

# Downregulation of Fes inhibits VEGF-A-induced chemotaxis and capillary-like morphogenesis by cultured endothelial cells

Shigeru Kanda<sup>a, c, \*</sup>, Hiroshi Kanetake<sup>b</sup>, Yasuyoshi Miyata<sup>b</sup>

<sup>a</sup>Department of Molecular Microbiology and Immunology, Division of Endothelial Cell Biology, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan

<sup>b</sup>Department of Urology, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan

<sup>c</sup>Department of Experimental and Clinical Laboratory Medicine, National Hospital Organization, Nagasaki Hospital, Nagasaki, Japan

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## Abstract

The aim of this study was to determine whether the downregulation of endogenous Fes by siRNA in cultured endothelial cells affects vascular endothelial growth factor-A (VEGF-A)-induced chemotaxis and capillary-like morphogenesis, which are considered as angiogenic cellular responses *in vitro*. VEGF-A-treatment induced autophosphorylation of Fes in cultured endothelial cells. LY294002, a phosphoinositide 3-kinase inhibitor, significantly inhibited VEGF-A-induced chemotaxis and capillary-like morphogenesis. Downregulation of Fes attenuated these VEGF-A-induced cellular responses but LY294002 did not produce further inhibition of these responses. Downregulation of Fes neither affected VEGF-A-induced autophosphorylation of VEGF receptor 2 nor mitogen-activated protein kinase activation, but markedly decreased Akt activation. Taken together, our novel results indicate the involvement of Fes in VEGF-A-induced cellular responses by cultured endothelial cells.

**Keywords:** VEGF-A • Fes • siRNA • endothelial cell • chemotaxis • capillary-like morphogenesis

## Introduction

Non-receptor protein tyrosine kinase Fes, distinct from c-Src, c-Abl and other non-receptor protein tyrosine kinases [1, 2], is exclusively expressed in hematopoietic and vascular endothelial cells [3]. Fes is activated by oligomerization and sub-

sequent autophosphorylation [4, 5]. Expression of kinase-inactive Fes (KI-Fes) in cultured endothelial cells exerts dominant negative effect on endogenous Fes [6]. Using stable cell lines expressing KI-Fes, we demonstrated previously the involvement of Fes in chemotaxis of murine brain capillary endothelial cells (IBE cells) induced by fibroblast growth factor-2 (FGF-2) and angiopoietin 2 (Ang 2) [6, 7], and tube formation or capillary-like morphogenesis (*i.e.* morphological differentiation) induced by stromal cell-derived factor 1- $\alpha$  (SDF-1 $\alpha$ ) and sonic hedgehog (Shh) [8, 9]. Ang2-, SDF-1 $\alpha$ - and Shh-induced activation of phosphoinositide-3-kinase (PI3-kinase) is

\*Correspondence to: Shigeru KANDA, M.D., Ph.D.  
Department of Experimental and Clinical Laboratory  
Medicine, National Hospital Organization, Nagasaki  
Hospital, 41-6 Sakuragi-machi,  
Nagasaki 851-0251, Japan.  
Tel.: +81 95 823 2261  
Fax: +81 95 828 2616  
E-mail: skanda-jua@umin.net

dependent on c-Fes kinase activity, whereas FGF-2 activates Src within focal adhesions in a manner dependent on Fes activity [10]. These results suggest that Fes could be a potential target for antiangiogenic therapy designed to shutdown intracellular signals mediated by multiple pro-angiogenic factors simultaneously.

