

The Role of RPE Cell-Associated VEGF₁₈₉ in Choroidal Endothelial Cell Transmigration across the RPE

Haibo Wang,¹ Pete Geisen,¹ Erika S. Wittchen,² Bradley King,¹ Keith Burridge,² Patricia A. D'Amore,³ and M. Elizabeth Hartnett^{1,4}

PURPOSE. To determine the role of vascular endothelial growth factor 189 (VEGF₁₈₉) 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 VEGF₁₂₁, VEGF₁₆₅, and VEGF₁₈₉ was determined in human RPE from donor eyes, cultured human RPE in contact with CECs exposed to hydrogen peroxide (H₂O₂) 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 VEGF₁₈₉ expression. Migration of CECs across the RPE was determined using fluorescence microscopy.

RESULTS. VEGF₁₈₉ expression was increased in human RPE from aged compared with young donor eyes and from mouse RPE/choroids after laser to induce CNV. VEGF₁₈₉ 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 VEGF₁₈₉; VEGFR2 and Rac1 activities were also increased in these CECs. When CECs were cocultured with RPE silenced for VEGF₁₈₉, 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 VEGF₁₈₉ 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 properties¹⁰ 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 VEGF₁₈₉ (VEGF₁₈₈), which is predominantly cell associated, VEGF₁₂₁ (VEGF₁₂₀), which is soluble, and VEGF₁₆₅ (VEGF₁₆₄),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

From the Departments of ¹Ophthalmology and ²Cell and Developmental Biology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina; ³Schepens Eye Research Institute, Boston, Massachusetts; and ⁴Department of Ophthalmology, Moran Eye Center, University of Utah, Salt Lake City, Utah.

Supported by National Institutes of Health Grants R01 EY017011 (MEH) and EY015435 (PAD); Macula Society, Retina Research Foundation, Mills and Margaret Cox Endowment (MEH); and National Institutes of Health Grants GM029860 (KB) and 3-R01-GM029860-28S (KB).

Submitted for publication March 29, 2010; revised May 27 and July 26, 2010; accepted August 22, 2010.

Disclosure: **H. Wang**, None; **P. Geisen**, None; **E.S. Wittchen**, None; **B. King**, None; **K. Burridge**, None; **P.A. D'Amore**, None; **M.E. Hartnett**, None

Corresponding author: M. Elizabeth Hartnett, 65 Mario Capecchi Drive, Moran Eye Center, University of Utah, Salt Lake City, UT 84132; me.hartnett@hsc.utah.edu.

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 VEGF₁₈₉ 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 VEGF₁₈₉ 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 VEGF₁₈₉ in CEC transmigration. Our results reveal that RPE-derived VEGF₁₈₉ 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 VEGF₁₈₈ 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 (hRPE) were isolated from donor eyes (Advanced Bioscience Resources, Alameda, CA) of 15- to 18-week-old fetuses, following a previously published protocol²⁵; passages 1 to 3 hRPE 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- μ m 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, hRPE was incubated with H₂O₂. In others, ARPE-19 cells were transfected with VEGF₁₈₉ siRNA, as described below. Twenty-four hours after contact and indicated treat-

ments, 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 hRPE cells were plated as for coculture assays on inserts (Transwell; Corning) with 8- μ m 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.

VEGF₁₈₉ Knockdown

VEGF₁₈₉ 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 μ g 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 μ L; Applied Biosystems) contained 20 ng cDNA, 8 μ L mix (TaqMan PCR MasterMix; Applied Biosystems), and 1 μ M forward primer, 1 μ M reverse primer, and 1 μ M 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 μ m; OcuLight GL, Iridex, CA) were applied around the optic nerve. Seven days after laser injury, mice were euthanized 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 VEGF₁₂₁, VEGF₁₆₅, and VEGF₁₈₉ 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 VEGF₁₆₅ (cycle threshold [CT] value, 30) was higher than that of VEGF₁₂₁ and VEGF₁₈₉ (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 VEGF₁₈₉, 23-fold for VEGF₁₂₁, and 9-fold for VEGF₁₆₅. 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 VEGF₁₈₉ 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 VEGF₁₈₉ 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 VEGF₁₈₉ in RPE compared with noncontacting coculture or solo RPE culture ($P = 0.0028$), but there was little change in VEGF₁₂₁ and VEGF₁₆₅ expression in these conditions. There was no significant change in expression of VEGF splice variants in CECs (Fig. 1E). To determine the expression of VEGF₁₈₉ 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 VEGF₁₈₈ ($P = 5.5 \times 10^{-6}$).

