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VEGFA Activates Erythropoietin Receptor and Enhances VEGFR2-Mediated Pathological Angiogenesis

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Clinical and animal studies implicate erythropoietin (EPO) and EPO receptor (EPOR) signaling in angiogenesis. In the eye, EPO is involved in both physiological and pathological angiogenesis in the retina. We hypothesized that EPOR signaling is important in pathological angiogenesis and tested this hypothesis using a rat model of oxygen-induced retinopathy that is representative of human retinopathy of prematurity. We first determined that EPOR expression and activation were increased and that activated EPOR was localized to retinal vascular endothelial cells (ECs) in retinas at postnatal day 18 (p18), when pathological angiogenesis in the form of intravitreal neovascularization occurred. In human retinal microvascular ECs, EPOR was up-regulated and activated by VEGF. Lentiviral-delivered shRNAs that knocked down Müller cell–expressed VEGF in the retinopathy of prematurity model also reduced phosphorylated EPOR (p-EPOR) and VEGFR2 (p-VEGFR2) in retinal ECs. In human retinal microvascular ECs, VEGFR2-activated EPOR caused an interaction between p-EPOR and p-VEGFR2; knockdown of EPOR by siRNA transfection reduced VEGF-induced EC proliferation in association with reduced p-VEGFR2 and p-STAT3; however, inhibition of VEGFR2 activation by siRNA transfection or semaxanib (SU5416) abolished VEGFA-induced proliferation of ECs and phosphorylation of VEGFR2, EPOR, and STAT3. Our results show that VEGFA-induced p-VEGFR2 activates EPOR and causes an interaction between p-EPOR and p-VEGFR2 to enhance VEGFA-induced EC proliferation by exacerbating STAT3 activation, leading to pathological angiogenesis. *(Am J Pathol 2014, 184: 1230–1239; http://dx.doi.org/10.1016/j.ajpath.2013.12.023)*

Retinopathy of prematurity (ROP) is an important cause of vision loss and blindness in infants worldwide.[1] Because of the limited ability to study human preterm infant eyes, models have been established in which newborn animals that normally vascularize their retinas after birth are exposed to oxygen stresses that lead to retinal features similar to human ROP.[2] Based on such models of oxygen-induced retinopathy (OIR) and on observations in human infants, ROP has been described as having two phases.[3,4] In phase I, infants experience delayed physiological retinal vascular development and sometimes vasoattenuation from high oxygen.[5] Phase II is characterized by aberrant disordered developmental angiogenesis in the form of vasoproliferative intravitreal neovascularization (IVNV).[2] Several angiogenic agonists and inhibitors have been recognized as potentially involved in human ROP. Of these, the most studied is vascular endothelial growth factor A (VEGFA).[6–9]

Besides being involved in human pathological angiogenic eye disease,[10] VEGFA is also important in retinal vascular development.[11,12] Inhibition of the bioactivity of VEGFA in preterm infants with severe ROP reduced the IVNV of phase II,[13] but reports of persistent avascular retina and recurrent pathological angiogenesis raised concern.[14] Furthermore, neutralizing VEGFA with an antibody similar to that used in human preterm infants with severe ROP initially reduced IVNV in the rat ROP model, but caused recurrent pathological angiogenesis in association with up-regulation of several angiogenic agonists, including erythropoietin (EPO).[15]

EPO is known mainly for hematopoiesis, and it has been used to treat anemia.[16] However, a growing body of evidence indicates that EPO has other biological effects, including neuroprotective,[17–19] antiapoptotic,[19,20] antioxidative,[20,21]

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and angiogenic[22–24] properties. Evidence supporting the role of EPO in angiogenesis comes from clinical and animal studies. In clinical studies, proliferative diabetic retinopathy and severe ROP have been associated with increased EPO. In proliferative diabetic retinopathy, vitreous EPO was increased,[25] and a promoter polymorphism in the *EPO* gene resulting in increased production of EPO was associated with severe diabetic retinopathy in a largely European-American population.[26] In ROP, greater risk of severe ROP was associated with EPO treatment for anemia of prematurity.[27,28] In a mouse OIR model, hyperoxia down-regulated EPO expression in the retina and decreased vascular stability in association with vaso-obliterated retina,[29] and, after relative hypoxia, retinal EPO was increased and contributed to IVNV.[30] EPO was also identified as a target in OIR in a study using a transgenic mouse in which hypoxia inducible factor 2α (HIF-2α; *EPAS1*, alias HLF) was knocked down,[31] and EPO synergistically increased VEGFA-induced human retinal microvascular endothelial cell (hRMVEC) proliferation.[15] However, EPO also promoted physiological retinal vascularization in a rat OIR model.[32] Thus, the evidence is mixed, in that EPO has been associated with both physiological and pathological retinal angiogenesis. EPO is now being considered as a neuroprotective agent to promote cognitive development in preterm infants.[33] Greater understanding is needed regarding EPO and EPO receptor (EPOR) signaling in ROP and developmental angiogenesis.

In the present study, we used a rat ROP model[34] in which VEGFA is overexpressed by postnatal day 12 (p12) and causes IVNV at p18.[35] Our research group previously found that the VEGFA signal is detected in Müller cells and developed a method using a lentiviral vector that targets Müller cells and knocks down VEGFA *in vivo*, thereby inhibiting IVNV without interfering with pup growth or serum VEGFA.[36] The present investigation of the role of EPOR adapted lentiviral vector rat model. In phase I, EPOR activation was lower than in phase II, when VEGFA expression and VEGFR2 expression and activation were increased.[37] In hRMVECs, VEGFA up-regulated and activated EPOR and (through crosstalk between activated VEGFR2 and EPOR) increased the activation of STAT3 to enhance angiogenesis. Our present findings support the hypothesis that VEGFA activates and causes an interaction between EPOR and VEGFR2 to contribute to pathological angiogenesis.

