Modulation of VEGF-Induced Retinal Vascular Permeability by Peroxisome Proliferator-Activated Receptor-β/δ

Sandra Suarez,[1] Gary W. McCollum,[2] Colin A. Bretz,[1] Rong Yang,[2] Megan E. Capozzi,[3] and John S. Penn[1–3]

[1]Department of Cell and Developmental Biology, Vanderbilt University School of Medicine, Nashville, Tennessee, United States
[2]Department of Ophthalmology and Visual Sciences, Vanderbilt University School of Medicine, Nashville, Tennessee, United States
[3]Department of Molecular Physiology and Biophysics, Vanderbilt University School of Medicine, Nashville, Tennessee, United States

Correspondence: Sandra Suarez, The Vanderbilt Eye Institute, 1215 21st Avenue South, 8000 Medical Center East, North Tower, Nashville, TN 37232-8808, USA; sandra.suarez@vanderbilt.edu.

Submitted: February 27, 2014
Accepted: October 27, 2014

Citation: Suarez S, McCollum GW, Bretz CA, Yang R, Capozzi ME, Penn JS. Modulation of VEGF-induced retinal vascular permeability by peroxisome proliferator-activated receptor-β/δ. *Invest Ophthalmol Vis Sci*. 2014;55:8232–8240. DOI:10.1167/iovs.14-14217

Purpose. Vascular endothelial growth factor (VEGF)-induced retinal vascular permeability contributes to diabetic macular edema (DME), a serious vision-threatening condition. Peroxisome proliferator-activated receptor β/δ (PPARβ/δ) antagonist/reverse agonist, GSK0660, inhibits VEGF-induced human retinal microvascular endothelial cell (HRMEC) proliferation, tubulogenesis, and oxygen-induced retinal vasculopathy in newborn rats. These VEGF-induced HRMEC behaviors and VEGF-induced disruption of endothelial cell junctional complexes may well share molecular signaling events. Thus, we sought to examine the role of PPARβ/δ in VEGF-induced retinal hyperpermeability.

Methods. Transendothelial electrical resistance (TEER) measurements were performed on HRMEC monolayers to assess permeability. Claudin-1/Claudin-5 localization in HRMEC monolayers was determined by immunocytochemistry. Extracellular signal-regulated protein kinases 1 and 2 (Erk 1/2) phosphorylation, VEGF receptor 1 (VEGFR1) and R2 were assayed by Western blot analysis. Expression of VEGFR1 and R2 was measured by quantitative RT-PCR. Last, retinal vascular permeability was assayed in vivo by Evans blue extravasation.

Results. Human retinal microvascular endothelial cell monolayers treated with VEGF for 24 hours showed decreased TEER values that were completely reversed by the highest concentration of GSK0660 (10 μM) and PPARβ/δ-directed siRNA (20 μM). In HRMEC treated with VEGF, GSK0660 stabilized tight-junctions as evidenced by Claudin-1 staining, reduced phosphorylation of Erk1/2, and reduced VEGFR1/2 expression. Peroxisome proliferator-activated receptor β/δ siRNA had a similar effect on VEGFR expression and Claudin-1, supporting the specificity of GSK0660 in our experiments. Last, GSK0660 significantly inhibited VEGF-induced retinal vascular permeability and reduced retinal VEGFR1and R2 levels in C57BL/6 mice.

Conclusions. These data suggest a protective effect for PPARβ/δ antagonism against VEGF-induced vascular permeability, possibly through reduced VEGFR expression. Therefore, antagonism/reverse agonism of PPARβ/δ siRNA may represent a novel therapeutic methodology against retinal hyperpermeability and is worthy of future investigation.

Keywords: vascular permeability, PPARβ/δ, VEGF

Diabetic retinopathy (DR) is a progressive, neurovascular inflammatory disease, leading to extensive retinal damage that often results in blindness.[1,2] Diabetic macular edema (DME) is the leading cause of vision loss in DR,[3–5] and it results from compromise of the blood retinal barrier (BRB) and/or impaired fluid removal from the retina. Vascular endothelial growth factor (VEGF) is a potent endothelial cell mitogen that induces endothelial cell (EC) migration and tube formation.[6] A vast number of studies indicate that it also induces compromise of the BRB, resulting in retinal hyperpermeability, and ultimately DME.[7,8,9–13] Anti-VEGF therapies have been tested for efficacy against DME in humans.[14–17] Of these, Ranibizumab (lucentis), a humanized monoclonal antibody against VEGF, was approved by the Food and Drug Administration for treatment of DME, and has shown modest vision improvements in some patients.[18] However, multiple repeated intravitreal injections are required to achieve these outcomes, carrying the risk of iatrogenic endophthalmitis and other putative risks.[19,20] Additionally, experimental evidence has emerged suggesting that VEGF blockade may lead to exacerbation of vasoproliferative disease after cessation of initial anti-VEGF therapy.[21] Hence, the need for development of other therapies against DME persists.

The inner BRB, consisting of the endothelium lining the lumina of the retinal vasculature, has selective barrier properties that depend on the formation of tight and adherens junctions between adjacent EC.[22] Studies have shown that Claudins-1 and -2 are essential structural components of EC tight-junctions.[23–29] The pathogenesis of DME correlates with tight-junction disorganization and/or internalization, and changes in Claudin expression have been observed in several pathologies associated with increased vascular permeability.[30,31]

Peroxisome proliferator-activated receptors (PPARs) are transcription factors and members of the steroid nuclear hormone receptor family.[32,33] Three PPAR isoforms have been

characterized: PPARα (NR1C1), PPARβ/δ (NR1C2), and PPARγ (NR1C3).[33–35] All PPAR subtypes are constitutively expressed in the whole retina.[36,37] Peroxisome proliferator-activated receptor β/δ is ubiquitously expressed and was first recognized for its role in lipid metabolism and glucose homeostasis.[38] However, an increasing number of studies suggest additional biologic activities in cell proliferation, differentiation, and inflammation.[39,40] Very little is known about any putative biologic function of PPARβ/δ in DR.

In a previous study we observed that the PPARβ/δ antagonist/reverse agonist, GSK0660, inhibited VEGF-induced human retinal microvascular endothelial cell (HRMEC) proliferation, tubulogenesis, and oxygen-induced retinal vasculopathy in newborn rats.[33,41,42] These results led us to hypothesize that GSK0660 also may show efficacy against other VEGF-induced HRMEC behaviors, such as hyperpermeability, because, akin to VEGF-induced HRMEC proliferation, it is signaled by molecular events downstream from VEGF receptor (VEGFR) activation. Hence, the effects of the selective PPARβ/δ antagonist/inverse agonist, GSK0660, were assessed on VEGF-induced HRMEC paracellular permeability by performing TEER measurements and by assessing retinal permeability in mice receiving intravitreal injections of VEGF. To investigate the mechanism of action of PPARβ/δ on these cell behaviors, we also investigated the following effects of PPARβ/δ inhibition in HRMECs treated with VEGF: localization of Claudin-1, VEGF signal activation through extracellular signal-regulated protein kinases 1 and 2 (Erk 1/2) phosphorylation, and VEGF expression.

Materials and Methods

Human Retinal Microvascular Endothelial Cell Culture

Primary cultures of HRMECs (Cell Systems, Kirkland, WA, USA) were seeded into tissue culture flasks coated with attachment factor (Cell Signaling, Danvers, MA, USA). Human retinal microvascular ECs were grown and cultured in phenol red-free endothelial basal medium (EBM; Lonza, Walkersville, MD, USA) supplemented with 10% fetal bovine serum (FBS), 1× antibiotic/antimycotic solution, and endothelial cell growth supplements (EGM SingleQuots; Lonza), hereafter referred to as growth medium. When experimental conditions required serum-free medium, EBM with no FBS or growth supplements was used. All cultures were incubated at 37°C, 5% CO_2, and 95% relative humidity (20.9% oxygen). Passages 6 to 8 were used for these experiments.

