

# IQGAP1, a Novel Vascular Endothelial Growth Factor Receptor Binding Protein, Is Involved in Reactive Oxygen Species–Dependent Endothelial Migration and Proliferation

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**Abstract**—Endothelial cell (EC) proliferation and migration are important for reendothelialization and angiogenesis. We have demonstrated that reactive oxygen species (ROS) derived from the small GTPase Rac1-dependent NAD(P)H oxidase are involved in vascular endothelial growth factor (VEGF)–mediated endothelial responses mainly through the VEGF type2 receptor (VEGFR2). Little is known about the underlying molecular mechanisms. IQGAP1 is a scaffolding protein that controls cellular motility and morphogenesis by interacting directly with cytoskeletal, cell adhesion, and small G proteins, including Rac1. In this study, we show that IQGAP1 is robustly expressed in ECs and binds to the VEGFR2. A pull-down assay using purified proteins demonstrates that IQGAP1 directly interacts with active VEGFR2. In cultured ECs, VEGF stimulation rapidly promotes recruitment of Rac1 to IQGAP1, which inducibly binds to VEGFR2 and which, in turn, is associated with tyrosine phosphorylation of IQGAP1. Endogenous IQGAP1 knockdown by siRNA shows that IQGAP1 is involved in VEGF-stimulated ROS production, Akt phosphorylation, endothelial migration, and proliferation. Wound assays reveal that IQGAP1 and phosphorylated VEGFR2 accumulate and colocalize at the leading edge in actively migrating ECs. Moreover, we found that IQGAP1 expression is dramatically increased in the VEGFR2-positive regenerating EC layer in balloon-injured rat carotid artery. These results suggest that IQGAP1 functions as a VEGFR2-associated scaffold protein to organize ROS-dependent VEGF signaling, thereby promoting EC migration and proliferation, which may contribute to repair and maintenance of the functional integrity of established blood vessels. (*Circ Res.* 2004;95:276-283.)

**Key Words:** IQGAP1 ■ reactive oxygen species ■ vascular endothelial growth factor ■ endothelial cell ■ cell migration

Endothelial cells (ECs) are pivotal in the regulation of vascular functions including cell adhesion, inflammatory responses, vasoactivity, and macromolecular permeability. Regeneration of the endothelium after vascular damage is important in limiting atherogenesis.<sup>1</sup> EC activation, migration, and proliferation are important for endothelial wound repair and neovascularization, a process by which new blood vessels are formed from preexisting vessels.<sup>2</sup> The underlying molecular mechanisms are incompletely understood.

Vascular endothelial growth factor (VEGF) stimulates EC migration and proliferation primarily through the VEGF type 2 receptor (VEGFR2, KDR/Flk-1), thereby contributing to angiogenesis *in vivo*.<sup>3</sup> In ECs, VEGF binding initiates dimerization and transphosphorylation (autophosphorylation) of tyrosine residues in the cytoplasmic kinase domain of VEGFR2, which is followed by activation of key signaling

enzymes involved in angiogenesis/neovascularization including mitogen activated proteins (MAP) kinases and Akt.<sup>4</sup> VEGF also promotes mobilization and recruitment of endothelial progenitor cells into ischemic sites, which contribute to neovascularization.<sup>5,6</sup> Moreover, VEGF is upregulated and promotes regeneration of ECs in balloon-injured arteries.<sup>7,8</sup> We and others demonstrated that VEGF stimulates an increase in reactive oxygen species (ROS) generation via activation of the small GTPase Rac1-dependent NAD(P)H oxidase and that ROS participate in VEGFR2-mediated signaling, EC migration, and proliferation.<sup>9–11</sup> Relatively little is known about the detailed molecular pathways linking VEGFR2 activation with these Rac-mediated redox-sensitive responses in ECs.

In preliminary studies using a yeast two hybrid system, we screened a human aortic cDNA library for proteins that bind

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the cytoplasmic domain of VEGFR2 and identified a partial sequence of the cDNA for IQGAP1. IQGAP1 is a scaffolding protein that is involved in cellular motility and morphogenesis<sup>12</sup> by interacting directly with cytoskeletal, cell adhesion, and signal transduction proteins including calmodulin,<sup>13,14</sup> activated Cdc42 and Rac1,<sup>15</sup> actin,<sup>16</sup>  $\beta$ -catenin,<sup>17,18</sup> E-cadherin,<sup>14,19</sup> and the microtubule plus end binding protein CLIP-170.<sup>20</sup> IQGAP1 derives its name from the presence of four calmodulin-binding IQ motifs on each of its two identical subunits,<sup>16,21</sup> as well as a region with sequence similarity to the catalytic domain of Ras GTPase activating proteins (Ras-GAPs).<sup>22</sup> Although originally posited to be a GAP based on its amino acid sequence similarity to Ras-GAP, subsequent *in vitro* analysis revealed that IQGAP1 binds to active (GTP-bound) Rac1, which in turn inhibits its intrinsic GTPase activity, thereby increasing active Rac1.<sup>15,23</sup> Recent evidence shows that IQGAP1 is involved in regulating cell migration in a Rac1/Cdc42-dependent manner in MCF-7 cells.<sup>23</sup>

In the present study, we demonstrate that IQGAP1 is robustly expressed in ECs and binds to VEGFR2. Pull-down assays show that IQGAP1 directly interacts with active VEGFR2. In cultured ECs, VEGF stimulation rapidly promotes recruitment of Rac1 to the IQGAP1 that inducibly associates with VEGFR2. The formation of the Rac1, IQGAP1, VEGFR2 complex also results in the tyrosine phosphorylation of IQGAP1. VEGF-stimulated ROS production, its downstream ROS-dependent Akt phosphorylation, endothelial migration, and proliferation are inhibited by IQGAP1 siRNA, suggesting that IQGAP1 is a critical component of VEGF signaling. IQGAP1 and phosphorylated VEGFR2 accumulate and colocalize at the leading edge in actively migrating ECs. Moreover, we found that IQGAP1 expression is dramatically increased in the VEGFR2-positive regenerating EC layer in the balloon-injured rat carotid artery. These results suggest that IQGAP1 may function as a scaffold protein to organize ROS-dependent VEGF signaling, thereby promoting EC migration and proliferation, which may contribute to the regeneration of ECs after vascular injury.

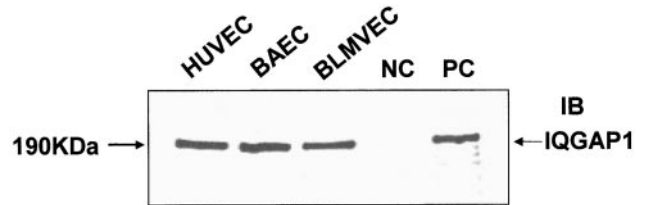
## Materials and Methods

Materials, cell culture, yeast two-hybrid screening, *in vitro* GST pull-down assay, synthetic siRNA and its transfection, measurements of intracellular H<sub>2</sub>O<sub>2</sub> levels, scratch wound assay, modified Boyden chamber assay, *in vitro* proliferation assay, confocal immunofluorescence microscopy, balloon injury and immunohistochemical staining, and statistical analyses are described in the expanded Materials and Methods section in the online data supplement available at <http://circres.ahajournals.org>. Immunoprecipitation and immunoblotting were performed as described previously.<sup>24</sup>

## Results

### IQGAP1 Is Expressed in ECs and Binds to VEGFR

To identify signaling molecules that associate with VEGFR2, the VEGFR2<sub>cyto</sub> was used as a bait to screen a human aorta cDNA library in the yeast two-hybrid system. Positive clones identified in this screen encoded a partial sequence of cDNA for IQGAP1<sup>22</sup> (data not shown). Subsequently, to examine whether IQGAP1 protein is expressed in cultured ECs, we



**Figure 1.** IQGAP1 is expressed in various ECs. HUVEC, BAEC, and BLMVEC lysates were immunoblotted with anti-IQGAP1 antibody. Blots are representative of three experiments. NC indicates negative control (cell lysates from *E. coli*); PC, positive control (recombinant full-length His-tagged IQGAP1 protein).

