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Critical role of c-Jun N-terminal kinase in regulating bFGF-induced angiogenesis *in vitro*

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Shen Kaikai[1], Sheng Yuchen[2], Ji Lili[1,3,*] and Wang Zhengtao[1,3,†]

[1]The MOE Key Laboratory for Standardization of Chinese Medicines and The SATCM Key Laboratory for New Resources and Quality Evaluation of Chinese Medicines, Institute of Chinese Materia Medica, Shanghai University of Traditional Chinese Medicine, Shanghai 201210; [2]Department of Pharmacology, Shanghai Institute of Pharmaceutical Industry, Shanghai 200437; and [3]Shanghai R&D Centre for Standardization of Chinese Medicines, Shanghai 201210, China

*Ji Lili, The MOE Key Laboratory for Standardization of Chinese Medicines and The SATCM Key Laboratory for New Resources and Quality Evaluation of Chinese Medicines, Institute of Chinese Materia Medica, Shanghai University of Traditional Chinese Medicine, 1200 Cailun Road, Shanghai 201210, China. Tel: 86-21-51322506, Fax: 86-21-51322519, email: lily0913@yahoo.cn

†Wang Zhengtao, The MOE Key Laboratory for Standardization of Chinese Medicines and The SATCM Key Laboratory for New Resources and Quality Evaluation of Chinese Medicines, Institute of Chinese Materia Medica, Shanghai University of Traditional Chinese Medicine, 1200 Cailun Road, Shanghai 201210, China. Tel: 86-21-51322507, Fax: 86-21-51322519, email: wangzht@hotmail.com

Angiogenesis, the process of new blood vessels formation, is a critical step for wound healing, tumour growth and metastasis, diabetic retinopathy, psoriasis, etc. The present study was designed to investigate whether c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) is critical for regulating basic fibroblastic growth factor (bFGF)-induced angiogenesis in human umbilical vein endothelial cells (HUVECs). Our results showed that bFGF-induced HUVECs proliferation, migration and tube formation with a concentration-dependent manner. Further results showed that bFGF induced the phosphorylation of JNK/SAPK at 15 min. Both JNK/SAPK inhibitor SP600125 and JNK/SAPK peptide inhibitor 420116 could inhibit bFGF-induced HUVECs proliferation, migration and tube formation, so did JNK/SAPK-specific siRNA. Moreover, when HUVECs were stimulated with bFGF, upstream signals of JNK/SAPK, SEK1/MKK4 and MKK7 were both activated at 2 min. In summary, our results indicate that JNK/SAPK signal pathway plays an important role in regulating bFGF-mediated angiogenesis in HUVECs, which may therefore be a new therapeutic approach for the treatment of angiogenesis-associated diseases.

Keywords: angiogenesis/bFGF/HUVECs/JNK/SAPK.

Abbreviations: bFGF, basic fibroblastic growth factor; EGF, epidermal growth factor; ERK1/2, extracellular signal-regulated kinase 1 and 2; FGF, fibroblastic growth factors; HUVECs, human umbilical vein endothelial cells; JNK/SAPK, c-Jun N-terminal kinase/stress-activated protein kinase; MAPKs, mitogen-activated protein kinases; MMP, matrix metalloproteinase; PDGF, platelet-derived growth factor; PI3K, phosphatidylinositol 3-kinase; PLCγ, phospholipase Cγ; TGF, transforming growth factors; u-PA, urokinase-type plaminogen activatior; VEGF, vascular endothelial growth factor.

Many studies report that angiogenesis contributes to the occurrence and development of various pathological states, including tumour growth and metastasis, diabetic retinopathy, psoriasis, age-related macular degeneration, rheumatoid arthritis, etc. (*1*). The suppression of this abnormal angiogenesis may provide therapeutic strategies for the treatment of those diseases. Thus, exploring the signals regulating angiogenesis has been aroused more and more attentions throughout the world.

A number of angiogenic factors derived from surrounding pathological environment can stimulate the abnormal activation of endothelial cells and then initiate angiogenesis. From which, growth factors have been well-characterized in pathological angiogenesis (*2*, *3*), such as vascular endothelial growth factor (VEGF), basic fibroblastic growth factor (bFGF), platelet-derived growth factor (PDGF), transforming growth factors (TGF) α and β. Notably, bFGF has been described as a multipotent cytokine that regulates pathological angiogenesis by binding to cell surface FGF receptors in the presence of heparin proteoglycans (*4–7*).

It is well-established that bFGF activates multiple downstream signal transduction pathways, including mitogen-activated protein kinases (MAPKs), in various cell types (*8–10*). The MAPKs family consists of extracellular signal-regulated kinases 1 and 2 (ERK1/2), p38 and c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) (*11*, *12*). In particular, activated MAPKs have been detected in many pathological angiogenesis including tumour progression and metastasis, diabetic retinopathy and age-related macular degeneration, suggesting the possibility that MAPKs may play a role in angiogenesis-associated diseases (*13–15*). Recent evidences show that various anti-angiogenic compounds can abrogate VEGF- and bFGF-induced angiogenesis via inhibiting the activation of Raf/MEK/ERK signalling cascade, demonstrating the involvement of ERK1/2 in angiogenesis (*16–18*). Also, p38 is the critical modulator

of actin cytoskeleton remodelling required for the migration of HUVECs (*19*). As for the JNK/SAPK, it participates in angiogenesis via inhibiting the secretion of VEGF and matrix metalloproteinase (MMP), affecting the cell–cell interaction via down-regulating bFGF-mediated surface cadherin (*20*, *21*). However, whether JNK/SAPK is directly involved in bFGF-induced angiogenic responses in HUVECs is not very clear. The present study is designed to investigate whether JNK/SAPK signalling pathway is involved in bFGF-induced angiogenesis *in vitro*.

