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Vascular Biology, Atherosclerosis and Endothelium Biology

Mitogen-Activated Protein 3 Kinase 6 Mediates Angiogenic and Tumorigenic Effects via Vascular Endothelial Growth Factor Expression

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Genome-wide screening using a small interfering RNA (siRNA) library has revealed novel molecules that are involved in a wide range of physiological responses. The expression of vascular endothelial growth factor (VEGF) is increased under hypoxic conditions, and plays a crucial role in tumor angiogenesis and tissue responses to ischemia. Here, we used a siRNA expression vector library to elucidate molecules that modify VEGF expression. Screening using an siRNA library revealed that MAPKKK6 (MEKK6/MAP3K6) regulates VEGF expression under both normoxic and hypoxic conditions *in vitro*, although the biological function of MAP3K6 remains unknown. Attenuation of VEGF expression by MAP3K6 inhibition was demonstrated by transient transfection of double-stranded RNA as well as by stable transfection of short hairpin RNA-expressing vectors against MAP3K6. Conditioned medium of MAP3K6-knocked down cells attenuated both endothelial proliferation and capillary network formation in a VEGF-dependent manner *in vitro*. In addition, tumor cells with down-regulation of MAP3K6 expression showed significant suppression of tumor growth *in vivo*, which was accompanied by significant repression of vessel formation and VEGF expression in these tumors. The results of this study suggest that MAP3K6 regulates VEGF expression in both normoxia and hypoxia, and that regulation of VEGF by MAP3K6 may play a crucial role in both angiogenesis and tumorigenesis. ***(Am J Pathol 2009, 174:1553–1563; DOI: 10.2353/ajpath.2009.080190)***

Vascular endothelial growth factor (VEGF) is a well-known angiogenic growth factor that is up-regulated in most tumor cells,[1–4] and whose receptors are highly expressed in almost all tumors. Administration of antibody against VEGF has been shown to inhibit tumor growth in clinical cancer therapies.[5,6] The angiogenic effect of VEGF is mediated both by the induction of endothelial cell proliferation and the stimulation of endothelial cell migration.[7–9] VEGF also serves as a prosurvival factor for endothelial cells.[9,10] VEGF expression is induced in response to hypoxia or ischemic damage in various organs[11–13]: as a potent stimulator of angiogenesis, VEGF restores the vascular supply to cells, thereby improving oxygen supply.

Hypoxia is one of the signals for blood vessel invasion into a tumor and is believed to be critical to initiation of the angiogenetic cascade.[14] Hypoxia triggers the change in oxygen-regulated gene expression via the activation of the basic helix-loop-helix-PAS transcriptional factors.[15] The hypoxia-inducible factors (HIFs) activate genes encoding proteins that mediate adaptive responses (eg, angiogenesis), including VEGF, by binding to a *cis*-acting DNA regulatory sequence, the hypoxia response element (HRE).[16] However, the induction of VEGF expression in hypoxic conditions is not fully HIF-dependent.[17–19] In evidence of this, the tyrosine kinase inhibitor gefitinib decreased VEGF expression in SQ20B squamous cell carcinoma by both HIF-dependent and -independent mechanisms,[18] whereas in colon cancer cells, the hypoxic induction of VEGF was only partially blocked by HIF-1α knock-down.[19]

RNA interference (RNAi) was originally described as a powerful tool for the inhibition of gene expression in *Caenorhabditis elegans* and *Drosophila*. The use of genome-

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wide RNAi screening in *C. elegans* using libraries of *in vitro*-transcribed long double-stranded (ds) RNAs has proven extremely useful in gene discovery and functional annotation in various processes, including early embryonic development, lethality, sterility, genome instability, and longevity.[20–22] Until recently, however, loss-of-function genetic screening in mammalian cells was hampered because dsRNAs also have nonspecific inhibitory effects, which are known as the interferon response. Although the use of synthetic siRNAs allows cells to escape the interferon response,[23] vector-based RNAi is also useful in maintaining RNAi activity for much longer periods in stable transfection studies.[24–28]

In this study, we attempted to identify novel regulatory components of the VEGF expression pathway by screening 320 short hairpin RNA expression vectors that target genes for kinases and transcription factors. We identified MAP3K6 as a novel regulator of VEGF expression. Although MAP3K6 was found to weakly activate JNK,[29] its biological validity has not been well documented. Our data demonstrate that regulation of VEGF expression by MAP3K6 plays a crucial role in angiogenesis.

Materials and Methods

Cell Culture

HEK293T and HeLa S3 cells were cultured in Dulbecco's modified Eagle's medium (Nissui, Tokyo, Japan) buffered with 25 mmol/L HEPES (Sigma, St. Louis, MO) at pH 7.4, supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C under a humidified atmosphere of 5% CO_2/95% air. Hypoxic conditions were established at 0.2% O_2 with an Anaerocult A mini pack (Merck KGaA, Darmstadt, Germany), and at 1.0% by exposure to 1.0% O_2/5% CO_2, with the balance as nitrogen, in an APM-30D multigas incubator (Astec, Fukuoka, Japan). Rat kidney vascular endothelial cells[30,31] were cultured in RPMI 1640 medium containing 10% fetal bovine serum and 10% NuSerum (BD Biosciences, San Jose, CA).

Construction of Reporter Plasmids

To construct an optimal vector that reflects VEGF expression in a hypoxic milieu, we constructed a luciferase reporter vector by incorporating the promoter region of human VEGF into the pGL4-basic plasmid (Promega, Madison, WI), termed pGL4-VEGFp. To obtain a 2.7-kbp human VEGF promoter region, human genomic DNA was amplified in a two-step polymerase chain reaction (PCR). In the first step, PCR amplification was done using (forward, 5′-ACCTCCACCAAACCACAGCAACAT G-3′ and reverse, 5′-TCGCACGCACGTCCCCAGCCG-3′) synthetic oligonucleotides, with the annealing/extension step set at 68°C for 360 seconds. In the second step, the first PCR product was amplified using forward, 5′-GCGCTCGAGTCCCATTCTCAGTCCATGCCTCCAC-3′ and reverse, 5′-AAGTCCATGGTTTCGGAGGCCCGACCG-3′ synthetic oligonucleotides, with the annealing/extension step set at 68°C for 360 seconds. The obtained fragment was digested with *Xho*I and *Nco*I, and then cloned into pGL4-basic at *Xho*I through *Nco*I sites. The insert was confirmed to be identical to the human VEGF promoter by sequence analysis.

