Serine Proteinase Inhibitor SERPINA3K Suppresses Corneal Neovascularization Via Inhibiting Wnt Signaling and VEGF

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Purpose. To evaluate the antineovascularization effects and investigate the possible mechanisms of SERPINA3K, a member of serine proteinase inhibitor family, using a specific rat model of suture-induced corneal neovascularization.

Methods. A rat corneal suture model was set up and SERPINA3K was topically administered three times daily for 7 days. The clinical indications were evaluated on days 2, 5, and 7, including area of neovascularization and inflammation index. The eyeballs were collected after day 7 and the following examinations were performed: histologic investigation, immunostaining, Western blot, and quantitative real-time PCR assay. Wnt3a, a Wnt pathway ligand, was added to cultured human umbilical vein endothelial cells (HUVEC), followed by detecting cell migration and Western blot. Meanwhile, an in vitro $VEGF_{165}$-stimulated HUVEC model was applied and the following measurements were conducted: cell proliferation, cell migration, and tube formation.

Results. SERPINA3K significantly suppressed corneal neovascularization and inhibited corneal inflammation. SERPINA3K downregulated the levels of β-catenin, nonphospho-β-catenin, and transcription factor 4 (TCF4), but upregulated the level of phospho-β-catenin of the corneas induced by suture. SERPINA3K also decreased the gene expression and protein level of VEGF. Meanwhile, induction of Wnt3a increased the cell migration, activated the Wnt signaling, and upregulated VEGF in cultured HUVEC, which were antagonized by SERPINA3K. In addition, SERPINA3K significantly inhibited $VEGF_{165}$-induced cell proliferation and migration of HUVEC, SERPINA3K also specifically suppressed the $VEGF_{165}$-induced tube formation of HUVEC.

Conclusions. SERPINA3K has therapeutic potential for corneal neovascularization. The underlying mechanism may be through inhibiting Wnt signaling pathway and VEGF.

Keywords: corneal neovascularization, SERPINA3K, Wnt signaling pathway

Corneal neovascularization is associated and accompanied with numerous of ocular surface diseases, such as inflammatory or infectious disorders and so on. Corneal neovascularization is the main cause leading to loss of vision and blindness.[1,2] It is considered that a balance exists between pro-angiogenic and antiangiogenic factors in the cornea. Corneal neovascularization occurs when the balance is tilted toward angiogenesis in pathological conditions.[1,3] Various types of treatments were reported to effectively suppress corneal neovascularization including medical and surgical therapies.[4–6] However, the etiology of corneal neovascularization is not completely identified. Further investigations are also needed to find the new ideal therapies and explore the therapeutic mechanisms.

Wnt signaling plays an important role in the regulation of fundamental aspects of development during cell fate specification, proliferation, survival, and overall organogenesis.[7–10] Recent reports demonstrated that Wnt system impacts the vascular morphogenesis in the embryo and in organ-specific endothelial cell differentiation. Disorder of Wnt signaling pathway will influence vascular development and lead to various vascular diseases. Further studies have revealed that the capacity of Wnt signaling affects the angiogenesis in a variety of pathological diseases such as angiogenesis in cancer. There are also reports that Wnt signaling plays roles in the neovascularization of eyes, particularly in retina, while the full mechanism remains unknown.[11,12]

β-catenin is the major mediated protein of Wnt pathway. In absence of Wnt ligands, cytoplasmic β-catenin is phosphorylated and degraded by a multiprotein destruction complex and Wnt targeted genes are repressed. Activation of the canonical Wnt pathway involves stabilization of β-catenin through binding of Wnt ligands to the cell-surface receptors including Frizzled (Fz) family receptors and low-density lipoprotein receptor-related proteins (LRP5/6). The nonphosphorylated form of β-catenin (nonphospho-β-catenin) then accumulates in the cytoplasm and is translocated into the nucleus, and then β-catenin will bind and activate transcription factor/lymphoid

enhancer-binding factor (TCF/LEF) family transcription factors and regulate the expression of downstream targeted genes including VEGF, an important factor for angiogenesis.[13–15]

SERPINA3K, also named kallikrein-binding protein (KBP), is a member of serine proteinase inhibitors. It is reported to be predominantly produced in the liver and be identified in retina and vitreous body. SERPINA3K binds to tissue kallikrein and inhibits its activity specifically.[16,17] SERPINA3K has been demonstrated to be an anti-neovascularization protein in retinal neovascularization.[18] Studies revealed that SERPINA3K plays the antineovascularization roles through binding of the LRP6 and inhibits Wnt signaling pathway, which is independent of its interactions with the kallikrein-kinin system.[18,19] We previously demonstrated that SERPINA3K has anti-inflammation and antineovascularization effects in the rat corneas with alkali-burn, a general chemical injury to corneas, while the underlying mechanism of antineovascularization needs to be further investigated.[20]

In this present study, we focused on and evaluated the antineovascularization effects of SERPINA3K on the corneas, using the corneal suture, which is a more specific and dominant model to mainly investigate the corneal neovascularization and further investigated the possible mechanisms. We elucidated and focused on the roles of Wnt signaling pathway and VEGF with either in vivo or in vitro models. It was a continuation of our previous work to offer a fresh perspective and better understanding of the etiology of corneal neovascularization and provide new evidence for its novel potential therapy.

Materials and Methods

Materials

The CCK-8 assay kits were purchased from Dojindo Company (Tokyo, Japan). The matrigel basement membrane matrix was purchased from Becton Dickinson and Company (Franklin Lakes, NJ, USA). The antibodies of phospho-β-catenin, non-phospho-β-catenin, and transcription factor 4 (TCF4) were purchased from Cell Signaling Technology, Inc., (Danvers, MA, USA). The polymorphonuclear leucocyte (PMN) antibody was purchased from Fitzgerald (Acton, MA, USA). The antibody of VEGF was purchased from Abcam (Cambridge, MA, USA) and the antibody of β-actin was purchased from Sigma Aldrich Corp. (St. Louis, MO, USA). The Wnt3a peptide was purchased from Abcam (Cambridge). The recombinant human $VEGF_{165}$ protein was purchased from R&D Systems, Inc. (Minneapolis, MN, USA).

Rat Corneal Suture Model

Thirty Wistar rats (male, 180–220 g) were purchased from Shanghai SLAC laboratory animal center (Shanghai, China). The rats were kept in standard environment throughout the study as follows: room temperature 25°C ± 1°C, relative humidity 60% ± 10%, and alternating 12 hour light–dark cycles. Food and water were available ad libitum. All animal experiments were performed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the experimental protocol was approved by the experimental animal ethics committee of Xiamen University (Xiamen, Fujian, China).

The rat corneal suture was applied to induce corneal neovascularization. After induction of general anesthesia by intraperitoneal injection with pentobarbital (40–50 mg/kg), topical application of 0.5% proparacaine ophthalmic solution was added. Three corneal sutures (10-0 nylon) were conducted in the cornea between corneal center and the limbus at the 12, 4, and 8 o'clock position, respectively. Topical norfloxacin was applied immediately after suture surgery. The rats were carefully maintained and the eyes were observed daily after surgery.

In Vivo Experimental Procedure

After the suture model was set up, the eyes of rats were carefully examined and six rats were not used in the experiments due to the failure of surgery. The remaining 24 normal and successfully sutured rats were randomly divided into four groups ($n = 6$ in each group): (1) normal or control group without suture and any treatment, (2) suture plus BSA (0.25 μg/μL) as positive control group, (3) suture plus SERPINA3K (0.05 μg/μL) group, and (4) suture plus SERPINA3K (0.25 μg/μL) group. All topical administration was applied at volume of 10 μL per eye each time and given three times (8 AM, 3 PM, 10 PM) daily for 7 days. During the experiment, the clinical evaluations of corneal neovascularization and inflammation were performed by a single masked experienced ophthalmologist with slit-lamp microscopy on days 2, 5, and 7. After administration for 7 days, all rats were killed and the whole corneas were dissected for histologic analysis, immunostaining, Western blot, and quantitative real-time PCR assay following the methods described below.

