

Cystatin C Expression is Promoted by VEGFA Blocking, With Inhibitory Effects on Endothelial Cell Angiogenic Functions Including Proliferation, Migration, and Chorioallantoic Membrane Angiogenesis

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Background—Vascular development, including vasculogenesis and angiogenesis, is involved in many diseases. Cystatin C (CST3) is a commonly used marker of renal dysfunction, and we have previously reported that its expression level is associated with variations in the gerbil circle of Willis. Thus, we hypothesized that CST3 may affect endothelial function and angiogenic capacity. In the current study, we sought to determine the influence of CST3 on endothelial function and explore its potential regulatory pathway.

Methods and Results—We analyzed CST3 and vascular endothelial growth factor A (VEGFA) levels in different developmental stages of gerbils using ELISAs and immunofluorescence (to examine the relationship between CST3 and VEGFA). We used a real-time cell analyzer, cytotoxicity assays, and the chorioallantoic membrane assay to investigate the function of CST3 in endothelial cells and the chorioallantoic membrane. Additionally, we used Western blotting to explore the downstream targets of CST3. The expression levels of both CST3 and VEGFA were at their highest on day 10 of the embryonic stage. CST3 inhibited endothelial cell proliferation, migration, tube formation, and permeability, as well as vascular development in the chorioallantoic membrane. Blocking of VEGFA dose-dependently increased CST3 expression in arterial and venous endothelial cells. Furthermore, overexpression and knockdown of CST3 significantly affected the protein levels of p53 and CAPN10 (calpain 10), suggesting that CST3 might play a role in vascular development through these proteins.

Conclusions—CST3 may be associated with vascular development and angiogenesis, and this effect could be promoted by blocking VEGFA. (*J Am Heart Assoc.* 2018;7:e009167. DOI: 10.1161/JAHA.118.009167.)

Key Words: cystatin C • stroke • vascular endothelial function • vascular endothelial growth factor

Vascular development, including vasculogenesis and angiogenesis, is an extremely complex process. Vasculogenesis is the de novo formation of blood vessels from mesoderm-derived hemangioblasts, while angiogenesis is the subsequent formation of vessels from pre-existing vasculature by sprouting and intussusceptive microvascular growth of

endothelial cells.¹ Vascular development is associated with many types of disease, such as cancer,² type 2 diabetes mellitus,³ and cardiovascular and cerebrovascular disease.^{4,5}

The circle of Willis (CoW) is a primary arterial collateral structure connecting the brain's hemispheric circulation.⁶ Studies have shown that various deformities in the CoW occur in a majority of humans.⁷ Previously, we used the observation of different CoW patterns in gerbils as a means to explore genes associated with vascular development. We found that 4 genes (CST3 [cystatin C], GNAS [guanine nucleotide-binding protein G (s) subunit alpha], GPX4 [glutathione peroxidase-4], and profilin2) may be associated with CoW variations,⁸ which implies that these 4 genes might be linked to vascular development. We also investigated the serum levels CST3, GPX4, PFN2, and vascular endothelial growth factor A (VEGFA) in a gerbil cerebral ischemia model with different CoW patterns. The results indicated that serum CST3 levels were related to malformation of the CoW, and that these levels varied with different time points (Figure S1). We hypothesized that CST3 may be associated with vascular

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Clinical Perspective

What Is New?

- We found that endogenous or exogenous cystatin C (CST3) could inhibit endothelial cell proliferation, migration, and angiogenesis via P53 and CAPN10 (calpain 10).
- Both CST3 and vascular endothelial growth factor A reached maximal expression levels on embryonic day 10 of the gerbil brain and heart, suggesting they are involved in vascular development and that day 10 (embryo) is an important time point in this process.

What Are the Clinical Implications?

- CST3 could play a direct role in the pathogenesis of vascular disease via its inhibitory effect on the function of endothelial cells and blood vessel formation, which suggests that CST3 might serve as a potential drug target for vascular disease.

development and, thus, chose to investigate this protein further in the current study.

CST3 is a low molecular weight (≈ 13.3 kDa) protein⁹ produced by nucleated cells, and is the most important extracellular inhibitor of cysteine proteinases.¹⁰ Imbalance between cysteine proteinases and cystatins can result in connective tissue remodeling.¹¹ As an inhibitor of cathepsins, CST3 contributes to endothelial cell (EC) tubule formation¹² and shows angiogenic characteristics *in vitro*.¹³ It is well known that angiogenesis is essential for tumor growth.¹⁴ It has been reported that decreased CST3 expression is associated with poor prognosis for breast cancer,¹⁵ while a high CST3 level is an independent indicator of poor prognosis for patients with nasopharyngeal carcinoma.¹⁶ Recently, interest in the role of CST3 in vascular diseases has been growing.^{17–21} Unfortunately, there is minimal direct evidence that CST3 influences vasculogenesis and angiogenesis. There has also been research into CST3 in the area of renal disease. CST3 levels can be used to predict acute renal injury in cancer patients receiving cisplatin,²² and CST3 has been used as a marker for estimated glomerular filtration rate.²³ Thus, CST3 is a well-characterized marker of renal dysfunction, is increased in patients suffering from renal disease, and may, therefore, affect endothelial function and the angiogenic capacity of the vasculature in these patients.

Both vasculogenesis and angiogenesis are required for migration of endothelial progenitor cells and ECs.²⁴ Therefore, if CST3 influences endothelial cell migration and proliferation, this may be evidence of a role of CST3 in vasculogenesis and angiogenesis.

Vasculogenesis and vascular development involves various molecular signaling pathways, such as extracellular regulated protein kinases (ERK) signaling,²⁵ Notch signaling,²⁶ Rho

GTPase signaling,²⁷ and VEGF signaling.²⁸ VEGF and the VEGF receptor (VEGFR) are components of 1 of the main angiogenic signaling pathways.^{29,30} In particular, VEGFA and its receptor VEGFR2 are the main targets of currently used antiangiogenic agents.³¹ The main targets of VEGFA are ECs.³² The VEGF/VEGFR pathway initiates signaling cascades that promote EC growth, migration, and differentiation.³¹ VEGF expression was found to be correlated with CST3 levels in patients with esophageal carcinoma.³³ VEGF can activate the ERK1/2 pathway to induce matrix metalloproteinase (MMP)-2/9 expression,³⁴ and CST3 is cleaved by MMP-2,³⁵ which is a potent pluripotent angiogenic stimulator. MMP-2 degrades and inactivates VEGF-binding inhibitory proteins and releases VEGF.³⁶ However, the relationship between CST3 and VEGFA in vascular development is unclear.

Here, we investigate the effects of CST3 and VEGFA on EC proliferation and migration using a real-time cell analyzer (RTCA), 3-(4,5-dimethyl-thiazolyl)-2,5-diphenyl-SH-tetrazolium bromide (MTT) assays, ELISAs, and immunofluorescence staining. We also examined the interaction between CST3 and VEGFA in endothelial cells, its expression level in different developmental stages of gerbils, and its effect on chorio-allantoic membrane (CAM) vascular development.

Methods

All our data and methods of analysis will be made available to other researchers for purposes of reproducing the results or replicating the procedure. All supporting data are available within this article.

Ethics Statement

All experiments and animal care were conducted in accordance with the Guidelines of Capital Medical University Animal Experiments and the Experimental Animals Management Committee. The protocol was approved by the Animal Experiments and Experimental Animal Welfare Committee of Capital Medical University (Permit Number: AEEI-2017-032).

Cell Culture

Human umbilical vein ECs (HUVECs) obtained from a colleague (Capital Medical University) were cultured in RPMI-1640 medium (Hyclone). Rat brain microvessel ECs (RBMECs) obtained from ScienCell were cultured in EC medium (ScienCell) for the first 5 generations, and then cultured in RPMI-1640 medium. The medium was changed every 2 to 3 days. The CST3 and VEGFA (165) proteins (PROSPEC) and blocking peptides for CST3 and VEGFA

(ABGENT), were used in our experiments. These blocking peptides, also called neutralizing antibodies, block the active site of proteins, or influence protein activity, and are widely used in protein activity blocking experiments.^{32,37} The proteins and peptides were dissolved in water before use.

Lentiviral Vector Construction and Infection for CST3 Overexpression and Knockdown by shRNA Interference

Lentiviral vectors were used for overexpression and knockdown of CST3. CST3 complementary DNA (cDNA) (Accession: BT006839.1) was cloned into the pLVX-mCMV-ZsGreen-PGK-Puro vector. Lentivirus-mediated CST3 shRNA (-CCGAGCCA GCAACGACAT GTACCACTCG AGTGGTACAT GTCGTTGCTG GCTTTTTT-) was cloned into pLVX-shRNA2-Puro. Viruses were produced as per the manual instructions. HUVEC cells were transduced for 24 hours with recombinant lentivirus in the presence of 10 µg/mL polybrene. After transduction, the cells were cultured for 72 hours. Overexpression and knockdown of CST3, as well as the transduction efficiency of the CST3 (shCST3) constructs, were verified by expression of GFP as determined by flow cytometric analyses, and subsequently confirmed by reverse transcription-polymerase chain reaction and Western blotting.

