Bradykinin potentially stimulates cell proliferation in rabbit corneal endothelial cells through the ZO-1/ZONAB pathway

LIXIAN HE[1*], ZHOU ZHOU[2*], YI SHAO[3], ZHEN YANG[4], SHUANGSHUANG ZHOU[1], XUEXIANG ZOU[1], YING ZHOU[1] and GANG TAN[1]

[1]Department of Ophthalmology, The First Affiliated Hospital of University of South China;
[2]Medical College, University of South China, Hengyang, Hunan 421001;
[3]Department of Ophthalmology, The First Affiliated Hospital of Nanchang University,
Jiangxi Province Clinical Ophthalmology Institute, Nanchang, Jiangxi 330006;
[4]Department of Cardiology, The First Affiliated Hospital of Sun Yat-sen University,
Guangzhou, Guangdong 510080, P.R. China

Abstract. Bradykinin (BK) has been demonstrated to induce proliferation in several types of cell in *ex vivo* corneas. However, the mechanisms underlying the action of BK on corneal endothelial cells (CECs) remain largely unknown. The present study aimed to investigate the effect of BK on rabbit corneal endothelial cell (RCEC) proliferation, and assess the involvement of the zonula occludens-1(ZO-1)/ZO-1associated nucleic acid binding protein (ZONAB) pathway. Cell proliferation and cell cycle distribution was analyzed following treatment with BK (0.01, 0.1,1.0 or 10.0 μM) for the indicated time intervals (24, 48, 72 and 96 h), or following BK treatment combined with transfection of ZONAB-small interfering (si)RNA for 72 h. In addition, the expression of tight junction ZO-1, nuclear ZONAB, proliferating cell nuclear antigen(PCNA) and cyclin D1 were evaluated using western blotting or immunofluorescence. BK treatment was demonstrated to induce time- and concentration-dependent cell proliferation and cell cycle progression, along with the upregulation of tight junction ZO-1 and nuclear ZONAB, as well as PCNA and cyclin D1 protein expression. Furthermore, knockdown with ZONAB-siRNA inhibited cell proliferation, induced cell cycle arrest and downregulated PCNA and cyclin D1 protein expression. ZONAB knockdown therefore successfully reversed the increase in proliferation induced by BK treatment. Taken together, these results suggested that BK stimulated RCEC proliferation, potentially via the ZO-1/ZONAB pathway. The signaling paradigm disclosed in the present study potentially serves as an important therapeutic target for cornea regeneration and transplantation.

Introduction

Corneal endothelial cells (CECs) form a single monolayer on the posterior surface of the cornea and serve a pivotal function in the regulation of stromal hydration and the maintenance of corneal transparency (1). Adult human CECs are G_1-arrested, leading to a decline of endothelial cell density and subsequent endothelial dysfunction and loss of vision, particularly following injury, aging and surgery (2-4). The conventional approach involves transplantation of healthy donor CECs, but a current global shortage of donor corneas necessitates other options and demands the development of novel therapeutic agents and strategies to induce cell proliferation in the corneal endothelium.

Bradykinin (BK), a nonapeptide, is a major effector of the kallikrein-kinin system that demonstrates a wide range of biological activities, being involved in inflammation, pain, angiogenesis and cell proliferation (5-12). In ocular tissues, BK receptors (B1 and B2 receptors) are abundantly distributed and may trigger ocular allergies and inflammatory responses on the ocular surface (13). BK has been demonstrated to promote cell proliferation through the B2 receptor and epidermal growth factor receptor (EFGR) in *ex vivo* corneas, including bovine corneal endothelial cells (8), canine/human corneal epithelial cells (9,10) and corneal keratocytes or fibroblasts in the Statens Seruminstitut Rabbit (11,12). However, this phenomenon has not been reported in rabbit CECs, and the exact cellular mechanisms underlying BK-induced proliferation in CECs remain unknown.

Tight junctions (TJs), which are major components of the cell junctional complex, are essential for the barrier

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Correspondence to: Professor Gang Tan, Department of Ophthalmology, The First Affiliated Hospital of University of South China, 69 Chuanshan Road, Hengyang, Hunan 421001, P.R. China
E-mail: tangang99@hotmail.com

*Contributed equally

function of epithelium, epithelial proliferation and differentiation (14,15). Zonula occludens-1 (ZO-1) is a key TJ-associated protein that links junctional membrane proteins to the cytoskeleton (14). ZO-1-associated nucleic-acid-binding protein (ZONAB) is a Y-box transcription factor that is recruited to TJs by binding to the Src homology 3(SH3) domain of ZO-1 (14-16). ZONAB interacts with ZO-1 and regulates the transcriptional activity of cell cycle genes, including cyclin D1 and proliferating cell nuclear antigen (PCNA), that modulate cell cycle progression and cell proliferation (16-18). The ZO-1- and ZONAB-associated pathway (ZO-1/ZONAB pathway) has been demonstrated to regulate proliferation in epithelial cells derived from the renal proximal tubule and retinal pigment epithelium (RPE) (16-20). However, little is known about the effect of ZO-1 and ZONAB on CECs; the involvement of the ZO-1/ZONAB pathway in BK-stimulated cell proliferation remains to be examined.

Therefore, the purpose of the present study was to explore the effect of BK on cell proliferation in cultured rabbit corneal endothelial cells (RCECs), and to determine the contribution of the ZO-1/ZONAB pathway to BK-induced RCEC proliferation. To the best of our knowledge, the present study is the first to demonstrate BK-stimulated cell proliferation and cell cycle progress in RCECs, and that the underlying mechanisms involved the activation of the ZO-1/ZONAB signaling pathway.

Materials and methods

Animals. A total of 34 New Zealand white rabbits (Experimental Animal Center, University of South China, Hengyang, China; weight, 1.5-2.0 kg; age, 50 days) were employed in the present study. Rabbits were housed in individual cages under standard conditions (room temperature at 25-27˚C, humidity at 45-55% with 12 h light/dark cycle) with free access to standard laboratory chow and sterile acidified water. All experimental protocols were conducted in accordance with the Experimental Animal Regulations established by The Ministry of Science and Technology of the People's Republic of China, and the Guidelines for the Care and Use of Laboratory Animals published by the National Institutes of Health (Bethesda, MD, USA) (21). The study received ethical approval from the ethics committee of the University of South China.

