The Role of RPE Cell-Associated $VEGF_{189}$ in Choroidal Endothelial Cell Transmigration across the RPE

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Purpose. To determine the role of vascular endothelial growth factor 189 ($VEGF_{189}$) in choroidal endothelial cell (CEC) migration across the retinal pigment epithelium (RPE) and to explore the molecular mechanisms involved.

Methods. Using real-time PCR, the expression of VEGF splice variants $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$ was determined in human RPE from donor eyes, cultured human RPE in contact with CECs exposed to hydrogen peroxide (H_2O_2) or hypoxia, and RPE/choroid specimens from mice treated with laser to induce choroidal neovascularization (CNV). Activation of VEGF receptors (VEGFRs), phosphoinositol 3-kinase (PI-3K) or Rac1 was measured in CECs cocultured in contact with RPE exposed to peroxide or silenced for $VEGF_{189}$ expression. Migration of CECs across the RPE was determined using fluorescence microscopy.

Results. $VEGF_{189}$ expression was increased in human RPE from aged compared with young donor eyes and from mouse RPE/choroids after laser to induce CNV. $VEGF_{189}$ was also upregulated in human RPE challenged with peroxide, hypoxia, or cultured in contact with CECs. CEC migration across RPE was greater after RPE exposure to peroxide to induce $VEGF_{189}$; VEGFR2 and Rac1 activities were also increased in these CECs. When CECs were cocultured with RPE silenced for $VEGF_{189}$, VEGFR2 and Rac1 activities in CECs were significantly reduced, as was CEC migration across the RPE. Inhibition of Rac1 activity significantly inhibited CEC transmigration without affecting PI-3K activity.

Conclusions. RPE-derived cell-associated $VEGF_{189}$ facilitates CEC transmigration by Rac1 activation independently of PI-3K signaling and may have importance in the development of neovascular AMD. (*Invest Ophthalmol Vis Sci.* 2011;52:570–578) DOI:10.1167/iovs.10-5595

Age-related macular degeneration (AMD) is a leading cause of nonreversible blindness worldwide.[1] Vision loss most often occurs in advanced forms, which are atrophic AMD (geographic atrophy) and neovascular AMD. In atrophic AMD, there is loss of the photoreceptors, retinal pigment epithelium (RPE), and choriocapillaris in the outer retina, whereas in neovascular AMD, blood vessels from the choroid grow into Bruch's membrane, the subretinal space, and neurosensory retina. Neovascular AMD accounts for 80% of the severe central vision loss (legal blindness) in AMD.

The steps involved in the development of neovascular AMD are complex and incompletely understood. From clinicopathologic studies, it appears that >50% of vision-threatening neovascular AMD occurs when choroidal endothelial cells (CECs) are induced to migrate toward the RPE and make contact with the RPE and its extracellular matrix. After contact with RPE, CECs can migrate across the RPE into the neurosensory retina, where choroidal neovascularization (CNV) develops.[2,3] The normal outer neurosensory retina lacks blood vessels, and the new blood vessels that develop often leak and bleed, causing vision loss. Thus, the migration of CECs across the RPE and the development of CNV in the neurosensory retina are important events leading to severe vision loss from neovascular AMD.

The RPE barrier, which is composed of a monolayer of polarized epithelial cells linked by tight junctions, is important in several processes necessary for fine visual acuity.[4] There is evidence that under normal conditions the RPE barrier compartmentalizes angiogenic agonists predominantly by secreting them basally, whereas inhibitors are secreted apically.[5] In aging eyes, it has been postulated that the RPE becomes less able to handle its metabolic load[6,7] and stressors such as light, hypoxia,[8] and inflammation,[9] leading to RPE barrier compromise.[10,11] In addition, these stressors have also been shown to result in the increased expression of angiogenic factors.[12] We previously reported that RPE-CEC contact led to reduced RPE barrier properties[10] and facilitated CEC migration across the RPE, induced by vascular endothelial growth factor (VEGF).[13]

VEGF-A (hereafter referred to as VEGF) is one angiogenic factor produced by RPE. Five splice variants or isoforms of VEGF in humans and three in mice are alternatively spliced from a single gene, and each has different biological functions and bioavailability.[14–16] The most studied human splice variants (mouse analogs in parentheses) are $VEGF_{189}$ ($VEGF_{188}$), which is predominantly cell associated, $VEGF_{121}$ ($VEGF_{120}$), which is soluble, and $VEGF_{165}$ ($VEGF_{164}$),[17] which has intermediate properties. Experimental studies using genetically modified mice indicated that VEGF signaling was important in the formation of CNV in AMD.[16,18–21] Clinical experience reveals that inhibition of all splice variants of VEGF with a humanized monoclonal antibody against VEGF led to improved visual acuity in approximately 40% of cases.[22] However, concern is raised about the safety of using agents to block all VEGF functions because VEGF is also a survival factor for CEC and RPE.[23,24] Given that VEGF has beneficial effects, it would be desirable to develop a strategy to inhibit only its pathologic functions.[1]

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We studied the role of cell-associated $VEGF_{188/189}$ (term includes the mouse and human analogs) in neovascular AMD. Specifically, we hypothesized that RPE-derived $VEGF_{189}$ would be upregulated in response to certain stressors or early events that occur in advanced AMD and that this splice variant would facilitate CEC migration across RPE, a critical step in the development of vision-threatening neovascular AMD. To test this hypothesis, we used a coculture model that mimicked the physiologic positions of RPE and CECs and controlled for contact between the two cell types. We determined the expression of $VEGF_{189}$ in young and old human donor RPE specimens and in mouse RPE after treatment with laser to induce CNV. We investigated the molecular mechanisms and the role of $VEGF_{189}$ in CEC transmigration. Our results reveal that RPE-derived $VEGF_{189}$ plays a critical role in facilitating CEC migration across the RPE by activating VEGFR2 and Rac1 in CECs in a pathway that appears independent of the phosphoinositol 3-kinase (PI-3K) signaling pathway.

METHODS

Animals

Adult transgenic mice bred to express only the cell-associated $VEGF_{188}$ splice variant on a C57Bl/6 background were used to isolate murine RPE ($mRPE_{188/188}$).[19] Appropriate controls were age-matched wild-type mice of the same genetic background (mRPE-WT). RPE and CECs were isolated and grown in culture, as described. All animal experiments were approved by the Institutional Animal Care and Use Committees of the University of North Carolina and the Schepens Eye Research Institute in accordance with National Institutes of Health guidelines for the care and use of laboratory animals and adhering to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Human RPE RNA Extraction

Posterior eyecups removed from human eyes within 24 hours of death were obtained from the North Carolina Eye Bank, and RPE were immediately collected for RNA extraction. All studies involving human eyes were done adhering to the tenets of the Declaration of Helsinki for research involving human tissue.

