SRPK1 Inhibition Modulates VEGF Splicing to Reduce Pathological Neovascularization in a Rat Model of Retinopathy of Prematurity

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Submitted: January 10, 2013
Accepted: May 19, 2013

Citation: Gammons MVR, Dick AD, Harper SJ, Bates DO. SRPK1 inhibition modulates VEGF splicing to reduce pathological neovascularization in a rat model of retinopathy of prematurity. *Invest Ophthalmol Vis Sci.* 2013;54:5797–5806. DOI:10.1167/iovs.13-11634

Purpose. We tested the hypothesis that recombinant human VEGF-A_{165}b and the serine arginine protein kinase (SRPK) inhibitor, SRPIN340, which controls splicing of the VEGF-A pre-mRNA, prevent neovascularization in a rodent model of retinopathy of prematurity (ROP).

Methods. In the 50/10 oxygen-induced retinopathy (50/10 OIR) model that exposes newborn rats to repeated cycles of 24 hours of 50% oxygen alternating with 24 hours of 10% oxygen, pups received intraocular injections of SRPIN340, vehicle, $VEGF_{165}$b, anti-VEGF antibody, or saline. Whole mounts of retinas were prepared for isolectin immunohistochemistry, and preretinal or intravitreal neovascularization (PRNV) determined by clock hour analysis.

Results. The anti-VEGF antibody ($P < 0.04$), rh$VEGF_{165}$b ($P < 0.001$), and SRPIN340 ($P < 0.05$) significantly reduced PRNV compared with control eyes. SRPIN340 reduced the expression of proangiogenic $VEGF_{165}$ without affecting $VEGF_{165}$b expression.

Conclusions. These results suggest that splicing regulation through selective downregulation of proangiogenic VEGF isoforms (via SRPK1 inhibition) or competitive inhibition of VEGF signaling by rh$VEGF_{165}$b has the potential to be an effective alternative to potential cyto– and neurotoxic anti-VEGF agents in the treatment of pathological neovascularization in the eye.

Keywords: angiogenesis, splicing, VEGF, retinopathy of prematurity

Retinopathy of prematurity (ROP) is a potentially blinding disease that alters the normal development of retinal blood vessels in premature infants leading to retinal neovascularization (NV).[1,2] During the initial phase, the hyperoxic postnatal environment[3] reduces growth factor production, and induces vasoattenuation leading to a impaired retinal vascular development.[4–6] Subsequently, vasoproliferation and preretinal NV (PRNV) results, which can progress to retinal NV. Retinal NV predisposes the infant to intravitreal hemorrhages, retinal detachment, and subsequent visual loss.[7]

The development and severity of ROP is a multifactorial process. Factors, including hypoxic/hyperoxic tissue,[8] hypercarbia, metabolic acidosis,[9] temporal development, and gene expression, combine with clinical care to impact ROP pathogenesis.[10] During the second phase of ROP, vascular endothelial growth factor-A (VEGF-A, hereafter referred to as VEGF) a key regulator of angiogenesis,[11] is upregulated,[12] stimulating NV in ROP.[13] Not surprisingly, VEGF has been identified as an attractive target for the development of novel therapeutics targeting pathological ocular angiogenesis, but anti-VEGF agents, such as ranibizumab or bevacizumab, are likely to prevent the endogenous survival effects of VEGF-A, and VEGF blockade has been shown to induce retinal neurodegeneration.[14,15]

The human *VEGF* gene is organized into eight exons and seven introns[16] spanning a coding region of approximately 14 kilobases.[17] VEGF is spliced alternatively to form two families of isoforms that differ in structure and function. $VEGF_{xxx}$ isoforms are noted for their proangiogenic activity[18–21] and $VEGF_{xxx}$b isoforms conversely are noted for their antiangiogenic activities.[22,23] A key difference between these families is an altered terminal six amino acid sequence of Ser-Leu-Thr-Arg-Lys-Asp (SLTRKD; Exon8b), compared with Cys-Arg-Lys-Pro-Arg-Arg (CDKPRR; Exon8a) in the $VEGF_{xxx}$ family.[24] It has been suggested that the differences in these terminal six amino acids and, thus, protein tertiary structure, are key to determining the antiangiogenic activity of $VEGF_{xxx}$b isoforms, preventing robust VEGFR-2 phosphorylation, recruitment of co-receptor neuropilin-1 (NP-1) and downstream signalling.[25,26] The auxiliary splicing factor, serine-rich splicing factor-1 (SRSF1), interacts with an exonic sequence enhancer (ESE) upstream of the VEGF exon 8a proximal splice site promoting $VEGF_{xxx}$ splicing.[27–29] SRSF1 is phosphorylated by SRPK1 in the cytoplasm enabling its nuclear translocation[30–32] and, once in the nucleus, SRSF1 can bind pre-mRNA and affect alternative splicing. SRPK1 has been identified as a target to prevent SRSF1 phosphorylation, proangiogenic VEGF upregulation, and angiogenesis in vivo.[27–29] The SRPK1 small molecule inhibitor, SRPIN340,[33] has been shown to inhibit splicing to $VEGF_{165}$, but not $VEGF_{165}$b in retinal pigmented epithelial cells, and promote a modest, but significant reduction in ocular neovascularization in a murine oxygen-induced retinopathy model (OIR).[28]