Cell culture. Isolation and establishment of RCECs was performed as previously described, with modifications (22,23). Briefly, the rabbit corneal buttons were obtained following enucleation. Corneal endothelia with Descemet's membrane were dissected and peeled off under a stereoscopic dissecting light microscope (SMZ800; Nikon Corporation, Tokyo, Japan). Cells were then incubated in disaggregating solution (300 U type I collagenase and 1% antibiotic/antimycotic) in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 3 h at 37˚C in 5% CO_2. The medium was changed every other day. When cells reached confluence (within 10-14 days), they were enzymatically detached with 0.25% trypsin (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) and subcultured. RCECs that had been passaged 2-4 times were used for the following experiments.

Small interfering (si)RNA preparation, screening and transfection. Three siRNA duplexes targeting ZONAB (GenBank accession ID: AF171061.1) were designed using the siRNA Target Finder and Design Tool (http://www.ambion.com; Ambion; Thermo Fisher Scientific, Inc.) and National Center for Biotechnology Information Basic Local Alignment Search Tool. Another scrambled sequence siRNA, with no homology to the rabbit ZONAB gene, was used as a siRNA negative control (NC-siRNA). All siRNAs were commercially synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The sequences of each siRNA targeting ZONAB, as well as the scramble control were presented in Table I.

Transient siRNA transfection was performed using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocols, due to the high transfection efficiency and low cytotoxicity of lipofectin transfection (24). In short, three siRNA sequences or the scrambled control siRNA complex (5 μl of each at the concentration of 20 μM, diluted with diethyl pyrocabonate-treated water) with transfection reagents were added to cultured cells at 50% confluence. After 48 h, mRNA or protein was extracted to detect the transfection efficiency. The ZONAB-siRNA that had the maximum inhibition rate was selected and used for further *in vitro* experiments.

BK administration and experimental groups. In the present study, cells in the logarithmic growth phase were incubated with various concentrations (0.01, 0.1, 1.0 and 10.0 μM) of BK (Abcam, Cambridge, MA, USA) for the indicated time intervals (24, 48, 72 and 96 h) at 37˚C. The time intervals and concentrations of BK were selected based on the results of previous studies and were confirmed to effectively induce cell proliferation (9-12). When the cultures reached confluence, cells were plated onto 12-well culture plates (1 ml/well) for the measurement of ZO-1 and ZONAB protein expression. RCECs were plated onto 24-well plates (0.5 ml/well) for the examination of cell growth and morphology under a phase-contrast microscope (CH2; Olympus Corporation, Tokyo, Japan).

In order to investigate the causal function of ZONAB-associated signaling in BK-induced cell proliferation, RCECs were treated with BK alone, or BK treatment was combined with ZONAB-siRNA transfection. The cells were randomly divided into 6 groups (n=8 each) as follows: Control group (neither BK treatment nor ZONAB-siRNA transfection), BK group (treated with 1.0 μM BK), NC-siRNA group (transfected with NC-siRNA), ZONAB-siRNA group (transfected with the ZONAB-siRNA sequence), NC-siRNA+BK group (1.0 μM BK was administrated to cells transfected with NC-siRNA) and ZONAB-siRNA+BK group (1.0 μM BK was administrated to cells transfected with the ZONAB-siRNA sequence). Cell samples were collected at 72 h. MTT assays were performed to detect cell proliferation, and cell cycle distribution was analyzed using flow cytometry, as subsequently described. ZO-1, ZONAB, PCNA and cyclin D1 protein expression was detected by western blotting, together with immunofluorescence assay for ZONAB.

Cell proliferation assay. Cells (5x10³/plate) were loaded in 96-well plates, maintained in DMEM with 10% FBS, and then treated with 0.3% dimethyl sulfoxide (DMSO)

Table I. siRNA and RT-PCR primer sequences.

A, siRNA sequences used for ZONAB silencing

ZONAB siRNA1	5'-GAAUCAACAAGCAGCCAAUUTdTdT-3' (sense)
	5'-AUUGGCUGCUUGUUGCUUCTTdTdT-3' (antisense)
ZONAB siRNA2	5'-GAUCGGAGAGAUGAAGGAUUTdTdT-3' (sense)
	5'-AUCCUUCAUCUCUCCGAUCTT dTdT-3' (antisense)
ZONAB siRNA3	5'-GGAAUUUGAUGUGGUGGAAUUTdTdT-3' (sense)
	5'-UUCCACCACAUCAAAUUCCTTdTdT-3' (antisense)
Scramble control	5'-CGAGGAGACUUCCGAAUCUAUdTdT-3' (sense)
	5'-ACGUGACACGUUCGGAGAATTdTdT-3' (antisense)

B, Primer sequences used for RT-PCR analysis of ZONAB

ZONAB	5'-GCCATCAAGAAGAATAACCCACG-3' (forward)
	5'-GCGTAACGACTCCCTTCCACA-3' (reverse)
β-actin	5'-GTTCGAGACCTTCAACACCCC-3' (forward)
	5'-CCGGCCAGCCAGGTCCAGA-3' (reverse)

RT-PCR, reverse transcription polymerase chain reaction; siRNA, small interfering RNA; ZONAB, ZO-1-associated nucleic-acid-binding protein.

or BK (0.01, 0.1, 1.0 and 10.0 μM), or BK (1.0 μM) with ZONAB-siRNA or NC-siRNA transfection as aforementioned. At each exact time point (24, 48, 72 and 96 h), cells were treated with MTT reagent (10 μl) for 4 h at 37°C and then with 100 μl DMSO overnight at 37°C. Absorbance at 490 nm was measured usinga Bio-Rad microplate reader (Model-680; Bio-Rad Laboratories, Inc., Hercules, CA, USA). Wells containing culture medium but no cells served as controls. All experiments were repeated five times to ensure consistent results.

