CALL FOR PAPERS | Cellular Circadian Rhythms

Nestin-mediated cytoskeletal remodeling in endothelial cells: novel mechanistic insight into VEGF-induced cell migration in angiogenesis

Zhen-wei Liang,1* Zheng Wang,1* Hui Chen,2,4 Cen Li,1 Ti Zhou,1 Zhonghan Yang,1 Xia Yang,1 Yanfang Yang,1 Guoquan Gao,1,3* and Weibin Cai1,4*  
1Department of Biochemistry, Zhongshan Medical School, Sun Yat-sen University, Guangzhou, Guangdong Province, China; 2Department of Obstetrics and Gynecology, Sun Yat-sen Memorial Hospital of Sun Yat-sen University, Guangzhou, Guangdong Province, China; 3Key Laboratory of Functional Molecules from Marine Microorganisms (Sun Yat-sen University), Department of Education of Guangdong Province, Guangzhou, Guangdong Province, China; and 4Center for Disease Model Animals, Sun Yat-sen University, Guangzhou, China, Guangzhou, Guangdong Province, China

Submitted 18 April 2014; accepted in final form 4 December 2014

Liang ZW, Wang Z, Chen H, Li C, Zhou T, Yang Z, Yang X, Yang Y, Gao G, Cai W. Nestin-mediated cytoskeletal remodeling in endothelial cells: novel mechanistic insight into VEGF-induced cell migration in angiogenesis. Am J Physiol Cell Physiol 308: C349–C358, 2015. First published December 10, 2014; doi:10.1152/ajpcell.00121.2014.—Nestin is highly expressed in poorly differentiated and newly formed proliferating endothelial cells (ECs); however, the role of this protein in angiogenesis remains unknown. Additionally, the cytoskeleton and associated cytoskeleton-binding proteins mediate the migration of vascular ECs. Therefore, the aim of the present study was to determine whether VEGF regulates the cytoskeleton, as well as other associated proteins, to promote the migration of vascular ECs. The coexpression of nestin and CD31 during angiogenesis in alkali-burned rat corneas was examined via immunohistochemical analysis. Western blot analyses revealed that the exposure of human umbilical vein endothelial cells (HUVECs) to hypoxia promoted nestin expression in vitro. Additionally, nestin silencing via siRNA significantly inhibited many of the processes associated with VEGF-induced angiogenesis, including tube formation and the migration and proliferation of HUVECs. Moreover, FITC-phalloidin labeling revealed that F-actin filaments were successfully organized into microfilaments in VEGF-treated cells, suggesting a network rearrangement accomplished via F-actin that contrasted with the uniform and loose actin filament network observed in the siRNA-nestin cells. The results of the present study highlight the key role played by nestin in activated HUVECs during angiogenesis. The inhibition of the ERK pathway suppressed the nestin expression induced by VEGF in the HUVECs. Therefore, our study provides the first evidence that nestin-mediated cytoskeleton remodeling in ECs occurs via filopodia formation along the cell edge, facilitating both filopodia localization and cell polarization and ultimately promoting HUVEC migration via VEGF induction, which may be associated with ERK pathway activation.

angiogenesis; nestin; VEGF; cytoskeleton

ANGIOGENESIS POSITIVELY IMPACTS physiological processes such as embryonic development, growth, and tissue regeneration and also plays an important role in pathological processes involving the development of new capillaries from existing vessels (16). VEGF, the primary angiogenic growth factor, has been shown to play a significant role in neovascularization (NV). Indeed, VEGF facilitates endotheliocyte proliferation and migration and regulates capillary permeability via binding to VEGF receptors (flt-1 and flk-1/KDR) (7, 13, 18). VEGF proteins have been implicated in pathological angiogenesis but also play a role in normal revascularization, tissue regeneration, hemangiectasis, clotting, and platelet secretion. Suppressive therapy accomplished via VEGF targeting may influence normal physiology, a possibility that warrants further study (27, 33).