Knowledge gained from models of retinal diseases have yielded much of what we know about physiological and pathological blood vessel growth in the retina.[10] There currently are two widely accepted OIR models for the study of ROP, the murine 75% hyperoxia model developed by Smith et al.[34] and the alternating 50/10% oxygen rat model developed

by Penn et al.[35] Both models consistently reproduce retinal NV, but the 50/10 model in rat is regarded as a more clinically robust model of human ROP.[10,36,37] Like infants suffering from zone II ROP, pups exposed to the 50/10 OIR develop peripheral retinal avascularity, arterial tortuosity, and preretinal NV.[37–40] The neovascularization occurs as budding at the ends of the vessels in zone II, similar to that seen in humans, rather than diffuse widespread proliferative angiogenesis throughout the intermediate region of the retina. Furthermore, increased VEGF expression in ROP in the 50/10 OIR model,[38,41] which is linked mechanistically to the progression of PRNV, is also seen at the mRNA level during human ROP.[42] During this study, we investigated the splicing of VEGF during OIR, and tested the specific hypothesis that rhVEGF$_{165}$b and SRPIN340 would have antiangiogenic effects in the rat OIR model of human ROP. Such a strategy could have the potential clinical benefit of limiting PRNV without the potential adverse long-term effects of ranibizumab.

Materials and Methods

Isolation of Primary Cells and Cell Culture

Isolations were performed under cell culture hoods in class II facilities using aseptic technique, sterile instruments, and autoclaved solutions. Umbilical cords for human umbilical vein endothelial cell (HUVEC) isolation were obtained with patient consent from St. Michael's Hospital, Bristol, UK. Cords were stored in 1× PBS and 1% PenStrep (Invitrogen, Carlsbad, CA). HUVEC were isolated by collagenase treatment of human umbilical veins as described.[43] HUVEC were cultured in EBM-2 supplemented with EGM-2 BulletKit (Lonza, Walkersville, MD). Primary human RPE isolations were performed on human donor globes obtained within 24 hours postmortem from the Bristol Eye bank (Bristol Eye Hospital, Bristol, UK). All human tissues were obtained with ethical approval from the Bristol Research Ethics Committee in accordance with the declaration of Helsinki as revised in 2008. Retinas with choroid-RPE sheets were removed to a Petri dish, finely chopped, and digested in 5 mL Dulbecco's Modified Eagle Medium (DMEM):F12(1:1) + GlutaMax (Gibco, Carlsbad, CA) supplemented with 0.3mg/mL collagenase for 15 minutes at 37°C. Digested choroid-RPE sheets were added to 30 mL media (DMEM:F12 + GlutaMax) supplemented with 10% fetal bovine serum (FBS), 0.5% PenStrep (Invitrogen) and spun at 251*g* for 10 minutes to pellet cells. Supernatent was aspirated off, pellet resuspended in 4 mL media supplemented with 25% FBS (Gibco), and transferred to a T25 flask (Greiner Bio One, Frickenhausen, Germany). Cells were grown in cell culture flasks (Greiner Bio One) and split at 80% confluence.

Migration Assay

A series of chemoattractant solutions were made in triplicate with 1 nM VEGF$_{165}$ and varying concentrations of VEGF$_{165}$b (0, 0.1, 0.2, 0.3, 0.5, and 1nM). Anti-VEGF$_{165}$b antibody (clone 56/1, R&D Systems, Minneapolis, MN) was added at 0.12 μg/mL to 0.3 nM VEGF$_{165}$b. Full growth media and serum-free media, were used as positive and negative controls, respectively. Chemoattractant solutions were added to 24-well plates (500 μL/well) and HUVECs, previously serum starved for 15 to 16 hours, were seeded at 100,000 cells/insert in serum-free media. Cells were left to migrate for 6 hours, washed and fixed in 4% paraformaldehyde for 15 minutes. Inserts were stained for Hoescht and nuclei counted at using a 40× objective.

Semi-Quantitative Reverse Transcriptase (RT)-PCR for VEGF in Human Cells

Conventional PCR was used to detect VEGF$_{165}$ and VEGF$_{165}$b mRNA. A total of 5% to 10% of the cDNA was added to a reaction mixture containing 2× PCR Master Mix (Promega, Madison, WI), primers (1 μM each) complementary to exon 7b (5′-GGC AGC TTG AGT TAA ACG AAC-3′) and the 3′UTR of exon 8b (5′-ATG GAT CCG TAT CAG TCT TTC CTG G-3′), and DNase/RNase free water. All samples were run in parallel with negative controls (water and cDNA without reverse transcriptase [-RT]) and positive controls (VEGF$_{165}$ in a plasmid expression vector [pcDNA] and VEGF$_{165}$b pcDNA). The reaction mixture was thermo-cycled (PCR Express; Thermo Electron Corporation, Basingstoke, UK) 30 to 35 times, denaturing at 95°C for 60 seconds, annealing at 55°C for 60 seconds, and extending at 72°C for 60 seconds. PCR products were separated on 2.5% agarose gels containing 0.5 μg/mL ethidium bromide (BioRad, Hercules, CA) and visualized under an ultraviolet transilluminator (BioRad).