Materials and Methods

Reagents

Phospho-specific rabbit polyclonal antibodies against ^{202}Thr and ^{204}Tyr dual-phosphorylated p44/42 MAPK (ERK1/2), ^{180}Thr and ^{182}Tyr dual-phosphorylated p38, ^{257}Ser and ^{261}Tyr dual-phosphorylated SEK1/MKK4, ^{271}Ser and ^{275}Thr dual-phosphorylated MKK7, total p44/42 MAPK (ERK1/2), p38, JNK/SAPK, SEK1/MKK4, MKK7, β-actin and mouse polyclonal antibodies against ^{183}Thr and ^{185}Tyr dual-phosphorylated JNK/SAPK, JNK/SAPK siRNA and control siRNA were all purchased from Cell Signaling Technology (Danvers, MA, USA). Peroxidase-conjugated goat anti-Rabbit IgG (H+L) and peroxidase-conjugated goat anti-Mouse IgG (H+L) were purchased from Jackson ImmunoResearch (West Grove, PA, USA). Cell culture reagents and lipofectamine RNAiMAX transfection reagent were purchased from Invitrogen (Invitrogen, Carlsbad, CA, USA). Von Willebrand factor was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Human recombinant bFGF was purchased from PeproTech (Rocky Hill, NJ, USA). MAPK inhibitors (SP600125, SB203580 and PD98059) were purchased from Alexis Biochemicals (Alexis, San Diego, CA, USA). Peptide inhibitor of JNK/SAPK (catalog no. 420116) and negative peptide inhibitor of JNK/SAPK (catalog no. 420118) were purchased from Calbiochem (Calbiochem, San Diego, CA, USA). Other reagents unless indicated were from Sigma Chemical Co. (St. Louis, MO, USA).

Cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated from fresh umbilical cord veins, identified as positively immunofluorescent staining for von Willebrand factor antigen, as previously described method (*22*). Isolated endothelial cells were cultured in M199 medium supplemented with 20% [v/v] heat-inactivated foetal bovine serum (FBS), 10 ng/ml epidermal growth factor (EGF), 30 μg/ml endothelial cell growth supplement (ECGS), 5 U/ml heparin, 100 U/ml penicillin and 100 μg/ml streptomycin in humidified atmosphere of 95% air and 5% CO_2 at 37°C. Experiments were performed on cell cultures of the third to sixth passages.

Cell viability assay

HUVECs (2×10^4 cells/well) were pre-treated with or without MAPK inhibitors for 15 min and then treated with or without bFGF (10 ng/ml) for 48 h. After treatments, cells were incubated with 500 μg/ml 3-(4, 5-dimethylthiazol-2-yl) 2, 5-diphenyltetrazolium bromide (MTT) for 4 h. The functional mitochondrial succinate dehydrogenases in survival cells can convert MTT to formazan that generates a blue colour. At last the formazan was dissolved in 10% SDS–5% iso-butanol–0.01 M HCl. The optical density was measured at 570 nm with 630 nm as a reference and cell viability was normalized as a percentage of control.

Transwell migration assay

The 8-μM pore transwells (Millipore, MA, USA) were coated with 0.1% gelatin for 1 h at 37°C. HUVECs were incubated with M199 containing 0.1% BSA for 4 h at 37°C and then were treated with or without MAPK inhibitors for 15 min at 37°C. At last, HUVECs were seeded at a density of 4×10^4 cells/well into the top chamber of transwells. The bottom chamber of transwells was filled with M199 containing 0.1% BSA with or without bFGF (10 ng/ml). After 8 h of incubation, the cells on the top surface of the membrane (non-migrated) were scraped with a cotton swab, while the cells on the bottom surface of the membrane (migrated) were fixed with cold 4% paraformaldehyde for 30 min. After that, those migrated cells were stained with 0.1% crystal violet for 30 min and observed under the microscope. Images were taken using an inverted microscope (Olympus, CK40, Japan) and the migrated cells from five randomly chosen fields were quantified by manual counting. Migrated cells were normalized as a percentage of untreated cells (control).

Tube formation assay

The 96-well plates were coated with 30 μl cold liquid matrigel per well and incubated at 37°C for 45 min to promote solidification. HUVECs were incubated with M199 containing 0.1% BSA for 4 h and then treated with or without MAPK inhibitors for 15 min at 37°C. At last, cells were seeded at a density of 1×10^4 cells/well into the previous 96-well plates and incubated with M199 containing 0.1% BSA with or without bFGF (10 ng/ml) at 37°C for 4 h. Images were recorded by an inverted microscope (Olympus, CK40, Japan) and tubes forming intact networks were counted.

siRNA transfection

HUVECs were cultured in 60 mm plates (for cell proliferation, migration and tube formation) or 35 mm plates (for western blot analysis) with growth medium without antibiotics. Overnight, JNK/SAPK siRNA and control siRNA (no silencing) were transfected into HUVECs via lipofectamine RNAiMAX according to the manufacturer's recommendations.

Western blot analysis

After treatments, cells were lysed in lysis buffer containing 50 mM Tris (pH 7.5), 1 mM EDTA, 150 mM NaCl, 20 mM NaF, 0.5% NP-40, 10% glycerol, 1 mM phenylmethylsulphonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A. Proteins were separated by SDS–PAGE and blots were probed with appropriate combination of primary antibodies and HRP conjugated secondary antibodies. Membranes were stripped in 62.5 mM Tris (pH 6.7), 20% SDS and 0.1 M 2-mercaptoethanol for 30 min at 50°C to repeated immunoblotting.

Statistical analysis

For all experiments, data were presented as means ± SEM. Statistical comparisons were subjected to an analysis of variance (ANOVA) and LSD-test using SPSS version 11.5 and $P < 0.05$ was considered as statistically significant difference. All figures were performed using SigmaPlot version 10.0 software program.