Cell Culture

Human fetal RPE (hfRPE) were isolated from donor eyes (Advanced Bioscience Resources, Alameda, CA) of 15- to 18-week-old fetuses, following a previously published protocol[25]; passages 1 to 3 hfRPE were used in experiments. Human CECs were isolated from donor eyes from the North Carolina Eye Bank, Inc. (Winston-Salem, NC). Primary CECs were isolated as previously described.[13] Passages 2 through 5 cells were used in experiments. ARPE-19 cells were obtained from ATCC (Rockville, MD) and grown in Dulbecco's modified Eagle's medium/F12 (Invitrogen) (DMEM/F-12) plus 10% FBS and penicillin-streptomycin. Cells below passage 18 were used for experiments. Primary mouse RPE (mRPE) was isolated using a modified protocol, as previously described by Gibbs et al.[26] Passages 3 to 5 cells were used in experiments. Cells were confirmed as RPE and not endothelial cells or fibroblasts by positive pan-cytokeratin staining (Abcam, Cambridge, MA).

Coculture Studies

For biochemical assays, CECs were grown on inserts (Transwell; Corning, Corning, NY) inserts with 1-μm diameter pores that were too small to allow cell migration but still allowed CEC cell processes to make contact with the basal aspects of the RPE grown on the underside of the inserts.[27] In some experiments, hfRPE was incubated with H_2O_2. In others, ARPE-19 cells were transfected with $VEGF_{189}$ siRNA, as described below. Twenty-four hours after contact and indicated treatments, CECs were collected from the tops of the inserts (Transwell; Corning) and were processed for PI-3K and Rac1 activity assays and VEGFR2 phosphorylation, as described in the following sections. The total protein was determined using the BCA protein assay (Thermo Scientific, Pittsburgh, PA), and equivalent amounts were used for each assay. PI-3K and Rac1 activity assays were performed as previously described using immunoprecipitation to probe phospho-Akt1 and GST-PBD pull-down to detect active Rac1.[27] Phospho-VEGFR2 was measured, as previously described,[28] by immunoprecipitation to detect phospho-VEGFR2. Western blot analysis was developed with enhanced chemiluminescence (ECL; Amersham, Piscataway, NJ) and analyzed using gel analysis software (Un-Scan-It; Silk Scientific, Orem, UT).

Transmigration Assays

Transmigration was measured as previously described.[13] ARPE-19 or hfRPE cells were plated as for coculture assays on inserts (Transwell; Corning) with 8-μm pores. Before plating, CECs were fluorescently labeled with cell-labeling solution (Vybrant Dio, V22886; Molecular Probes, Invitrogen, Carlsbad, CA) in accordance with the manufacturer's protocol. Labeled CECs were then plated into each insert. Forty-eight to 72 hours after plating, migrated CECs were counted using fluorescence microscopy.

$VEGF_{189}$ Knockdown

$VEGF_{189}$ gene silencing was performed in ARPE-19 cells using custom-designed small interfering RNA (siRNA; AAAUCAGUUCGAGGAAAG-GTT (sense) and CCUUUCCUCGAACUGAUUUTT (antisense) purchased from Ambion (Austin, TX). Nontargeting control siRNA was used as a negative control (4390843; Applied Biosystem, Foster City, CA). ARPE-19 cells were transfected with the siRNA duplex (JetPRIME; Polyplus Transfection, Illkirch, France) and then were plated onto the undersides of inserts (Transwell; Corning). Twenty-four hours later, ARPE-19 cells were plated onto the inserts (Transwell; Corning) for coculture and transmigration assays.

Inhibition of Rac1 Activity

Rac1 inhibition was achieved by expression of Rac binding domain of Rac1 effector POSH (GFP-POSH-RBD) in CECs, as described previously,[27] and were compared with control GFP alone. Briefly, GFP-POSH-RBD or GFP was transfected into CECs using DNA transfection (JetPRIME; Polyplus Transfection) before CECs were plated onto inserts.

Real-Time Quantitative PCR

Total RNA was extracted (RNeasy Mini Kit; Qiagen Valencia, CA). Assays were performed using the real-time PCR system (7500; Applied Biosystems). Briefly, 1 μg total RNA was reverse-transcribed into cDNA using a cDNA kit (High Capacity; Applied Biosystem) according to the manufacturer's protocol. Each reaction (TaqMan, 16 μL; Applied Biosystems) contained 20 ng cDNA, 8 μL mix (TaqMan PCR MasterMix; Applied Biosystems), and 1 μM forward primer, 1 μM reverse primer, and 1 μM probe. All the samples were analyzed for human β-actin expression in parallel as an internal control. Gene expression was normalized to the expression level of β-actin. Primers and probes were as follows: human VEGF121, 5′-CAT AGG AGA GAT GAG CTT CC-3′ (forward), 5′-CCT CGG CTT GTC ACA TTT TTC T-3′ (reverse), FAM-CA GCA CAA CAA ATG TGA ATG CAG ACC A-TAMRA (probe); for human VEGF165, 5′-CAT AGG AGA GAT GAG CTT CC-3′ (forward), 5′-AAG GCC CAC AGG GAT TTT CT-3′ (reverse), FAM-CA GCA CAA CAA ATG TGA ATG CAG ACC A-TAMRA (probe); for human VEGF189, 5′-CCA AAG AAA GAT AGA GCA AGA C-3′ (forward), 5′-AGG ACT TAT ACC GGG ATT TCT-3′ (reverse), FAM-TG CCC CTT TCC CTT TCC TCG AAC TG-TAMRA (probe).

Laser-Injury CNV Model

The laser-induced choroidal neovascularization (CNV) model was carried out as previously described.[29] Three to six spots of laser photocoagulation

(532 nm, 200 mW, 100 ms, 75 μm; OcuLight GL, Iridex, CA) were applied around the optic nerve. Seven days after laser injury, mice were euthanatized and posterior eyecups were harvested and kept in tissue storage reagent (RNAlater; Ambion) at −80°C for RNA extraction. The mRNA of VEGF splice variants was analyzed by RT-PCR.

