the large differences in the formulation of each medium.

Na^+/K^+-ATP and AQP1 are associated with the pump functions of CECs [29-30]. The endothelial pump function prevents corneal stroma swelling by removing excess stromal fluid *via* the activity of bicarbonate-dependent Mg^{2+}-ATPase and Na^+/K^+-ATPase. Except as sodium and bicarbonate pumps, aquaporins also participate in fluid movement across the endothelium. Aquaporins are integral membrane proteins that act as water-selective channels. Several isoforms of aquaporins have been identified and one of these, AQP1, is expressed in CECs and lens epithelial cells [31]. AQP1 expression is reduced in human corneas with endothelial disease, but not in human corneas with corneal disease of a non-endothelial nature [32]. Our study indicated that the pump function of CECs changed slightly after cultivation in CSC-CM and BEPC-CM, and intercellular adherent junctions were enhanced.

CECs also have a barrier function *via* tight junctions (ZO-1)[29]. All CMs in our study had positive effects on the barrier function of CECs, especially BMSC-CM.

Collagen VIII, a component of Descemet's membrane, is secreted by endothelial cells [33]. CSC-CM and BMSC-CM result in notably increased collagen VIII secretion, and BEPC-CM also increased this secretion. The majority of CECs and stromal cells of the mammalian eye are derived from the neural crest [34]. The expression of NSE, which occurs principally in neuronal tissues, is used to identify these cell types [35]. CECs are derived from the neural crest, and thus express NSE. After expansion in the four culture media, CECs maintained NSE expression, which exhibited a slight increase, indicating that the cells are CECs. Although these characteristics are not exclusive to CECs, they can be used together to define the functional characteristics of CECs *in vitro*. Based on the upregulation of vimentin and PAX6 expression, we inferred that the self-renewal ability was enhanced as CECs were expanded in the four CMs and that CECs expressed stem-cell-like properties. Additional experiments are necessary to analyze the multiplication capacity of CECs, such as CCK8 analyses and an examination of the expression of Ki-67. Among the four CMs, CSC-CM had the greatest effect on the multiplication capacity of CECs.

Taken together, our findings indicate that CSC-CM, BEPC-CM, and BMSC-CM all stimulate the proliferation of human CECs by enhancing various cell function, but also maintain the characteristic differentiation phenotypes necessary for endothelial functions. CECs maintained in CSC-CM acquire stem-cell-like properties, which may enable the regeneration of CECs into a functional corneal endothelium. These findings are the first evidence that when treated with CSC-CM, CECs retain proliferative potential with the capacity to be fully differentiated. Thus, a combination of a tissue-engineered human corneal endothelium coupled with surgical procedures presents a possible roadmap for the treatment of endothelial dysfunctions.

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REFERENCES

1 Tan DT, Dart JK, Holland EJ, Kinoshita S. Corneal transplantation. *Lancet* 2012;379(9827):1749-1761.

2 Feng MT, Price MO, Price FW Jr. Update on Descemet membrane endothelial keratoplasty (DMEK). *Int Ophthalmol Clin* 2013;53(2):31-45.

3 Maier P, Reinhard T, Cursiefen C. Descemet stripping endothelial keratoplasty--rapid recovery of visual acuity. *Dtsch Arztebl Int* 2013;110 (21):365-371.

4 Nanavaty MA, Wang X, Shortt AJ. Endothelial keratoplasty versus penetrating keratoplasty for Fuchs endothelial dystrophy. *Cochrane Database Syst Rev* 2014;2:CD008420.

5 Arenas E, Esquenazi S, Anwar M, Terry M. Lamellar corneal transplantation. *Surv Ophthalmol* 2012;57(6):510-529.

6 Joyce NC. Cell cycle status in human corneal endothelium. *Exp Eye Res* 2005;81(6):629-638.

7 Baum JL, Niedra R, Davis C, Yue BY. Mass culture of human corneal endothelial cells. *Arch Ophthalmol* 1979;97(6):1136-1140.

8 Nakahara M, Okumura N, Kay EP, Hagiya M, Imagawa K, Hosoda Y, Kinoshita S, Koizumi N. Corneal endothelial expansion promoted by human bone marrow mesenchymal stem cell-derived conditioned medium. *PloS One* 2013;8(7):e69009.

9 Kyurkchiev D, Bochev I, Ivanova-Todorova E, Mourdjeva M, Oreshkova T, Belemezova K, Kyurkchiev S. Secretion of immunoregulatory cytokines by mesenchymal stem cells. *World J Stem Cells* 2014;6(5):552-570.

10 Shao C, Chen J, Chen P, Zhu M, Yao Q, Gu P, Fu Y, Fan X. Targeted transplantation of human ubilical cord blood endothelial progenitor cells with immunomagnetic nanoparticles to repair corneal endothelium defect. *Stem Cells Dev* 2015;24(6):756-767.

11 Shao C, Fu Y, Lu W, Fan X. Bone marrow-derived endothelial progenitor cells: a promising therapeutic alternative for corneal endothelial dysfunction. *Cells Tissues Organs* 2011;193(4):253-263.