The migration of ECs is a notable and key step in angiogenesis; however, the precise endothelial cell (EC) receptor signaling molecule responsible for this migration has not been identified. Although numerous factors affect EC migration, the influence of VEGF has received considerable attention from researchers, as VEGF is widely used as a signaling molecule for the induction of EC migration (36, 37, 44). Additional studies have shown that VEGF induces the expression of integrin αvβ3 and αvβ5 and various cathepsins from ECs, which mediate both EC migration and angiogenesis (1, 14). Both the cytoskeleton and cytoskeleton-binding proteins represent the internal basis for the migration of vascular ECs. Both the redistribution of the cytoskeleton and changes in the cytoskeleton’s configuration occur in ECs, processes induced via signaling molecules, such as growth factors, chemokines, and extracellular matrix proteins, resulting in cell polarization (11, 32, 43). Additionally, actin polymerization at the anterior of the cell provides the impetus that pushes the cell membrane forward (8, 11, 15). However, it is unclear whether VEGF regulates the cytoskeleton and other associated proteins to improve vascular EC migration.

Nestin, a class VI intermediate filament protein, is a cytoskeletal protein that associates with class III intermediate filaments, including vimentin, desmin, and α-internexin, to form a heterodimer (12, 30). Nestin, a marker of nerve stem cells (NSCs), is expressed in multiple cells and organs, particularly in neural precursor cells, during the embryonic period (2, 24). However, nestin is overexpressed in ECs in response to pathological angiogenesis, as it is often observed in various neoplasms (9, 35, 39), acute pancreatitis (20), glycosuria (31), and nephropathy (22). Nestin is expressed in proliferative cells (40) and stimulates endothelial proliferation and migration by acting as an endothelial survival factor (45). Although both

http://www.ajpcell.org

0363-6143/15 Copyright © 2015 the American Physiological Society
VEGF and nestin play important roles in angiogenesis, it is not clear whether a regulatory relationship exist between these proteins (34). The expression of nestin and the precise molecular mechanism by which this protein activates human umbilical vein endothelial cells (HUVECs) are not clearly understood. The aim of the present study was to characterize the effects of nestin on the proliferation, migration, and cytoskeleton remodeling of HUVECs stimulated by VEGF. The results of this study will be important in identifying and in evaluating the progress of angiogenesis and may provide the first evidence of VEGF-induced EC migration in angiogenesis via nestin-mediated cytoskeleton remodeling. Therapeutic strategies targeting nestin might represent promising safe and efficient treatments for cancer and many other diseases.

MATERIALS AND METHODS

Animal handling and the corneal alkali burn model. The rat model utilizing alkali-burned corneas was described as developed previously, with some modifications (10). Briefly, female Sprague-Dawley rats (weighing 150–230 g) were purchased from the Laboratory Animal Center of Guangdong Province (Guangzhou, Guangdong, China). Ten rats were randomly divided into two groups containing five rats each. Tobramycin eye drops were administered twice daily to establish the model. The rats were anesthetized via an intraperitoneal injection of 5% chloral hydrate (40 mg/kg). Filter paper (2 mm in diameter) was soaked in 220 μl of 1 mol/l NaOH and applied to the central corneas of both eyes for 40 s. The ocular surface was subsequently rinsed with 50 ml of sterilized phosphate-buffered saline (PBS). A circular, gray burned point was detected at the center of the eye. The protocol was approved by the Ethics Committee for Animal Experimentation at the Sun Yat-sen University of Medicine. All procedures conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication, 8th ed., 2011).

Cell culture and hypoxic treatment. The HUVECs were cultured according to a previously described protocol (6). The cells were isolated from the umbilical cord using a 0.25% trypsin solution in PBS and subsequently cultured in serum-free medium (SFM) medium supplemented with 50 μg/ml EC growth supplement (ECGS; BD), 15% fetal bovine serum (FBS; HyClone), 0.5% heparin (DINGGUO, Beijing, China), 100 U/ml streptomycin, and 100 U/ml penicillin (4). Human retinal capillary endothelial cells (HRCECs) were isolated from donor eyes and obtained from Zhongshan Ophthalmic Center of SUN Yat-sen University, China, using a protocol described previously (5). HRCECs were cultured in MEM supplemented with 10% fetal bovine serum, 0.5% heparin, 1% insulin, 100 U/ml streptomycin, and 100 U/ml penicillin. Cell passages 2–6 were used for the experiments. Regarding the hypoxic exposure required for some of the experiments, the HUVECs were cultured in an atmosphere of 1% oxygen-5% CO₂-94% nitrogen at 37°C for the indicated intervals (6).