Flow cytometry. Confluent cells, treated for 72 h, were collected using 0.25% trypsin, fixed with 70% absolute ethyl alcohol at 4°C overnight, washed twice in 3 ml PBS, and stained in darkness with PBS containing propidium iodide (50 μg/ml; Roche Diagnostics Co., Ltd., Shanghai, China) at 4°C for 1 h and RNAse (100 μg/ml; Thermo Fisher Scientific, Inc.) at 37°C for 30 min. The samples were then analyzed using a FACSCanto II flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Based on DNA content, the percentage of cells in each stage of the cell cycle (G_0/G_1, S and G_2/M phases) was calculated using ModFit LT 3.0(Verity Software House, Inc., Topsham, ME, USA).

Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). Total RNA was extracted using an RNeasy Mini kit (Invitrogen; Thermo Fisher Scientific, Inc.), and RT was performed using a High Capacity Reverse Transcription kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. PCR primers targeting ZONAB were designed and synthesized by Sangon Biotech Co., Ltd and are presented in Table I. Thermocycler conditions were as follows: 10 min of initial activation at 95°C, followed by 40 cycles of 15 sec denaturation at 95°C, and 1 min annealing and extension at 60°C. The identity of each PCR product was confirmed by size determination using 2% agarose gels followed by ethidium bromide staining and the PCR marker, using an EC3 Imaging System (BioImaging Systems; UVP, Inc., Upland, CA, USA). The intensities of the bands were densitometrically quantified using Quantity One 1D software (version 4.6.9, Bio-Rad Laboratories, Inc., Hercules, CA, USA), and β-actin was used as an internal reference in each reaction.

Western blotting. Confluent cells were washed, scraped, collected, and centrifuged at 226,000 x g for 1 h at 4°C to yield whole cell extract. Nuclear ZONAB protein and cytoplasmic ZO-1 protein were extracted using a Bicinchoninic acid protein assay (Nanjing KeyGen Biotech Co., Ltd.) according to the manufacturer's protocol. Samples (50 μg protein) were denatured, subjected to 10% SDS-PAGE, and transferred to nitrocellulose membranes. Subsequently, the membrane was blocked with 5% non-fat milk in TBST (containing 0.2% Tween-20, 20 mmol/l Tris-HCl, and 150 mmol/l NaCl, pH 7.14; non-fat milk, Bio-Rad Laboratories, Inc., Hercules, CA, USA; Tris, Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 1 h at 37°C, then incubated withanti-ZO-1 (1:500; cat. no. ab-61357, Abcam), anti-ZONAB (1:1,000; cat. no. 40-2800; Invitrogen; Thermo Fisher Scientific, Inc.), anti-PCNA (1:1,000; cat. no. 60097-1-Ig; ProteinTech Group, Inc., Chicago, IL, USA), anti-cyclin D1 (1:1,000; cat. no BS-0623R; BIOSS, Beijing, China) and anti-β-actin (1:1,000; cat. no. 60008-1-Ig; ProteinTech Group, Inc.) at 4°C overnight. The membrane was washed three times, blocked with 5% non-fat milk in TBST and then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody at a final dilution of 1:6,000 (cat. no. SA00001-2;
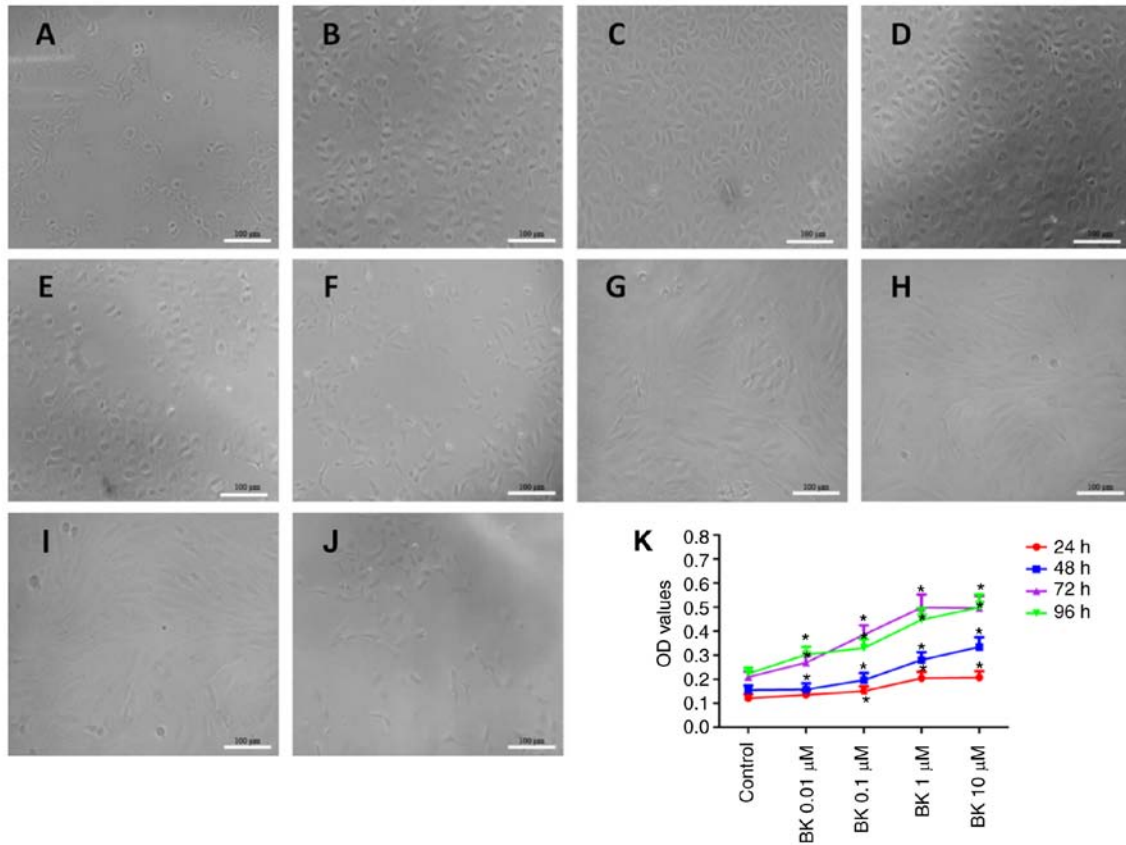
Figure 1. Time- and concentration-dependence of BK-induced proliferation in RCECs. Representative photos of RCECs under phase-contrast microscopy, following treatment with various concentrations of BK are presented (magnification, x100; scale bars, 100 µm). (A) Treatment without BK for 72 h; (B) treatment with 0.01 µM BK for 72 h; (C) treatment with 0.1 µM BK for 72 h; (D) treatment with 1.0 µM BK for 72 h; (E) treatment with 10.0 µM BK for 72 h; (F) treatment without BK for 96 h; (G) treatment with 0.01 µM BK for 96 h; (H) treatment with 0.1 µM BK for 96 h; (I) treatment with 1.0 µM BK for 96 h; (J) treatment with 10.0 µM BK for 96 h. (K) Analysis of cell proliferation by MTT assay. Data are expressed as the mean ± standard deviation (n=5). *P<0.05 vs. control group. BK, bradykinin; RCECs, rabbit corneal endothelial cells; OD, optical density.