Cytotoxicity Assay

We performed MTT (Solarbio) assays to examine the cytotoxicity of CST3 and VEGFA proteins (100 ng/mL, PROSPEX), CST3 and VEGFA blocking peptides (100 µg/µL, ABGENT), and the lentiviral vector. Water was used as the control for both proteins and peptides. Target cells (HUVEC or RBMEC) were resuspended in medium at a density of 2000 cells/well and were allowed to adhere for 6 hours. Wells containing 100-µL medium alone (without cells) were used as negative controls. MTT assays were performed every 6 hours after treatment with the targeted protein, for 48 hours. The results for the negative control were used as a baseline. Each experiment was repeated 3 times, and the results are presented as a percentage of viable cells as calculated by the following equation: (mean absorbance of experimental well/mean absorbance of positive control well) × 100 = percentage of viable cells.

RTCA Assay

We used the RTCA system (ACEA, USA) to explore the role of CST3 in endothelial cell migration and proliferation. RTCA is a system that has emerged as a non-invasive and label-

free approach to dynamic monitoring of changes in cell populations, such as cell proliferation, death, migration, and receptor-mediated signaling, on a cellular level.³⁸ RTCA uses E-plates and an RTCA-Dual Purpose instrument to monitor cell proliferation by measuring the cell index, which is proportional to the number of cells. Cells were seeded in E-plates at a density of 1000 HUVECs/well and 2000 RBMECs/well. The E-plates were then transferred to the RTCA-Dual Purpose instrument for automated real-time monitoring under standard incubator conditions. Cell index measurements were collected every 5 minutes. Cellular migration and invasion were also monitored using the RTCA system on cell invasion-and-migration (CIM)-plates instead of E-plates. Cell migration activity was monitored with the impedance readouts. Migration assays were performed by seeding cells in the upper chambers of the CIM-plates in serum-free medium at a density of 10 000 cells/well. The bottom chambers of the CIM-plates were filled with serum-containing medium to promote migration across the membranes along the serum gradient. After seeding, the CIM-plates were transferred into the RTCA-Dual Purpose instrument for real-time readouts. Protein (100 ng/mL) or blocking peptide (100 ng/mL) was administered after cells had been cultured for 6 hours, and data were collected by real-time readouts.

ELISAs

The supernatant of the HUVEC or RBMEC culture medium, after being treated with CST3/VEGFA protein or CST3/VEGFA blocking peptide, was analyzed using ELISA. The concentrations of CST3 and VEGFA in the EC supernatant were determined using a human or rat cystatin C (Cyagen) or VEGFA (R&D) kit in accordance with the manufacturers' instructions. All experiments were performed following the instructions in the kit.

Chick CAM Assay

A total of 27 fertilized chicken eggs (9 groups, 3 eggs/group) were incubated at 38.5°C and 80% humidity. On the 5th day of incubation, a square window was carved in each shell. Filter paper disks saturated with 1 ng protein/blocking peptide or water (control) were placed on the areas between preexisting vessels, after which the embryos were incubated for an additional 5 days. After the second incubation, the arterial branches in each treatment group were photographed using a digital camera system (Paull). The effect of each agent was determined by changes in the relative numbers of arterial branches. Each experiment was performed 3 times.

Endothelial Cell Permeability

To explore the effect of CST3 on endothelial cell permeability *in vitro*, an endothelial permeability assay was performed.³⁹ HUVECs (2×10^5) were seeded onto polycarbonate cell culture inserts of a 24-well Transwell system (Costar) and treated with either CST3 or VEGFA at a concentration of 200 ng/mL, until they formed a complete monolayer. Then FITC-BSA was added to the upper chamber and the fluorescence was evaluated in the lower chamber after adding FITC-BSA for 24 hours (excitation wavelength, 488 nm; emission wavelength, 525 nm).

Immunofluorescence

A total of 1×10^4 cells was seeded on a glass slide that had been previously treated with type I procollagen to promote cell adhesion. After reaching 70% confluency, cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton x-100 in PBS, and blocked with 10% donkey serum (Santa Cruz Biotechnology). After 1 hour, the cells were incubated in a primary antibody against CST3 (Abcam, ab24327, UK) at 4°C overnight. Thereafter, cells were washed 5 times with PBS at 3 minutes intervals, and further incubated with Alexa Fluor/FITC-conjugated secondary antibodies for 1 hour. Hoechst33342 was added in the last 10 minutes. The over-stained cells were washed 4 times with PBS at 5-minutes intervals. Finally, the cells were visualized with a confocal fluorescence microscope.

Quantitative Polymerase Chain Reaction

Fifteen each of gerbil embryos (prenatal), brains, and hearts (postnatal) were used for real-time quantitative polymerase chain reaction analysis (3 for each time point). The HUVECs were treated with VEGF protein or blocking peptide and were also analyzed with qPCR. HUVECs were resuspended in medium at a density of 2000 cells/well and were allowed to adhere for 6 hours. Total RNA was extracted from HUVEC cells using TRIzol reagent (Tiangen). We synthesized cDNA using FastQuant RT Kit (Tiangen) following manufacturer's instructions. An iQ5 thermal cycler (Bio-Rad, USA) was used to perform qPCR as follows: pre-denaturation at 95°C for 15 minutes, 40 cycles of denaturation at 95°C for 10 seconds, annealing and extension at 60°C for 35 seconds, and 71 cycles of melt curve analysis at 60°C for 10 seconds.

CST3 primer sequences: F- CAACAAAGCCAGCAACGACA, R- TCTTGGTACACGTGGTTCGG.

β -actin primer sequences: F- AGAGGGAAATCGTGCCTGAC, R- CAATAGTGATGACCTGGCCGT.

Protein Extraction and Western Blotting

Proteins were extracted from the samples using Proteins Extraction Kit (CWBIO, China) and quantified with BCA-Reagents (CWBIO, China). Proteins were separated by SDS-PAGE at 160 V on 12% gel (CWBIO, China) for 1 hour and then transferred to a 0.22- μ m nitrocellulose filter membrane) at 200 mA for 3 hours. The primary antibodies were diluted

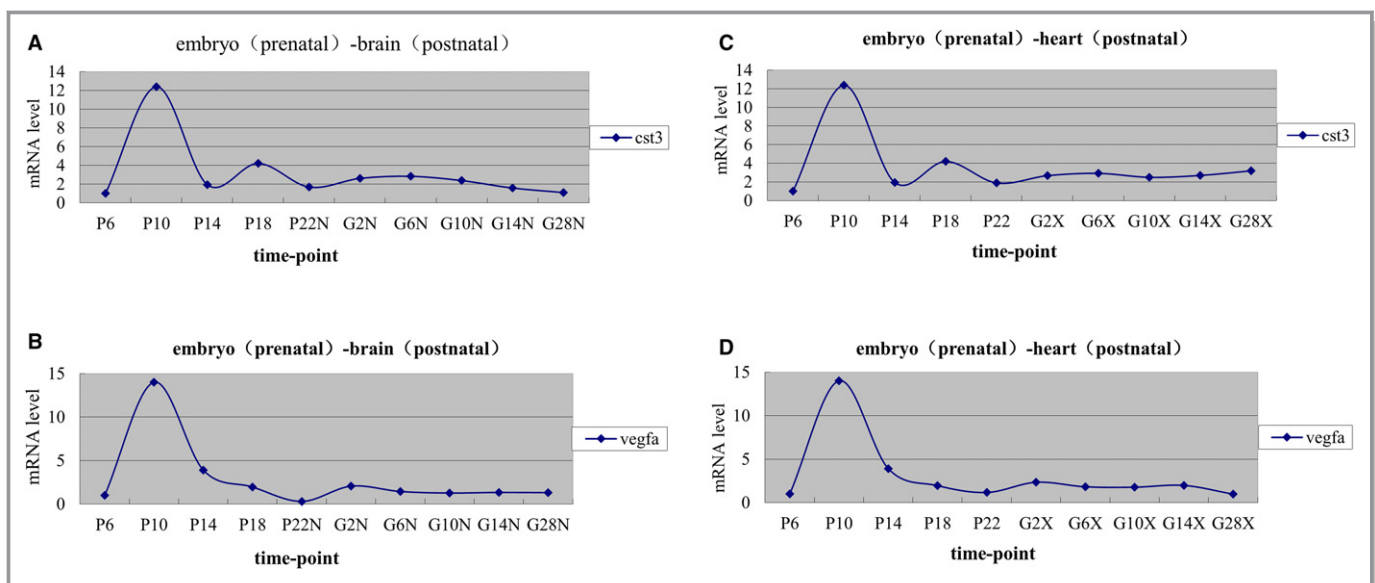


Figure 1. The mRNA levels of CST3 and VEGFA in different developmental stages of gerbils (embryo [prenatal], brain and heart [postnatal]). **A and B**, mRNA levels of CST3 and VEGFA in different developmental stages of gerbil brains. **C and D**, mRNA levels of CST3 and VEGFA in different developmental stages of gerbil hearts. P-embryo (prenatal), G#N-brain (postnatal), G#X-heart (postnatal). Sample sizes: n=3. CST3 indicates cystatin 3; VEGFA, vascular endothelial growth factor A.

as follows: CST3 (Santa, sc-16989, USA) was diluted 1:100, p53 (Abcam, ab131442, UK), CAPN10 ([Calpain 10] Abcam, ab28226, UK) and GAPDH (Abcam, ab181602, UK) were diluted 1:1000. Secondary antibodies were diluted 1:5000. The membranes were washed completely and visualized with enhanced chemiluminescence immunoblotting detection reagents (Thermo Fisher Scientific, USA). Semiquantitative results were normalized to the housekeeping gene GAPDH after gray scanning.