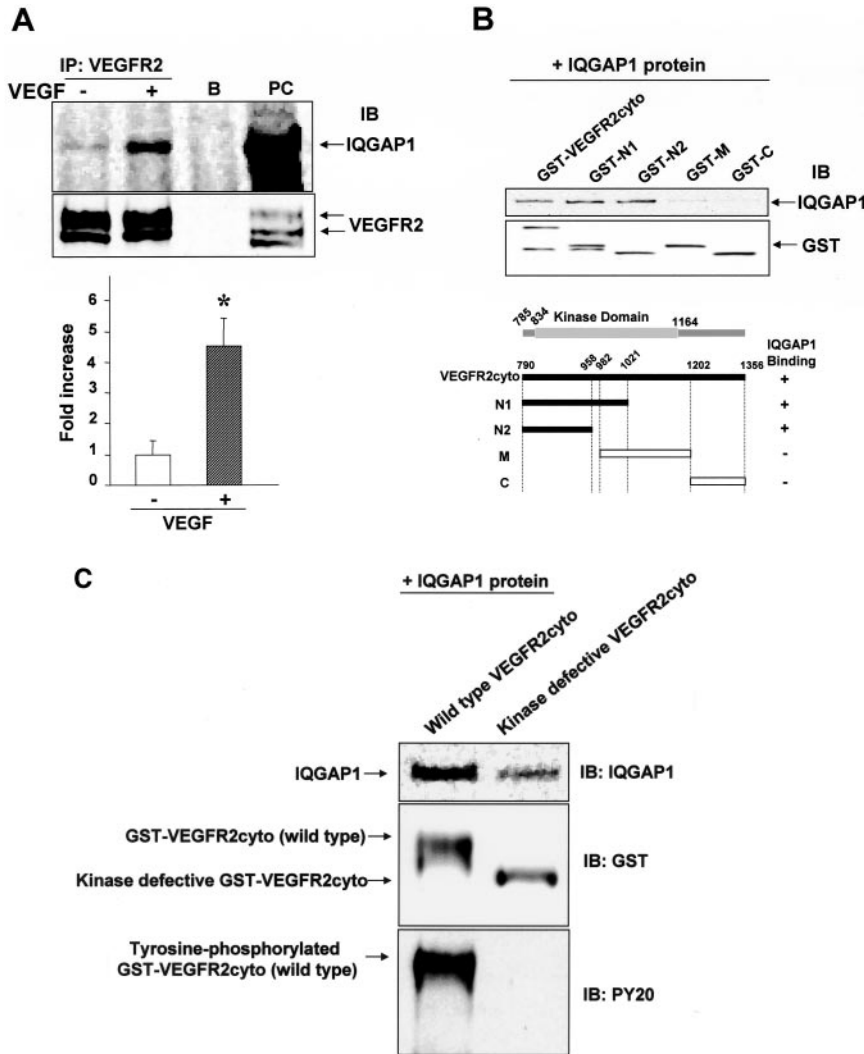
performed immunoblot analysis using antibody raised against IQGAP1 and found that anti-IQGAP1 antibody reacted in cell lysates of HUVECs, BAECs, and BLMVECs with a 190-kDa protein (Figure 1), which is consistent with the previously reported molecular mass of IQGAP1 in other cell systems.

To gain insights into the potential physical interaction of IQGAP1 and VEGFR2 in ECs, we performed coimmunoprecipitation assays in HUVEC lysates. As shown in Figure 2A, VEGFR2 and IQGAP1 were slightly bound in basal state and VEGF stimulation for 5 minutes caused a significant increase in the amount of IQGAP1 in the complex immunoprecipitated with anti-VEGFR2 antibody. Similarly VEGF-induced formation of the IQGAP1-VEGFR2 complex was observed also in BAECs and BLMVECs (data not shown).

To examine whether IQGAP1 directly interacts with VEGFR2, we also performed pull-down assays using GST-VEGFR2<sub>cyto</sub> and its various deletion mutants with purified IQGAP1 protein<sup>16</sup> (Figure 2B). As shown in Figure 2B, the purified IQGAP1 protein bound to GST-VEGFR2<sub>cyto</sub> (aa 790 to 1356), -N1 (aa 790 to 1021), and -N2 (aa 790 to 958), but not to GST-M (aa 982 to 1202) or -C (aa 1202 to 1356). These results suggest that IQGAP1 directly binds a region of VEGFR2 within the kinase domain comprising amino acids 790 to 958 of the receptor. Since IQGAP1 association with VEGFR2 was promoted after VEGF stimulation, we next examined whether IQGAP1 binds more preferentially to the active form of VEGFR2. For this purpose, we performed pull-down assays using constitutively tyrosine phosphorylated GST-VEGFR2<sub>cyto</sub> (Figure 2C, lower panel) and a kinase defective mutant GST-VEGFR2<sub>cyto</sub> (K866R) purified from Sf9 cells. As shown in Figure 2C, the purified IQGAP1 protein bound more strongly to the wild-type GST-VEGFR2<sub>cyto</sub> than VEGFR2<sub>cyto</sub> (K866R).

### VEGF Stimulates the Recruitment of Rac1 to IQGAP1 and the Tyrosine Phosphorylation of IQGAP1 in ECs

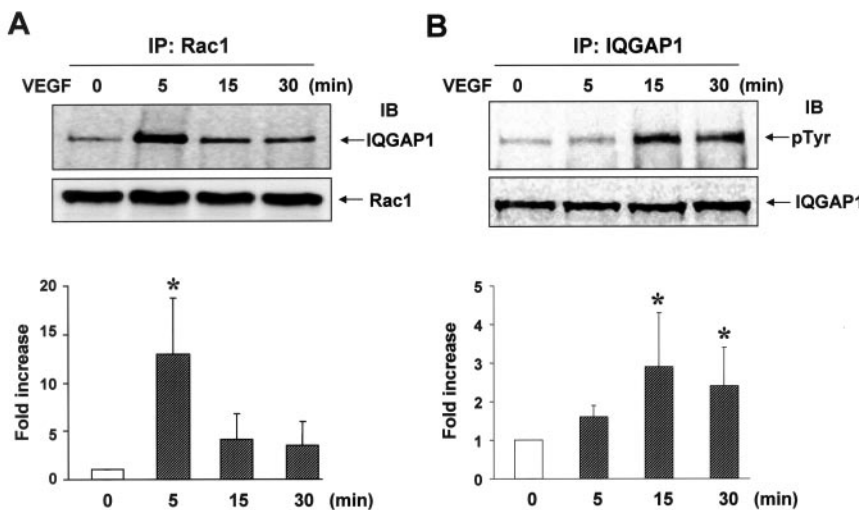
Purified IQGAP1 has been shown to bind to the GTP-bound form of Rac1, which in turn inhibits the intrinsic GTPase activity of Rac1,<sup>15</sup> thereby increasing levels of active Rac1 in cells.<sup>23</sup> Because VEGF stimulation rapidly activates Rac1,<sup>10,11</sup> we posited that VEGF would promote Rac1 association with IQGAP1 in ECs. As shown in coimmunoprecipitation experiments with HUVECs, Rac1 and IQGAP1 were slightly bound basally and this association was significantly enhanced within 5 minutes of VEGF stimulation (Figure 3A). This result is consistent with the fact that VEGF activates Rac1.