ProteinTech Group, Inc.) for 1 h at 37˚C. An enhanced chemiluminescence system (Pierce; Thermo Fisher Scientific, Inc.) was used for measuring the protein-antibody complexes. The blots were quantified by Quantity One 1-D software (version 4.6.9; Bio-Rad Laboratories, Inc.) and β-actin was used as the control.

Immunofluorescence assay. Confluent cells were fixed in 4% paraformaldehyde in PBS, pH 7.2, for 10 min at 37˚C, then permeabilized in 0.1% Triton X-100 (Sigma-Aldrich; Merck KGaA) in PBS for 5 min at room temperature; rinsed three times in PBS, and blocked for 30 min in PBS with 10% goat serum (Sigma-Aldrich; Merck KGaA) at 37˚C. Primary antibody staining was performed at 4˚C overnight with the following antibodies at a 1:50 dilution: Anti-ZO-1 and anti-ZONAB. The sections were then incubated with an Alexa Fluor® 594-conjugated goat anti-mouse secondary antibody at a 1:100 dilution (cat. no. SA00006-3; ProteinTech Group, Inc.) for 2 h at room temperature in the dark. Following DAPI staining at 37˚C for 10 min, cells were imaged using an inverted fluorescence microscope (TE2000U Eclipse; Nikon Corporation).

Statistical analysis. Statistical analysis was performed using SPSS 17.0 statistical software (SPSS, Inc., Chicago, IL, USA)

for Windows. All data are expressed as the mean ± standard deviation), and analyzed via one-way analysis of variance followed by the post hoc Bonferroni's t-test where appropriate. P<0.05 was considered to indicate a statistically significant difference.

Results

BK treatment induces RCECs proliferation in a time- and concentration-dependent manner. Under an inverted microscope, cells treated with 0.1-1.0 µM BK were revealed to have formed a monolayer with a mosaic arrangement, cellular morphology was normal at 72 h (Fig. 1A-D). However, at 96 h, cells were irregular in shape with thin, long, neurite-like processes, and cell extensions and increased detachment were observed (Fig. 1F-J). In addition, BK increased cell density in a concentration-dependent manner when treated with 0.1-1.0 µM BK, while cell growth was significantly inhibited following 10.0 µM BK treatment (Fig. 1A-E). Thus, BK-induced proliferation of RCECs was demonstrated to be time- and concentration- dependent.

Next, BK-induced cell proliferation was analyzed using an MTT assay. As presented in Fig. 1K, exposure of RCECs to BK at 0.1-1.0 µM resulted in a concentration-dependent increase in optical density (OD) values, while 10.0 µM BK
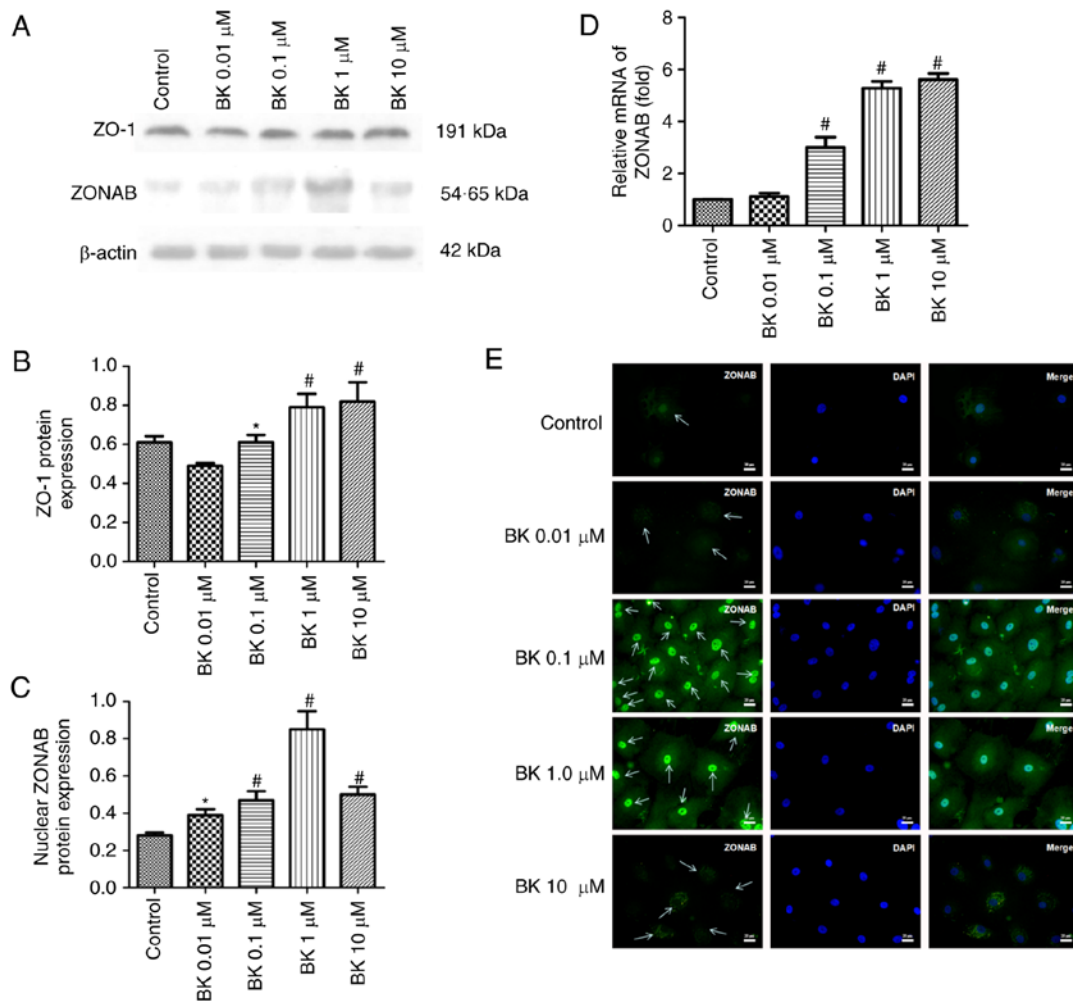
Figure 2. BK upregulated the expression of tight junction ZO-1 and nuclear ZONAB in rabbit corneal endothelial cells. (A) Representative western blotting images for ZO-1 and ZONAB, with quantification of (B) ZO-1 protein expression and (C) nuclear ZONAB protein expressions. (D) Relative ZONAB mRNA levels, as determined by reverse transcription polymerase chain reaction. (E) The localization of ZONAB was evaluated using immunofluorescence. Nuclei were counterstained with DAPI. White arrows indicate ZONAB-positive cells stained green (magnification, x400; scale bars, 20 μm). Data are expressed as the mean ± standard deviation (n=4). *P<0.05 vs. control group; **P<0.01 vs. control group. BK, bradykinin; ZO-1, zonula occludens-1; ZONAB, ZO-1-associated nucleic-acid-binding protein.

