Angiopoietin-1 Induces Krüppel-like Factor 2 Expression through a Phosphoinositide 3-Kinase/AKT-dependent Activation of Myocyte Enhancer Factor 2*

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Angiopoietin-1 (Ang1) regulates both vascular quiescence and angiogenesis through the receptor tyrosine kinase Tie2. We and another group have recently shown that Ang1 and Tie2 form distinct signaling complexes at cell-cell and cell-matrix contacts and further demonstrated that the former selectively induces expression of Krüppel-like factor 2 (KLF2), a transcription factor involved in vascular quiescence. Here, we investigated the mechanism of how Ang1/Tie2 signal induces KLF2 expression to clarify the role of KLF2 in Ang1/Tie2 signal-mediated vascular quiescence. Ang1 stimulated KLF2 promoter-driven reporter gene expression in endothelial cells, whereas it failed when a myocyte enhancer factor 2 (MEF2)-binding site of KLF2 promoter was mutated. Depletion of MEF2 by siRNAs abolished Ang1-induced KLF2 expression, indicating the requirement of MEF2 in KLF2 induction by Ang1. Constitutive active phosphoinositide 3-kinase (PI3K) and AKT increased the MEF2-dependent reporter gene expression by enhancing its transcriptional activity and stimulated the KLF2 promoter activity cooperatively with MEF2. Consistently, inhibition of either PI3K or AKT and depletion of AKT abrogated Ang1-induced KLF2 expression. In addition, we confirmed the dispensability of extracellular signal-regulated kinase 5 (ERK5) for Ang1-induced KLF2 expression. Furthermore, depletion of KLF2 resulted in the loss of the inhibitory effect of Ang1 on vascular endothelial growth factor (VEGF)-mediated expression of vascular cell adhesion molecule-1 in endothelial cells and VEGF-mediated monocyte adhesion to endothelial cells. Collectively, these findings indicate that Ang1/Tie2 signal stimulates transcriptional activity of MEF2 through a PI3K/AKT pathway to induce KLF2 expression, which may counteract VEGF-mediated inflammatory responses.

Angiopoietin-1 (Ang1) is a ligand for endothelium-specific receptor tyrosine kinase Tie-2. Gene-targeting analyses of either Ang1 or Tie2 in mice reveal an essential role of Ang1/Tie2 signaling in developmental vascular formation (1–3). In adult vasculature, Ang1/Tie2 signal maintains quiescence of mature blood vessels by enhancing vascular integrity and endothelial survival (4–6). However, Tie2 signaling is also involved in physiological and pathological angiogenesis, as opposed to the maintenance of vascular quiescence (4, 5, 7–9). As to this question, we and Alitalo’s group (10, 11) have recently clarified that the distinct localization of Tie2 in the presence or absence of cell-cell contact determines the specificity of downstream signaling of Tie2. Ang1 induces trans-association of Tie2 at endothelial cell-cell contacts, whereas Tie2 is anchored to extracellular matrix by Ang1 in the absence of cell-cell contacts. Trans-associated Tie2 bridged by Ang1 and extracellular matrix-anchored Tie2 by Ang1 induce distinct signaling pathways preferable for vascular quiescence and angiogenesis via AKT and extracellular signal-regulated kinase (ERK) 1/2, respectively. By performing DNA microarray analysis, we also revealed that a distinct set of genes is regulated by Ang1 in the presence or absence of cell-cell contacts. Among them, Krüppel-like factor 2 (KLF2), Krüppel-like factor 2; COMP, cartilage oligomeric matrix protein; MEF2, myocyte enhancer factor 2; ERK, extracellular signal-regulated kinase; PI3K, phosphoinositide 3-kinase; VEGF, vascular endothelial growth factor; VEGRF2, VEGF receptor 2; VCAM-1, vascular cell adhesion molecule-1; GFP, green fluorescent protein; GST, glutathione S-transferase; HUVEC, human umbilical vein endothelial cell; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HDAC, histone deacetylase; PCAF, p300/CBP-associated factor; CBP, CREB-binding protein; CREB, cAMP-response element-binding protein; BSA, bovine serum albumin; siRNA, small interfering RNA; MOPS, 4-morpholinepropanesulfonic acid; wt, wild type; mut, mutant; Luc, luciferase; CA, constitutive active.

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The abbreviations used are: Ang1, angiopoietin-1; KLF2, Krüppel-like factor 2; COMP, cartilage oligomeric matrix protein; MEF2, myocyte enhancer factor 2; ERK, extracellular signal-regulated kinase; PI3K, phosphoinositide 3-kinase; VEGF, vascular endothelial growth factor; VEGRF2, VEGF receptor 2; VCAM-1, vascular cell adhesion molecule-1; GFP, green fluorescent protein; GST, glutathione S-transferase; HUVEC, human umbilical vein endothelial cell; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HDAC, histone deacetylase; PCAF, p300/CBP-associated factor; CBP, CREB-binding protein; CREB, cAMP-response element-binding protein; BSA, bovine serum albumin; siRNA, small interfering RNA; MOPS, 4-morpholinepropanesulfonic acid; wt, wild type; mut, mutant; Luc, luciferase; CA, constitutive active.

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pel-like factor 2 (KLF2) was selectively induced by Ang1 in the presence of cell-cell contacts (10).