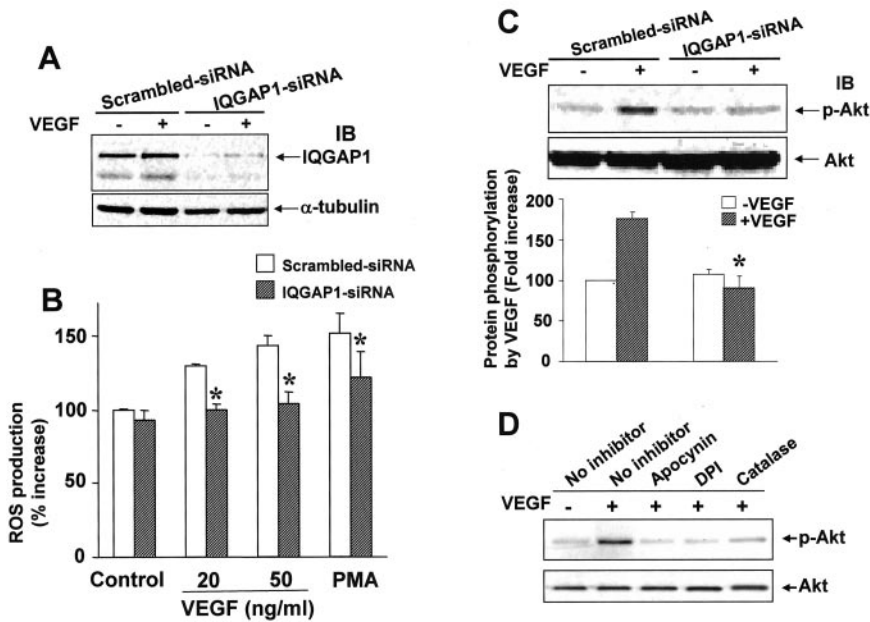
**Figure 2.** IQGAP1 directly binds to VEGFR2. A, IQGAP1 coimmunoprecipitates with VEGFR2 in HUVECs. Growth-arrested HUVECs were stimulated with (+) or without (-) VEGF (20 ng/mL) for 5 minutes. Lysates were immunoprecipitated (IP) with anti-VEGFR2 antibody, followed by immunoblotting (IB) with anti-IQGAP1 antibody or anti-VEGFR2 antibody. B indicates buffer blank; PC, positive control (cell lysates before immunoprecipitation). Bottom graphs represent averaged data (n=4), expressed as a fold increase in the extent of association over that in unstimulated cells. \*P<0.05 for changes induced by VEGF versus unstimulated cells. B, GST-conjugated VEGFR2 cytoplasmic domain fusion protein (GST-VEGFR2cyto) and its various deletion mutants (GST-N1, N2, M, and C) were expressed and purified from *E. coli*. GST fusion proteins bound to IQGAP1 protein were separated by SDS-PAGE, followed by immunoblotting with anti-IQGAP1 antibody or anti-GST antibody to show equal loading of GST fusion proteins. Representative blots (Top) and the schematic model of VEGFR2 deletion mutant construct (Bottom) are shown. C, GST-VEGFR2cyto (wild-type) and its kinase-defective mutant GST-VEGFR2cyto (K866R) were purified from Sf9 cells. GST fusion proteins bound to IQGAP1 protein were separated and immunoblotted with anti-IQGAP1 antibody or anti-GST antibody or anti-phosphotyrosine (pTy) antibody. For the GST blot, the differential mobility of GST wild-type VEGFR2cyto and the GST kinase-defective VEGFR2cyto probably reflects the intrinsic tyrosine phosphorylation of the wild-type protein (C, bottom).

To investigate further the possibility that IQGAP1 is a component of VEGF signaling, we next examined whether IQGAP1 is tyrosine phosphorylated after VEGF stimulation in HUVECs. As shown in Figure 3B, immunoprecipitated IQGAP1 was immunoreactive on Western blots probed with

an anti-tyrosine antibody, and the level of tyrosine phosphorylated IQGAP1 increased substantially within 15 minutes after VEGF stimulation (3.2-fold increase, P<0.05). The tyrosine phosphorylation of IQGAP1 may be important for activation of downstream VEGF signaling events in ECs.



**Figure 3.** VEGF stimulates Rac1 recruitment to IQGAP1, and tyrosine phosphorylation of IQGAP1 in ECs. Growth-arrested HUVECs were stimulated with 20 ng/mL of VEGF for indicated times (min). A, Lysates were immunoprecipitated (IP) with anti-Rac1 antibody, followed by immunoblotting (IB) with anti-IQGAP1 antibody or anti-Rac1 antibody. B, Effects of VEGF on IQGAP1 tyrosine phosphorylation in ECs. Lysates were immunoprecipitated (IP) with anti-IQGAP1 antibody, followed by immunoblotting (IB) with anti-phosphotyrosine (pTy) antibody or anti-IQGAP1 antibody. Bar graph represents averaged data, expressed as a fold increase in the extent of association of Rac1 with IQGAP1 (A) or in tyrosine phosphorylation of IQGAP1 (B) over that in unstimulated cells (control). Values are the mean±SE for 3 independent experiments. \*P<0.05 vs control.



**Figure 4.** Involvement of IQGAP1 in VEGF-induced  $H_2O_2$  production and Akt phosphorylation in ECs. HUVECs were transfected with scrambled siRNA (control) or IQGAP1 siRNA. A, Lysates were immunoblotted with anti-IQGAP1 antibody to confirm the knockdown effect of siRNA. B, Effect of IQGAP1 siRNA on VEGF-stimulated  $H_2O_2$  production. Cells were incubated with DCF-DA for 2.5 hours and stimulated with 20 or 50 ng/mL VEGF or 1  $\mu$ mol/L PMA for 5 minutes. Graphs represent averaged data, expressed as fold increase in the relative increase in DCF-DA fluorescence intensity over that in unstimulated cells transfected with scrambled siRNA. C, Effect of IQGAP1 siRNA on VEGF-stimulated Akt phosphorylation. Cells were stimulated with 20 ng/mL of VEGF for 15 minutes, and immunoblotted with anti-phospho-Akt antibody or anti-Akt antibody. Bar graph represents averaged data, expressed as percent increase by VEGF (100% = control). In B and C, values are the mean  $\pm$  SE for 3 independent experiments. \* $P < 0.05$  for increase by

VEGF or PMA (for B) in cells transfected with IQGAP1 siRNA vs scrambled siRNA. D, Effects of antioxidants on VEGF-stimulated Akt phosphorylation. Cells were preincubated with vehicle, apocynin (1 mmol/L), DPI (10  $\mu$ mol/L) for 30 to 60 minutes, or with catalase (1000 U/mL) for 12 hours and then stimulated with 20 ng/mL VEGF for 15 minutes, and immunoblotted with anti-phospho-Akt antibody or anti-Akt antibody.

