VEGF Stimulates HDAC7 Phosphorylation and Cytoplasmic Accumulation Modulating Matrix Metalloproteinase Expression and Angiogenesis

Chang Hoon Ha, Bong Sook Jhun, Hung-Ying Kao, Zheng-Gen Jin

Objective—Histone acetylation/deacetylation plays an important role in the control of gene expression, tissue growth, and development. In particular, histone deacetylases 7 (HDAC7), a member of class Iia HDACs, is crucial in maintaining vascular integrity. However, whether HDAC7 is involved in the processes of vascular endothelial signaling and angiogenesis remains unclear. Here, we investigated the role of HDAC7 in vascular endothelial growth factor (VEGF) signaling and angiogenesis.

Methods and Results—We show for the first time that VEGF stimulated phosphorylation of HDAC7 at the sites of Ser178, Ser344, and Ser479 in a dose- and time-dependent manner, which leads to the cytoplasmic accumulation of HDAC7. Using pharmacological inhibitors, siRNA, and adenoviruses carrying dominant-negative mutants, we found that phospholipase Cγ/protein kinase C/protein kinase D1 (PKD1)-dependent signal pathway mediated HDAC7 phosphorylation and cytoplasmic accumulation by VEGF. Infection of ECs with adenoviruses encoding a mutant of HDAC7 specifically deficient in PKD1-dependent phosphorylation inhibited VEGF-induced angiogenic gene expression, including matrix metalloproteinases MT1-matrix metalloproteinase (MMP) and MMP10. Moreover, HDAC7 and its targeting genes were involved in VEGF-stimulated endothelial cell migration, tube formation, and microvessel sprouting.

Conclusions—Our results demonstrate that VEGF stimulates PKD1-dependent HDAC7 phosphorylation and cytoplasmic accumulation in endothelial cells modulating gene expression and angiogenesis. (Arterioscler Thromb Vasc Biol. 2008;28:1782-1788)

Key Words: VEGF ■ histone deacetylase 7 ■ protein kinase D ■ gene expression ■ endothelial cells ■ angiogenesis

Angiogenesis is essential for normal physiological processes such as organ development and wound healing. It is also critical to pathological processes such as tumor growth, atherosclerosis, rheumatoid arthritis, and diabetic retinopathy.1–3 Vascular endothelial growth factor (VEGF) is crucial for many angiogenic processes both in normal and pathological conditions.4–7 The binding of VEGF to its cognate receptors triggers intracellular signal cascades to mediate endothelial gene expression, cell migration, and angiogenesis.4–7 In particular, VEGF receptor 2 (VEGFR2)-mediated phospholipase Cγ (PLCγ)/protein kinase C (PKC) pathway regulates the activation of extracellular-regulated kinases and angiogenesis.4–7 Recently, we have further shown protein kinase D1 (PKD1), a novel serine/threonine protein kinase,9–12 is phosphorylated in endothelial cells (ECs) in response to VEGF, which mediates VEGF-induced activation of extracellular-regulated kinases and endothelial cell proliferation.13 However, the direct downstream targets of PKD1 in VEGF signaling are not fully understood.

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Histone acetylation/deacetylation plays an important role in the control of gene expression.14,15 Histone acetyltransferases stimulate transcription through acetylation of histones, resulting in relaxation of nucleosomes; and histone deacetylases (HDACs) deacetylates histone and repress transcription by condensing the chromatin. Recently we have shown that HDAC5, a member in class Iia HDAC family, is involved in VEGF-induced gene expression and angiogenesis.16 Interestingly, HDAC7, also belongs to class Iia HDAC family, has been shown to maintain vascular integrity by repressing matrix metalloproteinase 10 (MMP10).17 Specifically, both global deletion and vascular EC-specific deletion of HDAC7 gene in mice resulted in the defects of endothelial cells-cells contacts and consequent dilation and rupture of blood vessels.17 Silencing HDAC7 in ECs impairs cell migration and tube formation.18 HDAC7 also functions as a signal-dependent repressor of gene transcription during T-cell
development. However, the role of HDAC7 in VEGF signaling and function remains largely unclear.

Here, we demonstrate for the first time that VEGF stimulates phosphorylation of HDAC7 on Ser178/344/479 residues in ECs via PKD1-dependent pathway, and subsequent nuclear exclusion of HDAC7 and myocyte enhance factors 2 (MEF2) transcriptional activation. Importantly, PKD1-HDAC7 pathway modules VEGF-induced gene expression of matrix metalloproteinases and angiogenesis.