KLF2 is a zinc finger family of transcription factor functioning in both vascular smooth muscle cells and endothelial cells and is, therefore, essential in developmental vascular formation (12–15). The KLF2 knock-out mice exhibit impaired blood vessel formation attributable to the lack of smooth muscle cell recruitment (12). KLF2 expression in endothelium is induced by laminar shear stress and is thought to act as a molecular transducer of laminar shear stress (16–18). In the adult human vasculature, KLF2 expression is found at laminar segments of blood vessels and is decreased at branched points, which are more prone to develop atherosclerotic lesions (18), suggesting the role of KLF2 as a flow-mediated atheroprotective factor. Consistently, more than 15% of flow-regulated genes are dependent upon flow-mediated KLF2 induction (19).

It has been reported that laminar shear stress induces KLF2 expression via an ERK5–myocyte enhancer factor 2 (MEF2) signaling pathway (19, 20). The MEF2 family of transcription factors is composed of four members (MEF2A, MEF2B, MEF2C, and MEF2D) and is known as a regulator of vascular functions (21–23). Therefore, KLF2 may act downstream of MEF2 to regulate vascular functions.

KLF2 controls endothelial functions by negatively regulating inflammation and angiogenesis, thereby contributing to the maintenance of vascular quiescence (24–27). KLF2 inhibits cytokine-mediated induction of pro-inflammatory targets such as vascular cell adhesion molecule-1 (VCAM-1) and E-selectin (24, 25). In addition to anti-inflammatory action, KLF2 also down-regulates expression of vascular endothelial growth factor (VEGF) receptor 2 (VEGFR2), leading to the inhibition of VEGF-induced angiogenesis and hyperpermeability (26, 27). Similarly, Ang1 functions as an anti-inflammatory and anti-permeability factor (6, 29). Ang1 inhibits VEGF-stimulated leukocyte adhesion to endothelium by reducing expression of cell adhesion molecules such as VCAM-1 and E-selectin (30). Furthermore, Ang1 counteracts VEGF-induced hyperpermeability in vitro and in vivo (29, 31–33). Our previous data that Ang1 induced KLF2 expression (10) and the common roles by Ang1 and KLF2 for vascular quiescence prompted us to test our hypothesis that Ang1/Tie2 signal may maintain the vascular quiescence through KLF2 induction and to investigate how KLF2 is induced by Ang1/Tie2 signaling.

In this study, we found that Ang1/Tie2 signal stimulates transcriptional activity of MEF2 through a phosphoinositide 3-kinase (PI3K)/AKT pathway to induce KLF2 expression. Moreover, we revealed that Ang1-induced signaling functionally competes with VEGF-induced inflammatory responses.

**EXPERIMENTAL PROCEDURES**

**Reagents, Antibodies, siRNAs, and Recombinant Protein—**

Ang1 and cartilage oligomeric matrix protein (COMP)-Ang1 were prepared as described before (34). VEGF was purchased from R&D Systems. Wortmannin and AKT inhibitor IV were obtained from Calbiochem. We generated anti-KLF2 monoclonal antibody against amino acids 2–34 of human KLF2. Anti-green fluorescent protein (GFP) antibody was prepared as described before (35). Other antibodies were purchased as follows: anti-tubulin, anti-ERK5, anti-FLAG (M2), and anti-β-actin from Sigma-Aldrich; anti-MEF2 from Santa Cruz Biotechnology; anti-phospho-AKT, anti-AKT, anti-phospho-ERK1/2, and anti-ERK1/2 from Cell Signaling Technology; horseradish peroxidase-coupled sheep anti-mouse and anti-rabbit IgG from GE Healthcare Life Sciences; and Alexa Fluor 488-labeled secondary antibody from Molecular Probes. Stealth siRNAs targeting the genes indicated below were purchased from Invitrogen: human KLF2 (HSS145585, HSS145587), human ERK5 (HSS140815), human AKT1 (validated stealth RNA interference: 12935-001), human AKT2 (validated stealth RNA interference: 12937-40), human MEF2A (HSS106435, HSS106436), human MEF2C (HSS106438, HSS106439), and human MEF2D (HSS106441, HSS106442). Glutathione S-transferase (GST) fusion protein containing transactivating domain of MEF2C (GST-MEF2C) was prepared as described before (36).

**Plasmids and Adenoviruses—**

A luciferase reporter plasmid containing the proximal 221-bp region of KLF2 promoter (KLF2wt–Luc) was kindly provided by M. K. Jain (Case Western Reserve University). The MEF2-binding site of the KLF2wt–Luc plasmid was mutated using the QuikChange site-directed mutagenesis kit (Stratagene) using the KLF2wt–Luc vector as a template. Expression plasmids encoding MEF2C and constitutive active mutants of AKT (pCEFL–myrAKT) and PI3K-γ (pcDNA3–PI3K-CAAX) and a luciferase reporter plasmid containing a single MEF2-binding site (pGL3–MEF2) have already been described (36–38). A cDNA encoding full-length MEF2C amplified by PCR using pCEFL–GST–MEF2C as a template was inserted into p3xFLAG CMV10 vector (Sigma-Aldrich) or cloned into pCMV–BD vector (Stratagene) to construct the plasmid expressing a Gal4 DNA-binding domain (DBD)–MEF2C fusion protein (Gal4/MEF2C). Plasmids encoding Gal4/MEF2C mutant proteins (Thr–293, Thr–300, Ser–378 (phosphorylation sites by ERK5 and p38), and Thr–404 (potential phosphorylation site by AKT)) were replaced with Ala, and 6 Lys residues (Lys–116, Lys–119, Lys–234, Lys–239, Lys–252, Lys–264; acetylation sites by p300) replaced with Arg were generated using the QuikChange site-directed mutagenesis kit. An expression vector encoding FLAG-tagged HDAC5 was generously obtained from C. Grozinger (Harvard University). Other vectors were purchased as follows: pRL–SV40 and pRL–TK from Promega Corp.; pEGFP–C1 from Clontech; and pFR from Stratagene. Recombinant adenovirus vectors encoding GFP and constitutively active form of AKT were kindly provided by H. Kurose (Kyushu University) and Y. Fujio (Osaka University), respectively.