treatment resulted in relatively limited proliferation, reflected by the decrease of OD values. These results indicated that BK treatment at 1.0 μM significantly increased cell viability and induced RCEC proliferation.

BK treatment increases the expression of the tight junction ZO-1 and nuclear ZONAB during RCECs proliferation. To determine the involvement of ZO-1 and ZONAB in the regulation of RCEC proliferation, the localization and transcription levels of ZONAB and ZO-1 were assessed. Based on immunofluorescence analysis of RCECs, ZONAB was revealed to be primarily located with the nucleus or nuclear membrane, and there was high luminescence for ZONAB in these areas of cells following treatment with 0.1-1.0 μM BK and of cells in the control group, but not cells treated with 0.01 μM BK. In cells exposed to 10 μM BK, ZONAB was primarily localized within the cytoplasm and was excluded from the nuclear region (Fig. 2E). These observations are consistent with the data from western blotting and RT-PCR. Compared with the control group, BK increased nuclear ZONAB mRNA and protein expression in a concentration-dependent

manner (0.01-1.0 μM BK; P<0.05 or P<0.01; Fig. 2C and D). Similarly, this BK-induced concentration-dependent effect was also was observed for ZO-1 protein (0.1-10.0 μM BK; P<0.05 or P<0.01). These data suggested that ZO-1 and ZONAB are crucial components in the regulation of cell proliferation in RCECs, and are potentially associated with BK-induced proliferation.

Knockdown with ZONAB siRNAs induces significant downregulation of ZONAB mRNA and protein. Next, RCECs were transfected with small ZONAB-directed RNA duplexes to induce RNA interference. RT-PCR data (Fig. 3A) and western blotting analysis (Fig. 3B) suggested that transient transfection of three different regions of ZONAB resulted in efficient reduction of ZONAB mRNA and protein expression, while the negative control RNA duplex had no effect. In particular, transfection of the second sequence, ZONAB-siRNA2, efficiently decreased ZONAB mRNA expression with a reduction of ~85% in RCECs (P<0.01; Fig. 3A). This sequence was therefore used for the remaining experiments.
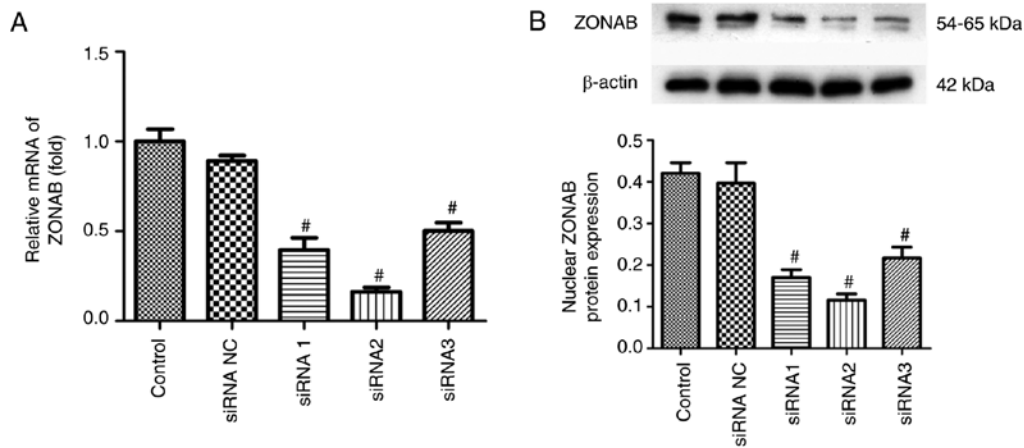
Figure 3. Knockdown of ZONAB expression in RCECs transfected with siRNAs. (A) Reverse transcription polymerase chain reaction and (B) western blotting analysis showed revealed that transfection of ZONAB siRNAs efficiently inhibited the expression of ZONAB mRNA and protein in RCECs, respectively. In particular, transfection with ZONAB-siRNA2 achieved an ~85% reduction of ZONAB mRNA. Data are expressed as the mean ± standard deviation (n=6). **P<0.01 vs. control group. ZONAB, ZO-1-associated nucleic-acid-binding protein; RCECs, rabbit corneal endothelial cells; siRNA, small interfering RNA.