Vascular endothelial growth factor (VEGF) is a family of closely related polypeptides, comprising VEGF-A, -B, -C, -D and -E, and placental growth factor [11, 12]. Their specific receptor tyrosine kinases are VEGF receptor (VEGFR)-1, -2 and -3 [13]. We have previously shown that a prototype VEGF, VEGF-A, activated Fes through VEGFR-2, but not through VEGFR-1, in porcine aortic endothelial cells [14]. In these cells, Fes contributed to VEGF-A-promoted activation of PI3-kinase. However, expression of KI-Fes failed to exhibit dominant negative effect on VEGF-A-activated PI3-kinase because other signaling molecules, such as VEGFR-2, Src and insulin-receptor substrate 1, seemed to compensate the loss of Fes kinase activity [14]. Accordingly, chemotaxis and capillary-like morphogenesis by VEGF-A-treatment were not inhibited by the expression of KI-Fes [14]. These results suggest that inhibition of endogenous Fes activity may not sufficiently suppress VEGF-A-driven angiogenesis. Since VEGF-A plays central roles in tumour angiogenesis [15], it is important to know whether targeting Fes is a suitable strategy for antiangiogenic tumour therapy. Fes has a unique amino-terminal domain containing two coiled-coil domains and a Src-homology 2 (SH2) domain, it is plausible that these domains provide a scaffold function to transduce signals in the absence of Fes tyrosine kinase activity. To address this issue, we examined the effect of the downregulation of Fes protein using small interfering RNA (siRNA) on VEGF-A-promoted cellular responses by endothelial cells.

## Materials and methods

### Materials

Recombinant human VEGF-A (165 amino acids) was obtained from PeproTech (London, UK). Type I collagen gel was purchased from Cohesion Technologies Inc. (Palo Alto, CA, USA). Growth factor-reduced Matrigel<sup>®</sup> matrix was purchased from BD Bioscience (Bedford, MA, USA). Anti-Fes

antibody (N-16), anti-VEGFR-2 antibody, anti-Akt monoclonal antibody, anti-ERK1 antibody and anti-phosphotyrosine antibody (PY99) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-phospho-mitogen-activated protein kinase (MAPK) polyclonal antibody and anti-phospho Akt 1 (pS473) were obtained from Cell Signaling Technology, Inc., (Beverly, MA, USA). HiPerFect<sup>®</sup> transfect reagent, negative control siRNA and human Fes siRNA (Hs\_FES\_6\_HP Validated siRNA) were purchased from Qiagen (Tokyo, Japan). The PI3-kinase inhibitor LY294002 was obtained from Merck Company (Tokyo). LY294002 was dissolved in dimethyl sulfoxide (DMSO) and the final concentration of DMSO in the culture medium was always 0.1% (Vo/Vo). As a vehicle, 0.1% DMSO was added to the cells without treatment with LY294002.

### Cell culture

Human umbilical vein endothelial cells (HUVECs) and their culture medium were obtained from Cambrex (Walkersville, MD, USA). The cells were cultured in endothelial cell basal medium-2 (EBM-2) supplemented with 2% foetal bovine serum, 10 ng/ml VEGF-A, 20 ng/ml FGF-2, 10 ng/ml endothelial growth factor, 10 ng/ml insulin-like growth factor-I, 50 mg/ml ascorbic acid, 100 ng/ml heparin and 10 pM dexamethasone.

### Preparation of siRNA-treated HUVECs

HUVECs were transfected with siRNA using HiPerFect<sup>®</sup> reagent as described previously [16]. In brief, either negative control or Fes siRNA was transfected into HUVECs. After 2 or 3 days, the cells were used for the chemotaxis assay or the capillary morphogenesis assay. Alternatively, the cells were serum and growth factor-starved for 2 hrs and then either stimulated or unstimulated with VEGF-A, lysed and used for immunoblotting.

### Chemotaxis assay

Chemotaxis assay was performed as described previously [17]. Briefly, the membranes of Transwell membrane filters (Corning Coster Japan, Tokyo) were coated with type I collagen gels. Cells suspended in EBM-2 containing 0.5% FBS were seeded onto the upper well of Transwell inserts, and test samples were added to the medium in the lower well. Four hours later, cells that migrated onto the lower surface of the membranes were counted under microscopy.