Evaluation of Neovascularization and Inflammation

Images were captured using a slit-lamp microscope (Kanghua Science & Technology Co., Chongqing, China), and the corneal neovascularization was quantified as previously described.[20] Briefly, the cornea was divided into four quadrants and the vessel length of each quarter (L_i, $i = 1$–4) was measured. The cornea neovascularization area (A) was calculated using the equation

$$A = \sum_{i=1-4} 3.1416 \times \left\{R^2 - (R - L_i)^2\right\}, \qquad (1)$$

where $R = 3.5$ mm, the radius of rat cornea.

Inflammatory response was evaluated by slit lamp and the inflammatory index was analyzed as previously described.[20] mainly based on the following three parameters: ciliary hyperemia (absent, 0; present but less than 1 mm, 1; present between 1 and 2 mm, 2; present more than 2 mm, 3); central corneal edema (absent, 0; present with visible iris details, 1; present without visible iris details, 2; present without visible pupil, 3); and peripheral corneal edema (absent, 0; present with visible iris details, 1; present without visible iris details, 2; present with no visible iris, 3). The final inflammatory index data were obtained by summing the scores of the different parameters divided by a factor of nine.

Purification of SERPINA3K

The SERPINA3K/Pet28 plasmid was introduced into *Escherichia coli* strain BL21. The purification protocol of SERPINA3K was followed as described previously.[20] The purity of recombinant SERPINA3K was detected by SDS-PAGE and the endotoxin levels were examined using a limulus amebocyte kit (Biowhittaker, Walkeraville, MD, USA). Activity of the purified protein was examined by CCK-8 assay using primary human umbilical vein endothelial cells (HUVEC).

Cell Culture Procedures

Primary HUVEC were isolated from fresh human umbilical cord veins by 0.25% EDTA-Tripsin (Invitrogen, Carlsbad, CA, USA) treatment as described previously with modifications.[21] This investigation was carried out according to the principles

outlined in the Declaration of Helsinki for medical research involving human subjects (2008). All the tissue donors signed a written informed consent and the Institutional Medical Ethics Committee of Xiamen University approved the study protocol. Tightly confluent monolayers of HUVEC from third to sixth passage were used in all the experiments. The cells were cultured in M200 medium supplemented with low serum growth supplement (LSGS) and 1% penicillin. The culture dish was placed in a CO_2 regulated incubator with 5% CO_2. Medium was replaced every 2 days. The cultured HUVEC were used in the following experiments as described below.

When cells were cultured to 75% confluency, the culture medium was replaced with Wnt3a conditional medium or Wnt3a plus SERPINA3K. After treatment for 24 hours, cells were collected for following tests. For $VEGF_{165}$ treatment, the pattern was the same with the Wnt3a treatment as described above.

Cell Viability

The CCK-8 assay was performed to assess the viability of cells as described in our previous study.[22] Briefly, the HUVECs were inoculated in 96-well flat-bottomed plates at a concentration of 8000 cells per well. The HUVEC were cultured to 75% confluency for cell viability test. The media was replaced by 10% CCK-8 constituted in basic culture media. After incubation of 4 hours in incubator, the solution was directly detected. The absorbance was detected spectrophotometrically at 570 nm with a Bio Tek EXL800 microplate reader (Bil-Tek Instrument, Highland Park, Winooski, VT, USA).

Endothelial Cell Scratch Wound Assay

After the HUVECs grew to approximately 100% confluency in 96-well plates, the cells were wounded with pipette tips and washed with PBS. M200 medium containing 0.5% LSGS was added into the wells with or without 20 ng/mL VEGF or 500 ng/mL Wnt3a and various concentrations of SERPINA3K. DAPI (4′,6-diamidino-2-phenylindole; Vector, Burlingame, CA, USA) was added and incubated for 3 minutes before the image was taken. Images of the closing wound were acquired by microscope at 0 and after 24 hours of incubation. The migration area was detected and analyzed using the ImageJ 1.46r image analysis software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). Three independent experiments were performed.

Tube Formation Assay

The tube formation assay was performed as described previously with modifications.[23] Briefly, the growth factor-reduced Matrigel (BD Biosciences, Bedford, MA, USA) was pipetted into 96-well plates (100 uL Matrigel per well) and polymerized for 30 minutes at 37°C. Human umbilical vein endothelial cells were placed onto the layer of Matrigel (15,000 cells per well) in M200 added with 20 ng/mL $VEGF_{165}$ or different concentration of SERPINA3K. After 6 hours of incubation in the carbon dioxide cell incubator, the cells were photographed using an inverted microscope (magnification ×40; Olympus, Tokyo, Japan). The perimeter of HUVEC formational tubes were measured and analyzed by ImageJ software. Each experiment was repeated three times.

Histologic Staining and Immunostaining

After surgery for 7 days, the rat global tissues were collected, embedded in optimal cutting temperature compound (OCT; Tissue-Tek Miles, Inc., Elkhart, IN, USA) and then frozen at –80°C and kept for use. Sections with thickness of 6 μm were cut with a cryotome (CM 1850UV; Leica Microsystems AG, Wetzlar, Germany) and fixed in acetone at –20°C for 10 minutes before the following assays were conducted.

For hematoxylin and eosin (H&E) staining, the sections were stained with the alum hematoxylin for 3 to 5 minutes, differentiated with 0.3% acid alcohol for 2 to 3 seconds, and rinsed in running tap water for 15 minutes. After staining with eosin for 2 minutes, the sections were dehydrated, cleared, and mounted.

For immunofluorescent staining, the sections were blocked in 2% BSA for 1 hour and incubated at 4°C overnight with the β-catenin antibody (1:150). After incubation in AlexaFluor488-conjungated IgG (1:1000), samples were counterstained with DAPI, mounted and photographed using a confocal laser scanning microscope (Fluoview 1000; Olympus).

For immunohistochemical staining, the endogenous peroxides activity was quenched with 0.6% hydrogen peroxide for 30 minutes. After incubating with 2% BSA, the antibodies of TCF4 (1:400) and PMN (1:8000) were applied and incubated at 4°C overnight. Then the sections were further incubated with biotinylated anti-rabbit IgG (1:50) using Vectastain Elite ABC kits (Vector Laboratories, Inc., Burlingame, CA, USA) according to the manufacturer's protocol. After rinsing with PBS, the reaction product was developed with diaminobenzidine (DAB), the peroxidase substrate for 60 seconds and mounted with mounting medium (H-5000; Vector) and examined under a light microscope (Nikon Eclipse 50i; Tokyo, Japan).

Western Blot

Proteins of corneas or cells were extracted with cold RIPA buffer. Equal amounts of proteins of cell lysates were subjected to electrophoresis on 8% SDS-PAGE and then electrophorectially transferred to PVDF membranes. After blocking in 1% BSA for 1 hour, the membranes were incubated with primary antibodies of nonphospho-β-catenin (1:1000), phospho-β-catenin (1:1000), VEGF (1:1000), and β-actin (1:10,000) as loading control. After three washes with Tris-buffer saline with 0.05% Tween-20 for 10 minutes each, the membranes were incubated with HRP-conjugated goat anti-rabbit IgG (1:10,000; Bio-Rad, Hercules, CA, USA) for 1 hour at room temperature. The specific bands were visualized by enhanced chemiluminescence reagents (Lulong, Inc., Xiamen, China) and recorded by the transilluminator (ChemiDox XRS; Bio-Rad).