12 Pinnamaneni N, Funderburgh JL. Concise review: Stem cells in the corneal stroma. *Stem Cells*2012;30(6):1059-1063.

13 Hatou S, Yoshida S, Higa K, Miyashita H, Inagaki E, Okano H, Tsubota K, Shimmura S. Functional corneal endothelium derived from corneal stroma stem cells of neural crest origin by retinoic acid and Wnt/β-catenin signaling. *Stem Cells Dev* 2013;22(5):828-839.

14 Okumura N, Nakano S, Kay EP, Numata R, Ota A, Sowa Y, Sakai T, Ueno M, Kinoshita S, Koizumi N. Involvement of cyclin D and p27 in cell proliferation mediated by ROCK inhibitors Y-27632 and Y-39983 during wound healing of corneal endothelium. *Invest Ophthalmol Vis Sci* 2014;55 (1):319-329.

15 Peh GS, Toh KP, Wu FY, Tan DT, Mehta JS. Cultivation of human corneal endothelial cells isolated from paired donor corneas. *PloS One* 2011;6(12):e28310.

16 Deng Y, Bi X, Zhou H, You Z, Wang Y, Gu P, Fan X. Repair of critical-sized bone defects with anti-miR-31-expressing bone marrow stromal stem cells and poly (glycerol sebacate) scaffolds. *Eur Cell Mater* 2014;27:13-24; discussion 24-25.

17 Ni N, Zhang D, Xie Q, Chen J, Wang Z, Deng Y, Wen X, Zhu M, Ji J, Fan X, Luo M, Gu P. Effects of let-7b and TLX on the proliferation and differentiation of retinal progenitor cells *in vitro*. *Sci Rep* 2014;4:6671.

18 Xia J, Liu H, Fan X, Hu Y, Zhang Y, Wang Z, Zhou X, Luo M, Gu P. An in vitro comparison of two different subpopulations of retinal progenitor cells for self-renewal and multipotentiality. *Brain Res* 2012;1433:38-46.

19 Miyawaki T, Uemura A, Dezawa M, Yu RT, Ide C, Nishikawa S, Honda Y, Tanabe Y, Tanabe T. Tlx, an orphan nuclear receptor, regulates cell numbers and astrocyte development in the developing retina. *J Neurosci* 2004;24(37):8124-8134.

20 Peh GS, Beuerman RW, Colman A, Tan DT, Mehta JS. Human corneal endothelial cell expansion for corneal endothelium transplantation: an overview. *Transplantation* 2011;91(8):811-819.

21 Levis HJ, Kureshi AK, Massie I, Morgan L, Vernon AJ, Daniels JT. Tissue Engineering the Cornea: The Evolution of RAFT. *J Funct Biomater* 2015;6(1):50-65.

22 Joyce NC. Proliferative capacity of corneal endothelial cells. *Exp Eye Res* 2012;95(1):16-23.

23 Kinoshita S, Amano S, Inoue Y, Ohashi Y, Takahashi H, Tsubota K, Nishida K. Grading for corneal endothelial damage. *Nihon Ganka Gakkai Zasshi* 2014;118(2):81-83.

24 Siu GD, Young AL, Jhanji V. Alternatives to corneal transplantation for the management of bullous keratopathy. *Curr Opin Ophthalmol* 2014;25(4): 347-352.

25 Zhang J, Patel DV. The pathophysiology of Fuchs' endothelial dystrophy-a review of molecular and cellular insights. *Exp Eye Res* 2015; 130:97-105.

26 Engelmann K, Bednarz J, Valtink M. Prospects for endothelial transplantation. *Exp Eye Res* 2004;78(3):573-578.

27 Wang B, Wu J, Ma M, Li PP, Yu J. Ursolic acid inhibits corneal graft rejection following orthotopic allograft transplantation in rats. *Nan Fang Yi Ke Da Xue Xue Bao* 2015;35(4):530-535.

28 West-Mays JA, Dwivedi DJ. The keratocyte: corneal stromal cell with variable repair phenotypes. *Int J Biochem Cell Biol* 2006;38 (10): 1625-1631.

29 Lai JY, Chen KH, Hsu WM, Hsiue GH, Lee YH. Bioengineered human corneal endothelium for transplantation. *Arch Ophthalmol* 2006;124 (10): 1441-1448.

30 Joyce NC. Proliferative capacity of the corneal endothelium. *Prog Retin Eye Res* 2003;22(3):359-389.

31 Verkman AS. Role of aquaporin water channels in eye function. *Exp Eye Res* 2003;76(2):137-143.

32 Macnamara E, Sams GW, Smith K, Ambati J, Singh N, Ambati BK. Aquaporin-1 expression is decreased in human and mouse corneal endothelial dysfunction. *Mol Vis* 2004;10:51-56.

33 Chen KH, Azar D, Joyce NC. Transplantation of adult human corneal endothelium ex vivo: a morphologic study. *Cornea* 2001;20(7):731-737.

34 Gage PJ, Rhoades W, Prucka SK, Hjalt T. Fate maps of neural crest and mesoderm in the mammalian eye. *Invest Ophthalmol Vis Sci* 2005;46(11): 4200-4208.

35 Bohnke M, Vogelberg K, Engelmann K. Detection of neurone-specific enolase in long-term cultures of human corneal endothelium. *Graefes Arch Clin Exp Ophthalmol* 1998;236(7):522-526.