

# CXCL16 induces angiogenesis in autocrine signaling pathway involving hypoxia-inducible factor 1 $\alpha$ in human umbilical vein endothelial cells

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**Abstract.** Chemokine (C-X-C motif) ligand 16 (CXCL16) is a new angiogenic factor inducing angiogenesis via extracellular signal-regulated kinases pathway. To further understand the molecular mechanism underlying CXCL16-induced angiogenesis, we explored involvement of other relevant pathways in CXCL16-induced angiogenesis. In the present study, we investigated the mechanisms underlying CXCL16-induced angiogenesis in human umbilical vein endothelial cells (HUVECs). CXCL16 promoted HUVEC proliferation, tube formation and migration. Enzyme-linked immunosorbent assay revealed that CXCL16 induced vascular endothelial growth factor secretion from HUVECs. Western blot analysis showed that CXCL16 increased the level of hypoxia-inducible factor 1 $\alpha$ , p-extracellular signal-regulated kinases (ERK), p-p38 and p-Akt dose- and time-dependently. ERK-, p38- and Akt-selective inhibitors significantly suppressed HUVEC proliferation, migration, tube formation and hypoxia-inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ) expression induced by CXCL16. Furthermore, CXCL16 peptides induced CXCL16 secretion via ERK, p38 and Akt pathways, which was suppressed by HIF-1 $\alpha$ -selective inhibitor PX12. Our data suggest that

CXCL16 induces angiogenesis in autocrine manner via ERK, Akt, p38 pathways and HIF-1 $\alpha$  modulation.

## Introduction

Chemokine (C-X-C motif) ligand 16 (CXCL16) is a member of CXC chemokine family, initially identified in dendritic cells in T cell zone and cells in the splenic red pulp (1). Later, the expression of CXCL16 in macrophages (2,3), dendritic (4), liver sinusoidal endothelial (5) and cancer cells (6-8) was observed. There are two forms of CXCL16: membrane-bound CXCL16 inducing firm adhesion in chemokine (C-X-C motif) receptor 6 (CXCR6) expressing cells (9), and soluble CXCL16 inducing migration in CXCR6 expressing cells (10,11). CXCL16 is involved in progress of many diseases (12-14), including cancer (6,7). For instance, higher expression of CXCL16 reduced the overall survival of cancer patients (6); CXCR6 is involved in cancer the cell proliferation and invasion *in vitro* (15). Our previous research showed that CXCR6 contributed to breast cancer cell migration via the regulation of hypoxia-inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ) under hypoxia (16).

The effect of CXCL16 in cancer cell is well defined, while the effect of CXCL16 on angiogenesis is rarely studied. Previous research has reported that CXCL16 promotes human umbilical vein endothelial cell (HUVEC) proliferation, chemotaxis, and tube formation by activating ERK pathway *in vitro* (17). Whereas, the role of other pathways and their involvement in CXCL16-induced angiogenesis is not clear. It has been observed that CXCL16 improves the human aortic smooth muscle cells proliferation and adhesion by activating the PI3K/Akt pathway (18) whereas the binding of dextran sulfate sodium to CXCL16 activates the p38 pathway in murine peritoneal macrophages (19). Moreover, the activation of p38 and Akt pathways induces angiogenesis (20-22).

In the present study, we investigated the molecular mechanisms underlying CXCL16-induced angiogenesis. Our results revealed that CXCL16 induced angiogenesis and HIF-1 $\alpha$  expression by activating the ERK, p38 and Akt pathways in HUVECs. Furthermore, we found that CXCL16 increased

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VEGF and CXCL16 secretion by modulating HIF-1 $\alpha$  in HUVECs.

## Materials and methods

**Cell culture.** HUVECs were cultured in complete medium consisted of M199 medium, 20% FBS, 50 ng/ml endothelial cell growth supplement (ECGS) (both from Sigma) and 100 ng/ml heparin. Cells were incubated in humidified atmosphere with 5% CO<sub>2</sub> at 37°C. HUVECs at the 6-9th passage were used for experiments.

**Cell proliferation assay.** HUVECs were seeded into a 24-well plate in complete medium and cultured overnight. After the replacement of medium with M199 supplemented with 1% FBS and 100 ng/ml heparin, HUVECs were pre-treated with 2.5  $\mu$ M FR180204 (ERK-selective inhibitor), 5  $\mu$ M SB202190 (p38 MAP kinase-selective inhibitor), 5  $\mu$ M LY294002 (selective inhibitor of phosphatidylinositol 3-kinase) or 10  $\mu$ M PX12 (HIF-1 $\alpha$ -selective inhibitor) (all from Tocris Bioscience) for 1 h. Then cells were exposed to 0, 1, 10 and 100 ng/ml CXCL16 polypeptide (PeproTech) for 48 h. Cells were then counted in five randomly selected fields per group under a microscope.

CXCL16 was dissolved in phosphate-buffered saline (PBS); FR180204, SB202190, LY294002 and PX12 were dissolved in dimethylsulfoxide (DMSO).

**Cell migration assay.** Transwell chamber migration system with polycarbonate membranes (8.0- $\mu$ m pore size; Corning) was used for cell migration assay (23). The medium used in the migration assay consisted of M199, 1% FBS and 100 ng/ml heparin. HUVECs (1 $\times$ 10<sup>4</sup> cells/ml) were seeded into the upper chamber with 2.5  $\mu$ M FR180204, 5  $\mu$ M SB202190, 5  $\mu$ M LY294002 or 10  $\mu$ M PX12. Medium with CXCL16 (100 ng/ml) and FBS (10%) was added into the lower chamber. HUVECs (1 $\times$ 10<sup>4</sup> cells/ml) were seeded into the upper chamber along with various concentrations of CXCL16 (1, 10 or 100 ng/ml) added into the lower chamber. The cell migration system was incubated at 37°C for 6 h. Cells were fixed with 4% paraformaldehyde, stained with 0.2% cresyl violet solution and counted in five randomly selected fields.