### Capillary-like morphogenesis assay

HUVECs suspended in EBM-2 medium containing 0.5% FBS with or without the indicated treatments were seeded

onto growth factor-reduced Matrigel and cultured for up to 24 hrs [9]. To quantify the length of capillary-like structures, three different phase-contrast photomicrographs ( $\times 4$  objectives) per well were taken, and the length of each capillary-like structure was measured using NIH image software (version 1.64). The length of capillary-like structures of control siRNA-treated HUVECs cultured in the presence of 100 ng/ml of VEGF-A was set to 1.0.

## Immunoprecipitation and immunoblotting

Immunoprecipitation and immunoblotting were performed as described previously [16]. The indicated proteins were immunoprecipitated with specific antibodies and separated onto SDS-PAGE. For examination of loaded protein for Fes downregulation, 10% of the total cell lysate was separated onto SDS-PAGE. To detect activation of mitogen-activated protein kinase (MAPK), total cell extracts prepared by the direct addition of boiled SDS-sample buffer to the cells were used. After transfer of the proteins onto polyvinylidene difluoride membranes, the blots were probed with the indicated primary antibodies followed by detection through enhanced chemiluminescence reaction and exposure to X-ray film. Stripping was conducted between the two probing.

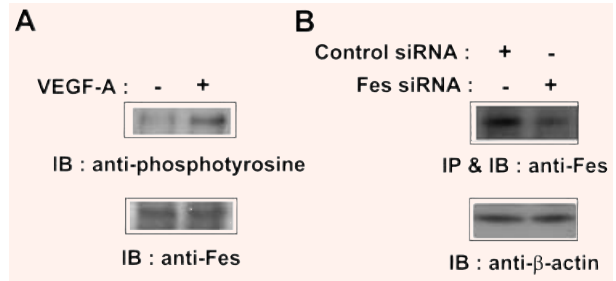
## Statistical analysis

Values are presented as mean capillary length  $\pm$  SD. Differences between two groups were determined by Mann–Whitney’s U-test. Differences were considered significant when the *P*-value was less than 0.05.

## Results

### Downregulation of Fes protein attenuates VEGF-A-induced chemotaxis and capillary-like morphogenesis by HUVECs

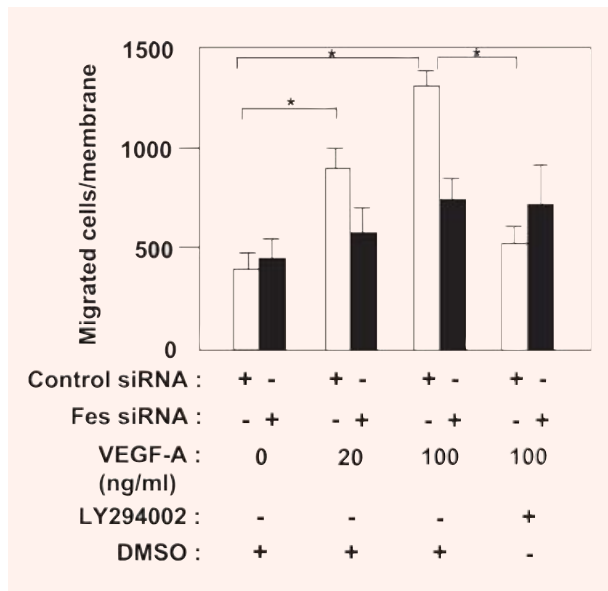
We first examined whether VEGF-A activates endogenous Fes in HUVECs. As shown in Figure 1A, VEGF-A-treatment induced autophosphorylation of Fes in HUVECs, suggesting that Fes acts as a signaling molecule in VEGF-A-treated HUVECs. We then examined the effect of Fes siRNA on Fes protein expression in HUVECs. As shown in Figure 1B, treat-