**Cell Culture, Transfection, siRNA-mediated Protein Knockdown, and Adenovirus Infection—**

Human umbilical vein endothelial cells (HUVECs) were cultured as described previously (10) and used for experiments before passage 7. HUVECs were placed on collagen-coated plates at a density of 2,000 cells/cm² and 40,000 cells/cm² and cultured overnight to obtain sparse and confluent cell cultures, respectively. U937 cells, a human monocyte-like cell line, were cultured in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum, 50 units/ml penicillin, and 50 µg/ml streptomycin. HUVECs were transfected using Lipofectamine 2000 reagent (Invitrogen) and Lipofectamine Plus reagent (Invitrogen) according to the manufac-
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for 3 h. After the stimulation, total RNA was purified using TRIzol (Invitrogen). Quantitative real-time reverse transcription (RT)-PCR was carried out using Quantitect SYBR Green RT-PCR kit (Qiagen) as described before (10). For each reaction, 100 ng of total RNA was transcribed for 10 min at 50°C followed by a denaturing step at 95°C for 5 min and 40 cycles of 10 s at 95°C and 30 s at 60°C. Fluorescence data were collected and analyzed using Mastercycler ep realplex (Eppendorf). The primers used for amplification were as follows: for human KLF2, 5′-CTCACACAAAGGTTGCATCTG-3′ and 5′-CCGGTTGCTTTCGAGTGTG-3′; for human VCAM1, 5′-CAAATCCTTGTATGTGCTTCTC-3′ and 5′-TGGACCTTTGCTCAACG-3′; for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-ATGGGAAAGGTGAGGTC-3′ and 5′-GGGTGCTATTGATGGCAAC-3′. For normalization, expression of human GAPDH was determined in parallel as an endogenous control.

Detection of KLF2 Protein Expression—To examine the KLF2 protein expression induced by COMP-Ang1, confluent HUVECs plated on a collagen-coated dish were starved in Humedia-EB2 medium (Kurabo) containing 0.5% fetal calf serum for 12 h and stimulated with 400 ng/ml COMP-Ang1 for the periods as indicated in the figure legends. After the stimulation, the cells were washed once with ice-cold phosphate-buffered saline, harvested by scraping, and pelleted by centrifugation at 4,000 × g for 10 min at 4°C. The cell pellets were then lysed at 4°C in radiolabelled precipitation buffer containing 50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and 1× protease inhibitor mixture. The cell lysates were subjected to SDS-PAGE and Western blot analysis as described above (39).

Detection of ERK5, AKT, and ERK1/2 Activities—Sparsely and confluent HUVECs were transfected with siRNA duplexes using Lipofectamine RNAiMAX reagent (Invitrogen) and used for the experiments 48–72 h after transfection. HUVECs were infected with adenovirus vectors at the appropriate multiplicity of infection. Forty-eight hours after infection, the cells were used for experiments.

Real-time Reverse Transcription-PCR—Sparse and confluent HUVECs were starved in medium 199 containing 1% BSA for 6 h and stimulated with COMP-Ang1 (C-Ang1) at the concentrations indicated at the bottom (ng/ml) for 3 h. The cells were subjected to real-time RT-PCR analysis to determine the expression level of KLF2 mRNA as described in the figure legends. After the stimulation, total RNA was extracted and subjected to real-time RT-PCR analysis to determine the expression level of KLF2 mRNA as described under "Experimental Procedures." Bar graphs show relative mRNA levels of KLF2 normalized to that of GAPDH. KLF2 mRNA levels are expressed relative to that in the untreated cells and shown as means ± S.D. of five independent experiments. C, confluent HUVECs were starved in HuMedia EB2 medium containing 0.5% fetal calf serum for 12 h and stimulated with COMP-Ang1 (for the periods indicated at the top (h)). Cell lysates were subjected to Western blot analysis with anti-KLF2 (top panel) and anti-tubulin (bottom panel) antibodies. D, confluent HUVECs were stimulated with vehicle (control), 600 ng/ml Ang1 (Ang1), and COMP-Ang1 (C-Ang1) for 1 h. KLF2 mRNA levels were determined as described in A. Values are expressed relative to that in the control cells and shown as means ± S.D. of six independent experiments. Significant differences between two groups (A) or from the control (B and D) are indicated as **, p < 0.01 or ***, p < 0.001. n.s. indicates no significance between two groups.