Further, to construct an optimal vector that responds maximally in a hypoxic milieu, we constructed a second luciferase reporter vector by incorporating seven tandem repeats of HIF-responsive elements (HREs) and a human minimal CMV promoter (hmCMVp) into the pGL3-basic plasmid (Promega), to give pHRE-Luc.[32] In brief, complementary oligonucleotides containing a HRE and *Nhe*I recognition site at both ends (5′-CTAGCCCACAGTGCATACGTGGGCTTCCACAGGTCGTCTG-3′ and 5′-CTAGCAGACGACCTGTGGAAGCCCACGTATGCACTGTGGG-3′) were synthesized, annealed, and cloned into pGL3-basic at *Nhe*I through *Bgl*II sites in tandem repeats, directly or via blunt-ended ligation. A synthetic fragment composed of hmCMVp was inserted at the *Bgl*II-*Hind*III site.

Construction of siRNA Expression Plasmids

The short hairpin type of a siRNA (shRNA) expression plasmid library was purchased from iGENE Therapeutics Inc. (Tsukuba, Ibaraki, Japan).[25] In brief, the vector includes a human U6 promoter, a puromycin resistance gene, and *Bsp*MI cloning sites and is termed pcPUR hU6. Synthetic oligonucleotides in which sense and antisense nucleotides were connected by an 11-base hairpin loop were formed as a single chain. After annealing, DNA fragments were ligated into the *Bsp*MI sites of pcPUR hU6. Target sites of oligonucleotides for each gene were predicted by an algorithm developed by the manufacturer.

Screening of siRNA Library by Transient Transfection of Reporter and siRNA Expression Vectors

HEK293T cells (1×10^5) were cultured in a 96-well plate in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum and 1% antibiotics. Mixtures of three plasmids, ie, 400 ng of siRNA library, 400 ng of pGL4-VEGFp, and 3 ng of pCMV-*Renilla* were co-transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) as described in the manufacturer's protocol. Forty-eight hours after transfection, fresh medium was added to the cells followed by exposure to 0.2% O_2. After 24 hours of incubation, the cells were assayed by a dual luciferase assay system (Promega), with luciferase activity normalized by *Renilla* gene activity. The pcPUR hU6 vector containing a targeted sequence against the HIF-1α gene and seven tandem thymidine repeats (T7) served as positive and negative control, respectively. Screening was performed in duplicate. Hit clones were considered to be those which suppressed VEGF reporter activity in comparison with the negative control in three independent experiments.

Enzyme-Linked Immunosorbent Assay (ELISA)

VEGF concentration in the culture medium was determined by an ELISA (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. The data were adjusted by the total protein amount of the cells.

Quantitation of mRNA Expression by PCR Analysis

Total RNA was isolated using Isogen (Nippon Gene, Tokyo, Japan) and reverse-transcribed with an Im-Prom II reverse transcription kit (Promega). A measure of one-twentieth (v/v) cDNA was used as a template for subsequent quantification. PCR was run on an iCycler (Bio-Rad, Hercules, CA) using iQt SYBR Green PCR supermix (Bio-Rad). The relative amount of VEGF and MAP3K6 gene expression was calculated and corrected for that of ribosomal protein LS28. The sets of primers for VEGF, MAP3K6, and LS28 were: forward, 5′-TCTGAGCAAGGCCCACAGGGA-3′ and reverse, 5′-CCCTGATGAGATCGAGTACATCTT-3′; forward, 5′-CTGCTGCTTCCTCTATGCACT-3′ and reverse, 5′-CCCGTCTCCGTGTACTCATAA-3′; and forward, 5′-ATGGTCGTGCGGAACTGC-3′ and reverse, 5′-TTGTAGCGGAAGGAATTGCG-3′, respectively.

dsRNA Synthesis and Transient Expression

Two sets of double-strand RNA (siPerfect) for MAP3K6 and the negative control dsRNA were generated by the manufacturer (RNAi Co., Ltd., Tokyo, Japan) according to an original algorithm developed by it. Two hundred and fifty pmol of dsRNA were transfected into 5.0×10^5 HeLa S3 cells using Lipofectamine 2000 (Invitrogen) in a six-well plate, as described in the manufacturer's protocol. Twenty-four hours after transfection, cells were placed under normoxic or hypoxic conditions (1% O_2) for 24 hours.

shRNA Stable Transfection

The pcPUR hU6 vector targeted against the MAP3K6 gene was transfected into HeLa S3 cells using Lipofectamine 2000 (Invitrogen). Forty-eight hours after transfection, cells were selected by exposure to 1 μg/ml of puromycin for 120 hours. After selection, surviving colonies of cells were isolated and resuspended in fresh medium. Transfected cells having the pcPUR hU6 vector containing seven tandem repeats of thymidine (T7) served as control.

Mouse MAP3K6 Transient Transfection

To obtain a full length of mouse MAP3K6 cDNA, IMAGE clone (BC120565) was purchased from Open Biosystems (Huntsville, AL). Mouse MAP3K6 cDNA was isolated by *Eco*RI and *Xho*I digestion from pCR-XL-TOPO, and was subcloned in pCDNA3.1-TOPO. Transfection of this expression plasmid into HeLa S3 cells was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Western Blot Analyses

Expression of MAP3K6, VEGF, and ASK-1 in HeLa S3 cells as well as that of MAP3K6 and VEGF in the tumors was monitored by Western blot analysis. The tumors were collected in lysis buffer (50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1% Nonidet P-40, 1 mmol/L dithiothreitol, 2 μg/ml leupeptin, 2 μg/ml pepstatin A) and homogenates were prepared manually using a tissue homogenator (Niti-on, Chiba, Japan). Sixty μg of whole cell extracts or 40 μg of tumor homogenates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions and electrophoretically transferred to a nylon membrane. The membranes were blocked with 5% nonfat dry milk in TBS Tween-20 (0.1%, v/v) at room temperature for 1 hour. For MAP3K6 detection, the membranes were incubated with 1:100 anti-human MAP3K6 primary antibody (Abnova, Taipei, Taiwan) at 4°C overnight followed by incubation with horseradish peroxidase-conjugated anti-mouse IgG. For the detection of VEGF and ASK-1, the membranes were incubated with 1:300 anti-human VEGF(147) primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and 1:250 anti-human ASK-1 (H-300) primary antibody (Santa Cruz Biotechnology) at 4°C overnight, respectively. Hybridizations with horseradish peroxidase-conjugated anti-rabbit IgG were performed the following day. Immunoreactive protein was visualized by a chemiluminescence protocol (ECL; Amersham, Arlington Heights, IL). Electric densities were measured using an image processing and analysis program, Image J software (National Institutes of Health, Bethesda, MD).