**Fig. 1. (A)** VEGF-A induces autophosphorylation of Fes in HUVECs. HUVECs grown in 15-cm dishes were serum- and growth factor-starved for 2 hrs. Orthovanadate at 100  $\mu$ M was added to the cells and 10 min later, the cells were either stimulated or left unstimulated with 200 ng/ml of VEGF-A for 15 min. Cells were washed, lysed, and Fes was immunoprecipitated. Autophosphorylation of Fes was examined by immunoblotting. **(B)** Downregulation of Fes by siRNA. HUVECs grown in 10 cm dishes were transfected with either control siRNA or Fes siRNA at 20 nM and three days later, Fes was immunoprecipitated from 95% of lysate, separated by SDS-PAGE, and Fes protein was visualized by immunoblotting. The remaining 5% of lysate was examined for  $\beta$ -actin expression by immunoblotting as a loading control. Similar results were obtained in two independent experiments.

ment of HUVECs with Fes siRNA downregulated the expression of Fes protein by up to 78%.

Next, we examined the effect of downregulation of Fes protein on chemotaxis and capillary-like morphogenesis by HUVECs. As shown in Figure 2, PI3-kinase inhibitor LY294002 significantly inhibited the chemotaxis of control siRNA-treated HUVECs toward VEGF-A. In cells treated with Fes siRNA, chemotaxis toward VEGF-A was attenuated. Treatment with VEGF-A did not significantly increase the process and such cells showed a similar degree of chemotaxis even in the presence of LY294002, suggesting that downregulation of Fes may effect VEGF-A-induced activation of PI3-kinase. VEGF-A-treated capillary-like morphogenesis was also impaired by treatment of HUVECs with Fes siRNA (Fig. 3). LY294002 inhibited VEGF-A-induced capillary-like morphogenesis by HUVECs treated with control siRNA, but not with Fes siRNA. Another human Fes siRNA (Hs\_FES\_5\_HP Validated siRNA from Qiagen) also downregulated Fes in HUVECs and similar effects on VEGF-A-induced chemotaxis and capillary-like morphogenesis were observed (data not shown).



**Fig. 2.** Treatment of HUVECs by Fes siRNA inhibits chemotaxis toward VEGF-A. Culture media containing VEGF-A at indicated concentrations, with or without 5  $\mu$ M LY294002, were placed in the lower wells of Transwell culture plates. HUVECs were pretreated with either 20 nM control siRNA or Fes siRNA for 66 hrs, suspended in EBM-2 supplemented with 0.5% FBS with or without LY294002 at 5  $\mu$ M, and seeded into the upper wells. The number of HUVECs that migrated onto the lower surface of Transwell inserts was counted. Data are mean numbers  $\pm$  SD for quadruplicate wells. The results were reproduced in two independent experiments.