RNA Isolation and Quantitative Real Time PCR

Total RNA was extracted from the corneas using TRIzol reagent (Invitrogen). Reverse transcription was performed with Oligo 18T primers and reverse transcription reagents according to the manufacturer's protocol (TaKaRa, Shiga, Japan). Quantitative real-time PCR was performed with VEGF mRNA special primers: 5′-ATTGAGACCCTGGTGGAC′ (forward) and 5′-CCTATGTGCTGGCTTTGG-3′ (reverse). Polymerase chain reaction reactions were performed on a BIO-RAD CFX-96 Real Time system with SYBR Premix Ex Taq (TaKaRa) at 95°C for 10 minutes, followed by 45 cycles of 95°C for 10 seconds, 57°C for 30 seconds, and 75°C for 10 seconds, after which melt curve analysis was performed at once from 65°C to 95°C. All reactions were performed in triplicate and the average cycle threshold (Ct) values greater than 38 were treated as negative.

Statistical Analysis

The data of CCK-8 assay, tube formation, cell migration, Western blot, and quantitative real-time PCR were analyzed by one-way ANOVA, followed by a post hoc analysis Tukey test to compare the differences between the groups or by a

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Student's *t*-test. *P* less than 0.05 was considered statistically significant.

Results

Antineovascularization Effects of SERPINA3K in the Cornea

We selected and applied rat corneal suture, which is a more specific and dominant model to mainly investigate the corneal neovascularization or angiogenesis. We first examined the clinical indication of corneal neovascularization. It was demonstrated that numerous of new vessels invaded the suture area from limbal region in BSA-treated group on day 7 (Fig. 1B, as arrow pointed). The rats treated with SERPINA3K at both low (0.05 μg/μL) and high (0.25 μg/μL) dosages showed apparently less corneal neovascularization (Figs. 1C, 1D, as arrows pointed), compared with BSA-treated group (Fig. 1B). The data of quantified measurement of neovascularization area revealed that SERPINA3K at low (0.05 μg/μL) and high (0.25 μg/μL) dosages significantly reduced the corneal neovascularization on days 5 and 7, compared with BSA-treated group (Fig. 1E), suggesting an antagonizing effect of SERPINA3K on the corneal neovascularization.

Anti-Inflammatory Effects of SERPINA3K in the Cornea

We also detected and analyzed corneal inflammation simultaneously. The data of quantified measurement of inflammatory index revealed that SERPINA3K at low (0.05 μg/μL) and high (0.25 μg/μL) dosages significantly reduced inflammation on days 5 and 7, compared with BSA-treated group (Fig. 2A).

Meanwhile, histologic examination of H&E staining showed that BSA-treated group had more inflammatory cell infiltration in the corneal stroma (Fig. 2B), while SERPINA3K decreased inflammatory cell infiltration (Fig. 2B). SERPINA3K also attenuated the suture-induced thickening of the cornea, suggesting an improved corneal edema. We next observed and confirmed the inflammatory cells in the cornea by immunohistolchemical staining with PMN antibody, which is a marker of neutrophils. It showed that dramatic PMN positive staining cells were found in stroma in BSA-treated group, while SERPINA3K reduced neutrophils (Fig. 2B). These results indicated that SERPINA3K suppressed corneal inflammation induced by saturation.

SERPINA3K Blocked Wnt Signaling Pathway in the Cornea

To elucidate the mechanism underlying the effects of SERPINA3K on corneal neovascularization in the suture model, we focused on the role of SERPINA3K on the Wnt signaling pathway since SERPINA3K was reported to be an inhibitor of Wnt pathway,[18,19] by detecting the factors of Wnt pathway: β-catenin, nonphospho-β-catenin, phospho-β-catenin, and TCF4 with immunostaining, Western blot, and quantitative real-time PCR assay.

It was demonstrated that corneal saturation induced dramatic stronger immunostaining signals of β-catenin in the corneal epithelium and stroma in BSA-treated group, compared with the control (healthy) group, while SERPINA3K (0.05 μg/μL) blocked the upregulation of β-catenin induced by suture (Fig. 3A). Likewise, the Western blot results showed that the level of nonphospho-β-catenin was significantly increased in BSA-treated group after saturation and SERPINA3K significantly decreased the level of nonphospho-β-catenin (Fig. 3B). Interestingly, the data of Western blot also demonstrated that

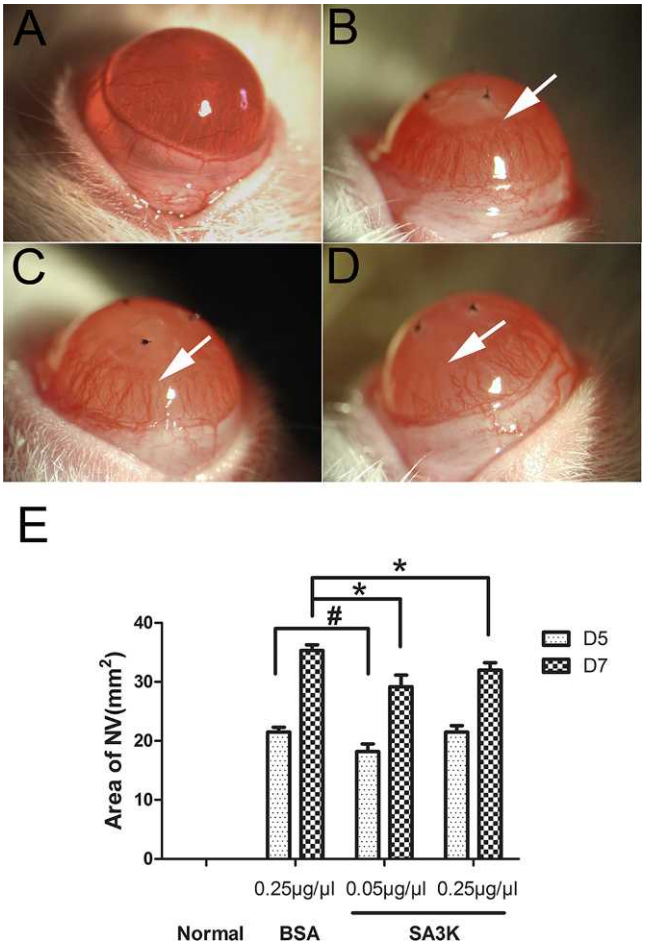

Figure 1. Antineovascularization effects of SERPINA3K in the cornea. (**A**–**D**) Representative micrographs of neovascularization on day 7 after saturation. (**A**) Group of normal or control rat without saturation or any treatment, (**B**) group of saturation plus BSA (0.25 μg/μL), (**C**) group of saturation plus SERPINA3K (0.05 μg/μL), and (**D**) group of saturation plus SERPINA3K (0.25 μg/μL). The new vessels were seen in corneas (**B**) and the new vessels were reduced in (**C**, **D**) (as *arrows* pointed). (**E**) The statistic comparison of the data of neovascularization among the above four groups on day 5 and 7 (data are presented as mean ± SEM, $n = 6$ in each group, $^{*}P < 0.05$, $^{\#}P < 0.05$; SA3K, SERPINA3K).

the level of phospho-β-catenin, which represents the degradation of β-catenin and inactivation of Wnt pathway, was reduced in BSA-treated group and SERPINA3K upregulated phospho-β-catenin back to normal level (Fig. 3C). Furthermore, we also measured the alteration of targeted factor of Wnt pathway: TCF4, using immunohistochemical staining with antibody of TCF4. It was shown that the immnunosignals of TCF4 were stronger in the corneal epithelium and stroma of BSA-treated group, compared with control group and it was apparently weaker in SERPINA3K-treated group (as arrows showed in Fig. 3D).