### IQGAP1 Is Involved in VEGF-Stimulated ROS-Dependent Signaling in ECs

Because ROS are important mediators for VEGF signaling in ECs, we next examined, using siRNA to knockdown endogenous IQGAP1, whether IQGAP1 is involved in VEGF-stimulated ROS production and activation of the key downstream signaling enzyme Akt. As shown in Figure 4, transfection of HUVECs with IQGAP1 siRNA, but not control scrambled siRNA, significantly reduced endogenous IQGAP1 expression (Figure 4A) and inhibited VEGF-induced  $H_2O_2$  production, as measured at 5 minutes after stimulation (Figure 4B). Furthermore, phorbol myristate acetate (PMA) stimulation for 5 minutes induced a rapid increase in DCF-DA fluorescence, which was also significantly inhibited by IQGAP1 siRNA, suggesting that IQGAP1 acts on common targets which are involved in ROS production and are activated by both VEGF and PMA. Similar effects were obtained after 30 minutes stimulation with VEGF and PMA (data not shown). Moreover, VEGF-stimulated phosphorylation of Akt was significantly inhibited by IQGAP1 siRNA, but not by scrambled siRNA (Figure 4C). Importantly, we found that VEGF-induced phosphorylation of Akt was inhibited by various antioxidants including apocynin, DPI, and catalase, suggesting that Akt is a redox-sensitive kinase in the VEGF signaling pathway (Figure 4D). Taken together these results indicate that IQGAP1 is involved in ROS-dependent VEGF signaling in ECs.

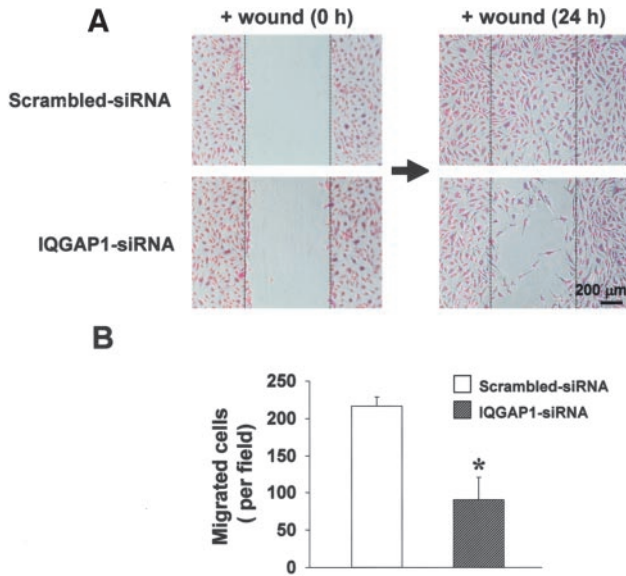
### IQGAP1 Is Involved in VEGF-Stimulated Cell Migration and Proliferation in ECs

To examine the functional roles of IQGAP1 in VEGF-induced cell migration, we performed both wound scratch and modified Boyden chamber assays using IQGAP1 siRNA. For

the wound scratch assay, which resembles reendothelialization and primarily measures migration of cells,<sup>25</sup> confluent monolayers of HUVECs were transfected with IQGAP1 siRNA or control scrambled siRNA and were wounded in the presence of VEGF, and the cells migrating into the wounded area were compared after 24 hours. As shown in Figure 5, IQGAP1 siRNA, but not control siRNA, significantly inhibited cell migration toward the wound area (68% inhibition,  $P < 0.01$ ). Moreover, modified Boyden chamber assays also demonstrated that transfection of IQGAP1 siRNA, but not scrambled control siRNA, inhibited VEGF-stimulated cell migration, in a dose-dependent manner, in BLMVECs (Figure 6A). Importantly, similar results were obtained in HUVECs. IQGAP1 siRNA had no effect on either migration in the basal state or the migratory response induced by sphingosine 1-phosphate (S1P), the effect of which is independent of ROS<sup>11</sup> (Figure 6B), confirming the specificity of IQGAP1 siRNA. Similarly, IQGAP1 siRNA, but not scrambled control siRNA, significantly blocked VEGF-stimulated cell proliferation without affecting the basal state in HUVECs (Figure 6C). Taken together, these data strongly suggest that IQGAP1 is involved in VEGF-stimulated EC migration and proliferation.

### IQGAP1 Colocalizes With Activated VEGFR2 at the Leading Edge in Actively Migrating ECs

To gain insight into the role of the IQGAP1 association with VEGFR2 in VEGF-stimulated EC migration, we examined whether IQGAP1 colocalizes with VEGFR2 in actively migrating ECs after wounding. As shown in Figure 7, in confluent monolayers of unstimulated HUVECs, IQGAP1 and basally phosphorylated VEGFR2 are mainly localized at the cell margins. In contrast, after wounding in the presence



**Figure 5.** Involvement of IQGAP1 in wound scratch-induced repair process of ECs. HUVECs were transiently transfected with scrambled siRNA (control) or IQGAP1 siRNA for 96 hours. Monolayer confluent cells were growth-arrested and scraped with a 200  $\mu$ L pipette tip in the presence 50 ng/mL VEGF to stimulate the EC migration toward the wound area. A, Images were captured immediately after rinsing at 0 hour and at 24 hours after the wounding in siRNA-transfected cells. B, Bar graph represents averaged data, expressed as cell number per 4 fields. Values are the mean  $\pm$  SE for 3 independent experiments. \* $P < 0.05$  vs control.

of VEGF to stimulate directed cell migration into the open space, both IQGAP1 and autophosphorylated VEGFR2, as detected by phospho-specific VEGFR2 antibody raised against pY1054, one of major autophosphorylation sites of VEGFR2,<sup>26</sup> accumulated and colocalized at the leading edge. These results suggest that VEGF-stimulated EC migration is associated with the spatially restricted activation of VEGFR2 and IQGAP1.

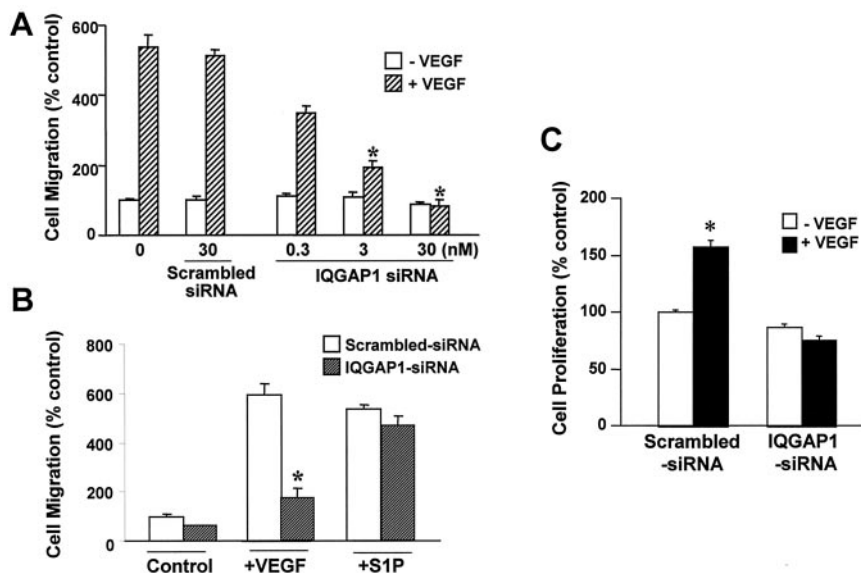
### IQGAP1 Expression Is Dramatically Increased in the VEGFR2-Positive Regenerating EC Layer in Balloon-Injured Rat Carotid Artery

EC migration is a key event for endothelial wound repair that is crucial in limiting vascular damage due to injury of the artery wall. To assess the role of IQGAP1 in the vasculature in vivo, we examined the expression of IQGAP1 in balloon-injured rat carotid artery. As shown in Figure 8, IQGAP1 protein expression was dramatically increased in the luminal regenerating endothelial layers, which was associated with the increase in expression of VEGFR2 at 4 weeks after balloon-injured arteries compared with uninjured control. IQGAP1 expression was slightly induced at injured lesion of ECs at 1 week after balloon injury, which gradually increased up to 4 weeks after injury (data not shown). Given the functional role of IQGAP1 in EC migration, these data suggest that IQGAP1 is involved in the repair process of ECs after vascular injury.