Methods
A detailed methods description is included in the supplemental materials (available online at http://atvb.ahajournals.org). Experiments were performed in primary bovine aortic ECs (BAECs) and human umbilical vein ECs (HUVECs). HUVECs were used for the experiments with small interfering RNA (siRNA) that specifically target on genes in human cells. HUVECs were transfected with 21-mer siRNA complexed to Lipofectamine 2000 (Invitrogen). BAECs and HUVECs were infected with adenoviruses encoding HA-HDAC7 wild-type (WT) and S178/344/479A mutant (S/A), and YFP-tagged HDAC7-WT. The immunofluorescence studies for HDAC7 subcellular localization were performed in fixed cells. mRNA was measured by reverse transcription polymerase chain reaction (RT-PCR) using TaqMan (Applied Biosystems). Cell migration was analyzed under wound closure. Capillary-like tube formation in Matrigel (BD Biosciences) for in vitro angiogenesis was performed, and microvessel sprouting was analyzed by aortic ring assay for ex vivo angiogenesis.

Results
VEGF Stimulates HDAC7 Phosphorylation in Endothelial Cells
To examine the potential role of HDAC7 in VEGF signaling, we first studied HDAC7 phosphorylation in ECs in response to VEGF. BAECs were stimulated with VEGF (20 ng/mL) at indicated time, and the phosphorylation of HDAC7 in cell lysates was determined by Western blots using phospho-specific HDAC7 antibodies, which recognizes the HDAC7 phosphorylated at Ser178, Ser344, or Ser479, respectively. We observed that VEGF rapidly induced HDAC7 phosphorylation within 2 minutes, and the activation reached a maximum after 15 minutes and still high at 90 minutes (Figure 1A). Because of similar pattern among 3 sites for HDAC7 phosphorylation in response to VEGF, hereafter we only show the results for HDAC7 phosphorylation at Ser479. We also observed that VEGF induced HDAC7 phosphorylation within 2 minutes, and the activation reached a maximum after 15 minutes and still high at 90 minutes (Figure 1A). Because of similar pattern among 3 sites for HDAC7 phosphorylation in response to VEGF, hereafter we only show the results for HDAC7 phosphorylation at Ser479. We also observed that VEGF induced HDAC7 phosphorylation in a dose-dependent manner. VEGF induced HDAC7 phosphorylation at a concentration as low as 1 ng/mL and achieved maximal activation at 10 to 100 ng/mL (Figure 1B). The induced phosphorylation of HDAC7 by VEGF was not limited on BAECs because VEGF also stimulated HDAC7 phosphorylation in a similar time- and dose-dependent manner in HUVECs (data not shown).

PKD1 Mediates HDAC7 Phosphorylation by VEGF
Next we studied the signal pathway(s) for VEGF-induced HDAC7 phosphorylation. Using selective neutralizing antibodies, we found that VEGFR2 but not VEGFR1 mediated VEGF-stimulated HDAC7 phosphorylation in ECs (supplemental data S1). Using specific pharmacological inhibitors, we further observed that PLCγ/PKC-dependent pathway, but not the phosphoinositide 3-kinase (PI3K)-dependent pathway, was required for VEGF-stimulated HDAC7 phosphorylation (supplemental data S2).

Recently we have demonstrated that PKC activation by VEGF leads to PKD1 phosphorylation and activation in ECs. Thus we decided to examine the potential role of PKD1 in VEGF-induced HDAC7 phosphorylation. A PKD inhibitor Go6976 significantly inhibited VEGF-induced HDAC7 phosphorylation in a dose-dependent manner (Figure 2A), suggesting the potential involvement of PKD in phosphorylation of HDAC7 by VEGF. To specifically define the role of PKD1 in VEGF-induced HDAC7 phosphorylation, we knocked down endogenous PKD1 in HUVECs using siRNA specifically targeting on human PKD1 as previously described. Silencing PKD1 by siRNA significantly inhibited VEGF-induced HDAC7 phosphorylation (Figure 2B), indicating that PKD1 is required for HDAC7 phosphorylation by VEGF in ECs.
To further determine whether PKD1 activation mediates HDAC7 phosphorylation by VEGF, we generated and tested adenoviruses expressing GFP-tagged PKD1 wild-type (Ad-GFP-PKD1-WT) and PKD1 kinase-negative (K612W) mutant (Ad-GFP-PKD1-KN). The dominant negative nature of this ATP-binding site mutant PKD1-KN has been previously characterized.26,27 Infection of BAECs with Ad-GFP-PKD1-WT and Ad-GFP-PKD1-KN resulted in robust expression of these PKD1 (Figure 2C). In contrast to that Ad-GFP-PKD1-WT enhanced basal and VEGF-induced HDAC7 phosphorylation, Ad-GFP-PKD1-KN significantly reduced VEGF-induced HDAC7 phosphorylation, compared to that in the control infected with adenoviruses encoding GFP alone. Together, these data demonstrate an essential role of PKD1 for VEGF-induced HDAC7 phosphorylation in ECs.