VEGF₁₈₉ Effect on CEC Migration across RPE

As shown in Figures 1B and 1D, preferential upregulation of VEGF₁₈₉ occurs when RPE is treated with H_2O_2 or grown in

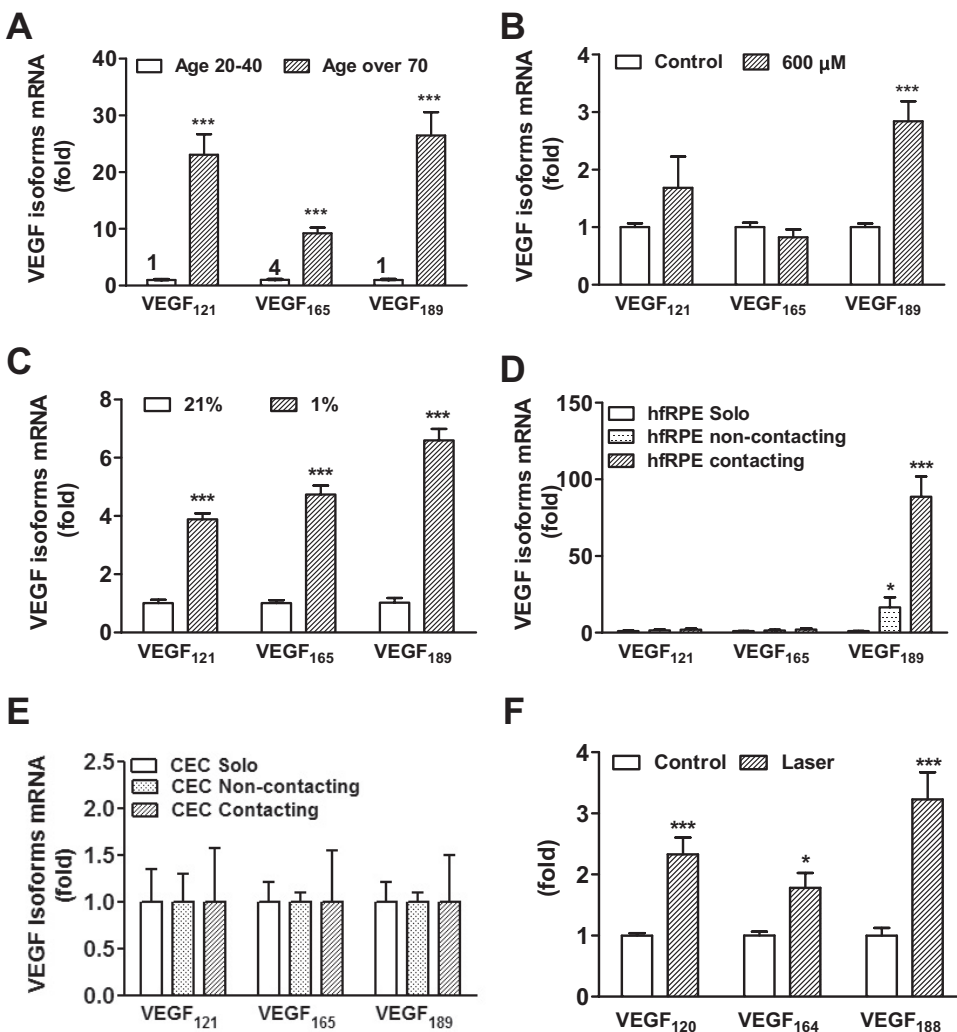


FIGURE 1. Expression of VEGF isoforms in human and mouse RPE. The expression of VEGF isoforms was measured by RT-PCR in (A) human RPE from young (>20 years old) and old (>70 years old) donors. $***P < 0.0001$ vs. young ($n = 3$); the number on the top of the bar means mRNA expression level of VEGF splice 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 \pm SEM.