Scratch Wound Assay (Wound Scratch Assay)

HUVECs (50 000) were cultured in a 6-well plate until 90% confluent. HUVECs were serum-starved (1% FBS+ DMEM) for 24 hours. Scratch wounds were created using a 1000- μ L pipette tip. After 24 hours, the cells were observed by microscope at 5 \times magnification and images of the scratched area were captured to determine wound closure. Average scratch area was quantitated using NIH ImageJ software.

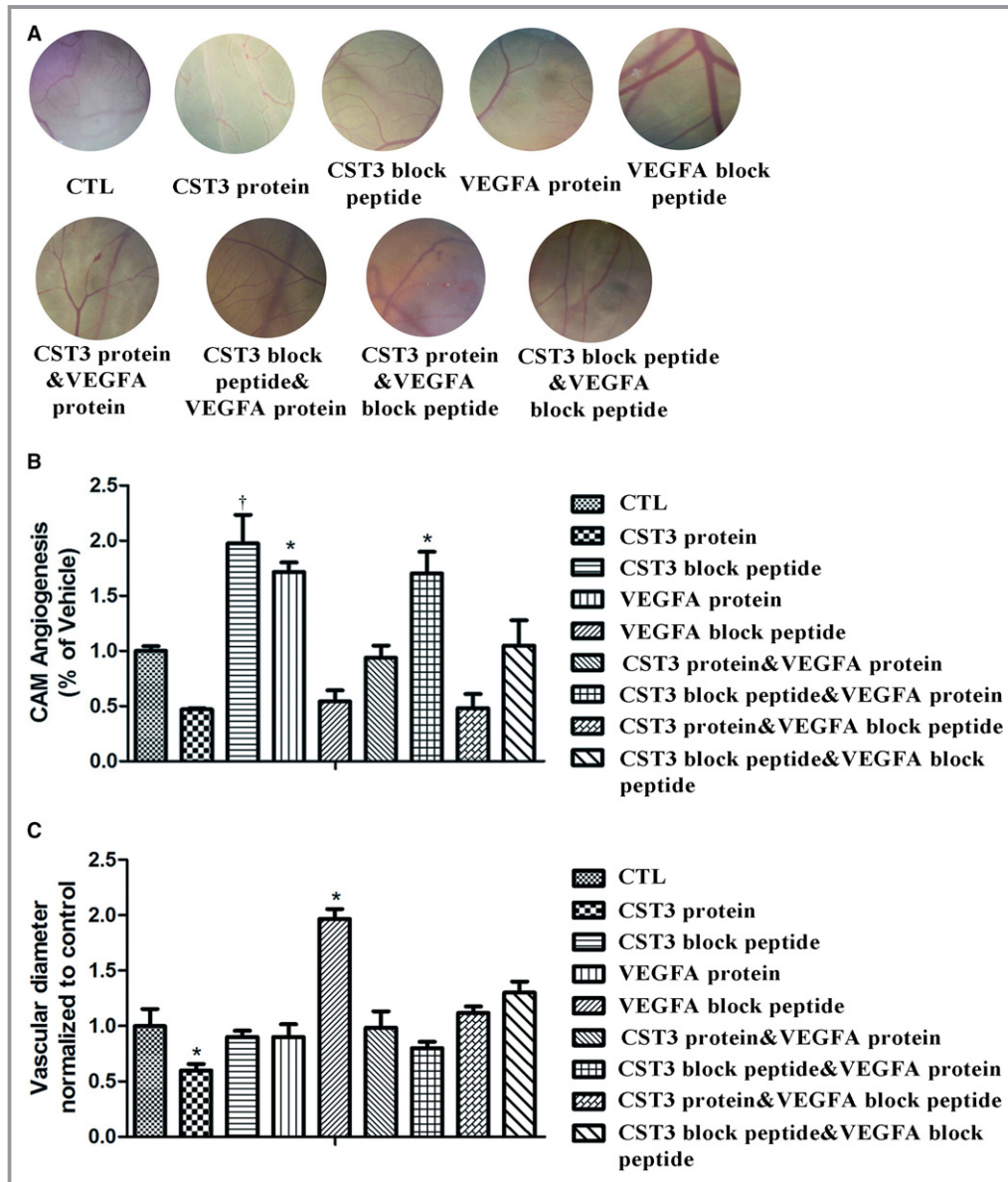


Figure 2. CAM assays. **A**, The effect of CST3 on vascular development in the CAM assay. **B and C**, The statistical results of the CAM assay. Sample sizes: n=3. Comparisons between different groups were conducted using one-way ANOVA. Bar charts show the mean \pm SEM. CAM indicates chorioallantoic membrane; CTL, control; CST3, cystatin 3; VEGFA, vascular endothelial growth factor A. * $P\leq 0.05$ and [†] $P\leq 0.01$ showed significant difference.

Transwell Assay

HUVECs were placed on an 8- μ m membrane in the upper chamber. Medium with proteins or blocking peptides were placed in the lower chamber. HUVECs that had not migrated were retained in the top chamber and were carefully removed by cotton swabs. HUVECs that migrated to the bottom side of the membrane were stained using MTT. After solubilization of MTT with dimethyl sulfoxide, absorbance was measured at 490 nm.

Statistical Analysis

Statistical analysis was performed using SPSS 16.0 (SPSS Inc, USA). After normality test and variance homogeneity test on measurement data, comparisons between different groups were conducted using Student *t* test, one-way ANOVA and repeated measures ANOVA (Tukey). Bar charts showed the mean \pm SEM; “*” ($P\leq 0.05$) and “+” ($P\leq 0.01$) showed significant difference.

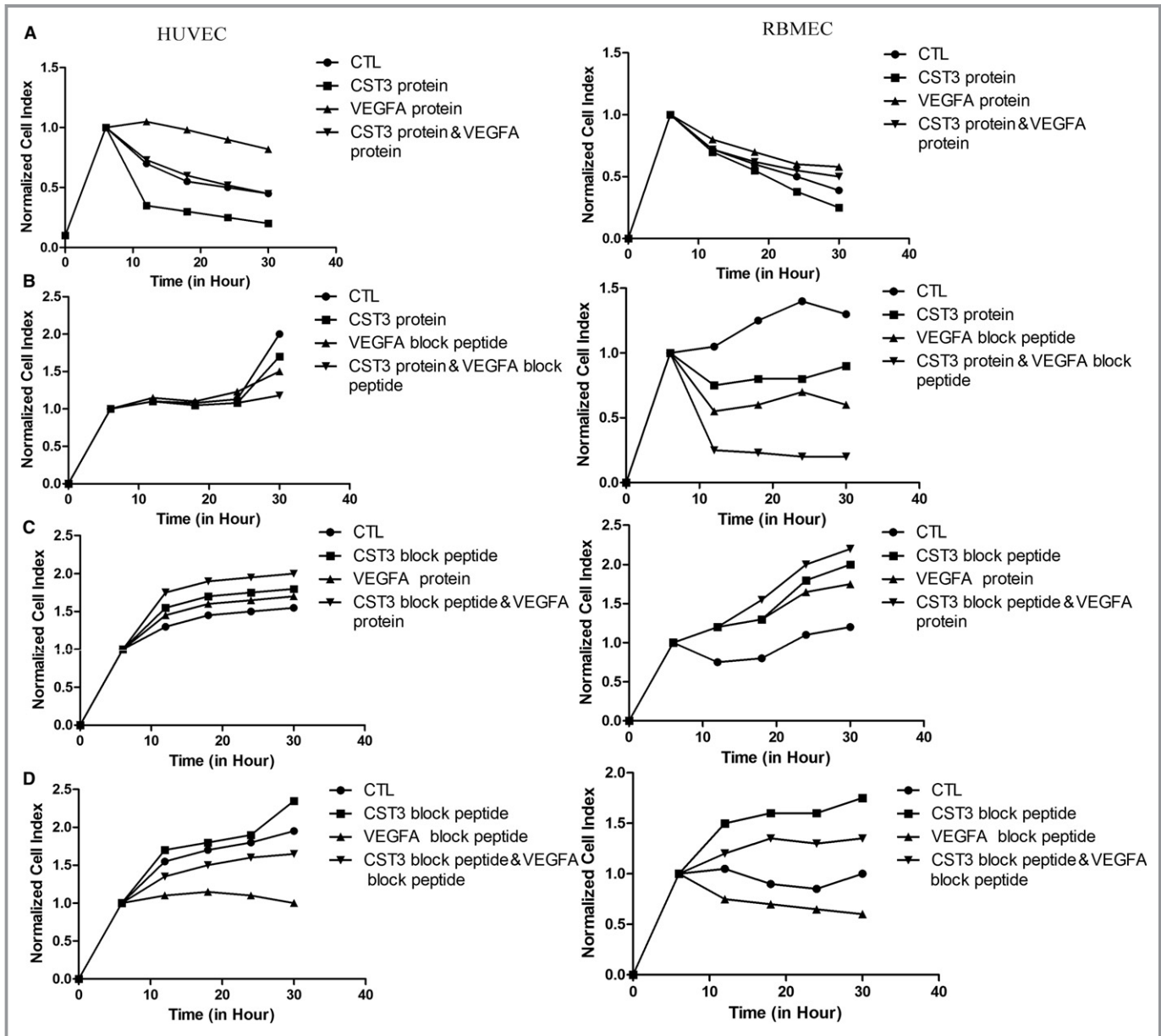


Figure 3. Cell proliferation ability as determined by RTCA analysis. **A**, HUVEC and RBMEC proliferation following treatment with CST3 and/or VEGF proteins. **B**, HUVEC and RBMEC proliferation following treatment with CST3 protein and/or VEGF blocking peptide. **C**, HUVEC proliferation following treatment with CST3 blocking peptide and/or VEGF protein. **D**, HUVEC and RBMEC proliferation following treatment with CST3 and/or VEGF blocking peptides. Sample sizes: n=3. Comparisons between different groups were conducted using repeated measures ANOVA. CTL indicates control; CST3, cystatin 3; VEGFA, vascular endothelial growth factor A. HUVECs indicate human umbilical vein endothelial cells; RBMECs, rat brain microvessel endothelial cells, RTCA, real-time cell analyzer; VEGFA, vascular endothelial growth factor A.