Results

bFGF-induced proliferation, migration and tube formation in HUVECs

The proliferation, migration and tube formation of endothelial cells represent each stage for the formation of new blood vessels during angiogenesis, respectively. As shown in Fig. 1A, stimulation with bFGF resulted in an increase of 1.6-fold higher proliferation than the basal level. Additionally, the result in Fig. 1B showed that bFGF also stimulated the migration of HUVECs in a concentration-dependent manner and the concentration of bFGF at 1 ng/ml displayed the strongest stimulatory ability. Further result in Fig. 1C showed that bFGF could stimulate tube formation of HUVECs in the concentration-dependent manner.

bFGF-induced the phosphorylation of ERK1/2, p38 and JNK/SAPK in HUVECs

Next, we investigated the effects of bFGF (50 ng/ml) on the phosphorylation of ERK1/2, p38 and JNK/SAPK in HUVECs. As shown in Fig. 2, the bFGF-induced maximum phosphorylation of JNK/SAPK was at 15 min. Also, bFGF strongly induced the

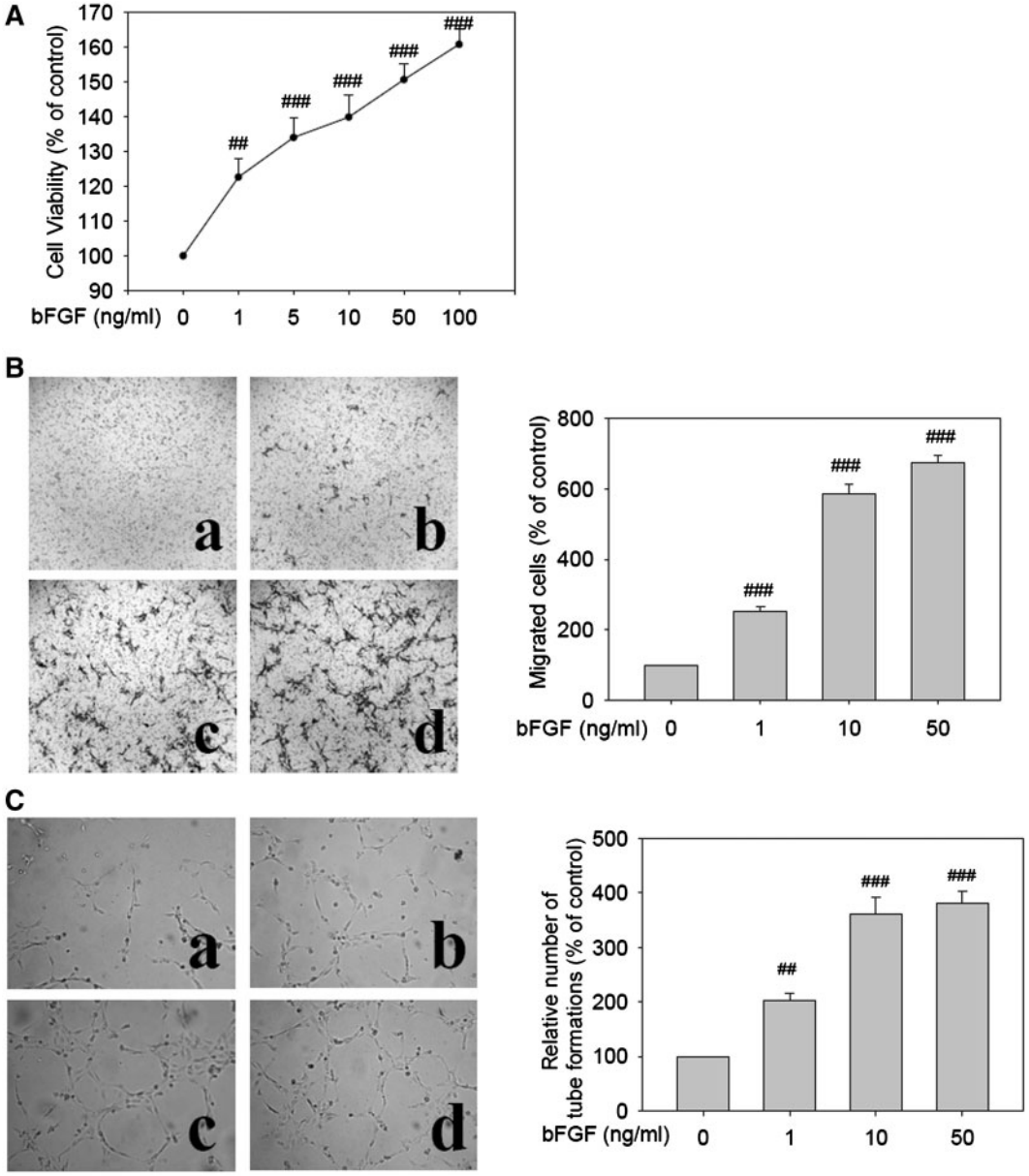

Fig. 1 bFGF-induced proliferation, migration and tube formation in HUVECs. (A) HUVECs were incubated with M199 containing 1% FBS and treated with different concentrations of bFGF (0–100 ng/ml) for 48 h. The survival cells were determined by MTT assay. (B) HUVECs were incubated with M199 containing 0.1% BSA for 4 h, then seeded in the topper chambers of transwell at the density of 4×10^4 cells/well. The bottom chamber was supplemented with or without different concentrations of bFGF (0–50 ng/ml). After 8 h of incubation, the migrated cells passed through the membrane and were quantified by manual counting the cells that crossed the membrane with an inverted microscope at ×100 magnification. (C) HUVECs were incubated with M199 containing 0.1% BSA for 4 h, placed in the matrigel-coated 96-well plates at the density of 1×10^4 cells/well and then supplemented with or without different concentrations of bFGF (0–50 ng/ml). After 4 h, pictures were taken under an inverted microscope at ×100 magnification. The tubular structures of HUVECs were quantified by manual counting. a: control, b: bFGF 1 ng/ml, c: bFGF 10 ng/ml, d: bFGF 50 ng/ml. Data were means ± SEM from three independent experiments. $^{##}P < 0.01$; $^{###}P < 0.001$ versus control.

phosphorylation of ERK1/2 and p38 at 5 and 2 min, respectively, with the continuous activation to 15 min and then the phosphorylation slightly decreased. The phosphorylation of p38 and ERK 1/2 induced by bFGF was earlier than JNK/SAPK.