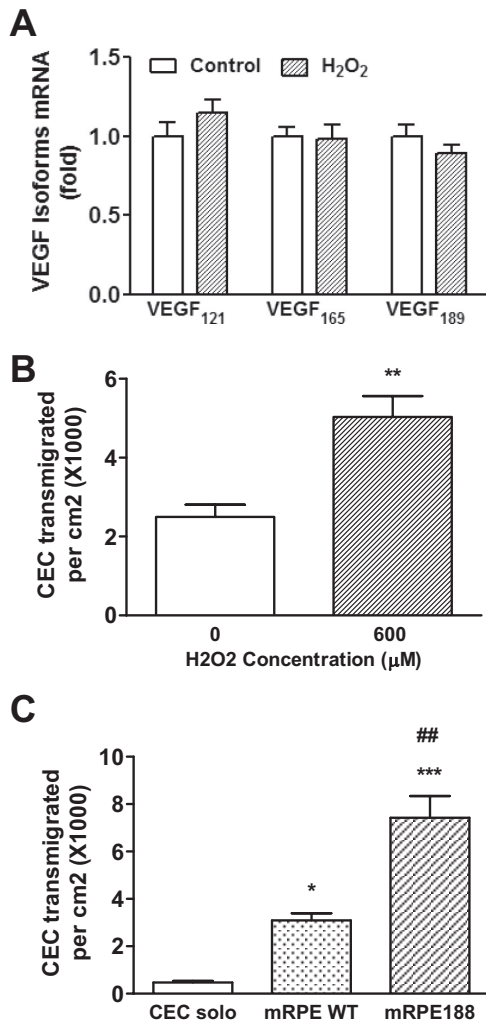


FIGURE 2. RPE-derived VEGF_{189/188} facilitates CEC migration across RPE. (A) Expression of VEGF splice variants in solo CECs treated with or without H₂O₂ ($n = 3$); CEC transmigration was measured in cocultures of RPE and CEC. (B) CEC transmigration was measured when the cocultured hRPE was incubated in the presence or absence of 600 μ M H₂O₂ for 16 hours. H₂O₂ treatment of hRPE 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 \pm 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. hRPE 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, H₂O₂ (to a final concentration of 600 μ M) was added to the well containing the RPE. VEGF₁₈₉ mRNA was upregulated in hRPE treated with H₂O₂ (Fig. 1B). The expression of VEGF splice variants in solo CECs treated with H₂O₂ was unaffected compared with control (Fig. 2A). H₂O₂ treatment led to a 2-fold increase in CEC migration across the hRPE (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 VEGF₁₈₈ (mRPE_{188/188}) com-

pared with RPE that expressed all VEGF splice variants (mRPE WT) (Fig. 2C; $P = 0.011$).

To confirm the effect of RPE-derived cell-associated VEGF₁₈₉ on CEC migration across RPE, we depleted endogenous VEGF₁₈₉ in ARPE-19 cells using siRNA. Two different siRNA sequences were designed to target VEGF₁₈₉, and a random sequence nontargeting siRNA was used as a negative control. To test the efficiency of VEGF₁₈₉ 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 VEGF₁₈₉ knocked down the expression of VEGF₁₈₉ without affecting the expression level of VEGF₁₂₁ and VEGF₁₆₅. 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 VEGF₁₈₉-silenced ARPE-19 was decreased approximately 40% compared with controls (6960.78 \pm 2248.45/cm² transmigrated CECs across VEGF₁₈₉-silenced RPE vs. 10,261.51 \pm 1187.32/cm² transmigrated CECs across control RPE) (Fig. 3B; $P = 0.0098$).

VEGF₁₈₉ Effect on Phosphorylated VEGFR2 in CECs

To test our hypothesis that RPE-derived VEGF₁₈₉ by H₂O₂ 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

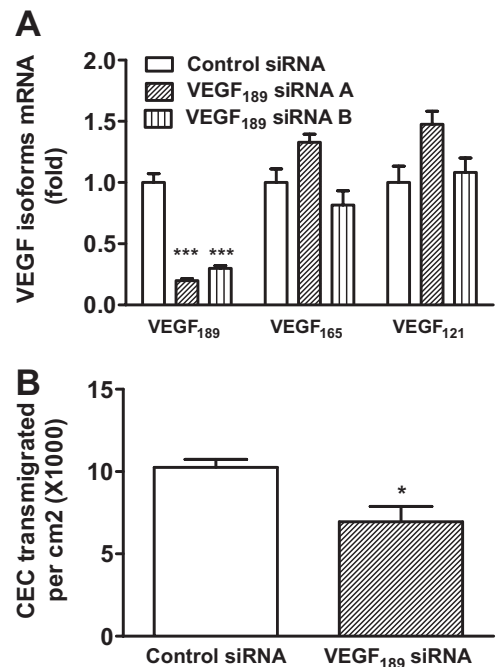


FIGURE 3. Knockdown of VEGF₁₈₉ in RPE decreases CEC transmigration. (A) mRNA levels of VEGF isoforms in ARPE-19 cells transfected with VEGF₁₈₉ siRNA were measured by RT-PCR. Nontargeting siRNA was used as a negative control. Both VEGF₁₈₉ siRNA sequences A and B specifically reduced VEGF₁₈₉ while not 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 VEGF₁₈₉ by siRNA. CEC transmigration was decreased when cocultured with ARPE-19 with reduced VEGF₁₈₉. * $P < 0.05$ vs. control siRNA ($n = 6$). All data are described as mean \pm SEM.