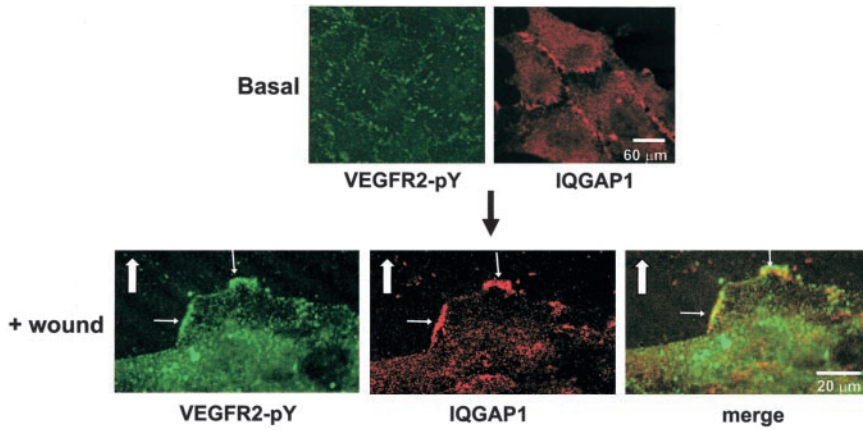
### Discussion

IQGAP1 contains multiple protein-interacting domains that mediate binding to target molecules including F-actin,<sup>21</sup> calmodulin,<sup>13,14</sup> Cdc42 and Rac1,<sup>13,15</sup> actin,<sup>16</sup>  $\beta$ -catenin,<sup>17,18</sup> E-cadherin,<sup>14,19</sup> and the microtubule tip binding protein CLIP-170.<sup>20</sup> The diversity of IQGAP1 binding partners suggests that IQGAP1 functions as a particularly robust scaffold that regulates multiple signaling networks and the actin cytoskeleton.<sup>12,27,28</sup> In the present study, we demonstrate that IQGAP1 is a VEGFR2 binding protein in ECs. The interaction is functional because IQGAP1 siRNA significantly inhibits VEGF-stimulated ROS production, Akt activation, EC migration, and proliferation. Moreover, we also found that IQGAP1 expression is dramatically upregulated in VEGFR2-positive regenerating EC layers after vascular injury, a finding that is consistent with a putative role of IQGAP1 in endothelial regeneration.

IQGAP1 is expressed robustly in ECs from several sources (Figure 1) and coimmunoprecipitated with VEGFR2 (Figure 2A). Treatment of ECs with VEGF markedly increases over



**Figure 6.** IQGAP1 is involved in VEGF-induced cell migration and proliferation in ECs. BLMVECs (A) and HUVECs (B and C) were transiently transfected with scrambled siRNA (control) or IQGAP1 siRNA for 96 hours. A and B, Cell migration was measured by the modified Boyden chamber method after stimulation with vehicle (-), 50 ng/mL of VEGF, or 10  $\mu$ mol/L sphingosine 1-phosphate (S1P) (for B) for 6 (B) to 7 hours (A). Migrated cells through Transwell pores were quantified by counting under a light microscope. Bar graph represents averaged data, expressed as cell number counted per 4 fields ( $\times 200$ ). C, Cell proliferation stimulated with or without 20 ng/mL VEGF was determined by cell number after plating cells in 0.2% FBS containing culture medium for 48 hours. Values are the mean  $\pm$  SE for 3 independent duplicate experiments. \* $P < 0.05$  for effects of VEGF in cells transfected with IQGAP1 siRNA vs scrambled siRNA.



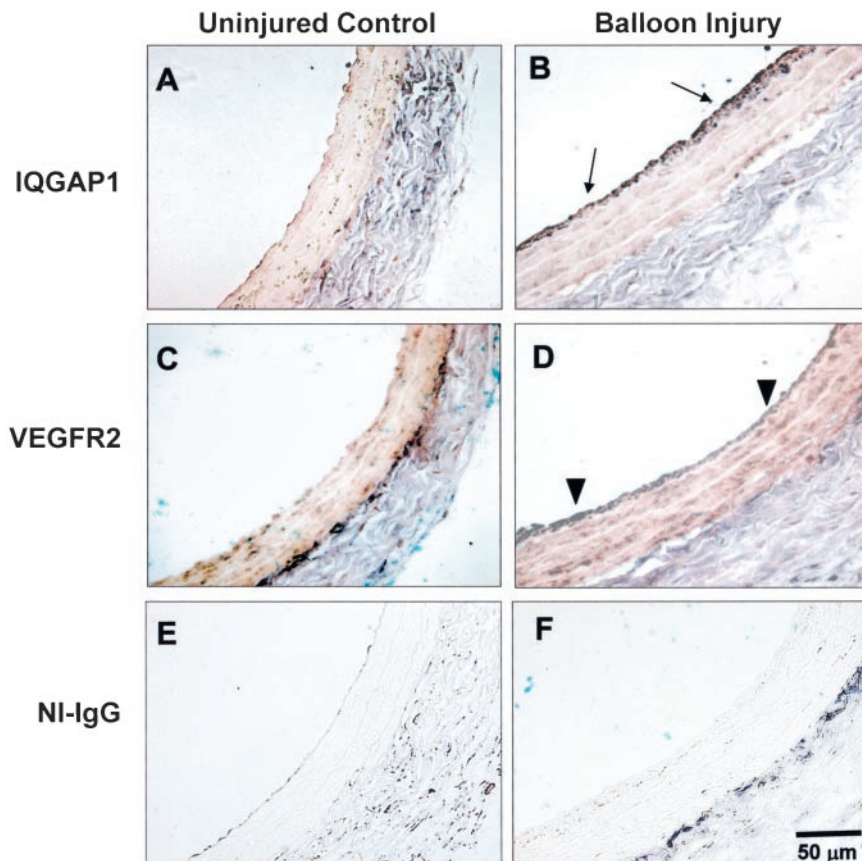
(green) and anti-IQGAP1 antibody (red). Small white arrows point to the leading edge and white arrows point to direction of scraping. IQGAP1 and autophosphorylated VEGFR2 colocalize at the leading edge after VEGF stimulation. Results are representative of 3 independent replicates of immunofluorescence images.

**Figure 7.** IQGAP1 colocalizes with auto-phosphorylated VEGFR2 at the leading edge in actively migrating ECs. Top, Growth-arrested confluent monolayer of HUVECs were fixed and stained with rabbit anti-phospho-VEGFR2 (pY1054) antibody (VEGFR2-pY; green) or mouse anti-IQGAP1 antibody (red) followed by anti-rabbit FITC-conjugated and anti-mouse Rhodamine Red X-conjugated secondary antibodies. IQGAP1 and basally phosphorylated VEGFR2 are mainly localized at cell margins in unstimulated ECs. Bottom, Growth-arrested HUVECs were wounded by scraping with a sterile rubber policeman and stimulated with 50 ng/mL VEGF for 8 hours. Cells were fixed and double-labeled with anti-VEGFR2-pY antibody

basal levels the amount of IQGAP1 in the complex brought down by anti-VEGFR2. Two lines of evidence using pull-down assays suggest that the IQGAP1-VEGFR2 interaction is direct. First, recombinantly and bacterially constructed VEGFR2 cytoplasmic tail sequences within the kinase domain corresponding to the amino acid sequence 790 to 958 interacted directly with purified IQGAP1 (Figure 2B). Secondly, the wild-type GST-VEGFR2<sub>cyto</sub> purified from Sf9 cells is tyrosine phosphorylated (and presumably active) and binds purified IQGAP1 more robustly than does the GST-

kinase defective VEGFR2<sub>cyto</sub> mutant that is not tyrosine phosphorylated (Figure 2C). Thus, these data also support inferentially the notion that the direct IQGAP1-VEGFR2 interaction is functional, and the interaction requires the kinase domain and is enhanced by activation. The weak basal interaction of IQGAP1 and VEGFR2 seen in ECs and the substantial increase with VEGF stimulation (Figure 2A) can be explained in this light.