Preparation of Conditioned Media (CM)

The angiogenic effect of VEGF on the endothelial cells was assessed using CM. Control HeLa and MAP3K6 stably knocked-down HeLa S3 cells were grown to confluence in 100-mm culture dishes. After changing the medium to Dulbecco's modified Eagle's medium with 10% fetal bovine serum, the cells were cultured under hypoxic conditions (1% O_2) for an additional 24 hours. Supernatants were then collected, centrifuged to remove cellular debris, and passed through a 0.22-μm pore filter (Millipore, Tokyo, Japan).

Cultured Endothelial Cell Proliferation and Capillary Network Formation

Endothelial cell proliferation in the CM was examined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reduction to formazan (MTS assay, Promega). Cells were seeded at 2.0×10^4 on 96-well culture plates and incubated overnight. The medium was then aspirated and replaced by the CM, and the cells were incubated for an additional 24

hours. After this incubation, the cells were loaded with MTS reagent, and cell proliferation was measured using a microtiter plate reader at 492 nm.

Capillary network formation was examined by Matrigel assays (BD Biosciences). ECs (2.0×10^4) were seeded on four-well Lab-Tek chamber slides (Nalge Nunc International, Naperville, IL) coated with Matrigel, and cultured in the CM for 8 hours under isolation from control and MAP3K6 knock-down cells. After incubation, capillary network formation was observed with a phase-contrast microscope and quantitatively evaluated by measuring capillary tube length. A total of 20 ng/ml of recombinant VEGF purchased from R&D Systems was added to the CM isolated from MAP3K6 knock-down cells.

Tumor Xenograft Assay

BALB/cAJc-nu/nu male mice, 7 to 8 weeks of age, were purchased from CLEA Japan (Tokyo, Japan) and inoculated subcutaneously on the flank with 5×10^6 of control or MAP3K6 stably knock-down HeLa S3 cells. Tumor volumes were measured at the indicated days after transplantation with electronic calipers (Mitutoyo, Kanagawa, Japan) and calculated using the formula length (mm) $\times$ width (mm) $\times$ height (mm)/2 and expressed in mm^3. Tumor weight was measured at 28 days after initiation of transplantation. All experiments were performed in accordance with the guidelines of the Committee on Ethical Animal Care and Use at the University of Tokyo.

Vessel Density Analysis

For measurement of microvessel density in the tumors, frozen sections from xenograft tissues were fixed in acetone for 1 minute and allowed to air-dry. After 3 hours of incubation at room temperature with anti-mouse CD31 monoclonal antibody (BD Biosciences) at 1:20 dilution, the sections were incubated with biotinylated secondary antibodies at a 1:1000 dilution for 1 hour, followed by a 30-minute incubation with horseradish peroxidase-conjugated avidin D (Vector Laboratories, Burlingame, CA). Color was developed by incubation with diaminobenzidine (Wako Pure Chemical Industries, Tokyo, Japan) and the sections were counterstained with Mayer's hematoxylin (Wako Pure Chemical Industries). Quantification of tumor vessel counts was performed in a blinded manner with 10 randomly selected fields at a $\times$100 magnification per section.

VEGF Expression Analysis in the Tumors

VEGF expression in the tumors was also monitored by immunohistochemical analysis. The tumors at 14 days were fixed in 10% neutralized formalin after removal, and then processed, embedded in paraffin, and cut into 3-μm sections. The sections were incubated with primary antibody against human VEGF(A20) (Santa Cruz Biotechnology). After overnight incubation at 4°C with the primary antibody at 1:100 dilution, the sections were incubated with horseradish peroxidase-conjugated secondary antibodies at 1:500 dilution for 1 hour. Color was developed by incubation with diaminobenzidine (Wako Pure Chemical Industries) and hydrogen peroxide and the sections were counterstained with Mayer's hematoxylin (Wako Pure Chemical Industries).

Phospho-MAPK Array

A Proteome Profilter antibody array of human phosphonomitogen-activated protein kinases (MAPKs) and other serine/threonine kinases was purchased from R&D Systems. The array was a nitrocellulose membrane on which capture antibodies against a variety of MAPKs and other serine/threonine kinases had been spotted in duplicate, including ERK1/2/3, JNK1/2/3, p38α/β/δ/γ, RSK1/2, GSK-3α/β, Akt1/2, MSK2, HSP27, and p70 S6 kinase. Control and MAP3K6 stably knocked-down HeLa S3 cells were cultured in hypoxic conditions for 45 minutes or 24 hours. Three hundred μg of protein from whole lysates of the cells were hybridized with the antibody array according to the manufacturer's protocol. Relative changes in phosphorylation state between two samples were measured with Image J software.

Statistical Analysis

Statistical analyses were performed using Stat-View software (ver. 5.0; SAS Institute, Cary, NC). Comparisons among groups were done using analysis of variance, with Bonferroni/Dunn correction. Nonparametric data were analyzed with the Mann-Whitney test. P values of <0.05 were considered statistically significant.

Results

Establishment of VEGF Reporter Assay

A VEGF-reporter vector was obtained using a 2.7-kbp fragment of the human VEGF gene located in its 5′-flanking promoter region. To validate the assay, HEK293T cells were transiently transfected with VEGF reporter vector and were subjected to a graded oxygen content series for 24 hours. A decrease in oxygen concentration affected reporter activity, with a fivefold increase seen at concentrations of 0.2% (Figure 1A). Co-expression with the VEGF reporter and shRNA expression vector of HIF-1α, which mediates various hypoxic responses, significantly ameliorated the hypoxic response of the reporter compared with control, ie, VEGF reporter-transfected cells without the shRNA expression vector. Moreover, co-expression with the VEGF reporter and shRNA expression vector against nonrelated genes, such as GFP or the tandem repeated sequence of T (T7), did not affect reporter activity (Figure 1B). These results indicated that the 2.7-kbp fragment of the human VEGF promoter can be used to monitor the hypoxic response to VEGF expression.