VEGF Stimulates HDAC7 Cytoplasmic Accumulation in Endothelial Cells Through PKD1-Dependent Phosphorylation

To gain insight into the functional significance of HDAC7 phosphorylation in VEGF-mediated signaling events, we studied the effect of VEGF on HDAC7 subcellular localization in ECs with HDAC7 fused with YFP. BAECs were infected with adenovirus expressing YFP-tagged HDAC7-WT, and then exposed to 20 ng/mL VEGF for indicated times. YFP-HDAC7 localization in cells was analyzed by fluorescence microscope. Without the treatment of VEGF, YFP-HDAC7 was located primarily in the nuclei of ECs (Figure 3A). In response to VEGF stimulation, YFP-HDAC7 in the cells was undergoing time-dependent nucleocytoplasmic shuttling. Cytoplasmic accumulation of HDAC7 was observed at 30 minutes after the application of VEGF and reached maximum at 2 hours, in which almost all YFP-HDAC7 was accumulated in the cytoplasm from nucleus. Exported YFP-HDAC7 was sustained in cytoplasm for several hours, and then gradually accumulated in the nucleus after 12-hour VEGF stimulation. At 24 hours after VEGF treatment, almost all of YFP-HDAC7 accumulated in the nuclei from cytoplasm. These results clearly show that VEGF stimulates dynamic nucleocytoplasmic shuttling of HDAC7 in ECs.

Consistent with a role of PLCγ/PKC pathway in regulation of HDAC7 phosphorylation in response to VEGF, we found that the inhibition of PLCγ and PKCs also abolished VEGF-induced HDAC7 cytoplasmic accumulation (supplemental data S3). However, PI3K inhibitor LY294002, calcium chelator BAPTA/AM, or calmodulin-dependent kinase inhibitor KN93 had no effect on HDAC7 cytoplasmic accumulation in response to VEGF (supplemental data S3). In agreement with the critical role of PKD1 in VEGF-induced HDAC7 phosphorylation, PKD inhibitor Go6976 and siRNA PKD1 blocked VEGF-induced HDAC7 cytoplasmic accumulation (Figure 3B and 3C).

To determine whether PKD1-dependent phosphorylation of HDAC7 at Ser178/344/479 is required for HDAC7 cytoplasmic accumulation, we studied subcellular localization of YFP-HDAC7-S/A mutant, in which serine178/344/479 residues were replaced with alanine. ECs were infected with Ad-YFP-HDAC7-WT and Ad-YFP-HDAC7-S/A. In basal condition without VEGF stimulation, both YFP-HDAC7-WT and YFP-HDAC7-S/A were localized in the nuclei of ECs (Figure 3D). After VEGF stimulation for 2 hours, YFP-HDAC7 was accumulated in the cytoplasm. In contrast, YFP-HDAC7-S/A remained in the nuclei after VEGF stimulation, suggesting that these 3 serines are essential for VEGF-induced cytoplasmic accumulation of HDAC7. In addition, YFP-HDAC7-S/A was also resistant to shuttling in response to PKC activator PMA stimulation in ECs (data not shown). Similar results were observed when ECs were infected with Ad-HA-HDAC7-WT and Ad-HA-HDAC7-S/A.
followed with immunofluorescence staining with anti-HA antibodies (Figure 3E). Collectively, these results demonstrate that PKD1-dependent phosphorylation of HDAC7 mediates its cytoplasmic accumulation in ECs in response to VEGF.

**PKD1-HDAC7 Pathway Modulates VEGF-Induced MEF2 Transcriptional Activation and Expression of MMPs**

HDAC7 have been shown to interact with MEF2 transcription factors resulting inhibition of MEF2-dependent gene expression as well as apoptosis in T cells.28 To examine whether HDAC7 modulates VEGF-induced MEF2 activation, we examined the effect of HA-HDAC7-S/A on VEGF-induced MEF2 transcriptional activation in ECs. Cotransfection of HA-HDAC7-S/A with 3xMEF2 luciferase plasmids abolished MEF2 transcriptional activation in response to VEGF (supplemental data S4).

It has been reported that MEF2-dependent gene MMP10 is induced in HDAC7 knockdown HUVECs.17 However, whether VEGF stimulates MMP10 via PKD1-HDAC7 pathway is unknown. Thus, we examined the expression of MMP10 with RT-PCR. We found that VEGF strongly stimulated MMP10 mRNA expression in a time-dependent manner, peaking at 1 hour (Figure 4A). Furthermore, we also found that VEGF stimulated expression of membrane type MMP 1 (MT1-MMP), which plays a crucial role in regulation of angiogenesis.29 Interestingly, both Ad-HA-HDAC7-S/A and Ad-GFP-PKD1-KN significantly inhibited such an induction of MMP10 and MT1-MMP by VEGF (Figure 4B), indicating a critical role of PKD1-dependent HDAC7 phosphorylation for VEGF induction of MMP10 and MT1-MMP expression in ECs.
HDAC7 and MT1-MMP Are Involved in VEGF-Induced EC Migration and Tube Formation

