

# SKLB1002, a potent inhibitor of VEGF receptor 2 signaling, inhibits endothelial angiogenic function *in vitro* and ocular angiogenesis *in vivo*

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**Abstract.** Ocular angiogenesis is a major cause of severe vision loss, which can affect several parts of the eye, including the retina, choroid and cornea. Vascular endothelial growth factor receptor 2 (VEGFR2) inhibitors have demonstrated great potential for treating ocular angiogenesis and SKLB1002 is a potent inhibitor of VEGF receptor 2 signaling. The present study investigated the effects of SKLB1002 administration on ocular angiogenesis. SKLB1002 administration did not show obvious cytotoxicity and tissue toxicity at the tested concentrations. In an alkali-burn corneal model, SKLB1002 administration significantly decreased the mean length and number of new corneal blood vessels. SKLB1002 administration significantly reduced endothelial cell proliferation, migration and tube formation *in vitro*. Mechanistically, SKLB1002 inhibited endothelial angiogenic functions by blocking the phosphorylation of ERK1/2, JNK and p38. Thus, selective inhibition of VEGFR-2 through SKLB1002 administration is a promising therapy for ocular angiogenesis.

## Introduction

Pathological angiogenesis occurs in several parts of the eye, including retina, choroid and cornea. It is a major cause of vision

loss in numerous ocular diseases, such as diabetic retinopathy, age-related macular degeneration and keratitis. Corneal neovascularization (CoNV) usually occurs in the inflammatory or infectious ocular surface diseases (1,2). It is characterized by the invasion of the capillaries from the pericorneal limbal vascular plexus into avascular cornea tissue. CoNV can lead to corneal scarring, edema and inflammation, which eventually affects visual acuity and worsens the prognosis of subsequent penetrating keratoplasty (3,4). A balance exists between the angiogenic factors and anti-angiogenic factors in the cornea (5,6). CoNV will occur when the balance is disturbed (7,8). CoNV is associated with increased expression of angiogenic factors and decreased expression of anti-angiogenic factors (9).

Numerous growth factors and cytokines have been reported to be involved in the angiogenic process. Among them, vascular endothelial growth factor (VEGF) is a critical angiogenic factor. VEGF is upregulated in the inflamed and vascularized corneas (10). VEGF is a secreted growth factor induced by hypoxia or inflammatory stimulation and plays an important role in endothelial angiogenic functions, including cell proliferation, migration and new tube formation (11,12). VEGF usually exerts its biological effects by binding to and activating its major receptor, VEGF receptor 2 (VEGFR2) (13-15). Thus, inhibition of VEGFR-2 signaling is a promising strategy for treating CoNV.

SKLB1002, derived from quinazoline, is a small molecule that displays potent and specific inhibition of VEGFR2 tyrosine kinase activity. It can inhibit VEGF-induced phosphorylation of VEGFR2 kinase and the downstream protein kinases (16,17). A previous study has demonstrated that SKLB1002 inhibits angiogenesis and may be a potential drug candidate for anti-cancer therapy (18). The present study investigated the role of SKLB1002 endothelial angiogenic function *in vitro* and ocular angiogenesis *in vivo*. The results revealed that SKLB1002 can significantly inhibit CoNV induced by alkali-burn *in vivo* and inhibit endothelial angiogenic functions *in vitro*.

## Materials and methods

**Corneal alkali-burn mouse model.** In total, 40 male ICR mice (age, 12 weeks; weight 30±2 g) were chosen to build CoNV

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model, and were purchased from Nanjing Qinglongshan Experimental Animal Center. The mice were raised under standard conditions (temperature,  $22\pm 2^{\circ}\text{C}$ ; humidity,  $50\pm 5\%$ ) with a controlled 12-h light/dark cycle and had free access to water and standard laboratory chow. The mice were anesthetized by intraperitoneal injection of chloral hydrate at a dose of 350 mg/kg. No signs of peritonitis were observed during the experiment. Corneal alkali-burn was performed by applying a 2.5 mm diameter filter paper soaked with 1 mol/l NaOH on the corneal center for 25 sec. After the filter paper was removed, the eye was rinsed with the sterilized saline for 1 min. An eyedrop of SKLB1002 (0.05 mg/ml) or sodium carboxymethyl cellulose (CMC-Na; 0.5%) was used on the surface of cornea 3 times each day. Corneal neovascularization was observed using a slit lamp. The animals were euthanized by cervical dislocation.

**Histopathological analysis.** Hematoxylin and eosin (H&E) staining was performed to detect the histopathological change of cornea. The mice were treated with SKLB1002, CMC-Na (0.5%), or PBS 3 times per day. The eyeballs were collected and fixed in 4% paraformaldehyde (Beyotime Institute of Biotechnology) at  $4^{\circ}\text{C}$  for 24 h. Then, the eyeballs were dehydrated by immersion in a series of increased concentrations of alcohol, embedded in paraffin wax and sectioned at 5- $\mu\text{m}$  thickness. The paraffin sections were deparaffinized by xylene for 30 min and rehydrated by an alcohol gradient. After washing with  $\text{ddH}_2\text{O}$ , the sections were stained with hematoxylin at room temperature for 10 min. Then, the sections were washed with  $\text{ddH}_2\text{O}$  and stained with eosin at room temperature for 1 min. After washing with  $\text{ddH}_2\text{O}$ , the sections were dehydrated using alcohol and mounted using resinene. The images were captured using a light microscope at magnification,  $\times 10$ .