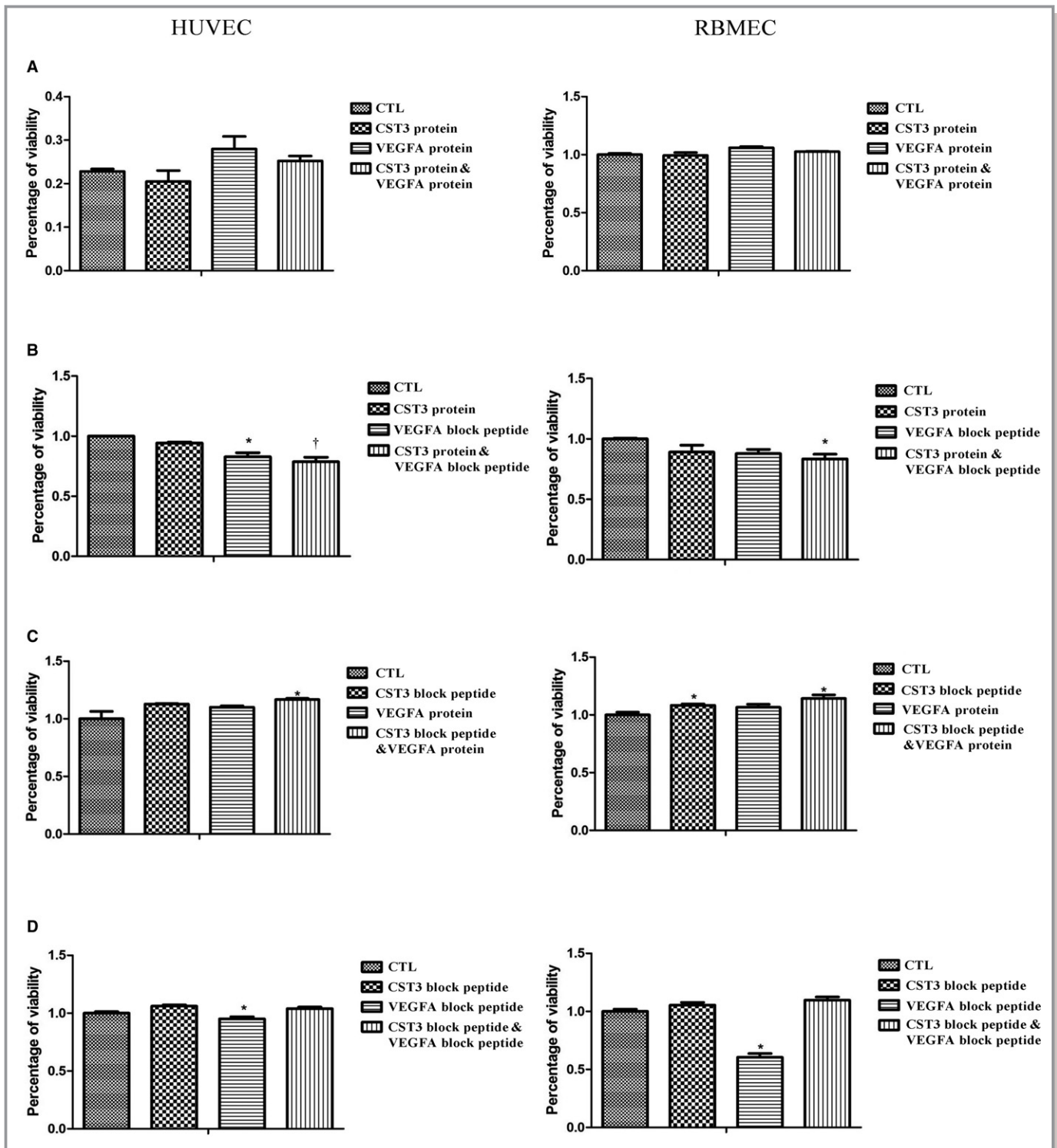


Figure 4. Cytotoxicity as determined by the MTT test. **A**, Percentage of viable HUVECs and RBMECs treated with CST3 and/or VEGF proteins. **B**, Percentage of viable HUVECs and RBMECs treated with CST3 protein and/or VEGF blocking peptide. **C**, Percentage of viable HUVECs and RBMECs treated with CST3 blocking peptide and/or VEGF protein. **D**, Percentage of viable HUVECs and RBMECs treated with CST3 and/or VEGF blocking peptide. Sample sizes: n=8. Comparisons between different groups were conducted using one-way ANOVA. Bar charts show the mean±SEM. CTL indicates control; CST3, cystatin 3; HUVEC, human umbilical vein endothelial cells; MTT, 3-(4,5-dimethyl-thiazolyl)-2,5-diphenyl-SH-tetrazolium bromide; RBMECs, rat brain microvessel endothelial cells; VEGFA, vascular endothelial growth factor A. * $P \leq 0.05$ and † $P \leq 0.01$ showed significant difference.

Results

Effects of CST3 and VEGFA on Vascular Development in Gerbils and CAMs

To understand the relationship between CST3 and angiogenesis, we investigated the role of CST3 in gerbil embryo development and CAM vascular development using VEGFA as a positive control. The results showed that both CST3 and VEGFA reached maximal expression levels on day 10 of the

embryo stage (Figure 1), suggesting that day 10 (embryo) is an important time point for cerebrovascular development in gerbils. In the CAM assay, the number of vessels was significantly increased in CAMs treated with VEGFA protein ($P=0.002$), CST3 blocking peptide ($P=0.021$), and VEGFA protein/CST3 blocking peptide ($P=0.024$) (Figure 2A and 2B). Conversely, CST3 protein and VEGFA blocking peptide/CST3 protein significantly decreased the number of vessels in CAMs. However, in the VEGFA blocking peptide group, vessel