Human Retinal Microvascular Endothelial Cell Transfection

For transfection, HRMECs were cultured in six-well dishes and 1 mL fresh media was added to each well 30 minutes before treatment. For each well, 4 μL 20 μM small interfering RNA (siRNA) oligos (Qiagen, Limburg, The Netherlands; Supplementary Material), 9 μL Targefect Solution A (Targeting Systems, El Cajon, CA, USA), and 18 μL Virofect (Targeting Systems) were added to 250 μL Optimem (Life Technologies, Carlsbad, CA, USA) in a separate tube, and inverted between the addition of each reagent. Mixed reagents were incubated at 37°C for 25 minutes before being added to each well. Cells were incubated with transfection reagents for 12 hours, before being washed and treated with fresh media. Experimental treatments began 24 hours after transfection. Knockdown efficiency and other quality control aspects of our siRNA experiments are shown in Supplementary Figure S1.

Transendothelial Cell Electric Resistance

All resistance measurements were performed with an epithelial volt ohm meter (World Precision Instruments, Sarasota, FL, USA) using STX2 chopstick electrodes (World Precision Instruments). We coated 0.4-μM polycarbonate membrane 12-mm transwell inserts for 12-well tissue culture plates (Costar, Corning, NY, USA) with a solution of human fibronectin (Sigma-Aldrich Corp., St. Louis, MO, USA) dissolved in PBS at a concentration of 50 μg/mL for 6 hours at 25°C. The fibronectin solution was aspirated and allowed to dry. Human retinal microvascular endothelial cells were suspended in MCDB-131 complete medium (VEC Technologies, Rensselaer, NY, USA) and plated in the multiwall inserts. The cells were cultured for an additional 5 to 7 days until the TEER was greater than or equal to 20 ohm × cm^2. The cells were pretreated with 10 μM GSK0660 (Tocris Biosciences, Minneapolis, MN, USA) or vehicle (0.1% dimethyl sulfoxide [DMSO]) in MCDB-131 complete medium for 24 hours. The cells were treated with 2% serum MCDB-131 + 10 μM GSK0660 or 0.1% DMSO for 1 hour and then treated with increasing doses of human recombinant VEGF (Invitrogen, Grand Island, NY, USA; 25, 50, or 75 ng/mL) for 24 hours. In other experiments, using the same culture conditions to establish TEER greater than or equal to 20 ohm × cm, HRMECs were transfected with control siRNA or sequence-specific siRNA directed against PPARβ/δ in 10% FBS for 24 hours. The medium was changed to 2% FBS for 1 hour, followed by treatment with vehicle (PBS) or 75 ng/mL VEGF for 24 hours. Resistance measurements were performed at 0, 4, 8, 12, and 24 hours after VEGF treatment.

Immunocytochemistry

Human retinal microvascular endothelial cells were seeded at a density of 2 × 10^4 cells/cm^2 on four-chamber slides (Lab Tek, Scotts Valley, CA, USA) coated with attachment factor in Dulbecco's modified Eagle's medium low glucose (1×) media (Invitrogen). After the cells formed confluent monolayers, they were pretreated with GSK0660 for 24 hours, serum starved for 2 hours, and treated with VEGF (75 ng/mL) for 24 hours. In other experiments, HRMECs were transfected with control siRNA or sequence-specific siRNA directed against PPARβ/δ for 24 hours, serum starved for 2 hours, then treated with 75 ng/mL VEGF. Following each treatment period, cells were fixed in methanol at −20°C for 15 minutes and antigens were detected by immunofluorescence staining. After the fixation period, cells were blocked with 10% donkey serum/PBS (Millipore, Billerica, MA, USA) for 30 minutes at room temperature. Slides were then incubated overnight with primary antibody, rabbit anti-Claudin-1 (Invitrogen), and rabbit anti-Claudin-5 (Santa Cruz, Dallas, TX, USA) diluted in 1% donkey serum in PBS with Tween 20 (PBST). After washing with PBS, secondary antibody, Alexa Fluor goat anti-rabbit IgG H+L (Invitrogen) was added for 1 hour at room temperature. Cells were then washed in PBST and 4′,6-diamidino-2-phenylindole stain was applied (Sigma-Aldrich Corp.). Last, chamber slides were washed and cells were embedded using Fluorogel with Tris buffer (Electron Microscopy Science, Hatfield, PA, USA) and examined by fluorescence microscopy (Olympus AX70; Tokyo, Japan).

Western Blot Analysis

Protein lysates were collected from HRMECs using the Qproteome Mammalian Protein Prep Kit (Qiagen). Protein concentrations of the supernatants were determined with the BCA kit (Thermo Scientific, Rockford, IL, USA). Twenty microliters (25 μg) were mixed with 5 μL NuPAGE LDS SampleBuffer (4×) (Invitrogen) and heated at 95°C for 10

minutes. The samples were resolved by SDS-PAGE and were transferred to 0.45 μm nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). Nitrocellulose membranes were blocked with TBST-1% bovine serum albumin (Sigma-Aldrich Corp.) and were probed with primary antibodies, phospho-p44/42 MAPK (p-Erk1/2) and p44/42 MAPK (Erk1/2) (Cell Signaling). Goat anti-rabbit IgG-horseradish peroxidase (HRP; Millipore) secondary antibody was applied to the membranes and developed using enhanced chemiluminescence (ECL; Amersham, Piscataway, NJ, USA). Each Western blot was repeated at least three times. For VEGFR1/2 Western blots, retinas were dissected and placed in 200μL radio-immunoprecipitation assay buffer (Sigma-Aldrich, Corp.). Samples were sonicated, homogenized, and prepared for Western blot analysis (see conditions mentioned previously). Blots were probed with anti-VEGFR1 (Abcam, Cambridge, UK) and anti-VEGFR2 (Cell Signaling) primary antibodies. Goat anti-rabbit IgG-HRP (Millipore) secondary antibody was applied to membranes and developed using enhanced chemiluminescence.

Messenger RNA HRMEC Expression

Human retinal microvascular endothelial cells were cultured and treated as necessary. Total RNA was isolated from cells using an RNeasy mini kit (Qiagen). cDNAs were reverse transcribed using the High-Capacity cDNA Archive Kit according to the manufacturer's instructions (Life Technologies). Quantitative real-time PCR (qRT-PCR) was performed by co-amplification of PPARβ/δ, PPARα, VEGFR1, or VEGFR2 versus β-actin, using gene-specific TaqMan Gene Expression Assays according to the manufacturer's instructions (Thermo Scientific). Expression data was analyzed by the comparative Ct method.

Evans Blue

All experiments were approved by the Vanderbilt University Institutional Animal Care and Use Committee and were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Four- to 6-week old male C57BL/6 mice were procured from Charles River (Wilmington, MA, USA). Mice received an intravitreal injection of PBS (2 μL), VEGF (2 μL at 50 ng/mL) plus vehicle (0.1% DMSO in PBS) or VEGF plus GSK0660 (10 μM); 24 hours later, mice were anesthetized and injected by tail vein with 1 mL/kg 3% Evans blue dissolved in 0.9% saline. Forty minutes after tail vein injection, mice were anesthetized with ketamine and xylazine and perfused using 0.9% saline. After perfusion, retinas were dissected, weighed, and placed in 200 μL formamide for 18 hours at 70°C to extract Evans blue. The following day, the retinas were centrifuged at 21,130*g* for 45 minutes and removed from the formamide. The A_{620} of the samples and standard curve was measured using a plate reader. Results are expressed as ng Evans blue/mg tissue.