Taken together, the results above suggested that corneal suture induced activation of Wnt signaling pathway in the cornea and SERPINA3K blocked the activation of Wnt pathway induced by corneal suture.

SERPINA3K Downregulated VEGF in the Cornea

VEGF is a key factor of proneovascularization or pro-angiogenesis. We measured the gene expression and protein level of VEGF by quantitative real-time PCR assay and Western

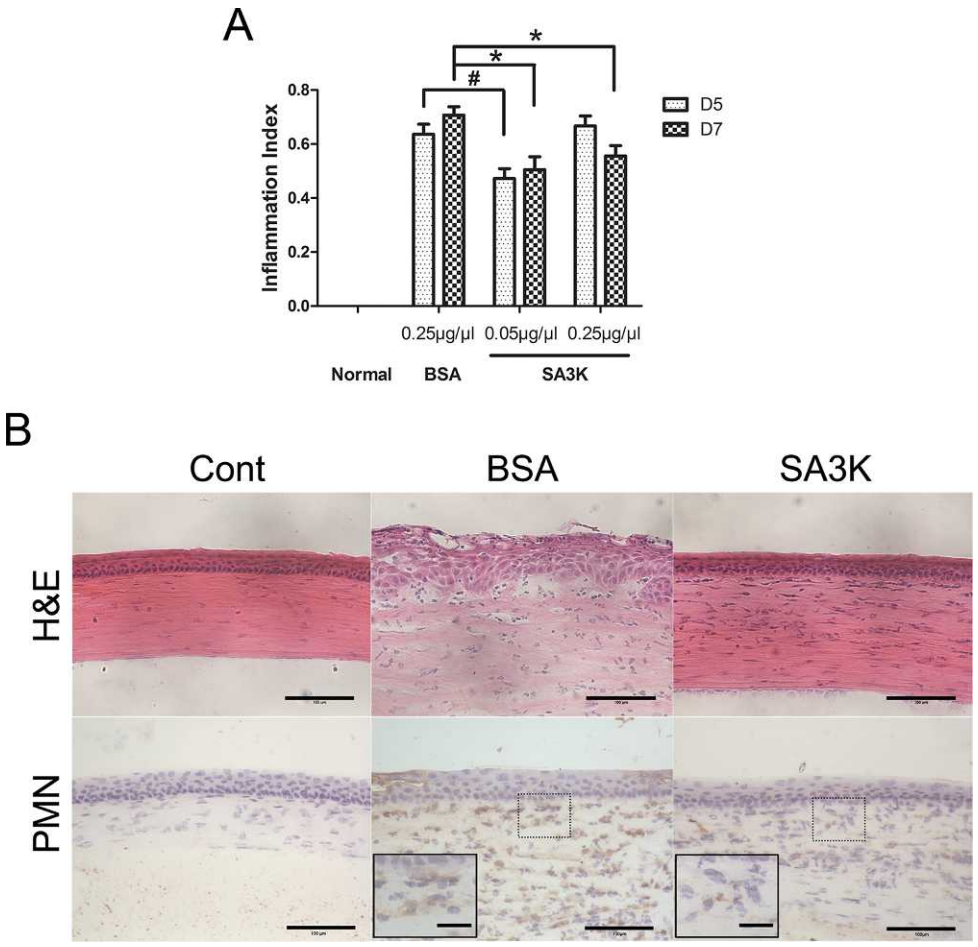

Figure 2. Anti-inflammatory effects of SERPINA3K in the cornea. (**A**) The statistic analysis of clinical inflammatory index data among groups on day 5 and 7. The rats were divided into four groups: normal or control rat without saturation or any treatment; saturation plus BSA (0.25 μg/μL); saturation plus SERPINA3K (0.05 μg/μL), and saturation plus SERPINA3K (0.25 μg/μL). Data are presented as mean ± SEM, $n = 6$ in each group, $^{*}P < 0.05$, $^{\#}P < 0.05$. (**B**) Representative images of H&E staining and immunohistochemical staining with antibody of PMN of central cornea on day 7 after saturation. The *insets* represented higher magnification of the *dotted area*. From *left* to *right* are group of control: normal or control rat without saturation or any treatment, group of BSA: saturation plus BSA (0.25 μg/μL) and group of SERPINA3K: saturation plus SERPINA3K (0.25 μg/μL; *upper panel*, H&E staining, *lower panel*, PMN staining.) The increased thickness of cornea and increased inflammatory cells were seen in the stroma and the inflammation was ameliorated by SERPINA3K. *Scale bar*: 100 μm (SA3K, SERPINA3K).

blot. Western blot data revealed that the protein level of VEGF was significantly elevated in BSA-treated group, compared with control group (Figs. 4A, 4B). In contrast, SERPINA3K significantly downregulated the level of VEGF induced by suture (Figs. 4A, 4B). In addition, it was shown by the data of quantitative real-time PCR assay that corneal suture significantly increased the gene expression of VEGF and SERPINA3K suppressed the gene expression of VEGF induced by suture (Fig. 4C).

SERPINA3K Suppressed the Activation of Wnt Pathway In Vitro

To confirm the blocking effect of SERPINA3K on the Wnt pathway activation, we performed a series of in vitro experiments of Wnt activation in cultured HUVECs. We selected Wnt3a, which is a ligand of Wnt pathway to induce the activation of Wnt signaling pathway. The cultured HUVECs were exposed to conditioned medium containing 500 ng/mL Wnt3a or the medium with Wnt3a plus SERPINA3K at concentrations of 80 and 160 nM.

We first detected the cell migration by the scratch wound test. It showed that Wnt3a increased the cell migration of HUVECs, while SERPINA3K at concentrations of 80 and 160 nM suppressed the increase of cell migration induced by Wnt3a (Fig. 5A).

We also measured changes of the factors of Wnt pathway in cultured HUVECs. It was demonstrated by Western blot that the level of nonphospho-β-catenin, the critical protein in Wnt pathway, was upregulated in HUVEC when exposed to Wnt3a conditional medium, and SERPINA3K inhibited the upregulation of nonphospho-β-catenin induced by Wnt3a (Fig. 5B). Meanwhile, the level of phospho-β-catenin, an indicator of the degradation of β-catenin and inactivation of Wnt pathway, showed the opposite trend to nonphospho-β-catenin (Fig. 5C). Furthermore, Wnt3a induced upregulation of protein level of VEGF, and this upregulating effect was blocked by SERPINA3K (Fig. 5C). These results indicated that SERPINA3K blocked the Wnt pathway activation and VEGF in vitro.

SERPINA3K Inhibited $VEGF_{165}$-Induced Model In Vitro

We further performed an in vitro experiment to determine the antineovascularization effects of SERPINA3K and confirm the inhibitory effects of SERPINA3K on VEGF, using cultured

