IQGAP1 Mediates VE-Cadherin–Based Cell–Cell Contacts and VEGF Signaling at Adherence Junctions Linked to Angiogenesis

Minako Yamaoka-Tojo, Taiki Tojo, Ha Won Kim, Lula Hilenski, Nikolay A. Patrushev, Lynn Zhang, Tohru Fukai, Masuko Ushio-Fukai

Objective—Vascular endothelial growth factor (VEGF) induces angiogenesis by stimulating reactive oxygen species (ROS) production primarily through the VEGF receptor-2 (VEGFR2). One of the initial responses in established vessels to stimulate angiogenesis is loss of vascular endothelial (VE)-cadherin–based cell–cell adhesions; however, little is known about the underlying mechanisms. IQGAP1 is a novel VEGFR2 binding protein, and it interacts directly with actin, cadherin, and β-catenin, thereby regulating cell motility and morphogenesis.

Methods and Results—Confocal microscopy analysis shows that IQGAP1 colocalizes with VE-cadherin at cell–cell contacts in unstimulated human endothelial cells (ECs). VEGF stimulation reduces staining of IQGAP1 and VE-cadherin at the adherens junction without affecting interaction of these proteins. Knockdown of IQGAP1 using siRNA inhibits localization of VE-cadherin at cell–cell contacts, VEGF-stimulated recruitment of VEGFR2 to the VE-cadherin/β-catenin complex, ROS-dependent tyrosine phosphorylation of VE-cadherin, which is required for loss of cell–cell contacts and capillary tube formation. IQGAP1 expression is increased in a mouse hindlimb ischemia model of angiogenesis.

Conclusions—IQGAP1 is required for establishment of cell–cell contacts in quiescent ECs. To induce angiogenesis, it may function to link VEGFR2 to the VE-cadherin containing adherens junctions, thereby promoting VEGF-stimulated, ROS-dependent tyrosine phosphorylation of VE-cadherin and loss of cell–cell contacts. (Arterioscler Thromb Vasc Biol. 2006;26:1991-1997.)

Key Words: angiogenesis ■ cell–cell adherions ■ IQGAP1 ■ reactive oxygen species ■ vascular endothelial growth factor ■ VE-cadherin

Vascular endothelial growth factor (VEGF) induces angiogenesis by stimulating endothelial cell (EC) migration and proliferation primarily through the VEGF type2 receptor (VEGFR2, KDR/Flk-1).1 VEGF binding initiates autophosphorylation of VEGFR2, which is followed by activation of diverse key angiogenic enzymes such as MAP kinases and Akt.1 One of the initial responses of quiescent ECs to induce angiogenesis is the loosening of cell–cell contacts, which is followed by migration of ECs to form capillary tube networks that become functional capillaries.2 The molecule primarily responsible for cell–cell adhesions of ECs is the transmembrane homophilic adhesion molecule, vascular endothelial (VE)-cadherin.3 The cytoplasmic domain of VE-cadherin binds to β-catenin, which in turn is linked to the actin cytoskeleton via α-catenin.3 This linkage between VE-cadherin–based adherens junctional complex and the actin cytoskeleton contributes to the strong adhesion. Furthermore, deletion or cytosolic truncation of VE-cadherin impairs remodeling and maturation of the vascular networks, and it inhibits VEGF-stimulated Akt phosphorylation induced by formation of a VEGFR2/VE-cadherin/β-catenin/phosphatidylinositol 3-kinase (PI3 kinase) complex.4 Tyrosine phosphorylation of the VE-cadherin complex is another mechanism that regulates the stability of cell–cell junctions,5–7 which is in part mediated through reactive oxygen species (ROS).8,9 We demonstrated that ROS derived from Rac1-dependent NAD(P)H oxidase play an important role in VEGF signaling and angiogenesis in ECs and in vivo.10,11 Thus, the VE-cadherin–based endothelial adherens junction is a potential site for initial activation of VEGFR2-mediated, ROS-dependent signaling linked to angiogenesis. However, underlying regulatory mechanisms are incompletely understood.