Materials and Methods

Rat ROP Model

The rat ROP model used in this study has been described previously.[34] Within 6 hours of birth, newborn Sprague–Dawley pups and dam (Charles River Laboratories International, Wilmington, MA) were placed into a regulated oxygen environment (OxyCycler; BioSpherix, Lacona, NY) in which oxygen was cycled between 50% and 10% every 24 hours for 14 days, and then into ambient room air (RA) for 4 days. Phase I, delayed physiological retinal vascular development, occurred at p14 and phase II, IVNV, at p18. All animal studies were performed in compliance with the University of Utah (incorporating the current Guide for the Care and Use of Laboratory Animals) and the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Visual Research. For each study, at least three different litters were analyzed for immunohistochemistry, quantitative real-time PCR (qPCR), or Western blotting; all litters had between 12 and 14 pups.

For knockdown of Müller cell VEGFA, we used a lentivirus with a CD44 promoter that drives GFP and VEGFA shRNA when embedded within a microRNA 30 context. A subretinal injection of 1 μL of lentivirus (1×10^9 viral particles per milliliter) with either VEGFA shRNA or control luciferase shRNA was delivered at the beginning of the 50% oxygen cycle of the ROP model, on p8, as described previously.[36] Each pup received the same type of lentivector in each eye, to reduce the potential for confounding from crossover effects. Eyes were processed for immunohistochemistry of phosphorylated VEGFR2 (p-VEGFR2), or EPOR (p-EPOR) and lectin.

Retinal Section Preparation and Staining

Anterior segments were removed from eyes after 10 minutes of fixation in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA); retinas were carefully dissected and placed into 4% paraformaldehyde for another 15 minutes, followed by overnight incubation in 30% sucrose. After immersion in optimal cutting temperature compound [Tissue-Tek OCT (Sakura Finetek, Torrance, CA); Electron Microscopy Sciences, Hatfield, PA], retinas were cut into 12-μm cryosections. Sections were incubated with rabbit anti–p-VEGFR2 (1:50; Abcam, Cambridge, MA) or anti–p-EPOR (1:50; Santa Cruz Biotechnology, Dallas, TX) overnight at 4°C. After rinsing, sections were incubated for 1 hour with FITC-conjugated goat anti-rabbit secondary antibody (1:200; Jackson ImmunoResearch Laboratories, West Grove, PA.) and Alexa Fluor 594–conjugated antibody for isolectin B4 (lectin) (1:500; Life Technologies, Carlsbad, CA), to stain vessels. Sections stained without primary antibodies were used as controls. Labeling for all sections was performed during the same experiment session. Images were captured using confocal microscopy (Olympus IX81; Olympus, Tokyo, Japan) at ×20 or ×40 magnification.

Cell Culture, Transfection, and Proliferation Assay

hRMVECs (Cell Systems, Kirkland, WA) were maintained in EGM-2 endothelial growth medium (Lonza, Walkersville, MD) supplemented with 5% fetal bovine serum. Cells of passage 3 to 5 were used for experiments. Confluent cells in six-well plates were starved for 16 hours and then treated

with 20 ng/mL VEGFA or PBS for 24 hours for qPCR or were treated with VEGFA for 30 minutes for p-VEGFR2, p-EPOR, and p-STAT3.

For siRNA transfection, hRMVECs at 70% confluency were transfected in six-well plates with siRNAs targeting human EPOR, VEGFR2, or STAT3 (Life Technologies) using Lipofectamine 2000 reagent (Life Technologies). A silencer selective negative control siRNA was used as control.

For cell proliferation assays, hRMVECs were plated into 96-well plates at a density of 5000 cells per well. At 24 hours after transfection with siRNA, cells were starved in serum-free medium for 24 hours and then were incubated with 20 ng/mL VEGFA or control PBS for another 24 hours. To inhibit VEGFR2 or STAT3 activation, cells were pretreated with 5 μmol/L of the VEGFR2 tryosine-kinase inhibitor semaxanib (SU5416), 10 μmol/L of the JAK2/STAT3 inhibitor AG490 (both from Sigma-Aldrich, St. Louis, MO), or dimethyl sulfoxide (DMSO) vehicle control for 1 hour before addition of VEGFA. Cell number was measured with a Vybrant MTT cell proliferation assay kit (Life Technologies, Carlsbad, CA).

RNA Isolation and qPCR Analysis

Total RNA of cells was extracted by TRI Reagent (Sigma-Aldrich). For dissected retinas, total RNA was extracted using an RNeasy mini kit (Qiagen, Valencia, CA). RNA was quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA). cDNA was generated with the use of a high-capacity cDNA archive kit (Life Technologies). qPCR was performed on a Mastercycler ep *realplex* system (Eppendorf, Hauppauge, NY) with the use of SYBR Green master mix (Roche Diagnostics, Indianapolis, IN) and primers synthesized by the core research facility of the University of Utah. Expression levels for VEGFA were normalized to the mean value of internal control β-actin. The primers (forward and reverse, respectively) were rat EPOR 5′-CATTCTCGTCCTCATCTCACTG-3′ and 5′-AACTCATTCTCTGGGCTTGG-3′, and human EPOR 5′-CCCCAAGTTCGAGAGCAAAG-3′ and 5′-GGTAGGAGAAGCTGTAGTTGC-3′.

Protein Extraction and Western Blot Analysis

Dissected retinas and hRMVECs were lysed in radioimmunoprecipitation assay buffer with 1:100 protease inhibitors (Roche Diagnostics, Indianapolis, IN) and 2 mmol/L sodium orthovanadate and were homogenized and centrifuged at 15,700 × *g* for 10 minutes at 4°C. Total protein in the supernatant fluid was quantified by a bicinchoninic acid assay (Pierce BCA; Thermo Fisher Scientific, Waltham, MA). Membranes were incubated overnight at 4°C with primary antibodies to p-STAT3 and total STAT3 (1:1000; Cell Signaling Technology, Danvers, MA), p-EPOR and total EPOR (1:500; Santa Cruz Biotechnology), and p-VEGFR2 and total VEGFR2 (1:500; Santa Cruz Biotechnology). Blots were visualized, and the relative densities of bands were calculated as described previously,[32] using gel analysis software (UN-SCAN-IT gel, version 6.1; Silk Scientific, Orem, UT). Relative activities were calculated as the ratio of phosphorylated to total protein or to β-actin and are expressed as fold difference compared with control. At least six samples from each group were measured and analyzed.