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FIGURE 1. Ang1 induces KLF2 expression in confluent HUVECs. A, sparse (open bars) and confluent (closed bars) HUVECs were starved in medium 199 containing 1% BSA for 6 h and stimulated with COMP-Ang1 (C-Ang1) at the concentrations indicated at the bottom (ng/ml) for 1 h. After the stimulation, total RNA was extracted and subjected to real-time RT-PCR analysis to determine the expression level of KLF2 mRNA as described under "Experimental Procedures." Bar graphs show relative mRNA levels of KLF2 normalized to that of GAPDH. KLF2 mRNA levels are expressed relative to that in confluent cells stimulated with 400 ng/ml COMP-Ang1. Data are shown as means ± S.D. of six independent experiments. B, confluent HUVECs starved for 6 h were stimulated with 400 ng/ml COMP-Ang1 for the periods indicated at the bottom (h) (COMP-Ang1 was used at the concentration of 400 ng/ml throughout the following experiments). KLF2 mRNA levels were analyzed by real-time RT-PCR as described in A. Values are expressed relative to that in the unstimulated cells and shown as means ± S.D. of five independent experiments. C, confluent HUVECs were starved in HuMedia EB2 medium containing 0.5% fetal calf serum for 12 h and stimulated with COMP-Ang1 (for the periods indicated at the top (h)). Cell lysates were subjected to Western blot analysis with anti-KLF2 (top panel) and anti-tubulin (bottom panel) antibodies. D, confluent HUVECs were stimulated with vehicle (control), 600 ng/ml Ang1 (Ang1), and COMP-Ang1 (C-Ang1) for 1 h. KLF2 mRNA levels were determined as described in A. Values are expressed relative to that in the control cells and shown as means ± S.D. of six independent experiments. Significant differences between two groups (A) or from the control (B and D) are indicated as **, p < 0.01 or ***, p < 0.001. n.s. indicates no significance between two groups.
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FIGURE 2. MEF2 is responsible for Ang1-induced KLF2 expression. A, structures of KLF2wt-Luc and KLF2mut-Luc reporter constructs are shown. B, confluent HUVECs were transfected with either KLF2wt-Luc (left) or KLF2mut-Luc (right) reporter constructs together with pRL-SV40 vector. After transfection, the cells were stimulated without (−) or with (+) COMP-Ang1 (C-Ang1) in 1% BSA-containing medium 199 for 24 h. The medium was replaced with the freshly prepared same medium every 6 h. After the stimulation, the cells were collected, and the lysates were assayed for firefly and Renilla luciferase activities as described under “Experimental Procedures.” The data represent firefly luciferase activity normalized by the Renilla luciferase activity present in each cellular lysate. Values are expressed as a percentage relative to that observed in the KLF2wt-Luc transfected cells treated with vehicle and shown as mean ± S.D. of three independent experiments. C, confluent HUVECs transfected with control siRNA (control) or with two independent MEF2 siRNA mixtures (MEF2#1 and MEF2#2: each mixture contains siRNAs targeting MEF2A, MEF2C, and MEF2D) were stimulated as described in the legend for Fig. 1 (panel D). KLF2 mRNA levels were determined as described in the legend for Fig. 1 (panel A). Values are expressed relative to that in the control siRNA-transfected cells treated with vehicle and shown as means ± S.D. of three independent experiments. D, confluent HUVECs transfected with control siRNA or with MEF2 siRNA mixture (MEF2#1) were starved and stimulated with vehicle (−) or COMP-Ang1 (+) as described in the legend for Fig. 1 (panel C). Cell lysates were subjected to Western blot analysis with anti-KLF2 (top panel), anti-MEF2 (middle panel), and anti-tubulin (bottom panel) antibodies. In B and C, significant differences between two groups are indicated as **, p < 0.01, or *** p < 0.001. n.s. indicates no significance between two groups.

FIGURE 3. Ang1-induced KLF2 expression does not require ERK5. A, sparse (left) and confluent (right) HUVECs were starved in medium 199 containing 1% BSA for 6 h and stimulated with vehicle (−) or COMP-Ang1 (C-Ang1) (+) for 15 min. To measure the ERK5 activity, in vitro kinase assay was performed using anti-ERK5 immunoprecipitates from the corresponding cell lysates as described under “Experimental Procedures.” **P-labeled substrates are shown at the top (MEF2C (kinase)). In parallel, cell lysates were subjected to Western blot analysis with anti-ERK5 (ERK5), anti-phosphoAKT (pAKT), anti-AKT (AKT), anti-phosphoERK1/2 (pERK1/2), and anti-ERK1/2 (ERK1/2) antibodies. B, confluent HUVECs transfected with control siRNA (left) or ERK5 siRNA (right) were starved and stimulated with vehicle (−) or COMP-Ang1 (+) as described in the legend for Fig. 1 (panel D). KLF2 mRNA levels were determined and expressed as described in the legend for Fig. 2 (panel C). Values are shown as means ± S.D. of five independent experiments. Significant differences between two groups are indicated as ***, p < 0.001. C, confluent HUVECs transfected with control or ERK5 siRNA were starved and stimulated as described in the legend for Fig. 1 (panel C). Cell lysates were subjected to Western blot analysis with anti-KLF2 (top panel), anti-ERK5 (middle panel), and anti-tubulin (bottom panel) antibodies.

The ERK5 activity, in vitro kinase assay was performed as described previously (40). Briefly, endogenous ERK5 was immunoprecipitated from aliquots of cell lysate with anti-ERK5 antibody at 4 °C for 3 h, and the immunocomplexes were recovered with protein G-Sepharose beads (GE Healthcare Life Sciences). Beads were washed three times with phosphate-buffered saline containing 1% Nonidet P-40 and 2 mM sodium vanadate, once with washing buffer containing 100 mM Tris at pH 7.5 and 0.5 mM LiCl and once with kinase reaction buffer containing 12.5 mM MOPS at pH 7.5, 12.5 mM β-glycerophosphate, 7.5 mM MgCl₂, 0.5 mM EGTA, 0.5 mM sodium vanadate, and 0.5 mM sodium fluoride. Samples were then resuspended in 15 μl of kinase reaction buffer containing 3 μg of GST-MEF2C, 1 μCi of [γ-32P]ATP, and 20 μM cold ATP and incubated at 37 °C for 90 min. 32P-labeled substrates were separated by SDS-PAGE and detected by autoradiography. To evaluate the phosphorylation of AKT and ERK1/2, aliquots of cell lysate were subjected to Western blot analysis with anti-phospho-AKT and anti-phospho-ERK1/2 antibodies, respectively. The total contents of ERK5, AKT, and ERK1/2 in each cell lysate were also assayed in a parallel run using corresponding antibodies.