Using a yeast 2-hybrid system, we recently identified IQGAP1 as a novel VEGFR2 binding protein.12 IQGAP1 is a scaffold protein that interacts directly with actin, E-cadherin, β-catenin, active Rac1/Cdc42, calmodulin, and the microtubule plus end binding protein, CLIP-170,13–15 thereby regulating actin cytoskeleton, cell–cell adhesion, cellular motility...
and morphogenesis. IQGAP1 is a downstream effector of active Rac\textsuperscript{16,17} and acts as anti-GAP through a GAP-related domain, thereby increasing GTP-bound Rac\textsuperscript{16,18}. In mouse fibroblasts, IQGAP1 localizes at sites of cell–cell contact and overexpression of IQGAP1 reduces E-cadherin-mediated cell–cell adhesion via interacting with β-catenin, thereby releasing α-catenin from the cadherin/catenin complex.\textsuperscript{19} Knockdown of IQGAP1 using siRNA reduces the accumulation of actin filaments, E-cadherin and β-catenin at sites of cell–cell contact in MDCKII cells.\textsuperscript{20} These results suggest negative and positive roles of IQGAP1 for cell–cell adhesions in epithelial cells. We previously demonstrated that IQGAP1 plays an essential role in VEGF-stimulated ROS production and VEGFR2-mediated EC migration and proliferation.\textsuperscript{12} However, a specific role of IQGAP1 in VE-cadherin-mediated cell–cell adhesions as well as VEGF-induced loss of cell–cell contacts linked to angiogenesis is unknown.

The present study demonstrates that IQGAP1 colocalizes and forms a complex with VE-cadherin at the site of cell–cell contacts in unstimulated confluent human umbilical vascular endothelial cells (HUVECs). VEGF stimulation reduces the staining of VE-cadherin and IQGAP1 at cell margins without affecting their complex formation. Using IQGAP1 siRNA, we found that IQGAP1 is required for establishment of VE-cadherin-based cell–cell contacts in quiescent ECs. We also suggest that IQGAP1 may function as a scaffold protein to link VEGFR2 to the VE-cadherin/β-catenin complex at the adherens junctions, thereby promoting VEGF-stimulated ROS-dependent tyrosine phosphorylation of VE-cadherin and its downstream Akt phosphorylation, which may contribute to angiogenesis.

Materials and Methods

Materials
Antibodies to VEGFR2, IQGAP1, phosphotyrosine, VE-cadherin, α-catenin, β-catenin, Akt, and α-tubulin were from Santa Cruz. Anti-phospho-Akt antibody was from Cell Signaling. Human recombinant VEGF\textsubscript{165} was from R&D Systems and BRB Preclinical Repository. Oligofectamine was from Invitrogen Corp. Carboxy-H2–2′,7′-dichlorofluorescein diacetate (DCF-DA) was from Molecular Probes. All other chemicals and reagents were from Sigma.

Cell culture, measurement of intracellular H\textsubscript{2}O\textsubscript{2} levels, confocal immunofluorescence microscopy, synthetic siRNA and its transfection, immunoprecipitation and immunoblotting, tube formation assay in 3-dimensional type I collagen gels, mouse ischemic hindlimb reperfusion, and histological analysis, and statistical analyses are described in the Material and Methods section in the online data supplement (see http://atvb.ahajournals.org).

Results

Subcellular Localization of IQGAP1 and VE-Cadherin and Their Association during VEGF Stimulation in ECs

To examine the role of IQGAP1 in localization of VE-cadherin at adherence junctions in confluent monolayers of HUVECs, we found that IQGAP1 is required for establishment of VE-cadherin-based cell–cell contacts in quiescent ECs. We previously demonstrated that IQGAP1 plays an essential role in VEGF-stimulated ROS production and VEGFR2-mediated EC migration and proliferation.\textsuperscript{12} However, a specific role of IQGAP1 in VE-cadherin-mediated cell–cell adhesions as well as VEGF-induced loss of cell–cell contacts linked to angiogenesis is unknown.

Figure 1. Subcellular localization of IQGAP1 and VE-cadherin and their association during VEGF stimulation in ECs. A, Growth-arrested HUVECs were stimulated with VEGF (50 ng/mL) for 1 hour, and double-stained with anti-VE-cadherin (red) and IQGAP1 antibody (green), followed by anti-mouse Rhodamine Red X- and anti-rabbit fluorescein isothiocyanate (FITC)-conjugated secondary antibodies, respectively. NC indicates negative control, staining with nonimmune IgG. White arrows show VEGF-induced decrease in VE-cadherin staining at cell–cell contact (red) and increase in IQGAP1 staining at the perinucleus (green). All fluorescence images were taken at 5 different fields/well, and images are representative of more than 3 independent experiments. B, HUVECs were stimulated with VEGF (50 ng/mL) and lysates were immunoprecipitated (IP) with anti-IQGAP1 antibody, followed by immunoblotting (IB) with anti-VE-cadherin or IQGAP1 antibody. Blots are representative of 3 independent experiments.