**Cell culture.** Human umbilical vein endothelial cells (HUVECs) were obtained from Lonza Group, Ltd. (cc-2159) and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) at  $37^{\circ}\text{C}$  with 5%  $\text{CO}_2$  in a humidified atmosphere.

**Cell viability assay.** MTT assay was performed to detect cell viability. HUVECs were seeded onto 96-well plate (3,000-4,000/well) and treated with different concentrations of SKLB1002 for 48 h. HUVECs were also treated with p38 inhibitor SB203580 (10  $\mu\text{M}$ ; cat. no. S1863; Beyotime Institute of Biotechnology), ERK inhibitor U0126 (10  $\mu\text{M}$ ; cat. no. S1901; Beyotime Institute of Biotechnology) or JNK inhibitor SP600125 (10  $\mu\text{M}$ ; cat. no. S1876; Beyotime Institute of Biotechnology) for 1 h, and incubated with VEGF (10 ng/ml), VEGF + SKLB1002 or left untreated at  $37^{\circ}\text{C}$  for 48 h. Then, MTT (5 mg/ml, Beyotime Institute of Biotechnology) was added at  $37^{\circ}\text{C}$  for 4 h. After removing the medium, the crystals were dissolved with isopropanol and determined at 570 nm wavelength using a microplate reader.

**Cell proliferation assay.** Ki67 staining was performed to determine cell proliferation. In brief, HUVECs were seeded onto 24-well plate. Following the required treatment, they were washed with PBS buffer, fixed in 4% paraformaldehyde

(Beyotime Institute of Biotechnology) at room temperature for 15 min and blocked in 5% BSA (Biofroxx GmbH) at  $37^{\circ}\text{C}$  for 0.5 h. Ki67 antibody (1:200; Abcam; cat. no. ab16667) was added to each well overnight at  $4^{\circ}\text{C}$ . The plate was placed at room temperature to rewarm for 1 h and incubated with the secondary antibody for 3 h. The nuclei were stained with DAPI (1:1,000; Biosharp) at room temperature for 10 min. The plate was imaged using a fluorescence microscope.

**EdU incorporation assay.** After the required treatment, the cells were cultured with EdU medium (50  $\mu\text{M}$ , 300  $\mu\text{l}$ /well; Guangzhou RiboBio Co., Ltd.) at  $37^{\circ}\text{C}$  for 4 h. Cells were fixed with 4% paraformaldehyde (Beyotime Institute of Biotechnology) at room temperature for 30 min and incubated with 0.5% Triton X-100 for 10 min. After washing with PBS for three times, cells were incubated with Apollo Dye solution (Guangzhou RiboBio Co., Ltd.; cat. no. C10310) at  $37^{\circ}\text{C}$  for 1 h in the dark. Then, the cells were incubated with 0.5% Triton X-100 at room temperature for 15 min. Cell nuclei were stained by Hoechst 33342 at room temperature for 30 min in the dark. The images were captured using a fluorescent microscope at magnification,  $\times 40$ .

**Flow cytometry.** HUVECs ( $5\times 10^5$  cells/well in 6-well plates) were incubated with or without SKLB1002 at  $37^{\circ}\text{C}$  for 24 h. They were harvested using a 0.05% trypsin solution, washed twice with PBS and centrifuged at  $1,200\times g$  at  $4^{\circ}\text{C}$  for 5 min. Then, cell suspension was stained with fluorescein isothiocyanate-labeled Annexin V (BD Pharmingen; Becton, Dickinson and Company) and counterstained with propidium iodide (PI; BD Pharmingen; Becton, Dickinson and Company) at room temperature for 10 min in the dark. Finally, the percentage of apoptotic HUVECs were determined using a flow cytometer (cytoFLEX; Beckman Coulter, Inc.) and analyzed using CytExpert 2.3 (Beckman Coulter, Inc.).

**Scratch wound healing assay.** HUVECs were seeded onto a 6-well plate. After they reached  $>90\%$  confluence, a 10  $\mu\text{l}$  pipette tip was used to make a straight line in the middle of confluent monolayer. The floating cell debris was washed with PBS buffer and the injured cell monolayers were cultured in serum-free medium. The injured area was observed and the images captured at different time points (0, 24 and 48 h).

**Transwell migration assay.** HUVECs were seeded onto the upper Transwell inserts (Corning, Inc.) at  $2\times 10^4$ /well and allowed for migrating through the hole for 10 h. Meanwhile, serum-free media was added into the upper insert and the complete media (10% FBS in DMEM) was put into the lower chamber as the chemoattractant. These non-migrated cells were removed by cotton swabs. The migrated cells were fixed with methyl alcohol at room temperature for 15 min and stained with 0.5% crystal violet solution at room temperature for 30 min. Finally, the stained cells were counted under a light microscope and images were captured at magnification,  $\times 20$ .