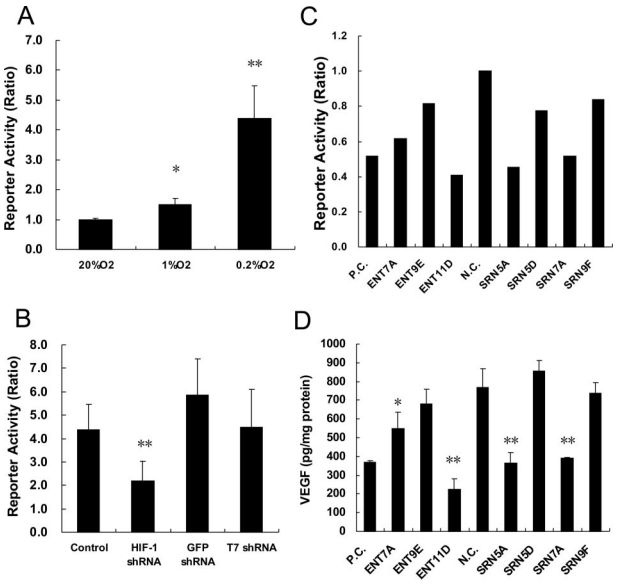

Figure 1. Construction of the VEGF reporter system and siRNA library screening in HEK293T cells. **A:** A luciferase reporter conjugated with 2.7 kbp of the human VEGF promoter region sensed hypoxia in an oxygen tension-dependent manner. $^{*}P < 0.05$, $^{**}P < 0.01$ versus 20% O_2. **B:** Reporter activities were suppressed by transfection of HIF-1 shRNA expression vector under hypoxic conditions (0.2% O_2) compared with the control. Further, a shRNA expression vector against nonrelated genes, namely GFP and a tandem repeat of thymidine (T7), did not affect these reporter activities. The data were obtained from three independent experiments and are shown as mean ± SD ($n = 5$). $^{**}P < 0.01$ versus control. **C:** In the first screening, HEK293T cells seeded in 96-well plates were co-transfected with a reporter vector and shRNA expression plasmids. The reporter vector was conjugated with 2.7 kbp of human VEGF promoter (pGL4-VEGFp), and shRNA expression plasmids were constructed against various kinases and transcription factors. Cells were incubated for 48 hours to allow target knock-down, and reporter activity was measured after further induction of hypoxia for 24 hours. The graph shows relative reporter activity compared with negative controls, with reporter activity of the negative control set at 1. The shRNA expression vector containing a targeted sequence against the HIF-1α gene and T7 served as positive control (P.C.) and negative control (N.C.), respectively. **D:** In the second screening, VEGF content in medium from cells that were cultured in the same conditions as above were measured by ELISA. The data were obtained by triplicate transfection of the target shRNA expression vector and are shown as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$ versus negative control.

siRNA Library Screening

For our screening analysis, we selected 320 vectors designed against genes for kinases and transcription factors. We selected shRNA against HIF-1α and T7 to serve as a positive and negative control, respectively. HEK239T cells were co-transfected with the VEGF reporter vector and a shRNA expression vector. Screening was performed in duplicate and quantitatively scored. We identified several shRNA expression vectors that modified expression of the VEGF reporter vector after 24 hours of incubation under hypoxic conditions. Figure 1C shows clones that suppressed VEGF reporter activity compared with the negative control in three independent experiments. To confirm the inhibitory effects of these shRNA expression vectors, we measured VEGF content in the CM by ELISA (Figure 1D). Results showed that knock-down of ENT7A, ENT11D, SRN5A, or SNR7A significantly suppressed both VEGF reporter activity and VEGF expression (Supplemental Table S1 available at *http://ajp.amjpathol.org*). Of these four molecules, ENT11D and SRN5A were more effective than the others. We selected SRN5A (mitogen-activated kinase kinase kinase 6, also called MAP3K6/MEKK6) for further biological analysis because MAP3K6 weakly activates JNK, although the biological function of this molecule remains primarily unknown.

Attenuation of VEGF Expression by Transient Transfection of MAP3K6 dsRNA

To confirm the effect of this kinase, we performed transient transfection of dsRNA into HeLa S3 cells instead of the shRNA expression vector using two distinct synthesized dsRNAs that target different sites of the MAP3K6 gene. Quantitative PCR analysis showed that both dsRNA significantly reduced MAP3K6 (70 to 73% reduction, Figure 2A) and VEGF mRNA expression (28 to 34% reduction, Figure 2B) after 24 hours of incubation under hypoxic conditions. In addition, a decrease in MAP3K6 expression in dsRNA-transfected cells at the protein level was confirmed by Western blot analysis (Figure 2C). On ELISA, VEGF content in the CM showed a significant decrease on the introduction of MAP3K6 dsRNA after 24 hours of incubation under hypoxic conditions (30 to 41% reduction, Figure 2D). Intriguingly, MAP3K6 dsRNA also produced a significant decrease in VEGF content in CM from cells under normoxic conditions (21 to 23% reduction).

Attenuation of VEGF Expression by Stable Transfection of MAP3K6 shRNA

To further analyze the contribution of MAP3K6 to VEGF expression, we established cells in which MAP3K6 was stably knocked down. shRNA expression vector against MAP3K6 was stably transfected in HeLa S3 cells, and three independent clones were obtained using the puromycin selection system. Western blot analysis of cell lysates from two independent clones confirmed the down-regulation of MAP3K6 expression in the cell lines compared to control vector transfectants, ie, stably transfected cells with the shRNA expression vector against T7 (Figure 3A). In one of the clones (clone number 2), down-regulation of MAP3K6 was also confirmed at the mRNA level by quantitative real-time PCR analysis (72% reduction, Figure 3B). We then measured VEGF content in the CM by ELISA. As shown in Figure 3C, MAP3K6 shRNA-expressing cells exposed to hypoxia showed a significant reduction in VEGF expression (34% reduction). In addition, cells also showed a significant decrease in VEGF expression by MAP3K6 shRNA under normoxic conditions (41% reduction, Figure 3C). These results are consistent with those in cells transiently transfected with dsRNA. A decrease in mRNA expression of MAP3K6 and reduction of VEGF expression in another clone of MAP3K6 knock-down (clone number 1) was also confirmed by quantitative PCR analysis and ELISA, respectively (Figure 3D). In addition, to rescue the phenomenon, we transiently transfected the mouse ortholog of MAP3K6