Equal cDNA loading was determined by PCR with GAPDH primers (forward: 5′-CAC CCA CTC CTC CAC CTT TGA C-3′; reverse: 5′-GTC CAC CAC CCT GTT GCT GTA G-3′). Primers result in one amplicon at approximately 112 base pairs (bp) after thermo cycling 30 times, denaturing at 94°C for 45 seconds, annealing at 65°C for 45 seconds and extending at 72°C for 60 seconds.

PanVEGF and VEGF$_{xxx}$b ELISA

One μg/mL pan-VEGF capture antibody (Duoset VEGF ELISA DY293; R&D Systems) was incubated overnight at room temperature. The plates were blocked (Superblock; Thermo Scientific, Waltham, MA), and serial dilutions of recombinant human (rh)VEGF$_{165}$ or rhVEGF$_{165}$b standards (ranging from 4 ng/mL to 16.25 pg/mL) were added, incubated alongside sample lysates, typically diluted 1:10. The plate was incubated for one hour at 37°C with shaking, washed, and incubated with 100 μL/well of either biotinylated goat anti-human VEGF (0.1 μg/ml; R&D Systems) or mouse anti-human VEGF$_{165}$b (0.25 μg/mL) for one further hour at 37°C. After washing, 100 μL/well of horseradish peroxidase (HRP)-conjugated streptavidin (1:200; R&D Systems) was added and plates were left at room temperature for 20 minutes.

The plates were washed, and color change induced with substrate A and B (DY999; R&D Systems) for 20 minutes under light protection. The reaction was stopped by addition of 100 μL/well of 1M H_2SO_4 and the absorbance was read immediately in an ELISA plate reader (Opsys MR system plate reader; Dynex Technologies, Chantilly, VA) at 450 nm with a control reading at 570 nm. Revelation Quicklink 4.25 software also was used to calculate a standard curve from mean absorbance values of standards enabling the estimation of VEGF concentration for each sample.

Western Blotting

Subconfluent cells were lysed in RIPA lysis buffer supplemented with protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). A total of 30 μg of total protein was resuspended in sample buffer, heated at 95°C for 5 minutes, and subjected to SDS-PAGE under reducing conditions. Subsequently, proteins were electrotransferred for 2 hours at 4°C to polyvinylidene fluoride (PVDF) membranes. The membranes then were exposed to primary antibodies (VEGF A20, 1:1000 2.5% milk [sc-152]; anti-VEGF$_{xxx}$b, [clone 56/1] 1:1000 5% BSA), washed in Tris-buffered saline/0.3% Tween 20, (TBS-T) and incubated with a secondary peroxidase-conjugated antibody at a 1:10,000

dilution. Signals were detected by enhanced chemoluminescence (ECL) substrate.

50/10 OIR Model

Female Sprague Dawley (SD) rats and their newborn litters were placed in the oxygen chamber within 4 hours of birth, or raised in normoxia. Combined litters of between 14 and 17 pups were exposed to alternating 24-hour cycles of hyperoxia (50% O_2) and hypoxia (10% O_2). Pups received intraocular injection of 25 ng SRPIN340 or vehicle (saline + 0.05% dimethyl sulfoxide [DMSO]) at p12; or 25 ng $VEGF_{165}b$, 1μg anti-VEGF (G6-31), or saline at p14. In a previous study of OIR, mouse IgG (control for G6-31) was compared to saline injection and showed no significant difference (data not shown). At p14 pups were removed from the chamber and returned to normoxia until p20. Contralateral eyes were injected with saline controls. On day 14, 17, or 20 pups were culled, unfixed retinas were taken for protein extraction, and fixed retinas were dissected, stained, and flat mounted. The 50/10 OIR model develops vascular tortuosity at P12 and PRNV at P18. Moreover, the peripheral retina remains avascular at P20. Survival rates were in excess of 90% for hyperoxia/hypoxia-exposed neonatal rats and there was no obvious maternal oxygen toxicity. Rats reared in room air were used as untreated normal controls. All animals were treated according to the institutional guidelines regarding animal experimentation, and the ARVO Animal Statement for the Use of Animals in Ophthalmic and Vision Research.

Intraocular Injection

SD rat pups were anesthetized with an intraperitoneal injection of a mixture of 50 mg/kg ketamine and 0.5 mg/kg medetomidine at time points ranging from P5 to P8 for normal vasculature studies, and P12 to P14 during OIR. Intraocular injections were administered using a 35-gauge needle (P5–P8, 1 μL injection volume) or 33-gauge needle (P12–P14, 2.5 μL injection volume), each injection was sustained for a duration of one minute to minimize loss of solution. Injections resulting in intravitreal hemorrhaging or clouding of the lens were not included within the study.