Statistical Analysis

Data were analyzed with commercial software (GraphPad Prism 6; GraphPad, La Jolla, CA, USA) using ANOVA with Fisher's least significant difference post hoc analysis. Values of P less than 0.05 were considered statistically significant.

Results

GSK0660 Inhibits VEGF-Induced Permeability in HRMEC Monolayers

Confluent HRMEC monolayers were treated with vehicle (sterile water + 0.1% BSA) or VEGF at 25, 50, and 75 ng/mL concentrations. The TEER was significantly decreased at 8 hours at all VEGF concentrations, compared with control monolayers (0.0001). The trend toward decreased resistance continued and at 24 hours dose-responsively ranged from 19% to 48% ($P <$ 0.0001) compared with controls (Fig. 1A). Resistance values of confluent HRMECs treated with 75 ng/mL of VEGF and 10 μM GSK0660 were not significantly different from those of control cells, indicating a complete stabilization of the HRMEC monolayers and inhibition of VEGF-induced permeability by GSK0660 (10 μM); GSK0660 was not effective at the 1-μM dose (Fig. 1B). To test for possible PPARβ/δ-independent effects of GSK0660, we transfected HRMECs with sequence-specific PPARβ/δ-directed siRNA and measured TEER over 24 hours of VEGF treatment. Peroxisome proliferator-activated receptorβ/δ knockdown corrected VEGF-induced decreases in TEER, achieving statistical significance at 8, 12, and 24 hours (Fig. 1C). Statistical analysis showed that, like 10 μM GSK0660, PPARβ/δ-directed siRNA caused full recovery of control resistance levels.

GSK0660 Inhibits VEGF-Induced Claudin-1 Plasma Membrane Delocalization

Human retinal microvascular endothelial cells were treated with 75 ng/mL VEGF and/or 10 μM GSK0660 for 24 hours. Claudin-1 plasma membrane localization was disrupted in VEGF-treated cells when compared with control conditions. Treatment with GSK0660 in combination with VEGF treatment mitigated VEGF-induced Claudin-1 plasma membrane disruption (Figs. 2A–C). Peroxisome proliferator-activated receptorβ/δ-directed siRNA also blocked VEGF-induced claudin-1 disorganization at the cell borders. These data indicate that the observed GSK0660 stabilization of Claudin-1 is PPARβ/δ-specific. Notably, Claudin-5 localization was not affected by VEGF (Figs. 3A–C).

GSK0660 Inhibits VEGF-Induced Erk 1/2 Phosphorylation

Human retinal microvascular endothelial cells treated with VEGF (75 ng/mL) for 30 minutes showed a 4-fold increase in phosphorylation of Erk 1/2. Cells treated with 10 μM GSK0660 alone resulted in phosphorylated levels of Erk 1/2 similar to vehicle (0.1% DMSO). GSK0660 at 10 μM inhibited VEGF-induced Erk 1/2 phosphorylation as shown in Figure 4A. Peroxisome proliferator-activated receptorβ/δ-directed siRNA knockdown in HRMECs also inhibited VEGF-induced Erk 1/2 phosphorylation, indicating that the similar finding observed in GSK0660-treated HRMECs was indeed specific to PPARβ/δ (Fig. 4C). Quantification of three independent Western blots is demonstrated in Figures 4B and 4D.

GSK0660 and PPARβ/δ-Directed siRNA Knockdown Decreases VEGFR Expression in HRMECs

Vascular endothelial growth factor R1 and R2 mRNA levels were assayed in HRMECs treated with 10 μM GSK0660 or transfected with 20 μM PPARβ/δ-directed siRNA. In GSK0660-treated cells, VEGFR1 and R2 expression were decreased approximately 55% and 45%, respectively, at 6 hours (Fig. 5A). Vascular endothelial growth factor R1 and R2 were decreased by approximately 90% after treatment with PPARβ/δ-directed siRNA (Fig. 5B).

GSK0660 Inhibits VEGF-Induced Retinal Vascular Permeability in C57BL/6 Mice

Extravasation of retinal Evans blue (μg/mg of wet retinal tissue) was significantly increased in C57BL/6 mice receiving intraoc-