**Tube formation assay.** Tube formation assay was performed as previously described (23), 96-well plates were coated with 50  $\mu$ l/well growth factor reducing Matrigel (Becton, Dickinson and Company) and incubated at 37°C for 2 h. HUVECs (1 $\times$ 10<sup>4</sup> cells/well) were incubated with CXCL16 (0, 1, 10 or 100 ng/ml) at 37°C for 12 h. Or, HUVECs (1 $\times$ 10<sup>4</sup> cells/well) were incubated with 2.5  $\mu$ M FR180204, 5  $\mu$ M SB202190 or 5  $\mu$ M LY294002 in the presence of CXCL16 (100 ng/ml) for 24 h. Or, HUVECs (1 $\times$ 10<sup>4</sup> cells/well) were treated with or without 10<sup>-5</sup> M topotecan in the presence of CXCL16 (1 or 100 ng/ml) for 12 h. Tubes were counted in five randomly selected fields per group under a microscope.

**Western blotting.** HUVECs were treated with 100 ng/ml of CXCL16 for 0, 10, 20, 30, 40 and 50 min in a time-dependent study and with different concentrations of CXCL16 (0, 1, 10 and 100 ng/ml) for 30 min in a dose-dependent study; or with 100 ng/ml of CXCL16 for 0, 3, 6, 9, and 12 h; or with

100 ng/ml of CXCL16 in the presence of DMSO, 2.5  $\mu$ M FR180204, 5  $\mu$ M SB202190 or 5  $\mu$ M LY294002, respectively. Conditioned medium was collected and concentrated with Amicon Ultra-15 (with Ultracel-10; Millipore). Cells were washed with ice-cold PBS and then lysed with RIPA buffer (Beyotime). Total protein concentration was evaluated with the BCA protein assay reagent kit (Pierce, Rockford, IL, USA). Denatured samples (40  $\mu$ g/lane) were separated by 10-15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Transferred membranes were probed with murine anti-human antibodies: anti-CXCL16 antibody (R&D Systems), anti-HIF-1 $\alpha$  antibody (Sigma), anti-p-ERK1/2 antibody, anti-p-Akt antibody, anti-p-p38 MAPK antibody, anti-ERK1/2 antibody, anti-Akt antibody, anti-p38 antibody (all from Cell Signaling Technology) and anti-actin antibody (Sigma). Horseradish peroxidase (HRP)-linked anti-rabbit IgG (Cell Signaling Technology) or HRP-linked anti-mouse IgG (Sigma) was used as the secondary antibody. Immunoreactive proteins on the membrane were visualized by enhanced chemiluminescence western blotting detection reagents (Millipore, USA).

**Real-time quantitative PCR.** HUVECs were pre-treated with or without 10  $\mu$ M PX12 for 1 h, followed by the treatment of 100 ng/ml of CXCL16 for 3 or 6 h; or followed by incubation under normoxia or hypoxia for 6 h. Total cell RNA was isolated with TRIzol<sup>®</sup> reagent (Invitrogen). Total RNA (2  $\mu$ g) was used for first-strand cDNA synthesis with RevertAid First Strand cDNA Synthesis kit (Fermentas). Primers for RT-PCR sequences were as follows: HIF-1 $\alpha$  forward, 5'-CTCACCCA ACGAAAAATTACAGAA-3' and HIF-1 $\alpha$  reverse, 5'-ATTGA GTGCAGGGTCAGCACTAC-3' (24); CXCL16 forward, 5'-GGCAGCGTCACTGGAAGTTGTTAT-3' and CXCL16 reverse, 5'-ACCGATGGTAAGCTCTCAGGTGTT-3';  $\beta$ -actin forward, 5'-GAGCGGGAAATCGTGCGTGACATT-3' and  $\beta$ -actin reverse, 5'-GAAGGTAGTTTCGTGGATGCC-3' (25). iQ<sup>TM</sup> SYBR-Green Supermix (Bio-Rad Laboratories) was used as a fluorescent dye to detect the presence of double-stranded DNA. The amount of mRNA was normalized to an internal control  $\beta$ -actin mRNA. Relative mRNA levels were normalized to control.

**VEGF enzyme-linked immunosorbent assay.** HUVECs were exposed to 100 ng/ml of CXCL16 for 0, 3, 6, 9, 12, 24 and 30 h. At each time point, conditioned medium were collected and concentrated at 8,000 rpm at 4°C for 1 min. The supernatant was collected and stored at -70°C. The enzyme-linked immunosorbent assay (ELISA) was performed according to the manufacturer's instruction for human VEGF.

**Statistical analysis.** All data represent at least three independent experiments and are expressed as mean  $\pm$  SEM. Student's t-test was used for statistical analysis.

