

# Nuclear Translocation of Phosphorylated STAT3 Is Essential for Vascular Endothelial Growth Factor-induced Human Dermal Microvascular Endothelial Cell Migration and Tube Formation\*

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**Vascular endothelial growth factor (VEGF) is a potent, multifunctional, endothelial-cell-specific growth factor. It stimulates proliferation and migration of endothelial cells. Characterization of intracellular signal transduction after VEGF and VEGF receptor (VEGFR) interaction has demonstrated the involvement of the mitogen-activated protein kinase pathway. However, several studies indicated that signal transducers and activators of transcription (STAT) is another important pathway downstream of VEGF/VEGFR interaction. Therefore, we studied the role of STAT3 in the migration and tube formation of the human dermal microvascular endothelial cells (HDMEC). HDMEC expressed phosphorylated forms of STAT1, STAT3, and STAT5, and a marked increase of phosphorylated STAT3 in the nuclear fraction after addition of VEGF was observed by Western blot and immunohistochemical staining. To verify the functional implication of STAT3 phosphorylation in HDMEC migration, we introduced a dominant-negative STAT3 using adenovirus vector system. Dominant-negative STAT3 abolished the VEGF-induced nuclear translocation of phosphorylated STAT3 and inhibited HDMEC migration completely. Dominant-negative STAT3 also suppressed VEGF-induced HDMEC tube formation on Matrigel and on collagen gel. These data demonstrate that STAT3 and its phosphorylation are involved in the downstream pathway of VEGF/VEGFR interaction and regulate VEGF-induced HDMEC migration and tube formation.**

Vascular endothelial growth factor (VEGF)<sup>1</sup> is a member of the platelet-derived growth factor superfamily and an endothelial-cell-specific growth factor. It stimulates vasodilation and cell proliferation, increases permeability and migration, and

promotes endothelial cell survival (1–4). VEGF plays an important role as a regulator of blood-vessel growth and development (5–14). VEGF exerts its effects by interacting with two high affinity membrane receptors, VEGF receptor (VEGFR) 1 (Flt-1) (15, 16) and VEGFR2 (Flk-1/KDR) (17, 18). VEGFR1 and VEGFR2 are tyrosine kinase receptors that trans-phosphorylate and, in turn, phosphorylate on specific tyrosine residues of SH2 domain-containing signaling molecules (19, 20). VEGFR1 undergoes weak ligand-dependent tyrosine phosphorylation, whereas VEGFR2 has a strong response. This difference in signal transduction properties corresponds to diverse functions of VEGF (16, 19). Namely, VEGFR1 mediates cell migration and differentiation, whereas VEGFR2 mediates cell proliferation and survival (7, 8, 16, 21, 22).

It has been suggested that the major signaling pathway downstream of VEGF/VEGFR is the serine/threonine kinase mitogen-activated protein kinase (20). However, an involvement of signal transducers and activators of transcription (STAT) proteins in the VEGF signaling pathway has also been reported (23). STAT proteins are activated in response to a number of cytokines, growth factors, and hormones (24–26). After the binding of ligands to their receptors, STAT proteins are activated, dimerize, translocate to the nucleus, and bind to specific target gene promoters. So far, seven STAT proteins (STAT1, -2, -3, -4, -5A, -5B, and -6) have been identified.

Induction of endothelial cell migration is one of the major biological functions of VEGF. Interestingly, a recent report showed that STAT3 plays crucial roles in the migration of keratinocytes, the hair cycle, and wound healing, using keratinocyte-specific STAT3 knockout mice (27). Therefore, we hypothesized that VEGF-induced endothelial cell migration involves the STAT3 signaling pathway. To test this hypothesis, we treated cultured human dermal microvascular endothelial cells (HDMEC) with recombinant VEGF and studied the role of STAT3 on HDMEC migration and tube formation using an adenovirus vector carrying a dominant negative STAT3. We show here the first evidence that STAT3 and its phosphorylation regulates VEGF-induced HDMEC migration and tube formation.