Effects of MAPKs inhibitors on bFGF-induced proliferation, migration and tube formation in HUVECs

As bFGF could induce the phosphorylation of JNK/SAPK, ERK1/2 and p38, thus, we further investigated the roles of MAPKs in angiogenesis induced by bFGF. All those inhibitors had no significant cytotoxicity in HUVECs by themselves (shown in Supplementary Fig. S3). The result in Fig. 3A showed that SP600125 and PD98059 but not SB203580 inhibited bFGF-induced HUVECs proliferation. Further result in Fig. 3B showed that all the MAPK inhibitors could inhibit bFGF-induced HUVECs migration and of which the inhibition of SP600125 was the best. In addition, the result in Fig. 3C showed that SP600125 completely inhibited bFGF-induced tube formation in

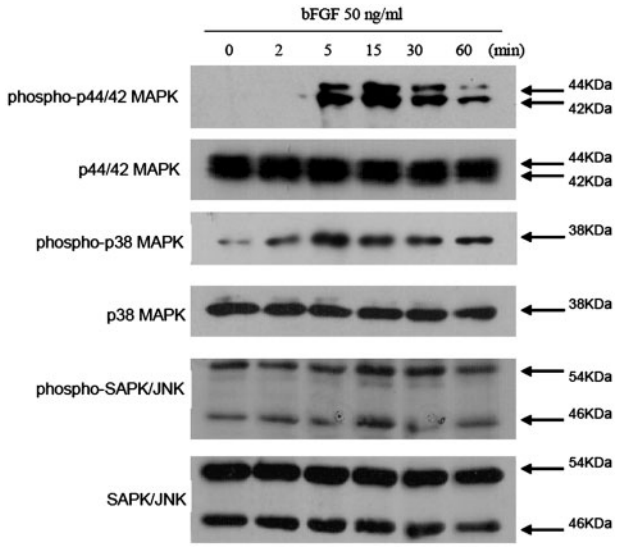

Fig. 2 bFGF-induced the phosphorylation of ERK1/2, p38 and JNK/SAPK in HUVECs. HUVECs were incubated in M199 containing 1% FBS for 6 h and then treated with bFGF (50 ng/ml) for the indicated time. The phosphorylated forms of ERK1/2, p38 and JNK/SAPK and the total amount of ERK1/2, p38 and JNK/SAPK were visualized by immunoblotting using specific antibodies. The results represented at least three repeats.

HUVECs and PD98059 also blocked the tube formation, while SB203580 failed to do so.

Effects of a JNK/SAPK peptide inhibitor on proliferation, migration and tube formation induced by bFGF in HUVECs

In order to further confirm the role of JNK/SAPK in angiogenesis induced by bFGF, a JNK/SAPK peptide inhibitor 420116 and a negative JNK/SAPK peptide inhibitor 420118 were applied in the further experiment. The result in Fig. 4A showed that 420116 led to the efficient down-regulation of the phosphorylation of JNK/SAPK, meanwhile, both 420118 and 420116 had no directly cytotoxic effect on HUVECs by themselves. Further, 420116 could inhibit bFGF-induced HUVECs proliferation, migration and tube formation as compared with 420118, which further confirms that JNK/SAPK plays a crucial role in bFGF-induced angiogenesis.

Effects of JNK/SAPK siRNA on proliferation, migration and tube formation induced by bFGF in HUVEC

Next, JNK/SAPK-specific siRNA was futher applied to observe its effect on bFGF-induced angiogenic response *in vitro*. Our results in Fig. 5 showed that down-regulation of JNK/SAPK by specific siRNA inhibited bFGF-induced cells proliferation, migration and tube formation in HUVECs compared with HUVECs transfected with control siRNA.

The phosphorylation of JNK/SAPK upstream signals induced by bFGF in HUVECs

Last, we investigated whether bFGF (50 ng/ml) could induce the phosphorylation of SEK1/MKK4 and MKK7, which were both upstream signals of JNK/SAPK. As shown in Fig. 6, bFGF could induce the phosphorylation of SEK1/MKK4 at 2 min and of which the stimulation for 5 min reached the peak. Moreover, the phosphorylation of MKK7 induced by bFGF was at 2 min, with the continuous activation to 60 min.

Discussion

Our results firstly demonstrated that bFGF could induce cell proliferation, migration and tube formation in HUVECs. The family of fibroblast growth factors (FGFs) majorly consists of 22 structurally related polypeptides, including the two prototypes, FGF-1 (acidic FGF) and FGF-2 (basic FGF). The bFGF is a potent mitogen, which acts as a survival factor for the newly formed vessels involved in pathological angiogenesis (*23*). Many established drugs, such as thalidomide, indomethacin and spironolactone, have anti-angiogenic properties against bFGF-induced angiogenesis (*24–26*). Therefore, advance in the understanding of intracellular signalling pathways in the bFGF-mediated angiogenesis is very interesting and noticeable.