IQGAP1 Is Required for Localization of VE-Cadherin at Cell–Cell Contacts

To examine the role of IQGAP1 in localization of VE-cadherin at adherence junctions in confluent monolayers of ECs, HUVECs were transfected with IQGAP1 siRNA. As shown in Figure 2, IQGAP1 siRNA, but not scrambled siRNA, almost completely knocked down IQGAP1 protein without affecting VE-cadherin protein expression. Moreover, IQGAP1 siRNA had no effect on expression of IQGAP2 or β-catenin protein (data not shown), further confirming the
specificity of IQGAP1 siRNA. IQGAP1 siRNA markedly reduced VE-cadherin staining at sites of cell–cell contact, resulting in small gaps between adjacent cells in basal and VEGF-stimulated ECs. These results suggest that IQGAP1 is required for proper localization of VE-cadherin at the adherens junctions and for VE-cadherin-mediated cell–cell adhesions in ECs.

**IQGAP1 Is Required for VEGF-Induced Association of VEGFR2 With VE-Cadherin/β-Catenin Complex**

VEGF stimulation promotes formation of VEGFR2/VE-cadherin/β-catenin complex, but their interaction is not direct. We previously demonstrated that VEGF induces direct interaction of VEGFR2 with IQGAP1 in HUVECs. Because IQGAP1 directly binds to E-cadherin and β-catenin, we examined whether IQGAP1 is involved in VEGF-induced formation of VEGFR2/VE-cadherin/β-catenin complex in ECs. As shown in Figure 3, VE-cadherin was co-immunoprecipitated with α-catenin and β-catenin in unstimulated confluent HUVECs. VEGF stimulation rapidly promoted recruitment of VEGFR2 to and dissociation of α-catenin from the VE-cadherin/β-catenin complex, which was significantly inhibited by IQGAP1 siRNA. These results suggest that IQGAP1 may function as a scaffold to link VEGFR2 to the adherens junctions through binding to VEGFR2 and VE-cadherin/β-catenin complex, thereby dissociating α-catenin from the adherens junctional complex, and contributing to VEGF-stimulated loss of cell–cell contacts in ECs.

**IQGAP1 Is Required for ROS-Dependent Tyrosine Phosphorylation of VE-Cadherin**

Because tyrosine phosphorylation of VE-cadherin is required for VEGF-induced dissociation of cell–cell contacts in ECs, we examined whether IQGAP1 is involved in this response. As shown in Figure 4A, VEGF stimulation induced a significant increase in tyrosine phosphorylation of VE-cadherin and IQGAP1 within 5 minutes. These increases were significantly inhibited by IQGAP1 siRNA. Basal phosphorylation of VE-cadherin was rather enhanced in IQGAP1 siRNA-transfected cells, presumably because of the reduction of VE-cadherin–mediated cell–cell adhesions induced by IQGAP1 depletion (Figure 2). Under this condition, IQGAP1 siRNA significantly inhibited VEGF-stimulated Akt phosphorylation (Figure 4B), which is a downstream response of formation of the VEGFR2/VE-cadherin/β-catenin complex. These results suggest that IQGAP1-mediated formation of VEGFR2/VE-cadherin/β-catenin complex (Figure 3) may be involved in VEGF-induced tyrosine phosphorylation of VE-cadherin as well as Akt phosphorylation in ECs. Because we found previously that IQGAP1 is involved in VEGF-induced increase in ROS production, we examined the role of ROS in phosphorylation of VE-cadherin by VEGF. IQGAP1 siRNA inhibited VEGF-stimulated ROS production (Figure 4C), and VEGF-stimulated tyrosine phosphorylation of VE-cadherin was significantly inhibited by H2O2 scavenger, polyethylene glycol (PEG)-catalase, and thiol antioxidant, NAC (Figure 4D). PEG only had no effects (data not shown). These results suggest that IQGAP1-mediated rapid increase

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**Figure 2.** Role of IQGAP1 in localization of VE-cadherin at cell–cell contacts in ECs. HUVECs were transfected with scrambled or IQGAP1 siRNAs. Left panel, Lysates were immunoblotted with anti-IQGAP1 or VE-cadherin antibody. Right panel, Cells were stimulated with or without VEGF (50 ng/mL) for 1 hour and stained with anti-VE-cadherin antibody. White arrows show VE-cadherin staining at the perinucleus by IQGAP1 siRNA. Results are representative of 3 independent experiments.

**Figure 3.** Role of IQGAP1 in VEGF-induced association of VEGFR2 with VE-cadherin/β-catenin complex. HUVECs transfected with scrambled or IQGAP1 siRNAs were stimulated with VEGF (50 ng/mL). Lysates were immunoprecipitated (IP) with anti-VE-cadherin antibody, followed by immunoblotting (IB) with anti-VEGFR2, α-catenin, β-catenin, IQGAP1, or VE-cadherin antibody. NC indicates negative control (buffer blank); PC, positive control (HUVEC total cell lysates). The graphs represent averaged data, corrected for total VE-cadherin loading, expressed as fold change of interaction from basal (set to 1). *P<0.05 vs untreated control in scrambled siRNA transfected cells (n=3).
in ROS may participate in the initial activation of VEGF signaling at the VE-cadherin-based adherens junction in ECs.