**Tube formation assay.** The tube formation assay was performed to detect the angiogenic ability of HUVECs. The 24-well plate was frozen in advance, thawed Matrigel (BD Biosciences;

Becton, Dickinson and Company) was added onto the bottom of wells and incubated at 37°C for 1 h to solidify. After the required treatment, HUVECs were cultured onto these wells at  $1 \times 10^5$ /well. The tube formation was observed by a light microscope and the tube length was calculated using ImageJ 1.52p software (National Institutes of Health).

**Protein extraction and western blot analysis.** After the required treatment, HUVECs were collected and lysed in RIPA lysis buffer (Beyotime Institute of Biotechnology). Following centrifugation at  $10,000 \times g$  at 4°C for 15 min, the supernatant was collected and the concentration of protein was determined using a BCA Protein Assay kit (Thermo Fisher Scientific, Inc.). The extracted protein (30  $\mu$ g/lane) was separated on 10% SDS-PAGE and transferred onto the PVDF membranes (EMD Millipore). After blocking with 5% non-fat milk at room temperature for 0.5 h, the membranes were incubated with ERK1/2 (1:1,000; 9102), p-ERK1/2 (1:1,000; 4370), JNK (1:1,000; 9252), p-JNK (1:1,000; 9251), p38 (1:1,000; 9212), p-p38 (1:1,000; 9215), or GAPDH (1:1,000; 2118; all from Cell Signaling Technology, Inc.) overnight at 4°C. After washing with TBST (containing 0.05% Tween) for 3 times, the PVDF membranes were incubated with the HRP-conjugated secondary antibody (1:1,000; Beyotime Institute of Biotechnology) at room temperature for 3 h. The results of blots were visualized using an ECL detection system (Nanjing KeyGen Biotech Co., Ltd.) and the densitometry was measured using ImageJ 1.52p software (National Institutes of Health).

**Statistical analysis.** All experiments were repeated at least 3 times. All quantitative data were presented as mean  $\pm$  standard error of the mean. Statistical significance was calculated using unpaired or paired Student's *t* test or one-way ANOVA followed by Bonferroni post hoc test.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**SKLB1002 administration has no obvious cytotoxicity in vitro and tissue toxicity in vivo.** Corneal tissues were administered SKLB1002 (0.05 mg/ml), CMC-Na solution (0.5%), or PBS (Control) for 7 days. The histological change of the cornea was observed using H&E staining. Compared with the control group, no obvious morphologic change was observed after the administration of SKLB1002 or CMC-Na (Fig. 1A). MTT assay was performed to determine whether SKLB1002 had cytotoxicity *in vitro*. The result demonstrated that SKLB1002 had no obvious cytotoxicity on HUVECs ranging from 1 to 100 nm (Fig. 1B). Flow cytometry assays through Annexin V-FITC/PI double labeling revealed that compared with the control group, SKLB1002 administration did not lead to an increased percentage of apoptotic HUVECs (Fig. 1C). Collectively, these results show that SKLB1002 administration has no obvious cytotoxicity *in vitro* and tissue toxicity *in vivo*.

**Topical application of SKLB1002 eyedrop inhibits CoNV.** To determine whether SKLB1002 administration plays an inhibitory role in CoNV, a CoNV model was first constructed

through alkali-burn injury and then the injured corneas were treated with SKLB1002 eyedrops (0.05 mg/ml) or CMC-Na solution (0.5%) 3 times per day. The results demonstrated that new corneal blood vessels appeared at 1 day and peaked at 7 days after alkali-burn injury. The image of the anterior segment was taken using a slit lamp at 7 days after alkali-burn injury. There was no neovascularization in normal cornea (Ctrl group). Furthermore, alkali-burn injury induced an increased number and length of pathological corneal blood vessels (Alkali group). Compared with Alkali group, the number and the length of new corneal blood vessels was significantly reduced after the administration of SKLB1002 (Alkali + SKLB1002 group), but not CMC-Na (Alkali + CMC-Na group; Fig. 2). Collectively, the above-mentioned results suggest that SKLB1002 administration can suppress CoNV *in vivo*.

**SKLB1002 administration suppresses endothelial angiogenic function in vitro.** To determine the effect of SKLB1002 administration on HUVEC viability, HUVECs were pre-treated with SKLB1002 and then treated with or without VEGF (10 ng/ml). VEGF treatment significantly increased the viability of HUVECs. However, pre-treatment with SKLB1002 (10 or 50 nM) significantly reduced VEGF-induced increase in HUVEC viability (Fig. 3A). EdU incorporation assay and Ki67 immunofluorescence staining demonstrated that pre-treatment with SKLB1002 significantly decreased the proliferation ability of HUVECs (Fig. 3B and C). Transwell migration assay and scratch wound healing assay demonstrated that pre-treatment of SKLB1002 significantly reduced the migration ability of HUVECs (Fig. 3D and E). Matrigel tube formation assay demonstrated that VEGF-mediated tube formation ability was interrupted after the administration of SKLB1002 (Fig. 3F).