Statistical Analysis

Significant differences between treatment groups were determined by one-way analysis of variance using the Newman−Keuls multiple comparison post hoc test or a two-way analysis of variance for grouped comparison. A minimum value of $P < 0.05$ was considered statistically significant. For proliferation assays, experiments were performed three times with $n = 3$ per condition in each experiment. For qPCR and protein analyses, six individual samples were used (sometimes pooled from retinas of pups from the same group).

Results

EPOR Expression and Activation Are Increased in Retinal Vascular Endothelium in Association with IVNV in Rat ROP Model

We have previously reported that exogenous EPO restored developmental retinal vascularization by 40%.[32] In addition, pathological angiogenesis recurred in the rat ROP model after treatment with anti-VEGFA antibody, in association with increased retinal EPO.[15] These observations suggested a role for EPO in angiogenesis associated with the ROP model. To assess EPO signaling, we measured the expression of retinal EPO and EPOR at several time points, including phase I (p14) and phase II (p18) in the rat ROP model and in RA-raised control pups of the same developmental ages. Compared with RA, EPO protein was significantly decreased in the ROP model at p14 and p18 (Figure 1A); this is in contrast to retinal VEGFA, which is significantly increased at both p14 and p18 in the ROP model.[38] Also, compared with RA, EPOR protein was significantly increased in the ROP model at p14 and p18 (Figure 1B). To determine whether EPOR was activated in the ROP model, Western blots were performed using anti−p-EPOR antibody. p-EPOR was increased in the retina in the ROP model at p18, but not at p14, compared with RA (Figure 1, C and D).

To determine the localization of EPOR activation, cryosections from RA and ROP retinas from p18 pups were colabeled with p-EPOR and lectin to stain vascular cells and, in adjacent sections, with p-VEGFR2 and lectin. p-EPOR colabeling with lectin was not strong at p18 in RA sections, but colabeled vessels were apparent at p18 in the

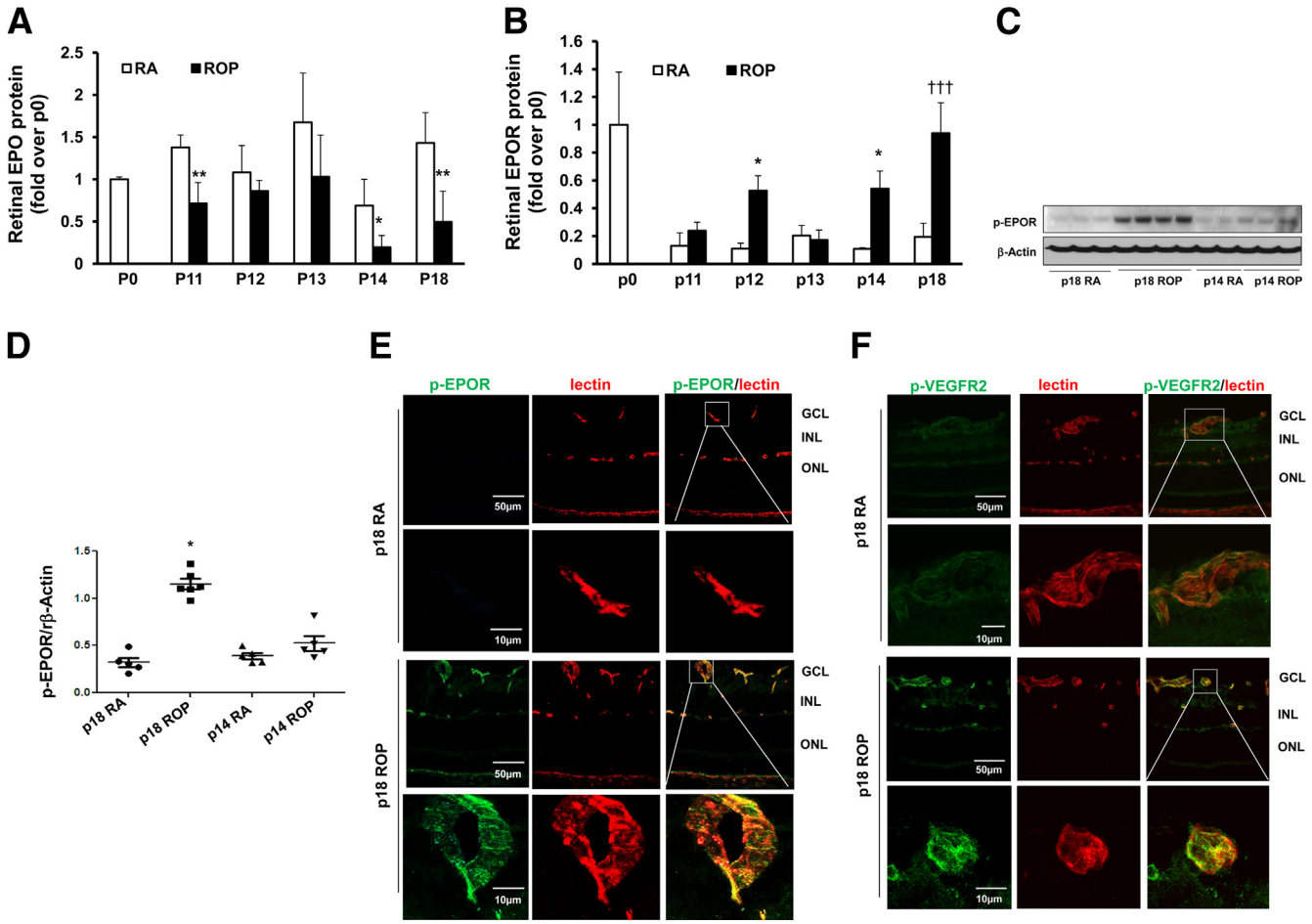

Figure 1 EPOR expression and activation increased in vascular endothelium in rat ROP model. **A** and **B:** Protein analyses for EPO (**A**) and EPOR (**B**) in retinas from rat pups raised in room air (RA) and ROP model pups at time points from baseline to p18. **C** and **D:** Representative Western blots of p-EPOR in retina at p14 and p18 (**C**) and quantification of gels (**D**). **E** and **F:** Immunohistochemical staining of p-EPOR (green) (**E**) or p-VEGFR2 (green) (**F**) colabeling with lectin (red) in retinal cryosections at p18. The **boxed** region in each **upper** row corresponds to the adjacent image at higher magnification in the **lower** row. Negative controls are shown in Supplemental Figure S1A. Data are expressed as means ± SD (**A** and **B**) or as individual data points with means ± SD (**D**). $^{*}P < 0.05$, $^{**}P < 0.01$ versus RA at the same developmental age. $^{\dagger\dagger\dagger}P < 0.001$, overall analysis of variance. Scale bars: 50 μm (**E** and **F**, upper rows); 10 μm (**E** and **F**, lower rows). GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.