Next we asked whether HDAC7-dependent regulation of MT1-MMP is implicated in the processes of angiogenesis. Using the assay of cell migration during wound closure process, we found that VEGF-induced EC migration was substantially inhibited by Ad-HA-HDAC7-S/A (Figure 5A and supplemental data S5). Knockdown of MT1-MMP by siRNA in HUVECs also significantly inhibited VEGF-induced EC migration during wound closure (Figure 5B and supplemental data S6). In the assay of in vitro angiogenesis, the capability of primary ECs to form capillary-like tube structures was investigated on cultivation on Matrigel and quantified by measuring the length of capillary-like tube structure.30 VEGF-induced capillary-like tube formation in Matrigel was significantly attenuated by Ad-HA-HDAC7-S/A and MT1-MMP siRNA (Figure 5C and 5D, and supplemental data S7 and S8).

PKD1-HDAC7 Pathway Is Critical for VEGF-Induced Aorta Ring Angiogenesis

To define the role of PKD1-HDAC7 pathway in angiogenesis in intact vessels, we used aorta ring assay for ex vivo angiogenesis.24,25 VEGF increased the number of microvessels sprouting from aortic rings isolated from mice (Figure 6), and infection of Ad-PKD1-KN and Ad-HDAC7-S/A markedly inhibited VEGF-induced microvessel sprouting (Figure 6A).

Discussion

The major findings of this study are that VEGF stimulates PKD1-dependent HDAC7 phosphorylation and cytoplasmic accumulation in ECs, and that PKD1-HDAC7 pathway is involved in VEGF-induced MT1-MMP and MMP10 expression, EC migration, tube formation, and microvessel sprouting. Because MT1-MMP gene expression is implicated in angiogenesis in vivo,29 our data suggest that PKD1-HDAC7 pathway is likely to play an important role in VEGF signaling and angiogenesis in physiological and pathological conditions.

Acetylation of chromatin proteins and transcription factors is part of a complex signaling system that is largely involved in the control of gene expression.14,15 Histone acetyltransferases and HDACs act in an opposing manner to control the acetylation state of nucleosomal histones. Class IIα HDACs including HDAC4, HDAC5, HDAC7, and HDAC9 have been shown to act as signal-responsive repressors of cardiac hypertrophy, skeletal muscle differentiation, and bone development.31–33 We recently reported that HDAC5 was involved in VEGF signaling and gene expression.16 In addition to HDAC5, HDAC7 has been implicated in regulation of vascular integrity because HDAC7-deficient mice died in embryonic stages because of vascular leakage.17 HDAC7 downregulates MMP10 expression in unstimulated ECs, and MMP10 induction plays a role in endothelial capillary-like tube formation.17 However, the role of HDAC7 in VEGF signaling remains unclear. In the present study, we showed that VEGF promotes phosphorylation of 3 serine 178/344/479 residues in HDAC7, which have been shown to be the docking sites for the 14-3-3 chaperone protein.34 Indeed, VEGF stimulated the association of HDAC7 and 14-3-3 in a phosphorylation-dependent manner because HDAC7 phosphorylation-defective mutant (HDAC7-S/A) failed to bind to 14-3-3 in ECs in response to VEGF (Supplemental data S3). Binding of 14-3-3 to HDAC7 disrupts its association with MEF2 transcriptional factors and triggers HDAC7 cytoplasmic accumulation, thus freeing MEF2 to activate subordinate genes that may govern EC growth and migration (Figure 6B). Consistent with this notion, we observed that VEGF induced...
cytoplasmic accumulation of HDAC7 translocated in response to VEGF stimulation in ECs, and increased MEF2 transcriptional activation. Mutation of these serine sites to alanine (HDAC7-S/A mutant) blocked VEGF-induced nucleo-cytoplasmic shuttling, which is consistent with previous report showing that all 3 phosphoserine sites are required for the localization of HDAC7.34 Furthermore, HDAC7-S/A mutant inhibited VEGF-stimulated increase in MT1-MMP and MMP10 expression, and EC migration and tube formation (Figure 6B). These findings suggest that HDAC7 is a transcriptional repressor for genes such as MT1-MMP and MMP10 that are involved in angiogenesis.