## Results

**Effects of CXCL16 on proliferation, migration and tube formation in HUVECs.** To study the effect of CXCL16 on angiogenesis *in vitro*, HUVECs were treated with 1-100 ng/ml of CXCL16 for 48 h for the cell proliferation assay, 6 h formi-

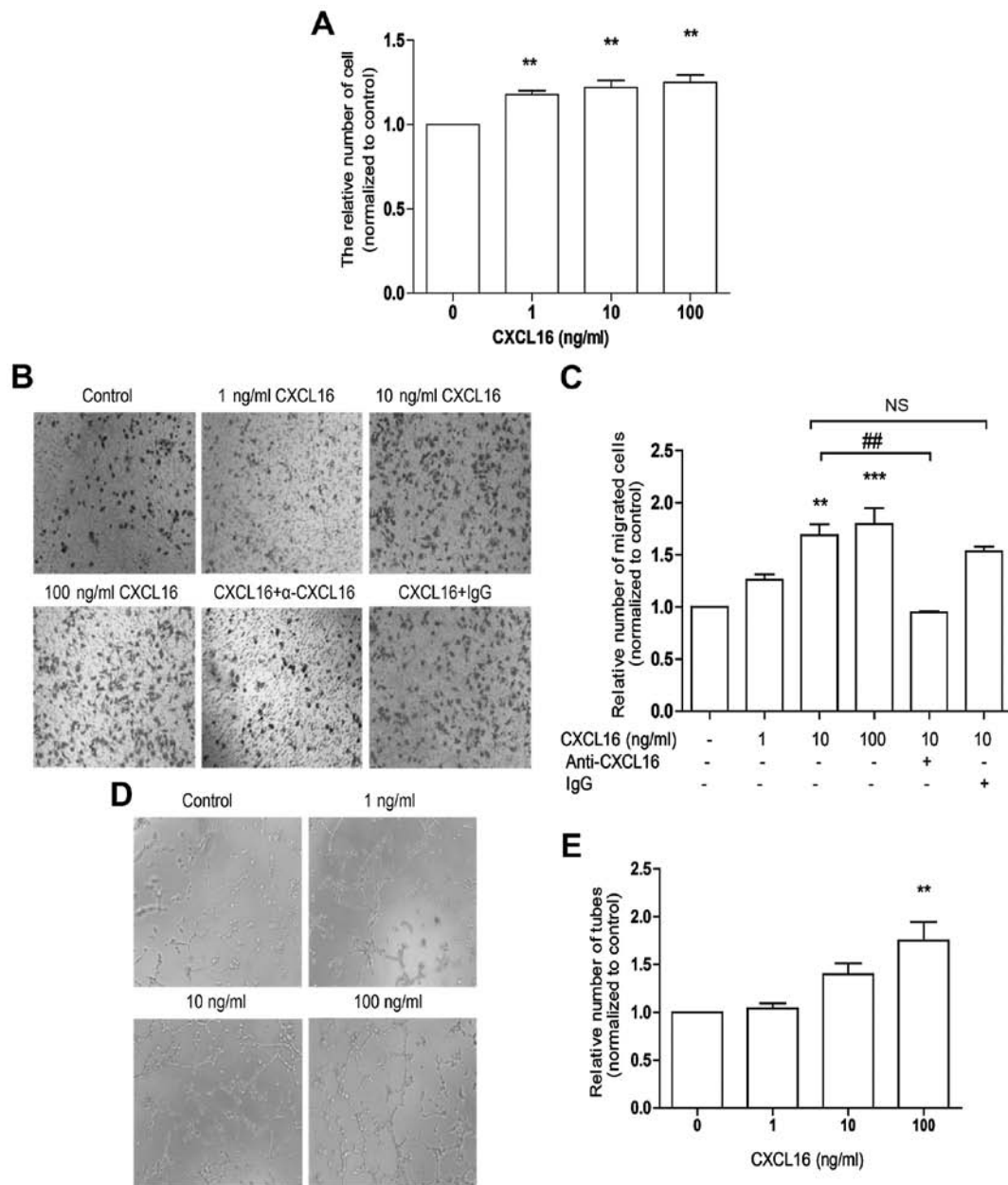


Figure 1. CXCL16 promotes angiogenesis in a dose-dependent manner. HUVECs were treated with different concentrations of CXCL16 (1, 10 and 100 ng/ml) for 48 h proliferation (A), 6 h migration (B and C), 6 h tube formation (D and E). Cells from five random fields per group were calculated under a microscope in B and D. \*\*P<0.01; \*\*\*P<0.001, compared with control; ##P<0.01.

gration assay and 12 h for tube formation assay. In line with previous research (17), our data showed that CXCL16 promoted HUVECs proliferation (Fig. 1A), migration (Fig. 1B and C) and tube formation (Fig. 1D and E) in a dose-dependent manner. Our data confirm that CXCL16 promotes HUVECs angiogenesis *in vitro*.

**Effects of CXCL16 on the activation of ERK, p-38 and Akt pathways in HUVECs.** In murine peritoneal macrophages and human aortic smooth muscle cells, CXCL16 activated ERK1/2, p38 and PI3K/Akt, respectively (18,19). To determine whether CXCL16 could activate ERK1/2, p38 and Akt pathways in HUVECs, we treated HUVECs with CXCL16 (100 ng/ml) for 0, 10, 20, 30, 40 and 50 min. Western blot results revealed that the phosphorylation levels of ERK1/2, p38 and Akt were

increased within 30 min and p-ERK1/2 and p-Akt were increased time-dependently (Fig. 2A).

After these findings, we studied the activation of ERK1/2, p38 and Akt pathways in HUVECs treated with different concentrations of CXCL16 (0, 1, 10 and 100 ng/ml) for 30 min. It was observed that p-ERK1/2 and p-Akt induced by CXCL16 were increased dose-dependently, whereas, p-p38 was increased in the presence of 100 ng/ml of CXCL16 (Fig. 2B).