Quantification of Pathology and Statistical Analysis

Flat mounted isolectin stained eyes were imaged and images merged to obtain whole retinal pictures. Clock hour analysis was performed on coded samples under the microscope and PRNV scored by two masked observers. ImageJ was used to measure the total retinal area, vascular area, and PRNV area of masked images. Tortuosity was calculated using Image J where the length of the vessel and the length of the line of best fit of the vessel path were calculated, and the two values expressed as a ratio. Statistical analysis was performed using GraphPad Prism software. Means are expressed ± SEM. Clock hours were analyzed using a two-sample Mann-Whitney rank sum test (two-sided) as the data were integral. Other analyses used Student's paired *t*-test and one-way ANOVA with Bonferroni post hoc unless otherwise stated.

Results

The 50/10 OIR Insult-Induced PRNV and Increased Proangiogenic VEGF Expression

Compared to normoxia raised age-matched controls, 50/10 OIR retinae stained with isolectin showed the presence of PRNV simulating a characteristic of ROP pathology (Figs. 1A, 1B). PRNV developed from ends of postcapillary venules before the vascular/avascular boundary, and are characterized as intensely stained swellings often merged together to produce a brush border of pathological angiogenesis. Retinae also showed increased tortuosity ($P < 0.05$, Fig. 1C) of retinal arteries, and a significant reduction in the area of vascularised retina ($P < 0.001$, Fig. 1D).

Protein extracted from retinae of normoxia raised (N) and 50/10 OIR raised (OIR) pups was assessed for VEGF expression. Total VEGF (A20; Santa Cruz Biotechnology, Inc., Dallas, TX) showed a clear upregulation in OIR samples compared to normoxic samples (Fig. 1E, left). However, assessment of the $VEGF_{xxx}b$ isoforms (56/1; R&D Systems) showed a reduction in the expression of certain isoforms. In addition to previously identified bands at 46 kDa (MW of VEGF dimer = 46 kDa), bands were observed at 23 kDa, the expected size of glycosylated $VEGF_{165}b$ monomers, and protein from retinas of rats exposed to OIR showed decreased expression of this product. In addition, other bands were observed in normoxic retinal protein, at approximately 33 kDa ($VEGF_{121}b$), and 16 kDa ($VEGF_{121}b$ monomer). These bands were absent from OIR retinal protein (Fig. 1E, right hand blot). Densitometry analysis revealed a significant upregulation of proangiogenic $VEGF_{165}$, but not $VEGF_{165}b$ (46 kDa band, $P < 0.05$, Student's *t*-test).

Recombinant Human $VEGF_{165}b$ Blocks $VEGF_{165}$ Mediated Cell Migration and Reduces PRNV Comparable to Anti-VEGF Treatment, Following OIR Insult

Endothelial cell migration is a critical component of angiogenesis and it has been reported that antiangiogenic $rhVEGF_{165}b$ can be used to prevent VEGF-mediated cell migration. Here, we have used proangiogenic and pro-migratory $rhVEGF_{165}$ (1 nM) as a chemoattractant to promote HUVEC migration. Recombinant human $VEGF_{165}b$ dose-dependently inhibited $VEGF_{165}$–mediated HUVEC migration, achieving significance ($P < 0.01$, one-way ANOVA with Dunnett's post hoc) at 0.2, 0.3, 0.5, and 1nM $VEGF_{165}b$ concentrations (Fig. 2A). When $VEGF_{165}$ and $VEGF_{165}b$ were combined to reduce migration by 56% compared to $VEGF_{165}$ alone, preincubation with anti-$VEGF_{xxx}b$ specific antibody (56/1; R&D Systems) abolished the inhibition on cell migration observed with $rhVEGF_{165}b$ treatment, returning $VEGF_{165}$-mediated migration to 83.1 ± 7.61% of $VEGF_{165}$ treatment alone ($P < 0.001$, unpaired *t*-test, Fig. 2B).

When tested in a mouse model of OIR, $rhVEGF_{165}b$ has been shown to be antiangiogenic to hypoxia-driven angiogenesis in the eye.[44] Here, we have tested $rhVEGF_{165}b$ in a rat model of OIR, a model that better reflects human ROP.[36] Intraocular injection of 25 ng $rhVEGF_{165}b$, (Fig. 3A), reduced PRNV in the eye following 50/10 OIR insult and was equivalent to anti-VEGF (G6-31; Hoffmann-La Roche, Nutley, NJ) treatment. Retinas from eyes treated with $rhVEGF_{165}b$ showed a significant reduction in the number of clock hours showing PRNV, 2.8 ± 0.31, compared to control eyes in the same pup, 4.1 ± 0.21 ($P < 0.001$, Mann-Whitney rank sum test, $n = 20$, power = 99.8%, Fig. 3A). Treatment with G6-31 also demonstrated a significant reduction in clock hours possessing PRNV, 4 ± 0.63, compared to 5.33 ± 0.33 in controls, respectively ($P < 0.05$, Mann-Whitney rank sums test). Further analysis revealed the total area of PRNV relative to total retinal area, was decreased in retinas from $rhVEGF_{165}b$ (49.4 ± 14.6%, $P < 0.01$) and G6-31 (57.5 ± 6.6%, $P < 0.05$) treated eyes relative to their respective control eyes. A positive correlation ($r_s = 0.71$, Spearman's rank correlation coefficient) between