Statistical Analysis

Significant differences between groups were determined by either one-way or two-way ANOVA. $P < 0.05$ was considered statistically significant.

Results

VEGF Splice Variants Expression in Human and Murine RPE

To determine the effect of RPE stressors relevant to human neovascular AMD, such as aging, hypoxia, and H_2O_2, we first measured the expression of VEGF splice variants $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$ in aged (>70 years) and young (20–40 years) human RPE obtained from eye bank eyes. As shown in Figure 1A, in the RPE of young donors, the expression of $VEGF_{165}$ (cycle threshold [CT] value, 30) was higher than that of $VEGF_{121}$ and $VEGF_{189}$ (CT value, 32). The expression of all VEGF splice variants was upregulated in RPE from older donor eyes compared with RPE from younger donors with an increase of approximately 26-fold for $VEGF_{189}$, 23-fold for $VEGF_{121}$, and 9-fold for $VEGF_{165}$. Next, hfRPE was challenged with hypoxia or oxidative stress. When hfRPE was incubated with different doses of H_2O_2 (at a final concentration of 50 μM-600 μM) for 16 hours, a selective increase in the expression of $VEGF_{189}$ occurred with 600 μM H_2O_2 (Fig. 1B). Incubation with 1% oxygen resulted in the upregulation of all splice variants, with the increase in $VEGF_{189}$ expression over room air slightly greater than that of the other splice variants (Fig. 1C).

The effect of RPE-CEC contact on the expression of VEGF splice variants was analyzed in hfRPE-CEC cocultures. As shown in Figure 1D, contact with CECs led to a much greater expression of $VEGF_{189}$ in RPE compared with noncontacting coculture or solo RPE culture ($P = 0.0028$), but there was little change in $VEGF_{121}$ and $VEGF_{165}$ expression in these conditions. There was no significant change in expression of VEGF splice variants in CECs (Fig. 1E). To determine the expression of $VEGF_{189}$ in CNV in vivo, VEGF splice variants were measured in isolated mouse RPE/choroids from wild-type mice treated with laser. All three splice variants were induced in retinas that had been treated with laser injury compared with nonlasered controls (Fig. 1F). There was a 3-fold increase in the mRNA expression of $VEGF_{188}$ ($P = 5.5 \times 10^{-6}$).

$VEGF_{189}$ Effect on CEC Migration across RPE

As shown in Figures 1B and 1D, preferential upregulation of $VEGF_{189}$ occurs when RPE is treated with H_2O_2 or grown in

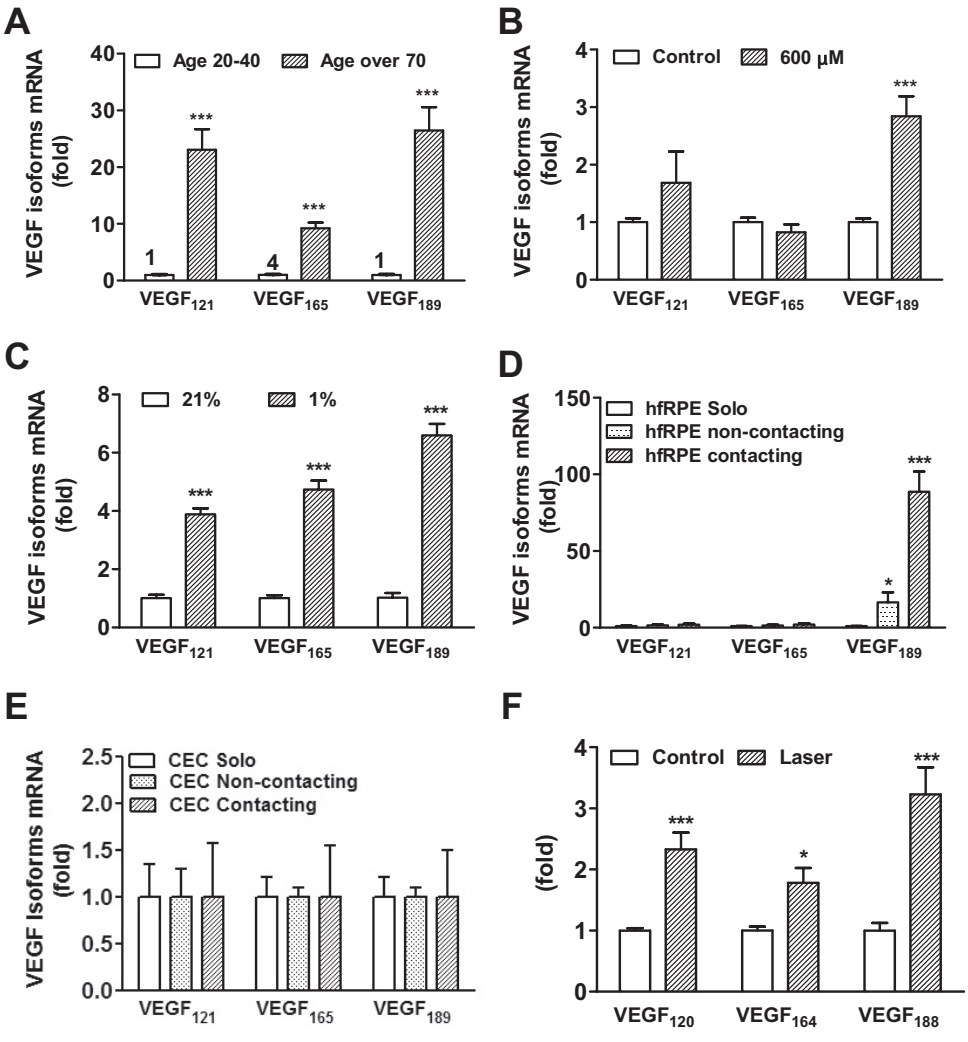

Figure 1. Expression of VEGF isoforms in human and mouse RPE. The expression of VEGF isoforms was measured by RT- PCR in (**A**) human RPE from young (>20 years old) and old (>70 years old) donors. $^{***}P < 0.0001$ vs. young ($n = 3$); the number on the top of the bar means mRNA expression level of VEGF splice variants for real-time PCR. (**B**) hfRPE treated with H_2O_2 (600 μM) for 16 hours. $^{***}P < 0.0001$ vs. control ($n = 6$). (**C**) hfRPE incubated under hypoxic conditions 1% O_2. $^{***}P < 0.0001$ vs. 21% O_2 ($n = 3$). (**D**) hfRPE solo, noncontact, and contact with CECs. $^{*}P < 0.05$, $^{***}P < 0.0001$ vs. solo ($n = 6$). (**E**) CECs solo, noncontact, and contact with hfRPE ($n = 3$). (**F**) Mouse RPE 7 days after laser injury CNV. $^{*}P < 0.05$ and $^{***}P < 0.0001$ vs. control ($n = 6$). All data are shown as mean ± SEM.