ROP model (Figure 1E). By contrast, p-VEGFR2 colabeled with lectin-stained vessels in both RA and ROP sections, and appeared qualitatively greater in the ROP model (Figure 1F and Supplemental Figure S1A). Taken together, these results indicate that, like VEGFR2, EPOR is activated in ECs in the ROP model during phase II, when IVNV occurs.

EPOR in ECs Is Up-Regulated and Activated by VEGFA

The oxygen levels used in the ROP model result in retinal hypoxia, as indicated by increased conjugated pimonidazole compared with RA.[39] Hypoxia induces HIF stabilization, which allows transcription of angiogenic factors,[38] including VEGFA[40] or EPO.[41] In the ROP model, retinal VEGFA is increased at p14, compared with RA counterparts.[37] By contrast, EPO protein was decreased during phase I (p14) (Figure 1A). Because both EPOR and VEGFR2 were activated in phase II, these findings raised the question of whether activation of EPOR in retinal ECs in the ROP model is affected by VEGFA. We therefore stimulated hRMVECs with VEGFA and measured EPOR expression and activation. Compared with PBS control, VEGFA increased EPOR mRNA expression (Figure 2A) and protein levels (Figure 2B) after overnight VEGFA treatment. VEGFA activated VEGFR2, beginning at 5 minutes of treatment (Supplemental Figure S2, A and B); EPOR activation peaked at 30 minutes of treatment (Figure 2C and Supplemental Figure S2, A and C), and STAT3 activation began at 30 minutes and peaked at 60 minutes of treatment (Supplemental Figure S2D). We therefore chose 30 minutes of stimulation with VEGFA for subsequent analyses of VEGFR2, EPOR, and STAT3 activation in the same cell lysate. Overnight treatment with 10 IU/mL EPO in hRMVECs also led to an increase in EPOR protein (Figure 2B), but the induction of EPOR by EPO was less than with VEGFA treatment; furthermore, 30 minutes of EPO treatment did not increase p-EPOR and p-VEGFR2 levels (Supplemental Figure S3).

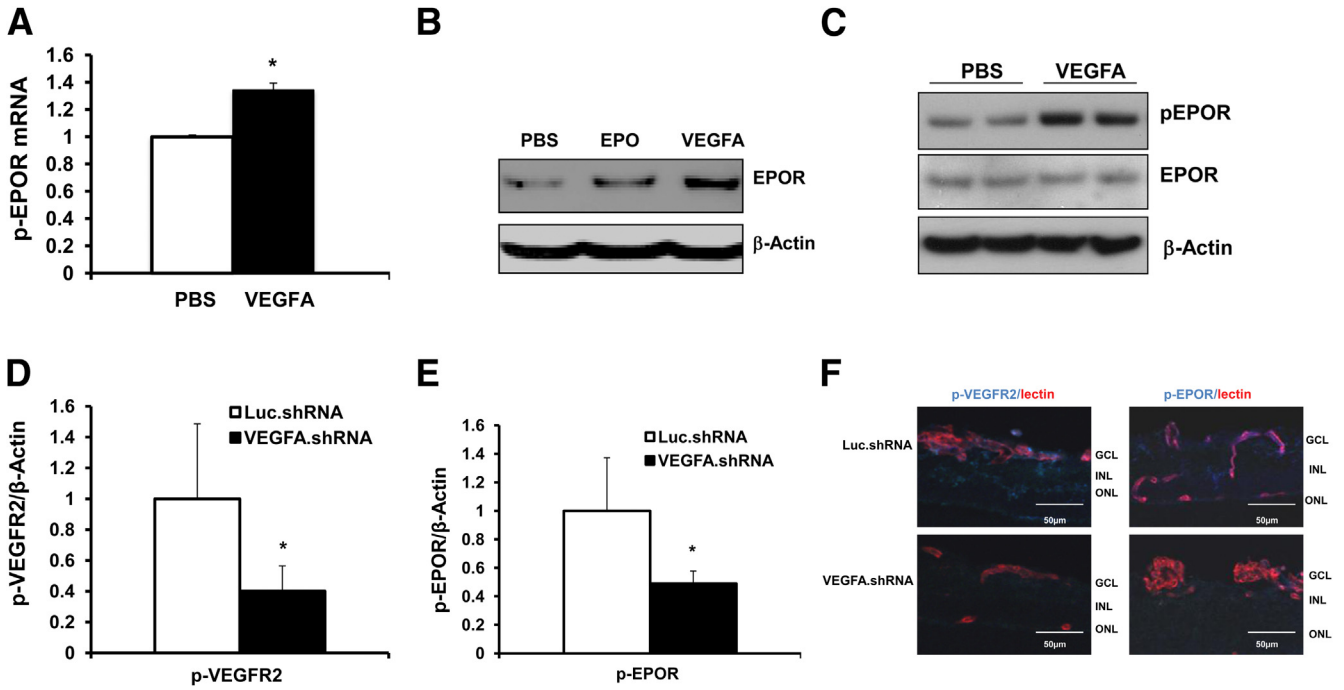

Figure 2 EPOR is up-regulated and activated by VEGFA *in vitro* and *in vivo*. **A** and **B:** qPCR of human EPOR (**A**) and Western blots of EPOR protein in hRMVECs stimulated by 20 ng/mL VEGFA or 10 IU/mL EPO for 18 hours (**B**). Human β-actin was used as an internal control. **C:** Western blots of p-EPOR and total EPOR in hRMVECs stimulated with 20 ng/mL VEGFA or PBS for 30 minutes. **D** and **E:** Quantification of Western blots of p-VEGFR2 (**D**) and p-EPOR (**E**) in retina. **F:** IHC of p-VEGFR2 or p-EPOR (blue) colabeled with lectin (red) in retinal sections from rat pups injected with lentivirus-delivered luciferase shRNA (Luc.shRNA) or VEGFA shRNA (VEGFA.shRNA) at p18 in rat ROP model. Negative controls are shown in Supplemental Figure S1B. Data expressed as means ± SD, representative of three or more independent experiments with $n = 2$-3 for each experiment. $^*P < 0.05$ versus control. Scale bar = 50 μm.