**SKLB1002 serves its anti-angiogenic function via the mitogen-activated protein kinase (MAPK) signaling pathway.** VEGF and VEGFR-2 usually serve their roles through activation of MAPK signaling. Western blotting was performed to investigate whether SKLB1002 administration could affect the activation of the MAPK signaling pathway. VEGF treatment led to increased expression levels of phosphorylated ERK1/2, JNK and p38. The increased expression of these proteins was markedly interrupted after SKLB1002 administration (Fig. 4A). It was thus concluded that SKLB1002 played its anti-angiogenic role in endothelial cells through inactivation of MAPK signaling as demonstrated in Fig. 4B. To further verify whether SKLB1002 performed its anti-angiogenic role in endothelial cells through MAPK signaling, HUVECs were treated with p38 inhibitor (SB203580), ERK inhibitor (U0126), JNK inhibitor (SP600125), or SKLB1002 and then incubated with or without VEGF. The results demonstrated that SKLB1002 administration decreased the viability of HUVECs (Fig. 4C) and reduced the migration and tube formation ability of HUVECs (Fig. 4D and E), showing similar effects as the combined effects of SB203580, U0126 and SP600125 administration on endothelial angiogenic functions. These results provided additional evidence that SKLB1002 plays its anti-angiogenic role in endothelial cells through the inactivation of MAPK signaling.

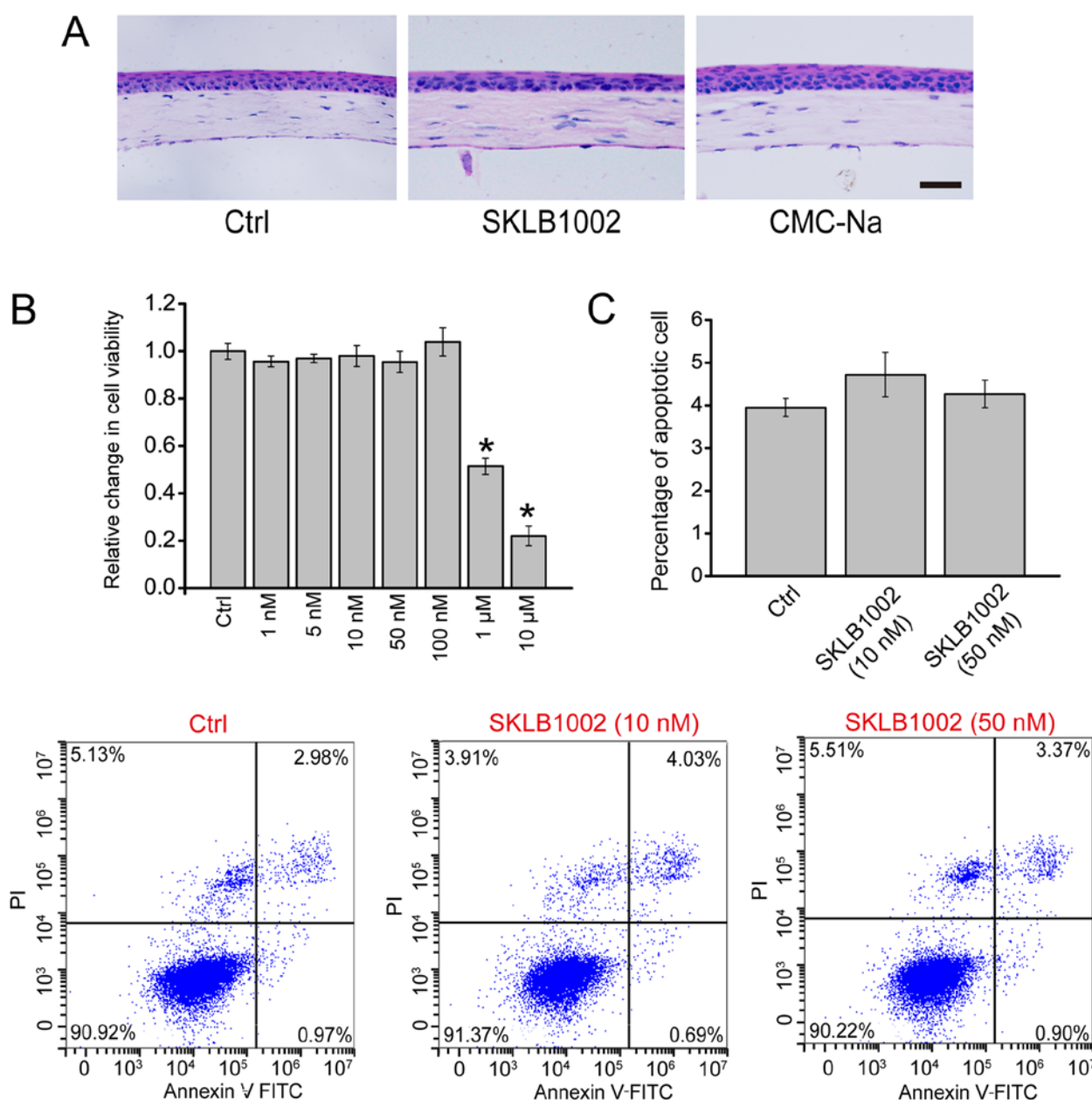


Figure 1. SKLB1002 administration has no obvious cytotoxicity *in vitro* and tissue toxicity *in vivo*. (A) Male ICR mice (12 weeks old) were administered SKLB1002, CMC-Na and PBS (Ctrl) for 7 days. The histological change of the cornea was determined by hematoxylin and eosin staining (n=3; Scale bar, 100  $\mu$ m). (B) HUVECs were administered different concentrations of SKLB1002 for 24 h or left untreated (Ctrl). MTT assays were performed to determine cell viability (n=3; P=0.024 and P=0.011, respectively). (C) Flow cytometry and quantification analysis were performed to determine the percentage of apoptotic HUVECs with SKLB1002 (10 and 50 nM, 24 h) or without SKLB1002 administration (Ctrl) using Annexin V-FITC/PI double staining (n=3). \*P<0.05 vs. Ctrl group. CMC-Na, sodium carboxymethyl cellulose; Ctrl, control; HUVECs, human umbilical vein endothelial cells; PI, propidium iodide.