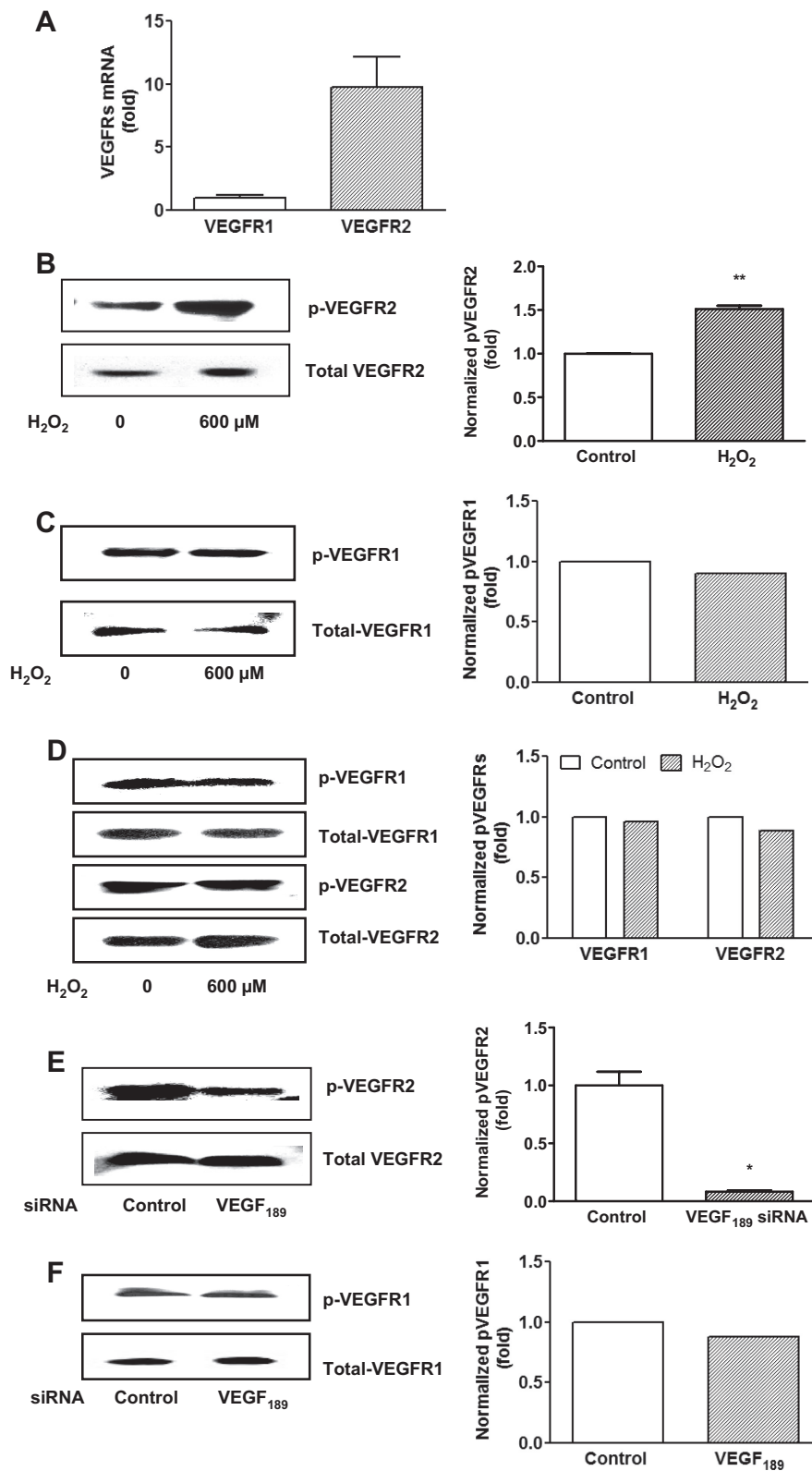


FIGURE 4. CEC contact with hRPE leads to VEGFR2 increased phosphorylation in CECs. (A) Expression of VEGFR1 and VEGFR2 in CECs ($n = 3$). Immunoprecipitation of phospho-VEGFR2 and VEGFR1 in CECs. CECs were grown in contact with hRPE. During the last 16 hours of contact, hRPE was incubated with 600 μM 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 VEGF₁₈₉ 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 VEGF₁₈₉ (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. hRPE was grown in contact with CECs for 24 hours, and, during the last 16 hours of incubation, hRPE, was incubated with H_2O_2 to induce the expression of VEGF₁₈₉ (Fig. 1B). After 16 hours of incubation, CECs were collected and analyzed for phosphorylated VEGFR1 and VEGFR2. Ty-

rosine phosphorylation of VEGFR2 in CECs was significantly increased when hRPE 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 H₂O₂ compared with control (Fig. 4D).

To determine whether the activation of VEGFR2 and VEGFR1 in CECs was due to H₂O₂-induced upregulation of RPE-associated VEGF₁₈₉, VEGF₁₈₉ 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 VEGF₁₈₉ (Fig. 4E), supporting the hypothesis that RPE-derived VEGF₁₈₉ activated VEGFR2 in CECs. However, VEGFR1 phosphorylation was unchanged under the same conditions (Fig. 4F).