### Downregulation of Fes affects VEGF-A-mediated activation of Akt

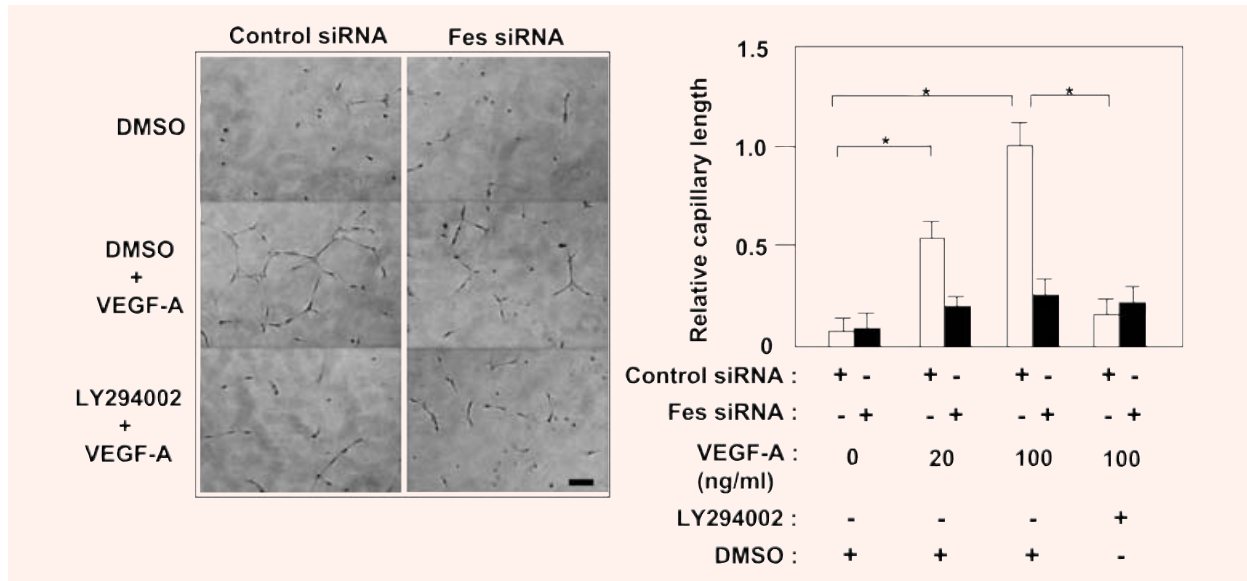
We first examined whether treatment with Fes siRNA attenuates VEGF-A-induced activation of VEGFR-2. As shown in Figure 4A, treatment with Fes siRNA did not decrease the autophosphorylation of VEGFR-2. We also examined VEGF-A-induced MAPK activation. In both control siRNA- and Fes siRNA-treated cells, VEGF-A treatment produced comparable activation of MAPK. Since Akt is an important downstream effector of PI3-kinase, we then examined the effect of Fes downregulation on VEGF-A-induced activation of Akt and found that Akt activation was impaired in Fes siRNA-treated cells. This observation suggests that downregulation of Fes seems to affect VEGF-A-induced activation of PI3-kinase/Akt pathway.

### Discussion

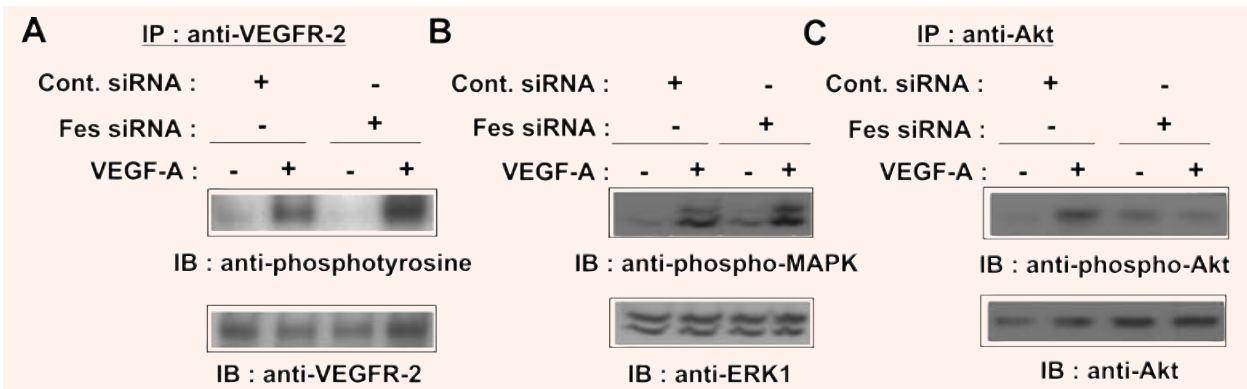
In the present study, we showed for the first time that downregulation of Fes in cultured endothelial cells attenuated VEGF-A-induced Akt activation with subsequent inhibition of chemotaxis and capillary-like morphogenesis. These findings suggest that downregulation of Fes protein may potentially inhibit VEGF-A-dependent tumour angiogenesis.