## EXPERIMENTAL PROCEDURES

**Cell Culture**—Human dermal microvascular endothelial cells (HDMEC) were isolated from human foreskin by modification of previously published methods (28). HDMEC were grown in medium 199 (Invitrogen, Tokyo, Japan) with 10% fetal calf serum (FCS), 10 ng/ml of recombinant human basic fibroblast growth factor (a generous gift from Kaken Pharmaceutical Inc., Tokyo, Japan), and kanamycin. HDMEC show typical cobblestone morphology of confluent monolayers in the absence of contaminating fibroblasts. Cells at the sixth passage were used for all experiments. These cells were pre-incubated in endothelial

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<sup>1</sup> The abbreviations used are: VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; STAT, signal transducers and activators of transcription; HDMEC, human dermal microvascular endothelial cell; FCS, fetal calf serum; EBM, endothelial basal medium; m.o.i., multiplicity of infection; JAK, Janus kinase; Ad, adenovirus vector; GFR, growth factor reduced.

basal medium (EBM) (Clonetics, San Diego, CA) with 2% FCS, hydrocortisone (1  $\mu\text{g/ml}$ ), and cAMP (25  $\mu\text{g/ml}$ ) for 24 h before each experiment.

**Proliferation Assay**—HDMEC ( $5 \times 10^3$  cells/well) were plated on 96-well tissue culture plates in EBM with 2% FCS and incubated at 37 °C for 4 h to allow the cells to adhere. Various concentrations of recombinant human VEGF (R&D Systems, Minneapolis, MN) were added to the wells. After 72 h, HDMEC mitogenesis was determined using a non-radioactive cell proliferation assay system consisting of tetrazolium and phenazine methosulfate. The  $A_{590}$  was measured spectrophotometrically using a plate reader (Eppendorf). Experiments were performed in triplicate.

**Immunoprecipitations and Western Blot Analysis**—Subconfluent HDMEC were treated with VEGF for 5 to 60 min. The cells were washed with cold PBS (–), and whole cell lysate was harvested on ice with a cell scraper in 400  $\mu\text{l}$  of lysis buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 120 mM NaCl, 10% glycerol, and 0.5% Nonidet P-40) containing proteinase inhibitors (proteinase inhibitor mixture; Sigma Chemical Co.). For immunoprecipitation, equivalent amount of lysate protein were incubated with precipitating antibodies bound to protein G-Sepharose beads (Amersham Biosciences) for 45 min at 4 °C. Immunoprecipitates were washed three times with the lysis buffer. After centrifugation, the whole cell lysates and immunoprecipitates were separated by 7.5% SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). The membrane was blocked and soaked with a 1:1000 dilution of primary antibody. The membrane was then washed and incubated with 1:2500 fluorescein-labeled goat anti-mouse IgG (Amersham Biosciences) for 1 h. The signal was amplified with an anti-fluorescein alkaline phosphatase conjugate followed by the addition of the fluorescent substrate Attophos (Amersham Biosciences). The membrane was scanned with a FluoroImager (Amersham Biosciences). The antibodies used in this study were anti-STAT1, anti-STAT2, anti-STAT3, anti-STAT4, anti-STAT5, anti-STAT6 (BD Transduction Laboratories, Lexington, KY), anti-phospho-STAT1 (New England Biolabs, Beverly, MA), anti-phospho-STAT3, anti-phospho-STAT5 (Cell Signaling, Beverly, MA), anti-phospho-JAK1 (BIOSOURCE International, Camarillo, CA), anti-phospho-JAK2 (BIOSOURCE International), and anti-phospho-Tyk2 (New England Biolabs).

**Migration Assay**—Migration was evaluated using a modified Boyden chamber assay described previously (29). Nucleopore polyvinylpyrrolidone-free polycarbonate membranes (8  $\mu\text{m}$ ; Neuro Probe, Inc., Gaithersburg, MD) were coated with type I collagen (Nitta Gelatin, Tokyo, Japan) for 30 min at room temperature and allowed to air dry. The filter was placed over a 48-well chamber containing various concentrations of VEGF in EBM with 2% FCS, hydrocortisone (1  $\mu\text{g/ml}$ ), and cAMP (25  $\mu\text{g/ml}$ ). After trypsinization,  $1 \times 10^4$  cells in 50  $\mu\text{l}$  of serum-free EBM were added to the wells in the upper chamber. The chamber was then placed in a humidified incubator at 37 °C for 7 h. Next, the upper surface of the filter was scraped to remove non-migratory cells. The filter was subsequently fixed in 10% buffered formalin for 30 min, washed with PBS, and stained with hematoxylin and eosin. The total number of cells per well was counted by microscopy.