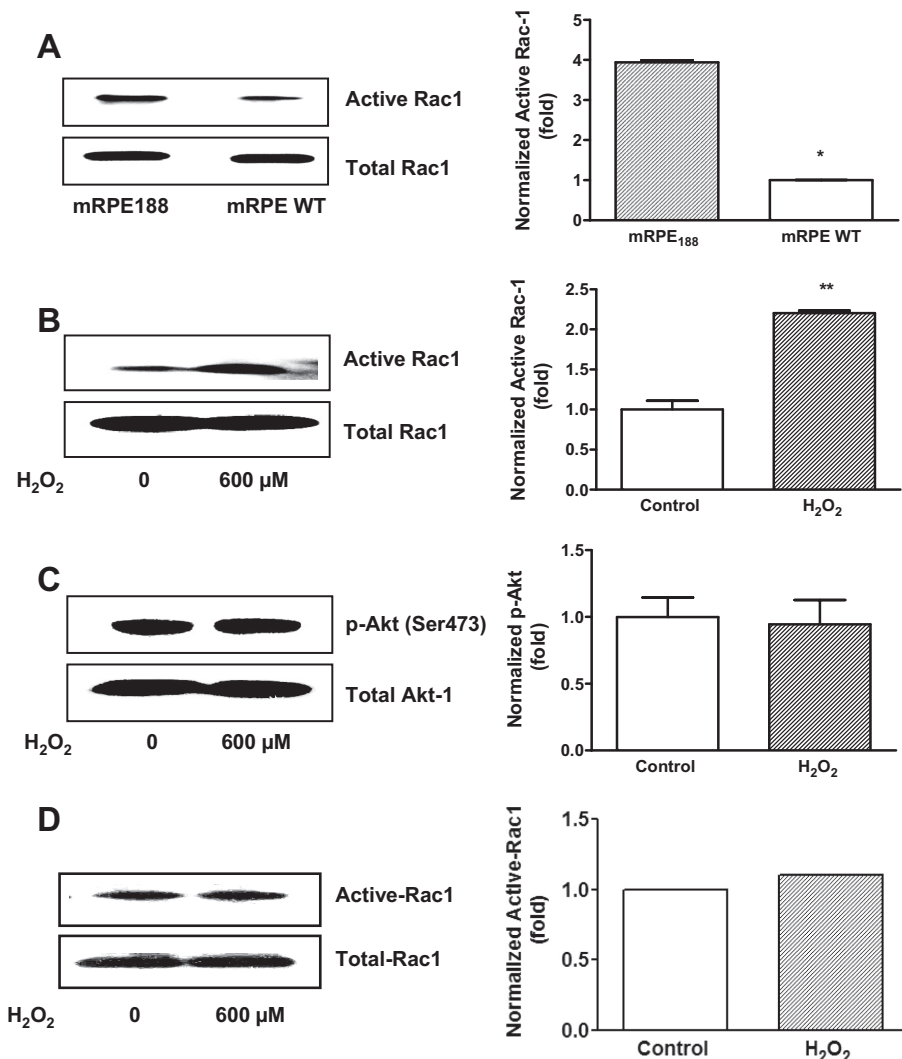
VEGF₁₈₉ Effect on CEC Transmigration and Activity of Rac1

Results indicated that oxidative stressor H₂O₂ upregulated VEGF₁₈₉ 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 VEGF₁₈₉ 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 hRPE preincubated with H₂O₂ 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 H₂O₂ compared with control (Fig. 5D), reducing the possibility that H₂O₂ treatment might have directly activated Rac1 in CECs.

To further examine the effect of RPE-derived VEGF₁₈₉ in the activation of CEC Rac1 activation, Rac1 activity assays were performed in CECs grown in coculture with ARPE-19 transfected with siRNA against VEGF₁₈₉. As shown in Figure 6A, Rac1 activity in CECs was decreased when grown in contact with coculture with ARPE-19 silenced for VEGF₁₈₉ compared with control siRNA. Akt1 phosphorylation in CECs was not affected by knockdown of VEGF₁₈₉ 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 VEGF₁₈₉, 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 hRPE-CEC coculture. During the last 16 hours of hRPE-CEC coculture, hRPE 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) hRPE treated with 600 μM H₂O₂ 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 hRPE treated with H₂O₂ 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 VEGF₁₈₉ by H₂O₂ 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 H₂O₂.