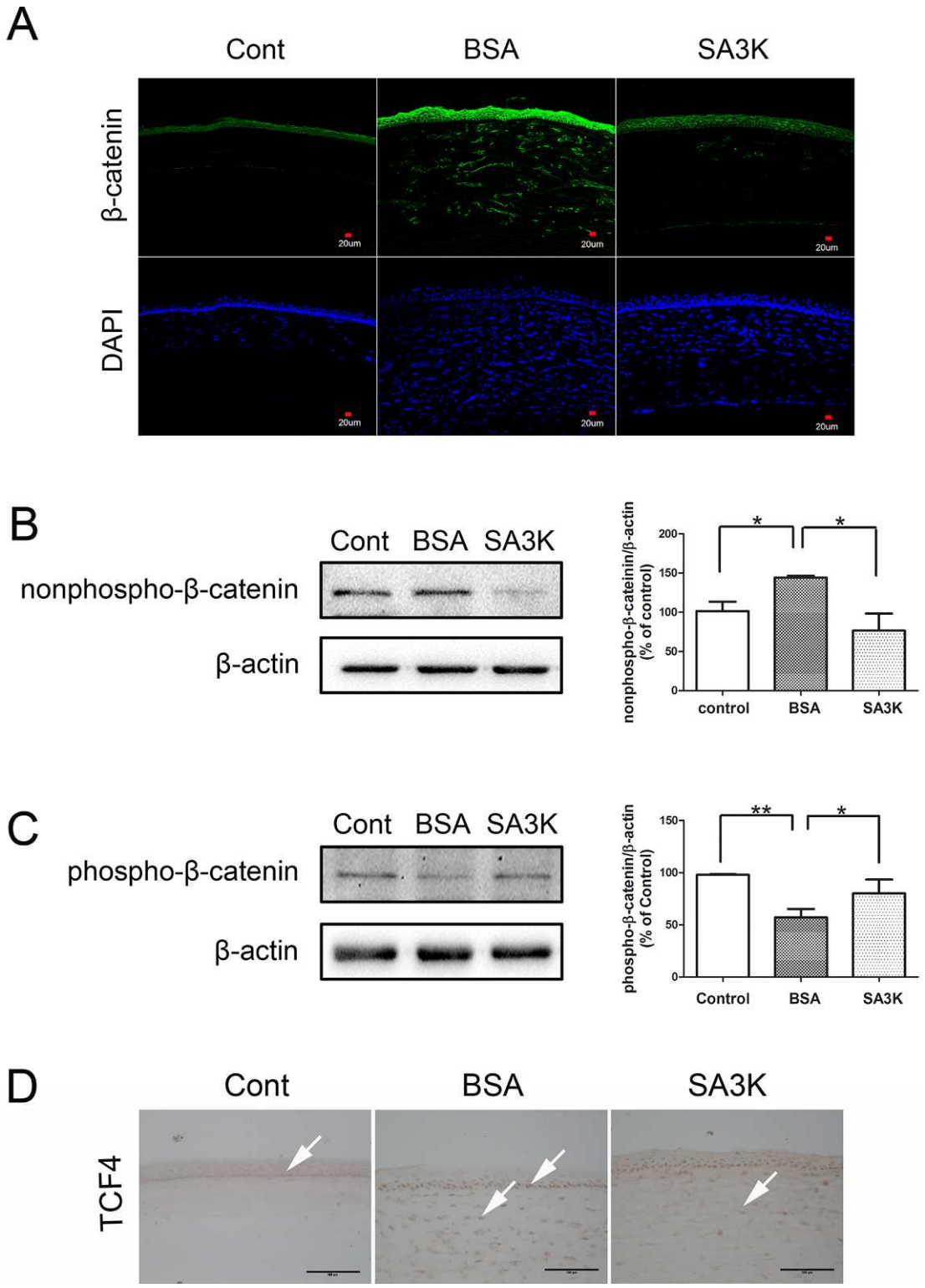

Figure 3. SERPINA3K blocked Wnt signaling pathway in the cornea. (**A**) Representative images of immunofluorescent staining with antibody of β-catenin. From *left* to *right* are group of control: normal or control rat without saturation or any treatment, group of BSA: saturation plus BSA (0.25 μg/μL) and group of SERPINA3K: saturation plus SERPINA3K (0.25 μg/μL; *upper panel*, *green*, β-catenin staining; *lower panel*, *blue*, DAPI nuclear staining). *Scale bar*: 20 μm. (**B**) Representative images and statistic analysis of Western blot with antibody of nonphospho-β-catenin. The groups were the same as in (**A**). Data are presented as mean ± SEM, $n = 5$ to 6 in each group, $^{*}P < 0.05$. (**C**) Representative images and statistic analysis of Western blot with antibody of phospho-β-catenin. The groups were the same as in (**A**). Data are presented as mean ± SEM, $n = 5$ to 6 in each group, $^{*}P < 0.05$; $^{**}P < 0.01$. *Scale bar*: 20 μm. (**D**) Representative images of immunohistochemical staining with antibody of TCF4. The groups were the same as in (A). *Scale bar*: 100 μm.

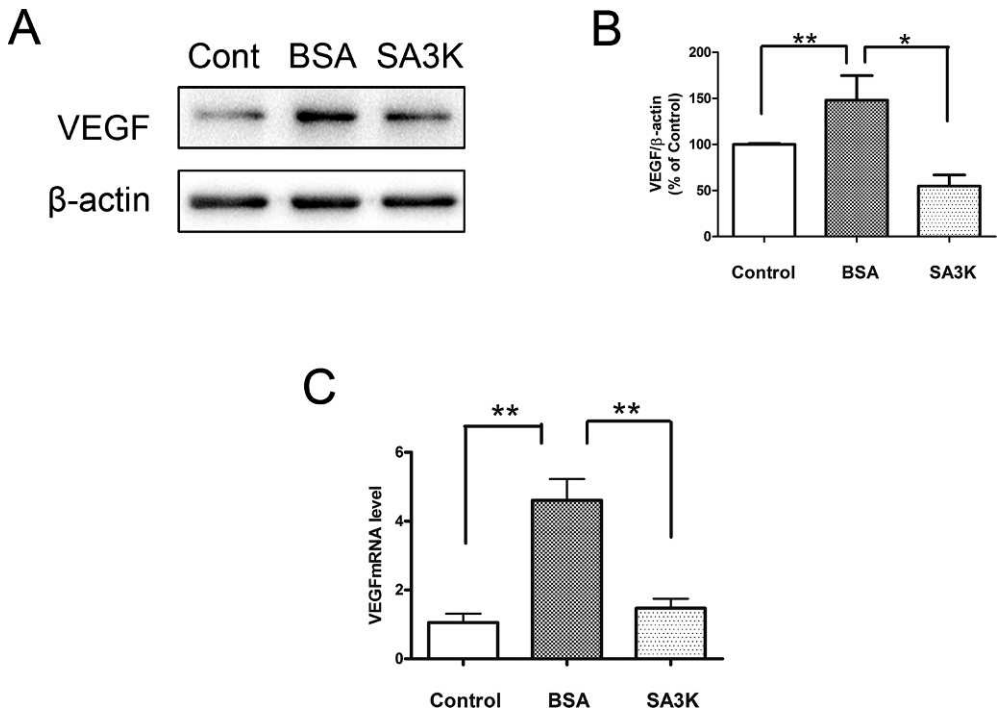

FIGURE 4. SERPINA3K downregulated VEGF in the cornea. (**A**, **B**) Representative images and analysis of Western blot with antibody of VEGF. From *left* to *right* are group of control: normal or control rat without saturation or any treatment, group of BSA: saturation plus BSA (0.25 μg/μL) and group of SERPINA3K: saturation plus SERPINA3K (0.25 μg/μL). Data are presented as mean ± SEM, $n = 6$ in each group, $^{*}P < 0.05$, $^{**}P < 0.01$. (**C**) Statistic analysis of quantitative real-time PCR assay of gene expression of VEGF. The groups were the same as in (**A**, **B**). Data are presented as mean ± SEM, $n = 5$ to 6 in each group, $^{**}P < 0.01$ (SA3K, SERPINA3K).

HUVEC stimulated by $VEGF_{165}$ as in vitro neovascularization model. We first investigated the cell viability of HUVEC after administration of $VEGF_{165}$ or $VEGF_{165}$ plus SERPINA3K. It was shown that the exposure of HUVEC to $VEGF_{165}$ increased the cell viability of HUVEC, while SERPINA3K at concentrations of 80 and 160 nM significantly decreased the cell viability of HUVEC induced by $VEGF_{165}$ (Fig. 6A).