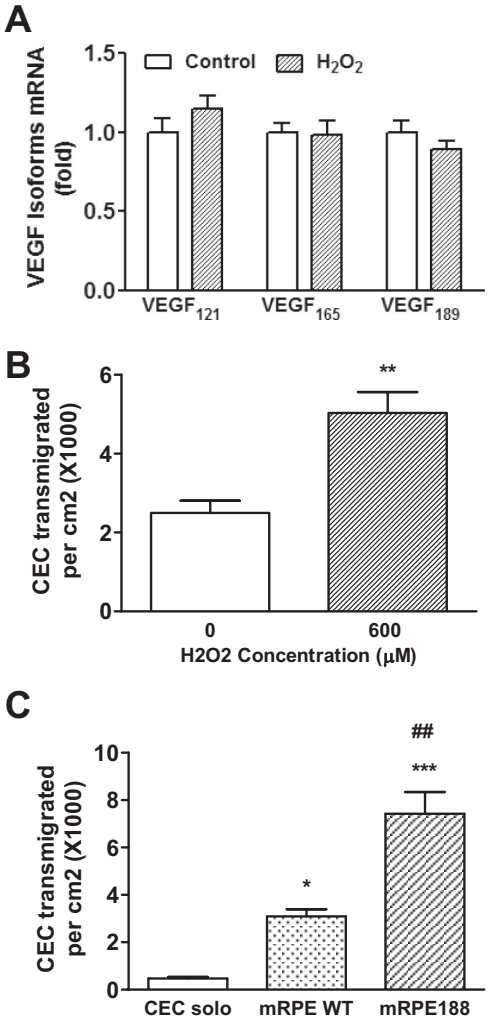

FIGURE 2. RPE-derived $VEGF_{189/188}$ facilitates CEC migration across RPE. (**A**) Expression of VEGF splice variants in solo CECs treated with or without H_2O_2 ($n = 3$); CEC transmigration was measured in cocultures of RPE and CEC. (**B**) CEC transmigration was measured when the cocultured hfRPE was incubated in the presence or absence of 600 μM H_2O_2 for 16 hours H_2O_2 treatment of hfRPE increased CEC transmigration. $^{**}P < 0.001$ vs. control ($n = 6$). (**C**) CEC migration across the filter when cultured alone (solo) or with mRPE-WT or $mRPE_{188/188}$. CEC transmigration was highest when cocultured with $mRPE_{188/188}$. $^{*}P < 0.05$ and $^{***}P < 0.0001$ vs. solo and $^{\#\#\#}P < 0.0001$ vs. mRPE ($n = 6$). All data are described as mean ± SEM.

contact with CECs. Thus, we next sought to determine whether this specific upregulation of cell-associated VEGF in RPE would facilitate CEC migration across the RPE. To test this possibility, RPE and CECs were plated for transmigration assays. hfRPE was plated on the underside of inserts (Transwell; Corning) and allowed to grow for 3 days before the addition of CECs and an additional 2 days of incubation. Sixteen hours before the termination of the experiment, H_2O_2 (to a final concentration of 600 μM) was added to the well containing the RPE. $VEGF_{189}$ mRNA was upregulated in hfRPE treated with H_2O_2 (Fig. 1B). The expression of VEGF splice variants in solo CECs treated with H_2O_2 was unaffected compared with control (Fig. 2A). H_2O_2 treatment led to a 2-fold increase in CEC migration across the hfRPE (Fig. 2B; $P = 0.0014$). Transmigration assays were also performed using murine $VEGF_{188/188}$ RPE ($mRPE_{188/188}$) and human CECs. Migration of CEC across RPE was higher with RPE that produced only $VEGF_{188}$ ($mRPE_{188/188}$) compared with RPE that expressed all VEGF splice variants (mRPE WT) (Fig. 2C; $P = 0.011$).

To confirm the effect of RPE-derived cell-associated $VEGF_{189}$ on CEC migration across RPE, we depleted endogenous $VEGF_{189}$ in ARPE-19 cells using siRNA. Two different siRNA sequences were designed to target $VEGF_{189}$, and a random sequence nontargeting siRNA was used as a negative control. To test the efficiency of $VEGF_{189}$ knockdown, the expression of VEGF splice variants was measured in ARPE-19 cells by real-time PCR 48 hours after transfection with siRNA. As shown in Figure 3A, both siRNA sequences against $VEGF_{189}$ knocked down the expression of $VEGF_{189}$ without affecting the expression level of $VEGF_{121}$ and $VEGF_{165}$. Given that siRNA sequence A led to a slightly greater knockdown, this siRNA sequence was used in transmigration assays. Twenty-four hours after siRNA transfection, ARPE-19 cells were plated onto the underside of the inserts (Transwell; Corning), and 4 hours later CECs were plated into the inserts. After 48 hours, CEC migration across the $VEGF_{189}$-silenced ARPE-19 was decreased approximately 40% compared with controls (6960.78 ± 2248.45/cm^2 transmigrated CECs across $VEGF_{189}$-silenced RPE vs. 10,261.51 ± 1187.32/cm^2 transmigrated CECs across control RPE) (Fig. 3B; $P = 0.0098$).