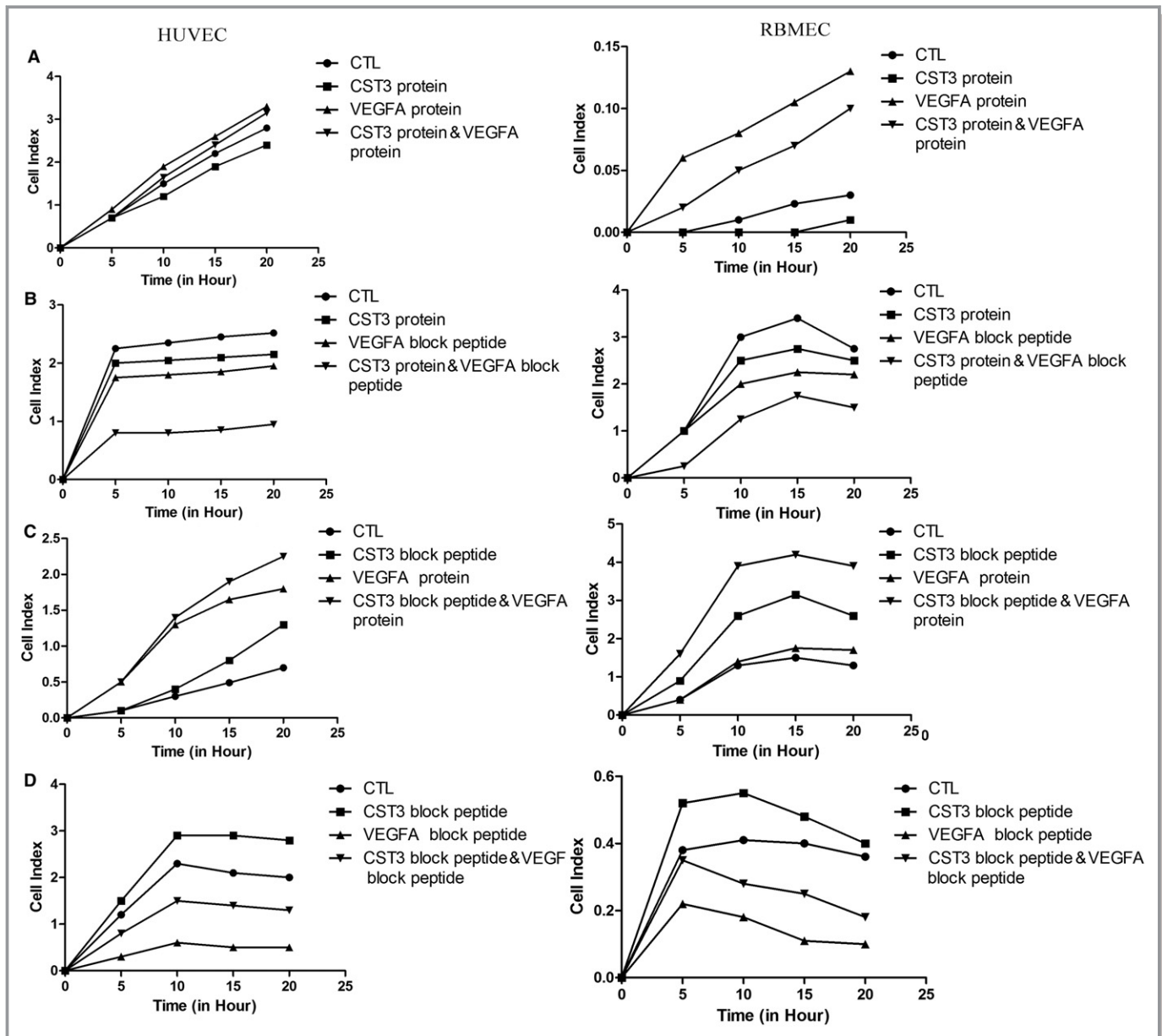


Figure 5. Cell migration ability as determined by RTCA analysis. **A**, Cell migration of HUVECs and RBMECs treated with CST3 and/or VEGF protein. **B**, Cell migration of HUVECs and RBMECs treated with CST3 protein and/or VEGF blocking peptide. **C**, Cell migration of HUVECs treated with CST3 blocking peptide and/or VEGF protein. **D**, Cell migration of HUVECs and RBMECs treated with CST3 and/or VEGF blocking peptide. Sample sizes: $n=3$. Comparisons between different groups were conducted using repeated measures ANOVA. CTL indicates control; CST3, cystatin 3, HUVEC, human umbilical vein endothelial cells; RBMECs, rat brain microvessel endothelial cells; RTCA, real-time cell analyzer.

diameter was greater (Figure 2A and 2C, $P<0.001$), and there were fewer smaller-diameter vessels. Furthermore, we found that CST3 could significantly decrease vessel diameter (Figure 2C, $P=0.011$). The effects on vessels in CAMs treated with proteins or peptides were neutralized (Figure 2A and 2B). These results indicate that CST3 decreases the number of vessels.

Function of CST3 in EC Proliferation and Migration

ECs play a crucial role in vascular development. We investigated the role of CST3 in the proliferation and migration of both venous and arterial ECs (HUVECs and RBMECs) by RTCA analysis.

RTCA results for cell proliferation

Cell proliferation was significantly decreased in HUVECs treated with CST3 protein and VEGFA blocking peptide ($P=0.045$) and was significantly increased in RBMECs treated with CST3 blocking peptide ($P=0.045$) and in both types of ECs treated with CST3 blocking peptide plus VEGFA protein (HUVECs, $P=0.035$; RBMECs, $P=0.038$). That is, cell proliferation decreased in both types of ECs treated with CST3 protein or VEGFA blocking peptide and increased in cells treated with VEGFA protein or CST3 blocking peptide. The effects on EC proliferation were neutralized following treatment with both proteins or blocking peptides. After simultaneous treatment with CST3 blocking peptide and VEGFA protein, the cell proliferation rates of the 2 types of ECs were higher than that for the VEGFA protein or the CST3 blocking peptide alone (Figure 3). In cells treated with CST3 protein and VEGFA blocking peptide, the proliferation rates of the 2 types of ECs were greater than that for those treated with either the VEGFA blocking peptide or CST3 protein (HUVECs, $P=0.002$; RBMECs, $P=0.0035$; Figure 3).

Cell viability, as tested by MTT assay

We investigated the effect of CST3 and VEGFA on cell viability. Cellular viability was significantly increased in RBMECs treated with CST3 blocking peptide ($P=0.032$) and in both EC types treated with CST3 blocking peptide and VEGFA protein (HUVECs, $P=0.032$; RBMECs, $P=0.038$; Figure 4). Significant decreases in viability were seen in RBMECs treated with VEGFA blocking peptide ($P=0.018$) and in both EC types treated with CST3 protein and VEGFA blocking peptide (HUVECs, $P=0.008$; RBMECs, $P=0.026$). Following treatment with 2 proteins or both blocking peptides, the effects on viability were neutralized. Upon simultaneous treatment with CST3 blocking peptide and VEGFA protein, the viability of both EC types was much higher than that in either the VEGFA protein or CST3 blocking peptide groups. Conversely,

simultaneous treatment with CST3 protein and VEGFA blocking peptide decreased viability of both EC types more than that in either treatment alone (Figure 4).

RTCA results for cell migration

We measured the effect of CST3 on EC migration using RTCA analysis (Figure 5). Cell migration was significantly decreased in both types of ECs treated with CST3 protein and VEGFA blocking peptide (HUVECs, $P=0.041$; RBMECs: $P=0.047$) and was significantly increased in RBMECs treated with CST3 blocking peptide and VEGFA protein ($P=0.029$). Following treatment with 2 proteins or both blocking peptides, the effects on migration were neutralized. Upon simultaneous treatment with CST3 blocking peptide and VEGFA protein, the cell migration rates of both types of ECs were much higher than that in either treatment alone. Conversely, cell migration was lower in cells treated with both CST3 protein and VEGFA blocking peptide compared with either treatment alone (Figure 5).

Permeability of HUVECs

We measured the effect of CST3 on permeability of HUVECs. The results showed that HUVEC permeability decreased significantly after treatment with either CST3 protein ($P=0.047$) or VEGFA blocking peptide ($P=0.040$) compared with control (Figure 6). In contrast, permeability significantly increased after treated with either CST3 blocking peptide ($P=0.040$) and VEGFA protein ($P=0.038$) compared with control (Figure 6). These data demonstrate that CST3 might prevent HUVEC permeability.

Blocking VEGFA Dose-Dependently Increases CST3 Expression

To investigate the relationship between CST3 and VEGFA, we performed ELISAs and found that VEGFA secretion was not

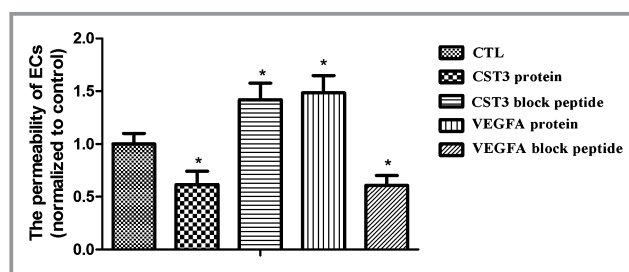


Figure 6. Permeability of HUVEC. Permeability of HUVEC treated with CST3 or VEGFA proteins or blocking peptides. Sample sizes: $n=8$. Comparisons between different groups were conducted using one-way ANOVA. Bar charts show the mean \pm SEM. CTL indicates control; CST3, cystatin 3; ECs, endothelial cells; HUVEC, human umbilical vein endothelial cell; VEGFA, vascular endothelial growth factor A. * $P\leq 0.05$ showed significant difference.