The binding of bFGF to its receptor induces dimerization and subsequent phosphorylation of the receptors through the activation of tyrosine kinase receptors, which leads to the activation of intracellular signalling molecules such as MAPKs, phosphatidylinositol 3-kinase (PI3K) and phospholipase Cγ (PLCγ) (*27*, *28*). MAPKs belong to a large family of serine/threonine kinases which are activated through signalling pathways triggered by multiple extracellular signals in mammalian cells. In general, ERK1/2 and p38 signalling pathways are essential for integrating signals that affect various cell functions. So far, previous studies have already demonstrated the crucial roles of ERK1/2 and p38 signalling pathways in bFGF-induced angiogenesis *in vitro* (*16–19*) and our results also illustrated the involvement of ERK1/2 and p38 signals in bFGF-induced angiogenesis.

The JNK/SAPK signal pathway is directly activated by diverse signals including growth factors, heat shock, osmotic shock, cytokines, protein synthesis inhibitors, antioxidants, ultraviolet radiation and DNA-damaging agents (*29*). Thus, the JNK/SAPK signalling cascade is crucial for the maintenance of cell homeostasis and controls many cellular processes, including cell growth, transformation, differentiation and apoptosis. Numerous studies have been performed to reveal a participation of JNK/SAPK in the processes of pathological angiogenesis. For instance, JNK/SAPK participates in bFGF-mediated down-regulation of surface cadherin, which may affect the cell–cell interaction between endothelial cells and then facilitate angiogenesis (*21*). Additionally, JNK/SAPK is involved in angiogenesis associated diseases through inducing the secretion of MMPs, urokinase-type plaminogen activator (u-PA) and VEGF, regulating endocytosis, nuclear translocation of VEGFR2 and MMP pathway and so forth (*30–35*). Our present results firstly demonstrated that bFGF could trigger the phosphorylation of