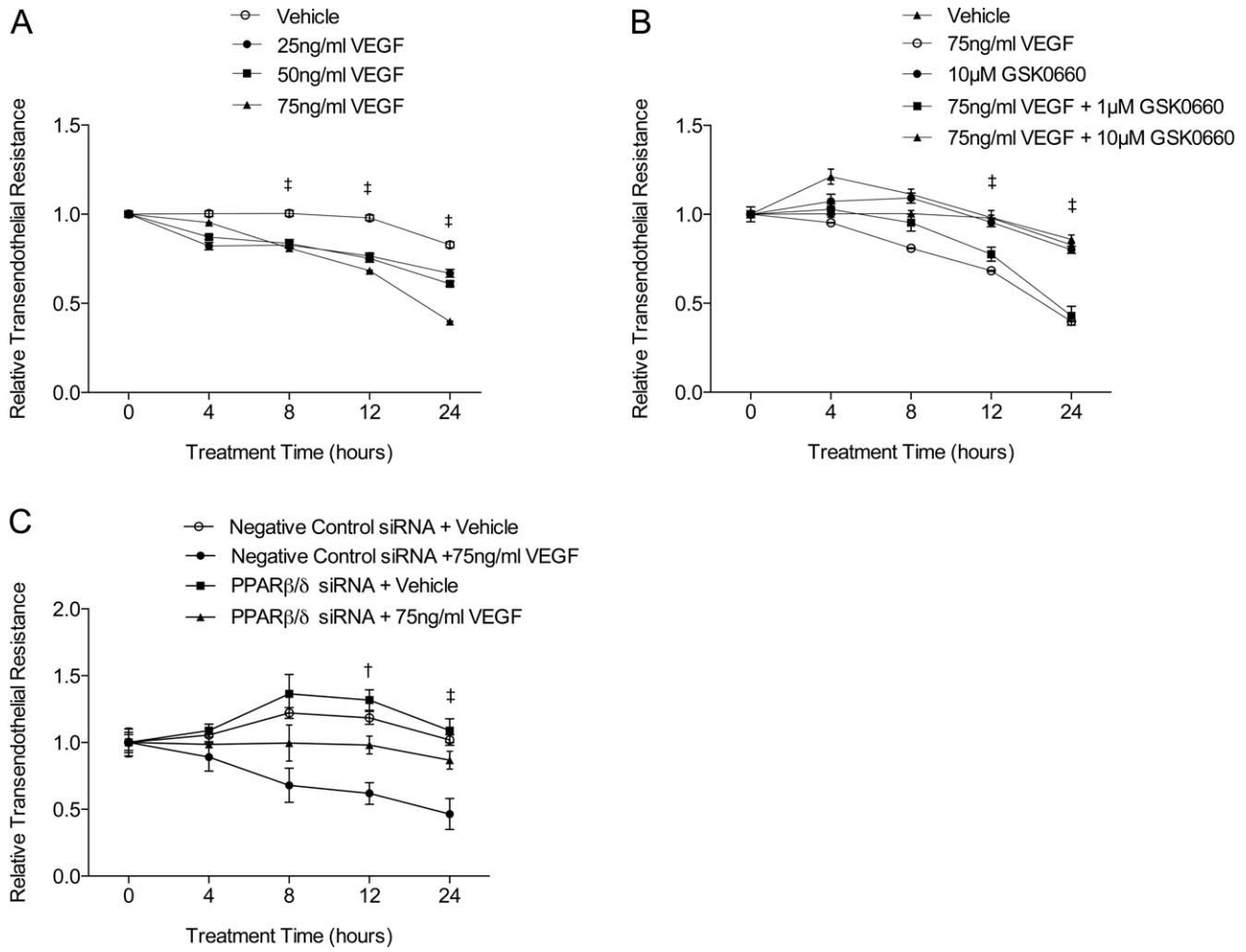

Figure 1. Both GSK0660 and PPARβ/δ-directed siRNA inhibit VEGF-induced permeability of HRMEC monolayers. (**A**) Human retinal microvascular endothelial cells were treated with vehicle (0.1% BSA), 25, 50, or 75 ng/mL VEGF for 24 hours, resulting in dose-dependent decreases in electrical resistance of HRMEC monolayers at 8, 12, and 24 hours; ‡$P < 0.0001$. (**B**) The GSK0660 completely blocked VEGF-induced decreases in electrical resistance of HRMECs (vehicle versus VEGF [75 ng/mL]; ‡$P < 0.0001$). There was no significant difference between monolayers treated with VEGF (75 ng/mL) + 10 μM GSK0660 and control monolayers not treated with VEGF. (**C**) Peroxisome proliferator-activated receptor β/δ–directed siRNA blocked VEGF-induced decreases in the electrical resistance in HRMEC monolayers. Vascular endothelial growth factor decreased the electrical resistance in HRMEC monolayers transfected (vehicle with negative control siRNA versus VEGF with negative control siRNA [75 ng/mL]; ‡$P < 0.0001$). There were no significant decreases in electrical resistance between monolayers transfected with PPARβ/δ-directed siRNA + VEGF (75 ng/mL) and controls not treated with VEGF.

ular injections of 50 ng/mL VEGF when compared with mice receiving vehicle ($P < 0.001$). Intraocular injection of 10 μM GSK0660 in addition to VEGF reversed this VEGF-induced retinal vascular hyperpermeability to control levels ($P < 0.001$) (Fig. 6). Evans blue data are representative of three independent experiments, each using at least three mice per treatment arm.

GSK0660 Reduces Retinal VEGFR1 and R2 Levels

The C57BL/6 mice received intraocular injections of vehicle, 50 ng/mL VEGF, 10 μM GSK0660, or the combination of both VEGF and GSK0660. After 24 hours, retinas were dissected, homogenized, lysed, and subjected to Western blot analysis. Compared with the vehicle, decreasing and increasing trends were observed with the GSK0660 and VEGF treatments, respectively, but neither was statistically significant. Retinal VEGFR1 was reduced by approximately 75% in the VEGF + GSK0660 group compared with VEGF group ($P < 0.05$). VEGF had no effect on retinal VEGFR2; although GSK06660 caused a trend toward lower VEGFR1 levels, the difference was not statistically significant when compared with the vehicle. Retinal VEGFR2 was reduced by approximately 75% in the VEGF + GSK0660 group compared with VEGF group ($P < 0.05$) (Fig. 7).

Discussion

We have previously reported that PPARβ/δ is expressed in HRMEC and its target genes, adipose differentiation-related factor and angiopoietin-like protein 4, are responsive to the selective antagonist/inverse agonist GSK0660.[33] The human GSK0660 IC_{50} value for PPARβ/δ is 0.155 μM, and greater than 10 μM for PPARs α and γ.[43] Therefore, at the concentrations tested in this study, selective pharmacologic manipulation of PPARβ/δ was observed.

In the current study, we treated HRMEC monolayers with VEGF and observed a significant decrease in TEER at 4 hours, continuing out to 24 hours, that was completely blocked by pretreating the HRMEC monolayers with GSK0660. Further-

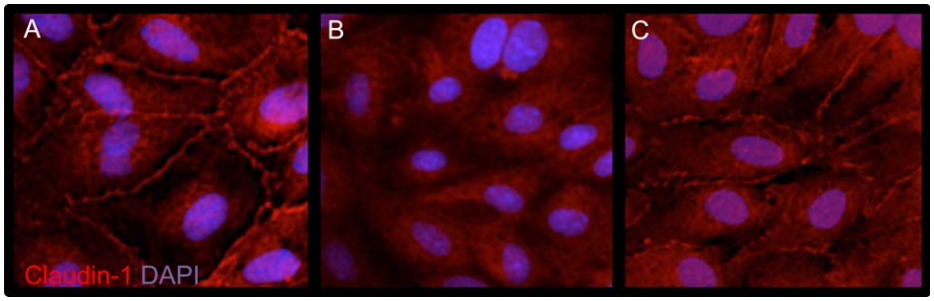

FIGURE 2. Plasma membrane localization of Claudin-1 is lost after VEGF treatment (24 hours), and GSK0660 (24 hours) restores this localization. Human retinal microvascular endothelial cells were cultured in (**A**) 10% FBS medium, (**B**) 75 ng/mL VEGF, or (**C**) 75 ng/mL VEGF plus 10 μM GSK0660.

more, PPARβ/δ-directed siRNA stabilized VEGF-induced decreases in TEER, suggesting that the effect of GSK0660 is PPARβ/δ-specific (Fig. 1). Other groups have shown that growth factor treatment of immortalized bovine retinal microvascular EC monolayers, resulted in the loss of Claudin-1 from their plasma membranes, correlating with decreased barrier function.[44] In the current study (Fig. 2), GSK0660 decreased the loss of Claudin-1 from plasma membranes of HRMECs challenged with VEGF; the same effect was recapitulated with PPARβ/δ siRNA (Fig. 3), indicating that the effect of GSK0660 was PPARβ/δ-specific. These data provide additional evidence for PPARβ/δ-dependent regulation of HRMEC barrier properties. Claudin-5 is another known protein constituent of tight-junction complexes, and in accordance with other studies VEGF did not affect Claudin-5 membrane localization (Fig. 3).[44]

These findings led us to hypothesize that the observed effects of PPARβ/δ manipulation might be due to a mitigating influence on VEGF signaling. The VEGF receptors, VEGFR1 and R2, are transmembrane tyrosine kinases that dimerize and autophosphorylate on binding VEGF, activating their intrinsic kinase activities. Subsequent phosphorylation of downstream signaling intermediates by VEGFR occurs translating into VEGF-dependent biologic functions, such as delocalization of tight-junctional complexes followed by increased vascular permeability.[6] We elected to use VEGF-induced Erk 1/2 phosphorylation, a signaling event immediately downstream from VEGFR phosphorylation, as a surrogate marker for activation of VEGF signaling in HRMECs. We observed a decrease in VEGF-induced Erk 1/2 phosphorylation after 24 hours of GSK0660 pretreatment or 24 hours of PPARβ/δ-directed siRNA (Fig. 4), immediately prompting us to investigate whether PPARβ/δ regulates VEGFR in HRMECs. We found that VEGFR1 and R2 expression were both decreased by GSK0660, as well as PPARβ/δ-directed siRNA (Fig. 5).