**IQGAP1 Is Involved in VEGF-Stimulated Tube Formation in Type I Collagen 3-Dimensional Culture of ECs**

Loss of cell–cell contacts in confluent monolayers of ECs triggers EC migration to form capillary vascular networks during angiogenesis. To assess the functional role of IQGAP1 in VEGF-induced angiogenesis in vitro, we examined whether IQGAP1 is involved in capillary tube formation using 3-dimensional culture of HUVECs in type I collagen gels. As shown in Figure 5, at 24 hours after overlaying the second collagen gel containing VEGF on confluent monolayer of HUVECs seeded on top of the collagen-coated wells, capillary tube-like structures were formed in scrambled siRNA-transfected cells. Thus, this model is valuable to study the initial mechanical events of angiogenesis. In contrast, VEGF-induced tube formation was not observed in IQGAP1 siRNA-transfected cells, suggesting that IQGAP1 plays an important role in angiogenesis in vitro.

**Induction of IQGAP1 Protein Expression in Mouse Ischemic Hindlimb Model of Angiogenesis**

To gain further insight into the role of IQGAP1 in angiogenesis in vivo, we examined the expression of IQGAP1 in a mouse hindlimb ischemia model in which angiogenesis is dependent at least in part on VEGF, VEGFR2, and NAD(P)H oxidase-derived ROS. Figure 6A using LDBF analysis demonstrates that hindlimb blood flow recovery was markedly decreased immediately after femoral artery ligation (day 0) and recovered to the level of that of the nonischemic limb by day 7. Western analysis also shows that IQGAP1 protein expression was significantly increased in the ischemic hindlimb tissues at 7 days after operation (Figure 6B) in association with the increase in VEGF expression (data not shown) compared with that in nonischemic sites. Immunocytochemical analysis of double staining for IQGAP1 and lectin showed that IQGAP1 protein and lectin-positive capillary ECs were dramatically increased and colocalized in the ischemic hindlimbs at 7 days after femoral ligation (Figure 6C). Similarly, VE-cadherin expression was increased in ischemic hindlimbs and partially colocalized with IQGAP1.
migration.12,24 One of the initial responses to stimulate EC
tial role in both VEGF-induced and wound injury-induced EC
angiogenesis. We also found that IQGAP1 expression is
IQGAP1, but not VE-cadherin, is also found in the cytosol in
cell–cell contacts in confluent monolayers of ECs. Of note,
colocalizes and associates with VE-cadherin at the sites of
co-immunoprecipitation assays, here we show that IQGAP1
sion in ECs is unknown. Using confocal microscopy and
analysis was performed in denatured condition, these factors
accessibility to the VE-cadherin antibody presumably caused
the disruption of cell–cell adhesion, which may reduce the
adherens junction; whereas it increased the staining of IQ-
GAP1, but not VE-cadherin, at the perinucleus area without
adherens junctions as a com-
plex without changing their protein expression. Thus, it is likely that perinuclear IQGAP1 protein which is
increased after VEGF stimulation may be translocated mainly
from the cytosol where VE-cadherin–unbound IQGAP1 lo-
calizes in basal state. Similar results are obtained for
β-catenin (unpublished observation). Our findings are con-
sistent with the previous reports that both VE-cadherin and
β-catenin are dissociated from adherens junctions as a com-
plex without changing their protein expression in HUVEC
monolayers in response to VEGF, thrombin, H2O2, and fluid
shear stress.5,25–27 This may be because of the possibility that
VE-cadherin staining using confocal microscopy in Triton
X-permeabilized and fixed cultured confluent ECs reflects
the disruption of cell–cell adhesion, which may reduce the
accessibility to the VE-cadherin antibody presumably caused
by conformational change and/or phosphorylation of VE-
cadherin induced by VEGF stimulation. Because Western
analysis was performed in denatured condition, these factors
might not be reflected. Moreover, knockdown of IQGAP1
using siRNA inhibits localization of VE-cadherin at cell–cell
contacts but causes its mislocalization (Figure III). These data indicate that IQGAP1 may be
involved in the process by which new blood vessels are
formed in vivo.