Migration of endothelial cells is critical for the formation of new blood vessels. We then evaluated the effects of SERPINA3K on the cell migration of cultured HUVEC using scratch wound test. As shown in Figures 6B and 6C, the cell migration was significantly enhanced by $VEGF_{165}$, whereas SERPINA3K at concentrations of 80 and 160 nM significantly inhibited the cell migration of HUVEC induced by $VEGF_{165}$.

Furthermore, a matrigel assay was performed and analyzed to investigate the effects of SERPINA3K on endothelial cells (ECs) tube formation in vitro. It demonstrated that an administration of $VEGF_{165}$ at 20 ng/mL for 24 hours in the conditioned medium induced an increase of the capillary-like structure formation of HUVEC, while SERPINA3K at concentrations of 80 and 160 nM significantly suppressed the tube formation induced by $VEGF_{165}$ (Figs. 6D, 6E).

All the data from $VEGF_{165}$-induced neovascularization model in vitro revealed that SERPINA3K had antagonizing effects on the new vessel tube formation and endothelial cell migration induced by VEGF, which is a proneovascularization promoting factor.

DISCUSSION

In our previous research, we applied an alkali burn–induced rat corneal neovascularization model to investigate the antineovascularization effects of SERPINA3K.[20] In our present study, we demonstrated and reconfirmed the antineovascularization effects of SERPINA3K using another specific corneal neovascularization model, the suture-induced corneal neovascularization model. Furthermore, in the investigation of underlying mechanistic, we found for the first time that the antineovascularization effects of SERPINA3K were through blocking Wnt signaling pathway. These finding will contribute to the better understanding of development and pathogenesis of corneal neovascularization and provide a novel potential therapeutic agent and new direction in the treatment of corneal neovascularization.

The neovascularization model, which we applied in this study is a more specific and dominant model to mainly investigate the corneal neovascularization, compared with alkali burn–induced cornea neovascularization model, which we used previously and that can cause more severe damage to the cornea and that is more complex in the mechanism. The corneal suture model has been used as a chronic inflammation and corneal neovascularization model as previously reported.[24,25] Corneal suture induces partial injury to the stroma and evoked a robust and nonresolving inflammatory response as a consequence of chronic injury and irritation.[26] Moreover, corneal suture model converses milder injury to the corneal epithelium, much more integrate epithelial layer and better transparency on cornea.[27] Neovascularization in the suture model was observed on day 2 after suture, became pronounced on day 7, and persisted as long as the sutures remain in the cornea.

Wnt signaling pathway is closely related with vessel development and angiogenesis,[12,13,28–31] including ocular neovascularization, such as choroidal neovascularization and retinal neovascularization.[18,19] Mutations in the genes of key

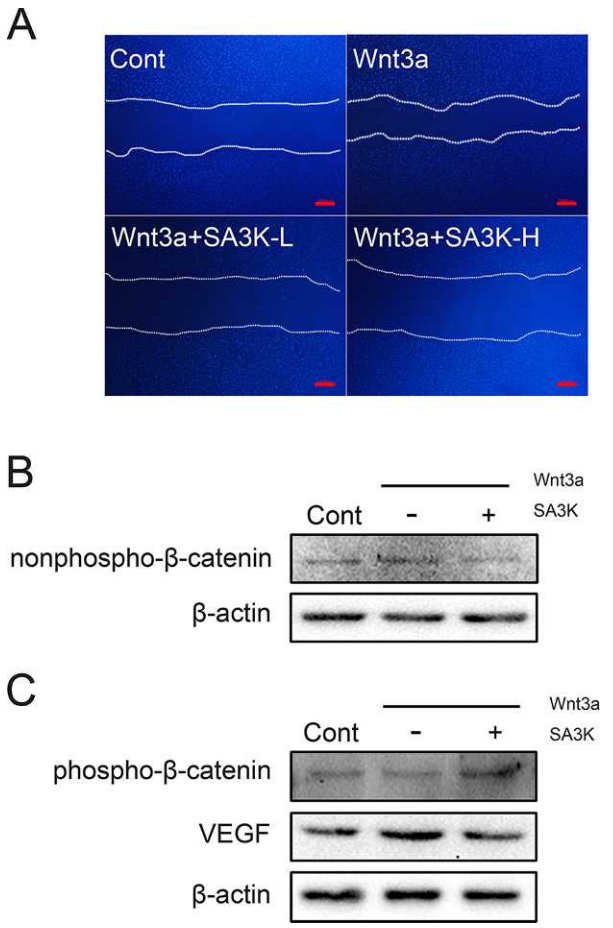

Figure 5. SERPINA3K suppressed the activation of Wnt pathway in vitro. The Wnt pathway was activated by administration of Wnt3a (500 ng/mL) in the conditioned medium of cultured HUVEC for 24 hours. (**A**) Representative images of migrated area of cultured HUVECs (*blue*, DAPI nuclear staining). Two concentrations of SERPINA3K were used: SERPINA3K-L (80 nM) and SERPINA3K-H (160 nM). (**B**, **C**) Representative images of Western blot data with antibody of nonphospho-β-catenin, phospho-β-cateninm, and VEGF, respectively. Control: without addition of Wnt3a and SERPINA3K; Wnt3a: treatment of 500 ng/mL Wnt3a for 24 hours and Wnt3a+ SERPINA3K: treatment of Wnt3a (500 ng/mL) plus SERPINA3K (160 nM) for 24 hours. *Scale bar*: 50 μm.

factors of Wnt pathway Fz4 and LRP5 have been found to associate with abnormal angiogenesis.[32,33] Activation of the Wnt signaling plays a pathogenic role in retinal vascularization in both human choroidal neovascularization and animal models of choroidal neovascularization.[14] In our present study, expression of β-catenin was obviously upregulated in the cornea of suture animal model. It is noteworthy that β-catenin expression presented a development from nothing to strong expression in stroma in normal and suture animal model respectively. It is well known that VEGF plays an important role in the angiogenesis or neovascularization. Vascular endothelial growth factor can be targeted and activated by the Wnt signaling pathway. Interestingly, we showed the upregulated level of VEGF in alkali burn–induced corneal neovascularization in our previous study. Here we found the same increased level of VEGF at gene expression and protein level after the suture. All the evidence demonstrated the activation of Wnt pathway in corneal neovascularization from two different animal experimental models, indicating a possible mechanism that Wnt activation plays roles in the corneal neovascularization.

SERPINA3K, an endogenic serpin, has been found to function as an antiangiogenic and an anti-inflammatory factor.[34,35] It was revealed that the antineovascularization effects of SERPINA3K acted through binding with LRP5/6 and inhibiting Wnt signaling pathway on retina.[18] SERPINA3K levels are decreased in retina of diabetic rats[36] and the expression of Wnt targeted gene such as VEGF and connective tissue growth factor (CTGF) are also regulated by SERPINA3K on retinal neovascularization. We had previously found the antineovascularization effects of SERPINA3K on alkali burn–induced corneal neovascularization, and SERPINA3K decreased the level of VEGF. In the present study, we showed that SERPINA3K downregulated the expression of β-catenin and other factors of Wnt pathway induced by saturation. The real-time PCR and Western blot data revealed that VEGF expression was also decreased by SERPINA3K after saturation. These in vivo data supported the primary demonstration that the mechanism of SERPINA3K's antineovascularization effect was through blocking Wnt signaling pathway.