**Collagen Endothelial Cell Tube Formation Assays**—Collagen gel was prepared according to a previous report (30). Seven volumes of type I collagen (3.1 mg/ml Vitrogen 100; Cohesion, Palo Alto, CA) were mixed with 1 volume of  $10\times$  medium 199, and 1 volume of 0.05 N NaOH, 200 mM HEPES, and 260 mM  $\text{NaHCO}_3$  on ice. The pH was adjusted to neutrality and 1 volume of distilled water was added. The mixture was quickly added to a 6-well plate and allowed to gel for 30 min at 37 °C. Collagen gels were washed twice with PBS (–). HDMEC were seeded on the surface of the collagen gel in medium 199 containing 10% FCS. When the cells reached confluence, the medium was replaced by EBM containing 2% FCS. After 24 h, the medium was changed with or without VEGF (33 ng/ml); after 48 h, tube formation was observed by phase contrast microscopy and recorded with a digital camera (Nikon E950, Tokyo, Japan).

**Matrigel Endothelial Cell Tube Formation Assays**—GFR Matrigel (BD Biosciences Discovery Labware, Bedford, MA) prepared from the Engelbreth-Holm-Swarm tumor was added to 24-well plates and allowed to gel for 30 min at 37 °C. HDMEC were seeded at a density of  $4 \times 10^4$  cells/well in 1 ml of EBM containing 2% FCS. After 24 h, tube formation was assessed as described above, and total tube length was calculated by Image-Pro Plus software (Media Cybernetics).

**Adenovirus Vector Construction and Infection**—The cosmid cassette pAxCAw, control Ad Ax1w, and the parent virus Ad5-dlX were all kind gifts from Dr. Izumu Saito (Tokyo University, Japan) (31). A fragment of STAT3F, a dominant-negative mutant with a phenylalanine substituted for the tyrosine phosphorylation site, was subcloned into

pAxCAw. Ad containing the CA promoter and STAT3F (AxSTAT3F) was generated by the COS-TPC method (31). The cosmid DNA was mixed with the *Eco*T22I-digested DNA-terminal protein complex of Ad5-dlX, and used to cotransfect 293 cells. Recombinant viruses were generated through homologous recombination in these cells. Virus stocks were prepared by a standard procedure (31). Concentrated, purified virus stocks were prepared by the CsCl gradient method, and the virus titer was checked with a plaque-formation assay. HDMEC were infected with adenovirus vectors at a multiplicity of infection (m.o.i.) of 15.

**X-Gal Staining**—HDMEC were infected with AdLacZ at m.o.i. 15. After 48 h, X-gal staining was performed by  $\beta$ -galactosidase staining kit (Active Motif, Carlsbad, CA) according to the manufacturer's instructions.

**Statistical Analysis**—The results are representative of three independent experiments. *p* values were calculated by a two-sided Student's *t* test (NS, not significant; \*, *p* < 0.05).

## RESULTS

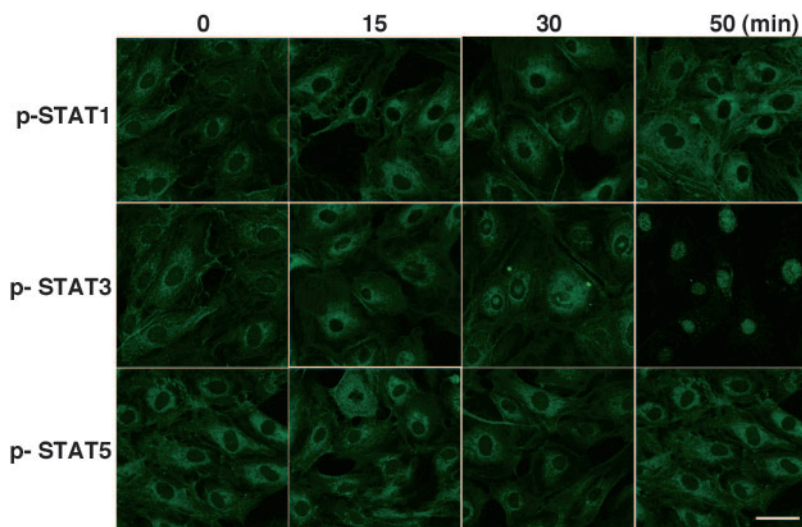
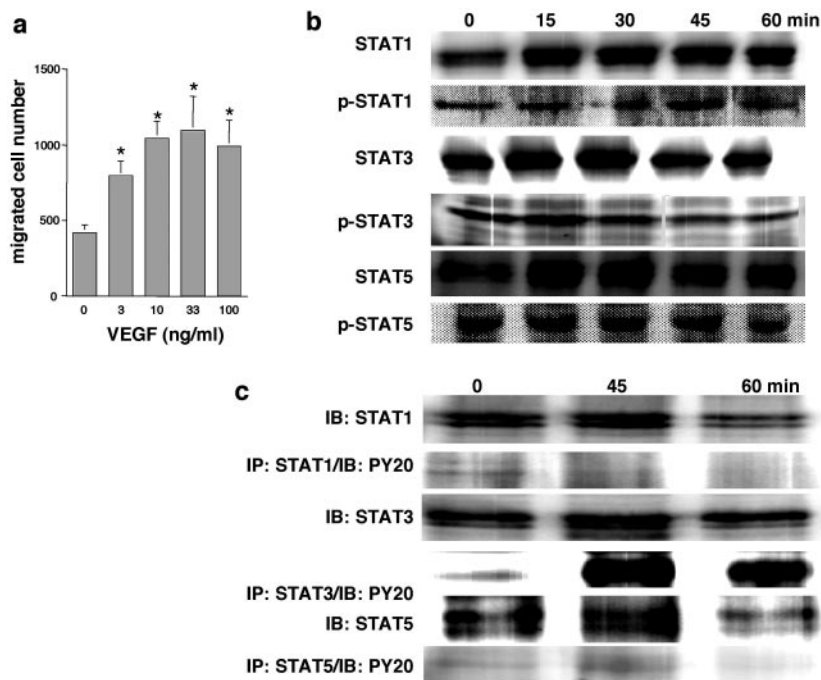
**VEGF-induced Endothelial Cell Motility**—HDMEC were seeded on type I collagen-coated filters, and then various concentrations of VEGF were added to the medium. After 7 h, VEGF enhanced HDMEC migration in a dose-dependent manner (Fig. 1a). At 3 ng/ml, VEGF enhanced HDMEC migration 2-fold, and at 33 ng/ml, migration was enhanced 2.7-fold compared with control. At a higher concentration of 100 ng/ml, HDMEC migration reached a plateau. Therefore, we used VEGF at 33 ng/ml to stimulate HDMEC in the following experiment.