Western blot analysis. The cells were washed three times with ice-cold PBS, harvested, and lysed for total protein extraction. Total proteins were extracted after boiling for 30 min, followed by centrifugation at 12,000 g for 10 min at 4°C. Equal amounts of proteins (100 μg) were separated through 8% SDS-PAGE and subsequently electroblotted onto polyvinylidene fluoride membranes (Bio-Rad Laboratories). After blocking with 7% skim milk in Tris-buffered saline/Tween buffer (TBST buffer) for 1 h, the membranes were incubated with anti-mouse antibodies against nestin (1:1,000 dilution; BD), p-ERK1/2 (dilution 1:2,000; Santa Cruz), ERK1/2 (dilution 1:2,000; Santa Cruz), and VEGF (1:1,000 dilution; Santa Cruz). The membranes were incubated with horseradish peroxidase-conjugated secondary antibodies and visualized using an ECL detection kit (Appygen, Beijing, China) according to the manufacturer’s instructions. The same membrane was stripped and rebotted with anti-mouse antibodies against β-actin (1:10,000 dilution; Sigma-Aldrich).

Transfection. HUVECs were transfected at 70% confluence for 36 h with 50 nM short interfering (si)RNAs targeting human nestin or a negative control siRNA (NC siRNA) using HiPerfect reagent (Qiagen). Briefly, the siRNA was complexed with 20 μl of transfection reagent and diluted with M199 to 120 μl. Complete fresh medium was added at 3 h after transfection, and the cells were further incubated for 24–36 h before the administration of subsequent treatments. The specific siRNA sequences: NES-homo-1233-A forward (5'-GUGCCAGCCUUUCUAAAGATT-3') and reverse (5'-UCUUAAAGAAAGGGUCACATT-3'); NES-homo-1702-B forward (5'-GGGAAGAGAAGGGAUCUUAAAT-3') and reverse (5'-UUUAAGACAGCUUUCCTCTTT-3'); NES-homo-2455-C forward (5'-CAGGAUCAGAUGACAAUATT-3') and reverse (5'-UCAUGAAGAUAUCACCCCTTT-3'); NES-homo-E(s)siRNApool: A,B,C,D mixture; Negative Control forward (5'-GGGACAGAUCUGC-UAAGA UdTdT-3') and reverse (5'-UCUUAAUGGCCAGGUGGUCGCTdTdT-3').

Cell viability assay. Cellular viability was evaluated using a CCK-8 kit (Dojindo Molecular Technologies). The silenced HUVECs were seeded onto 96-well plates at a density of 2.5 × 10³ cells/well, starved for 12 h, and subsequently exposed or sham exposed to VEGF for 24 h. The cells were washed twice with PBS and incubated with 100 μl of culture medium containing 10% CCK-8 solution for 4–6 h at 37°C. The absorbance was measured at 450 nm using a multimode microplate reader (Infinite M200, Tecan, Switzerland).

Cell apoptosis assay. The HUVECs were seeded onto six-well plates at a density of 2.5 × 10³ cells/well. After 24 h, the cells were transfected with siRNA for 48 h, followed by digestion with 0.05% trypsin and harvesting. The apoptotic cells were double-stained with annexin V-propidium iodide and analyzed via flow cytometry according to the manufacturer’s instructions. The cells treated with 10 mmol of colchicine were used as positive controls, and the cells treated with PBS and transfected with siRNA were used as negative controls.

Cell migration assay. Cell migration was examined using a modified Boyden chamber assay with 24-well chambers (pore size 8 μm; BD) (6). Approximately 500 μl of SFM medium containing 10% serum were added to the lower wells, and HUVECs were seeded at 3 × 10³ cells onto the upper wells containing 300 μl of SFM medium without serum. The chambers were subsequently incubated for 10 h at 37°C. The cells were fixed in 4% polyoxymethylene solution for 5 min and stained with 1% crystal violet. The migrated cells were manually calculated in three random microscopic fields using a fluorescence microscope.

Tube formation assay. A tube formation assay was performed as previously described. Briefly, 1.5 × 10³ cells per well were cultured on a 90 μl EC matrix containing 10 μl of 10× diluted buffer, followed by transfection with 50 nM siRNA-nestin for 8–16 h. The cells were fixed with 4% paraformaldehyde and photographed in three random fields at ×5 magnification using a digitized imaging system.