Several protein kinases have been identified to be responsible for phosphorylation and nuclear export of class IIa HDACs.15 Among them, PKD1, a downstream mediator of PLCγ/ PKC pathway,13 has emerged as a key regulator for HDAC5 and HDAC7 in several cell types. We and others have shown that PKD1 is involved in regulation of HDAC5 phosphorylation and nuclear export in cardiac myocytes and ECs.16,35 PKD1 also regulates HDAC7 phosphorylation and nuclear exclusion as well as gene expression in T cells and B cells.28,16 In this study, we provide strong evidence showing that HDAC7 is a PKD1 substrate in VEGF signaling. First, we found that HDAC7 phosphorylation and cytoplasmic accumulation was mediated through VEGF-stimulated VEGFR2-PLCγ-PKC pathway, the same pathway for VEGF activation of PKD1 which we have revealed previously.13 Furthermore, the inhibition of PKD1 by the pharmacological inhibitor siRNA and dominant-negative mutant blocked VEGF-induced HDAC7 phosphorylation cytoplasmic accumulation. In contrast, overexpression of PKD1-WT, which increases its kinase activity, enhanced HDAC7 phosphorylation and cytoplasmic accumulation even in the absence of VEGF stimulation. Concomitantly, the inhibition of PKD1 also abolished VEGF-induced MT1-MMP and MMP10 expression and VEGF-mediated microvessel sprouting, indicating that PKD1 plays an essential role in these processes.

Class IIa HDAC family members HDAC4, HDAC5, HDAC7, and HDAC9 are all expressed in HUVECs manifested by RT-PCR analysis (supplemental data S5). Whereas HDAC4 and HDAC9 were mainly localized in cytosol in COS7 cells and in ECs, HDAC5 and HDAC7 were primarily localized in the nucleus of the unstimulated cells. Constitutive active mutant PKD1-S/E (Ser744/748 were replaced with glutamate) stimulated both HDAC5 and HDAC7 nuclear export in COS7 cells and ECs (Supplemental data S6 and S7). Moreover, both HDAC5 and HDAC7 are involved in MEF2-dependent transcription in VEGF signaling.16 But the physiological relevance of having the two enzymes with similar functions is not clear. Our preliminary observations suggest that both HDAC5 and HDAC7 regulates MEF2-dependent gene NA4R1 (an orphan nuclear receptor, also called NUR77) in VEGF signaling,16 whereas HDAC7 but not HDAC5 suppresses MEF2-dependent gene MMP10 in ECs. It is possible that HDAC5 and HDAC7 may regulate different genes through collaborating with other transcription cofactors, such as HDAC3,27 a member in class I HDAC family. In addition, it has recently been shown that HDAC5 oligomerizes with HDAC4,38 so it is also possible that HDAC5 and HDAC7 may interact. Further studies are needed to clarify the redundancy and the specificity of HDAC5-dependent and HDAC7-dependent pathways.

In summary, our studies have demonstrated that VEGF stimulates HDAC7 phosphorylation and cytoplasmic accumulation in ECs through VEGFR2/PLCγ-PKC/PKD1 signaling pathway. Moreover, our experiments provided evidence of a critical role for PKD1 and HDAC7 in VEGF-induced MT1-MMP and MMP10 expression, EC migration, and in vitro angiogenesis. Thus, the present findings reveal a critical role of HDAC7 as a PKD1 substrate in VEGF signaling, and this discovery may implicate PKD1 and HDAC7 in mediating...
angiogenesis in vivo. As such, it could help us develop new strategies to control physiological and pathological angiogenesis.

Note
During the revision of this manuscript, Wang et al published a report, consistent with our data, showing that HDAC7 regulates VEGF-induced endothelial cell migration.

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Disclosures
None.

References
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VEGF Stimulates HDAC7 Phosphorylation and Cytoplasmic Accumulation
Modulating Matrix Metalloproteinase Expression and Angiogenesis

**Running title:** HDAC7 in VEGF signaling and angiogenesis

Chang Hoon Ha,¹ Bong Sook Jhun,¹ Hung-Ying Kao² and Zheng-Gen Jin¹

From the ¹Aab Cardiovascular Research Institute and Department of Medicine, University of Rochester Medical Center, Rochester, New York, USA; and the ²Department of Biochemistry, School of Medicine, Case Western Reserve University, Cleveland, Ohio, USA

Address correspondence to: Zheng-Gen Jin, The Aab Cardiovascular Research Institute/Department of Medicine, University of Rochester Medical Center, 601 Elmwood Avenue Box 679, Rochester, New York 14642. Phone: (585) 276-9783. Fax: (585) 276-9829. E-mail: zheng-gen_jin@urmc.rochester.edu.
Supplemental data

Materials and Methods

**Plasmid, Antibodies and Reagents**

VEGF, and neutralization antibodies against VEGFR1 and VEGFR2 were purchased from R&D System, Inc. Minneapolis, Minnesota). SU1498, U73122, LY294002, GF109203X (bisindolylmaleimide I), Ro-31-8425, Go6976, PMA (phorbol-12-myristate-13-acetate), KN93 and BAPTA/AM were from Calbiochem (San Diego, California). Phosphopeptide antisera against pS178, pS344, and pS479 of HDAC7 were generated previously. Anti-PKD1 and anti-HDAC7 antibodies were from Cell Signaling Technologies (Beverly, Massachusetts). Anti-β-actin antibodies were from Santa Cruz Biotechnology (Santa Cruz, California).