Luciferase Reporter Assay—Luciferase reporter assay was carried out as described before (36, 40). Confluent HUVECs plated on a collagen-coated 12-well plate were transfected with different expression vectors, together with reporter plasmids as described in the figure legends. The total amount of plasmid DNA was adjusted with empty vector. To examine the effect of COMP-Ang1, the cells were starved and stimulated as described in the figure legends. The cells were lysed using passive lysis buffer (Promega), and luciferase activities in cell extract were determined using a Dual-Luciferase assay system (Promega).

Detection of Subcellular Localization of FLAG-tagged HDAC5—Confluent HUVECs plated on a collagen-coated glass base dish were transfected with the plasmid encoding FLAG-tagged histone deacetylase (HDAC) 5. Twenty-four h after the transfection, the cells were starved in medium 199 containing 0.5% BSA for 6 h and subsequently stimulated with vehicle, COMP-Ang1, or VEGF for 3 h. After the stimulation, the cells were fixed and stained with anti-FLAG antibody as
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FIGURE 4. Ang1 does not induce nuclear export of HDAC5. A, confluent HUVECs plated on a collagen-coated glass base dish were transfected with the plasmid encoding FLAG-tagged HDAC5. After 24 h, the cells were starved in medium 199 containing 0.5% BSA for 6 h and stimulated with vehicle (control, upper panel), COMP-Ang1 (C-Ang1; middle panel), or 50 ng/ml VEGF (bottom panel) for 3 h. After the stimulation, the cells were fixed, immunostained with anti-FLAG antibody, and visualized with Alexa Fluor 488-conjugated secondary antibody. Alexa Fluor 488 and phase contrast images are shown at the left and right columns, respectively. B, nuclear export of HDAC5 by vehicle (control), COMP-Ang1 (C-Ang1), and VEGF observed in A was quantified. The number of cells expressing FLAG-tagged HDAC5 in the cytoplasm was counted and expressed as a percentage relative to the total number of cells expressing FLAG-tagged HDAC5. At least 100 cells were scored for each treatment. Values are expressed as means ± S.D. of three independent experiments. Significant difference from the control is indicated as ***, p < 0.001. n.s. indicates no significant difference from the control.

described before (10). Protein reacting with antibody was visualized with Alexa Fluor 488-conjugated secondary antibody. Alexa Fluor 488 and phase contrast images were recorded with an Olympus IX-81 inverted fluorescence microscope. The number of cells expressing FLAG-tagged HDAC5 in the cytoplasm among FLAG-tagged HADC5-expressing cells was counted. Nuclear export of HDAC5 was determined by the HDAC5 in the cytoplasm instead of the nucleus. At least 100 cells were scored for each experiment.

Monocyte Adhesion Assay—HUVECs transfected with control or KLF2 siRNA were placed on collagen-coated 24-well plates at the density of 40,000 cells/cm², cultured overnight, and starved in medium 199 containing 0.5% BSA for 2 h. The cells were then washed three times with prewarmed Hank’s buffered salt solution (Invitrogen) and fixed with 2% formaldehyde. Phase contrast and PKH67 fluorescent images were recorded with an Olympus IX-81 inverted fluorescence microscope. The adherent U937 cells were quantified by measuring fluorescent intensity at five randomly selected fields in each well using MetaMorph 6.1 software (Molecular Devices Corp.).

Statistical Analysis—The values are expressed as means ± S.D. Statistical significance was determined using one-way analysis of variance, two-way analysis of variance, or unpaired t test. p values < 0.05 were considered statistically significant.

RESULTS

Ang1 Induces KLF2 Expression in Confluent but Not Sparse Cultures of HUVECs—To first examine the KLF2 expression downstream of trans-associated Tie2 at cell-cell contacts and cell-substratum contact-anchored Tie2 in response to Ang1, HUVECs were stimulated with COMP-Ang1, a potent Ang1 variant, under either confluent or sparse culture condition. COMP-Ang1 concentration-dependently induced KLF2 mRNA expression in confluent HUVECs but not in the sparse cells (Fig. 1A). Consistently, KLF2 protein expression was upregulated upon stimulation with COMP-Ang1 (Fig. 1C). KLF2 mRNA and protein expression by COMP-Ang1 peaked at 1 h after the stimulation and then declined to the basal level by 6 h (Fig. 1, B and C). KLF2 mRNA level was increased in response to native Ang1 as well as COMP-Ang1 (Fig. 1D).

Taken together with the previously reported evidence that Ang1 induces trans-association of Tie2 in the presence of cell-cell contacts (10, 11), these results suggest that trans-association of Tie2 at cell-cell contacts is capable of inducing KLF2 expression.