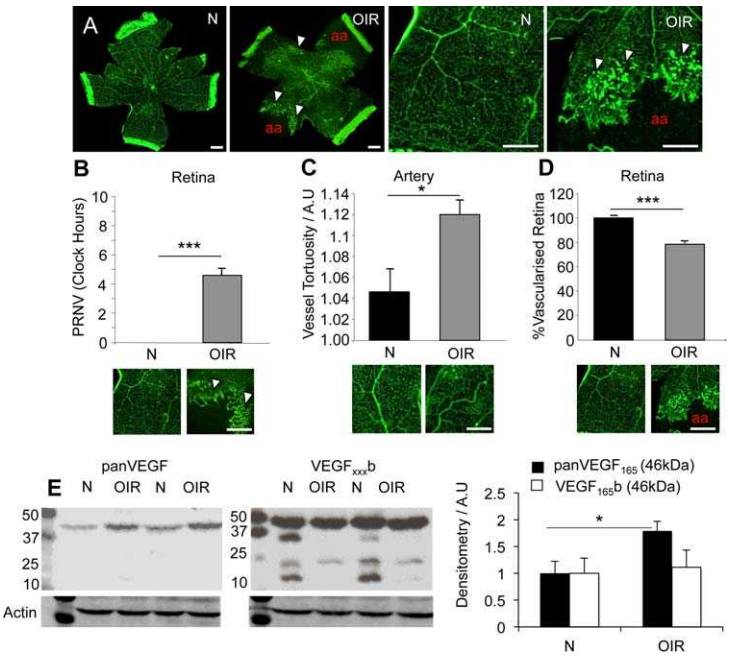

Figure 1. The 50/10 OIR insult induces pathologies reminiscent of human ROP. Litters of 12 to 14 Sprague Dawley pups were raised with their dams in either normoxia (N) or under conditions of fluctuating oxygen levels (OIR) between 50% and 10% every 24 hours, for the first 14 days of life (*white arrowheads* indicate PRNV, *aa* indicates avascular area. (**A**) Examples of whole flat mounted retinas from p20 rats were fixed and stained for isolectin-B4. Exposure to the 50/10 OIR conditions induced (**B**) PRNV ($P < 0.001$, unpaired *t*-test), (**C**) increased arterial tortuosity ($P < 0.05$, unpaired *t*-test), and (**D**) significantly decreased retinal vascular area compared to normoxic raised pups ($P < 0.001$, unpaired *t*-test). In addition, protein was extracted from P20 rats raised either in N or in OIR, and subjected to immunoblotting using panVEGF, $VEGF_{xxx}b$, or β-actin antibodies. (**E**) OIR increased total VEGF expression compared to normoxic controls (*left*); however, it reduced the number of $VEGF_{xxx}b$ splice isoforms detected (*right*). *Scale bar*: 1 mm.

PRNV area and number of clock hours showing PRNV was observed (data not shown). In this model we found $rhVEGF_{165}b$ had a consistently inhibitory effect on the development of pathological PRNV, similar to nonisoform specific pan VEGF inhibition (Fig. 3B). We also compared the effects of $rhVEGF_{165}b$ and G6-31 treatment on vessel tortuosity and avascular area. Although neither of the treatments affected the area of the avascular retina ($P > 0.05$, Fig. 3C), $rhVEGF_{165}b$ significantly reduced arterial tortuosity ($P < 0.05$, unpaired *t*-test, Fig. 3D).

$VEGF_{165}b$ expression was assessed in protein extracted from unfixed retinas of P20 OIR pups and saline injected eyes were compared to $VEGF_{165}b$ injected eyes. Recombinant human $VEGF_{165}b$ was detected in retinal protein from $VEGF_{165}b$-injected eyes as a monomer at 23 kDa even six days after its injection, suggesting its stability in the eye (Fig. 3E).

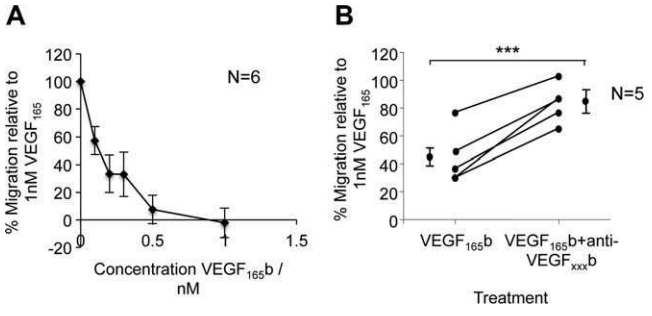

Figure 2. Recombinant human $VEGF_{165}b$ blocks $VEGF_{165}$ mediated cell migration. (**A**) HUVECs were left to migrate towards chemo-attractant solutions of 1nM $VEGF_{165}$ + varying concentrations of $VEGF_{165}b$. Increasing concentrations of $VEGF_{165}b$ inhibited the pro-migratory effect of $VEGF_{165}$, as seen by a reduction of % migration. (**B**) Preincubation with 0.12 ng/μL of a $VEGF_{165}b$ blocking antibody 4 hours previously successfully rescued the inhibitory action of $VEGF_{165}b$ over the pro-migratory $VEGF_{165}$, as seen by an increase of percentage migration relative to $VEGF_{165}$ alone (***$P < 0.001$, unpaired *t*-test).