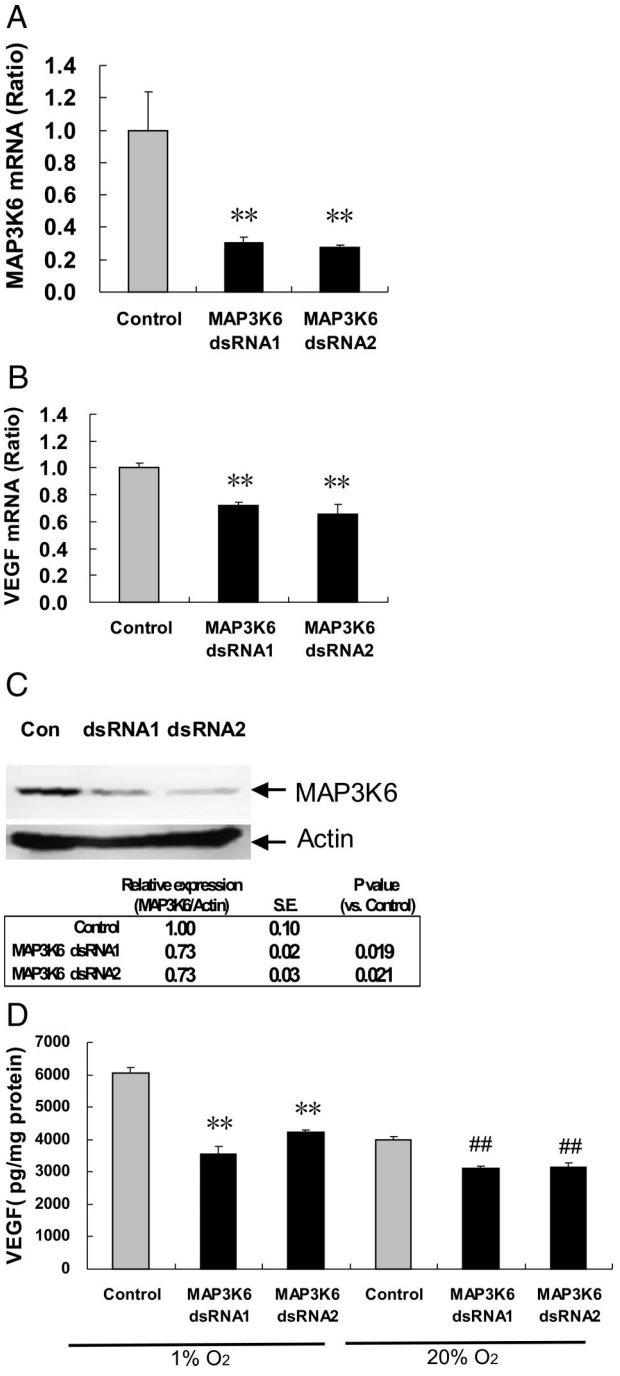

Figure 2. Induction of down-regulation of target gene and VEGF expression by transient transfection of MAP3K6 dsRNA. HeLa S3 cells were transiently transfected with dsRNA against MAP3K6. Gene expression was measured by quantitative PCR. Twenty-four hours after dsRNA transfection, cells were placed under hypoxic conditions (1% O_2) for 24 hours. Transient transfection of two different sequences of dsRNA against MAP3K6 decreased mRNA expression of MAP3K6 (**A**) and VEGF (**B**). The data were obtained from three independent experiments and are shown as mean ± SEM. $^{**}P < 0.01$ versus control. **C:** MAP3K6 protein expression was detected by Western blot analysis. Under hypoxic conditions, marked attenuation was observed on the transfection of two different sequences of dsRNA against MAP3K6. The table shows quantitative analysis by densitometry. The data were obtained from three independent experiments. **D:** VEGF content in the medium was measured by ELISA. Twenty-four hours after transfection, cells were placed under normoxic and hypoxic conditions for 24 hours. dsRNA against MAP3K6 significantly decreased VEGF protein expression in hypoxia and normoxia. The data are shown as mean ± SEM ($n = 5$). $^{**}P < 0.01$ versus the control in 1% O_2. $^{\#\#}P < 0.01$ versus control in 20% O_2.

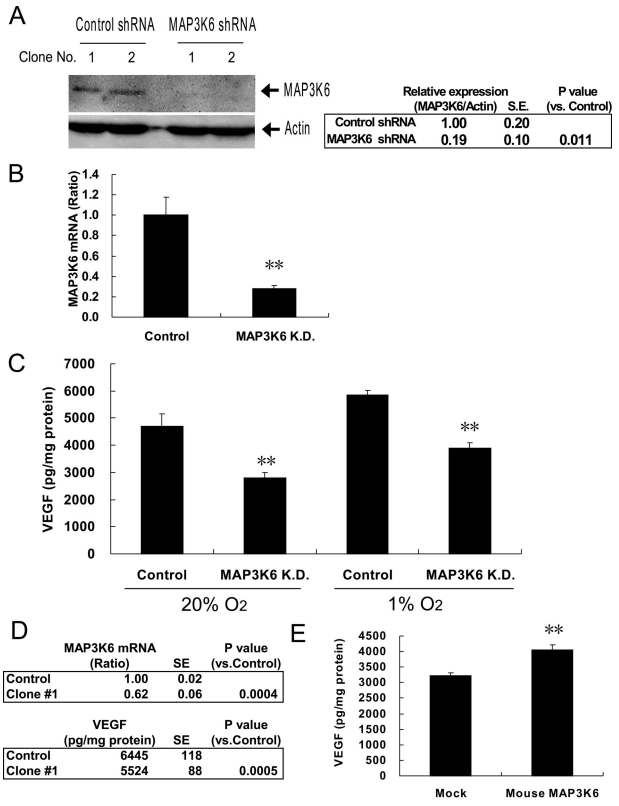

Figure 3. Induction of down-regulation of target gene and VEGF expression by stable transfection of shRNA expression vectors against MAP3K6. HeLa S3 cells were stably transfected with shRNA expression vector against MAP3K6. T7 shRNA-transfected cells served as a control. **A:** Under hypoxic conditions (1% O_2), Western blot analysis showed decreased MAP3K6 expression in two representative independent clones. The table shows quantitative analysis by densitometry. The data were obtained from three independent experiments. **B:** A decrease in mRNA expression of MAP3K6 in hypoxia was confirmed by quantitative PCR analysis ($n = 8$). **C:** VEGF content in the medium was measured by ELISA after 24 hours of incubation in normoxic and hypoxic conditions. Stable transfection of the shRNA expression vector against MAP3K6 decreased VEGF protein expression compared with the control. The data are shown as mean ± SEM ($n = 8$). **D:** Quantitative PCR analysis and ELISA confirmed a decrease in mRNA expression of MAP3K6 and repression of VEGF expression in other MAP3K6 knock-down cells (clone 1), respectively ($n = 6$). **E:** Transient transfection of the mouse ortholog of MAP3K6 into MAP3K6 knock-down cells reversed VEGF repression compared with mock-transfected cells. Cells were exposed under normoxic conditions (20% O_2) for 24 hours and VEGF content in the medium was measured by ELISA. The data are shown as mean ± SEM ($n = 6$). $^{**}P < 0.01$ versus control.

into MAP3K6 knock-down cells. Results showed reversed VEGF repression in these mouse MAP3K6 transfected cells but not in mock-transfected cells (Figure 3E). Taken together, these results indicated that MAP3K6 regulates VEGF expression under both hypoxic and normoxic conditions, ie, constitutive VEGF expression. The following results were obtained with clone number 2, but essentially the same results were observed with the other clones.