Discussion
Understanding the factors that regulate endothelial cell–cell
junctions is important for many pathophysiological processes
in which functional vascular integrity is compromised, such
as development of neovascularization during angiogenesis and
chronic inflammatory disorders. The present study shows that
IQGAP1 colocalizes and forms a complex with VE-cadherin
at the site of cell–cell contacts in unstimulated confluent
HUVECs, and VEGF stimulation reduces their localization at
the cell margin without affecting their complex formation.
Knockdown of IQGAP1 using siRNA inhibits localization of
VE-cadherin at cell–cell contacts as well as the following
VEGF-stimulated events: (1) recruitment of VEGF2 to and
the dissociation of α-catenin from the VE-cadherin/β-catenin
complex; (2) ROS-dependent tyrosine phosphorylation of
VE-cadherin, which is required for loss of cell–cell con-
acts8,9; and (3) capillary tube formation in 3-dimensional
collagen gels. We also found that IQGAP1 expression is
markedly increased in the increased in the mouse hindlimb ischemia model of
angiogenesis.

We previously demonstrated that IQGAP1 plays an essen-
tial role in both VEGF-induced and wound injury-induced EC
migration.12,24 One of the initial responses to stimulate EC
migration is the loosening of stable cell–cell contacts be-
tween ECs, and the molecule primarily responsible for
cell–cell adhesions of ECs is the VE-cadherin. Recent studies
reveal that IQGAP1 regulates E-cadherin-mediated cell–cell
adhesion both positively and negatively in epithelial cells.15
However, its role in VE-cadherin-mediated cell–cell adhe-
sion in ECs is unknown. Using confocal microscopy and
co-immunoprecipitation assays, here we show that IQGAP1
colocalizes and associates with VE-cadherin at the sites of
cell–cell contacts in confluent monolayers of ECs. Of note,
IQGAP1, but not VE-cadherin, is also found in the cytosol in
unstimulated HUVECs (Figure I and Figure I). VEGF
stimulation reduces IQGAP1 and VE-cadherin staining at the
adherens junction, whereas it increased the staining of IQ-
GAP1, but not VE-cadherin, at the perinucleus area without
changing their complex formation and protein expression.
Thus, it is likely that perinuclear IQGAP1 protein which is
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the disruption of cell–cell adhesion, which may reduce the
accessibility to the VE-cadherin antibody presumably caused
by conformational change and/or phosphorylation of VE-
cadherin induced by VEGF stimulation. Because Western
analysis was performed in denatured condition, these factors
might not be reflected. Moreover, knockdown of IQGAP1
using siRNA inhibits localization of VE-cadherin at cell–cell
contacts but causes its mislocalization to the perinucleus area
before and after VEGF stimulation, thereby reducing cell–
cell adhesions. Consistent with our result, Noritake et al20
reported that IQGAP1 siRNA reduces the accumulation of
E-cadherin and β-catenin at cell–cell contacts in MDCKII
cells. These results suggest that IQGAP1 is required for
proper localization of VE-cadherin at cell–cell contacts, and
for establishment of VE-cadherin-mediated cell–cell adhe-
sions in ECs.

It has been shown that VEGFR2 associates with VE-
cadherin/β-catenin complex after VEGF stimulation to acti-
vate VE-cadherin–dependent signaling including Akt in
ECs.4 However, interaction between VEGFR2 and VE-
cadherin/β-catenin is not direct. In the present study, we
show that IQGAP1 siRNA inhibits VEGF-induced recruitment of VEGFR2 to as well as dissociation of α-catenin from the VE-cadherin/β-catenin complex. Because IQGAP1 directly binds to activated VEGFR2,13 as well as to E-cadherin and β-catenin,19 these results suggest that IQGAP1 may function to link VEGFR2 to the adherens junctions through binding to VE-cadherin/β-catenin complex, thereby dissociating α-catenin from the adherens junctional complex, which in turn results in loss of cell–cell adhesions. Given that IQGAP1 siRNA inhibits VEGF-stimulated activation of Akt, and that disrupting stable cell–cell contacts is required to stimulate EC migration, it is conceivable that IQGAP1-dependent formation of the VEGFR2/VE-cadherin/β-catenin complex at the adherens junction is necessary for downstream activation of VEGFR2-mediated signaling leading to angiogenesis. It is important to characterize the interacting domains of IQGAP1 with its binding partners in future study.