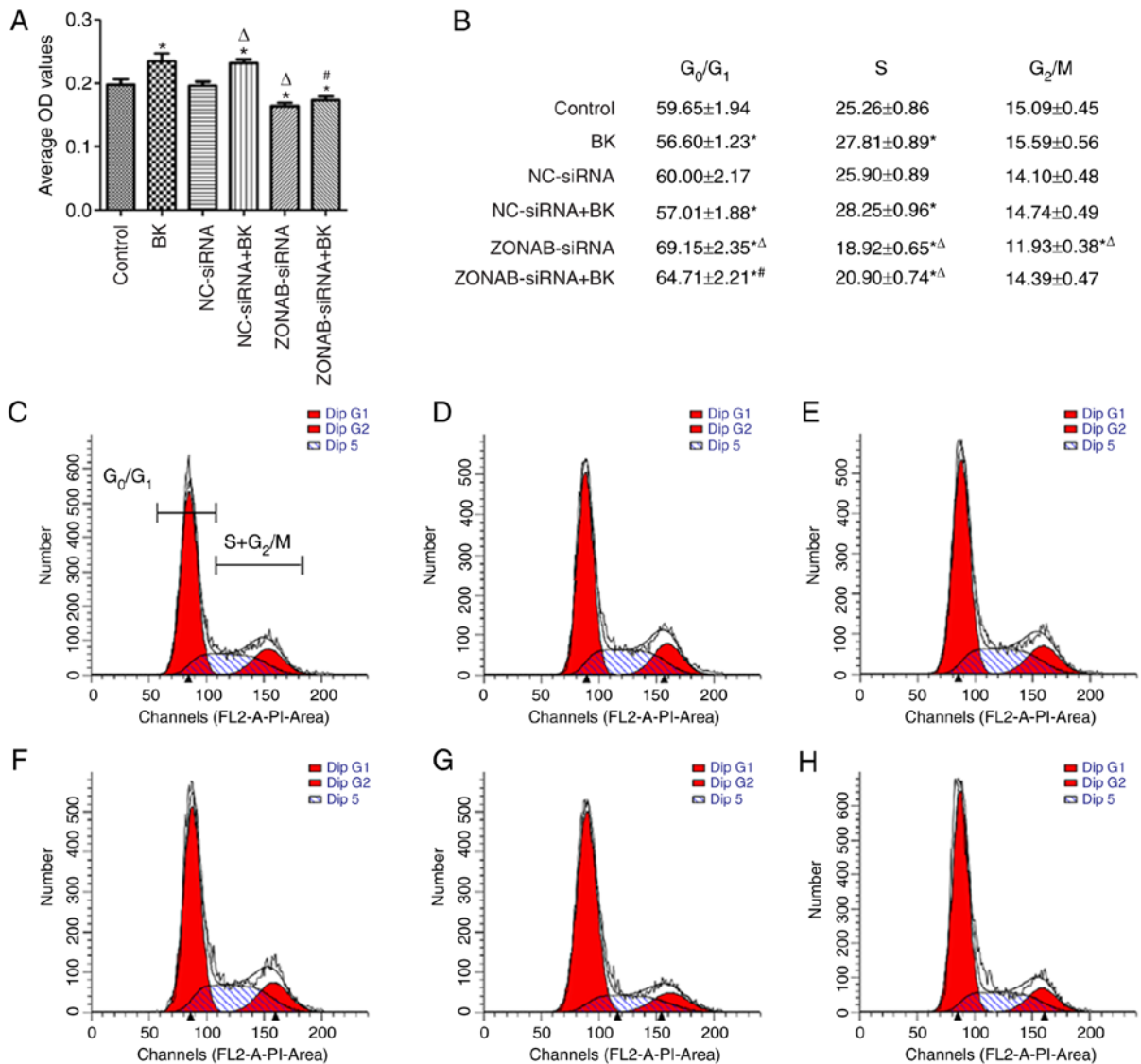|	G_0/G_1	S	G_2/M
Control	59.65±1.94	25.26±0.86	15.09±0.45
BK	56.60±1.23*	27.81±0.89*	15.59±0.56
NC-siRNA	60.00±2.17	25.90±0.89	14.10±0.48
NC-siRNA+BK	57.01±1.88*	28.25±0.96*	14.74±0.49
ZONAB-siRNA	69.15±2.35*Δ	18.92±0.65*Δ	11.93±0.38*Δ
ZONAB-siRNA+BK	64.71±2.21*#	20.90±0.74*Δ	14.39±0.47

Figure 4. Knockdown with ZONAB-siRNA reversed the effect of BK on cell proliferation and cell cycle progression. (A) Cell proliferation was determined by MTT assays. (B) Table of cell cycle distribution percentages of each group. Flow cytometric analysis of cell cycle progression of rabbit corneal endothelial cells in (C) the control group, (D) the BK group, (E) the NC-siRNA group, (F) the ZONAB-siRNA group, (G) the NC-siRNA+BK group and (H) the ZONAB-siRNA+BK group. Data are expressed as the mean ± standard deviation (n=5-6). *P<0.05 vs. control group; #P<0.05 vs. BK group; ΔP<0.05 vs. NC-siRNA group. ZONAB, ZO-1-associated nucleic-acid-binding protein; siRNA, small interfering RNA; BK, bradykinin; NC, negative control.
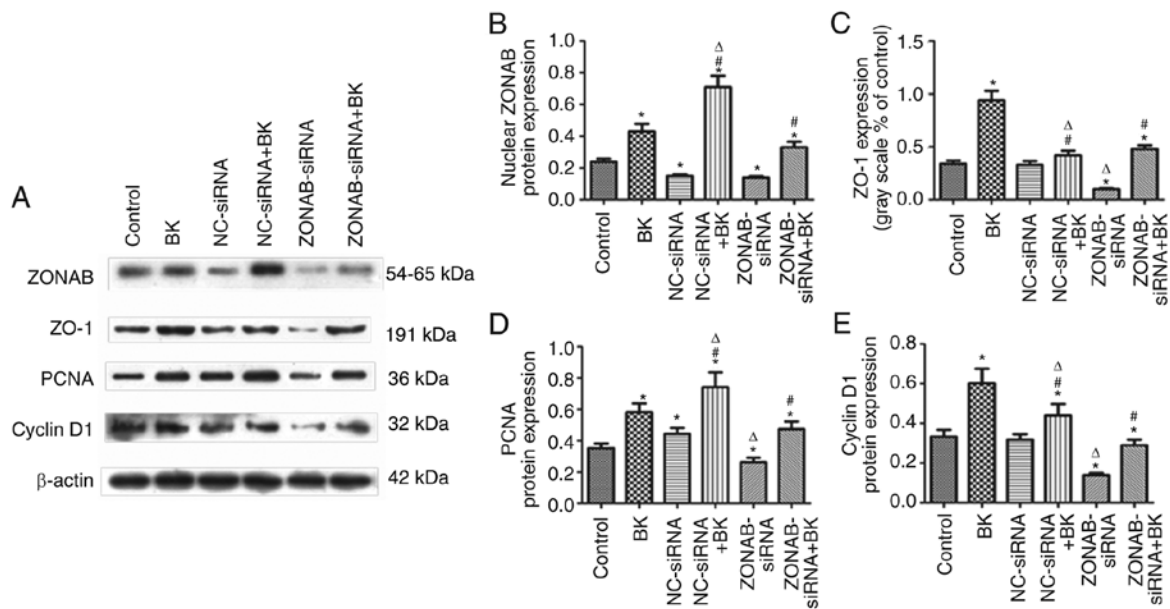
Figure 5. The involvement of the ZO-1/ZONAB pathway in BK-induced rabbit corneal endothelial cell proliferation. (A) Representative western blotting gel images. Quantification analysis of (B) ZO-1, (C) nuclear ZONAB, (D) PCNA and (E) cyclin D1. Protein expression was normalized to β-actin. Data are expressed as the mean ± standard deviation (n=5). *P<0.05, vs. control group; #P<0.05 vs. BK group; ΔP<0.05 vs. NC-siRNA group. ZO-1, zonula occludens-1; ZONAB, ZO-1-associated nucleic-acid-binding protein; siRNA, small interfering RNA; BK, bradykinin; PCNA, proliferating cell nuclear antigen; NC, negative control.