SRPK1 Inhibition Modulates Proangiogenic VEGF Expression and Reduces PRNV

The SRPK selective inhibitor, SRPIN340 dose-dependently and selectively reduced $VEGF_{165}$ expression, achieving maximal inhibition at 10 μM (Fig. 4A). Furthermore, treatment with 10 μM SRPIN340 altered VEGF protein expression in ARPE-19 and primary RPE cells. SRPIN340 significantly increased the expression of $VEGF_{xxx}b$ protein isoforms relative to total VEGF in ARPE-19 cells (Fig. 4Bi), which express low endogenous $VEGF_{xxx}b$ levels (Figs. 4Bi, 4Bii), and significantly reduced proangiogenic VEGF expression in primary RPE cells (Fig. 4Bii), which had significantly higher endogenous $VEGF_{xxx}b$ expression (Figs. 4Bi, 4ii).

SRPIN340 subsequently was tested in the 50/10 OIR model. SRPIN340 was injected two days earlier than $rhVEGF_{165}b$ to allow the splicing factor to take effect before the VEGF surge experienced on day 14.[45,46] A total of 25 ng SRPIN340 was injected intraocularly in a volume of 2.5 μl; saline controls were supplemented with 0.05% DMSO and injected at the same volume. Retinas from eyes treated with SRPIN340 showed a significant reduction of clock hours possessing PRNV compared to control eyes in the same pup ($P < 0.05$,

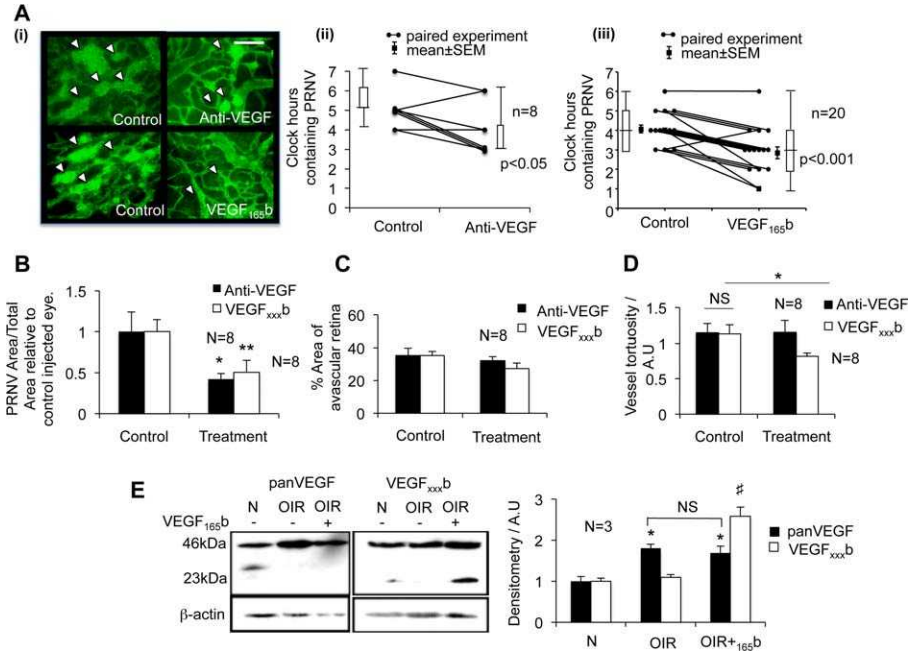

Figure 3. Recombinant human $VEGF_{165}b$ significantly reduces PRNV and vessel tortuosity in the rat 50/10 OIR model. Litters of 12 to 14 Sprague Dawley pups were raised with their dams under conditions of OIR every 24 hours for the first 14 days of life. At P14 pups received intraocular injections of either anti-VEGF antibody, G6-31 (1 μg), or $rhVEGF_{165}b$ (25 ng) in the ipsilateral eye, and control injections in the contralateral eye. (**Ai**) Examples of control and treated microvasculature shown (*white arrowheads* indicate PRNV). (**Aii**) G6-31 ($P < 0.05$, $n = 8$) and (**Aiii**) $rhVEGF_{165}b$ ($P < 0.001$, $n = 20$) significantly reduced the number of clock hours showing PRNV (Mann-Whitney *U* test), and (**B**) PRNV area relative to contralateral controls (paired *t*-test). (**C**) Neither G6-31 nor $rhVEGF_{165}b$ were capable of significantly reducing retinal avascular area ($P > 0.05$, paired *t*-test). However, (**D**) $rhVEGF_{165}b$ significantly reduced arterial vessel tortuosity. (**E**) Immunoblot for pan-VEGF and $VEGF_{165}b$ showing expression of VEGF dimers and monomers in uninjected, and expression in $rhVEGF_{165}b$-injected eyes six days after injection. Densitometry shows a significant upregulation of pan-VEGF, but not $VEGF_{165}b$, during OIR (*), and significantly increased $VEGF_{165}b$ after $rhVEGF_{165}b$ injection six days earlier (#). *, #$P < 0.05$. NS, not significant.