Endothelial Proliferation in CM from MAP3K6 Knock-Down Cells

To test whether VEGF reduction by MAP3K6 knock-down modulates endothelial cell proliferation, CM

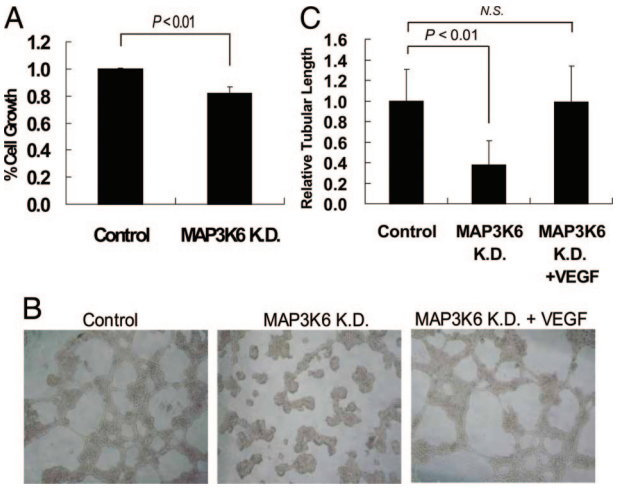

Figure 4. Endothelial proliferation and capillary network formation in CM from MAP3K6 knock-down cells. **A:** CM was obtained from control (T7 shRNA-transfected) and MAP3K6 knock-down HeLa S3 cells cultured under hypoxic conditions (1% O_2) for 24 hours. Cultured renal endothelial cells on 96-well culture plates were incubated in the CM for 24 hours. MTS assays showed significant attenuation of endothelial proliferation in the CM from MAP3K6 knock-down cells. The data are shown as the mean ± SEM ($n = 6$). **B:** Endothelial cells seeded on Matrigel for 8 hours in CM from control HeLa S3 cells and MAP3K6 knock-down HeLa S3 cells. CM from MAP3K6 knock-down cells induced a drastic decrease in capillary network formation compared with that from the control. Exogenous addition of 20 ng/ml of VEGF into CM from MAP3K6 rescued this capillary network dysformation. **C:** Average lengths of 20 randomly selected capillary structures under ×40 magnification. Control CM score was arbitrarily set at 1. The data are shown as mean ± SEM. Original magnifications, × 100.

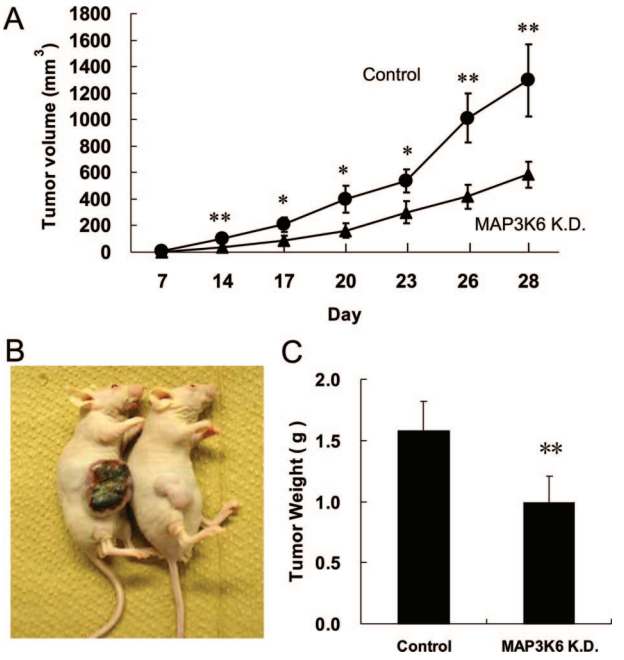

Figure 5. Growth rate and immunohistochemical analysis of MAP3K6 knock-down tumors. *In vivo* tumorigenesis was estimated by transplantation of HeLa S3 cells that stably expressed control or MAP3K6 shRNA. **A:** Growth curve of tumors. **B:** Representative examples of injected mice. **Left mouse**: control tumor; **right mouse**: MAP3K6 knock-down tumor. **C:** Tumor weight measured at the end of the study, presented as mean ± SEM (eight mice per group). $^{*}P < 0.05$; $^{**}P < 0.01$.

was prepared from stably transfected MAP3K6 knock-down and control HeLa S3 cells. Control cells were generated by stable transfection of the shRNA expressing vector against T7. Incubation of cultured endothelial cells with the CM from MAP3K6 knock-down cells showed a significant reduction in endothelial proliferation compared with the control (18% reduction, Figure 4A).

Capillary Network Formation on Matrigel

Capillary network formation was further evaluated using CM derived from stably transfected MAP3K6 knock-down and control HeLa S3 cells. The cells were exposed to hypoxia for 24 hours because tube-like structure formation of endothelial cells was relatively exaggerated in CM derived from hypoxia compared with that from normoxia (data not shown). When endothelial cells were seeded onto Matrigel at subconfluent density in the control CM, they developed tube-like structures at 8 hours. In contrast, endothelial cells in the CM from MAP3K6 knock-down cells did not show tube-like structure formation (Figure 4B). Quantification of capillary network formation by measurement of tube length revealed that the control CM promoted capillary network formation in endothelial cells. In contrast, endothelial cells in the CM from MAP3K6 knock-down cells showed significant suppression of network formation (63% suppression, Figure 4C). To validate the assay and determine conclusively that the effect of MAP3K6 knock-down was attributable to the inhibition of VEGF expression, recombinant VEGF were used. As shown in Figure 4, B and C, exogenous VEGF supplementation restored the attenuation of network formation in CM from MAP3K6 knock-down cells. These findings indicate that the decrease in VEGF in the CM from MAP3K6 knock-down cells affected not only endothelial proliferation but also capillary network formation.

Growth Rate of MAP3K6 Knock-Down Tumor

We further characterized the role of MAP3K6 using tumorigenesis assays conducted by MAP3K6 knock-down HeLa S3 cells. MAP3K6 knock-down HeLa S3 cells showed no difference in proliferation or apoptosis rate compared with control HeLa S3 cells (Supplemental Figure S1 available at *http://ajp.amjpathol.org*). However, MAP3K6 knock-down HeLa S3 cells generated tumors with a smaller volume than control HeLa S3 cells when injected subcutaneously in athymic nude mice (55% reduction; Figure 5, A and B). Further, consistent with the volume, the tumor weight of MAP3K6 knock-down cells at the end of the study was lower than that of control cells (37% reduction, Figure 5C). To exclude a possible effect of clonal variation on the smaller tumor size, we repeated these tumorigenesis assays with other transformed HeLa S3 cell clones. Results showed that other MAP3K6 knock-down cell clones also produced smaller tumors than control cells (data not shown).