In summary, these data suggest that GSK0660 stabilizes the TEER of HRMEC monolayers against VEGF challenge by reducing VEGFR1 and R2 levels. Consequently, reduced VEGFR mitigates postreceptor VEGF-induced signaling, increases tight-junction stability, and reduces paracellular permeability. However, we cannot rule out the possibility of a GSK0660-dependent effect on VEGF-induced degradation/internalization of adherens junction proteins. Adherens junctions also contribute to the barrier properties of ECs. Chen et al.[41] reported that VEGF-induced FAK phosphorylation facilitated VE-cadherin-β-catenin dissociation leading to the breakdown of

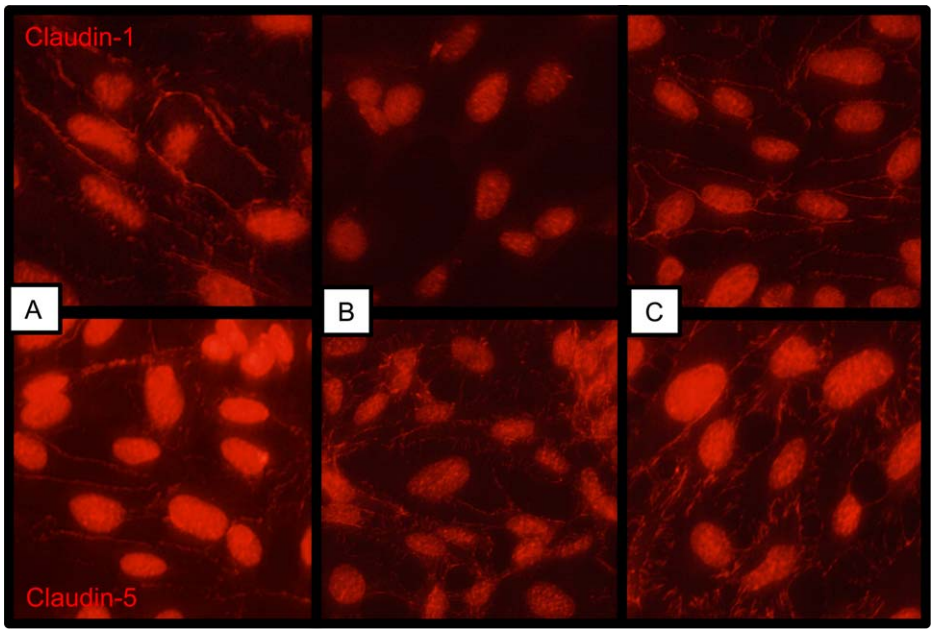

FIGURE 3. Plasma membrane localization of Claudin-1, but not Claudin-5, is lost after VEGF treatment (24 hours), and PPARβ/δ-directed siRNA restores membrane localization. Human retinal microvascular endothelial cells were cultured in (**A**) 10% FBS medium plus 20 μM negative control oligomer, (**B**) 20 μM negative control oligomer plus 75 ng/mL VEGF, or (**C**) 20 μM PPARβ/δ-directed siRNA plus 75 ng/mL VEGF. *Top*: Claudin-1, *bottom*: Claudin-5.

Investigative Ophthalmology & Visual Science

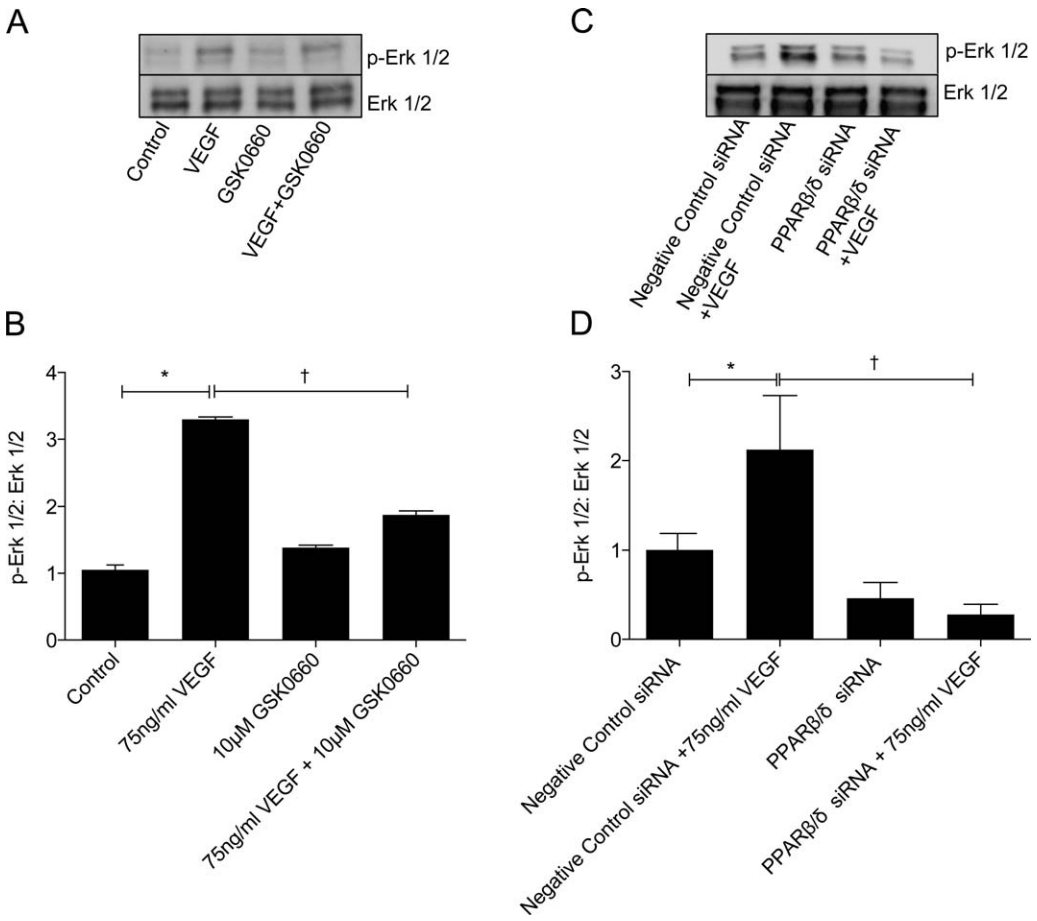

Figure 4. Both GSK0660 and PPARβ/δ-directed siRNA inhibit activation of VEGF signaling. Vascular endothelial growth factor (75 ng/mL) significantly upregulated phosphorylation of Erk 1/2 (p-Erk 1/2) when compared with control conditions. Treatment with (**A**) 10 μM GSK0660 or (**C**) 20 μM PPARβ/δ-directed siRNA significantly reduced VEGF-induced p-Erk 1/2. $^*P < 0.05$; $\dagger P < 0.01$. Quantification of three independent experiments is shown in (**B**, **D**).

adherens junctions. It would be interesting to investigate the effects of GSK0660 in this context in future studies.