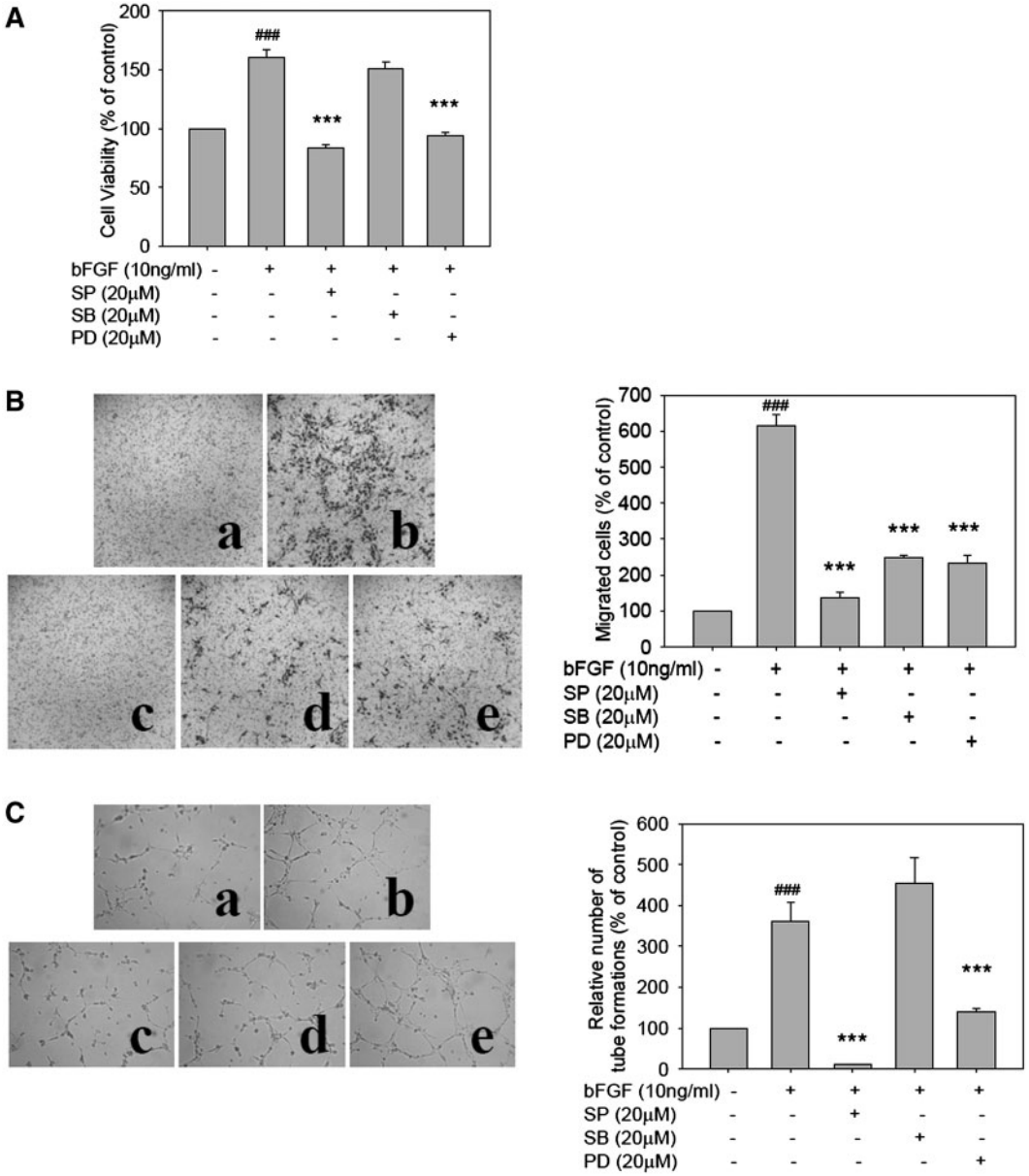

Fig. 3 Effect of MAPK-specific inhibitors (SP600125, SB203580 and PD98059) on bFGF-induced HUVECs proliferation, migration and tube formation. (A) HUVECs (2×10^4 cells/well) were incubated with M199 containing 1% FBS and pre-treated with MAPKs inhibitors (20 μM) for 15 min and then supplemented with or without bFGF (10 ng/ml) for 48 h. The survival cells were determined by MTT assay. (B) HUVECs were incubated with M199 containing 0.1% BSA for 4 h, pre-treated with MAPK inhibitors (20 μM) before placed in the topper chamber of transwells at the density of 4×10^4 cells/well and then supplemented with or without bFGF (10 ng/ml) in the bottom chamber of transwells. After 8 h of incubation, the migrated cells passed through the membrane and were quantified by manual counting the cells that crossed the membrane with an inverted microscope at ×100 magnification. (C) HUVECs were incubated with M199 containing 0.1% BSA and pre-treated for 15 min with MAPK inhibitors (20 μM) before placed in the matrigel-coated 96-well plates at the density of 1×10^4 cells/well and then supplemented with or without bFGF (10 ng/ml). After 4 h, pictures were taken under an inverted microscope at ×100 magnification. The tubular structures of HUVECs were quantified by manual counting. a: control, b: bFGF 10 ng/ml, c: SP600125 (SP) 20 μM + bFGF 10 ng/ml, d: PD98059 (PD) 20 μM + bFGF 10 ng/ml, e: SB203580 (SB) 20 μM + bFGF 10 ng/ml. Data were means ± SEM from three independent experiments. $^{###}P < 0.001$ versus control. $^{***}P < 0.001$ versus bFGF alone.

JNK/SAPK in HUVECs. Furthermore, the addition of specific inhibitors (SP600125 and 420116) and specific siRNA against JNK/SAPK diminished bFGF-induced angiogenic responses including proliferation, migration and tube formation in HUVECs (Figs 3–5). Also, the inhibition of JNK/SAPK via SP600125 and 420116 lead to the decrease of DNA synthesis in HUVECs via ^{3}H-thymidine incorporation assay (shown in Supplementary Fig. S1), which further confirmed the important role of JNK/SAPK in regulating bFGF-induced HUVECs proliferation. However, the effects of JNK inhibition by peptide and siRNA on migration and tube formation were better than on proliferation in HUVECs. We presumed JNK/SAPK is one of the numerous cellular signalling pathways involved in bFGF-induced cells proliferation and other than JNK/SAPK signalling pathways may be also involved. Two MEK family members, MKK4