Our research group has developed a lentivector gene therapy strategy to knock down overexpressed VEGFA in Müller cells in the rat ROP model.[36] Retinal VEGFA was reduced to levels observed in the retina of RA-raised pups, and VEGFR2 activation was inhibited in vascular ECs, in association with reduced phase II IVNV.[36] To assess whether knockdown of Müller cell–derived VEGFA reduces activated EPOR in the rat ROP model, we analyzed retinal lysates and retinal sections from p18 rat pup eyes in the ROP model that were knocked down for Müller cell VEGFA with a subretinal lentivector-driven shRNA. Compared with the control luciferase shRNA treatment, knockdown of Müller cell VEGFA reduced p-VEGFR2 (Figure 2D) in retinal lysates and reduced colabeling of p-VEGFR2 with lectin-stained ECs in retinal sections (Figure 2F), consistent with our previous findings.[36] In the same retinas, p-EPOR in retinal lysates (Figure 2E) and colabeling with lectin in sections were also significantly reduced, compared with the respective luciferase shRNA controls (Figure 2F and Supplemental Figure S1B). These findings provide strong evidence that inhibiting VEGFA reduces p-VEGFR2 and p-EPOR in vascular endothelial cells in the ROP model.

Activation of EPOR by VEGFA Enhances VEGFA-Mediated EC Proliferation

$EpoR^{-/-}$ rescued mice were reported to have a significant reduction in ischemia-induced VEGFA and VEGFR2 expression.[23] Our results also suggest that crosstalk exists, but that VEGFA–VEGFR2 signaling can regulate EPOR expression and activation. To assess crosstalk between EPOR and VEGFR2, we transfected hRMVECs with EPOR siRNA or control siRNA and then stimulated them with VEGFA or PBS (control). EPOR siRNA significantly reduced total EPOR in a dose-dependent pattern (Figure 3A), achieving maximum reduction at 50 pmol EPOR siRNA; this dose was used for the later experiments. We then determined whether knockdown of EPOR affects VEGFA-induced EC proliferation. Compared with hRMVECs transfected with control siRNA, VEGFA-induced hRMVEC proliferation was partially reduced after EPOR siRNA transfection (Figure 3B). By contrast, inhibition of VEGFR2 by the VEGFR2 inhibitor SU5416 (Figure 3C) or by siRNA transfection (Figure 3D) abolished VEGFA-induced proliferation. These results indicate that VEGFA-activated EPOR augments EC proliferation caused by stimulation with VEGFA, but that knockdown of EPOR does not fully abolish VEGFA-induced EC proliferation.

Activation of EPOR Interacts with VEGFR2 to Regulate STAT3 Activation

One means whereby EPOR can affect VEGFA-induced angiogenesis is through an interaction between the receptors. To determine whether there was an interaction between activated EPOR and VEGFR2, coimmunoprecipitation

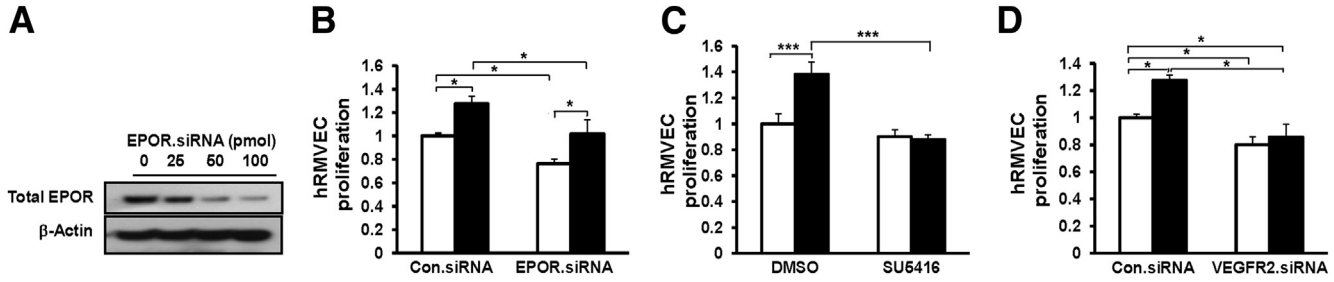

Figure 3 Knockdown of EPOR reduces VEGFA-induced EC proliferation, and inhibition of VEGFR2 activation inhibits it, in hRMVECs. **A:** Western blots of total EPOR in hRMVECs transfected with 0 to 100 pmol EPOR siRNA. **B**–**D:** VEGFA-induced proliferation assay in hRMVECs transfected with control siRNA (Con.siRNA) or EPOR siRNA (**B**), in hRMVECs treated with SU5416 (**C**), and in hRMVECs transfected with Con.siRNA or VEGFR2 siRNA (**D**). Data are expressed as means ± SD, representative of three or more independent experiments. White bars, PBS; black bars, VEGFA (**B**–**D**). $*P < 0.05$, $***P < 0.001$.

of EPOR and VEGFR2 was determined after stimulation with VEGFA or PBS control. After stimulation with VEGFA, both p-VEGFR2 and p-EPOR levels increased (Figure 4A), compared with control, and in the same cell lysate coimmunoprecipitation of VEGFR2 and p-EPOR likewise increased (Figure 4B). Activation of VEGFR2 led to reduced total VEGFR2 in lysates, which is believed to represent increased ubiquitinization of VEGFR2 on activation of the receptor.[42] To determine whether VEGFR2 activation is required for the interaction of p-EPOR and VEGFR2, hRMVECs were pretreated with the VEGFR2 inhibitor SU5416 for 1 hour before stimulation with VEGFA. SU5416 effectively blocked VEGFA-induced p-VEGFR2 and also p-EPOR (Figure 4A). In the same cell lysate, the VEGFA-induced coimmunoprecipitation of p-EPOR and VEGFR2 was abolished by pretreatment with the SU5416 (Figure 4B). hRMVECs transfected with EPOR siRNA failed to exhibit VEGFA-induced phosphorylation of EPOR or an interaction between p-EPOR and p-VEGFR2 (Figure 4C). These findings support the notion that an interaction between the receptors occurs in response to VEGFA and that activation of either receptor is needed for the interaction.