Ang1 Induces KLF2 Expression through MEF2—Next, we sought to delineate the signaling pathway responsible for Ang1-induced KLF2 expression. It has been reported that laminar shear stress and statins activate the KLF2 promoter via a single MEF2-binding site located at −120/−111 bp upstream from the transcriptional initiation site (19, 41, 42). Thus, we investigated the role of MEF2 in Ang1-induced KLF2 expression. HUVECs were transfected with either luciferase reporter plasmid in which the reporter is driven by the proximal 221-bp region (−221 bp upstream from the initiation site) of KLF2 promoter (KLF2wt-Luc) or by its MEF2-binding site-mutated promoter (KLF2mut-Luc) (Fig. 2A). COMP-Ang1 significantly induced KLF2 promoter-regulated reporter activity, whereas it failed to induce reporter activation when KLF2 promoter lacked MEF2-binding site (Fig. 2B), suggesting the indispensable role of MEF2 in Ang1-induced KLF2 expression. To further confirm the requirement of MEF2 for Ang1-induced KLF2 expression, we employed an siRNA technique to down-regulate MEF2 family proteins including MEF2A, MEF2C, and MEF2D. Depletion of these MEF2 proteins inhibited COMP-Ang1-induced both KLF2 mRNA expression and KLF2 protein expression (Fig. 2, C and D and supplemental Fig. S1). These findings
indicate that KLF2 expression induced by Ang1 depends upon MEF2.

Ang1-induced KLF2 Expression Is Not Mediated by the Signaling Pathways Involving ERK5 and HDAC5—ERK5 stimulates transcriptional activity of MEF2 factors by phosphorylating their transactivating domains (36, 43, 44). Previous reports indicate that KLF2 expression is severely impaired in ERK5-null mouse embryo (45) and that laminar shear stress stimulates ERK5 activity to induce MEF2-dependent KLF2 expression (19). Therefore, we examined whether Ang1 stimulates ERK5 activity under either confluent or sparse conditions to investigate the involvement of ERK5 in KLF2 induction by Ang1. COMP-Ang1 preferentially activated ERK5 as well as ERK1/2 in sparse HUVECs (Fig. 3A). However, stimulation of confluent cells with COMP-Ang1 did not enhance ERK5 kinase activity, although AKT was potently activated under this condition (Fig. 3A). These results suggest that Ang1 induces KLF2 expression independently of ERK5. Consistently, depletion of ERK5 by siRNAs did not affect COMP-Ang1-induced KLF2 mRNA and protein expression (Fig. 3, B and C).

Class II HDACs, consisting of HDAC4, HDAC5, HDAC7, and HDAC9, interact with MEF2 and repress the expression of MEF2 target genes (46). Phosphorylation of class II HDACs by calcium/calmodulin-dependent protein kinase and protein kinase D results in their nuclear exclusion, leading to the enhancement of MEF2 transcriptional activity (46–48). Therefore, we examined whether Ang1 induces nuclear export of class II HDACs to enhance MEF2 activity. COMP-Ang1 did not induce nuclear export of FLAG-tagged HDAC5, although VEGF did (Fig. 4), suggesting that Ang1-induced KLF2 expression does not depend upon the inhibition of class II HDACs.

A PI3K/AKT Pathway Is Responsible for Ang1-induced KLF2 Expression—Ang1 is able to induce KLF2 expression only under confluent condition as described above (Fig. 1A). Under this condition, an AKT pathway is preferentially activated by Ang1 (Fig. 3A). These evidences prompted us to investigate whether the AKT pathway is responsible for KLF2 induction by Ang1. COMP-Ang1-induced KLF2 expression was completely blocked by wortmannin and AKT inhibitor IV, inhibitors for PI3K and AKT, respectively (Fig. 5, A and B). Furthermore, we examined the involvement of AKT in Ang1-mediated KLF2 expression by depleting AKT using siRNAs for AKT. Depletion of AKT prevented both COMP-Ang1-induced KLF2 mRNA expression and COMP-Ang1-induced KLF2 protein expression (Fig. 5, C and D). These results indicate that a PI3K/AKT pathway is indispensable for Ang1-induced KLF2 expression.

A PI3K/AKT Pathway Stimulates Transcriptional Activity of MEF2—To clarify whether a PI3K/AKT pathway stimulates MEF2-dependent transcription, HUVECs were transfected with a plasmid expressing luciferase reporter gene under the control of a single MEF2 site (MEF2-Luc) together with the plasmid encoding constitutive active AKT (AKT-CA) or PI3K (PI3K-CA). The reporter gene activity was only slightly stimulated by AKT-CA or PI3K-CA (Fig. 6A). However, coexpression of MEF2C significantly enhanced AKT-CA- or PI3K-CA-induced reporter gene expression (Fig. 6A). To further investigate whether MEF2-dependent transcription stimulated by PI3K-CA and AKT-CA is ascribed to the enhanced transcriptional activity, we fused full-length MEF2C with DNA-binding domain of yeast Gal4 protein (Gal4/MEF2C) and tested the direct effect of AKT and PI3K on transcriptional activity of Gal4/MEF2C. AKT-CA and PI3K-CA potently stimulated the transcriptional activity of Gal4/MEF2C but not that of Gal4 (Fig. 6B). In addition, stimulation with COMP-Ang1 evoked Gal4/MEF2C-dependent reporter gene expression (Fig. 6C). Collectively, these findings indicate that Ang1/Tie2 signal stimulates ERK5 activity to induce MEF2-dependent KLF2 expression via a PI3K/AKT pathway.