Monoblast migration assay. HUVECs were seeded onto 24-well plates (10³ cells/well) for 24 h, followed by overnight serum starvation. The cells were subsequently washed three times with PBS, and 2 ml of SFM containing 10 ng/ml VEGF were added. The cells were treated with 10 mg/ml of VEGF for different times. Similar experiments were performed using HUVECs transfected with siRNA-nestin. The distance that the cells migrated was observed using phase contrast microscopy on an inverted microscope. Images of the center where the monolast was located were captured at regular intervals over the course of 24 h.

Wound healing assay. Cell migration was determined using a two-dimensional wound-scratch assay on 12-well plates. HUVECs were seeded onto 12-well plates (5 × 10³ cells/well) for 24 h, followed by overnight serum starvation. In each well, a straight lesion was created in the center of the cell monolayer using a sterile 10-μl pipette tip (400 μm wide). The wells were subsequently washed three times with PBS to remove the dead cells, and 2 ml of SFM containing

AJP-Cell Physiol • doi:10.1152/ajpcell.00121.2014 • www.ajpcell.org
10 ng/ml VEGF were added. Similar experiments were performed using HUVECs transfected with siRNA-nestin. The wounds were observed using phase contrast microscopy on an inverted microscope. Images were taken at regular intervals over the course of 24 h until wound closure was achieved.

**Light microscopy.** HUVECs were plated onto 2% gelatin-coated coverslips, serum starved for 12 h, and stimulated for 12 h with 10 ng/ml VEGF. The cells were washed three times with PBS, permeabilized for 20 min with 1% (vol/vol) Triton X-100, and washed three times with M-buffer solution. The cells were then fixed with 3% glutaraldehyde in PBS, washed three times with PBS, and stained with 0.2% Coomassie Blue. Similar experiments were performed using HUVECs transfected with siRNA-nestin. All slides were mounted and visualized using light microscopy.

**Immunohistochemical staining.** The eyes were enucleated at 14 days after alkali burning, fixed in cold acetone for 30 min, and subsequently sectioned into 10-μm-thick sections. The cryostat sections were incubated with rabbit anti-CD31 (Thermo Fisher Scientific) and mouse anti-nestin (BD Biosciences) for 14 h at 4°C, followed by incubation with a fluorescein-conjugated goat anti-rabbit IgG secondary antibody (Vector Laboratories) for 1 h at room temperature. DAPI was used for the counterstaining of nuclei.

The HUVECs were seeded onto 2% gelatin-coated coverslips, serum starved for 12 h, and stimulated for 12 h with 10 ng/ml VEGF. The cells were washed three times with PBS and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 10 min, followed by washing three times with PBS. The cells were subsequently permeabilized for 5 min in 0.1% (vol/vol) Triton X-100, washed three times with PBS, and blocked with normal goat serum for 30 min at room temperature. The cells were incubated with TRITC-phalloidin for 1 h (Invitrogen; 1:2,000), washed with PBS, and mounted using an 80% glycerol/20% water mix immersion. Similar experiments were performed using HUVECs transfected with siRNA-nestin. All slides were mounted and photographed using a fluorescence microscope (Axio Observer Z1; Carl Zeiss) equipped with appropriate filters.

**Statistical analysis.** The results are presented as arithmetic means ± SD. The data were normally distributed with homogeneous variances. Therefore, multiple group comparison testing was performed using SPSS 13.0 software and determined using a one-way ANOVA, followed by the Student-Newman-Keuls post-hoc test. A value of *P* < 0.05 was considered statistically significant.

**RESULTS**

**Nestin is expressed during corneal neovascularization.** Following surgery, a milky-white injured disc area was visible on the central cornea. In the following days, NV of the cornea was