Based on the findings that VEGFA-mediated EC proliferation was augmented through EPOR, we explored signaling pathways affected by EPOR or VEGFR2 activation. Previously, we used an ROP model with supplemental oxygen (ROP + SO model),[43] originally developed by Berkowitz and Zhang,[44] in which rat pups are placed into 28% instead of 21% oxygen after repeated fluctuations in oxygen concentration as a way to study the role of supplemental oxygen in ROP. We had found that the 28% oxygen reduces total retinal VEGFA, compared with the standard 21% oxygen model.[39] By using the 28% oxygen model, we could unmask signaling effects dominated by VEGFA-induced signaling and found that activation of STAT3 was important in phase II IVNV in the ROP + SO model.[43] In the present study, therefore, we focused on EC-activated STAT3.

Compared with control siRNA, EPOR siRNA inhibited VEGFA-induced p-EPOR (Figure 5, A and B) and p-STAT3 (Figure 5, E and F), and reduced p-VEGFR2 (Figure 5, C and D). Similarly, VEGFA-induced p-VEGFR2 (Figure 4A), p-EPOR (Figure 4A), and p-STAT3 (Figure 6, A and B) were reduced to baseline in hRMVECs pretreated with the VEGFR2 inhibitor SU5416. We further found that knockdown of VEGFR2 by siRNA transfection inhibited VEGFA-induced p-EPOR (Figure 6, C and E), but knockdown of STAT3 by siRNA did not affect VEGFA-induced p-VEGFR2 and p-EPOR (Figure 6, C–E). These findings provide evidence that p-VEGFR2 is required for VEGFA-induced p-EPOR. Furthermore, activation of p-STAT3 occurred downstream of p-VEGFR2 and p-EPOR. Inhibition of STAT3 with AG490 reduced VEGFA-induced

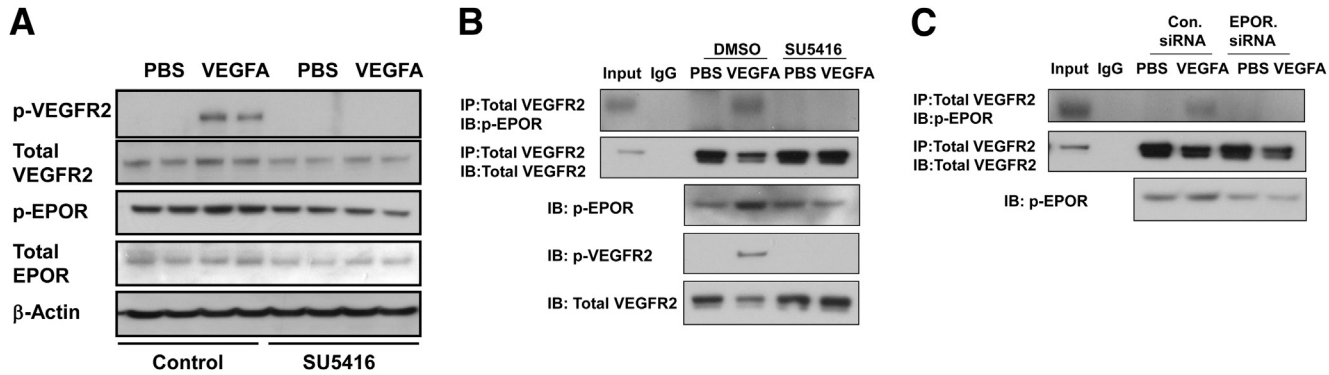

Figure 4 VEGFA-activated EPOR interacts with p-VEGFR2 in hRMVECs. **A:** Western blots of phosphorylated and total VEGFR2 and EPOR in hRMVECs treated with PBS or VEGFA in the presence of DMSO control or SU5416. **B** and **C:** Coimmunoprecipitation of p-EPOR and VEGFR2 in hRMVECs treated with PBS or VEGFA in the presence of DMSO control or SU5416 (**B**) or in hRMVECs transfected with Con.siRNA or EPOR.siRNA (**C**). Gels are representative of three or more independent experiments with $n = 2$ for each experiment. IB, immunoblot (Western blot); IP, immunoprecipitation.