$VEGF_{189}$ Effect on Phosphorylated VEGFR2 in CECs

To test our hypothesis that RPE-derived $VEGF_{189}$ by H_2O_2 and in contact with CECs triggers signaling to facilitate CEC migration through binding VEGFRs in CECs, we first confirmed that VEGFR1 and VEGFR2 were expressed in CECs using real-time PCR (Fig. 4A) showing a near 10-fold greater expression in

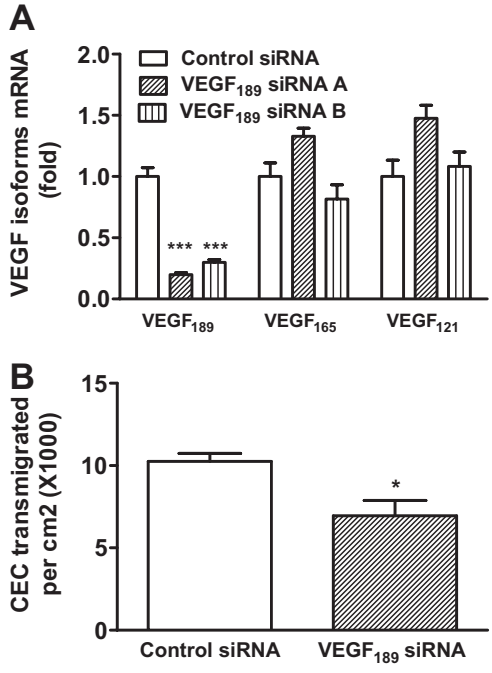

FIGURE 3. Knockdown of $VEGF_{189\ in}$ RPE decreases CEC transmigration. (**A**) mRNA levels of VEGF isoforms in ARPE-19 cells transfected with $VEGF_{189}$ siRNA were measured by RT-PCR. Nontargeting siRNA was used as a negative control. Both $VEGF_{189}$ siRNA sequences A and B specifically reduced $VEGF_{189}$ while not affecting expression of the other isoforms. $^{***}P < 0.0001$ vs. control ($n = 6$). (**B**) CEC transmigration assay was performed using ARPE-19 cell monolayer that had been depleted of $VEGF_{189}$ by siRNA. CEC transmigration was decreased when cocultured with ARPE-19 with reduced $VEGF_{189}$. $^{*}P < 0.05$ vs. control siRNA ($n = 6$). All data are described as mean ± SEM.

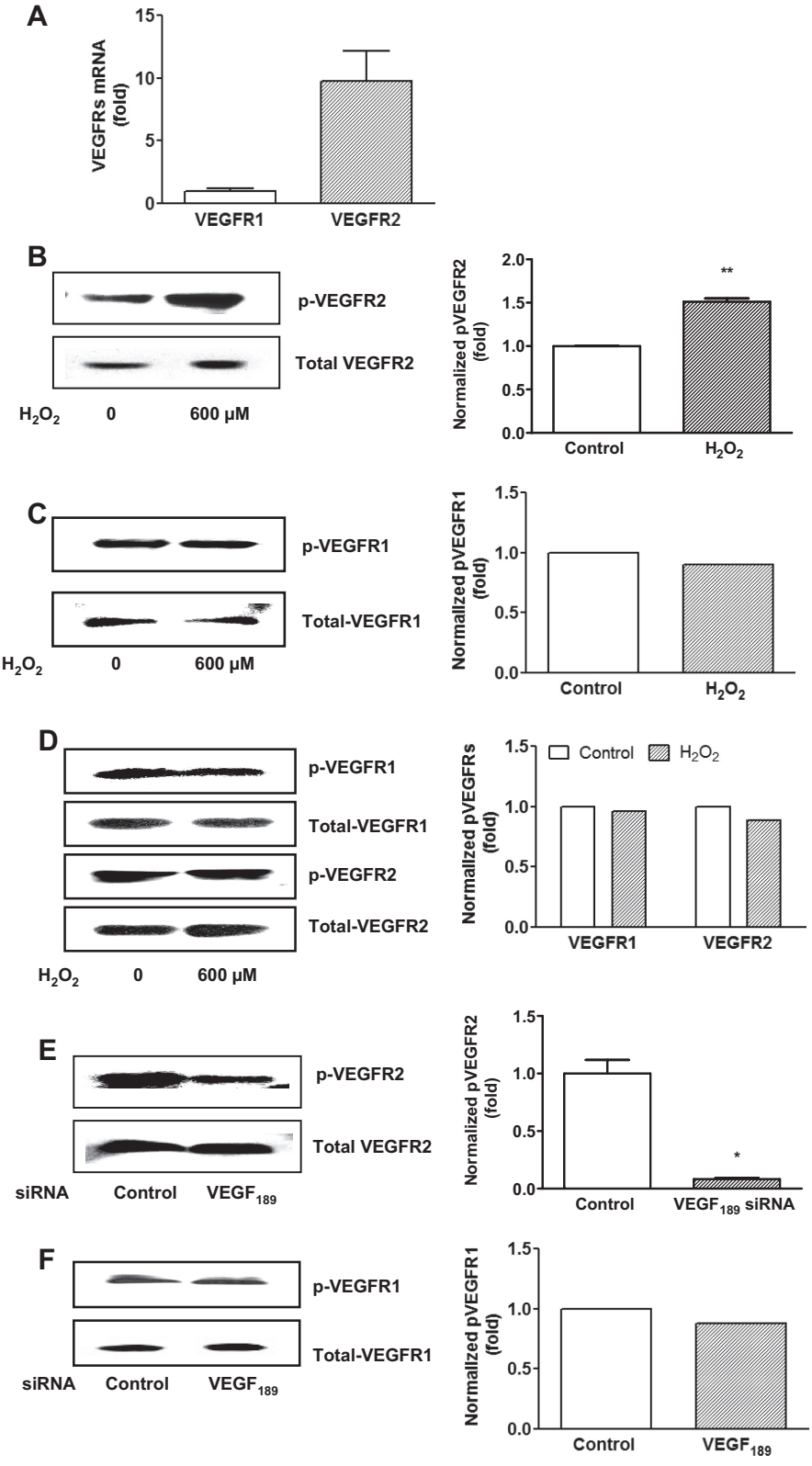

FIGURE 4. CEC contact with hfRPE leads to VEGFR2 increased phosphorylation in CECs. (**A**) Expression of VEGFR1 and VEGFR2 in CECs ($n = 3$). Immunoprecipitation of phospho-VEGFR2 and VEGFR1 in CECs. CECs were grown in contact with hfRPE. During the last 16 hours of contact, hfRPE was incubated with 600 μM H_2O_2. H_2O_2 treatment of RPE increased the phosphorylation of VEGFR2 in CECs (**B**) but did not affect VEGFR1 phosphorylation (**C**; representative blot shown). The bar graph on the *right* shows phospho-VEGFR2 band density normalized to total VEGFR2. $^{**}P < 0.001$ vs. control (PBS) ($n = 3$). (**D**) Representative blots showing that the phosphorylation of VEGFR1 and VEGFR2 was unchanged in solo CECs treated with H_2O_2. CECs grown in contact with ARPE-19 cells were transfected with $VEGF_{189}$ siRNA. After 24 hours of contact, CECs were collected for the detection of phospho-VEGFR2/total VEGFR2 and phospho-VEGFR1/total VEGFR1 by immunoprecipitation and Western blot analysis. VEGFR2 phosphorylation was decreased (**E**), but VEGFR1 phosphorylation was unchanged in CECs cocultured in contact with RPE with reduced $VEGF_{189}$ (**F**). The bar graph on the *right* shows phospho-VEGFR2 band density normalized to total VEGFR2. $^{*}P < 0.05$ vs. control siRNA ($n = 3$) and a representative blot of phospho-VEGFR1.