To further elucidate the activation of ERK1/2, p38 and Akt pathways induced by CXCL16 in HUVECs against the time frame, we treated HUVECs with CXCL16 (100 ng/ml) for 0, 3, 6, 9 and 12 h. Western blot results showed that all three pathways were activated within 3 h (Fig. 2C). However, the activation patterns of these pathways were different. ERK pathway was activated within 30 min (Fig. 2A) and lasted until

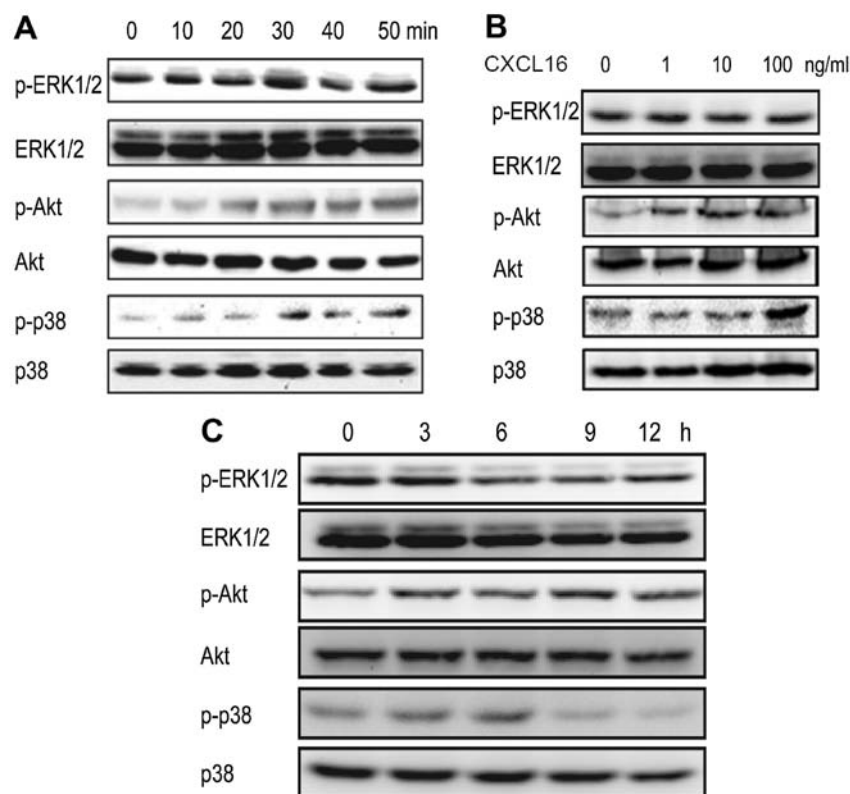


Figure 2. Activation of ERK1/2, Akt and p38 pathways induced by CXCL16. (A) CXCL16 activates ERK1/2, Akt and p38 in a time-dependent manner in HUVECs. HUVECs were treated with 100 ng/ml of CXCL16 for 0, 10, 20, 30, 40 and 50 min. p-ERK1/2, ERK1/2, p-Akt, Akt, p-p38 and p38 were checked by western blot analyses. (B) CXCL16 activates ERK1/2 and Akt in a dose-dependent manner. HUVECs were treated with 0, 1, 10 and 100 ng/ml of CXCL16 for 30 min. Phosphorylation of ERK1/2, Akt and p38 was analyzed by western blot analyses. (C) Long-term activation of ERK1/2, Akt and p38. HUVECs were treated with 100 ng/ml of CXCL16 for 0, 3, 6, 9 and 12 h and followed by western blot analysis.

3 h (Fig. 2C); Akt pathway activation reached its maximum level within 1 h and decreased at 12 h (Fig. 2C); the highest levels of p-p38 were observed at 6 h interval (Fig. 2C). The data indicated that CXCL16 activates ERK, p38 and Akt pathways in HUVECs.

**Inhibition of ERK, p38 and Akt pathways reduces HUVEC angiogenesis induced by CXCL16.** To confirm the involvement of ERK, p38 and Akt pathways in HUVEC-associated angiogenesis induced by CXCL16, we treated HUVECs with 100 ng/ml CXCL16 and FR180204 (inhibitor of ERK pathway) or SB202190 (inhibitor of p38 pathway) or LY294002 (inhibitor of PI3K/Akt pathway) in proliferation, migration and tube formation assays sequentially. We observed that FR180204, SB202190 and LY294002 markedly attenuated CXCL16-induced HUVEC proliferation (Fig. 3A) and tube formation (Fig. 3F and G), whereas LY294002 decreased HUVEC migration induced by CXCL16 (Fig. 3B and C) or FBS (Fig. 3D and E). It was also observed that SB202190 was effective only in cell migration induced by FBS (Fig. 3D and E). The data revealed the involvement of ERK, p38 and Akt signaling in CXCL16-induced HUVEC proliferation and tube formation and indicated that only Akt pathway was associated with HUVEC migration induced by CXCL16.

**Effect of CXCL16 on HIF-1 $\alpha$  expression, and CXCL16-stimulated HUVECs to secrete CXCL16 via ERK1/2, p38 and Akt/HIF-1 $\alpha$  pathway.** Topotecan is a chemotherapeutic

agent. We investigated whether topotecan has an influence in CXCL16-induced angiogenesis. Therefore, we treated HUVECs with different concentrations of CXCL16 and 1  $\mu$ M topotecan. Our findings in tube formation assays revealed that topotecan antagonized the positive effect of CXCL16 on tube formation *in vitro* (Fig. 4A). Our previous study showed that topotecan inhibits HIF-1 $\alpha$  expression *in vitro* (26). As shown in Fig. 3A, PX12 (HIF-1 $\alpha$  inhibitor) significantly attenuated HUVEC proliferation induced by CXCL16, indicating the regulation of HIF-1 $\alpha$  by CXCL16. Thus, we further investigated whether CXCL16 influenced HIF-1 $\alpha$  expression with western blotting and RT-PCR assays. Our results showed that CXCL16 increased HIF-1 $\alpha$  protein (Fig. 4B) and mRNA level at 3 h (Fig. 4D), however, the protein level of HIF-1 $\alpha$  was reduced after 6 h.