A PI3K/AKT/MEF2 Signaling Axis Stimulates the KLF2 Promoter—We further investigated the functional role of a PI3K/AKT/MEF2 pathway in Ang1-induced KLF2 expression. When HUVECs were transfected with the KLF2wt-Luc reporter gene along with the plasmid encoding GFP, AKT-CA, or PI3K-CA, AKT-CA and PI3K-CA induced more luciferase expression than GFP (Fig. 6D). The stimulatory effects of
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FIGURE 6. A PI3K/AKT pathway stimulates transcriptional activity of MEF2, leading to KLF2 expression. A, confluent HUVECs were cotransfected with pGL3-MEF2 and pRL-TK vectors, together with the plasmid encoding either GFP or MEF2C as indicated at the bottom. The expression vector for GFP, Akt-CA, or PI3K-CA was also included in the transfection mixture as shown at the left. The cells were lysed 24 h after the transfection. Luciferase activities were assayed and presented as described in the legend for Fig. 2 (panel A). Data are expressed as a percentage relative to that observed in the cells expressing both PI3K-CA and MEF2C and shown as mean ± S.D. of four independent experiments. B, confluent HUVECs were cotransfected with pFR and pRL-TK vectors, along with the plasmid expressing either Gal4 (left) or Gal4-MEF2C (right) as indicated at the bottom. The expression vector for GFP, Akt-CA, or PI3K-CA was also included in the transfection mixture as shown at the left. After 24 h of incubation, luciferase activities were assayed and represented as described in the legend for Fig. 2 (panel B). Data are expressed as a percentage relative to that observed in the cells expressing both Gal4-MEF2C and PI3K-CA and shown as mean ± S.D. of three independent experiments. C, confluent HUVECs were transfected with the plasmid encoding Gal4-MEF2C together with pGal4-Luc and pRL-SV40 reporter plasmids were starved and stimulated as described in the legend for Fig. 2 (panel C). Luciferase activities were assayed and presented as described in the legend for Fig. 2 (panel B). Data are expressed as a percentage relative to that observed in the control cells and shown as mean ± S.D. of four independent experiments. D, confluent HUVECs were cotransfected with pRL-TK vector, along with either KLF2wt-Luc or KLF2mut-Luc reporter gene, and also with the expression vector for either GFP or MEF2C as indicated at the bottom. The plasmid encoding GFP, Akt-CA, or PI3K-CA was also included in the transfection mixture as shown at the left. Twenty-four h after the transfection, luciferase activities were assayed and presented as described in the legend for Fig. 2 (panel D). Data are expressed as a percentage relative to that in the cells transfected with KLF2wt-Luc along with the vector encoding GFP and shown as mean ± S.D. of four independent experiments. E, confluent HUVECs were infected without (-) or with adenoviruses encoding either GFP or Akt-CA as indicated at the top. Forty-eight h after infection, the cells were collected, and the lysates were subjected to Western blot analysis with anti-KLF2 (KLF2), anti-phosphoAKT (pAKT), anti-AKT (AKT), anti-GFP (GFP), and anti-β-actin (β-actin) antibodies. Significant differences between two groups (A, B, and D) or from the control (C) are indicated as **, p < 0.005; ***, p < 0.001, or ****, p < 0.0001.

AKT-CA and PI3K-CA on the reporter gene activity were significantly augmented by coexpression of MEF2C (Fig. 6D). AKT-CA and PI3K-CA did not stimulate the luciferase expres-

Ang1 Inhibits VEGF-induced Inflammatory Responses through KLF2—We finally addressed the significance of Ang1/Tie2-dependent KLF2 expression in the cells. KLF2 has potent

sion driven by the mutant promoter that lacks the MEF2-binding site (the KLF2mut-Luc), even in the presence of MEF2C (Fig. 6D). In addition, adenovirus-mediated overexpression ofAkt-CA potently induced both mRNA and protein expression of KLF2 in HUVECs (Fig. 6E and supplemental Fig. S2). Collectively, these results suggest that Ang1/Tie2 signal induces KLF2 expression through a PI3K/AKT/MEF2 signaling axis.

How does a PI3K/AKT signaling enhance transcriptional activity of MEF2? MEF2 activity is known to be regulated by its posttranslational modification such as phosphorylation and acetylation (49). We examined the effect of a PI3K/AKT pathway on posttranslational modification of MEF2C. We constructed the plasmids encoding Gal4/MEF2C mutants: 1) Gal4/MEF2C-ERK5/p38mut in which potential phosphorylation sites of MEF2C by p38 and ERK5 mitogen-activated protein (MAP) kinases (Thr-293, Thr-300, and Ser-387 corresponding to those in human MEF2C) were replaced by Ala (43) and 2) Gal4/MEF2C-AKT mutant in which consensus phosphorylation site of MEF2C by AKT (Thr-404 corresponding that in human MEF2C) was mutated to Ala; and 3) Gal4/MEF2C-6KR in which the 6 Lys residues in MEF2C acetylated by p300 (Lys-116, Lys-119, Lys-234, Lys-239, Lys-252, and Lys-264 corresponding to those in human MEF2C) were replaced by Arg (51). Using these plasmids, MEF2-dependent transcription was directly tested in the cells expressing PI3K-CA. Expression of PI3K-CA could stimulate transcriptional activity mediated by mutant MEF2C to a similar extent to wild type MEF2C (supplemental Fig. S3). These results suggest that the posttranslational modification by ERK5, p38, AKT, and p300 is not required for PI3K/AKT pathway-stimulated MEF2 transcriptional activity.
Ang1-induced KLF2 Expression via MEF2 Activation by PI3K/AKT

**DISCUSSION**

Ang1/Tie2 signal is involved in both angiogenesis and vascular quiescence in adult vasculature. Recently, we and another group have shown that endothelial cell-cell contacts specify downstream signaling pathways from Tie2 (10, 11). In the presence of cell-cell contacts, Ang1 induces trans-association of Tie2, leading to a preferential activation of the AKT pathway. In addition, we also found that KLF2 is specifically induced by trans-associated Tie2. Because KLF2 is a transcription factor involved in vascular quiescence, we hypothesized that KLF2 is responsible for Ang1/Tie2 signal-mediated vascular quiescence. To address this possibility, we tried to delineate the signaling pathways involved in Ang1-induced KLF2 expression. We found that Ang1/Tie2 signal stimulates transcriptional activity of MEF2 through a PI3K/AKT pathway, which in turn induces KLF2 expression.