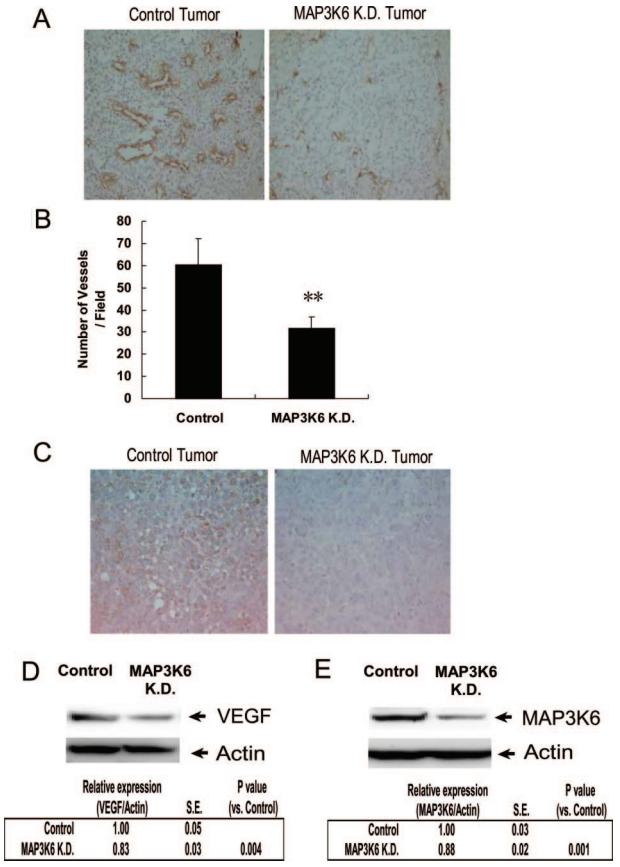

Figure 6. Microvessel formation and VEGF expression in MAP3K6 knock-down tumors. **A:** Tumors show microvessel formation. Representative fields of CD31 immunostaining in a HeLa tumor. **B:** Quantitative analysis of CD31-positive vessels showed significant attenuation of microvessel formation in tumors derived from the MAP3K6 knock-down cells. $^{**}P < 0.01$ versus control. The data are shown as the mean ± SEM ($n = 6$). **C:** VEGF expression in the tumor by immunostaining analysis. Representative fields of VEGF immunostaining in a HeLa tumor. **D:** VEGF expression in the tumor was confirmed by Western blot analysis. The table shows quantitative analysis by densitometry ($n = 4$). The data were obtained from three independent experiments. **E:** MAP3K6 expression in the tumor by Western blot analysis. The table shows quantitative analysis by densitometry ($n = 4$). The data were obtained from three independent experiments. Original magnifications: × 100 (**A**); × 400 (**C**).

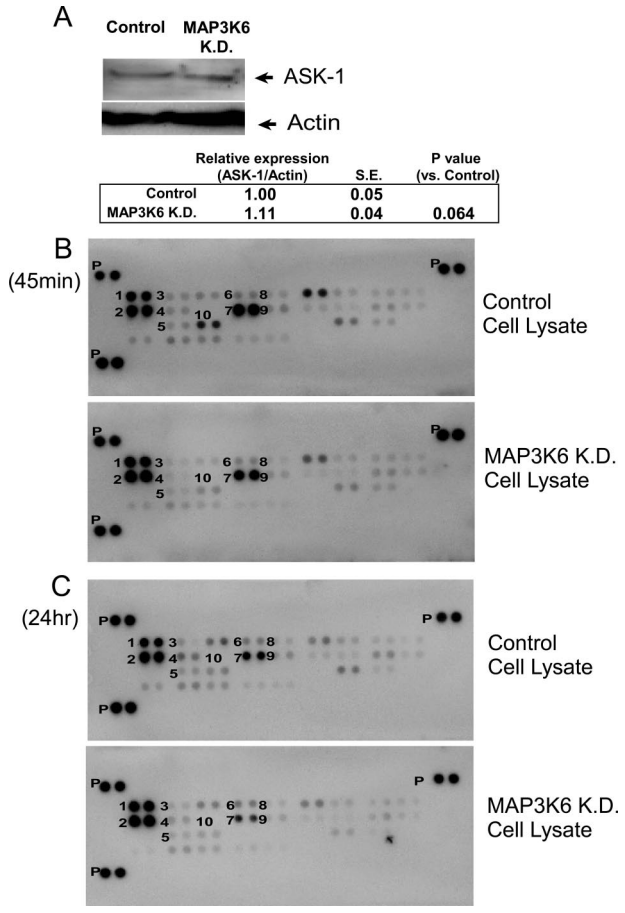

Figure 7. Phosphorylated kinase analysis and ASK-1 expression. **A:** Under hypoxic conditions (1% O_2), Western blot analysis showed that ASK-1 expression in MAP3K6 knock-down cells was not changed compared with that in the control. The table shows quantitative analysis by densitometry ($n = 6$). Phosphorylated kinase pattern in hypoxia was evaluated with kinase antibody arrays. The arrays were hybridized with 300 μg of protein of cell lysates. The cells were exposed to hypoxia (1% O_2) for 45 minutes (**B**) and 24 hours (**C**). Phosphorylated kinase pattern of control cells lysates and MAP3K6 knock-down cell lysates are shown in the **top** and **bottom panels**, respectively. Antibody was spotted in duplicate on the filter. Representative kinases are numbered. **1**, ERK1; **2**, ERK2; **3**, JNK1; **4**, JNK2; **5**, JNK3; **6**, p38γ; **7**, p38α; **8**, p38δ; **9**, p38β; **10**, MSK2; **P**, positive control.

Vessel Density and VEGF Expression in MAP3K6 Knock-Down Tumor

To investigate the role of MAP3K6 in tumor-associated angiogenesis, immunohistochemical analysis of CD31 was performed. The results revealed that the number of vessels in MAP3K6 knock-down tumors was significantly smaller than that in control tumors, showing that MAP3K6 knock-down suppressed angiogenesis in these tumors (48% suppression, Figure 6, A and B). To confirm the repression of VEGF in the tumors, we used immunohistochemical and Western blot analysis, and observed significant attenuation of VEGF expression in MAP3K6 knock-down tumors (Figure 6, C and D). Further, down-regulation of MAP3K6 expression in MAP3K6 knock-down tumors was also confirmed by Western blot analysis (Figure 6E). Taken together, these results provided evidence that MAP3K6 plays an important role in tumor-associated angiogenesis via the regulation of VEGF expression.