Regarding the data of CXCL16 activated ERK1/2, p38 and Akt pathways, we showed these pathways were correlated with HIF-1 $\alpha$  upregulation induced by CXCL16. Upon exposure of HUVECs to FR180204, SB202190, LY294002 and PX12 in the presence of CXCL16 for 6 h, we found that all the inhibitors reduced the improvement in HIF-1 $\alpha$  expression induced by CXCL16 at 6 h (Fig. 4E). These results implicated that CXCL16 increased HIF-1 $\alpha$  expression through ERK1/2, p38 and Akt pathways under normoxia.

All the results shown above were performed with the exogenous CXCL16 peptide with molecular weight of 10.1 kDa. We also investigated whether exogenous CXCL16 affected the expression of endogenous CXCL16 in the same way.

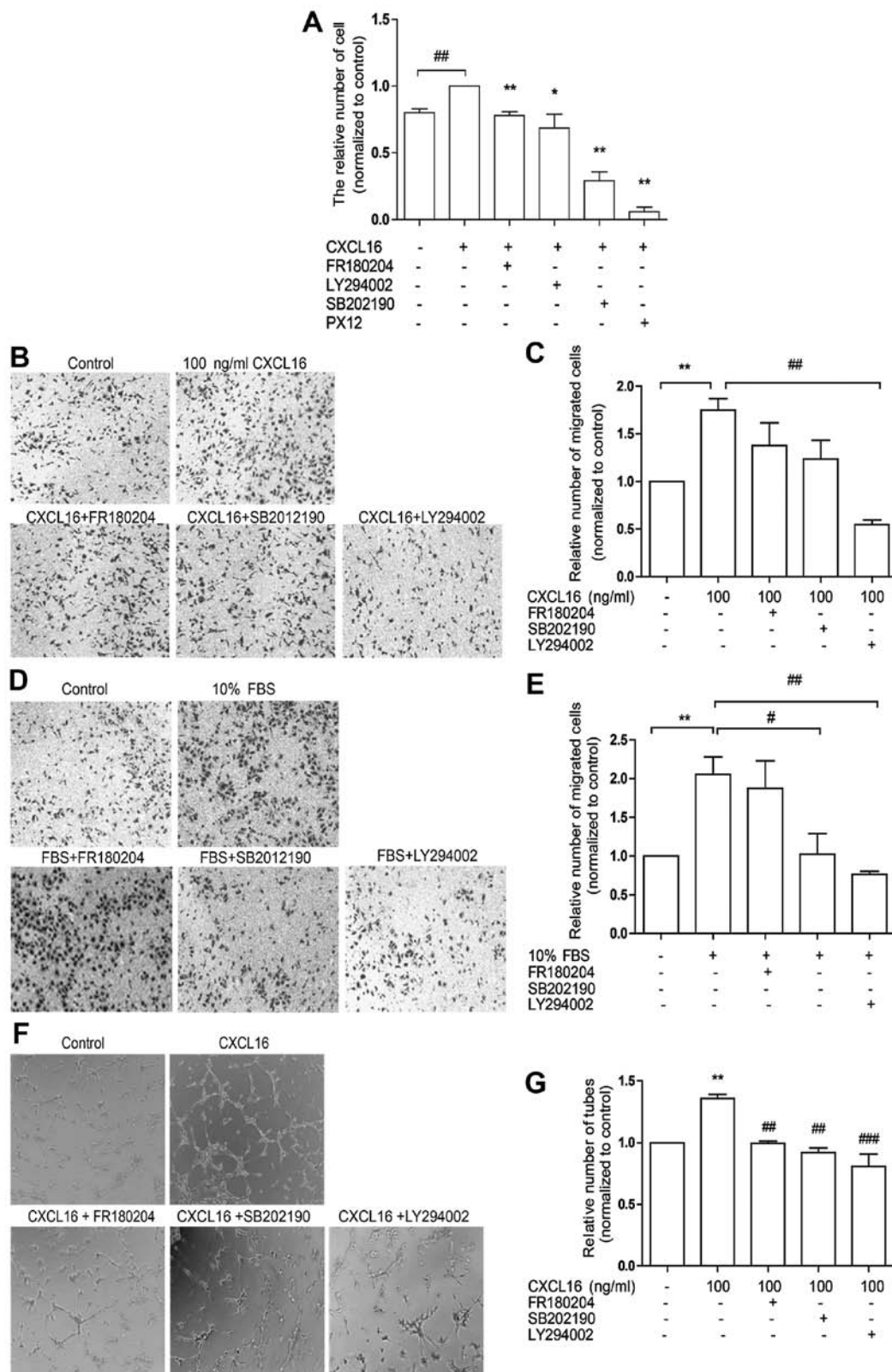


Figure 3. Involvements of ERK1/2, Akt and MAPK p38 pathways in angiogenesis induced by CXCL16. CXCL16 induces HUVECs proliferation (A) and tube formation (F) via ERK, Akt and p38 pathways. HUVECs were treated with 100 ng/ml of CXCL16 in the presence of 2.5  $\mu$ M FR180204, 5  $\mu$ M SB202190 and 5  $\mu$ M LY294002 for 48 (A) or 24 h (F). Cells were also treated with PX12 for 48 h (A). (B and C) Involvement of MAPK p38 pathway in HUVECs migration induced by CXCL16. (D and E) MAPK p38 and the Akt pathways are related to FBS-induced HUVECs migration. Cells from five fields/group were calculated under a microscope. The relative number of cells (A, B and D) and tubes (F) were normalized to control. \*\* $p$ <0.01, compared with control (PBS); \* $p$ <0.05, \*\*\* $p$ <0.001, compared with CXCL16 group.