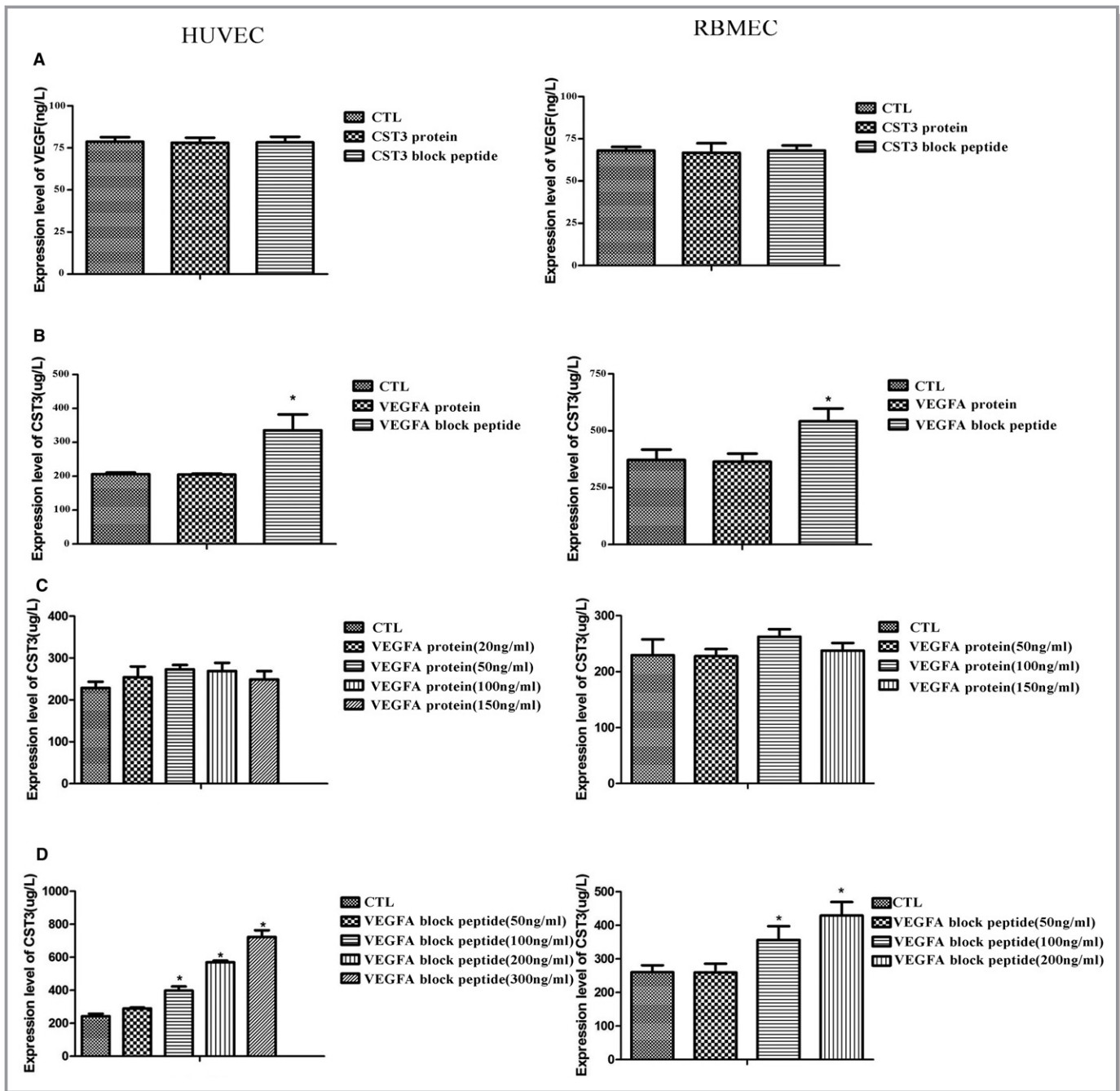


Figure 7. The regulatory relationship between CST3 and VEGFA explored by ELISA. **A**, VEGFA secretion in the cell culture media of HUVECs and RBMECs treated with CST3 protein and blocking peptide. **B**, CST3 secretion in cell culture media of HUVECs and RBMECs treated with VEGFA protein and CST3 blocking peptide. **C**, CST3 secretion in cell culture media of HUVECs and RBMECs treated with different VEGFA protein concentrations. **D**, CST3 secretion in cell culture media of HUVECs and RBMECs treated with different CST3 blocking peptide concentrations. CTL-control. Sample sizes: n=8. Comparisons between different groups were conducted using one-way ANOVA. Bar charts show the mean±SEM. CTL, indicates control; CST3, cystatin 3; HUVEC, human umbilical vein endothelial cells; RBMECs, rat brain microvessel endothelial cells; VEGFA, vascular endothelial growth factor A. * $P \leq 0.05$ showed significant difference.

significantly different in the cell culture media of HUVECs or RBMECs following treatment with CST3 protein or CST3 blocking peptide. There was also no difference in CST3 secretion level in the cell culture media of HUVECs or RBMECs following treatment with VEGFA protein or either

solvent. However, CST3 secretion was significantly, and dose-dependently, increased in the cell culture media of HUVECs ($P=0.041$) and RBMECs ($P=0.045$) treated with VEGFA blocking peptide (HUVEC-VEGFA-100 ng/mL, $P=0.032$; HUVEC-VEGFA-200 ng/mL, $P=0.011$; RBMEC-VEGFA-

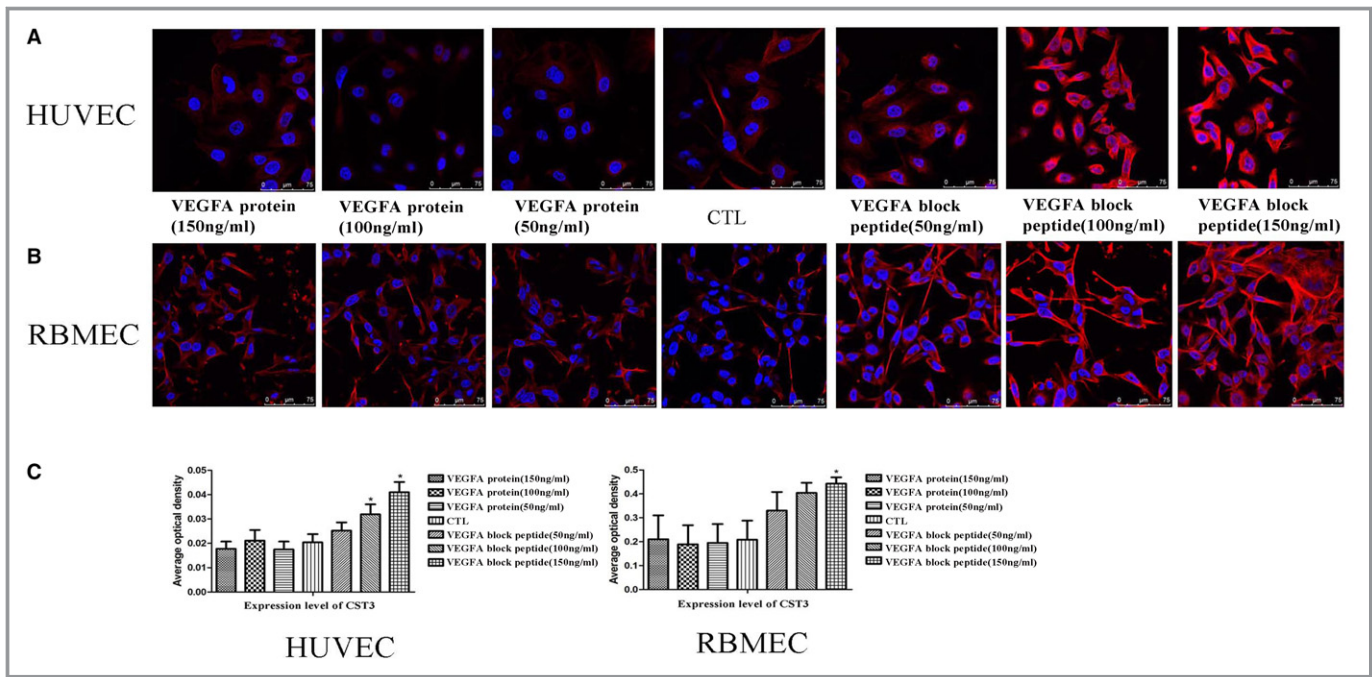


Figure 8. VEGFA blocking peptide increased CST3 protein expression level. **A**, Immunofluorescence staining of CST3 in HUVECs treated with different concentrations of VEGF protein and blocking peptide. **B**, Immunofluorescence staining of CST3 in RBMECs treated with different concentrations of VEGF protein and blocking peptide. **C**, The statistical results of immunofluorescence staining in HUVECs and RBMECs. Sample sizes: n=8. Comparisons between different groups were conducted using one-way ANOVA. Bar charts show the mean±SEM. CTL indicates control; CST3, cystatin 3; HUVEC, human umbilical vein endothelial cells; RBMECs, rat brain microvessel endothelial cells; VEGFA, vascular endothelial growth factor A. * $P \leq 0.05$ showed significant difference.

100 ng/mL, $P=0.045$; RBMEC-VEGFA-200 ng/mL, $P=0.033$; Figure 7).

To determine whether the effect occurred at the expression or secretion level, we analyzed the relationship between CST3 and VEGFA by immunofluorescence staining. The results

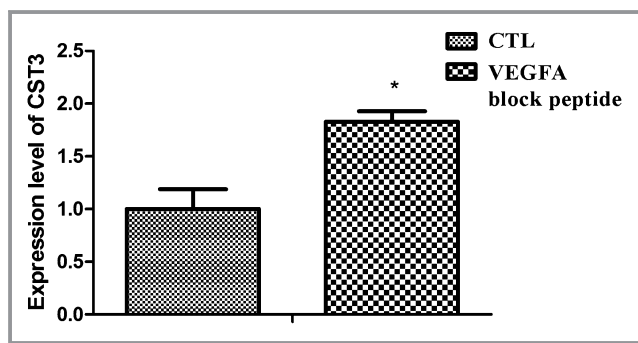


Figure 9. VEGFA blocking peptide increased CST3 mRNA of CST3 expression level. After treated with VEGF blocking peptide, the mRNA level of CST3 increased in HUVEC. CTL-control. Sample sizes: n=8. Comparisons between different groups were conducted using Student *t* test. Bar charts show the mean±SEM. CAM indicates chorioallantoic membrane; CTL, control; CST3, cystatin 3; HUVEC, human umbilical vein endothelial cell; VEGFA, vascular endothelial growth factor A. * $P \leq 0.05$ showed significant difference.

were consistent with the ELISA findings; CST3 expression levels did not significantly change in HUVECs or RBMECs treated with VEGFA protein, while they significantly and dose-dependently increased in HUVECs or RBMECs treated with VEGFA blocking peptide (HUVEC-VEGFA-100 ng/mL, $P=0.048$; HUVEC-VEGFA-150 ng/mL, $P=0.023$; RBMEC-VEGFA-150 ng/mL, $P=0.036$; Figure 8). These results indicate that blocking VEGFA increases CST3 protein expression level.