---

**Fig. 1. Nestin expression in endothelial cells (ECs) of alkali-induced corneal neovascularization.** A: generation of alkali-induced rat CNV. MVD in alkali-induced corneal neovascularization, as determined via immunocytochemistry and immunofluorescence (×200). HE, hematoxylin and eosin. B: nestin is significantly expressed during alkali-induced corneal neovascularization based on the results of the Western blot analysis. C: nestin is expressed in ECs during alkali-induced corneal neovascularization. Double immunostaining confirmed that nestin (green fluorescence) colocalizes with the endothelial marker CD31 (red fluorescence) and with DAPI (blue fluorescence).
observed. Slit lamp photos of the respective groups were captured on the 11th day following surgery. Disorganized vessels characteristic of NV were observed growing on the surfaces of the corneas of the treatment group following injury. Compared with the treatment group, no NV was observed on the corneas of the control group treated with PBS. Corneal opacity and ocular ciliary hyperemia were observed following alkali burning. Hematoxylin and eosin staining revealed the presence of disorganized vessels and inflammatory cells in the corneas of the alkali-treated group, whereas the corneas of the rats in the PBS-treated group were normal, immunofluorescence was utilized to determine the microvessel density of the corneas of the alkali-treated group (Fig. 1A). Taken together, these results suggested that the alkali burning of the cornea results in both severe inflammation and NV.

To characterize the extent of nestin expression during corneal NV, we detected VEGF and nestin expression in both groups using Western blotting in samples obtained from mice killed on the 11th day following alkali treatment. Our data confirmed that both nestin and vessels were lacking in the undamaged cornea. As shown in Fig. 1B, the level of nestin expression in the alkali-treated group was remarkably higher than in the PBS-treated control group. Double staining was performed to confirm the localization of nestin during corneal NV. At 11 days following alkali induction, nestin colocalized with CD31, an EC marker, in most of the areas characterized by corneal NV. By contrast, the level of nestin staining observed in the control group was clearly decreased (Fig. 1C).

Nestin expression in ECs is stimulated via either VEGF or hypoxia. Both nestin and VEGF play important roles in physiological angiogenesis in various diseases. Therefore, we determined whether VEGF regulates the expression of nestin in HUVECs. After HUVEC proliferation was arrested via serum deprivation, the cells entered a quiescent state. A Western blot analysis demonstrated that the HUVECs stimulated by VEGF exhibited markedly upregulated nestin expression in both dose (Fig. 2A)- and time-dependent manners (Fig. 2B). The same results were observed in the HRCECs (Fig. 2, C and D). Additionally, SU5416, an inhibitor of VEGFR2 (Flk-1), inhibited the upregulation of nestin via VEGF in HUVECs. We determined the level of nestin expression after the cells were pretreated with SU5416, and the results indicated that SU5416 inhibited VEGF-dependent nestin upregulation (Fig. 2E). Because hypoxia-induced endothelial activation is important in angiogenesis, we examined the effect of hypoxia on nestin expression in activated HUVECs. A subsequent
Fig. 3. Effects of nestin on the proliferation, apoptosis and migration of the HUVECs. A: comparison of the 5 siRNA-nestin sequences used to inhibit nestin expression in HUVECs (A-E; 5 different siRNA sequences). B: HUVECs were silenced using siRNA-nestin, and the viable cells were quantified using a CCK-8 assay. The data present the absorbance as a percentage of the control (means ± SD; n = 3; *P < 0.05 vs. the VEGF-treated group). C: apoptotic cells stained with annexin V and propidium iodide were quantified via flow cytometry (#P < 0.01 vs. control, the colchicine-treated group served as a positive-control group). D: migrated cells were observed using a modified Boyden chamber assay (×200). The data are presented as percentages of inhibition (n = 3; #P < 0.01, *P < 0.05, a: vs. the VEGF-treated group, b: vs. the control group).
Western blotting analysis demonstrated that nestin expression was significantly increased in ischemia-stimulated HUVECs (Fig. 2F), whereas the cells cultured under normoxic conditions exhibited gradual reduction in their levels of nestin expression over time, as well as a lack of growth factor expression. Nestin was downregulated in HUVECs subjected to SU5416 treatment, an effect induced by hypoxia (Fig. 2G). Taken together, these results suggest that nestin is involved in NV via VEGF regulation.