Encouraged by the results we obtained with HRMEC monolayers, we proceeded to assay permeability in the in vivo setting. We found GSK0660 reduced VEGF-induced retinal hyperpermeabilty in mice as measured by Evans blue dye extravasation (Fig. 6). As a proof of concept, GSK0660 was administered by intravitreal injection to enhance its bioavailablity; we did not opt for systemic administration because GSK0660 has a short half-life.[43] Future studies should

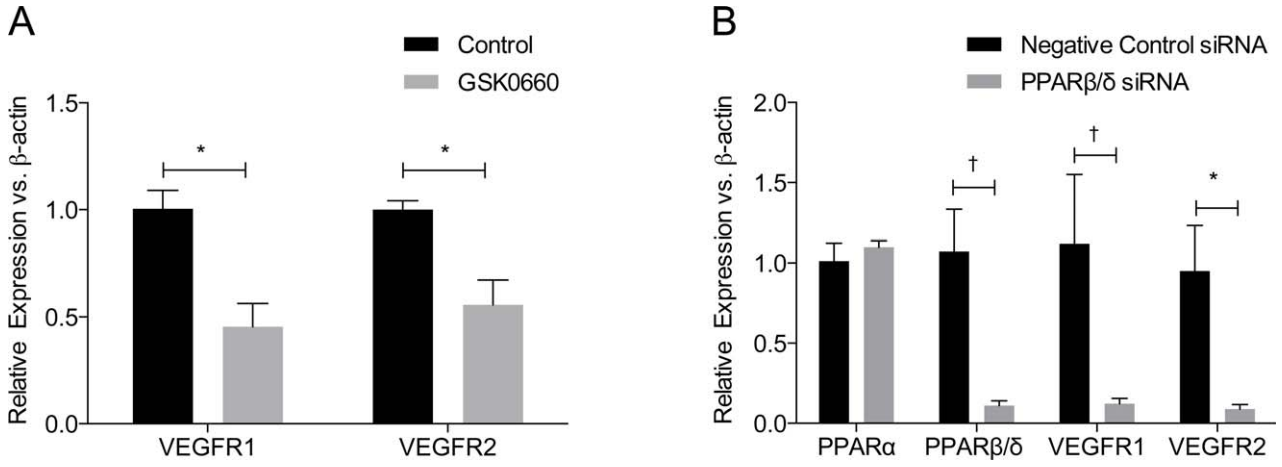

Figure 5. GSK0660 or PPARβ/δ-directed siRNA inhibit constitutive expression of VEGFR1 and VEGFR2 in HRMECs. Human retinal microvascular endothelial cells were cultured in 10% FBS plus (**A**) 10 μM GSK0660 for 6 hours or (**B**) 20 μM negative control siRNA oligomers or 20 μM PPARβ/δ-directed siRNA for 24 hours. Expression of VEGFR1 and VEGFR2 was measured using qRT-PCR. $^*P < 0.05$; $\dagger P < 0.01$.

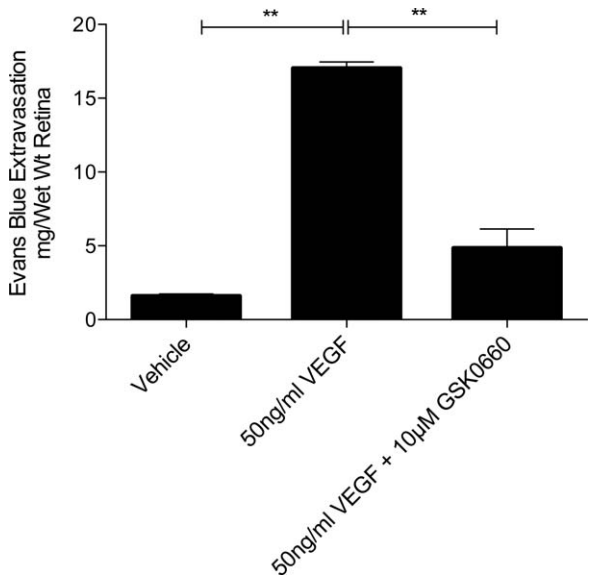

FIGURE 6. GSK0660 inhibits VEGF-induced retinal vascular permeability. Intraocular injection of 50 ng/mL VEGF significantly increased Evans blue extravasation when compared with vehicle injection. Treatment with 10 μM GSK0660 reduced VEGF-induced retinal extravasation. Permeability was analyzed via Evans blue extravasation after 24 hours of 50 ng/mL VEGF and/or 10 μM GSK0660 injections. Data are normalized to the vehicle group. $^{**}P < 0.001$.

incorporate systemic administration perhaps using GSK0660 derivatives with increased biologic stability, as intravitreal injection carries the associated risks of endophthalmitis, cataractogenesis, and glaucoma.[42,45–52]

Our in vitro experiments indicated that GSK0660-dependent PPARβ/δ antagonism/reverse agonism stabilizes the barrier properties of HRMEC monolayers against VEGF challenge by reducing the VEGFR protein levels. These results led us to investigate whether GSK0660 could also reduce retinal VEGFR levels within the time-course of our VEGF-induced retinal permeability experiments. Mice administered VEGF + GSK0660 by intravitreal injection showed statistically significant decreases in retinal VEGFR1 and R2 protein levels 24 hours later when compared with controls. Administration of GSK0660 alone showed downward trends in VEGFR1 and R2 levels that did not reach statistical significance (Fig. 7). Based on these results, we propose that the observed GSK0660-dependent reduction in retinal VEGFR1 and R2 accounts for the observed efficacy against VEGF-induced retinal permeability. Our proposition is supported by studies depicting the roles of VEGFR1 and R2 in VEGF-induced permeability.[53,54] In one of these, neutralizing antibodies targeting VEGFR1 or R2 demonstrated efficacy against retinal permeability; in another, these antibodies were protective against VEGF-induced TEER decreases in EC monolayers.[54,55]

Stephen et al.[56] reported that PPARβ/δ agonists increase VEGFR1 expression in breast cancer and human umbilical vein cells. Other groups have also shown that treatment with PPARβ/δ agonists can suppress VEGFR2 but only at extremely high concentrations.[57] However, no PPARβ/δ antagonist/inverse agonist was tested in that study and to our knowledge, this is the first report describing the negative regulation of