**Expression Profile of STAT in HDMEC and Translocation of Phosphorylated STAT3 into the Nucleus by VEGF**—Because STAT protein expression differs depending on cell types, we investigated STAT protein expression in HDMEC. Western blot analysis showed that HDMEC expressed STAT1, STAT3, and STAT5, but not STAT2, STAT4, or STAT6 (Fig. 1b). Next, we examined whether STAT1, -3, and -5 are phosphorylated in response to stimulation with VEGF. Although phosphorylated STAT1, -3, and -5 were detected in HDMEC, their levels of phosphorylation were not altered by VEGF treatment of HDMEC (Fig. 1b). Because phosphorylated STAT proteins translocate into the nucleus, we examined the expression of phosphorylated STAT1, STAT3, and STAT5 in the nuclear fraction. Phosphorylated STAT3 was increased remarkably in the nuclear fraction at 45 and 60 min after addition of VEGF (Fig. 1c). In contrast, VEGF did not affect the localization of phosphorylated STAT1 or -5 in HDMEC (Fig. 1c). To further confirm nuclear translocation of phosphorylated STAT3, we investigated the localization of phosphorylated STAT1, -3, and -5 in HDMEC immunohistochemically by confocal microscopy. Phosphorylated STAT3 was detected in the cytoplasm, but not in the nucleus of unstimulated HDMEC. However, phosphorylated STAT3 was detected predominantly in the perinuclear region or within the nuclear compartment 30 min after addition of VEGF. After 50 min, phosphorylated STAT3 was found mainly in the nuclear region (Fig. 2). Taken together, VEGF induced the translocation of phosphorylated STAT3 from the cytoplasm to the nucleus, although VEGF did not alter the total amount of phosphorylated STAT3 in HDMEC.

Because it has been reported that the phosphorylation of STAT is regulated by two pathways, the Janus kinase (JAK) and intrinsic tyrosine kinase of growth factor receptors (23), we examined whether HDMEC express JAK1, JAK2, and Tyk2. Western blot showed that HDMEC express JAK1, JAK2, and Tyk2; however, none of them were phosphorylated after addition of VEGF (data not shown).

**Inhibition of VEGF-induced Migration and Nuclear Translocation of STAT3 by a Dominant-negative Mutant of STAT3**—Because STAT3 deficiency is lethal in mice during early embryogenesis (32), it is actually impossible to analyze the role of STAT3 about skin angiogenesis using mouse model. In this

**FIG. 1. VEGF-induced migration and STAT expression in HDMEC.** *a*, HDMEC seeded on type-I collagen-coated filter, migrated in a dose-dependent manner after the addition of VEGF. HDMEC migration was increased 2-fold by 3 ng/ml VEGF. VEGF optimally induced HDMEC migration 3-fold over control at 33 ng/ml. *b*, Western blot analysis show that phosphorylated STAT1, -3, and -5 are not altered after VEGF stimulation in whole cell lysates. IB, immunoblot; IP, immunoprecipitation. *c*, only phosphorylated STAT3 is dramatically induced in nuclear fraction. p-STAT, phosphorylated STAT; PY20, phosphotyrosine. \*, significantly different ( $p < 0.05$ ) from control.