It has been reported that there are 19 Wnt ligands, most of which bind with LRP5/6 and the Fz receptors activate Wnt signaling pathway.[37] Previous study showed that SERPINA3K blocks the Wnt pathway activation induced by Wnt ligands, but not by LiCl in ARPE19 cells.[18] Here we used another endothelia cell, HUVECs, and demonstrated the same blocking effects of SERPINA3K on Wnt3a, a ligand of Wnt pathway, induced activation of Wnt pathway, including the expression of critical protein β-catenin and targeted gene VEGF. Endothelial cell migration is an essential step in angiogenesis[38] and is regulated by Wnt signaling.[39] In our study, the migration of HUVEC cells increased when treated with Wnt3a, and suppressed by SERPINA3K at both low and high concentrations. These results provide further evidence that SERPINA3K plays a role of antiangiogenesis in endothelial cells through blocking Wnt signaling pathway.

Vascular endothelial growth factor, a key factor in angiogenesis, was applied in our study as an inducer of in vitro angiogenesis. The angiogenic isoforms are known as $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, and $VEGF_{189}$ in humans.[40] Vascular endothelial growth $factor_{165}$ is one of the most abundant isoforms and has been extensively studied. In our $VEGF_{165}$-induced in vitro angiogenesis model, SERPINA3K apparently reduced the cell viability and cell migration of HUVEC. Furthermore, we also identified the antiangiogenesis effect of SERPINA3K on HUVEC by a three-dimensional tube formation assay. Vascular endothelial growth factor was widely known as the downstream target gene of Wnt pathway, and was reported to be regulated by SERPINA3K. However, as a secretary protein, $VEGF_{165}$ was considered to induce cellular responses mediated by two high affinity type III tyrosine kinase receptors, VEGF-R2 (KDR/Flk1) and VEGF-R1 (Flt-1), and two receptors of the semaphoring receptor family, neuropilin-1 and neuropilin-2.[41,42] The mechanism of SERPINA3K's inhibition on the stimulating effects of $VEGF_{165}$ on HUVEC migration and tube formation may be through the complex signaling network including VEGF receptors, exception of Wnt pathway, while it needs further investigation.

SERPINA3K is known as a specific inhibitor of tissue kallikrein and a potent angiogenic inhibitor on retina. Our previous and present experimental evidence has suggested that SERPINA3K is a potent angiogenic inhibitor on corneal neovascularization using different chemical burn and surgical suture models. Here, we, for the first time, revealed that the mechanism of corneal neovascularization is associated with the activation of Wnt signaling pathway, providing a new insight for the better understanding of the pathogenesis of corneal neovascularization. Our reconfirmed beneficial antiangiogenic

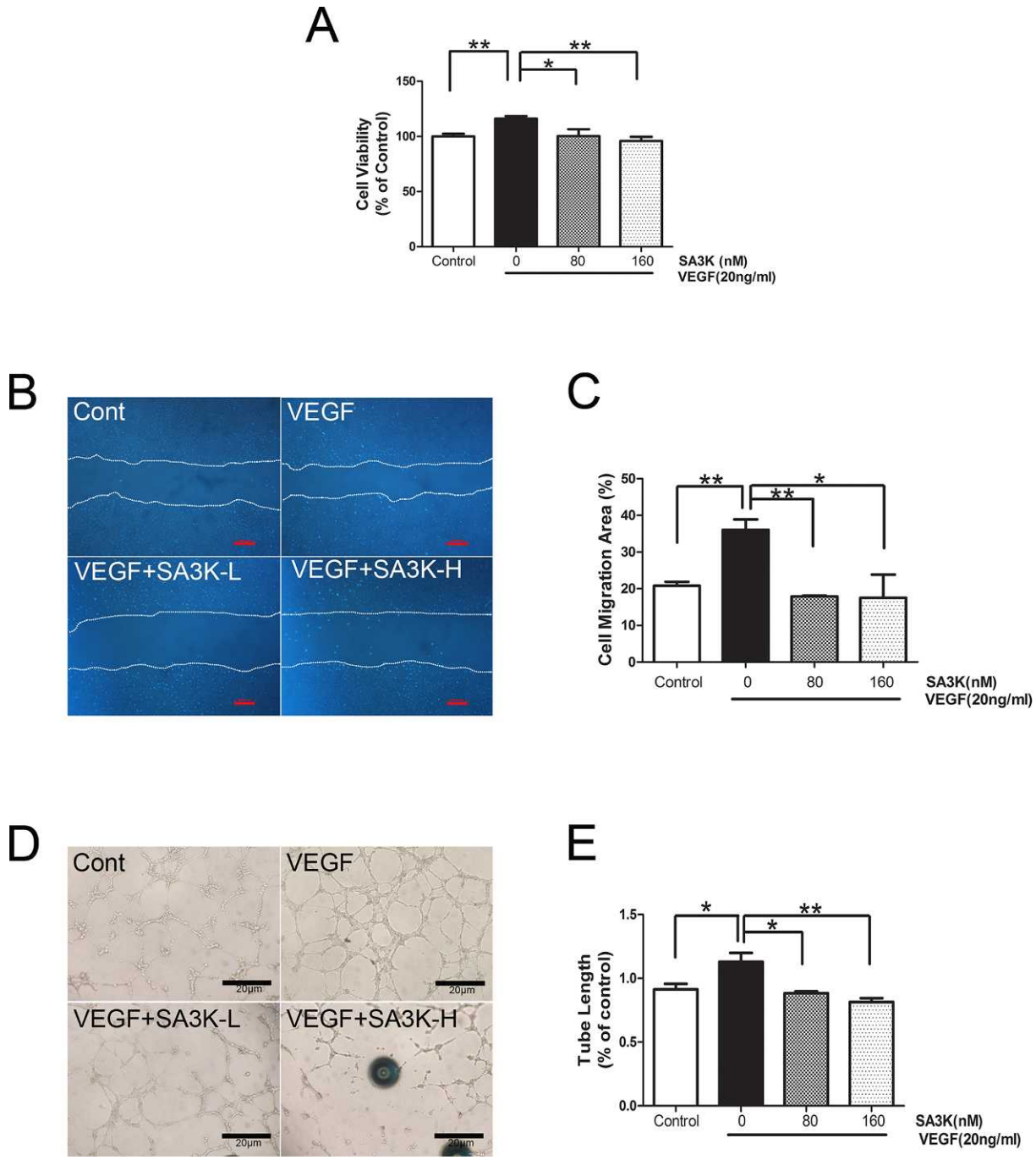

Figure 6. SERPINA3K inhibited $VEGF_{165}$-induced model in vitro. An administration of $VEGF_{165}$ at 20 μg/mL was given to conditioned medium of cultured HUVEC for 24 hrs. Two concentrations of SERPINA3K were used: SERPINA3K-L (80 nM) and SERPINA3K-H (160 nM). The cell viability, cell migration, and tube formation were detected and analyzed. (**A**) Statistic analysis of the data of cell viability of HUVECs (data are presented as mean ± SEM, $n = 4$–5 in each group, $^{*}P < 0.05$, $^{**}P < 0.01$). (**B**) Representative images of the migrated area of HUVEC (*blue*, DAPI nuclear staining). (**C**) Statistic analysis of the data of cell migration of HUVEC (data are presented as mean ± SEM, $n = 4$ in each group, $^{*}P < 0.05$, $^{**}P < 0.01$). (**D**) Representative images of the tube formation of HUVEC. (**E**) Statistic analysis of the data of tube formation (data are presented as mean ± SEM, $n = 3$ in each group, $^{*}P < 0.05$, $^{**}P < 0.01$). *Scale bar*: 20 μm.

effect of SERPINA3K on corneal neovascularization and the novel identification of SERPINA3K's role of blocking Wnt signaling pathway in the corneal neovascularization suggest that SERPINA3K has therapeutic potential for corneal neovascularization and the Wnt pathway may become an important target of antiangiogenic therapy for corneal neovascularization.