**Cell Culture, transfection, and report assay**

BAECs were purchased from Clonetics and were cultured in medium 199 supplemented with 10% fetal bovine serum as described previously. HUVECs were isolated from human umbilical veins and grown in medium 200 with LSGS (Cascade Biologics, Inc.) as described previously. Confluent cells cultured in 60-mm dishes were serum-starved for 24 h and exposed to VEGF as indicated. For the inhibitor studies, cells were pretreated with various inhibitors for 30 min in serum-depleted medium. COS7 cells were purchased from ATCC and cultured in DMEM medium supplemented with 10% fetal bovine serum.
Transfections for reporter assay were performed with Electroporation (Bio-Rad). All transfections were performed in triplicate and are represented as the mean of at least three independent experiments. Cells were stimulated 8 h before harvesting, and reporter assays were performed according to the dual luciferase reporter assay's recommendations (Promega).

The siRNA duplex targeting PKD1 and the scrambled siRNA control (a non-targeting siRNA pool) were purchased from Dharmacon, Inc. The sequences of siRNA against human and bovine PKD1 are sense GAAAGAACGCUCUCAACAUCUU and antisense 5’-P GAUGUUGAAGCGUUCUUUCUU. The sequences of siRNA against human MT1-MMP are GAACAAAUACUGGAAAUUCUU and antisense 5’-PGAAUUUCCAGUAUUUGUUCUU. siRNA transfections of siRNA were performed using lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol as described previously. VEGF stimulation was performed 48 h after siRNA transfection.

**Adenovirus Constructs and Infection**

Adenovirus constructs encoding human VEGFR2 kinase inactive mutant (VEGFR2-K868M) were kindly provided by Dr. Masabumi Shibuya (University of Tokyo, Japan). Adenoviruses encoding HA-tagged HDAC7 wild type (Ad-HA-HDAC7-WT) and S178/479A mutant (Ad-HA-HDAC7-S/A), YFPagged HDAC7-WT (Ad-YFP-HDAC7-WT) and YFP-tagged HDAC7-S/A mutant (Ad-YFP-HDAC7-S/A), GFP-tagged PKD1 wild type (Ad-GFP-PKD1-WT) and its kinase-negative mutant (Ad-GFP-PKD1-KN) were generated from plasmids using ViraPower Adenoviral Expression System (Invitrogen) according to the manufacturer’s protocol. Adenovirus containing β-
galactosidase (LacZ) or GFP was used as a control. The infection of endothelial cells with recombinant adenovirus was performed as described previously. Briefly, endothelial cells cultured in 60-mm dishes were infected with recombinant adenovirus at the indicated multiplicity of infection (MOI) for 24 hours in a growth medium, and then treated with or without inhibitors followed by the application of VEGF or PMA.

**Immunoprecipitation and Western Blot Analysis**

Cells were harvested in lysis buffer and clarified by centrifugation as described previously. The protein concentrations in the lysates were determined using the Bradford method (BioRad, Hercules, California). The immunoprecipitation were performed according to standard protocols as described previously. The immune complex samples or total cell lysates were resolved on SDS-PAGE according to standard protocols. For Western blots, the protein samples from total cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membrane and incubated with appropriate primary antibodies. After incubating with fluorescence-conjugated secondary antibodies, immunoreactive proteins were visualized by Odyssey Infrared Imaging System (LI-COR Biotechnology, Nebraska). Densitometric analyses of immunoblots were performed with Odyssey software (LI-COR Biotechnology). Results were normalized by arbitrarily setting the densitometry of control sample to 1.0.

**HDAC7 subcellular localization Immunofluorescence study**

For analysis of subcellular localization of HDAC7 in endothelial cells, BAECs or HUVECs were infected with adenoviruses encoding YFP-HDAC7-WT, YFP-HDAC7-
S/A, HA-HDAC7-WT, HA-HDAC7-S/A, GFP-PKD1-WT or GFP-PKD1-KN on gelatin-coated 35 mm dishes containing medium 199 supplemented with 10% fetal bovine serum, 1% MEM vitamins, 1% MEM amino acids solution, and penicillin-streptomycin. After overnight culture, the cells were cultured in serum-free medium for 18 hours. The cells were stimulated with VEGF or PMA for indicated time or exposed to various inhibitors for 30 min prior to stimulation with the VEGF or PMA for 3 hours. The cells were washed with PBS and fixed with 3.7% formaldehyde in PBS. Images were captured at a magnification of x40 or x60, using a fluorescence microscope (Olympus BX51)\textsuperscript{11}.