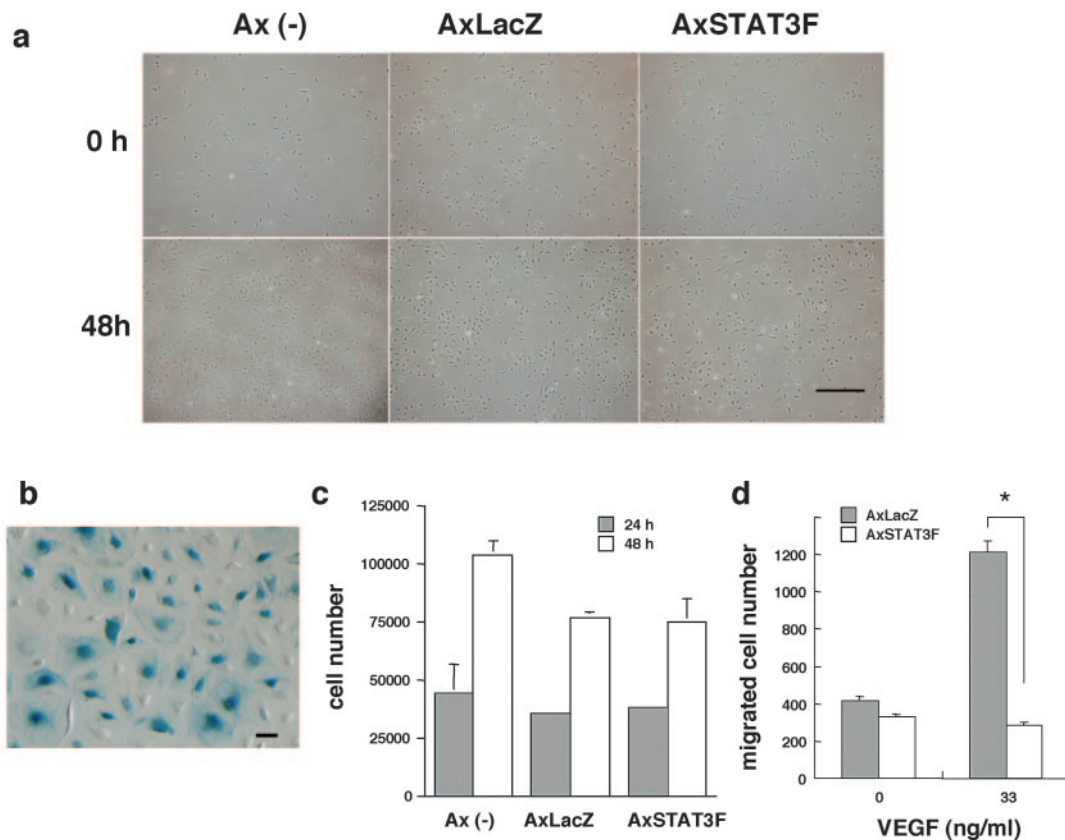


**FIG. 2. Translocation of phosphorylated STAT3.** STAT3 immunoreactivity was detectable only in the cytoplasm in unstimulated HDMEC. After 15 to 30 min of VEGF stimulation, phosphorylated STAT3 was detected predominantly at the perinuclear region or within the nuclear compartment. After 50 min, phosphorylated STAT3 was detected only in the perinuclear region. Scale bar, 5  $\mu$ m.

study, we used an adenovirus vector containing a mutant STAT3 that has a dominant-negative effect. Adenovirus vector has an advantage over other methods of transfection for endothelial cells. Transfection efficiency of adenovirus vector for endothelial cell is almost 100%. To assess the requirement for STAT3 phosphorylation in VEGF-induced endothelial cell migration directly, we exploited the effect of a dominant-negative mutant of STAT3 named STAT3F. In this mutant, the tyrosine residue at amino acid position 705 is mutated to phenylalanine. This results in the blockade of Tyr705 phosphorylation of STAT3, which is required for dimerization and nuclear translocation (33). Ad expressing  $\beta$ -galactosidase (AxLacZ) was used as controls to exclude the effect of Ad itself. We infected HDMEC with AxSTAT3F or control Ad at an m.o.i. of 15. AxSTAT3F as well as AxLacZ showed no significant morphological change (Fig. 3a). We confirmed gene expression in almost all of HDMEC using control virus (AxLacZ) at same m.o.i. (*i.e.* 15) by X-gal staining (Fig. 3b), and AxSTAT3F had no effect on HDMEC proliferation (Fig. 3c). However, AxSTAT3F inhibited the VEGF-induced HDMEC migration by 100% (Fig. 3d). Furthermore, AxSTAT3F inhibited nuclear translocation