IQGAP1 was originally posited to be a GAP based on its amino acid sequence similarity to Ras-GAP;<sup>22</sup> however,



**Figure 8.** Upregulation of IQGAP1 in the VEGF receptor 2 (VEGFR2)-positive regenerating ECs layer in balloon-injured rat carotid artery. Balloon-catheter injury of rat carotid arteries was induced as described in Experimental Procedures. The left common carotid artery wall was injured with an embolectomy balloon catheter (2F Fogarty), and the right common carotid artery served as uninjured control. Carotid arteries were cut into cross-sectional segments, and sections (5- $\mu$ m thick) were immunohistologically stained with a rabbit anti-IQGAP1 antibody (Santa Cruz, 1:500 dilution) or a mouse anti-VEGFR2 antibody (Santa Cruz, 1:50 dilution) using DAB substrate kit (Vector Laboratory). Image was displayed in a high-resolution monitor and digitized by a video frame ( $\times 400$ ). Representative sections show strong immunoreactive staining for IQGAP1 or VEGFR2 in the regenerated EC layers at 4 weeks after balloon injury (B and D). Nonimmune IgG (NI-IgG) shows no specific staining. A, C, and E, Serial sections obtained from uninjured right common carotid artery; B, D, and F, Serial sections from artery obtained 4 weeks after injury.

subsequent studies revealed that purified IQGAP1 binds to active Rac1 through a GAP-related domain (GRD), thereby suppressing the intrinsic GAP activity of the GTPase.<sup>15</sup> Thus, IQGAP1 stabilizes and increases the amount of active, GTP-bound Rac1.<sup>23</sup> In ECs, Rac1 is involved in VEGF-mediated activation of NAD(P)H oxidase.<sup>11</sup> We have previously shown that VEGF rapidly stimulates Rac1 translocation from the cytosol to the EC plasma membrane, which is essential to the activation of NAD(P)H oxidase, and that the resulting ROS are involved in VEGF2 autophosphorylation and angiogenic-related effects of VEGF.<sup>11</sup> In the present study, we show that VEGF stimulation promotes association of Rac1 with IQGAP1 (Figure 3A), which inducibly associates with VEGFR2 in ECs (Figure 2A). Given that VEGFR2 localizes at the plasma membrane, IQGAP1 may function as a VEGFR2-associated scaffolding protein that recruits active Rac1 to the plasma membrane in close proximity to VEGFR2, thereby facilitating the spatial specificity of activation of ROS-dependent VEGF signaling. Moreover, we also found that VEGF stimulation induces tyrosine phosphorylation of IQGAP1 beginning within 5 minutes (Figure 3B), further emphasizing that IQGAP1 is a critical component of VEGF signaling in ECs. We considered that tyrosine-phosphorylated IQGAP1 may provide the binding sites for the SH2- or phosphotyrosine-binding domain containing signaling molecules, thereby recruiting and forming multiple complexes with IQGAP1, which in turn, may facilitate the activation of downstream VEGF signaling events. We have found that IQGAP1 has 16 predicted tyrosine phosphorylation sites based on a computer-based sequence search; however, neither SH2 nor SH3 domain binding consensus sequences are found in the predicted phosphorylation sites. The implications of tyrosine phosphorylation of IQGAP1 are currently under investigation.

A functional role of IQGAP1 in ROS-dependent VEGF responses is demonstrated by the observations that IQGAP1 siRNA significantly inhibits VEGF-stimulated ROS production (Figure 4B) as well as cell motility and growth (Figures 5 and 6). In the present study, we used DCF-DA assay to measure ROS production. Although DCF fluorescence detects not only H<sub>2</sub>O<sub>2</sub> but also other peroxides, we have confirmed that VEGF-induced increase in DCF fluorescence is inhibited by catalase in HUVECs. Importantly, angiogenic S1P-stimulated EC migration is not affected by IQGAP1 siRNA (Figure 6B), consistent with its inability to produce ROS,<sup>11</sup> and supporting the specificity of the effects of IQGAP1 siRNA. Mataraza et al<sup>23</sup> reported that overexpression of IQGAP1 promotes cell migration in a Rac1- and Cdc42-dependent manner. We have previously demonstrated that overexpression of dominant-negative Rac1 almost completely blocks VEGF-stimulated ROS production, endothelial cell migration, and proliferation.<sup>11</sup> We also showed that endothelial cell migration and proliferation are ROS dependent because they are inhibited by antioxidant N-acetylcysteine as well as by transfecting antisense gp91phox oligonucleotide.<sup>11</sup> These results clearly suggest that Rac1-mediated increase in ROS derived from NAD(P)H oxidase are essential for the VEGF-induced angiogenic responses in ECs. The present study shows that VEGF binding to VEGFR2 in cultured ECs rapidly stimulates direct binding of IQGAP1 to the VEGFR2 and association of activated Rac1 with IQGAP1, which may enhance

and stabilize Rac1 activity.<sup>15,23</sup> These events are followed rapidly by increased ROS production (Figure 4B), ROS-dependent Akt phosphorylation (Figure 4D), endothelial cell migration and proliferation (Figures 5 and 6), all of which are significantly inhibited by IQGAP1 siRNA. Thus, these data suggest that IQGAP1 functions as a VEGFR2-associated scaffolding protein to organize ROS-dependent VEGF signaling leading to EC migration and proliferation. Because ROS formation induced by PMA, which also activates Rac1, is sensitive to IQGAP1 siRNA (Figure 4B), it is likely that Rac1-IQGAP1 interaction is important for regulating ROS production. To assess the functional role of Rac1 binding to IQGAP1 directly, we are currently investigating the effects of overexpression of mutant IQGAP1 that lacks the Rac1 binding domain on ROS-dependent VEGF signaling. Recently, IQGAP1 has been shown to be involved in EGF- as well as IGF-1-induced signaling in human breast epithelial cells<sup>29</sup>; thus, it is likely that IQGAP1 may be a common critical component of many growth factor signaling in various cell systems. However, the VEGFR2-IQGAP1 response seems not to be generalizable to all the angiogenic responses, because S1P-induced endothelial migration is not dependent on IQGAP1 (Figure 6B)