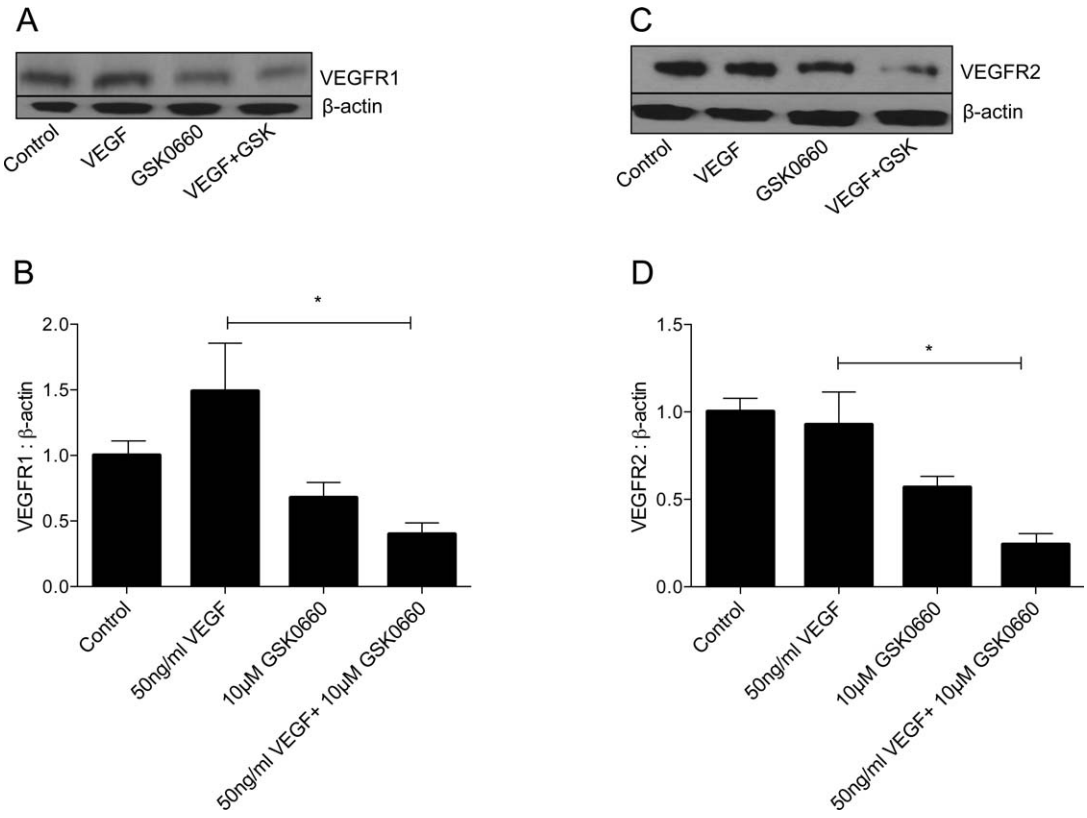

FIGURE 7. GSK0660 reduces retinal VEGFR1 and R2 levels. Mice were injected with 50 ng/mL VEGF and/or 10 μM GSK0660. Retinal levels of VEGFR1 (**A**) and VEGFR2 (**C**) were analyzed 24 hours later by Western blot analysis. *Bar graphs* represent quantification of at least two independent experiments (**B**, **D**). $^{*}P < 0.05$.

VEGFR by such an entity. The mechanisms of PPAR-dependent gene regulation are complex, varied, and often depend on the cell-type or tissue of interest.[40] The peroxisome proliferator response element (PPRE) is a consensus sequence that may facilitate PPAR binding to DNA cis control elements that participate in the transcriptional regulation of PPAR target genes. There are no known functional PPREs within the control and coding sequences of the human VEGFR1 and R2 genes.[58] However, this does not preclude a PPRE-dependent mechanism from an enhancer remotely located to either of these genes.[59] Furthermore, PPARβ/δ may mediate its biologic functions through PPRE-independent mechanisms.[34] The determination of the molecular cascade of events explaining the PPARβ/δ-dependent regulation of VEGFR is a subject that remains for future studies.

In conclusion, our data suggest the feasibility of PPARβ/δ antagonism/inverse agonism as a therapeutic methodology against retinal hyperpermeability and DME. Our data also indicate that GSK0660 blocks VEGF-induced permeability through the PPARβ/δ-dependent stabilization of EC tight-junctions, as evidenced by Claudin-1 staining in HRMECs. GSK0660 may produce these observed effects by the PPARβ/δ-dependent downregulation VEGFR1 and R2. Peroxisome proliferator-activated receptor β/δ antagonism may offer an improved alternative to the existing anti-VEGF therapies against DME in clinical use currently.

Acknowledgments

Supported by National Institutes of Health Grants R01 EY007533-27 (JSP), P30 EY008126 (Core Grant in Vision Research), and 3R01EY007533-26S1; an unrestricted grant from Research to Prevent Blindness, Inc.; and a grant from the OneSight Foundation.

Disclosure: **S. Suarez**, None; **G.W. McCollum**, None; **C.A. Bretz**, None; **R. Yang**, None; **M.E. Capozzi**, None; **J.S. Penn**, None

References

1. Antonetti DA, Klein R, Gardner TW. Diabetic retinopathy. *N Engl J Med*. 2012;366:1227–1239.
2. Zhang W, Liu H, Al-Shabrawey M, Caldwell RW, Caldwell RB. Inflammation and diabetic retinal microvascular complications. *J Cardiovasc Dis Res*. 2011;2:96–103.
3. Klein R, Klein BE, Moss SE, Cruickshanks KJ. The Wisconsin Epidemiologic Study of Diabetic Retinopathy. XV. The long-term incidence of macular edema. *Ophthalmology*. 1995;102:7–16.
4. Klein R, Klein BE, Moss SE, Linton KL. The Beaver Dam Eye Study. Retinopathy in adults with newly discovered and previously diagnosed diabetes mellitus. *Ophthalmology*. 1992;99:58–62.
5. Mohamed Q, Gillies MC, Wong TY. Management of diabetic retinopathy: a systematic review. *JAMA*. 2007;298:902–916.
6. Penn JS, Madan A, Caldwell RB, Bartoli M, Caldwell RW, Hartnett ME. Vascular endothelial growth factor in eye disease. *Prog Retin Eye Res*. 2008;27:331–371.
7. Senger DR, Galli SJ, Dvorak AM, Perruzzi CA, Harvey VS, Dvorak HF. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science*. 1983;219:983–985.
8. Miller JW, Le Couter J, Strauss EC, Ferrara N. Vascular endothelial growth factor a in intraocular vascular disease. *Ophthalmology*. 2013;120:106–114.
9. Sone H, Kawakami Y, Okuda Y, et al. Ocular vascular endothelial growth factor levels in diabetic rats are elevated before observable retinal proliferative changes. *Diabetologia*. 1997;40:726–730.
10. Duh E, Aiello LP. Vascular endothelial growth factor and diabetes: the agonist versus antagonist paradox. *Diabetes*. 1999;48:1899–1906.
11. Gilbert RE, Vranes D, Berka JL, et al. Vascular endothelial growth factor and its receptors in control and diabetic rat eyes. *Lab Invest*. 1998;78:1017–1027.
12. Hammes HP, Lin J, Bretzel RG, Brownlee M, Breier G. Upregulation of the vascular endothelial growth factor/vascular endothelial growth factor receptor system in experimental background diabetic retinopathy of the rat. *Diabetes*. 1998;47:401–406.
13. Boyer DS, Hopkins JJ, Sorof J, Ehrlich JS. Anti-vascular endothelial growth factor therapy for diabetic macular edema. *Ther Adv Endocrinol Metab*. 2013;4:151–169.
14. Runkle EA, Antonetti DA. The blood-retinal barrier: structure and functional significance. *Methods Mol Biol*. 2011;686:133–148.
15. Hofman P, Blaauwgeers HG, Tolentino MJ, et al. VEGF-A induced hyperpermeability of blood-retinal barrier endothelium in vivo is predominantly associated with pinocytotic vesicular transport and not with formation of fenestrations. Vascular endothelial growth factor-A. *Curr Eye Res*. 2000;21:637–645.
16. Scheppke L, Aguilar E, Gariano RF, et al. Retinal vascular permeability suppression by topical application of a novel VEGFR2/Src kinase inhibitor in mice and rabbits. *J Clin Invest*. 2008;118:2337–2346.
17. Aiello LP, Bursell SE, Clermont A, et al. Vascular endothelial growth factor-induced retinal permeability is mediated by protein kinase C in vivo and suppressed by an orally effective beta-isoform-selective inhibitor. *Diabetes*. 1997;46:1473–1480.
18. Stewart MW. Critical appraisal of ranibizumab in the treatment of diabetic macular edema. *Clin Ophthalmol*. 2013;7:1257–1267.
19. Englander M, Chen TC, Paschalis EI, Miller JW, Kim IK. Intravitreal injections at the Massachusetts Eye and Ear Infirmary: analysis of treatment indications and postinjection endophthalmitis rates. *Br J Ophthalmol*. 2013;97:460–465.
20. Sang DN, D'Amore PA. Is blockade of vascular endothelial growth factor beneficial for all types of diabetic retinopathy? *Diabetologia*. 2008;51:1570–1573.
21. McCloskey M, Wang H, Jiang Y, Smith GW, Strange J, Hartnett ME. Anti-VEGF antibody leads to later atypical intravitreous neovascularization and activation of angiogenic pathways in a rat model of retinopathy of prematurity. *Invest Ophthalmol Vis Sci*. 2013;54:2020–2026.
22. Gonzalez-Mariscal L, Betanzos A, Nava P, Jaramillo BE. Tight junction proteins. *Prog Biophys Mol Biol*. 2003;81:1–44.
23. Overgaard CE, Daugherty BL, Mitchell LA, Koval M. Claudins: control of barrier function and regulation in response to oxidant stress. *Antioxid Redox Signal*. 2011;15:1179–1193.
24. Lal-Nag M, Morin PJ. The claudins. *Genome Biol*. 2009;10:235.
25. Furuse M, Tsukita S. Claudins in occluding junctions of humans and flies. *Trends Cell Biol*. 2006;16:181–188.
26. Firth JA. Endothelial barriers: from hypothetical pores to membrane proteins. *J Anat*. 2002;200:541–548.
27. Furuse M, Fujita K, Hiiragi T, Fujimoto K, Tsukita S. Claudin-1 and -2: novel integral membrane proteins localizing at tight junctions with no sequence similarity to occludin. *J Cell Biol*. 1998;141:1539–1550.
28. Turksen K, Troy TC. Barriers built on claudins. *J Cell Sci*. 2004;117:2435–2447.
29. Nitta T, Hata M, Gotoh S, et al. Size-selective loosening of the blood-brain barrier in claudin-5-deficient mice. *J Cell Biol*. 2003;161:653–660.