Tyrosine phosphorylation of VE-cadherin is also critical for the loosening of cell–cell contacts in ECs.5–7 We demonstrate here that IQGAP1 siRNA significantly inhibits VEGF-stimulated tyrosine phosphorylation of VE-cadherin, whereas its response in basal state is rather enhanced presumably caused by the reduction of VE-cadherin-mediated cell–cell adhesions. Recently, VEGF-induced or Rac1-induced ROS have been shown to be involved in VE-cadherin tyrosine phosphorylation and loss of cell–cell contacts in ECs.8,9 We previously demonstrated that VEGF induces a rapid increase in ROS production via activation of Rac1-dependent NAD(P)H oxidase in ECs,10 which is mediated through IQGAP1.11 In line with these findings, the present study confirmed that IQGAP1 siRNA inhibits VEGF-stimulated increase in ROS production and that ROS inhibitors block tyrosine phosphorylation of VE-cadherin by VEGF. The mechanisms by which ROS mediate VE-cadherin phosphorylation remain unclear. Accumulating evidence suggests that ROS mediate the oxidation of critical cysteine residues in protein tyrosine phosphatases, thereby deactivating these enzymes, which results in increased tyrosine kinase activity.24–30 Although it is not known which protein tyrosine phosphatases may be involved, it is intriguing to note that SHP-2 has been shown to associate with VE-cadherin complex after thrombin stimulation in ECs31 and that VEGF stimulation promotes SHP-2 binding to IQGAP1/VEGFR2/VE-cadherin/β-catenin complex (authors’ unpublished observations). The precise underlying mechanism requires further investigation. Our present results are consistent with the possibility that VEGF-induced, IQGAP1-mediated formation of VEGFR2/VE-cadherin/β-catenin complex is important for ROS-dependent tyrosine phosphorylation of VE-cadherin at the adherens junction, thereby facilitating loss of cell–cell contacts.

Because loss of cell–cell adhesions is an initial key event for angiogenesis, we assessed the functional role of IQGAP1 in VEGF-induced angiogenesis using in vitro and in vivo models. Using 3-dimensional cultures in type I collagen gels, an in vitro model of angiogenesis,6 we demonstrate that VEGF-stimulated capillary-like tube formation is almost completely blocked in IQGAP1 siRNA-transfected HUVECs. We have shown that IQGAP1 is involved in VEGF-stimulated EC proliferation and migration,12 which may explain in part the mechanisms by which IQGAP1 regulates capillary tube formation in ECs. However, we cannot exclude the possibility that HUVEC data obtained in the present study may not be relevant to microvascular angiogenesis.

Study using mouse hindlimb ischemia model reveals that IQGAP1 is markedly increased in lectin-positive, newly formed capillary ECs and partially colocalizes with VE-cadherin in hindlimb tissues after ischemia. Because IQGAP1 is involved in VEGF-stimulated ROS production, loss of cell–cell contacts, EC migration, and proliferation as well as capillary tube formation in cultured ECs, the functional consequence of upregulation of IQGAP1 in neovascularization is consistent with the possibility that IQGAP1 may play an important role in postnatal angiogenesis, which is dependent on VEGF, VEGFR2, and NAD(P)H oxidase-derived ROS.11,22,23 The definitive role of IQGAP1 in ischemia-induced angiogenesis will require further investigation using IQGAP1−/− mice.

In summary, IQGAP1 plays an important role in establishment of VE-cadherin–based cell–cell contacts in quiescent ECs. It may also function as a scaffold protein to link VEGFR2 to the VE-cadherin/β-catenin complex at the adherens junctions, thereby promoting ROS-dependent tyrosine phosphorylation of VE-cadherin and loss of cell–cell contacts, which may contribute to postnatal angiogenesis. These findings suggest an essential role of IQGAP1 in organization of signaling at endothelial adherens junction and provide novel insight into IQGAP1 as an attractive therapeutic target for modulating development of neovascularization during angiogenesis.

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Disclosures
None.

References


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Material and Methods

Cell Culture- Human umbilical vein ECs (HUVECs) were from VEC Technologies (Rensselaer, NY) and were grown in endothelial cell growth medium (EGM-MV, Clonetics) containing 10% fetal bovine serum. Cells between passages 3 and 6 were used.

Measurement of intracellular $H_2O_2$ levels- HUVECs were loaded with 20 µM DCF-DA, and then exposed to VEGF (20 ng/ml). The DCF-DA fluorescence was measured, as reported previously $^{1,2}$, and was completely inhibited by catalase, confirming that the fluorescence signal mainly detects $H_2O_2$ $^{2}$.