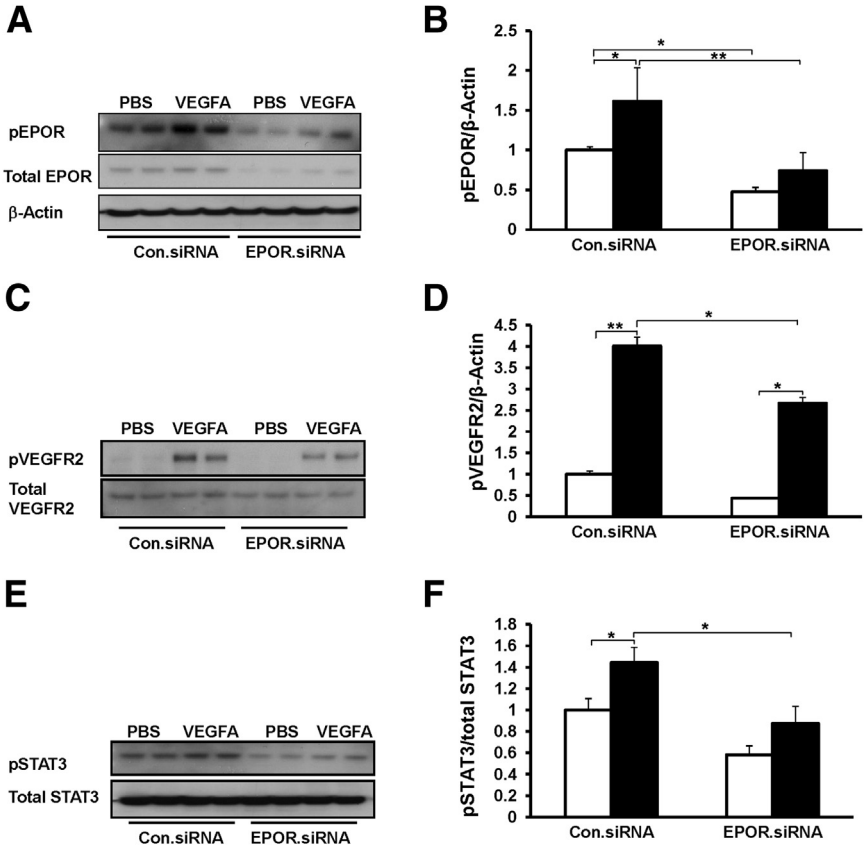

Figure 5 Knockdown of EPOR reduces VEGFA-induced p-VEGFR2 and inhibits VEGFA-induced p-EPOR and p-STAT3 in hRMVECs. Representative Western blots, with quantification of densitometry, for p-EPOR (**A** and **B**), p-VEGFR2 (**C** and **D**), and p-STAT3 (**E** and **F**) in hRMVECs transfected with Con.siRNA or EPOR.siRNA and treated with PBS or VEGFA. Data are expressed as means ± SD, representative of three or more independent experiments with $n = 2$ for each experiment. White bars, PBS; black bars, VEGFA (in panels **B**, **D**, and **F**). $^{*}P < 0.05$, $^{**}P < 0.01$.

hRMVEC proliferation to baseline (Figure 6F). Taken together, these findings provide evidence that VEGFA activates EPOR and induces an interaction between activated EPOR and VEGFR2, which augments angiogenesis through activation of STAT3.

Discussion

With the present study, we provide evidence that VEGFA activates VEGFR2, which then phosphorylates EPOR and forms an interaction with p-EPOR to exacerbate STAT3 activation and mediate the pathological angiogenesis seen in phase II ROP. We provide data using the relevant ROP model in rat showing that p-EPOR–labeled ECs were increased during phase II IVNV, but not in phase I. Furthermore, in the rat model with knockdown for Müller cell VEGFA, p-EPOR labeling in ECs was significantly reduced, compared with luciferase shRNA control. Knockdown of EPOR only partially reduced VEGFA-induced EC proliferation, whereas inhibition of VEGFR2 by either of two methods (siRNA transfection or tyrosine kinase inhibition) appeared to reduce VEGFA-induced EC proliferation to control levels. We further show that VEGFA induced an interaction between EPOR and VEGFR2, which triggered activation of STAT3 in ECs to induce EC proliferation. These findings suggest that EPOR may enhance VEGFR2 signaling and thus potentially lead to pathological angiogenesis in the setting of increased VEGFA, as in ROP, whereas in the physiological angiogenesis of normal development, VEGFA concentration is not as elevated and does not activate EPOR or cause its interaction with VEGFR2.

Recombinant EPO has been used to treat anemia of prematurity,[16] but several studies have indicated an association between severe ROP and EPO use.[28,45] EPO has since been shown to have other properties besides hematopoiesis,[46,47] including neuroprotection through interactions between EPOR and the β common receptor and angiogenesis in ischemic limb models through interactions between VEGFR2 and EPOR.[19,48] In terms of ROP, inhibition of phase II IVNV with a neutralizing antibody to VEGFA caused recurrent pathological angiogenesis in association with increased retinal EPO expression.[15] Studies in the mouse OIR model demonstrated that EPO is a target for phase II IVNV,[30,31] but can be protective if delivered as an intraperitoneal injection before hyperoxia-induced vaso-obliteration in phase I.[29] Furthermore, prolyl hydroxylase inhibitors can increase EPO and reduce phase I vaso-obliteration and phase II IVNV in the mouse OIR[49] and rat ROP[50] models. Increased size of the avascular retina has been associated with increased severity of ROP in several clinical studies,[51,52] suggesting that restoring physiological retinal vascularization and reducing avascular retina could reduce IVNV in severe ROP. Based on such studies, it was proposed that the timing of EPO administration is important and that EPO delivered early might protect against severe ROP. However, even though exogenous administration