Expression of Fes protein was downregulated by the treatment with Fes siRNA in HUVECs (Fig. 1 B). VEGF-A-induced chemotaxis and capillary-like morphogenesis by negative control siRNA-treated HUVECs were significantly inhibited by the treatment with LY294002. However, LY294002-treatment failed to inhibit VEGF-A-induced chemotaxis and capillary-like morphogenesis by Fes siRNA-treated HUVECs. These results suggest that VEGF-A-induced chemotaxis and capillary-like morphogenesis is at least partly regulated through PI3-kinase and Fes may be involved in the activation of this kinase. Indeed, downregulation of Fes neither inhibited VEGF-A-promoted autophosphorylation of VEGFR-2 nor activation of MAPK, but attenuated Akt phosphorylation. Thus, Fes plays significant roles in VEGF-A-promoted activation of PI3-kinase/Akt pathway and subsequent particular cellular responses, such as chemotaxis and capillary-like morphogenesis (Fig. 5).

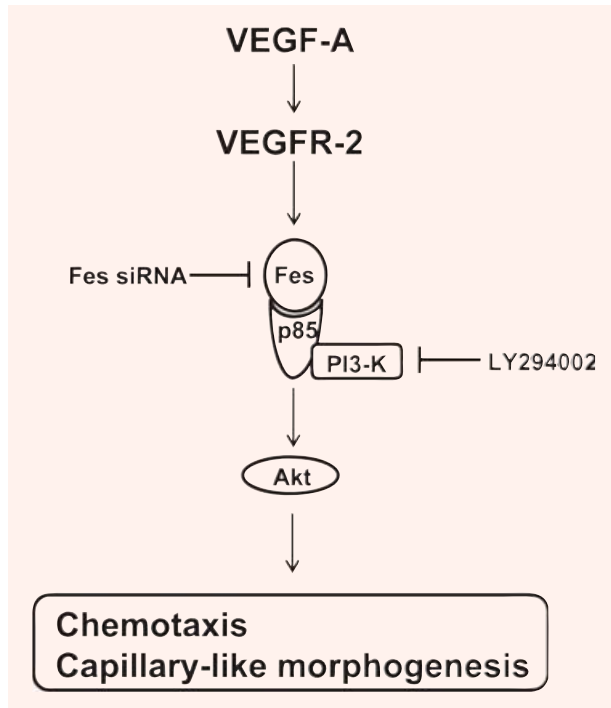
Tumour angiogenesis is not solely regulated by VEGF-A. In a preclinical model, chronic inhibition of VEGF-A signaling altered the characteristics of tumour growth from VEGF-A-dependent angiogenesis to FGF-2-dependent angiogenesis [18]. Hence, it would be clinically valuable to block the intracellular signaling molecules that commonly act downstream of a panel of pro-angiogenic factor receptors, because this strategy may shutdown the signals *via* multiple receptors simultaneously. Fes is involved in FGF-2- and Ang2-induced chemotaxis, and SDF-1 $\alpha$ - and Shh-induced morphological differentiation [6–10]. These data were obtained by the use of stable endothelial cell lines expressing KI-Fes, which exerts a dominant negative effect on endogenous Fes. Therefore, inhibition of Fes tyrosine kinase activity could be a potentially useful strategy against angiogenesis in cancer therapy. This inhibition may also be achieved by the use of small-molecular-weight synthetic protein kinase inhibitors, such as Gleevec. However, our previous study showed that expression of KI-Fes failed to exert dominant negative



**Fig. 3.** Treatment of HUVECs by Fes siRNA inhibits VEGF-A-induced capillary morphogenesis. HUVECs pretreated with either 20 nM control siRNA or Fes siRNA for 40 hrs were cultured on the surface of growth factor-reduced Matrigel in the presence or absence of VEGF-A at indicated concentrations with or without 5 μM LY294002 for 24 hrs. Photographs were taken under a phase-contrast microscope. Capillary length of control siRNA-treated cells cultured in the presence of 100 ng/ml of VEGF-A was set to 1.0. Values are expressed as mean ± SD of three images. Bar, 50 μm. Reproducible results were obtained from two independent experiments.