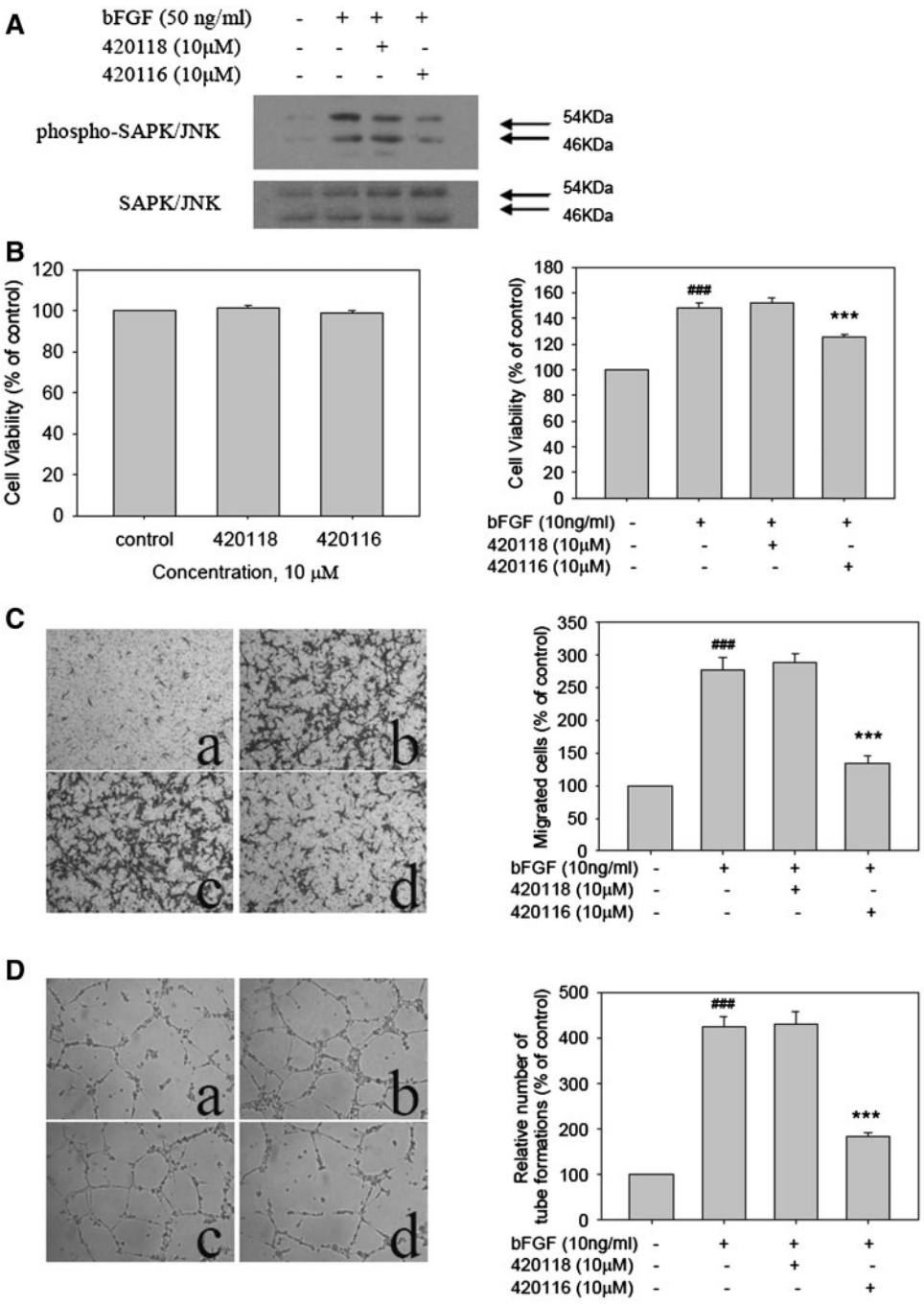

Fig. 4 Effects of a JNK/SAPK peptide inhibitor 420116 and a negative JNK/SAPK peptide inhibitor 420118 on bFGF-induced HUVECs proliferation, migration and tube formation. (A) After HUVECs treated with 420118 (10 μM) and 420116 (10 μM), the phosphorylated forms of JNK/SAPK and total amount of JNK/SAPK were detected by western blot analysis. The results represented at least three repeats. (B) HUVECs were seeded and treated with 420118 (10 μM) and 420116 (10 μM) for 48 h. The survival cells were determined by MTT assay. HUVECs (2×10^4 cells/well) were incubated with M199 containing 1% FBS and pre-treated with 420118 (10 μM) and 420116 (10 μM) for 15 min and then supplemented with or without bFGF (10 ng/ml) for 48 h. The survival cells were determined by MTT assay. (C) HUVECs were incubated with M199 containing 0.1% BSA for 4 h, pre-treated with 420118 and 420116 (10 μM) before placed in the topper chamber of transwells at the density of 4×10^4 cells/well and then supplemented with or without bFGF (10 ng/ml) in the bottom chamber of transwells. After 8 h of incubation, the migrated cells passed through the membrane and were quantified by manual counting the cells that crossed the membrane with an inverted microscope at ×100 magnification. (D) HUVECs were incubated with M199 containing 0.1% BSA and pre-treated for 15 min with 420118 (10 μM) and 420116 (10 μM) before placed in the matrigel-coated 96-well plates at the density of 1×10^4 cells/well and then supplemented with or without bFGF (10 ng/ml). After 4 h, pictures were taken under an inverted microscope at ×100 magnification. The tubular structures of HUVECs were quantified by manual counting. a: control, b: bFGF 10 ng/ml, c: 420118 10 μM + bFGF 10 ng/ml, d: 420116 10 μM + bFGF 10 ng/ml. Data were means ± SEM from three independent experiments. $^{###}P < 0.001$ versus control. $^{***}P < 0.001$ versus bFGF alone.