Immunoprecipitation and Immunoblotting- Growth-arrested cells with 0.5% FBS containing EBM media for 18-20 hr were stimulated with human recombinant VEGF at 37°C, and cells were lysed with 500 µl of ice-cold lysis buffer, pH 7.4 (50 HEPES, 5 EDTA, 50 NaCl [in mM], 1% Triton X-100, protease inhibitors (10 µg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin) and phosphatase inhibitors (50 sodium fluoride, 1 sodium orthovanadate, 10 sodium pyrophosphate [in mM]). For some experiments, the cell lysates were centrifuged at 12,000g at 4°C for 15 min and the supernatant was saved as a Triton-soluble fraction and the pellets were saved as the Triton-insoluble fraction. For immunoprecipitation, cell lysates (500 µg) were precipitated with antibody overnight at 4°C and then incubated with 25 µl of protein A/G-agarose beads for 2 hr at 4°C. Cell lysates (25 µg) or immunoprecipitates were separated using SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes, blocked for 1 hr in PBS containing 5% nonfat dry milk and 0.1% Tween 20, and incubated for 1 hr to overnight with primary antibodies as described previously $^{1}$. After incubation with secondary antibodies for 1 hr, proteins were detected by ECL chemiluminescence.

Isolation of membrane and cytosolic fractions- HUVECs were treated with ice-cold hypotonic lysis buffer (10 mM Tris pH 7.4, 1.5 mM MgCl2, 5 mM KCl, 1 mM DTT, 0.2 mM sodium vanadate, 1 mM PMSF, 1 µg/ml aprotinin, 1 µg/ml leupeptin) for 5 min. After drawing the lysate through a 1 ml syringe with several rapid strokes, the samples were centrifuged at 2,000 g at 4°C for 5 min. The supernatant was centrifuged at 100,000 g at 4°C for 90 min and the supernatant was saved as a “cytosolic” fraction. The pellets were
centrifuged at 14,000 rpm for 20 min at 4°C and the supernatant was saved as the “membrane” fraction, as described previously 3.

Confocal Immunofluorescence Microscopy- HUVECs growing on 0.1% gelatin-coated glass coverslips were stimulated with VEGF, and fixed with 4% paraformaldehyde in PBS, and permeabilized in 0.05% Triton X-100/PBS for 5 min. After blocking, cells were incubated with mouse monoclonal anti-VE-cadherin and/or rabbit anti-IQGAP1 antibody for 1 hr, and then incubated in either Rhodamine Red X (RRX)-conjugated goat anti-mouse IgG or FITC-conjugated goat anti-rabbit IgG for 1 hr. Images were taken using the confocal laser scanning imaging system Bio-Rad MRC-1024 as described previously 2,4,5. In double labeling experiments, FITC and RRX images were scanned sequentially and merged using the Bio-Rad LaserSharp software. Controls with no primary antibody (control) showed no fluorescence labeling. Single labeling showed identical staining to double labeling of IQGAP1/VE-cadherin.

Synthetic siRNA and its Transfection- RNA oligonucleotides were obtained from Sigma and annealed to be double-stranded, using a small-interference RNA (siRNA) construction kit (Ambion Inc.). The sequences of specific siRNA against IQGAP1 is; 5’-AAGGCCGAACTAGTGAAACTGCCTGTCTC-3’ and 5’-AACAGTTTCACTAGTTCGGCCCCTGTCTC -3’. The scrambled siRNA control is 5’-AAGTACCAAGGACGCGAATGTCCTGTCTC-3’ and 5’-AAACATTCGCCTCTTGGTACCCTGTCTC -3’.

We performed a Blast search and confirmed that the IQGAP1 and scrambled siRNA sequences have no overlap with other proteins. Moreover, we have previously shown that IQGAP1 siRNA has no effects on expression of Nox2, actin or α-tubulin 5, confirming the specificity of IQGAP1 siRNA used in the present study. HUVECs were grown to 60 % confluence in 100 mm dishes and transfected with 30 nM siRNA using Oligofectamine (Invitrogen), as described previously 2. Cells were used for experiments at 48 hr after transfection.

Tube formation assay of HUVECs in three dimensional (3D)-typeI collagen gels-
Type 1 collagen solution (Chemicon) in M199 medium (100 μl) was added to a 24-well plate
and incubated at 37°C for 1 hr as described 6. HUVECs were seeded on top of the collagen-coated wells at 1x10^5 per well and allowed to form confluent monolayers overnight at 37°C. Cells attached were overlaid with a second 100 μl collagen solution containing VEGF (20 ng/ml). After 24 hr, capillary-like structures on 3-D gels was visualized with an inverted phase-contrast microscope (200×) and images were taken with a Nikon digital camera. Eight random fields per well were imaged.

**Mouse Ischemic Hindlimb Model**- Study protocols were approved by the Animal Care and Use Committee of Emory University School of Medicine. Female C57BL/6J mice (8-9 weeks of age) were obtained from The Jackson Laboratory. Mice were subjected to unilateral hindlimb ischemia under anesthesia with intra-peritoneal administration of ketamine (87 mg/kg) and xylazine (13 mg/kg). The right superficial femoral artery was exposed and ligated proximally and distally with 5-0 silk ligatures, and excised. We measured hindlimb blood flow using a laser Doppler blood flow (LDBF) analyzer (Lisca AB) as described previously 7. To obtain mice thigh adductor muscle in ischemic hindlimbs for immunostaining, mice at 7 days after surgery were euthanized and perfused with saline and followed by 10% formalin through the left ventricle. In some experiments, mice hindlimbs were used for immunoblotting.