## Discussion

CoNV is characterized by the invasion of the new blood vessels into the cornea and is a major cause of blindness worldwide (19-21). However, the current therapeutic options have demonstrated limited or transitory results (22). The present study investigated the effects of SKLB1002 administration on the progression of CoNV. SKLB1002 administration can inhibit the progression of CoNV *in vivo* and regulate endothelial angiogenic functions *in vitro*.

Under normal condition, the transparency of the cornea is a prerequisite for vision (23). Pathological factors, such as infection, chemical burns, hypoxia and inflammatory molecules,

can interrupt the balance between angiogenic and anti-angiogenic factors in the cornea. VEGF has been reported as a major angiogenic factor during CoNV (24). The VEGF level in the vascularized cornea is significantly higher compared with the normal cornea (1,10,25). Anti-VEGF agents such as bevacizumab have been used for treating ocular angiogenesis, including CoNV. However, they require intravitreal injection and a relatively frequent dosing regimen (4-6 weeks) (26,27). In experimental models of neovascularization, anti-VEGF treatment became less effective at blocking vessel growth and regressing vessels as the neovascularization develops over time (8,28,29). In addition, subconjunctival injection of an anti-VEGF drug may lead to hemorrhage and damage patient



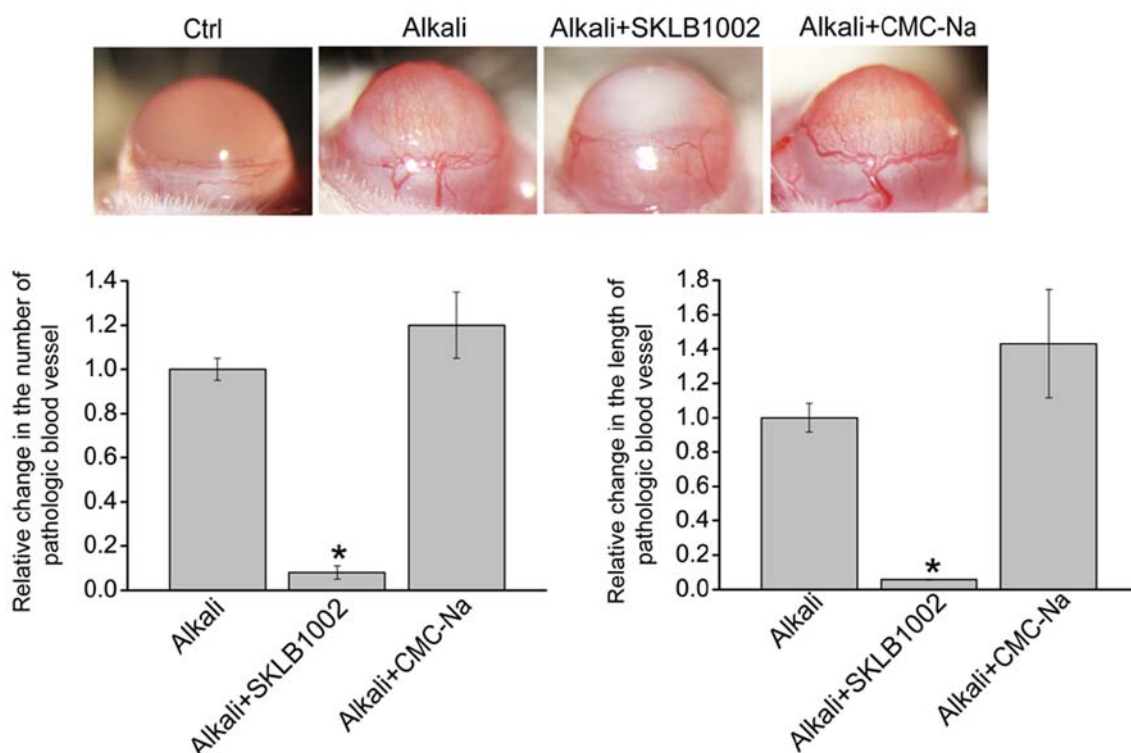


Figure 2. Topical application of SKLB1002 eyedrop inhibit CoNV. Male ICR mice (12 weeks old) were used to build CoNV model through alkali-burn injury. The eyedrops of SKLB1002 or CMC-Na were applied onto the injured cornea. At day 7 after alkali burn, CoNV was observed using a slit lamp. CoNV was quantified and normalized, including pathological vessel length and number.  $P=0.0047$  or  $0.0032$ , respectively. \* $P<0.05$  vs. alkali-burn injury group. CMC-Na, sodium carboxymethyl cellulose; CoNV, corneal neovascularization; Ctrl, control.

compliance. Thus, there remains a requirement to search for novel therapies for CoNV.