To further investigate the effect of VEGFA on CST3 expression, we performed real-time polymerase chain reaction. The results showed that the expression level of CST3 mRNA in HUVEC and RBMEC was significantly increased when the cells were treated with VEGFA blocking peptide ($P=0.029$) (Figure 9). Thus, we show that VEGFA blocking peptide increases CST3 mRNA expression level.

Proliferation and Migration of CST3 Overexpression and shRNA Interference ECs

To further confirm our findings, we developed ECs that either overexpressed CST3 or had CST3 knocked-down by shRNA (Figure 10A and 10B). The results showed that CST3 overexpression decreased EC proliferation and migration, while

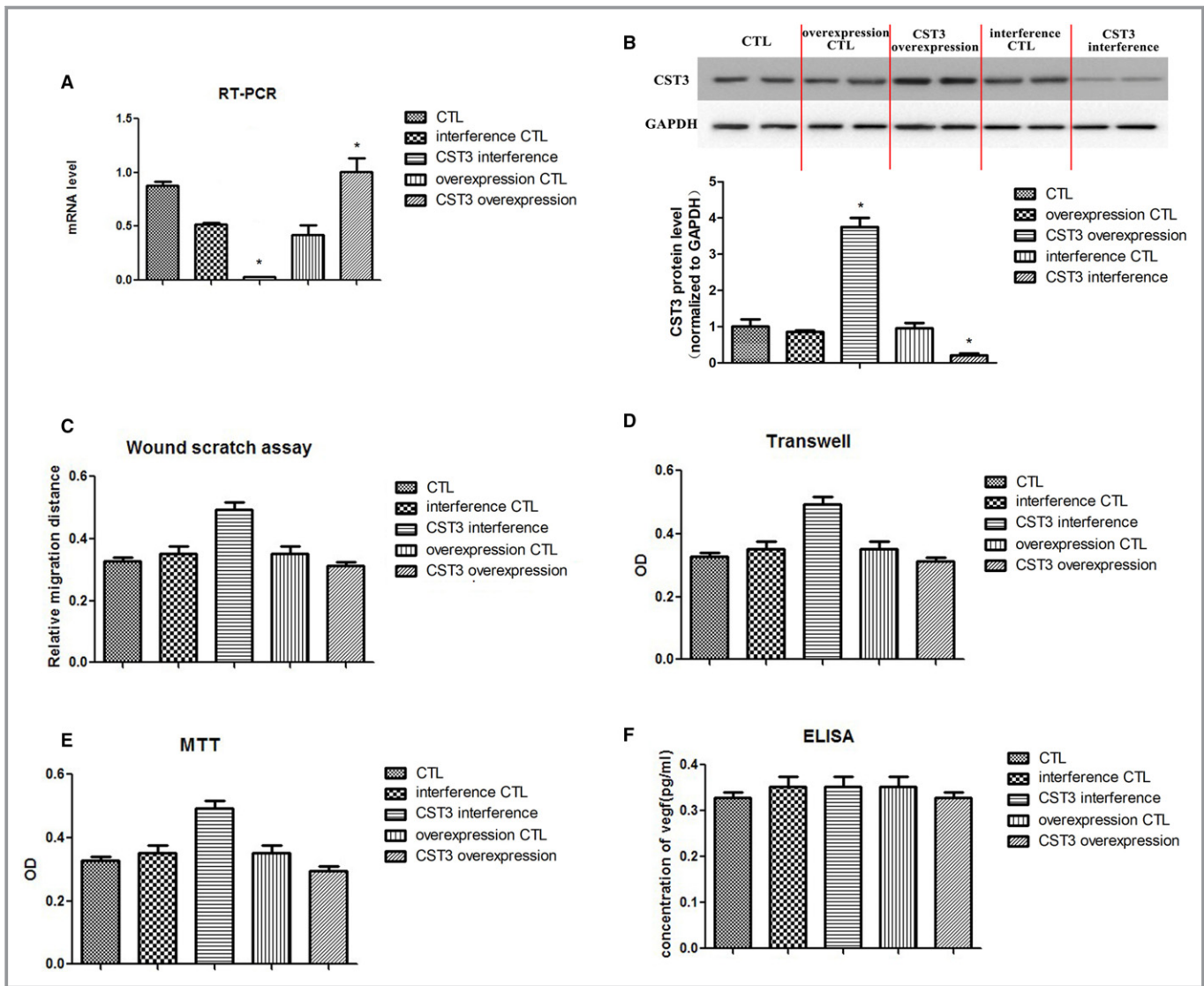


Figure 10. Proliferation and migration of CST3 overexpression and shRNA interference ECs. **A**, The mRNA levels of CST3 after lentiviral vector construction and infection in the CST3 overexpression and shRNA interference ECs. **B**, Protein levels of CST3 after lentiviral vector construction and infection in the CST3 overexpression and shRNA interference ECs. **C**, Migration ability of ECs by wound-scratch assay. **D**, Migration ability of ECs by transwell. **E**, Proliferation ability of ECs by MTT. **F**, VEGF content in culture medium of ECs. CTL-control. Sample sizes: $n=8$. Comparisons between different groups were conducted using one-way ANOVA. Bar charts show the mean \pm SEM. CTL indicates control; CST3, cystatin 3; ECs, endothelial cells; MTT, 3-(4,5-dimethyl-thiazolyl)-2,5-diphenyl-SH-tetrazolium bromide; RT-PCR, reverse transcription-polymerase chain reaction. * $P\leq 0.05$ showed significant difference.

CST3 knockdown increased them (Figure 10C through 10E). These results were consistent with those in the previous protein and peptide experiments.

Downstream Proteins of CST3

We used Western blotting to explore the downstream proteins of CST3. We selectively investigated proteins known to be associated with angiogenesis, proliferation, apoptosis, and metabolism. The results showed that overexpression of CST3 significantly increased the protein levels of p53 and CAPN10

($P=0.049$ and 0.002 , respectively), and knockdown of CST3 significantly decreased p53 and CAPN10 levels ($P=0.045$ and 0.033 , respectively) (Figure 11), which suggests that CST3 might play a role in vascular development through these proteins.

Discussion

CoW variations (Figure S2) are likely caused by variations in vascular development processes, and we found that CST3 reached maximum expression level on day 10 of the

embryonic stage in gerbils, similar to VEGFA. Therefore, we hypothesized that day 10 (embryo) was an important time point for cerebrovascular development in gerbils. Additionally, CST3 inhibited CAM vascular development and might therefore influence CoW patterns. VEGFA is known to induce HUVEC migration and proliferation⁴⁰ and increase the density of microvessels in the CAM.^{41,42} Our results were inconsistent with these findings in that VEGFA blocking peptide increased vessel diameter in CAMs (Figure 1C). Lu et al found that inhibition of the VEGF pathway promoted invasion of the glioblastoma multiforme phenotype in mouse models and in a group of glioblastoma multiforme patients treated with VEGF antibody. They demonstrated that VEGF blockade increased the survival benefit via MET signaling.⁴³ Therefore, inhibiting VEGFA might trigger another angiogenic pathway. Potente et al posited that damage to abnormal tumor vessels and decreased tumor microvasculature induced by antiangiogenic agents aggravates intratumor hypoxia and activates a prometastatic switch.⁴⁴ Therefore, our results may be the result of a compensatory effect of inhibiting the VEGF pathway. In our supplementary studies, vascular development in the CAM was greatly inhibited in the group treated with a VEGFA inhibitor (sunitinib malate, Figure S3), confirming our results.

CST3 decreases metastasis in some tissues,⁴⁵ suggesting that CST3 may affect cell migration. Gangoda et al showed that cathepsin inhibitors decreased the migratory potential of SK-N-BE2 cells.⁴⁶ Many previous reports have shown that inhibiting cathepsin S attenuated invasion, proliferation, and tubulogenesis in HUVECs, but had no effect on HUVEC migration⁴⁷ as other types of cathepsins may compensate for this effect. Moreover, serum CST3 levels are related to endothelial dysfunction in patients with metabolic syndrome.⁴⁸ Considering these data, we hypothesize that

CST3 will have some influence on ECs. In the embryo, new vessel formation occurs via assembly of mesoderm-derived endothelial precursors or angioblasts that differentiate into a primitive vascular labyrinth (vasculogenesis).⁴⁹ Then, vessel sprouting, mediated by EC proliferation and migration (angiogenesis), generates a network that remodels into arteries and veins.⁵⁰ Thus, ECs play a crucial role in vascular development. The findings from the present study confirmed our hypothesis and demonstrate that CST3 can inhibit HUVEC and RBMEC proliferation and migration. CST3 has also been reported to be associated with cardiovascular disease and peripheral artery disease,^{51–53} which may also be because of its effects on ECs.