Therefore, we treated HUVECs with 100 ng/ml of CXCL16 peptide for 0-12 h, and harvested the supernatant and cells for

western blot analysis. Our findings indicated that CXCL16 peptide increased endogenous CXCL16 secretion (Fig. 4B

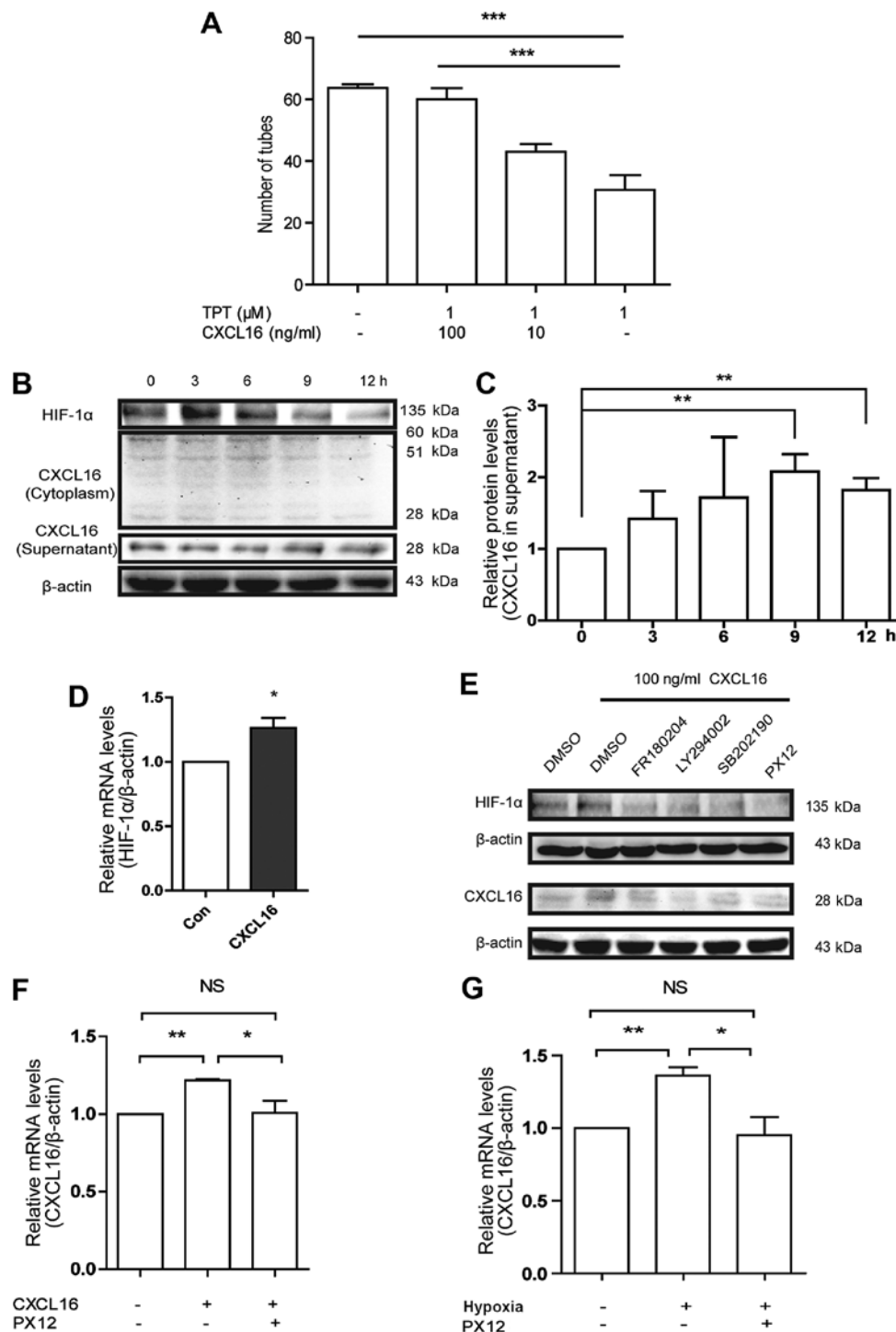


Figure 4. Effects of CXCL16 on HIF-1 $\alpha$  in HUVECs, and CXCL16-induced HUVECs to excrete CXCL16 by upregulating HIF-1 $\alpha$  through ERK, Akt and p38 pathways. (A) Involvement of HIF-1 $\alpha$  in CXCL16 induced tube formation in HUVECs. Cells were treated with 100 ng/ml CXCL16, or with TPT with different concentrations of CXCL16 (100, 1 and 0 ng/ml) for 6 h. Relative numbers of tubes were normalized to control (PBS). \*\*\* $p$ <0.001, compared with 100 ng/ml CXCL16 group. (B and C) CXCL16 increases HIF-1 $\alpha$  expression in HUVECs. Cells were treated with 100 ng/ml of CXCL16 for 0, 3, 6, 9 and 12 h. Total proteins from the cells were examined for HIF-1 $\alpha$  expression by western blot assays. Conditioned medium and cell lysates were collected from HUVECs treated with 100 ng/ml of CXCL16 for 0, 3, 6, 9 and 12 h. Proteins were examined for CXCL16 expression in cytoplasm and supernatant with murine anti-CXCL16 antibodies by western blot assays. (D) CXCL16 increases HIF-1 $\alpha$  mRNA in HUVECs. Cells were treated with 100 ng/ml of CXCL16 for 3 h and followed by mRNA extraction and subsequent qRT-PCR analysis. The mRNA level was normalized to control group. \* $p$ <0.05. (E) Cell lysates were collected from HUVECs treated with 100 ng/ml of CXCL16 in the presence of inhibitors against ERK, p38, Akt and HIF-1 $\alpha$  for 6 h. HIF-1 $\alpha$  and CXCL16 expressions were analyzed by western blot analyses. (F and G) CXCL16 induces CXCL16 secretion via the regulation of HIF-1 $\alpha$  in HUVECs. PX12 decreased CXCL16 mRNA level after CXCL16 peptide treatment for 6 h (F) or hypoxia treatment for 12 h (G). \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001.

and C) and mRNA level of CXCL16 at 6 h (Fig. 4F). These results suggested that CXCL16 is probably self-regulated and modulates angiogenesis in HUVECs.