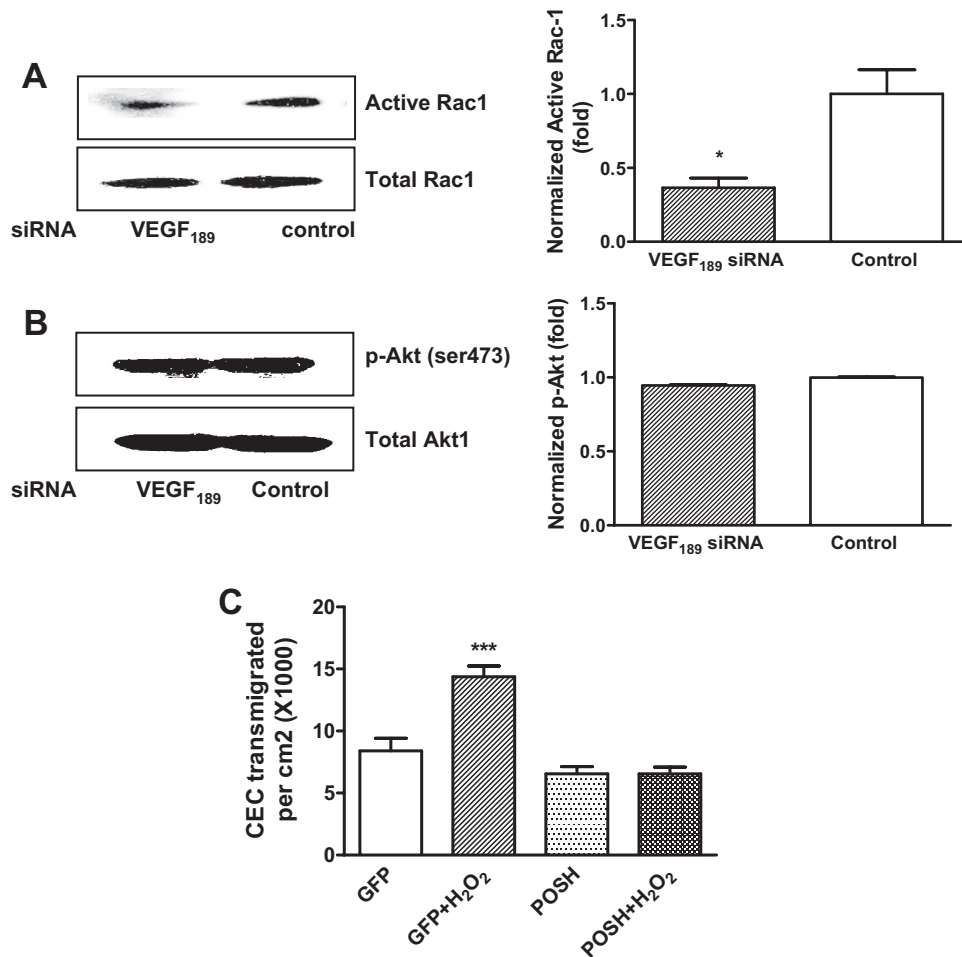


FIGURE 6. RPE-derived VEGF₁₈₉ stimulates CEC transmigration mediated by Rac1 activation. Activities of Rac1 (A) and PI-3K (B) were measured in CECs grown in contact with ARPE-19 cells with reduced VEGF₁₈₉. Twenty-four hours after contact, CECs were collected for the detection of active Rac1 and phospho-Akt by GST pull-down and immunoprecipitation. Whole cell lysates were used to detect total Rac1 and Akt by Western blot analysis. Knockdown of VEGF₁₈₉ 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 hRPE 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, hRPE was incubated with 600 μ M H₂O₂. CECs in which Rac1 activity was inhibited by expressing GFP-POSH did not exhibit increased transmigration when cocultured with H₂O₂-treated hRPE compared with cells expressing GFP alone. *** $P < 0.0001$ vs. GFP alone ($n = 6$).

challenged with 600 μ M H₂O₂. As shown in Figure 6C, and consistent with the results shown in Figure 2B, CEC transmigration increased significantly when hRPE were incubated with H₂O₂ ($P = 0.04$), but this effect was inhibited when Rac1 activity was decreased in CECs by transfection with GFP-POSH. These results provide support that VEGF₁₈₉ 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, VEGF₁₆₄ and VEGF₁₂₀ are the dominant splice variants, whereas VEGF₁₈₈ is virtually undetectable.³⁹ Our data from human eye samples showed that VEGF₁₆₅ was also the dominant splice variant in young adult RPE. However, in contrast to that found with VEGF₁₆₅, the change in mRNA expression levels of VEGF₁₈₉ and VEGF₁₂₁ were significantly increased in RPE from aged compared with young donor eyes. The observation that VEGF₁₈₉ was increased in RPE from aged donors suggested that VEGF₁₈₉ may play an important role in AMD. To mimic the stress relevant to human neovascular AMD in vitro, hRPE was cultured under hypoxic conditions or after exposure to H₂O₂. Notably, we observed that the expression of VEGF₁₈₉ was

preferentially upregulated in response to this stress. The up-regulation of VEGF₁₈₈ was also seen in RPE/choroids from mice that developed CNV after laser injury. These lines of evidence provide strong support that RPE-derived VEGF₁₈₉, 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 VEGF₁₈₉ 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 interactions¹³ 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 VEGF₁₈₉, 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 VEGF₁₈₉ knockdown indicates either that the small remaining amount of VEGF₁₈₉ resistant to knockdown is enough to trigger signaling in CECs or that other factors that are also partially cell associated, including VEGF₁₆₅, may be acting in parallel. We also showed that the RPE-derived VEGF₁₈₉ 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 migration⁴⁰⁻⁴² 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 integrins⁴⁴ 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 VEGF₁₈₉, such as VEGF₁₆₅, 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 VEGF₁₂₁ and VEGF₁₆₅, provide a chemotactic gradient for migrating CECs, which, on making contact with the RPE, lead to RPE barrier compromise¹⁰ and further upregulation of VEGF₁₈₉. 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.