Knockdown with ZONAB siRNAs abolishes the effect of BK on cell proliferation and cell cycle progression in RCECs. According to the MTT assay data, cells transfected with non-targeting control siRNA demonstrated proliferation capacities that were similar to those of the control group (P>0.05; Fig. 4A). Cells lines transfected with ZONAB-siRNA exhibited lower cell proliferation and OD values than those of the control group (P<0.05; Fig 4A). In contrast, a significant increase of OD value and proliferation capacity was observed in the BK-treated group compared with the control group (P<0.05; Fig. 4A). However, proliferation significantly decreased in cells transfected with ZONAB-siRNA in combination with BK compared with those treated with BK alone (P<0.05; Fig. 4A).

Furthermore, cell cycle distribution of RCECs was quantified by flow cytometry (Fig. 4B-H). Cell cycle analysis revealed that a significantly increased fraction of cells in the S phase and a significantly lower percentage of cells in the G_0/G_1 phases were present in cells treated with BK compared with the control group (P<0.05; Fig. 4B-D). BK pretreatment accelerated the G_1- to S-phase switch and enhanced DNA synthesis, thus inducing cell proliferation. Conversely, there were a larger fraction of cells arrested in G_0/G_1 phases and a reduction of the proportion of cells distributed in S phase in ZONAB-siRNA transfected cells compared with the control group (P<0.05; Fig. 4B, C and G), and a similar effect was observed in the ZONAB-siRNA+BK group compared with cells treated with BK alone (P<0.05; Fig. 4B, D and H). These results suggested that ZONAB-siRNA transfection induced cell cycle arrest in G_0/G_1 phases and inhibited cell mitosis, thus reversing BK-induced proliferation of RCECs.

Involvement of the ZO-1/ZONAB pathway in BK-induced proliferation of RCECs. Finally, the regulation of the ZO-1/ZONAB signaling cascade itself, from the upstream molecules (ZO-1 and ZONAB) to the downstream effectors (PCNA and cyclin D1) was investigated. PCNA and cyclin D1 are used as markers regulating cell proliferation and cell cycle progression in various types of cell (25,26). As demonstrated by western blotting, transfection with ZONAB-siRNA resulted in knockdown of ZONAB protein levels and inhibition of PCNA and cyclin D1 expression (P<0.05; Fig. 5), whereas non-targeting siRNA transfection did not alter PCNA or cyclin D1 protein levels (P>0.05; Fig. 5). BK pretreatment significantly increased the expression of PCNA and cyclin D1. In turn, transfection with ZONAB-siRNA inhibited BK-induced upregulation of PCNA and cyclin D1 (P<0.05; Fig. 5), and subsequently blocked BK-stimulated cell proliferation.

Discussion

The present study initially established the involvement of the ZO-1/ZONAB pathway in BK-induced RCECs proliferation. The data revealed that BK promoted cell proliferation and cell cycle progression in RCECs. BK treatment also resulted in the activation of signaling molecules in the ZO-1/ZONAB pathway, including the upregulation of tight junction ZO-1 and nuclear ZONAB, as well as PCNA and cyclin D1. Furthermore, knockdown with ZONAB-siRNA inhibited cell proliferation, induced cell cycle arrest and downregulated the PCNA and cyclin D1 protein expression. Pre-treatment with siRNA to knockdown ZONAB blocked the proliferation-promoting activity of BK. Taken together, these data indicated that BK treatment increased RCECs proliferation, at least in part due to the activation of the ZO-1/ZONAB pathway.

BK is a well-established mediator of exudative corneal wound healing, ocular allergy and pro-inflammatory responses

on the ocular surface (13). BK and its receptors, the B1 and B2 receptor, are present in the tissue homogenates of rabbit, swine and human eyes (27-29). BK interacts with its receptors on the cell surface to mediate a variety of biological effects, including cell proliferation. BK has been reported to induce proliferation of various types of cell in *ex vivo* corneas (8-12). However, the underlying mechanisms by which BK stimulates the proliferation of ocular cells remain to be fully understood. The majority of the biological functions of BK are mediated by the B2 receptor, which leads to an increase of intracellular $Ca^{2+}([Ca^{2+}]_i)$ mobilization, and tyrosine kinase and protein kinase C (PKC) activation via pertussis toxin (PTX)-insensitive G protein (8,9,12,30,31). Previous reportshave suggested that BK induces cell proliferation through stimulation of phosphoinositide turnover, $[Ca^{2+}]_i$-mobilization and diacylgylcerol production, which lead to increased DNA synthesis in human corneal epithelial cells and bovine CECs (8,9,12). However, pretreatment with HOE-140, a specific B2 receptor antagonist, attenuated the BK-induced increase in $[Ca^{2+}]_i$, suggesting that B2 receptors serve a crucial function in this process (8,9). Multiple previous studies have demonstrated that BK induces cell proliferation potentially through B_2 receptor coupling PTX-sensitive G protein/Ca^{2+}/PKC and EFGR/p42/p44 mitogen activated protein kinase (MAPK)-dependent pathways in various cell types (8-12,30-34). However, these mechanisms remained to be verified in CECs.