To determine the pathways involved in CXCL16 secretion, we treated HUVECs with CXCL16 (100 ng/ml) and FR180204, SB202190, LY294002 or PX12, respectively, for 12 h. These

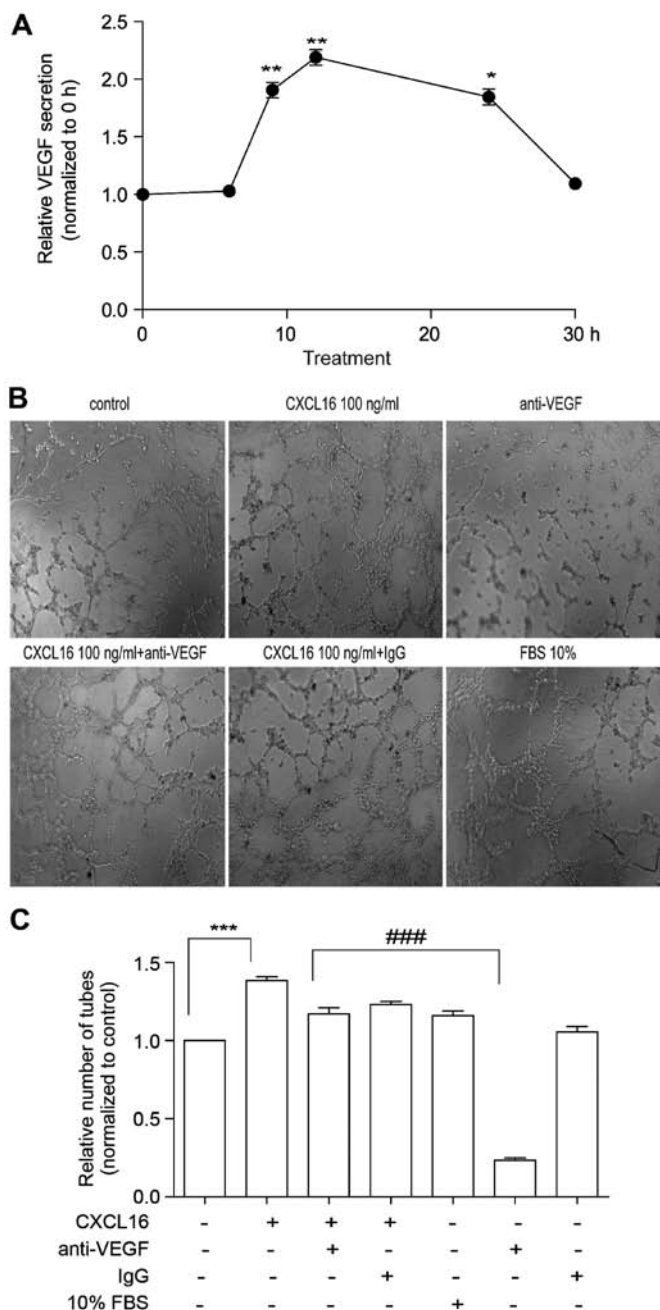


Figure 5. Effects of CXCL16 on tube formation are not due to VEGF secretion in HUVECs. (A) HUVECs were treated with 100 ng/ml of CXCL16 for 0, 3, 6, 9, 12, 24 and 30 h. Supernatants were collected and analyzed with VEGF ELISA kit in three repetitive wells. VEGF amount in the supernatants were normalized to control (0 h) and shown as mean  $\pm$  SEM. (B and C) CXCL16 induces HUVEC tube formation in autocrine manner and is not due to VEGF secretion. HUVECs were treated with 100 ng/ml of CXCL16 and, at the same time anti-VEGF antibody (R&D Systems) was added for 24 h. Cells from five random fields/group were calculated under a microscope. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ; ### $P < 0.001$ .

inhibitors reversed the increase of CXCL16 secretion induced by CXCL16 peptide (Fig. 4E). In addition, we found that PX12 significantly reduced mRNA level of CXCL16 induced by CXCL16 peptide (Fig. 4F). These results indicated that CXCL16 regulates its expression by itself via ERK1/2, p38 and Akt pathways and HIF-1 $\alpha$  regulation in HUVECs under normoxia.

To confirm the expression of CXCL16 via HIF-1 $\alpha$ , we pre-treated HUVECs with PX12 or DMSO for 1 h, and then incubated HUVECs under hypoxia or normoxia for 6 h; notably we found that hypoxia increased the CXCL16 mRNA level that was previously inhibited by PX12 (Fig. 4G). These findings suggested that CXCL16 induces HUVEC secretion under normoxia via activation of ERK, p38 and Akt signaling and involvement of HIF-1 $\alpha$ .