Phosphorylated Kinase Analysis

To identify the target kinases of MAP3K6, we first assessed ASK1 expression. Although the heterocomplex of ASK1/MAP3K5 and ASK2/MAP3K6 can themselves promote the stabilization of ASK1/MAP3K5 and ASK2/MAP3K6, ASK1 expression in MAP3K6 knock-down cells was not significantly different from that in the control (Figure 7A). Because the heterocomplex of ASK1/MAP3K5 and ASK2/MAP3K6 has been showed to be an activator of the JNK and p38 pathways, we further performed phospho-MAPK array studies, which can simultaneously detect the relative levels of phosphorylation of various kinases including JNK, p38, and ERK. Hybridizations were performed with control and MAP3K6 knock-down cell lysates that were exposed under hypoxic conditions for both short (45 minutes) and long (24 hours) periods. Comparison of corresponding signals on different arrays to determine the relative change in the phos-

phorylation state of specific serine/threonine kinases showed no differences between the two lysates, except for MSK2 (Figure 7, B and C). In particular, phosphorylation of MSK2 was significantly attenuated in MAP3K6 knock-down cell lysate, on short-term exposure (45 minutes) only (Figure 7B). In addition, phosphorylation of JNK seemed to be slightly attenuated in MAP3K6 knock-down cells and the possibility that JNK is regulated by MAP3K6 could not be fully excluded. However, because this difference was subtle, we speculate that this regulation may play no physiological role. Collectively, these results suggest that MAP3K6-mediated VEGF expression is independent of JNK, p38, and ERK activations.

Discussion

Mammalian siRNA libraries are now widely used to identify genes critical to a wide variety of biological processes, such as apoptosis, cell-cell interactions, and cellular stress responses.[25,33–36] In this study, we used siRNA library screening to explore molecules that regulate VEGF expression in hypoxia. To investigate HIF-independent regulation of VEGF, we generated a human VEGF promoter-conjugated reporter system. Our results showed that MAP3K6 is involved in VEGF expression in both hypoxic and normoxic conditions, ie, constitutive expression, as well as in tumorigenesis.

Various hits from the first screening of the VEGF promoter reporter system showed good correlation with the ELISA of VEGF, indicating that this system reflects VEGF gene expression well. Among several candidates, we chose to examine the biological role of MAP3K6 in endothelial cell function and cancer tumorigenesis because the physiological role of this molecule was primarily unknown. A major concern with use of siRNA in mammalian cells is off-target effects. To mitigate this problem, we designed two sets of dsRNA targeting different sites of MAP3K6 for transient transfection because of the significant potential for off-target effects elicited by a single set of siRNA. Transient expression of the two sets of dsRNA significantly suppressed not only MAP3K6 but also VEGF expression, indicating that MAP3K6 signaling is upstream of VEGF expression. We observed that MAP3K6 is involved in VEGF expression not only under hypoxic but also under normoxic conditions, suggesting that MAP3K6 participates in constitutive expression of VEGF.

MAP3K6 is a kinase that is involved in MAP kinase activation. Although several groups have reported the participation of the JNK/ERK/p38 pathways in VEGF expression,[37–41] it remains unknown whether MAP3K6 plays a role in these pathways. MAP3K6 was originally identified as a member of the serine/threonine protein kinase family by its interaction with MAP3K5/ASK1, a protein kinase that also activates c-Jun kinase (JNK) and p38 kinase. Although this kinase was found to weakly activate JNK but not ERK or p38,[29] its biological validity has not been well documented. Our results showed for the first time that MAP3K6 participates in the regulation of VEGF. Attenuation of VEGF expression by MAP3K6 inhibition was demonstrated by transient transfection with dsRNA as well as by stable transfection of shRNA-expressing vectors against MAP3K6. VEGF expression can be regulated by translational machinery via the binding of eukaryotic initiation factor-4E (eIF-4E) and internal ribosomal entry sites (IRES) to the 5′ UTR of VEGF mRNA.[42] With regard to MAP3K6, however, the mechanism of VEGF repression is assumed to act via transcriptional but not translational regulation because quantitative PCR analysis showed a significant reduction at the mRNA level. We speculate that MAP3K6 may directly phosphorylate a transcriptional factor that binds to the VEGF promoter. In this study, we found that phosphorylation of MSK2 was significantly attenuated in MAP3K6 knock-down cells on short-term exposure to hypoxia. MSK2 is required for the stress-induced phosphorylation of transcription factors CREB and ATF1 in primary embryonic fibroblasts.[43] The mitogen- and stress-induced phosphorylation of CREB at Ser133 has been linked to the transcription of several immediate early genes, including c-*fos*, *junB*, and *egr1*. However, because MSK2 is activated by either ERK1, ERK2, or SAPK2/p38 in primary fibroblasts,[44] the machinery does not seem to be identical in the case of this study. Of note, MSK2 was not attenuated on extended exposure to hypoxia (Figure 7C) and phosphorylated CREB did not significantly differ between MAP3K6 knock-down and control cells (Supplemental Figure S2 available at *http://ajp.amjpathol.org*), supporting this notion. Another possibility is that MAP3K6 activates some transcriptional factors via MSK2 activation. Although HIF-1α can be considered one candidate transcriptional factor, we confirmed that MAP3K6 knock-down has no effect on HIF-1α expression (data not shown). Identification of a direct target of MAP3K6 and comprehensive elucidation of the signal transduction pathway of VEGF regulation by MAP3K6 is beyond the scope of this study, but is an important subject for future investigation.

In addition to these findings under hypoxic conditions, knock-down of MAP3K6 suppressed basal VEGF expression under normoxic conditions also. This finding is supported by the results of our *in vivo* tumorigenesis experiments, in which the tumor growth rate of MAP3K6 knock-down cells was significantly lower than that of control cells at all time points, even before the extrapolated time at which the tumor would have grown sufficiently large to induce hypoxia inside the mass. VEGF is a critical regulator of tumor vascularization. VEGF-deficient embryonic stem cells injected into nude mice form significantly smaller, less vascularized tumors than tumors derived from wild-type control embryonic stem cells.[45] In a spontaneous tumor model, VEGF-deficient pancreatic islet tumors display a severe reduction in angiogenic switching and tumor growth.[46] A significant correlation between increased VEGF expression in the tumor and increased tumor microvascular density has been demonstrated in a number of reports.[47–49] In this study, we found that MAP3K6 knock-down physiologically affected tumor growth, which was accompanied by a lower density of vessels and decreased amount of VEGF in the tumors.

In conclusion, we screened a shRNA expression vector library and identified MAP3K6 as a novel regulator of VEGF expression. Our *in vivo* studies demonstrated a biological role of MAP3K6-mediated VEGF expression in angiogenesis.

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