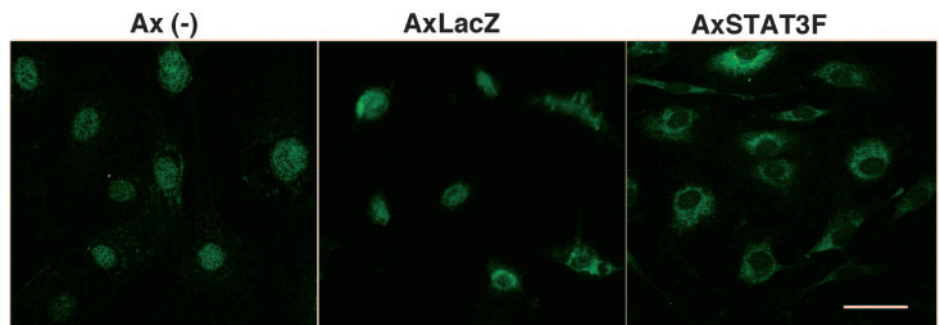
of phosphorylated STAT3 after VEGF addition as confirmed by confocal microscopy (Fig. 4).

**Blockade of Tube Formation on Matrigel and Collagen Gels by a Dominant-negative Mutant of STAT3**—On GFR-Matrigel, HDMEC began to rearrange or align themselves in an organized manner within 1 or 2 h without exogenous stimulation. In the absence of stimulation, HDMEC formed only a small number of short, incomplete tubes. The majority of the tubes were not linked to one another but remained close to the cell body from which they were derived. Untreated HDMEC formed significantly fewer tubes compared with stimulated cells. After a 12-h incubation, the cells treated with VEGF further differentiated into an expansive tube network, whereas most of the control cells remained as individual clusters or ovoid colonies. By 16 h, the boundaries of the stimulated tubes were sharply defined, elongated, and more extended. Furthermore, a majority of the HDMEC formed tubes. Overall, HDMEC treated with VEGF produced a more extensive network of interconnecting tubes compared with untreated HDMEC (Fig. 5a). The total tube length was calculated by Image-Pro Plus software. Compared with control, the total length of tubes was 1.7-fold longer



**FIG. 3. Morphometric change and inhibition of migration by AxSTAT3F.** *a*, adenovirus vector was used as a strategy to introduce mutant STAT3 into HDMEC at m.o.i. 15. Toxicity was not noticed. Scale bar, 50  $\mu$ m. *b*, Almost all of HDMEC expressed  $\beta$ -galactosidase 48 h after transfection of AxLacZ at m.o.i. 15. Scale bar, 5  $\mu$ m. *c*, AxSTAT3F did not influence on the proliferation of HDMEC compared with AxLacZ. *d*, AxSTAT3F inhibited VEGF-induced HDMEC migration by 100% compared with controls.

**FIG. 4. Inhibition of translocation of phosphorylated STAT3 by AxSTAT3F.** Confocal microscopy showed that AxSTAT3F (m.o.i. 15) inhibited nuclear translocation of phosphorylated STAT3 50 min after addition of VEGF. Scale bar, 5  $\mu$ m.