VEGFR2 mRNA than in VEGFR1 mRNA. We then measured the activation of both VEGFRs in CECs that had been grown in contact with RPE. hfRPE was grown in contact with CECs for 24 hours, and, during the last 16 hours of incubation, hfRPE, was incubated with H_2O_2 to induce the expression of $VEGF_{189}$ (Fig. 1B). After 16 hours of incubation, CECs were collected and analyzed for phosphorylated VEGFR1 and VEGFR2. Tyrosine phosphorylation of VEGFR2 in CECs was significantly increased when hfRPE was treated with 600 μM H_2O_2 (Fig. 4B). VEGFR1 phosphorylation was unchanged (Fig. 4C). As a control, to rule out the possibility that the increase in VEGFR2 phosphorylation in CECs occurred through a direct effect of H_2O_2 on CEC VEGFR2, we measured the phosphorylation of VEGFR1 and VEGFR2 in solo-cultured CECs treated with H_2O_2.

There was no significant change in VEGFR1 or VEGFR2 phosphorylation in solo CECs treated with H_2O_2 compared with control (Fig. 4D).

To determine whether the activation of VEGFR2 and VEGFR1 in CECs was due to H_2O_2-induced upregulation of RPE-associated $VEGF_{189}$, $VEGF_{189}$ was depleted by siRNA in ARPE-19 cells, and cells plated onto inserts 24 hours after transfection. CECs were plated into the inserts 4 hours later. After 24 hours of coculture, phosphorylation of VEGFR2 in CECs was decreased in cocultures in which ARPE-19 had been silenced for $VEGF_{189}$ (Fig. 4E), supporting the hypothesis that RPE-derived $VEGF_{189}$ activated VEGFR2 in CECs. However, VEGFR1 phosphorylation was unchanged under the same conditions (Fig. 4F).

$VEGF_{189}$ Effect on CEC Transmigration and Activity of Rac1

Results indicated that oxidative stressor H_2O_2 upregulated $VEGF_{189}$ in the RPE, facilitating CEC migration across RPE by activating VEGFR2 in CECs. We previously reported that PI-3K and Rac1 were activated in CECs grown in contact with RPE, and these signaling pathways were important in CEC migration across the RPE.[27] Therefore, we determined whether Rac1 and PI-3K signaling were triggered by RPE cell-associated $VEGF_{189}$ and whether they were necessary for CEC transmigration. Coculture assays were performed, and the activities of Rac1 and PI-3K were determined in CECs. Rac1 was activated in CECs grown in contact with $mRPE_{188/188}$ compared with wild-type mRPE (Fig. 5A). Coculture of CECs with hfRPE preincubated with H_2O_2 also led to the activation of Rac1 in CECs (Fig. 5B), but not to an increase in Akt1 phosphorylation (Fig. 5C). There was no difference in Rac1 activation in solo CECs treated with H_2O_2 compared with control (Fig. 5D), reducing the possibility that H_2O_2 treatment might have directly activated Rac1 in CECs.

To further examine the effect of RPE-derived $VEGF_{189}$ in the activation of CEC Rac1 activation, Rac1 activity assays were performed in CECs grown in coculture with ARPE-19 transfected with siRNA against $VEGF_{189}$. As shown in Figure 6A, Rac1 activity in CECs was decreased when grown in contact with coculture with ARPE-19 silenced for $VEGF_{189}$ compared with control siRNA. Akt1 phosphorylation in CECs was not affected by knockdown of $VEGF_{189}$ in ARPE-19 cells (Fig. 6B), consistent with results shown in Figure 5C. To determine whether Rac1 activation was necessary for CEC transmigration stimulated by RPE-derived $VEGF_{189}$, we used a construct containing the Rac-binding domain of Rac1 effector POSH (GFP-POSH-RBD), which we previously found inhibits Rac1 activity in CECs.[27] The construct and a control GFP construct were transfected into CECs before CEC plating. The transmigration assay was performed 48 hours after hfRPE-CEC coculture. During the last 16 hours of hfRPE-CEC coculture, hfRPE were