SKLB1002 is a novel potent inhibitor of VEGF receptor 2 signaling. It has no significant toxicity on mouse corneal structure *in vivo* and endothelial cell viability *in vitro*. An alkali-burn corneal angiogenesis model was used to investigate the role of SKLB1002 in anti-angiogenic effects. Eyedrops were used to deliver SKLB1002 onto the ocular surface, which have great advantage over the traditional subconjunctival injection because it is noninvasive and easily accepted by the patients. However, tear screening and nasolacrimal duct drainage may affect the bioavailability of SKLB1002 (30,31). To overcome this flaw, CMC-Na was used as the solvent of the eyedrops to increase the retention time of SKLB1002. CMC-Na is a hydrosoluble biopolymer derived from cellulose (18,32). CMC-Na has been widely used due to its high viscosity, non-toxicity and non-allergenicity (16). In addition, a previous study reported that CMC-Na can be used as a drug solvent to treat dry eye (17). Mouse eyes were injured through incubation with 1 mol/l NaOH to induce CoNV. After 7 days of alkali burn, the images of the anterior segment showed that the number and length of CoNV was attenuated in SKLB1002-administrated group.

Angiogenesis is a complicated process comprising the participation of multifarious cells and factors. Endothelial cells are the key regulators in the angiogenic cascade (33,34). Once the balance between angiogenic factors and anti-angiogenic factors is disturbed, endothelial cells are activated to proliferate, migrate and form the tubes (35-37). The present study demonstrated that SKLB1002 administration can inhibit the

VEGF-induced proliferation, migration and tube formation ability of HUVECs, suggesting a critical role of SKLB1002 in inhibiting endothelial angiogenic functions.

The molecular mechanism of SKLB1002 in anti-angiogenic effects was also investigated. VEGF-VEGFR-2 signaling has been reported to serve their roles in angiogenesis through the activation of MAPK signaling (38,39). MAPKs comprise the ERK1/2, JNK1/2/3 and p38 isoforms (40,41). ERK1/2 is involved in the regulation of HUVEC proliferation (42). VEGF-induced p38 change can affect cell migration (43,44). JNK plays important roles in the cell proliferation and apoptotic responses to cellular stresses (45). The present study demonstrated that SKLB1002 decreased the phosphorylation level of ERK1/2, JNK and p38. Given that ERK1/2, JNK and p38 have been demonstrated to be the critical regulators of cell proliferation, migration and tube formation (46), it is not surprising that SKLB1002-regulated MAPK signaling pathway is involved in the regulation of the angiogenic cascade.

In conclusion, the present study demonstrated that SKLB1002, a small-molecule inhibitor of VEGFR-2, exhibited anti-angiogenic effects on CoNV by blocking the MAPK signaling pathway. SKLB1002 administration can inhibit endothelial cell proliferation, migration and tube formation *in vitro*. Thus, selective inhibition of VEGFR-2 through SKLB1002 administration is a promising therapy for ocular angiogenesis. Angiogenesis can also contribute to the pathogenesis of other diseases, such as malignant, inflammatory, infectious and immune disorders. Anti-angiogenesis by SKLB1002 administration may offer new therapeutic opportunities for these disorders.