paired *t*-test, Fig. 5A). This was confirmed by PRNV area analysis showing a 33.3 ± 15.7% reduction in PRNV area compared to control eyes (Fig. 5B). In addition we assessed the effect of SRPIN340 administration on avascular area (Fig. 5C) and arterial tortuosity (Fig. 5D). SRPIN340 had no effect on either vascularity or tortuosity ($P < 0.05$, Student's *t*-test). Furthermore retinal protein was assessed for VEGF expression. VEGF expression significantly increased between P14 and P17, SRPIN340 injected eyes expressed significantly less VEGF compared to control, although $VEGF_{xxx}b$ isoform expression was unchanged (Fig. 5E). A single dose of SRPIN340, was capable of significantly reducing proangiogenic VEGF-mediated induction of PRNV, albeit modestly.

Total VEGF Blockade Retards Normal Vasculature Development

To determine whether anti-VEGF, $rhVEGF_{165}b$, or SRPIN340 affect the development of the normal retinal vasculature, developing eyes were injected at P5 (1 μL injection volume, concentration as before) and control eyes injected with mouse IgG, saline, or saline supplemented with 0.05% DMSO, respectively. Pups were culled at P8 and P12, retinae excised, stained, flat mounted, and avascular area assessed. Anti-VEGF treatment retarded growth of the vasculature leading to a significant increase in avascular area at P12 ($P < 0.05$, paired *t*-test). Conversely, 25ng $rhVEGF_{165}b$ and SRPIN340 failed to affect the developing retina at either time point. These results were reproduced when treatments were injected at P8 and pups culled at P12 (Supplementary Fig. S1).

Discussion

We have demonstrated selective upregulation of proangiogenic, but not antiangiogenic VEGF, while using Penn's 50/10 OIR model to stimulate neovascular growth by cyclically fluctuating oxygen levels.[35] The 50/10 OIR rat model is widely accepted as a clinically relevant model of ROP,[36] resulting in previously described PRNV, increased vessel width and tortuosity, as well an avascular retinal periphery.[35,37,38] We confirmed increased VEGF expression at P20, namely the $VEGF_{164}$ isoform,[41] coinciding with maximal PRNV.[47] To our knowledge, $VEGF_{xxx}b$ expression has not been investigated previously in this model, although it has been suggested that $VEGF_{165}b$ decreases during mouse OIR.[48] It is likely that the secondary anti-mouse IgG antibody used to detect the $VEGF_{xxx}b$ antibody (56/1; R&D Systems) also will bind to endogenous mouse IgG, which also runs at approximately 46 kDa. For this reason the use of rat tissue can be more illuminating for detection of $VEGF_{165}b$ by mouse antibodies that do not cross-react with rat IgG. Studies in the human fetal eye indicate that $VEGF_{165}b$ was not notable until vascular development neared completion, while $VEGF_{165}$ was very prominent as the vessels developed,[49] suggesting a developmental regulation of VEGF isoform expression and an ability of $VEGF_{xxx}b$ to "switch off" or prevent $VEGF_{xxx}$ activity. In physiological angiogenesis, for example, during follicular development[50] and wound healing,[51] proangiogenic VEGF isoforms are upregulated selectively. Alterations in the splicing of VEGF creating an "angiogenic switch" also have been observed in pathological situations where angiogenesis promotes disease progression. Proangiogenic isoform expression is upregulated selectively in numerous human cancers[23,24,52]