*Effects of CXCL16 on tube formation are not due to VEGF secretion in HUVECs.* As the secretion of VEGF is partially regulated by HIF-1 $\alpha$  in endothelial cells (27,28), we examined the secretion of VEGF from HUVECs treated with CXCL16. The HUVECs were exposed to CXCL16 (100 ng/ml) for different time periods (0, 3, 6, 9, 12, 24 and 30 h), followed by the detection of VEGF in the supernatant. ELISA results showed that the amount of VEGF was significantly increased at 9 h, reaching its maximum during 12 h and normalized at 30 h of CXCL16 treatment (Fig. 5A). The data indicate that CXCL16 promotes HUVECs to secrete VEGF possibly by regulating HIF-1 $\alpha$  expression.

We have found that CXCL16 can promote HUVECs to secrete VEGF, which can also promote tube formation. So we added CXCL16 100 ng/ml and, at the same time we added anti-VEGF antibody (R&D Systems) to verify whether the effect of CXCL16 on tube formation is due to VEGF secretion in HUVECs or due to some other reason. Notably, we found that the tube formation was reduced to a small extent (Fig. 5B and C). The data indicated that the effect of CXCL16 on tube formation was not due to VEGF secretion in HUVECs.

## Discussion

Recent research has shown that CXCL16 and its receptor play crucial roles in cancer growth and metastasis (6,15,16). Although many studies have confirmed the effect of CXCL16 in tumorigenesis, the effect of CXCL16 on angiogenesis was not clarified. The present study was aimed to investigate the signaling pathways of CXCL16 during angiogenesis. We demonstrated an autocrine signaling of CXCL16 that induced angiogenesis via activating ERK1/2, p38 and Akt pathways and upregulating HIF-1 $\alpha$  expression in HUVECs.

Akt phosphorylation is crucial in angiogenesis both *in vitro* and *in vivo* (20,29,30), and ERK and p38 pathways were also considered to be associated with angiogenesis (21,31,32). It has been found that CXCL16 activates ERK in HUVECs within 30 min (17). Consistent with these findings, activations of Akt, ERK and p38 by CXCL16 was also observed to be started within 30 min in HUVECs. However, the activation of ERK, Akt and p38 was decreased after 6 h, which could be due to the desensitization of CXCR6 after prolonged exposure to CXCL16. By treating HUVECs with FR180204 (inhibitor of ERK pathway), SB202190 (p-p38 inhibitor) and LY294002 (p-Akt inhibitor), we confirmed the involvement of ERK, p38 and Akt pathways in the angiogenesis induced by CXCL16 in HUVECs.

HIF-1 $\alpha$ , a transcription factor is usually found at low oxygen conditions and mediates cellular responses to hypoxia. Previous studies have suggested that cytokines and growth

factors, such as epidermal growth factor (33), insulin and interleukin-1 $\beta$  (34,35) induce HIF-1 $\alpha$  expression under normoxia. In the present study, we found that HIF-1 $\alpha$  was upregulated by CXCL16 at both protein and mRNA levels within 6 h under normoxia. However, at normoxia, HIF-1 $\alpha$  immediately degrades by cell type-specific regulation of von Hippel-Lindau tumor-suppressor protein (36). The Akt pathway is involved in the stabilization of HIF-1 $\alpha$  under normoxia (37) and regulation of HIF-1 $\alpha$  expression. ERK and p38 pathways are likely regulating HIF-1 $\alpha$  expression as well (38,39). Consistent with these observations, our data showed that SB202190 (p-p38 inhibitor) and LY294002 (p-Akt inhibitor) suppressed HIF-1 $\alpha$  expression induced by CXCL16.

HIF-1 $\alpha$  induces the expression of VEGF and CXCL12 in endothelial cells (38,39) under normoxia. In addition, CXCL16 improves secretion of VEGF in prostate cancer cells (15). Our data showed that CXCL16 induced HUVECs to secrete VEGF which reached the maximum level within 9 h under normoxia.

CXCL16 expression in HUVECs was measured. Since the CXCL16, used for treatment, was an exogenous polypeptide with a small molecular weight (10.1 kDa), we were able to distinguish endogenous soluble CXCL16 (30 kDa) from the peptide in western blotting and RT-PCR assays. We found that CXCL16 peptide improved the secretion of endogenous soluble CXCL16 from HUVECs. However, SB202190, FR180204 and LY294002 inhibited the secretion suggesting the involvement of ERK, p38 and Akt pathways in CXCL16 secretion induced by CXCL16 in HUVECs. Furthermore, we found that hypoxia induced CXCL16 mRNA was increased which was resisted by the HIF-1 $\alpha$  pathway inhibitor PX12. It can be due to high level of HIF-1 $\alpha$  induced by CXCL16. The soluble form of CXCL16 was kept in supernatant for 12 h, but HIF-1 $\alpha$ , potential upstream of CXCL16, was reduced after treatment with CXCL16 for 6 h, which may be due to the lower level of CXCR6 or CXCR6 desensitization. Thus, our data indicated that exogenous CXCL16 activated ERK, p38, and Akt/HIF-1 $\alpha$  pathway to induce CXCL16 secretion in HUVECs.

In solid tumor, cancer cells excreted CXCL16 to promote angiogenesis, and endothelial cells excreted VEGF under high level of CXCL16. However, CXCL16 exhibit a dual effect in atherosclerotic lesions; CXCL16 not only promotes endothelial cell survival but also guides the migration of macrophages into inflamed tissues, thus resulting in more serious inflammation.

In conclusion, the present study exhibited a novel signaling pathway of CXCL16 in HUVEC angiogenesis. CXCL16 promoted angiogenesis in autocrine signaling in HUVECs under normoxia, involving activation of ERK1/2, Akt and p38 pathways and subsequent upregulation of HIF-1 $\alpha$ . Moreover, CXCL16 increased VEGF secretion in HUVECs which is most probably via the regulation of HIF-1 $\alpha$ .

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