\textit{Nestin is involved in cell migration but not in the proliferation of HUVECs.} To determine the role played by nestin in the migration and proliferation of vascular ECs, we produced HUVECs lacking nestin expression via transfection, using siRNA-nestin sequences. siRNAs may induce unexpected and divergent changes in the gene expression of targeted proteins in mammalian cells. Therefore, the available sequences were chosen from five available candidates for RNAi experiments. The Western blot analysis revealed that the nestin levels in the

![Fig. 4. Effect of nestin silencing on tube formation and the cellular movement of the HUVECs. A: representative photographs of tube-like structures. B: phase-contrast micrographs of HUVECs at various times following monolayer wounding. C: dynamic observation of monoblasts using a living cell microscope imaging system at 3 point times (0, 12, and 24 h). The pictures were captured from the video records (refer to the Supplemental Video Materials).](http://ajpcell.physiology.org/)

\textit{AJP-Cell Physiol} • doi:10.1152/ajpcell.00121.2014 • www.ajpcell.org
HUVECs transfected with siRNA-A and siRNA-B were significantly decreased compared with the cells transfected with the other sequences (Fig. 3A). Therefore, we utilized only seq-A and seq-B, the most effective nestin siRNA sequences throughout the experiment. We performed cell apoptosis and cell migration assays to determine whether nestin depletion changes the viability of the HUVECs. As shown in Fig. 3, B and C, the downregulation of nestin expression had no apparent effect on either the proliferation or the apoptosis of the HUVECs (the cells treated with 10 mmol/l cholochine as a positive control). However, nestin silencing suppressed the chemotactic movement of the HUVECs that was induced by VEGF (10 ng/ml). As shown in Fig. 3D, VEGF apparently played a role in inducing the chemotaxis. The administration of siRNA-A and siRNA-B significantly reduced the number of migrating HUVECs compared with the control group, exerting an inhibitory effect on the cellular chemotaxis induced by VEGF (Fig. 3D). These findings suggest that the involvement of nestin in angiogenesis may be due in part to the effects of VEGF on the cellular chemotaxis of the HUVECs induced.

Nestin silencing inhibits tube formation and the cellular movement of the HUVECs. To determine the effect of nestin silencing on angiogenesis, we performed a tube formation assay, and the results indicated that HUVECs cultured in 0.2% serum undergo angiogenesis in vitro and that the cells transfected with siRNA-nestin were characterized by inhibited tube formation and cellular movement. As angiogenesis was visibly induced via VEGF at a concentration of 10 ng/ml, the effect exerted by nestin on angiogenesis was suppressed, reflecting an in vitro nestin deletion (Fig. 4A). These studies confirmed that VEGF is involved in angiogenesis via nestin regulation. To confirm nestin’s involvement in VEGF-induced migration, we silenced nestin expression in HUVECs to determine its role in HUVEC migration, using an in vitro scratch assay. As shown

![A](image1.png) ![B](image2.png)

Fig. 5. Effect of nestin on the polymerization, cytoskeletal remodeling, and localization of cytoskeletal proteins in the HUVECs. A: images showing the uniform and loose cytoskeleton distribution using Coomassie Brilliant Blue R250 under light microscopy (×200). B: immunolocalization of F-actin and vimentin using electron microscopy. The cells were counterstained with DAPI (blue; ×200).
in Fig. 4B, the HUVECs treated with VEGF exhibited significantly increased cell migration, and the two sides of the scratch merged after 24 h, whereas the nestin-silenced cells did not achieve wound closure.

To find more convincing evidence, supporting the promotion of nestin-mediated HUVEC movement by VEGF, a microscopic living cell culture system was used to directly track the HUVECs and observe for dynamic changes in cellular movement, the total distances traveled, and the travel patterns. The results indicated that both the migratory straight-line distance and the magnitude of the distance traveled in the VEGF-induced group were significantly increased compared with the negative control group during a 24-h period of continuous monitoring. However, in the nestin-silenced group, these effects were significantly attenuated compared with the VEGF-induced group (Fig. 4C).

Moreover, the uninterrupted video data clearly demonstrate both the full moving track and the motility of the HUVECs (refer to the Supplemental Video Materials; Supplemental Material for this article is available online at the Journal website).