Previous studies have shown that VEGF expression correlates with that of CST3 in patients with esophageal carcinoma.³³ Cathepsin S partially promotes ischemia-induced neovascularization via modulation of peroxisome proliferator-activated receptor- γ and VEGF/Akt signaling.⁴⁷ CST3 inhibits cathepsin S, which then activates VEGF/Akt signaling, suggesting that CST3 and VEGF exert opposite effects and that CST3 might inhibit VEGF. Conversely, Shan et al showed that VEGF induces MMP-2 expression³⁴ and that cystatin C was cleaved by MMP-2³⁶; however, whether VEGF could decrease cystatin C levels has not been confirmed. In this study, overexpression and knockdown of CST3 had the same effect as adding CST3 protein or CST3 blocking peptide. The role of CST3s as a secretory protein, and its internalization, might account for this result.^{54,55} However, a previous study reported that CST3 promotes tube formation in HUVECs and formation of branched blood vessels in CAMs,¹⁷ which seems to contradict our own observations. This may be because of differences in the experimental setup of both studies. Zou et al studied the effect of CST3 on the paracrine activity of PC12 cells in the context of HUVEC tube formation. They

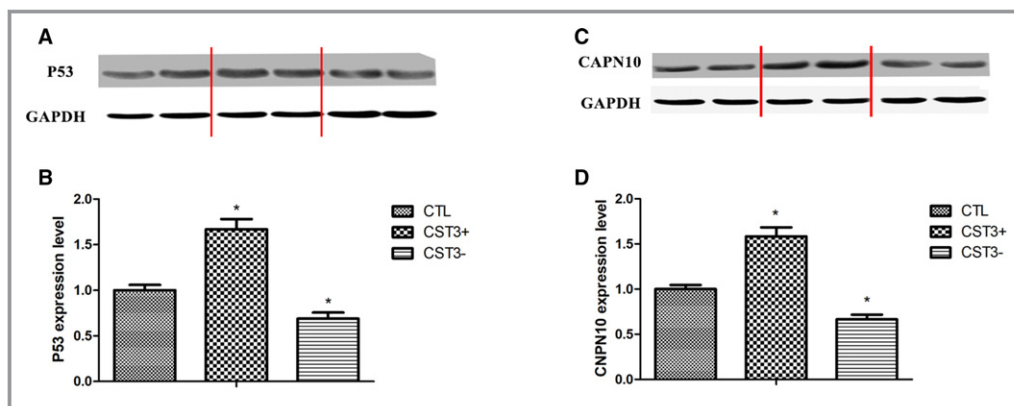


Figure 11. Effects of CST3 overexpression and knockdown on protein levels of P53 and CAPN10. **A and B,** Effects of CST3 overexpression and knockdown on protein levels of P53. **C and D,** Effects of CST3 overexpression and knockdown on protein levels of CAPN10. CTL-control. Sample sizes: n=8. Comparisons between different groups were conducted using Student *t* test. Bar charts show the mean \pm SEM. CTL indicates control; CST3, cystatin 3. * $P\leq 0.05$ showed significant difference.

used HUVECs cultured in 90% PC12 cell (CST3 overexpression)-conditioned media, whereas in this study, CST3 protein was administered to ECs, or overexpressed in ECs, directly. Their experimental conditions are more complicated and indirect than our own and were used because the authors had previously described that overexpression CST3 in PC12 could increase VEGF in the culture media. In addition, different subtypes of VEGF may also account for the different conclusions of Zou et al, as they did not show which subtypes or isoforms of VEGF they detected in their study. Mori et al found that p53 increases CST3 levels through a p53 binding sequence found in the first intron of CST3.¹⁵ Our results showed that CST3 also increased p53 levels, which may be as a result of positive feedback, but this hypothesis needs to be corroborated by further experiments. We further show that VEGFA inhibition increases the expression and secretion of CST3; however, we did not observe any change in CST3 expression or secretion after increasing the VEGFA concentration. This may be because constitutive CST3 expression was not be inhibited by VEGFA.

Some of the most important findings about CST3 are in the area of renal disease. CST3 can be used as a marker for estimated glomerular filtration rate²³ and a predictor of mortality in elderly patients with chronic kidney disease (CKD).⁵⁶ It has been reported that CST3 levels might be used to identify individuals with CKD who have the highest risk for complications.⁵⁷ CKD is characterized by a strong immune and inflammatory component that contributes to accelerated endothelial dysfunction, vascular inflammation, atherosclerosis, and calcification.^{58,59} In patients with CKD, ongoing endothelial damage in the capillary system of the renal medulla and accompanying vascular rarefaction are thought to be central processes contributing to progressive kidney damage.⁶⁰ In renal failure, endothelial dysfunction and atherosclerosis are universal, as are cardiovascular complications.⁶¹ Furthermore, microvascular disease is one of the factors contributing to atherosclerotic cardiovascular disease, prominent in patients with CKD,⁶² which also might be because of the effect of CST3 on angiogenesis. Taken together, these studies suggest that the change in CST3 seen in renal disease patients may be associated with endothelial dysfunction, which is consistent with our results, and suggests that CST3 influences endothelial cell function. Based on our own findings and those of other laboratories, we believe that CST3 could play a direct role in the pathogenesis of vascular disease via its inhibitory effect on the function of endothelial cells and blood vessel formation.

Conclusion

Collectively, our results suggest that CST3 might inhibit proliferation, migration, tube formation, and permeability of

ECs, as well as vascular development of CAMs through p53 and CAPN10. It is possible that these effects are promoted by blocking VEGFA signaling.

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Disclosures

None.

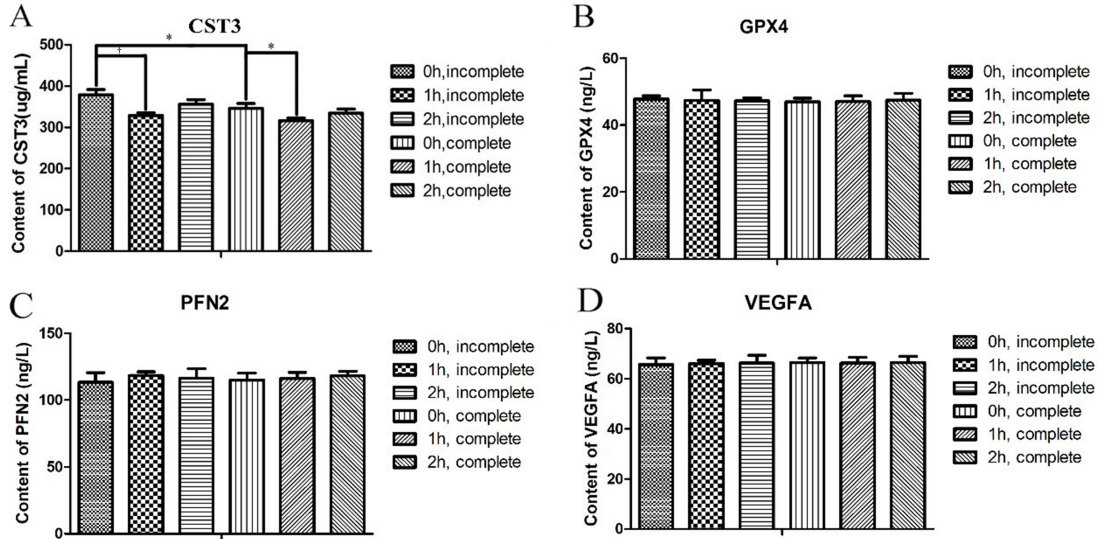
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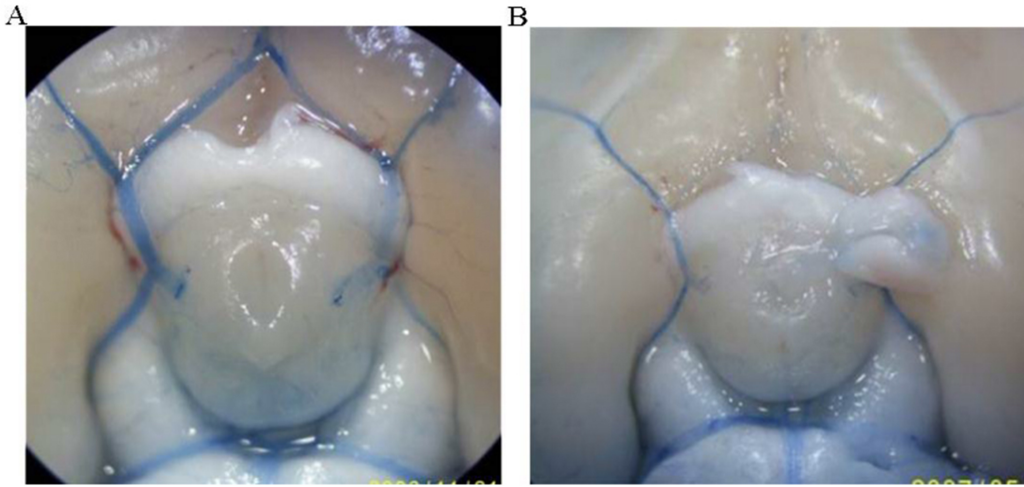
SUPPLEMENTAL MATERIAL

Figure S1. Serum concentrations of CST3 (A), GPX4 (B), PFN2 (C) and VEGFA (D) in cerebral ischemic gerbil with different CoW patterns (incomplete and complete) determined by ELISA at different time points of 0h, 1h, 2h.



Sample sizes: n=44. Comparisons between different groups were conducted using repeated measures ANOVA. Bar charts show the mean \pm SEM. Notes: “*” ($p \leq 0.05$) and “**” ($p \leq 0.01$) showed significant difference.

Figure S2. The referential typical picture of CoW.



A. The complete ACoA in the CoW. B. The incomplete ACoA in the CoW.

Figure S3. The effect of sunitinib malate (SM®) on vascular development.