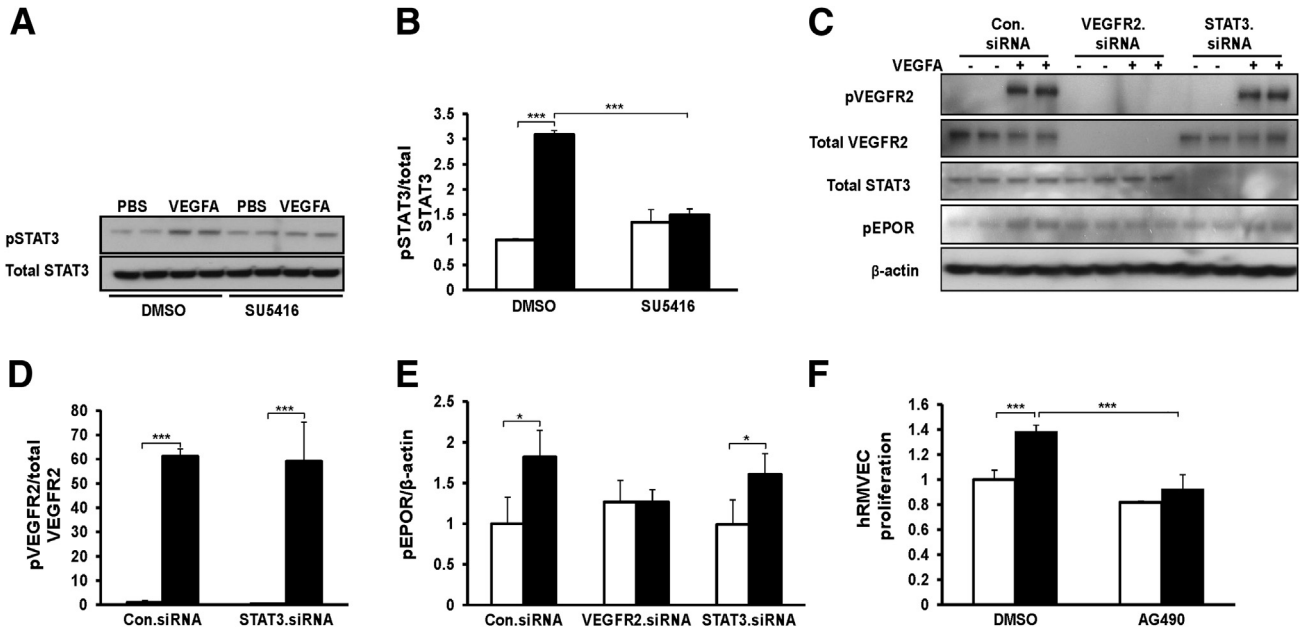

Figure 6 STAT3 is the downstream target of activated VEGFR2 and EPOR, and activation of STAT3 mediates VEGFA-induced EC proliferation. **A** and **B:** Representative Western blots (**A**) and quantification (**B**) of p-STAT3 and total STAT3 in hRMVECs treated with PBS or VEGFA in the presence of control DMSO or SU5416. Quantification of densitometry of p-STAT3 is normalized to total STAT3. **C**–**E:** Representative Western blots (**C**) with quantification (**D** and **E**) of p-VEGFR2, p-EPOR, total VEGFR2, and total STAT3 in hRMVECs transfected with Con.siRNA, VEGFR2.siRNA, or STAT3.siRNA and treated with PBS or with 20 ng/mL VEGFA for 30 minutes. Quantification of densitometry of p-VEGFR2 is normalized to total VEGFR2 (**D**) and that of p-EPOR is normalized to β-actin (**E**). **F:** Cell proliferation assay in hRMVECs treated with PBS or VEGFA in the presence of DMSO or AG490 Data are expressed as means ± SD, representative of three or more independent experiments with $n = 2$ for each experiment. White bars, PBS; black bars, VEGFA (in panels **B**, **D**, **E** and **F**). $^{*}P < 0.05$, $^{***}P < 0.001$.

of EPO restored physiological retinal vascularization in phase I of the ROP model,[32] phase II IVNV was not reduced (unpublished data). Also, in a multicenter clinical trial of darbepoietin (a derivative of erythropoietin) delivered systemically by weekly subcutaneous injections beginning immediately after birth to improve cognitive development in preterm infants ($n = 102$), darbepoietin neither increased nor reduced the occurrence of severe ROP.[33] The study population was small, but these findings show that timing of EPO administration is not the only consideration involved in ROP.

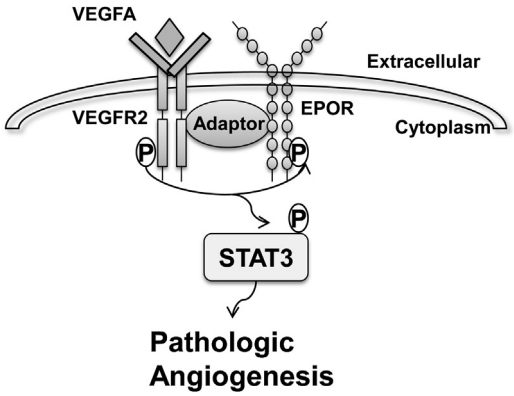

Figure 7 The hypothetical signaling pathway in pathological angiogenesis regulated by interactions of activated VEGFR2 and EPOR. VEGFA activates VEGFR2, which then phosphorylates EPOR and forms an interaction with p-EPOR to exacerbate STAT3 activation and mediate pathological angiogenesis as seen in phase II ROP.

Previous studies have indicated a role for EPOR in cancer cell growth[53] and in EPO–EPOR signaling in angiogenesis.[54] In EPOR knockout mice rescued by permitting EPO signaling of hematopoiesis ($Epor^{-/-}$ rescued mice), EPOR–EPOR signaling regulated angiogenesis by up-regulating VEGFA and its receptors in a hindlimb ischemia model, suggesting crosstalk between the signaling pathways.[23,55] In the present study, we found evidence that VEGFA can also up-regulate EPOR and activate EPOR through p-VEGFR2, leading to an interaction between the receptors and pathological angiogenesis. We previously reported that activation of STAT3 caused phase II IVNV in a model of ROP + SO.[43] Our present findings provide evidence that STAT3 is activated in ECs by VEGFA, leading to IVNV through an interaction between activated VEGFR2 and EPOR (which accounts for the phase II IVNV observed previously[43]). There are several potential mechanisms whereby STAT3 activation can increase IVNV. Activated STAT3 dimers can translocate to the nucleus and regulate transcription of genes involved in angiogenesis by binding to the gene promoters. In a previous study in the rat ROP model,[32] after repeated oxygen fluctuations, VEGFA activated STAT3 in Müller cells; after translocation to the nucleus, p-STAT3 dimers bound the EPO promoter and down-regulated expression of EPO, in association with delayed physiological retinal vascular development in phase I. Thus, VEGFA-activated STAT3 may also be involved in up-regulating EPOR in ECs by directly binding to the EPOR promoter.

Our findings also support the notion that VEGFA activates VEGFR2 (which in turn activates EPOR) in phase II ROP, potentially through another downstream signaling pathway or through an adaptor protein that facilitates an interaction between the receptors (Figure 7). Although current clinical trials show preliminary evidence that ROP is neither increased nor decreased when EPO derivatives are administered early,[33] the present study does not directly address whether exogenous EPO can safely be used in preterm infants. Our findings provide evidence of a potential role for EPOR and VEGFR2 interactions, induced by VEGFA in phase II IVNV in ROP and in other conditions associated with pathological angiogenesis.

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Supplemental Data

Supplemental material for this article can be found at *http://dx.doi.org/10.1016/j.ajpath.2013.12.023*.

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