Scaffold proteins function to assemble specific sets of signaling molecules and to target and/or localize these complexes to spatially defined cellular domains.<sup>30</sup> Previously, it has been shown that IQGAP1 localizes at cell-cell junctions in epithelial cells.<sup>31</sup> Endogenous or overexpressed IQGAP1 accumulates at the leading edge of migrating cells, suggesting a role of IQGAP1 as a determinant of polarized signal generation, which is important for directed cell migration.<sup>12,23,32</sup> As shown in Figure 7, in confluent monolayers of unstimulated ECs, both IQGAP1 and basally phosphorylated VEGFR2 are mainly localized at cell margins. After wounding and stimulating with VEGF to induce directed migration, autophosphorylated VEGFR2 accumulates and colocalizes with IQGAP1 at the leading edge. The role of IQGAP1 in directed migration probably reflects its ability to stimulate nucleation of branched actin filaments by Arp2/3 in motile lamellipodia (unpublished observations, 2004). The polarized distribution of active VEGFR2 at the leading edge during EC migration is intriguing, because this result contrasts with previous reports that G-protein-coupled chemoattractant receptors (*Dictyostelium* cAMP or mammalian C5a receptors) are uniformly distributed along the plasma membrane in highly polarized, chemoattractant-stimulated cells.<sup>33,34</sup> Given that VEGFR2 directly binds to IQGAP1, it is possible that IQGAP1 functions as a scaffold to bring relevant signaling molecules perhaps including the receptor itself to the leading edge in VEGF-stimulated, migrating ECs. Of note, it has been shown that there is a gradient of intracellular Rac1 activity with the highest levels at the leading edge in a wound-induced migrating cell model.<sup>35</sup> Furthermore, endogenous H<sub>2</sub>O<sub>2</sub> is generated at the wound edge in actively migrating ECs.<sup>36</sup> NAD(P)H oxidase inhibitors, O<sub>2</sub><sup>-</sup> scavengers or dominant-negative Rac1 block EC migration,<sup>11,36,37</sup> suggesting that the generation of H<sub>2</sub>O<sub>2</sub> resulting from the localized activation of VEGF2-Rac1-NAD(P)H oxidase may be essential to directed EC migration. Because IQGAP1 forms complexes with VEGFR2 and Rac1, these observations suggest that IQGAP1 may play an important role in targeting and/or localizing active VEGFR2 and active Rac1 at

the leading edge of migrating ECs, thereby promoting compartmentalized ROS production and directed cell migration.

Endothelial cell migration and proliferation are important for endothelial regeneration after vascular injury, and VEGF has been shown to participate in the repair process of ECs in balloon-injured arteries.<sup>7</sup> In the present study, we demonstrate that both IQGAP1 and VEGFR2 protein expression are dramatically increased in the luminal regenerated EC layers at 4 weeks after balloon-injured rat carotid artery (Figure 8). Although their biological/functional relationships in vivo remain unknown, based on our in vitro data, these results suggest that upregulation of IQGAP1 may contribute to the repair process of ECs after vascular injury.

In summary, the present study suggests that IQGAP1 functions as a VEGFR2-associated scaffolding protein to spatially organize ROS-dependent VEGF signaling, thereby promoting EC migration and proliferation. These mechanisms may contribute to the repair and maintenance of the functional integrity of established blood vessels as well as to the development of neovasculation, providing insight into a central role for IQGAP1 in vascular homeostasis.

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## Materials and Methods

*Materials-* Antibodies to VEGFR2, IQGAP1 (H-109), phosphotyrosine (PY20), Rac1 (C-11, for immunoprecipitation) and Akt were from Santa Cruz. Antibodies to IQGAP1, Rac1 (for immunoblotting) and phosphotyrosine (PY20) or phospho-VEGFR2 (pY1054) were from BD Biosciences Pharmingen. Anti-phospho- Akt antibody was from Cell Signaling. Mouse monoclonal anti-IQGAP1 antibody was produced as described previously <sup>1</sup>, and recombinant full-length His-tagged IQGAP1 protein was expressed and purified from Sf9 insect cells infected with baculovirus as described previously <sup>1</sup>. Human recombinant VEGF<sub>165</sub> was from R&D Systems and BRB Preclinical Repository. Carboxy-H<sub>2</sub>2',7'-dichlorofluorescein diacetate (DCF-DA) was obtained from Molecular probes. Transwell 24-well plates were from BD Biosciences. Enhanced chemiluminescence (ECL) Western Blotting Detection Reagents and nitrocellulose membranes (Hybond-ECL) were obtained from Amersham Biosciences Corp. Oligofectamine and Opti-MEMI Reduced-Serum Medium were from Invitrogen Corp. All other chemicals and reagents were from Sigma.

*Cell Culture-* Human umbilical vein ECs (HUVECs) were obtained from the Emory Skin Disease Research Center and were grown in endothelial cell growth medium (EGM-MV, Clonetics) containing 10% fetal bovine serum (FBS; Invitrogen) in endothelial basic medium (EBM), as described <sup>2</sup>. Experiments were performed using cells between passage 3 and 6. Bovine aortic endothelial cells (BAEC) were obtained from VEC technologies, Inc. and grown in Medium 199 (Invitrogen) containing penicillin (100 U/mL), streptomycin (100 mg/mL), vitamins, and 10% FBS. Experiments were performed using cells between passage 4 and 9. Bovine lung microvascular ECs (BLMVECs) were obtained from VEC technologies and grown in MCDB complete media containing antibiotics and FBS (VEC technologies). Experiments were performed using cells between passage 4 and 9. For Western blotting and immunoprecipitation experiments, cells were starved for 4 hours with serum free media before

agonist stimulation. For some experiments (cell migration, cell proliferation, ROS measurement), cells were incubated in EBM containing 0.5% FBS with supplement overnight.

*Yeast Two-hybrid Screening-* To identify VEGFR2 binding proteins, MATCHMAKER GAL4 Two-hybrid System 3 (Clontech) was used according to the manufacturer's instruction. This cDNA encoding the entire intracellular domain of murine VEGFR2 (VEGFR2cyto) kindly provided by Dr. Kevin Peters (Procter & Gamble Health Care Research Center) was subcloned into the yeast bait vector, pGBKT7 (Clontech). For library screening, a human aortic cDNA library (Clontech) constructed into the pACT2 vector was used as a prey and pGBKT7-VEGFR2cyto was used as bait. The library plasmids screened by  $\beta$ -galactosidase activity were recovered through *E. coli* transformation. The insert DNAs in the positive clones were sequenced.

*In vitro GST Pull-Down Assay-* GST-fusion proteins of VEGFR2cyto (GST-VEGFR2cyto, amino acids (aa) 790-1356) and its deletion mutants including VEGFR2-N1 (aa 790-1021); VEGFR2-N2 (aa 790-958); VEGFR2-M (aa 982-1202); and VEGFR2-C (aa 1202-1356) were generated by polymerase chain reaction, and ligated into a pGEX6P-1 vector, and expressed and purified from *E. coli*. For some experiments, GST-VEGFR2cyto and its kinase-defective mutant GST-VEGFR2cyto (K866R) were purified from Sf9 cells, as described previously<sup>3</sup>.

The primers of VEGFR2 domain constructs we used are as follows:

VEGFR2cyto; 5'- GACTCGAGAAGCGGGCCAATGGAGGGGAACTG -3' and 5'-  
GACTCGAGTTAAACAGGAGGAGAGCTCAGTGT -3', VEGFR2-N1; 5'-  
GACTCGAGAAGCGGGCCAATGGAGGGGAACTG -3' and 5'-  
AGAGGCGGCCGCTTACGATGCCAAGAACTCCATGCC -3' VEGFR2-N2; 5'-  
GACTCGAGAAGCGGGCCAATGGAGGGGAACTG -3' and 5'-  
AGAGGCGGCCGCTTAATCCACGGGATTGCTCCAC -3' VEGFR2-M; 5'-  
ATGAATTCAGCATCACCAGTAGCCAGAGCTCA -3' and 5'-  
GACTCGAGCATAACAGGAAACAGGTGAGGTAGG -3', and VEGFR2-C; 5'-

ATGAATTCGAGGAGGAGGAGGAAGTATGTGACCCCAA -3' and 5'-  
GACTCGAGTTAAACAGGAGGAGAGCTCAGTGT -3'.