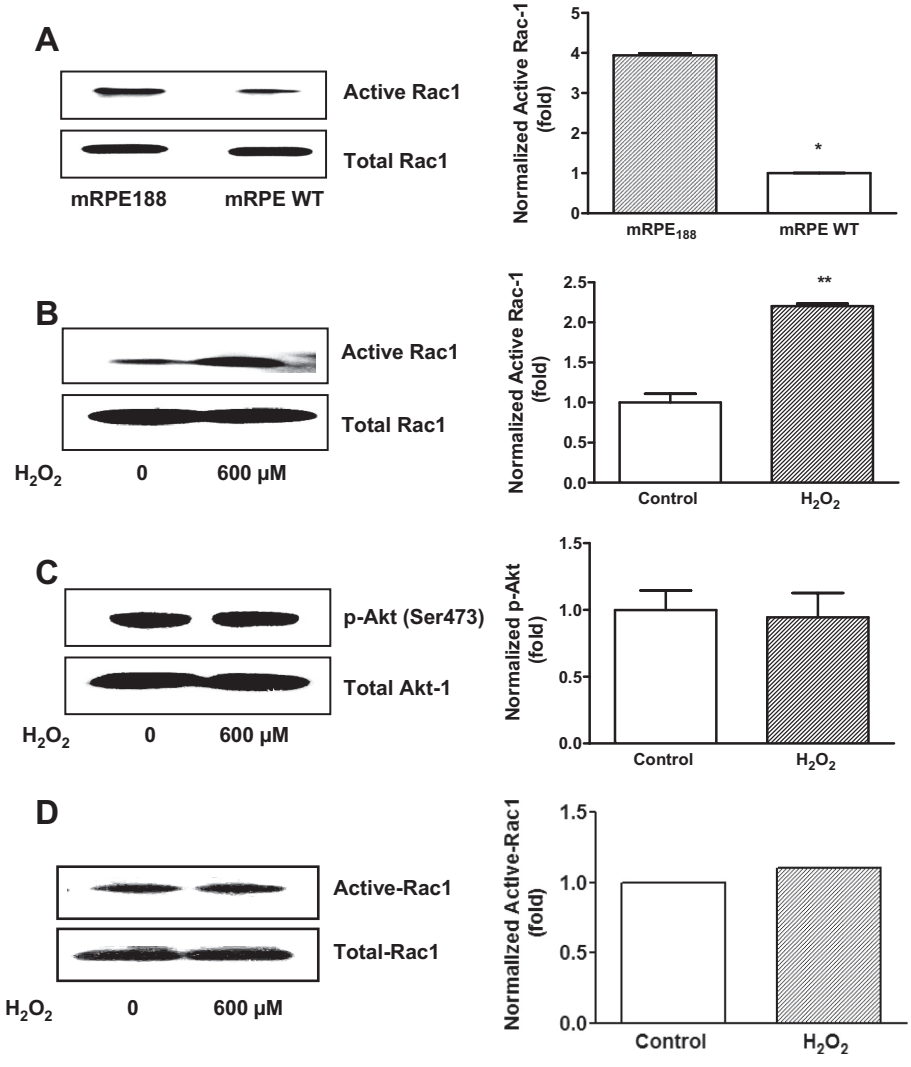

FIGURE 5. Coculture of CECs with RPE expressing elevated $VEGF_{189/188}$ leads to increased Rac1 activity. Rac1 activity assay was measured in CECs grown in contact with (**A**) mRPE-WT and mRPE188. The bar graph on the *right* shows active-Rac1 band density normalized to total Rac1. $^*P < 0.05$ vs. mRPEWT ($n = 3$). (**B**) hfRPE treated with 600 μM H_2O_2 for 16 hours. After 24 hours of contact, CECs were collected for the detection of active Rac1 and total Rac1 by GST-PBD pull-down and Western blot analysis, as described. Rac1 activity was increased with both treatments. The bar graph on the *right* shows active-Rac1 band density normalized to total Rac1. $^{**}P < 0.001$ vs. control (PBS) ($n = 3$). (**C**) PI-3K activity using phosphorylation of Akt-1 as a readout was measured in CECs grown in contact with hfRPE treated with H_2O_2 as in **B**. CECs were then collected for the detection of phospho-Akt and total Akt by immunoprecipitation and Western blot analysis as described in Experimental Procedures. Upregulation of $VEGF_{189}$ by H_2O_2 treatment of RPE does not affect phosphorylation of Akt-1 in CECs. The bar graph on the *right* shows phospho-Akt (Ser473) band density normalized to total Akt1. $P > 0.05$ vs. control (PBS) ($n = 3$). (**D**) Representative blot showing that Rac1 activity was unchanged in solo CECs treated with H_2O_2.

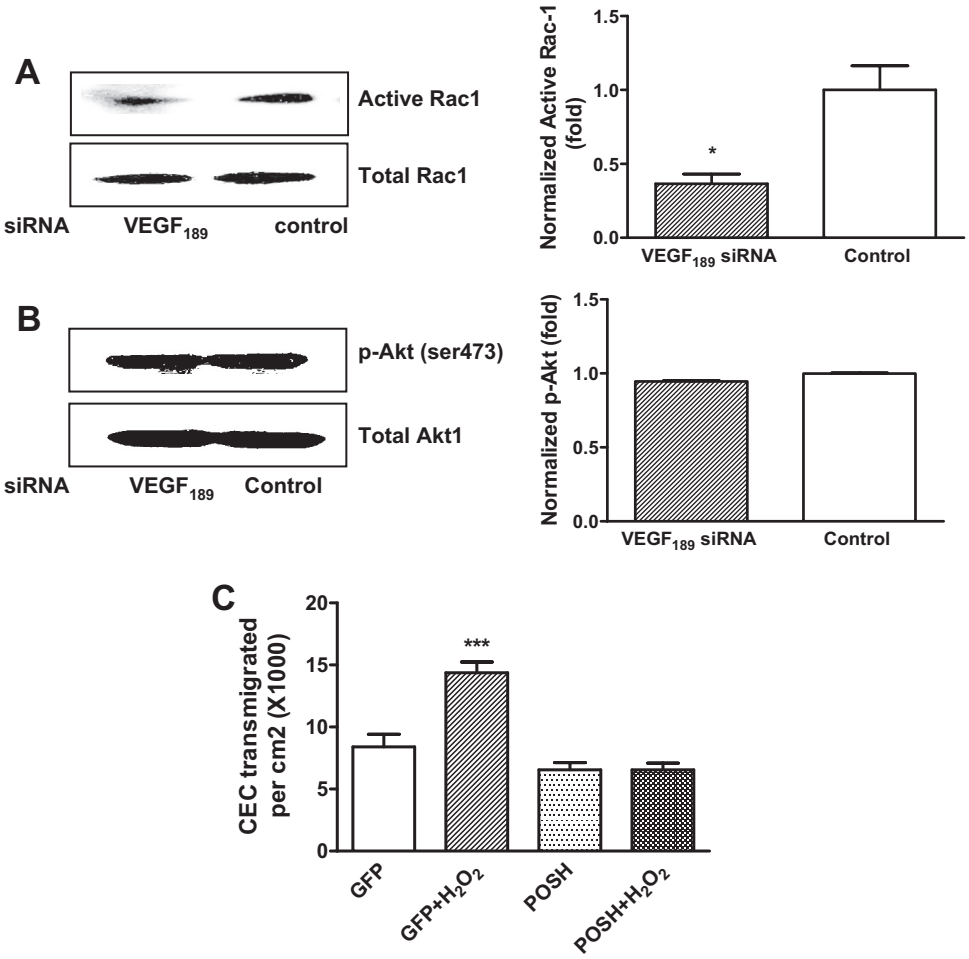

FIGURE 6. RPE-derived $VEGF_{189}$ stimulates CEC transmigration mediated by Rac1 activation. Activities of Rac1 (**A**) and PI-3K (**B**) were measured in CECs grown in contact with ARPE-19 cells with reduced $VEGF_{189}$. Twenty-four hours after contact, CECs were collected for the detection of active Rac1 and phospho-Akt by GST pull-down and immunoprecipitation. Whole cell lysates were used to detect total Rac1 and Akt by Western blot analysis. Knockdown of $VEGF_{189}$ decreased Rac1 activity compared with control siRNA, whereas p-Akt remained unchanged. The bar graph on the *right* shows active-Rac1 or phospho-Akt band density normalized to total Rac1 or total Akt1. $^{*}P < 0.05$ vs. control siRNA ($n = 3$). (**C**) CEC transmigration assay was performed using hfRPE and CECs in which Rac1 activity had been inhibited by transfection with GFP-POSH-RBD. Twenty-four hours after transfection, CECs were added, and transmigration was measured after 48 hours. During the last 16 hours of contacting coculture, hfRPE was incubated with 600 μM H_2O_2. CECs in which Rac1 activity was inhibited by expressing GFP-POSH did not exhibit increased transmigration when cocultured with H_2O_2-treated hfRPE compared with cells expressing GFP alone. $^{***}P < 0.0001$ vs. GFP alone ($n = 6$).