Accumulating evidence has demonstrated that the TJ-associated signaling proteins, ZO-1 and ZONAB, serve a vital role in cell proliferation, gene expression and differentiation, as reported in RPE cells and the renal proximal tubule (16-19,35-39). ZO-1 is a membrane-associated TJ adaptor protein and possesses several PDZ domains, one SH3 domain, and a domain homologous to yeast guanylate kinase (14,40). ZONAB is a Y-box transcription factor that modulates cell proliferation through its interaction with the SH3 domain of ZO-1. ZONAB is primarily distributed in the nucleus or nuclear membrane of proliferating cells, and drives the transcription of PCNA and cyclin D1 genes for the promotion of cell proliferation and cell cycle progression (16-18). However, in slowly or non-proliferating cells, nuclear ZONAB expression is reduced, and binding of ZONAB to ZO-1 results in cytoplasmic sequestration and inhibits the nuclear accumulation and transcriptional activity of ZONAB, resulting in reduced proliferation (14-20). In the present study, with BK pre-treatment, mRNA and protein levels of nuclear ZONAB were significantly upregulated in a concentration-dependent manner, suggesting the increase of ZONAB nuclear accumulation and transcriptional activity, thus resulting in RCEC proliferation.

In the present study, the subcellular localization of ZONAB was detected by immunofluorescence. High luminescence in the nucleus indicated ZONAB expression following treatment with 0.1-1.0 μM BK or without BK in the control group, but not following treatment with 0.01 μM BK. Treatment with 0.01 μM BK had no effect on ZONAB nuclear translocation, and the concentration-dependent effect of BK on the nuclear accumulation of ZONAB was not observed. Therefore, a wide range of BK concentrations (0.0001-10 μM), previously reported in human corneal epithelial cells (18), should be selected to determine the effect of BK (<0.01 μM) on ZONAB nuclear distribution. In addition, the subcellular localization

of ZO-1 was also analyzed. Based on previous immunofluorescence results, ZO-1 is primarily located at intercellular junctions and in the cytoplasm (16-18). While specific ZO-1 staining at intercellular junctions was not detected, ZO-1 was revealed to be distributed in the cytoplasm and nucleus (data not shown). The murine-derived monoclonal antibody against rabbit ZO-1 may have been non-specific; alternatively, ZO-1 may be sparsely distributed in the rabbit corneal endothelium. Even though the ZO-1 staining results of the present study were not satisfactory, the data suggested that the majority of ZONAB molecules bound to ZO-1 are associated with TJ, and the expression of TJ-associated molecules, ZO-1 and ZONAB, were affected by BK treatment.

Furthermore, the present study provided the evidence supporting the involvement of the ZO-1/ZONAB pathway during RCEC proliferation, as demonstrated by decreased cell proliferation, a greater fraction of cells arrested in G_0/G_1 phase and the downregulation of PCNA and cyclin D1 following ZONAB-siRNA transfection. These data are consistent with evidence from previous transgenic experiments. A study by Balda *et al* (16) demonstrated that depletion of ZONAB by RNA interference or ZO-1 overexpression reduced proliferation rates and final cell densities of Madin-darby canine kidney cells, while overexpression of ZONAB resulted in increased cell densities. Another investigation by Georgiadis *et al* (20) revealed that lentivirally-mediated overexpression of ZONAB or knockdown of ZO-1 resulted in an increased number of BrdU-positive cells and the induction of RPE proliferation. Kampik *et al* (37) revealed that knockdown of ZO-1 led to an average increase of 50% in human CECs density in corneal samples from donors >60 years old, while overexpression of ZONAB led to Ki67 upregulation but no significant increase in cell density. Taken together, these data suggest that ZO-1 and ZONAB are involved in signaling that modulates RCECs proliferation.

The present study investigated the function of the ZO-1/ZONAB pathway during BK-induced cell proliferation. The data revealed that transfection with ZONAB-siRNA reversed the proliferation-promoting effect of BK. Significant knockdown of ZONAB inhibited the transcriptional activity of the cell cycle genes PCNA and cyclin D1, thus attenuating BK-induced cell proliferation. Nevertheless, the exact mechanisms underlying BK-induced activation of ZO-1/ZONAB signaling remain to be fully elucidated. First, it is unclear whether the overexpression of ZONAB and knockdown of ZO-1 increase proliferation in the RCECs model utilized in the present study. Further transgenic research targeting ZO-1 and ZONAB is required. Second, the effect of ZO-1 and ZONAB on CECs differentiation remains unclear, and cellular mechanisms relevant to cell differentiation should be analyzed. Third, bioinformatics analysis of ZONAB siRNA is required to minimize off-target effects resulting from the introduction of individual siRNAs. Finally, ongoing experiments by our group should be replicated in human corneal endothelia, and focus on the crosstalk between the ZO-1/ZONAB pathway and the BK-mediated B_2 receptor-G protein/Ca^{2+}/PKC or EFGR-p42/p44 MAPK-dependent pathway. Further research is required to explore these areas.

In conclusion, the present study demonstrated that BK promoted RCECs proliferation and cell cycle progression, and the underlying mechanisms appeared to include the

ZO-1/ZONAB pathway. The signaling paradigm disclosed in the present study provide novel insights and, potentially, novel therapeutic targets for cornea regeneration and transplantation.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LH and ZZ contributed equally to the present study. GT conceived and designed the experiments; LH, ZZ, SZ XZ and YZ conducted the experiments; ZZ and SZ analyzed the data; LH wrote the paper; YS and GT revised the manuscript for critically important intellectual content. All the authors read and approved the paper.

Ethics approval and consent to participate

The present study received ethical approval from the Ethics Committee of University of South China (Hengyang, China).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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