Following expression of GST fusion proteins in *E. coli*, purification was performed by glutathione-Sepharose affinity chromatography. Production of the full-length of IQGAP1 wild type protein has been described previously<sup>1</sup>. For binding, full-length IQGAP1 was mixed with affinity beads coated with GST-fused VEGFR2 proteins in a binding buffer (150 mM NaCl, 50 mM Tris-HCl [pH 7.5], 1 mM EDTA, 1.5 mM MgCl<sub>2</sub>, and 0.1% NP-40). The reaction mixture was first incubated for 2 h in the buffer at 4°C. Glutathione-sepharose 4B was added to the reaction mixture, which was then incubated at room temperature for 30 min. The beads were then washed seven times with the buffer, and the bound proteins were eluted by the addition of 40 µl of 2X SDS sample buffer. The proteins recovered from the resin were separated in SDS polyacrylamide gel, blotted onto a nitrocellulose membrane, and reacted with a mouse monoclonal anti-IQGAP1 antibody.

*Immunoprecipitation and Immunoblotting*- Growth-arrested cells were stimulated with VEGF at 37°C, and cells were lysed with 500 µl of ice-cold lysis buffer, pH 7.4 ((in mM) 50 HEPES, 5 EDTA, 50 NaCl), 1% Triton X-100, protease inhibitors (10 µg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin) and phosphatase inhibitors ((in mM) 50 sodium fluoride, 1 sodium orthovanadate, 10 sodium pyrophosphate). For immunoprecipitation cell lysates (600 µg) were precipitated with antibody overnight at 4°C and then incubated with 20 µl of protein A/G-agarose beads for 1.5 h at 4°C. Cell lysates (25 µg) or immunoprecipitates were separated using SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes, blocked overnight in PBS containing 5% nonfat dry milk and 0.1% Tween 20, and incubated for overnight with primary antibodies as described previously<sup>4</sup>. After incubation with secondary antibodies, proteins were detected by ECL chemiluminescence.

*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., Austin, TX). The sequences of specific siRNA against IQGAP1 is; 5'-

AAGGCCGAACTAGTGAAACTGCCTGTCTC-3' and 5'-  
AACAGTTTCACTAGTTCGGCCCCTGTCTC -3'. The target region of IQGAP1 mRNA is  
4938-4959 base par. The scrambled siRNA control is 5'-  
AAGTACCAAGGACGCGAATGTCCTGTCTC -3' and 5'-  
AAACATTCGCGTCCTTGGTACCCTGTCTC -3'.

We performed a Blast search and confirmed that the IQGAP1 and scrambled siRNA sequences we used in this study have no overlap with other proteins. BLMVECs (or HUVECs) were seeded into 100 mm dishes one day prior to transfection. At the time of transfection with siRNA, the cells were about 60% confluent in 6 ml of complete MCDB medium (or EBM with supplements containing 10% FBS). Transfections of siRNA (at 0.3, 3, and 30 nM) were performed using Oligofectamine (Invitrogen) according to the manufacturer's protocol. VEGF stimulation was performed 96 h after transfection.

*Measurements of Intracellular H<sub>2</sub>O<sub>2</sub> Levels-* Intracellular H<sub>2</sub>O<sub>2</sub> levels in HUVECs were measured by using the DCF-DA fluorescence method as described previously with minor modification<sup>5,6</sup>. After siRNA transfection, HUVECs were seeded in 96 well plates. When cell confluency reached 60-70%, cells were starved with serum free media for 4 h. After aspiration of the media, EBM containing 1.6% FBS and 20 μM DCF-DA was added to the cells and incubated for 2.5 h at 37 °C. After aspiration of the media, cells were stimulated with VEGF (20 and 50 ng/ml) or PMA (1 μM) in HANKS' balanced salt media containing 1.6% FBS. Relative DCF-DA fluorescence was measured by fluorescence microplate reader.

*Scratch Wound Assay-* IQGAP1 or its scrambled control siRNA-transfected HUVECs were cultured on 0.1% gelatin coated 24-well plates. Confluent cell monolayers were scraped using sterilized 200-μl pipette tips and washed with serum free media, and stimulated with 50 ng/ml of VEGF for 20 h. Cells were fixed and stained using Diff-Quick (Harleco; EM Science). Images were captured immediately at 0 h and at 24 h after the wounding.

*Modified Boyden Chamber Assay-* Migration assays using HUVECs and BLMVECs with the Modified Boyden Chamber method were conducted in duplicate 24-well transwell chambers

as described previously <sup>2</sup>. The upper insert (8- $\mu$ m pores coated with 0.1% gelatin) containing BLMVECs suspensions ( $2 \times 10^6$  cells) transfected with IQGAP1 siRNA or scrambled control siRNA were placed in the bottom 24-well chamber containing fresh media with 0.2% FBS and stimulants. The chamber was incubated at 37°C for 6h (for HUVECs) and 7 h (for BLMVECs). The membrane was fixed and stained using Diff-Quick. Four random fields at x200 magnification were counted.

*Confocal Immunofluorescence Microscopy-* HUVECs on glass coverslips were rinsed quickly in ice-cold PBS, fixed in freshly prepared 4% paraformaldehyde in PBS for 10 min at room temperature, permeabilized in 0.05% Triton X-100 in PBS for 5 min, and rinsed sequentially in PBS, 50 mmol/L NH<sub>4</sub>Cl and PBS for 10 min each. After incubation for 1 hour in blocking buffer (PBS+3% BSA), cells were incubated with rabbit polyclonal anti-VEGFR2 [pY1054](BioSource) and/or mouse monoclonal anti-IQGAP antibody <sup>1</sup> for 1 h at room temperature, rinsed in PBS/BSA, and then incubated in either FITC-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch) or Rhodamine Red X (RRX)-conjugated goat anti-mouse IgG for 1 h at room temperature. Cells on coverslips were mounted onto glass slides in Vectashield (Vector Laboratories) and examined using the 488 and 543 nm lines of the argon ion and green HeNe lasers with 515/30 nm band pass and 585 nm long pass filters, respectively, in the confocal laser scanning imaging system Bio-Rad MRC-1024 as described previously <sup>7</sup>. In double labeling experiments, FITC and RRX images were scanned sequentially and merged using the Bio-Rad LaserSharp software. Controls with no primary antibody showed no fluorescence labeling and single label controls were performed in double labeling experiments.

*Balloon Injury and Immunohistochemical Staining-* Male Sprague-Dawley rats weighing 280-300 g were purchased from Taconic Farms. Balloon-catheter injury was induced as described previously <sup>8</sup> under anesthesia with ketamine (90 mg/kg) and xylazine (5 mg/kg). The left common carotid artery wall was injured with an embolectomy balloon catheter (Edwards Life Sciences) to induce neointimal formation as described previously, and the right common carotid artery served as control. Animals were sacrificed at 1, 2, and 4 weeks after balloon injury

with an overdose of pentobarbital (120 mg/kg) and subjected to whole body perfusion with 4% paraformaldehyde. The carotid arteries were removed, cut into cross-sectional segments, and embedded in paraffin. Representative sections were immunohistologically stained with a rabbit polyclonal anti-IQGAP1 antibody (H-109, 1:500 dilution) or a mouse monoclonal anti-VEGFR2 antibody (A-3, 1:50 dilution). Sections (5  $\mu$ m thick) were deparaffinized and blocked with BSA (1%) in PBS for 30 min. Primary antibody was added to slides and incubated for 1 h at room temperature. After washing, sections were incubated with biotinylated secondary antibody for 1 h. After washing, VECTASTAIN ABC-AP reagent (Vector Laboratories) was added to the sections, incubated for 30 min at room temperature, and developed with BCIP/NBT Alkaline Phosphatase substrate kit (Vector Laboratories). The image was displayed in a high-resolution monitor and digitized by a video frame.

*Statistical analyses-* All values are expressed as mean  $\pm$  SE. The significance of the differences between 2 groups was evaluated by Student's paired 2-tailed 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. Statistical significance was accepted at  $P < 0.05$ .

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