**RNA isolation and RT-PCR**

Total RNA was isolated from cultured endothelial cells using a Total RNA Isolation Kit (Qiagen). First-strand cDNA was synthesized with the SuperScript Preamplification System (Gibco-BRL). cDNA was amplified by PCR for 30 cycle (Applied Biosystem)\textsuperscript{11}. Primer sequences for human MT1-MMP are 5’-CGCTACGCCATCCAGGGTCTCAAA and 5’-CGGTCATCATCGGGCAGCACAAAA; and for human MMP10 are 5’-GTCACTTCAGCTCCTTTTCCT and 5’-ATCTTTGCGAAAAGGCAGGAACT. Human GAPDH served as an internal control. Experiments were repeated three times, in duplicate.

**Wound closure cell migration**

For the measurement of cell migration during wound closure\textsuperscript{12}, endothelial cells were seeded on 6-well plates coated with gelatin and grown to confluence. Cells were infected with recombinant adenovirus where indicated. Monolayers were then disrupted with a
cell scraper of ~1.2 mm and photographed at 0 h and 12 h after VEGF addition in a light microscope (Olympus CK40) equipped with a digital camera (Olympus DP11). The number of endothelial cells migrated into wounded area was quantified.

**Capillary-like tube formation assay for in vitro angiogenesis**

For *in vitro* angiogenesis assay, endothelial cells infected with adenoviruses as indicated were plated on a thin layer of Matrigel (BD Biosciences) at 5 x 10^4 cells/well of a 24-well plate in 1% FBS EC medium and incubated for 12 h at 37°C. The capillary-like tube structures were visualized by a light microscopy (Olympus CK40) at different time points and imaged with digital camera (Olympus DP11). The lengths of the tube-like structures were measured.

**Aortic ring assay for ex vivo angiogenesis**

Mouse aortic ring angiogenesis assays were performed as previously described. Essentially, thoracic aortas from mice (C57BL/6J, 8 weeks, male) were dissected and the periaortic fibroadipose tissue removed under a stereo dissection microscope using fine-tipped forceps and microdissection scissors. 1-mm long aortic rings were embedded in growth factor-reduced Matrigel supplemented with 20 U/mL heparin. The aortic rings were infected with recombinant adenovirus at 100 MOI for 24 h, and then treated with or without 20 ng/mL VEGF. Cultures were incubated in an incubator at 37°C, 5% CO2 for 8 days for optimal microvessel sprouting. Images of aortic rings were taken using an Olympus BX41 microscope (Olympus, Center Valley, PA).
**Data Analysis**

All data are presented as the means plus or minus standard error of the mean of at least 3 separate experiments. Differences between groups were tested for statistical significance using Student t test or analysis of variance (ANOVA). Statistical significance was set at $P$ less than 0.05.

**References**


Supplemental data Figure legends

Supplemental Figure S1. VEGFR2 but VEGFR1 mediates VEGF-induced HDAC7 phosphorylation. (A) HUVECs were pretreated with control mouse IgG, neutralizing antibodies against human VEGFR1 or VEGFR2 (all for 2 µg/ml) for 30 min and then stimulated with VEGF (20 ng/ml) for 15 min. (B) BAECs were pretreated with vehicle (DMSO) or VEGFR2 kinase inhibitors SU1498 (10 µM) for 30 min before exposed to VEGF. (C) BAECs were infected with adenoviruses expressing β-galactosidase (Ad-LacZ, control) or expressing VEGFR2-KN (Ad-VEGFR2-KN) at 100 MOI before exposed to VEGF. HDAC7 phosphorylation and expression in cell lysates were determined as described in Materials and Methods. Overexpression of VEGFR2-KN in endothelial cells was also shown by Western blots with anti-VEGFR2 antibody (C). Representative immunobLOTS were shown (n=3).

Supplemental Figure S2. PLCγ/PKC pathway, but not PI3K-dependent pathway, regulates VEGF-induced HDAC7 phosphorylation. BAECs were pretreated with the vehicle (DMSO as control), PLCγ selective inhibitor U73122 (3 µM, A), PI3K inhibitor LY294002 (10 µM, B), or PKC inhibitors GF109203X (1-10 µM, C), and then exposed to 20 ng/ml VEGF fro 30 min. HDAC7 phosphorylation and expression in cell lysates were determined. Representative immunobLOTS were shown (n=3).

Supplemental Figure S3. VEGF stimulates the association of HDAC7 and 14-3-3 in a phosphorylation-dependent manner in ECs. A, BAECs were infected with Ad-HA-
HDAC7-WT and then exposed to VEGF at indicated times. B, BAECs were infected with Ad-LacZ (control) or Ad-HA-HDAC7-WT or Ad-HA-HDAC7-S/A, and then exposed to VEGF for 2 hours. Co-immunoprecipitation assays were performed with anti-HA antibodies followed with western blots with anti-14-3-3 antibodies and anti-HA antibodies. Input: total cell lysates were immunoblotted with anti-14-3-3 antibodies. Representative blots were shown.