challenged with 600 μM H_2O_2. As shown in Figure 6C, and consistent with the results shown in Figure 2B, CEC transmigration increased significantly when hfRPE were incubated with H_2O_2 ($P = 0.04$), but this effect was inhibited when Rac1 activity was decreased in CECs by transfection with GFP-POSH. These results provide support that $VEGF_{189}$ facilitates CEC migration across the RPE, mediated by VEGFR2-induced activation of Rac1 in CECs.

DISCUSSION

VEGF-A is the most widely studied ligand of the VEGF family, and it plays an important role in the formation of CNV by several mechanisms,[30,31] including the release of matrix metalloproteinases,[32] endothelial cell survival,[33] CEC migration and proliferation,[34–36] increased permeability,[37] and integrin turnover with endothelial migration.[38]

In healthy young mouse RPE/choroidal specimens, $VEGF_{164}$ and $VEGF_{120}$ are the dominant splice variants, whereas $VEGF_{188}$ is virtually undetectable.[39] Our data from human eye samples showed that $VEGF_{165}$ was also the dominant splice variant in young adult RPE. However, in contrast to that found with $VEGF_{165}$, the change in mRNA expression levels of $VEGF_{189}$ and $VEGF_{121}$ were significantly increased in RPE from aged compared with young donor eyes. The observation that $VEGF_{189}$ was increased in RPE from aged donors suggested that $VEGF_{189}$ may play an important role in AMD. To mimic the stress relevant to human neovascular AMD in vitro, hfRPE was cultured under hypoxic conditions or after exposure to H_2O_2. Notably, we observed that the expression of $VEGF_{189}$ was preferentially upregulated in response to this stress. The upregulation of $VEGF_{188}$ was also seen in RPE/choroids from mice that developed CNV after laser injury. These lines of evidence provide strong support that RPE-derived $VEGF_{189}$, though minimally expressed in youth, is upregulated by stressors postulated to play a role in AMD, including hypoxia, oxidative stress, and contact between RPE and CECs before the development of neurosensory retinal CNV.

We next tested the hypothesis that RPE-derived $VEGF_{189}$ plays an important role in CEC transmigration. Contact between RPE and CECs is an important step preceding the development of vision-threatening neovascular AMD.[2,3] Given that there are no animal models or clinical studies that allow direct examination of the effects of contact between RPE and CECs, we used an in vitro coculture model to study the effects of RPE and CEC interactions[13] and contact on signaling pathways within each cell type and on CEC migration across the RPE. Results obtained with this model demonstrated that the expression of $VEGF_{189}$, compared with that of other VEGF splice variants, was preferentially upregulated in RPE grown in contact with CECs and that it contributed to CEC migration across the RPE. That there was only a 40% reduction in transmigration after $VEGF_{189}$ knockdown indicates either that the small remaining amount of $VEGF_{189}$ resistant to knockdown is enough to trigger signaling in CECs or that other factors that are also partially cell associated, including $VEGF_{165}$, may be acting in parallel. We also showed that the RPE-derived $VEGF_{189}$ binds to and activates VEGFR2 on CECs to trigger downstream signaling events facilitating CEC transmigration.

Rac1 is one of the small Rho family GTPases activated by guanine nucleotide exchange factors. In the GTP-bound activated state, Rac1 can modulate cell behavior through effector proteins. Rac1 is most often associated with cell motility and migration[40–42] as a key regulator of actin polymerization and reorganization in cell-membrane protrusions during directed endothelial cell migration.[43] Rac1 has been shown both to activate and to be activated by PI-3K. PI-3K, in turn, can be activated by multiple stimuli, such as integrins[44] and receptor growth-factor binding,[31] including VEGF.[45,46] Furthermore, it is an important mediator of signal transduction downstream of a variety of cell surface receptors, including VEGFR2.[47]

One well-known function of PI-3K is the regulation of cell migration. Endothelial cell chemotaxis is dependent on PI-3K activation of intracellular signaling cascades.[48] There are also other kinases, such as c-Jun N-terminal kinase (JNK),[49] p38, extracellular signal-regulated protein kinase (ERK), and mitogen-activated protein kinases (MAPKs), that play important roles in the regulation of cell movement,[50] and these pathways may also play a role in neovascular AMD. We previously identified a signaling pathway involving Rac1 and PI-3K that mediates CEC migration across an RPE monolayer in response to a VEGF gradient.[27] Here, we studied the splice variant most greatly upregulated in RPE in aged eyes and in response to contact with CECs, stressors relevant to human neovascular AMD, to specifically determine its role in possible PI-3K–triggered Rac1 activation and endothelial migration. Our results show that VEGF-induced CEC migration involves Rac1 but not PI-3K. In light of our previous findings,[27] these data suggest the existence of parallel pathways involving PI-3K through which splice variants other than $VEGF_{189}$, such as $VEGF_{165}$, might stimulate CEC transmigration. Our results do not preclude the role of other kinases, such as p38 MAPK or ERK, in CEC migration possibly involved downstream of Rac1.

Our findings suggest that stressors relevant to human neovascular AMD induce the expression of soluble and cell-associated VEGF splice variants in RPE. Increased levels of soluble VEGF splice variants, such as $VEGF_{121}$ and $VEGF_{165}$, provide a chemotactic gradient for migrating CECs, which, on making contact with the RPE, lead to RPE barrier compromise[10] and further upregulation of $VEGF_{189}$. VEGF2 phosphorylation in CECs leads to the activation of PI-3K and Rac1 and contributes to their transmigration.[27] This study provides evidence that RPE-derived cell-associated $VEGF_{188/189}$, which is upregulated by age and contact with CECs, may play an important role in the development of neurosensory retinal CNV in neovascular AMD.

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