MEF2 plays important roles not only in muscle development but also in regulation of blood vessels (21–23). MEF2C has been implicated as a regulator of endothelial integrity and permeability (21). Interestingly, MEF2C-deficient mice exhibit a similar defect in vascular development to KLF2-null mice (52). Our present data demonstrated that MEF2 is indispensable for Ang1-induced KLF2 expression. Therefore, defect of vascular development in Ang1-deficient mice might be partly due to the lack of MEF2-dependent KLF2 induction.

Several posttranslational modifications of MEF2 in the regulation of MEF2-mediated transcription has been reported (49). Among them, ERK5 regulates MEF2 transcriptional activity by phosphorylating its transcription activation domain (36, 43, 44). Parmar et al. (19) have reported that flow-mediated increase in KLF2 expression occurs via an ERK5/MEF2 pathway. Because ERK5 is essential for maintaining blood vessel integrity and is known to be stimulated by Ang1 (21, 22, 53), we examined whether ERK5 acts downstream of Ang1/Tie2 to induce KLF2 expression. Unexpectedly, Ang1 only stimulated the ERK5 activity in the absence of cell-cell contacts, and under this condition, KLF2 induction by Ang1 did not occur. Furthermore, knockdown of ERK5 did not prevent Ang1-induced KLF2 expression. Therefore, ERK5 appears to be dispensable for Ang1-induced KLF2 expression.

Our data show that Ang1/Tie2 signal utilizes a PI3K/AKT pathway instead of ERK5 to induce MEF2-dependent KLF2 expression. Similarly, it has been reported that a PI3K/AKT/
Ang1-induced KLF2 Expression via MEF2 Activation by PI3K/AKT

MEF2 pathway is used for insulin-like growth factor-induced myogenin expression (54). Huddleson et al. (42) have also shown that induction of KLF2 by shear stress requires a PI3K/chromatin-remodeling pathway. In contrast to our result, they claimed that AKT is not involved in this pathway, although it is activated by shear stress. We propose the involvement of AKT in Ang1-mediated KLF2 induction because 1) AKT inhibitor and knockdown of AKT both inhibit KLF2 mRNA and protein expression by COMP-Ang1; 2) AKT-CA induces the MEF2-dependent transcription and stimulates the KLF2 promoter cooperatively with MEF2; and 3) overexpression of AKT-CA induces both mRNA and protein expression of KLF2 in HUVECs. Currently, the reason for this discrepancy remains unclear, but it may be due to the different cell types used for the experiments. We performed the experiments with HUVECs, whereas they used an EOMA cell line. Alternatively, different stimuli such as Ang1 and shear stress may trigger distinct signaling pathways downstream of PI3K to stimulate KLF2 induction.

At present, a molecular link between AKT and MEF2 is still unknown. Recently, it has been reported that AKT directly phosphorylates transcriptional coactivator p300, leading to the association of MyoD with p300 and p300/CBP-associated factor (PCAF) (55). In addition, it has been also shown that p300 can physically interact with MEF2 as well as MyoD and enhances their transcriptional activity (28). Importantly, shear stress is shown to induce recruitment of p300 and PCAF into the KLF2 promoter (42). Therefore, the Ang1/Tie2/PI3K/AKT pathway may induce the association of MEF2 with transcriptional coactivators such as p300 and PCAF, thereby stimulating its transcriptional activity. p300 directly acetylates and stimulates MEF2 activity (51). However, PI3K-mediated activation of MEF2 is not mediated by such direct acetylation mechanism because transcriptional activity of acetylation-defective mutant of MEF2 could be stimulated by constitutive active PI3K (supplemental Fig. S3). Thus, further examination is required for clarifying the molecular link between AKT and MEF2.

KLF2 inhibits VEGF-induced angiogenesis and inflammation, possibly by maintaining vascular quiescence. Similarly, Ang1 counteracts VEGF-mediated inflammatory responses. Our present data suggest that inhibition of VEGF-mediated inflammatory responses by Ang1 depends upon KLF2. However, Ang1 also acts cooperatively with VEGF to induce angiogenesis in a certain situation (5, 7, 9). This opposite effect of Ang1 on VEGF-mediated responses may depend upon whether KLF2 is induced or not. In the presence of cell-cell contacts, Ang1/Tie2 signal is able to induce KLF2 expression and thereby inhibits VEGF-induced inflammation and angiogenesis. However, in the absence of cell-cell contacts, Ang1/Tie2 signal accelerates angiogenic signal because KLF2 is not induced in this condition, which accounts for the cooperation of Ang1 with VEGF. Therefore, KLF2 may be a key downstream factor from trans-associated Tie2 to maintain vascular quiescence.

In conclusion, we found that Ang1/Tie2 signal stimulates transcriptional activity of MEF2 through a PI3K/AKT pathway to induce KLF2 expression, thereby contributing to vascular quiescence.

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