Supplemental Figure S4. VEGF promotes cytoplasmic accumulation of HDAC7 through PLCγ/PKC-dependent pathway. BAECs were infected with Ad-YFP-HDAC7 at 50 MOI for 24 h, and then pretreated with various inhibitors the same as indicated in Figure 2S, or 20µM BAPTA/AM and 30µM KN93 for 30 min followed by the exposure of VEGF (20 ng/ml) for 3 h. The cells were fixed and YFP-HDAC7 distribution was analyzed by fluorescence microscopy. Representative images (magnification, x60) were from four independent experiments.

Supplemental Figure S5. Expression of class IIa HDACs in HUVECs. HUVEC mRNA was extracted from the cell lysates, and RT-PCR with the primers of HDAC4, HDAC5, HDAC7, HDAC9 and GADPH (internal control) were performed. The representative images were shown.

Supplemental Figure S6. PKD1 stimulates nuclear export of class II HDACs in COS7 cells. COS7 cells were co-transfected with Myc-HDAC4, GFP-HDAC5, YFP-HDAC7 and Myc-HDAC9 along with pcDNA (control) or HA-PKD1-S/E
(Ser744/Ser748 were replaced with glutamate, a constitutive active mutant). Subcellular localization of HDAC4, HDAC5, HDAC7 and HDAC9 in fixed cells were determined by indirect fluorescence immunocytochemistry or direct fluorescence image. Representative images are shown.

**Supplemental Figure S7. PKD1 stimulates nuclear export of class II HDACs in COS7 cells.** BAECs were co-transfected with Myc-HDAC4, GFP-HDAC5, YFP-HDAC7 and Myc-HDAC9 along with pcDNA (control) or HA-PKD1-S/E. Subcellular localization of HDAC4, HDAC5, HDAC7 and HDAC9 in fixed cells were determined by indirect fluorescence immunocytochemistry or direct fluorescence image. Representative images are shown.

**Supplemental Figure S8. PKD1-dependent HDAC7 phosphorylation contributes to VEGF-stimulated MEF2 transcription activation in endothelial cells.** BAECs were co-transfected with plasmids with 3xMEF2-luciferase report gene along with pCMV empty vector (as a control) or HA-HDAC7-S/A for 48 h and then stimulated with VEGF (20 ng/ml) for additional 16 h. MEF2 luciferase activity was determined as described in Materials and Methods. The graphs represent averaged data (means ± SEM, n=4), expressed as fold-change over basal. Each experiment was conducted in triplicate. *, p < 0.05 for the change of MEF2 activity versus control without VEGF stimulation; #, p < 0.05 vs. the group treated with VEGF alone.
Supplemental Figure S9. HDAC7 and MT1-MMP are involved in VEGF-induced endothelial cell migration. (A) BAECs were infected with Ad-LacZ (control), or Ad-HA-HDAC7-S/A and then measured for cell migration in response to VEGF in wound closure assay as described in Materials and Methods. (B) HUVECs were transfected with control siRNA or human MT1-MMP1 siRNA and then measured for migration in response to VEGF in wound closure assay. The representative images were shown (n=4).

Supplemental Figure S10. HDAC7 and MT1-MMP are involved in VEGF-induced endothelial tube formation. (A) HUVECs were infected with Ad-LacZ (control), or Ad-HA-HDAC7-S/A and then cultured in Matrigel and measured for tube formation in response to VEGF as described in Materials and Methods. (B) HUVECs were transfected with control siRNA or human MT1-MMP1 siRNA and then cultured in Matrigel and measured for tube formation in response to VEGF. The representative images for tube-like structure were shown (n=4).
A  Neutralization Antibodies

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|         | +   | +      | -      | +    | p-HDAC7 S479
|         |     |        |        |      | HDAC7 |

B  DMSO, SU1498

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C  LacZ, VEGFR2-KN

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Control  VEGF  U73122 + VEGF  Ro31-8220 + VEGF  
GF109203X + VEGF  LY294002 + VEGF  BAPTA/AM + VEGF  KN93 + VEGF

YFP-HDAC7
Myc-HDAC4  GFP-HDAC5  YFP-HDAC7  Myc-HDAC9

Control

PKD1-S/E
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![Bar graph showing MEF2 Luciferase Activity]

- pCMV
- pCMV + VEGF
- HA-HDAC7-S/A
- HA-HDAC7-S/A + VEGF

The graph indicates a significant difference in luciferase activity between control and treated groups. The bars with error bars represent the mean ± standard error of the mean.
A

0 h

Ad-LacZ

Ad-LacZ + VEGF

Ad-HA-HDAC7-S/A

Ad-HA-HDAC7-S/A + VEGF

12 h

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