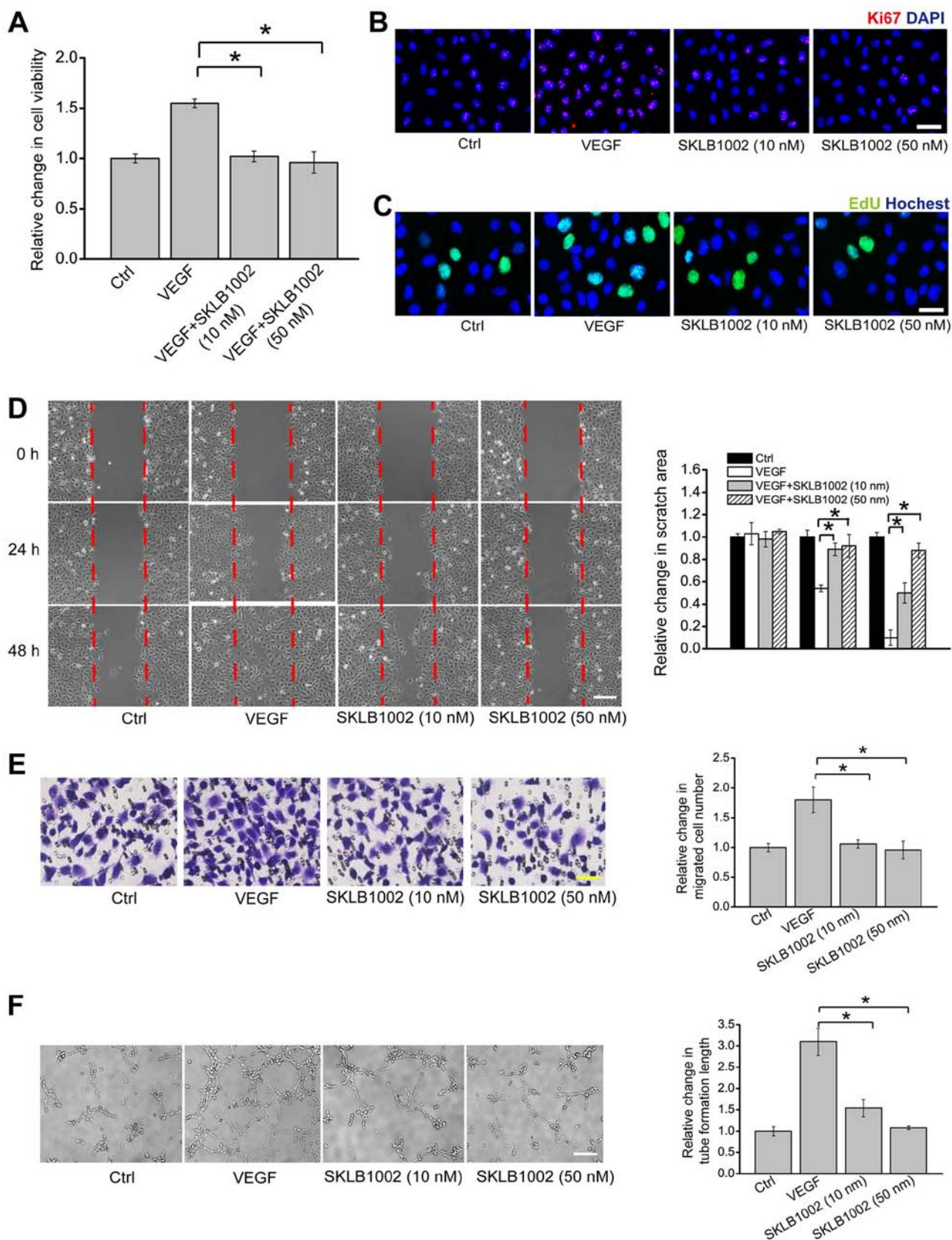


Figure 3. SKLB1002 administration suppresses endothelial angiogenic function *in vitro*. (A-C) HUVECs were cultured with VEGF (10 ng/ml), VEGF plus SKLB1002 (10 or 50 nM), or left untreated (Ctrl) for 24 h. Cell viability was determined by (A) MTT assay. Cell proliferation was detected by (B) Ki67 immunofluorescence staining and (C) EdU incorporation staining. HUVECs were cultured with VEGF (10 ng/ml), VEGF plus SKLB1002 (10 or 50 nM), or left untreated (Ctrl). (D) Scratch wound healing assay (n=3; scale bar, 100  $\mu$ m) and (E) Transwell assay (n=3; scale bar, 50  $\mu$ m) were performed to detect the migration of HUVECs. Quantification of migration was expressed as (D) the relative change of scratch area or (E) relative change of migrated cell number. HUVECs were seeded onto the Matrigel matrix and cultured at 37°C for 6 h. (F) The length of tube-like structures was captured by a light microscope and calculated using Image J software (n=3; scale bar, 50  $\mu$ m). All data were from  $\geq 3$  independent experiments. The significant difference ( $P < 0.05$ ) was evaluated by one-way ANOVA followed by Bonferroni post hoc test. HUVECs, human umbilical vein endothelial cells; VEGF, vascular endothelial growth factor; Ctrl, control.