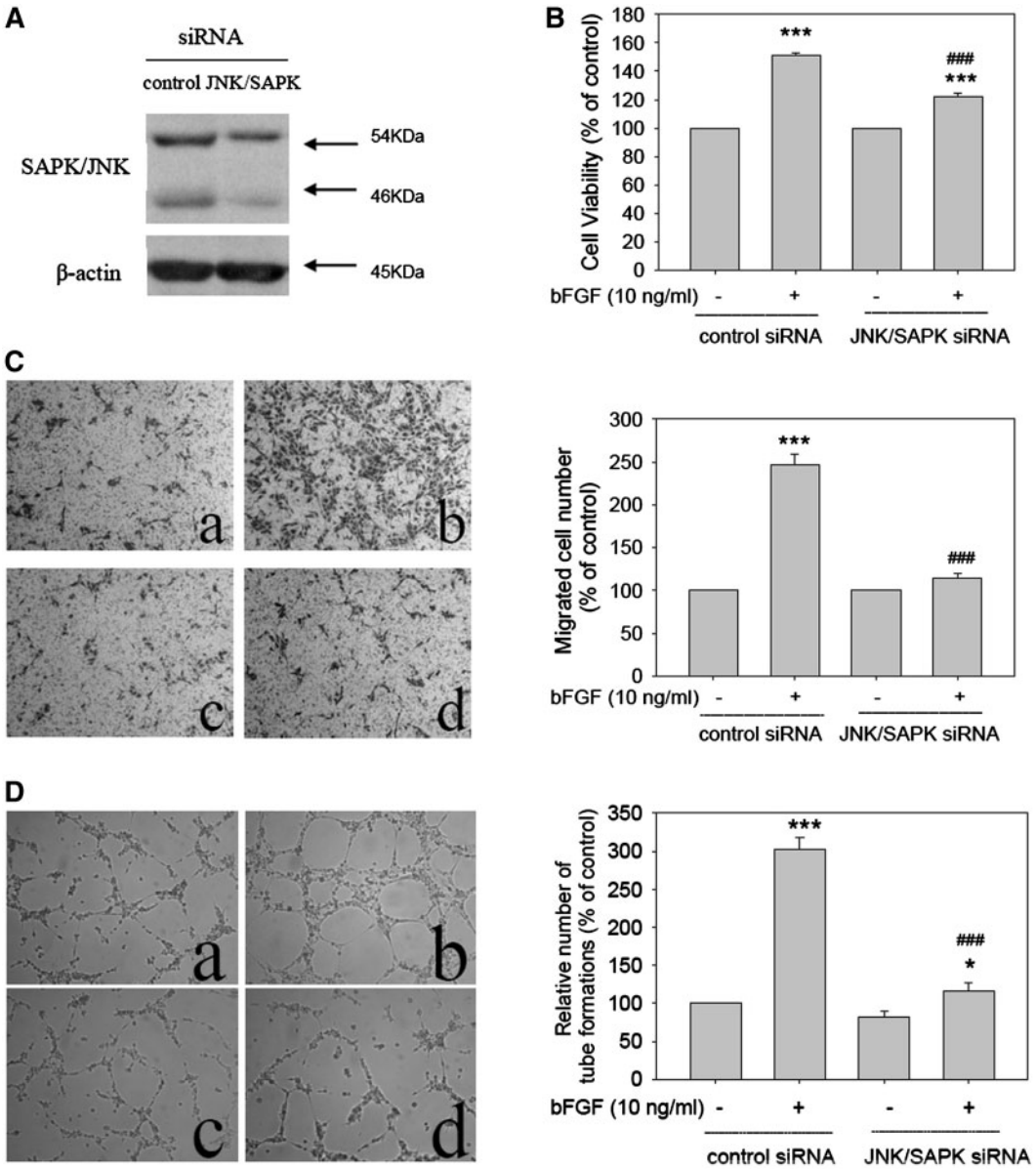

Fig. 5 Effects of JNK/SAPK siRNA on bFGF-induced cells proliferation, migration and tube formation in HUVECs. (A) HUVECs were transfected with JNK/SAPK siRNA and control siRNA. The total amount of JNK/SAPK and β-actin were detected by western blot analysis. The results represented at least three repeats. (B) HUVECs were transfected with JNK/SAPK siRNA and control siRNA, incubated with M199 containing 1% FBS at the density of 2×10^4 cells/well and supplemented with or without bFGF (10 ng/ml) for 48 h. The survival cells were determined by MTT assay. (C) HUVECs were transfected with JNK/SAPK siRNA and control siRNA, before placed in the topper chamber of transwells at the density of 4×10^4 cells/well and then supplemented with or without bFGF (10 ng/ml) in the bottom chamber of transwells. After 8 h of incubation, the migrated cells passed through the membrane and were quantified by manual counting the cells that crossed the membrane with an inverted microscope at ×100 magnification. (D) HUVECs were transfected with JNK/SAPK siRNA and control siRNA, placed in the matrigel-coated 96-well plates at the density of 1×10^4 cells/well and then supplemented with or without bFGF 10 ng/ml. After 4 h, pictures were taken under an inverted microscope at ×100 magnification. The tubular structure of HUVECs was quantified by manual counting. a: control siRNA; b: control siRNA stimulated with bFGF 10 ng/ml; c: JNK/SAPK siRNA, d; JNK/SAPK siRNA stimulated with bFGF 10 ng/ml. Data were means ± SEM from three independent experiments. $^{*}P < 0.05$; $^{***}P < 0.001$ versus control. $^{\#\#\#}P < 0.001$ versus cells transfected with control siRNA and stimulated with bFGF.

(SEK1, MEK4, JNKK1, SKK1) and MKK7 (MEK7, JNKK2, SKK4), have been implicated in JNK/SAPK pathways. JNK/SAPK is directly activated by the phosphorylation of tyrosine and threonine residues in a reaction catalysed by the dual specificity MAP kinase kinases MKK4 and MKK7 (*36*, *37*). Our results showed that SEK1/MKK4 and MKK7 were both activated by bFGF. All these results indicate that JNK/SAPK signalling pathway play an important role in directly regulating bFGF-induced angiogenic responses *in vitro*. Also, the present study and previous reports suggest a critical role of JNK/SAPK in regulating angiogenesis through complex mechanisms.

Furthermore, our results showed that SP600125 could inhibit bFGF-induced NIH3T3 cells proliferation and VEGF-induced HUVECs proliferation (shown in Supplementary Fig. S2). Those results suggest that the effect of JNK/SAPK on bFGF-induced

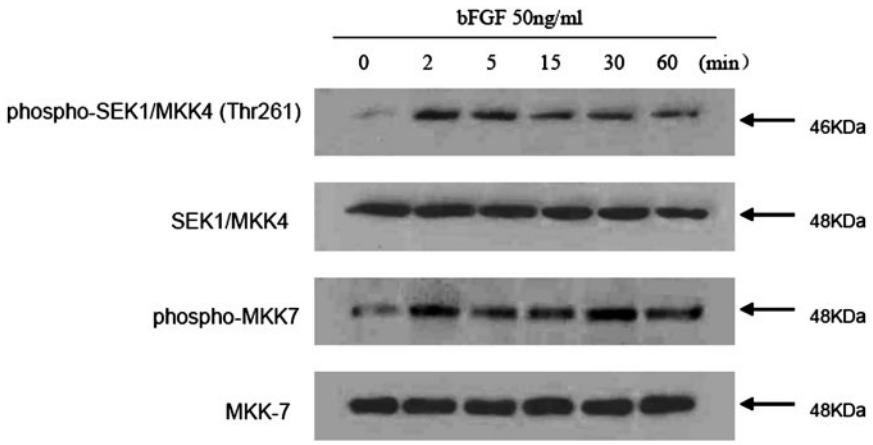

Fig. 6 Effects of bFGF on the phosphorylation of SEK1/MKK4 and MKK7 in HUVECs. HUVECs were incubated in M199 containing 1% FBS for 6 h and then treated with bFGF (50 ng/ml) for the indicated time. The phosphorylated forms of SEK1/MKK4 and MKK7 and the total amount of SEK1/MKK4 and MKK7 were visualized by immunoblotting using specific antibodies. The results represented at least three repeats.

HUVECs proliferation is not specific and JNK/SAPK may interfere with the similar signals induced by bFGF and VEGF in various cells, which needs further investigation. As FGFR and VEGFR are both tyrosine kinases, our results also suggest that JNK/SAPK may interfere with the similar signals involved in FGFR and VEGFR tyrosine kinase signal cascade.

Taken together, the findings of the current study for the first time indicate that JNK/SAPK signalling pathway directly interferes with bFGF-mediated HUVECs angiogenesis *in vitro*. This study also suggests that regulating JNK/SAPK signalling pathways may be a promising therapeutic approach for the treatment of angiogenesis associated diseases.

Supplementary Data

Supplementary Data are available at *JB* online.

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Conflict of interest

None declared.

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