**Histological analysis**- Hindlimb muscles were embedded in OCT compound (Sakura Finetek) and snap-frozen in liquid nitrogen. Seven-micrometer-thick sections in the ischemic and non-ischemic hindlimbs were double-stained with rabbit anti-IQGAP1 or VE-cadherin antibody and biotinylated *Griffonia simplicifolia* lectin (Vector Laboratories) to detect capillary ECs, followed by RRX-conjugated anti-rabbit secondary antibody and FITC-conjugated streptavidin (Jackson ImmunoResearch Lab), respectively 7. For quantification of IQGAP1 positive cells and lectin positive capillary ECs, cells were counted in 5 randomly selected high power fields (x400) with 3 independent samples and blinded investigators using the NIH image analysis.

**Synthetic siRNA and its Transfection**- RNA oligonucleotides were obtained from Sigma and annealed to be double-stranded using small-interference RNA (siRNA) construction kits (Ambion) as reported previously 2. HUVECs were seeded into 100 mm
dishes one day prior to transfection. At the time of transfection with siRNA, the cells were about 50-60% confluent in 6 ml of endothelial basal medium (EBM) with supplements containing 10% FBS. Transfections of siRNAs (30 nM) were performed using Oligofectamine (Invitrogen) according to the manufacturer’s protocol. VEGF stimulation was performed 72-96 hr after transfection.

Statistical Analyses- All values are expressed as mean ± SE. The significance of the differences between 2 groups was evaluated by an unpaired Student’s t test. The values in more than 3 groups were tested by one-way analysis of variance (ANOVA), and were followed by Scheffe’s F test. A probability value of 0.05 was considered significant.

References
7. Tojo T, Ushio-Fukai M, Yamaoka-Tojo M, Ikeda S, Patrushev NA, Alexander RW. Role of gp91phox (Nox2)-containing NAD(P)H oxidase in angiogenesis in response to

**Figure legend for Supplemental Figures:**

**Figure I: Association and protein expression of VE-cadherin and IQGAP1 before and after VEGF stimulation in HUVEC.** Confluent monolayer of HUVECs were stimulated with VEGF (20 ng/ml) for indicated time (min). **A,** Lysates were immunoprecipitated (IP) with anti-IQGAP1 antibody, followed by immunoblotting (IB) with anti-VE-cadherin or IQGAP1 antibody. Blots are representative of 3 independent experiments. **B and C,** Cells were separated for Triton-soluble and insoluble fractions (B) and for plasma membrane and cytosolic fractions (C) as described in Materials and Methods. Lysates were immunoblotted (IB) with anti-VE-cadherin or IQGAP1 antibody. Blots are representative of 3 independent experiments.

**Figure II: Role of IQGAP1 in VEGF-stimulated phosphorylation of Akt and ROS production in HUVEC.** HUVECs transfected with scrambled or IQGAP1 siRNAs were stimulated with VEGF (20 ng/ml). **A,** Lysates were IB with anti-phospho-Akt (pSer473) or Akt antibody. *P*<0.05 versus untreated, scrambled siRNA transfected cells (n=3). **B,** Cells were incubated with DCF-DA and stimulated with VEGF for 5 min. Bar graph represents averaged data, expressed as percent increase in DCF-DA fluorescence intensity by VEGF (100%).

**Figure III: Induction and colocalization of IQGAP1 and VE-cadherin in a mouse ischemic hindlimb model of angiogenesis.** Hindlimb ischemia was induced by the right femoral artery ligation as described in Materials and Methods. Immunofluorescence staining of non-ischemic and ischemic tissues with anti-IQGAP1 (green) and VE-cadherin (red) antibodies, followed by FITC-conjugated goat anti-rabbit IgG and RRX-conjugated goat anti-mouse IgG, respectively, at 7 days after ischemia. White arrows in merged image show co-localization of IQGAP1 and VE-cadherin.
Supplemental Figure I

A. IP: IQGAP1

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Supplemental Figure II

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![Western blot images with p-Akt and Akt markers]

Fold change

B

ROS production (% increase by VEGF)

![Bar graph showing ROS production comparison between Scrambled and IQGAP1 siRNA]

*
Supplemental Figure III

Ischemic

Non-ischemic

IQGAP1

VE-Cadherin

Merge

30 μm