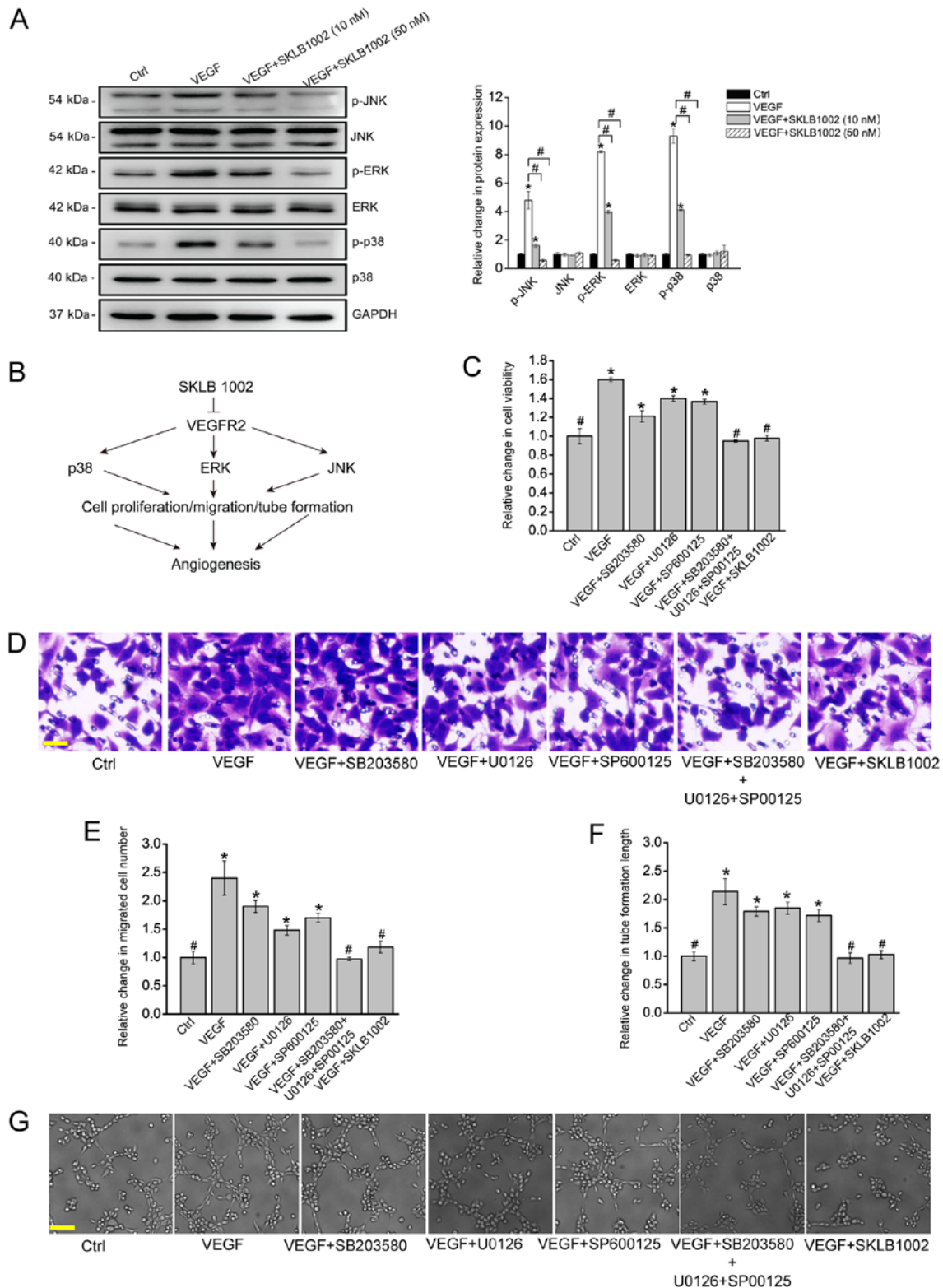


Figure 4. SKLB1002 performs its anti-angiogenic role via the MAPK signaling pathway. (A) HUVECs were incubated with or without SKLB1002 (10 or 50 nM) plus VEGF (10 ng/ml) or left untreated (Ctrl) for 24 h. HUVECs were collected and lysed. The expression levels of total ERK1/2, JNK, p38 and the phosphorylated forms of these proteins (p-ERK1/2, p-JNK and p-p38) were detected by western blotting. GAPDH was used as the internal control. (A) Representative immunoblots along with the densitometric quantitative results are shown. \* $P < 0.05$  vs. Ctrl group ( $P = 0.013, 0.028, 0.008, 0.016, 0.0052$  and  $0.018$ , respectively). # $P < 0.05$  vs. the marked groups. (B) A schematic image showing the potential mechanism of SKLB1002 in the regulation of endothelial angiogenic functions. HUVECs were pretreated with or without SB203580, U0126, or SP600125 and then incubated with VEGF (10 ng/ml), VEGF plus SKLB1002 (50 nM), or left untreated (Ctrl). Cell viability was determined by (C) MTT assay ( $n = 3$ ; \* $P = 0.017, 0.031, 0.023$  and  $0.027$ , respectively; # $P = 0.017, 0.022$  and  $0.026$ , respectively). (D) Transwell assay and (E) quantification analysis was performed to determine the migration of HUVECs ( $n = 3$ ; scale bar,  $50 \mu\text{m}$ ; \* $P = 0.005, 0.013, 0.022$  and  $0.015$ , respectively; # $P = 0.005, 0.008$  and  $0.014$ , respectively). HUVECs were seeded onto the Matrigel matrix and cultured at  $37^\circ\text{C}$  for 6 h. (F and G) The length of tube-like structures was captured by a light microscope and calculated using Image J software ( $n = 3$ ; scale bar,  $50 \mu\text{m}$ ; \* $P = 0.006, 0.017, 0.012$  and  $0.019$ , respectively; # $P = 0.006, 0.008$  and  $0.011$ , respectively). \* $P < 0.05$  vs. Ctrl group; # $P < 0.05$  vs. VEGF group. All data were from  $\geq 3$  independent experiments. The significant difference was evaluated by one-way ANOVA followed by Bonferroni post hoc test. MAPK, mitogen-activated protein kinase; HUVECs, human umbilical vein endothelial cells; VEGF, vascular endothelial growth factor; Ctrl, control; p, phosphorylated.



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## Availability of data and materials

All data generated or analyzed during this study are included in this published article.

## Authors' contributions

BY and QJ were the major contributors to the experimental design. QZ and ST established the animal models. CL, JL, XL and JY performed the western blot analysis. QZ and ST performed the cell culture. BY was involved in writing the manuscript and the analysis and interpretation of data. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

All experimental procedures adhered to the principles stated in the Guide for the Care and Use of Laboratory Animals (updated 2011; National Institutes of Health, Bethesda, MD, USA) and were approved by the Animal Care and the Use Committee of Nanjing Medical University.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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