References

- Penn JS, Madan A, Caldwell RB, et al. Vascular endothelial growth factor in eye disease. *Prog Retin Eye Res.* 2008;27:331-371.
- Hartnett ME, Elsner AE. Characteristics of exudative age-related macular degeneration determined in vivo with confocal and indirect infrared imaging. *Ophthalmology.* 1996;103:58-71.
- Stevens TS, Bressler NM, Maguire MG, et al. Occult choroidal neovascularization in age-related macular degeneration: a natural history study. *Arch Ophthalmol.* 1997;115:345-350.
- Marmor MF. From sea lemons to c-waves. *Cell Mol Neurobiol.* 1983;3:285-295.
- Slomiany MG, Rosenzweig SA. IGF-1-induced VEGF and IGFBP-3 secretion correlates with increased HIF-1 α expression and activity in retinal pigment epithelial cell line D407. *Invest Ophthalmol Vis Sci.* 2004;45:2838-2847.
- Green WR, Enger C. Age-related macular degeneration histopathologic studies: the 1992 Lorenz E. Zimmerman Lecture. *Ophthalmology.* 1993;100:1519-1535.
- Beatty S, Koh M, Phil M, et al. The role of oxidative stress in the pathogenesis of age-related macular degeneration. *Surv Ophthalmol.* 2002;45:115-134.
- Schlingemann RO. Role of growth factors and the wound healing response in age-related macular degeneration. *Graefes Arch Clin Exp Ophthalmol.* 2004;42:91-101.
- Grossniklaus HE, Ling JX, Wallace TM, et al. Macrophage and retinal pigment epithelium expression of angiogenic cytokines in choroidal neovascularization. *Mol Vis.* 2002;8:119-126.
- Hartnett ME, Lappas A, Darland D, et al. Retinal pigment epithelium and endothelial cell interaction causes retinal pigment epithelial barrier dysfunction via a soluble VEGF-dependent mechanism. *Exp Eye Res.* 2003;77:593-599.
- Luna JD, Chan CC, Derevanik NL, et al. Blood-retinal barrier (BRB) breakdown in experimental autoimmune uveoretinitis: comparison with vascular endothelial growth factor, tumor necrosis factor, and interleukin-1-mediated breakdown. *J Neurosci Res.* 1997;49:268-280.
- Kuroki M, Voest E, Amano S, et al. Reactive oxygen intermediates increase vascular endothelial growth factor expression in vitro and in vivo. *J Clin Invest.* 1996;98:1667-1675.
- Geisen P, McColm JR, Hartnett ME. Choroidal endothelial cells transmigrate across the retinal pigment epithelium but do not proliferate in response to soluble vascular endothelial growth factor. *Exp Eye Res.* 2006;82:608-619.
- Ishida S, Usui T, Yamashiro K, et al. VEGF164-mediated inflammation is required for pathological, but not physiological, ischemia-induced retinal neovascularization. *J Exp Med.* 2003;198:483-489.
- Usui T, Ishida S, Yamashiro K, et al. VEGF164(165) as the pathological isoform: differential leukocyte and endothelial responses through VEGFR1 and VEGFR2. *Invest Ophthalmol Vis Sci.* 2004;45:368-374.
- Lee S, Jilani SM, Nikolova GV, et al. Processing of VEGF-A by matrix metalloproteinases regulates bioavailability and vascular patterning in tumors. *J Cell Biol.* 2005;69:681-691.
- Tischer E, Mitchell R, Hartman T, et al. The human gene for vascular endothelial growth factor: multiple protein forms are encoded through alternative exon splicing. *J Biol Chem.* 1991;266:11947-11954.
- Stalmans I, Ng YS, Rohan R, et al. Arteriolar and venular patterning in retinas of mice selectively expressing VEGF isoforms. *J Clin Invest.* 2002;109:327-336.
- Carmeliet P, Ng YS, Nuyens D, et al. Impaired myocardial angiogenesis and ischemic cardiomyopathy in mice lacking the vascular endothelial growth factor isoforms VEGF164 and VEGF188. *Nat Med.* 1999;5:495-501.
- Ng YS, Rohan R, Sunday ME, et al. Differential expression of VEGF isoforms in mouse during development and in the adult. *Dev Dyn.* 2001;220:112-121.
- Ruhrberg C, Gerhardt H, Golding M, et al. Spatially restricted patterning cues provided by heparin-binding VEGF-A control blood vessel branching morphogenesis. *Genes Dev.* 2002;16:2684-2698.
- Ciulla TA, Rosenfeld PJ. Antivascular endothelial growth factor therapy for neovascular age-related macular degeneration. *Curr Opin Ophthalmol.* 2009;20:158-165.
- Li W, He Z, Li Y, Yanoff M. Vascular endothelial growth factor regulates both apoptosis and angiogenesis of choriocapillaris endothelial cells. *Microvasc Res.* 2000;59:286-289.
- Hoffmann S, Masood R, Zhang Y, et al. Selective killing of RPE with a vascular endothelial growth factor chimeric toxin. *Invest Ophthalmol Vis Sci.* 2000;41:2389-2393.
- Maminishkis A, Chen S, Jalickee S, et al. Confluent monolayers of cultured human fetal retinal pigment epithelium (hfRPE) exhibit morphology and physiology of native tissue. *Invest Ophthalmol Vis Sci.* 2006;47:3612-3624.
- Gibbs D, Williams DS. Isolation and culture of primary mouse retinal pigmented epithelial cells. *Adv Exp Med Biol.* 2003;533:347-352.
- Peterson LJ, Wittchen ES, Geisen P, et al. Heterotypic RPE-choroidal endothelial cell contact increases choroidal endothelial cell transmigration via PI-3K and Rac1. *Exp Eye Res.* 2007;84:737-744.
- Budd S, Byfield G, Martiniuk D, et al. Reduction in endothelial tip cell filopodia corresponds to reduced intravitreal but not intraretinal vascularization in a model of ROP. *Exp Eye Res.* 2009;89:718-727.