30. Mazzon E, Puzzolo D, Caputi AP, Cuzzocrea S. Role of IL-10 in hepatocyte tight junction alteration in mouse model of experimental colitis. *Mol Med*. 2002;8:353–366.
31. Wolburg H, Wolburg-Buchholz K, Kraus J, et al. Localization of claudin-3 in tight junctions of the blood-brain barrier is selectively lost during experimental autoimmune encephalomyelitis and human glioblastoma multiforme. *Acta Neuropathol*. 2003;105:586–592.
32. Desvergne B, Wahli W. Peroxisome proliferator-activated receptors: nuclear control of metabolism. *Endocr Rev*. 1999; 20:649–688.
33. Capozzi ME, McCollum GW, Savage SR, Penn JS. Peroxisome proliferator-activated receptor-beta/delta regulates angiogenic cell behaviors and oxygen-induced retinopathy. *Invest Ophthalmol Vis Sci*. 2013;54:4197–4207.
34. Tan NS, Michalik L, Desvergne B, Wahli W. Multiple expression control mechanisms of peroxisome proliferator-activated receptors and their target genes. *J Steroid Biochem Mol Biol*. 2005;93:99–105.
35. Bishop-Bailey D, Bystrom J. Emerging roles of peroxisome proliferator-activated receptor-beta/delta in inflammation. *Pharmacol Ther*. 2009;124:141–150.
36. Ciudin A, Hernandez C, Simo R. Molecular implications of the PPARs in the diabetic eye. *PPAR Res*. 2013;2013:686525.
37. Herzlich AA, Ding X, Shen D, Ross RJ, Tuo J, Chan CC. Peroxisome proliferator-activated receptor expression in murine models and humans with age-related macular degeneration. *Open Biol J*. 2009;2:141–148.
38. Bishop-Bailey D, Swales KE. The role of PPARs in the endothelium: implications for cancer therapy. *PPAR Res*. 2008;2008:904251.
39. Kilgore KS, Billin AN. PPARbeta/delta ligands as modulators of the inflammatory response. *Curr Opin Investig Drugs*. 2008; 9:463–469.
40. Peters JM, Gonzalez FJ. Sorting out the functional role(s) of peroxisome proliferator-activated receptor-beta/delta (PPARbeta/delta) in cell proliferation and cancer. *Biochim Biophys Acta*. 2009;1796:230–241.
41. Chen XL, Nam JO, Jean C, et al. VEGF-induced vascular permeability is mediated by FAK. *Dev Cell*. 2012;22:146–157.
42. Chieh JJ, Roth DB, Liu M, et al. Intravitreal triamcinolone acetonide for diabetic macular edema. *Retina*. 2005;25:828–834.
43. Shearer BG, Steger DJ, Way JM, et al. Identification and characterization of a selective peroxisome proliferator-activated receptor beta/delta (NR1C2) antagonist. *Mol Endocrinol*. 2008;22:523–529.
44. Deissler HL, Deissler H, Lang GK, Lang GE. VEGF but not PlGF disturbs the barrier of retinal endothelial cells. *Exp Eye Res*. 2013;115:162–171.
45. Jonas J, Heatley G, Spaide R, Varma R. Intravitreal triamcinolone acetonide and secondary ocular hypertension. *J Glaucoma*. 2005;14:168–171.
46. Jonas JB, Martus P, Degenring RF, Kreissig I, Akkoyun I. Predictive factors for visual acuity after intravitreal triamcinolone treatment for diabetic macular edema. *Arch Ophthalmol*. 2005;123:1338–1343.
47. Martidis A, Duker JS, Greenberg PB, et al. Intravitreal triamcinolone for refractory diabetic macular edema. *Ophthalmology*. 2002;109:920–927.
48. Greenberg PB, Martidis A, Rogers AH, Duker JS, Reichel E. Intravitreal triamcinolone acetonide for macular oedema due to central retinal vein occlusion. *Br J Ophthalmol*. 2002;86: 247–248.
49. Sutter FK, Simpson JM, Gillies MC. Intravitreal triamcinolone for diabetic macular edema that persists after laser treatment: three-month efficacy and safety results of a prospective, randomized, double-masked, placebo-controlled clinical trial. *Ophthalmology*. 2004;111:2044–2049.
50. Fintak DR, Shah GK, Blinder KJ, et al. Incidence of endophthalmitis related to intravitreal injection of bevacizumab and ranibizumab. *Retina*. 2008;28:1395–1399.
51. Jonas JB, Kamppeter BA, Harder B, Vossmerbaeumer U, Sauder G, Spandau UH. Intravitreal triamcinolone acetonide for diabetic macular edema: a prospective, randomized study. *J Ocul Pharmacol Ther*. 2006;22:200–207.
52. Moshfeghi DM, Kaiser PK, Bakri SJ, et al. Presumed sterile endophthalmitis following intravitreal triamcinolone acetonide injection. *Ophthalmic Surg Lasers Imaging*. 2005;36:24–29.
53. Olsson AK, Dimberg A, Kreuger J, Claesson-Welsh L. VEGF receptor signalling: in control of vascular function. *Nat Rev Mol Cell Biol*. 2006;7:359–371.
54. Huang H, Shen J, Vinores SA. Blockade of VEGFR1 and 2 suppresses pathological angiogenesis and vascular leakage in the eye. *PLoS One*. 2011;6:e21411.
55. Cai J, Wu L, Qi X, et al. Placenta growth factor-1 exerts time-dependent stabilization of adherens junctions following VEGF-induced vascular permeability. *PLoS One*. 2011;6:e18076.
56. Stephen RL, Gustafsson MC, Jarvis M, et al. Activation of peroxisome proliferator-activated receptor delta stimulates the proliferation of human breast and prostate cancer cell lines. *Cancer Res*. 2004;64:3162–3170.
57. Meissner M, Hrgovic I, Doll M, Kaufmann R. PPARdelta agonists suppress angiogenesis in a VEGFR2-dependent manner. *Arch Dermatol Res*. 2011;303:41–47.
58. Lemay DG, Hwang DH. Genome-wide identification of peroxisome proliferator response elements using integrated computational genomics. *J Lipid Res*. 2006;47:1583–1587.
59. West AG, Fraser P. Remote control of gene transcription. *Hum Mol Genet*. 2005;14:R101–R111.

Investigative Ophthalmology & Visual Science