Nestin was involved in the cytoskeletal remodeling in ECs. The results obtained in the present study suggest that nestin is involved in VEGF-induced cytoskeletal remodeling in HUVECs. To further determine the role of nestin, we applied Coomassie Brilliant Blue R250 to observe for changes in both the cytoskeletal protein and cell polarity. As shown in Fig. 5A, stress fibers were colored deep blue, but the color of the cytoplasmic background was weak under light microscopy. An increased number of filopodia were observed along the cell edge in the setting of VEGF stimulation, whereas if in the siRNA-nestin group, the filopodia exhibited directionless extension and were randomly interwoven into the reticular structures of the HUVECs. These preliminary results may indicate that nestin silencing reduces cell migration via the reduction of filopodia extension in HUVECs. Furthermore, we used FITC-phalloidin to stain F-actin and observed that the F-actin filaments in the VEGF-treated cells were well organized into microfilaments and exhibited an orderly rearrangement of the F-actin network, compared with the uniform and loose arrangement of the actin filaments in the siRNA-nestin cells. Previously, we demonstrated the dose-dependent expression of nestin in VEGF-stimulated HUVECs. Therefore, siRNAs can inhibit both filopodia localization and cell polarization. Taken together, these results suggest that nestin silencing inhibits filopodia localization and cell polarization and ultimately suppresses HUVEC migration. Additionally, we also observed that vimentin expression was significantly increased in the VEGF-induced group. However, vimentin expression was significantly decreased in the nestin-silenced group. Therefore, we hypothesized that vimentin expression was reduced in response to nestin silencing, as the functions of these proteins are closely related.

VEGF-induced nestin expression is mediated by the ERK pathway. Studies involving pharmacological inhibitors have suggested a role for the ERK-1/2 (also known as p42/44 MAPKs) in the physiological responses to VEGF in ECs (3, 23). To determine whether the ERK signaling pathway is involved in VEGF-induced nestin expression, we examined the levels of both nestin expression and ERK1/2 phosphorylation by adding U0126, which blocks ERK1/2 activation. As shown in Fig. 6, nestin expression was upregulated in HUVECs as a result of VEGF treatment, and this elevation was blocked by U0126, a specific inhibitor of ERK1/2 phosphorylation. Consistent with this observation, the level of active phosphorylated ERK1/2 in these cells was increased in the presence of VEGF. Therefore, the VEGF-mediated upregulation of nestin was mediated by the phosphorylation of ERK1/2.

DISCUSSION

Previous studies have demonstrated that nestin is involved in tumor angiogenesis and pterygium vasculature and may play a central role in the angiogenic response (28, 42). To further examine nestin expression in the setting of neovascularization, we established a mouse model of alkali burn-induced corneal neovascularization to assess molecular alterations and nestin variations. The localization of nestin was examined via immunofluorescence staining with the marker CD31, and the results indicated that nestin is clearly expressed in ECs during corneal neovascularization. These results are consistent with those of a previous study that demonstrated that nestin is specifically expressed in newly formed ECs. Additionally, alkali burn-induced corneal neovascularization significantly increased the expression of VEGF.

VEGF not only participates in embryo development but is also involved in the pathological angiogenesis of both cancer and intraocular neovascular disorders (21, 26). Angiogenesis and vessel maturation are complex but harmonious pro-

Fig. 6. Depletion of ERK-1/2 abolishes VEGF-induced nestin expression in the HUVECs. The HUVECs were preincubated with U0126 (8 μM) and then treated with 10 ng/ml VEGF. The HUVECs not treated with either VEGF or U0126 were used as the control group. All changes in nestin and p-ERK expression were determined via a Western blot analysis. The histograms represent means ± SD (n = 3; #P < 0.01, *P < 0.05 vs. the VEGF-treated group).
cesses that generate a series of chain reactions that activate the ligand receptor, and VEGF is the critical rate-limiting step in this process. Furthermore, VEGF promotes angiogenesis via the regulation of the proliferation and migration of vascular ECs (18).