29. Nozaki M, Sakurai E, Raisler BJ, et al. Loss of SPARC-mediated VEGFR-1 suppression after injury reveals a novel antiangiogenic activity of VEGF-A. *J Clin Invest*. 2006;116:422-429.
30. Klagsbrun M, D'Amore PA. Regulators of angiogenesis. *Annu Rev Physiol*. 1991;53:217-239.
31. Risau W. Mechanisms of angiogenesis. *Nature*. 1997;386:671-674.
32. Lamoreaux WJ, Fitzgerald MEC, Reiner A, et al. Vascular endothelial growth factor increases release of gelatinase A and decreases release of tissue inhibitor of metalloproteinases by microvascular endothelial cells in vitro. *Microvasc Res*. 1998;55:29-42.
33. Vinci MC, Visentin B, Cusinato F, et al. Effect of vascular endothelial growth factor and epidermal growth factor on iatrogenic apoptosis in human endothelial cells. *Biochem Pharmacol*. 2004;67:277-284.
34. Roeckl W, Hecht D, Sztajer H, et al. Differential binding characteristics and cellular inhibition by soluble VEGF receptors 1 and 2. *Exp Cell Res*. 1998;241:161-170.
35. Zachary I, Glikli G. Signaling transduction mechanisms mediating biological actions of the vascular endothelial growth factor family. *Cardiovasc Res*. 2001;49:568-581.
36. Ishibashi T, Hata Y, Yoshikawa H, et al. Expression of vascular endothelial growth factor in experimental choroidal neovascularization. *Graefes Arch Clin Exper Ophthalmol*. 1997;35:159-167.
37. Senger DR, Galli SJ, Dvorak AM, et al. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science*. 1983;219:983-985.
38. Ashton AW, Ware JA. Thromboxane A2 receptor signaling inhibits vascular endothelial growth factor-induced endothelial cell differentiation and migration. *Circ Res*. 2004;95:372-379.
39. Saint-Geniez M, Maldonado AE, D'Amore PA. VEGF expression and receptor activation in the choroid during development and in the adult. *Invest Ophthalmol Vis Sci*. 2006;47:3135-3142.
40. Ridley AJ, Schwartz MA, Burridge K, et al. Cell migration: integrating signals from front to back. *Science*. 2003;302:1704-1709.
41. Fukata M, Nakagawa M, Kaibuchi K. Roles of Rho-family GTPases in cell polarisation and directional migration. *Curr Opin Cell Biol*. 2003;15:590-597.
42. Itoh RE, Kurokawa K, Ohba Y, et al. Activation of Rac and Cdc42 video imaged by fluorescent resonance energy transfer-based single-molecule probes in the membrane of living cells. *Mol Cell Biol*. 2002;22:6582-6591.
43. Nobes C, Hall A. Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell*. 1995;81:53-62.
44. Keely P, Parise L, Juliano R. Integrins and GTPases in tumour cell growth, motility and invasion. *Trends Cell Biol*. 1998;8:101-106.
45. Zhao M, Bai H, Wang E, et al. Electrical stimulation directly induces pre-angiogenic responses in vascular endothelial cells by signaling through VEGF receptors. *J Cell Sci*. 2004;117:397-405.
46. Flaxenburg JA, Melter M, Lapchak PH, et al. The CD40-induced signaling pathway in endothelial cells resulting in the overexpression of vascular endothelial growth factor involves ras and phosphatidylinositol 3-kinase. *J Immunol*. 2004;172:7503-7509.
47. Gille H, Kowalski J, Yu L, et al. A repressor sequence in the juxtamembrane domain of Flt-1 (VEGFR-1) constitutively inhibits vascular endothelial growth factor-dependent phosphatidylinositol 3-kinase activation and endothelial cell migration. *EMBO J*. 2000;19:4064-4073.
48. Arefieva TI, Kuktina NB, Antonova OA, Krasnikova TL. MCP-1 stimulated chemotaxis of monocytic and endothelial cells is dependent on activation of different signaling cascades. *Cytokine*. 2005;31:439-446.
49. Okada Y, Saika S, Shirai K, et al. JNK MAPK signaling contributes in vivo to injury-induced corneal epithelial migration. *Ophthalmic Res*. 2009;42:185-192.
50. Huang C, Jacobson K, Schaller MD. MAP kinases and cell migration. *J Cell Sci*. 2004;117:4619-4628.