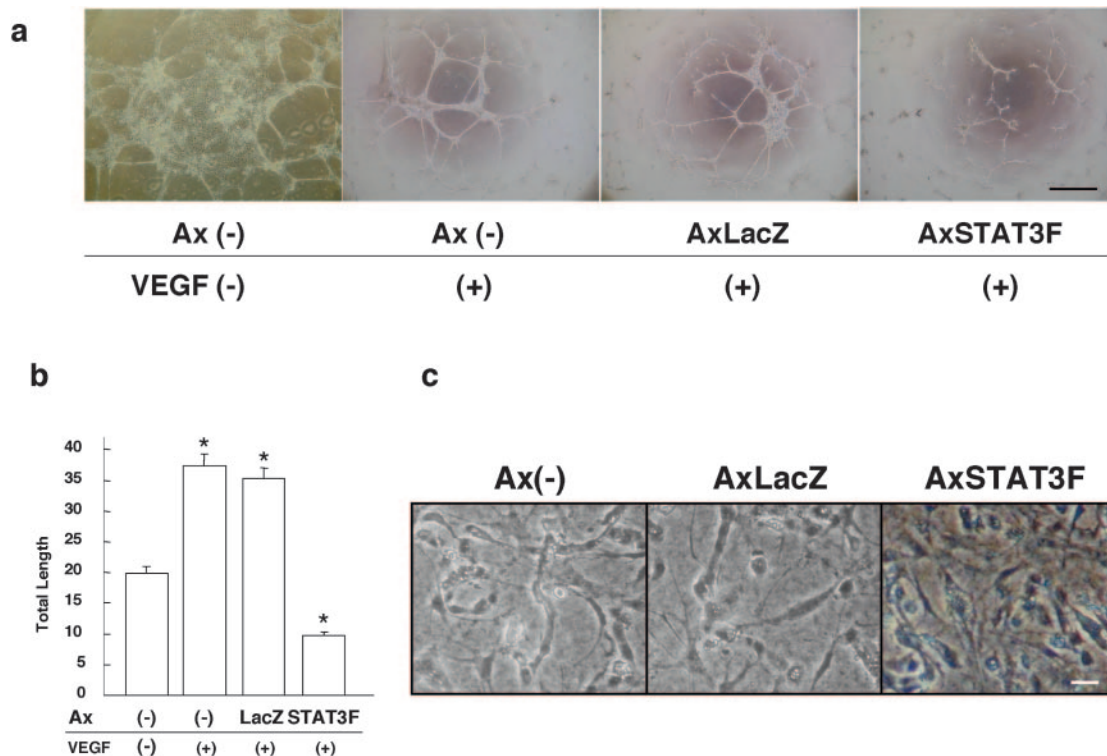
in VEGF-treated cells. These VEGF-induced tube formation was decreased by the treatment of dominant negative mutant form of STAT3, AxSTAT3F, by 74% (Fig. 5b). This result shows that VEGF-induced HDMEC tube formation occurred through the STAT3 signaling pathway.

To examine tube formation on an extracellular matrix that is inherently less conducive to rapid neovascularization, HDMEC were cultured on collagen gels in the presence or absence of stimulants. Confluent cultures of HDMEC on the collagen gels formed a monolayer of interlocking cells. Eight hours after the addition of VEGF, the cells became elongated and spindle shaped. After 48 h, numerous tubes were visible in the wells treated with VEGF. The control cells remained in the characteristic monolayer without any discernible tube growth. Like the tube formation on Matrigel, AxSTAT3F decreased VEGF-induced tube formation on collagen gel (Fig. 5c). This result shows that also in the VEGF-induced HDMEC tube formation on collagen gel was through STAT3 signaling pathway.

## DISCUSSION

Blood vessel regeneration is an important process in skin wound healing and regulated by various cytokines and growth factors. The most important of them is VEGF. It has been shown that blood vessel formation is directly induced by VEGF *in vivo* (34, 35) and that vascular endothelial cell migration and blood vessel regeneration are induced in a similar manner *in vitro* (36, 37). VEGF strongly induces migration of human umbilical vein endothelial cells, even at low concentrations compared with basic fibroblast growth factor (38). Therefore, induction of endothelial cell migration is one of the major functions of VEGF.

Intracellular signal transduction has been studied in many cytokines and growth factors, including VEGF. The major signaling pathway downstream of VEGF and VEGFR interaction has been supposed to be mitogen-activated protein kinase cascade (39). However, several groups have suggested the involvement of another signaling pathway, namely the STAT pathway



**FIG. 5. Blockade of tube formation on Matrigel and collagen gels by AxSTAT3F.** *a*, cells treated with VEGF formed an expansive tube network, whereas most of the control cells remained as individual clusters or ovoid colonies. AxSTAT3F (m.o.i. 15) treated cells showed faint or rudimentary tube network on Matrigel. Bar, 500  $\mu$ m. *b*, tube length was quantified by image analysis of digital pictures, using Image-Pro Plus software. Compared with controls, the calculated total length of tube growth was higher by 1.7-fold in VEGF-treated cells. AxSTAT3F decreased tube formation to 74% of untreated control values. *c*, effect of AxSTAT3F (m.o.i. 15) on HDMEC tube formation on collagen gel. HDMECs were seeded on collagen gel in 12-well plates and stimulated by VEGF. After 72 h, AxSTAT3F decreased VEGF-induced tube formation on collagen gel compared with controls. Scale bar, 5  $\mu$ m.