Nestin plays an important role in the differentiation and the development of the nervous system. Previous studies have shown that nestin may also be involved in angiogenesis, as this protein is highly expressed in poorly differentiated and potentially proliferative vascular ECs and endothelial progenitors; however, the mechanism underlying this phenomenon remains unclear (31, 39). Previous studies have indicated that VEGF, the VEGF receptor, and nestin are coexpressed in ECs during angiogenesis in tumor tissues (19, 46). Based on these results, we proposed that nestin may play an important role in the formation of new blood vessels, a process regulated primarily via VEGF. Indeed, a recent study demonstrated nestin expression in ovarian ECs, a process mediated by VEGF signaling (41). We used the inhibitor SU5416 to directly block VEGFR2, the primary receptor for VEGF-A. Following the blockage of the receptor, nestin stimulation through VEGF was significantly inhibited in a dose-dependent manner. Meanwhile, nestin was downregulated in the HUVECs treated with SU5416 in the setting of hypoxia; however, SU5416 did not completely suppress the hypoxia-induced expression of nestin in these HUVECs, which indicates that the effect of hypoxia on nestin expression is partially VEGF dependent. Therefore, we hypothesized that hypoxia may regulate nestin expression via other growth factors, such as transforming growth factor-β1, in addition to VEGF (38). A previous study demonstrated that VEGF regulates the directed migration of NSCs and induces the chemotaxis of NSCs (25, 47). Moreover, we observed the coexpression of CD31 and nestin in newly formed vessels. Taken together, these results suggested that nestin may participate in cell migration and proliferation in angiogenesis via VEGF. These results indicated that nestin silencing strongly inhibits its effects on migration, but no distinct inhibition of either proliferation or apoptosis was observed, suggesting that nestin is a key factor in migration during angiogenesis. The results of the monoblast migration assay and the wound healing assays indicated that the migration of VEGF-induced HUVECs was mediated via nestin.

Additionally, the immunolocalization assay demonstrated that vimentin, a type of intermediate filament, was observed in each of the groups and exhibited significantly different levels of expression during VEGF stimulation. A recent study demonstrated both the rearrangement and the polarization of F-actin in NSCs during both the spreading and the migration stimulated by VEGF (47). There is evidence that cytoskeleton proteins directly participate in migration during angiogenesis, a process during which cytoskeletal reorganization occurs in ECs and directly impacts cellular morphology and movement. It is known that VEGF is suppressed primarily via the inhibition of the ERK pathway (29). Moreover, EGF regulated nestin expression via the Ras-Raf–ERK signaling pathway, which was activated in reactive astrocytes (17). In the present study, U0126 treatment inhibited ERK phosphorylation and consequently suppressed the expression of nestin that was induced by VEGF in the HUVECs.

In conclusion, the results of the present study provide evidence that the ERK pathway plays an important role in VEGF-induced nestin expression in the setting of angiogenesis. Furthermore, VEGF primarily facilitates nestin expression to induce HUVEC migration via the formation of filopodia along the cell edge, facilitating both filopodia localization and cellular polarization and ultimately promoting HUVEC migration. In the past decade, several VEGF-based strategies for the targeting ECs with antiangiogenic agents have been developed. However, these treatments may evoke resistance or have adverse effects, highlighting the need for reliable biomarkers with which to monitor the efficacy of antiangiogenic therapy. As a supporting biomarker in the setting of antiangiogenic therapy, nestin is sensitive to cells in “activated, injured, or proliferative states.” Therefore, nestin may be a promising therapeutic target for the development of a safe drug that does not affect quiescent vessels in normal tissues and may also serve as a reliable biomarker with which to monitor antiangiogenic therapy.

ACKNOWLEDGMENTS

We are grateful to the Department of Obstetrics and Gynecology, Sun Yat-sen Memorial Hospital of Sun Yat-sen University for providing human umbilical cord samples.

GRANTS

This study was supported by National Nature Science Foundation of China Grants 81070746, 81172163, 81272338, 81272515, 81200706, and 81470015; National KeySci-Tech Special Project of China Grant 2013ZX09102-053; Guangdong Natural Science Foundation (1015100890100007, S201202001089, and Key Project 1025100890100009); Fundamental Research Funds for the Central Universities of China (Youth Program 09YKY73, 10YKY28); Changjiang Scholars and Innovative Research Team in University (985 Project PCSIRT 0947); and Guangzhou Science and Technology Project (2011J4100106, 2011Y1-00017-8). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: Z.-w.L., Z.W., H.C., C.L., T.Z., and Y.Y. performed experiments; Z.-w.L. and Z.W. analyzed data; Z.-w.L. and Z.W. interpreted results of experiments; Z.-w.L. and Z.W. prepared figures; Z.-w.L. and Z.W. drafted manuscript; Z.-w.L. and W.C. edited and revised manuscript; Z.Y., X.Y., G.G., and W.C. conception and design of research; G.G. and W.C. approved final version of manuscript.

REFERENCES

VEGF INDUCES NESTIN-MEDIATED CYTOSKELETON REMODELING


AJP-Cell Physiol • doi:10.1152/ajpcell.00121.2014 • www.ajpcell.org

Downloaded from http://ajpcell.physiology.org/ on July 6, 2017