**Fig. 4.** (A) Treatment of HUVECs by Fes siRNA does not affect VEGF-A-induced autophosphorylation of VEGFR-2. HUVECs treated with either control siRNA or Fes siRNA at 20 nM for 66 hrs were serum-starved for 2 hrs and either stimulated or left unstimulated with 200 ng/ml of VEGF-A for 10 min. Autophosphorylation of VEGFR-2 was examined by immunoprecipitation, followed by immunoblotting. (B) Treatment of HUVECs by Fes siRNA does not affect VEGF-A-induced activation of MAPK. HUVECs treated with either control siRNA or Fes siRNA were serum-starved for 2 hrs and either stimulated or left unstimulated with 200 ng/ml of VEGF-A for 10 min. Activated MAPK was detected by immunoblotting. (C) Treatment of HUVECs by Fes siRNA attenuates VEGF-A-induced activation of Akt. HUVECs treated with either control siRNA or Fes siRNA were serum-starved for 2 hrs and either stimulated or left unstimulated with 200 ng/ml of VEGF-A for 10 min. Activated Akt was detected by immunoprecipitation, followed by immunoblotting. Reproducible results were obtained from two independent experiments.



**Fig. 5.** Proposed signal transduction pathway leading to VEGF-A-induced chemotaxis and capillary-like morphogenesis by cultured endothelial cells. VEGF-A activates Fes through VEGFR-2 [14]. Treatment of endothelial cells with Fes siRNA or LY294002 attenuates VEGF-A-promoted Akt activation, resulting in the decreased chemotaxis or capillary-like morphogenesis.

effect on VEGF-A-induced chemotaxis and capillary-like morphogenesis [14]. This might be due to the compensatory mechanisms underlying multiple pathways other than Fes, such as Src and VEGFR-2 [14], to activate the PI3-kinase/Akt pathway driven by VEGF-A. PI3-kinase/Akt pathway regulates survival, motility and nitric oxide synthesis by endothelial cells [19]. Since Fes has several domains involved in the intermolecular association, such as SH2 domain, these domains could function as a scaffold to activate PI3-kinase independent of Fes kinase activity. SH2 domain of Fes binds to an autophosphorylation site in Fes, Y713 [20]. When Fes kinase is not activated or kinase activity is inhibited, its SH2 domain cannot associate with the autophosphorylation site. Unbound SH2 domain can bind to an adapter protein, Cas [21] and Cas binds to focal adhesion kinase [22], which could be associated with PI3-kinase [23]. Therefore, SH2 domain of Fes may be involved in the

activation of PI3-kinase independent of Fes activity. Also, Fes has a consensus SH2 binding motif for the p85 subunit of PI3-kinase (pYXXM) [1]. This motif includes Y633, which is not identified as an autophosphorylation site. It is however plausible that this tyrosine residue may be phosphorylated by other tyrosine kinases, which in turn creates a binding site for PI3-kinase. Because activation of class I PI3-kinases involves the binding to tyrosine phosphorylated proteins [24], these events may activate PI3-kinase independent of Fes kinase activity.

VEGF-A is a major pro-angiogenic factor in a variety of angiogenesis-dependent diseases. Thus, inhibition of VEGF-A-mediated signaling must be included in any antiangiogenic strategy. In the present study, we downregulated the expression of Fes protein in endothelial cells. Consequently, VEGF-A-induced chemotaxis and capillary-like morphogenesis were inhibited through impaired activation of Akt, suggesting that this strategy may affect the compensatory mechanism responsible for the activation of PI3-kinase by VEGF-A. Because small-molecular-weight kinase inhibitors that inhibit Fes activity are not available at present, downregulation of Fes in tumour tissue would be a favourable option in any novel antiangiogenic strategy.

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