(23, 40, 41), in VEGF-induced signal transduction, although the implication of STAT in VEGF function has not been fully elucidated. Sano *et al.* (27) have shown that the migration of keratinocytes induced by epidermal growth factor family growth factors was severely impaired in STAT3-disrupted keratinocytes. STAT3 is phosphorylated and activated by the interleukin-6 family, epidermal growth factor, platelet-derived growth factor, hepatocyte growth factor, granulocyte colony-stimulating factor, and leptin in various cell types (25, 42). These reports suggest the important role of STAT3 in cell migration. VEGF up-regulated HDMEC migration, but the downstream signaling pathway remained unclear. Therefore, we hypothesized that the STAT 3 might be involved in HDMEC migration and angiogenesis might be induced by VEGF.

To understand the role of STAT, it is important to clarify the mechanism of phosphorylation and nuclear translocation of phosphorylated STAT. Tyrosine phosphorylation of STAT is necessary but not sufficient for its transcriptional activity. Because STAT protein lacks a nuclear localization signal, it is supposed that chaperone proteins are required to assist the nuclear translocation of STAT (43). We showed that STAT1, STAT3, and STAT5 are expressed in HDMEC confirmed by Western blot and immunohistochemistry. STAT1, -3, and -5 were phosphorylated to some extent even at the steady state, and their levels were not altered by VEGF addition. However, phosphorylated STAT3, but not phosphorylated STAT1 and STAT5, is translocated into nucleus after VEGF stimulation, and impairment of translocation of phosphorylated STAT3 by a dominant-negative STAT 3 abolished VEGF-induced HDMEC migration and tube formation. These data indicate that translocation of phosphorylated STAT3 into nuclei is essential for triggering HDMEC migration. In aortic endothelial cells, phos-

phorylation of STAT1, STAT3, and STAT6 by VEGF was reported (23). In contrast to HDMEC, phosphorylated STAT1 and STAT6, but not phosphorylated STAT3, are translocated into nuclei (23). Therefore, this complicated relationship between phosphorylation and nuclear translocation in various STATs seems to contribute to diverse function corresponding to different cell types, even among endothelial cells.

Two pathways regulate the phosphorylation of STAT: phosphorylation by the Janus kinase and intrinsic tyrosine kinase of growth factor receptors (23). We found that HDMEC express JAK1, JAK2, and Tyk2 and that none of them were phosphorylated after addition of VEGF. In bovine aortic endothelial cells, involvement of the intrinsic tyrosine kinase activity of VEGFR2 was reported in STAT1 tyrosine phosphorylation (23). This suggests that intrinsic tyrosine kinase activity of VEGFR is a major pathway in STAT phosphorylation of endothelial cells.

VEGF and its two receptors, Flt-1 (VEGFR-1) and KDR/Flk-1 (VEGFR-2), have been demonstrated to be an essential regulatory system for blood vessel formation in mammals. KDR/Flk-1 ( $-/-$ ) homozygous mice died at embryonic day 8.5 (E8.5) from a severe deficiency in vascular formation associated with a strong hematopoietic impairment (7). Like KDR null mice, the Flt-1 ( $-/-$ ) homozygous mice also showed embryonic lethality at almost the same stage (E8.5–9.0) (8). On the other hand, the STAT3 $-/-$  mice embryos were being degenerated with no sign of mesoderm formation by E7.0. And by E7.5, STAT3 $-/-$  embryos were completely resorbed, indicating that STAT3 $-/-$  embryos die around E7.0, the day at which gastrulation initiates (32). It is suspected that the lethality might be the result of a defect in functions of visceral endoderm, such as nutritional insufficiency, although angiogenesis and vascular

formation have not been fully investigated. We show here that the dominant-negative STAT3 suppressed HDMEC tube formation on Matrigel and the VEGF-induced tube formation as well as HDMEC migration. These findings suggest that VEGF-KDR/Flt-STAT3 is one of the most important pathways in developmental angiogenesis.

In conclusion, we first demonstrated that the nuclear translocation of phosphorylated STAT3 is essential for VEGF-induced HDMEC migration and tube formation. This VEGF-induced tube formation was fully inhibited in cells expressing the dominant-negative mutant form of STAT3, AxSTAT3F, to levels below those of controls. It gives a new insight into the role of VEGF-STAT3 signaling in wound healing and angiogenesis of the skin as well as developmental angiogenesis.

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