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References

1. Chang JH, Gabison EE, Kato T, Azar DT. Corneal neovascularization. *Curr Opin Ophthalmol*. 2001;12:242–249.
2. Clements JL, Dana R. Inflammatory corneal neovascularization: etiopathogenesis. *Semin Ophthalmol*. 2011;26:235–245.
3. Chang JH, Garg NK, Lunde E, Han KY, Jain S, Azar DT. Corneal neovascularization: an anti-VEGF therapy review. *Surv Ophthalmol*. 2012;57:415–429.
4. Gupta D, Illingworth C. Treatments for corneal neovascularization: a review. *Cornea*. 2011;30:927–938.
5. Maddula S, Davis DK, Burrow MK, Ambati BK. Horizons in therapy for corneal angiogenesis. *Ophthalmology*. 2011;118:591–599.
6. Bock F, Regenfuss B, Cursiefen C. Antiangiogenic therapy at the ocular surface: when, what and why? [in German]. *Ophthalmologe*. 2011;108:230–236.
7. Gough NR. Focus issue: Wnt and beta-catenin signaling in development and disease. *Sci Signal*. 2012;5:eg2.
8. Clevers H, Nusse R. Wnt/beta-catenin signaling and disease. *Cell*. 2012;149:1192–1205.
9. MacDonald BT, Tamai K, He X. Wnt/beta-catenin signaling: components, mechanisms, and diseases. *Dev Cell*. 2009;17:9–26.
10. Rao TP, Kuhl M. An updated overview on Wnt signaling pathways: a prelude for more. *Circ Res*. 2010;106:1798–1806.
11. Chen J, Stahl A, Krah NM, et al. Wnt signaling mediates pathological vascular growth in proliferative retinopathy. *Circulation*. 2011;124:1871–1881.
12. Zhang B, Ma JX. Wnt pathway antagonists and angiogenesis. *Protein Cell*. 2010;1:898–906.
13. Dejana E. The role of wnt signaling in physiological and pathological angiogenesis. *Circ Res*. 2010;107:943–952.
14. Hu Y, Chen Y, Lin M, Lee K, Mott RA, Ma JX. Pathogenic role of the Wnt signaling pathway activation in laser-induced choroidal neovascularization. *Invest Ophthalmol Vis Sci*. 2013;54:141–154.
15. Zhang J, Yang Z, Li P, Bledsoe G, Chao L, Chao J. Kallistatin antagonizes Wnt/beta-catenin signaling and cancer cell motility via binding to low-density lipoprotein receptor-related protein 6. *Mol Cell Biochem*. 2013;379:295–301.
16. Chao J, Schmaier A, Chen LM, Yang Z, Chao L. Kallistatin, a novel human tissue kallikrein inhibitor: levels in body fluids, blood cells, and tissues in health and disease. *J Lab Clin Med*. 1996;127:612–620.
17. Murata M, Nakagawa M, Takahashi S. Expression and distribution of kallikrein-binding protein mRNA in rat ocular tissues. *Ophthalmologica*. 1998;212:334–336.
18. Zhang B, Abreu JG, Zhou K, et al. Blocking the Wnt pathway, a unifying mechanism for an angiogenic inhibitor in the serine proteinase inhibitor family. *Proc Natl Acad Sci U S A*. 2010;107:6900–6905.
19. Zhang B, Zhou KK, Ma JX. Inhibition of connective tissue growth factor overexpression in diabetic retinopathy by SERPINA3K via blocking the WNT/beta-catenin pathway. *Diabetes*. 2010;59:1809–1816.
20. Liu X, Lin Z, Zhou T, et al. Anti-angiogenic and anti-inflammatory effects of SERPINA3K on corneal injury. *PLoS One*. 2011;6:e16712.
21. Jaffe EA, Nachman RL, Becker CG, Minick CR. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *J Clin Invest*. 1973;52:2745–2756.
22. Zhou T, Zong R, Zhang Z, et al. SERPINA3K protects against oxidative stress via modulating ROS generation/degradation and KEAP1-NRF2 pathway in the corneal epithelium. *Invest Ophthalmol Vis Sci*. 2012;53:5033–5043.
23. Xu Y, Zhao H, Zheng Y, Gu Q, Ma J, Xu X. A novel antiangiogenic peptide derived from hepatocyte growth factor inhibits neovascularization in vitro and in vivo. *Mol Vis*. 2010;16:1982–1995.
24. Leedom AJ, Sullivan AB, Dong B, Lau D, Gronert K. Endogenous LXA4 circuits are determinants of pathological angiogenesis in response to chronic injury. *Am J Pathol*. 2010;176:74–84.
25. Cursiefen C, Maruyama K, Jackson DG, Streilein JW, Kruse FE. Time course of angiogenesis and lymphangiogenesis after brief corneal inflammation. *Cornea*. 2006;25:443–447.
26. Liclican EL, Nguyen V, Sullivan AB, Gronert K. Selective activation of the prostaglandin E2 circuit in chronic injury-induced pathologic angiogenesis. *Invest Ophthalmol Vis Sci*. 2010;51:6311–6320.
27. Jia C, Zhu W, Ren S, Xi H, Li S, Wang Y. Comparison of genome-wide gene expression in suture- and alkali burn-induced murine corneal neovascularization. *Mol Vis*. 2011;17:2386–2399.
28. Stefater JA III, Lewkowich I, Rao S, et al. Regulation of angiogenesis by a non-canonical Wnt-Flt1 pathway in myeloid cells. *Nature*. 2011;474:511–515.
29. Gore AV, Swift MR, Cha YR, et al. Rspo1/Wnt signaling promotes angiogenesis via Vegfc/Vegfr3. *Development*. 2011;138:4875–4886.
30. Zerlin M, Julius MA, Kitajewski J. Wnt/Frizzled signaling in angiogenesis. *Angiogenesis*. 2008;11:63–69.
31. Parmalee NL, Kitajewski J. Wnt signaling in angiogenesis. *Curr Drug Targets*. 2008;9:558–564.
32. Masckauchan TN, Kitajewski J. Wnt/Frizzled signaling in the vasculature: new angiogenic factors in sight. *Physiology (Bethesda)*. 2006;21:181–188.
33. Ye X, Wang Y, Cahill H, et al. Norrin, frizzled-4, and Lrp5 signaling in endothelial cells controls a genetic program for retinal vascularization. *Cell*. 2009;139:285–298.
34. Gao G, Shao C, Zhang SX, Dudley A, Fant J, Ma JX. Kallikrein-binding protein inhibits retinal neovascularization and decreases vascular leakage. *Diabetologia*. 2003;46:689–698.
35. Zhang B, Hu Y, Ma JX. Anti-inflammatory and antioxidant effects of SERPINA3K in the retina. *Invest Ophthalmol Vis Sci*. 2009;50:3943–3952.
36. Hatcher HC, Ma JX, Chao J, Chao L, Ottlecz A. Kallikrein-binding protein levels are reduced in the retinas of streptozotocin-induced diabetic rats. *Invest Ophthalmol Vis Sci*. 1997;38:658–664.
37. Nusse R. Wnt signaling in disease and in development. *Cell Res*. 2005;15:28–32.
38. Lamalice L, Le Boeuf F, Huot J. Endothelial cell migration during angiogenesis. *Circ Res*. 2007;100:782–794.
39. Samarzija I, Sini P, Schlange T, Macdonald G, Hynes NE. Wnt3a regulates proliferation and migration of HUVEC via canonical and non-canonical Wnt signaling pathways. *Biochem Biophys Res Commun*. 2009;386:449–454.
40. Tischer E, Mitchell R, Hartman T, et al. The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing. *J Biol Chem*. 1991;266:11947–11954.
41. Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. *Nat Med*. 2003;9:669–676.
42. Neufeld G, Cohen T, Gengrinovitch S, Poltorak Z. Vascular endothelial growth factor (VEGF) and its receptors. *FASEB J*. 1999;13:9–22.