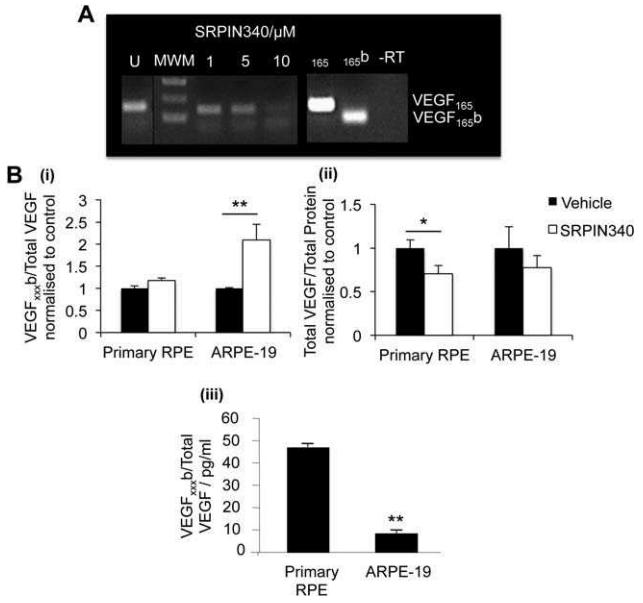

Figure 4. SRPK1 inhibition modulates proangiogenic VEGF expression in vitro. (**A**) Primary RPE cells were treated with varying concentration of SRPIN340 (1, 5, 10 μM) for 24 hours. RT-PCR was performed with primers spanning VEGF exon 7b and 8b. SRPIN340 switched the expression of VEGF isoforms to favor $VEGF_{165}b$ dose-dependently from 1 to 10 μM achieving significance at 5 μM ($P < 0.05$) and 10 μM ($P < 0.01$, one-way ANOVA, Dunnett's post hoc). (**B**) VEGF protein levels in primary RPE and ARPE-19 were assessed by ELISA. (**Bi**) SRPIN340 significantly increased the expression of $VEGF_{xxx}b$/Total VEGF in ARPE-19 cells ($P < 0.01$) and (**Bii**) significantly reduced proangiogenic VEGF in primary RPE cells ($P < 0.05$, Student's *t*-test). (**Biii**) $VEGF_{xxx}b$ expression was greater in primary RPE cells compared to ARPE-19 ($P < 0.01$).

and pathological eye disease, such as age-related macular degeneration.[53] Conversely $VEGF_{165}b$ is upregulated in systemic sclerosis, a disease characterized by a lack of angiogenesis.[54]

We confirmed that the $rhVEGF_{165}b$ used in our study can inhibit $VEGF_{165}$-induced cell migration, essential to angiogenesis.[55] Previous reports have shown $VEGF_{165}b$ overexpression,[56] and $rhVEGF_{165}b$ treatment inhibited $VEGF_{165}$-induced HUVEC and HMREC migration,[57,58] but our study also showed $rhVEGF_{165}b$ could be inhibited by preincubation with a $VEGF_{xxx}b$-specific antibody (Fig. 2B). These controls are important to show the activity of the $rhVEGF_{165}b$, as C'-terminal exopeptidase activity, or incomplete synthesis of the protein could lead to a truncated form of the protein, such as $VEGF_{159}$, which has been shown to have no antiangiogenic activity, and some angiogenic activity.[25] This also is the first evidence to our knowledge to show that $VEGF_{165}b$ antibodies can be neutralizing, in the same way that bevacizumab is neutralizing to all VEGF isoforms.[59] The ability of the anti-$VEGF_{165}b$ antibody to block the antimigratory properties of $VEGF_{165}b$ suggests that the C'-terminal region of $VEGF_{165}b$ may be involved in receptor binding, or at least binding to this region interferes with receptor interaction of the $VEGF_{xxx}$ isoforms.

To evaluate the importance of VEGF in this model a neutralizing antibody to all VEGF isoforms, G6-31 (the mouse monoclonal antibody that was a precursor of bevacizumab), was tested and compared to $rhVEGF_{165}b$ in rat. Human $VEGF_{165}b$ protein activity has been demonstrated when overexpressed in the mammary gland of transgenic mice,[60] and in the rabbit cornea and rat mesentery.[23] Previous reports have suggested that anti-VEGF therapy demonstrates a sustained and significant decrease in PRNV in the 50/10 OIR model.[41] A single intraocular injection of 1 μg G6-31 or 25 ng $rhVEGF_{165}b$ on day 14 significantly reduced the prevalence of PRNV on day 20 by clock hour analysis and by PRNV area analysis, described previously[61,62] (Figs. 3A, 3B). Of particular interest was that SRPIN340, a highly selective SRPK inhibitor,[33] significantly reduced PRNV compared to 0.05% DMSO controls when given as a single IVT injection on day 12 ($P < 0.05$). This demonstrates that selective downregulation of proangiogenic VEGF isoforms (Fig. 4), but not antiangiogenic isoforms, is capable of reducing PRNV (Fig. 5). Being upstream of VEGF, selective inhibition of SRPK1 and, therefore, the activity of SRSF1, may result in altered alternative splicing of other genes and, thus, introduce potential nonspecific effects, although initial toxicology tests showed gram quantities of SRPIN340 administered to animals resulted in no ill effect.[33] Other downstream targets of SRPIN340 currently are being investigated and it is possible that other angiogenic genes, similarly altered during OIR, also may be regulated by SRPK1. Modulation of SRPK1 activity, therefore, may have the potential benefit of resulting in a coordinated regulation of alternative splicing during disease progression.

Other studies have investigated whether anti-VEGF treatment affects other characteristics associated with ROP, microvessel density, and vessel tortuosity, and the latter has been linked to a poor prognosis in patients suffering from ROP.[63] In our study we observed that neither G6-31 nor $rhVEGF_{165}b$ was capable of increasing normal retinal vascularization (Fig. 3C), but we did note a significant reduction in vessel tortuosity for $rhVEGF_{165}b$ ($P < 0.05$, Student's *t*-test, Fig. 3D). SRPIN340 failed to affect either avascular area or vessel tortuosity (Figs. 5C, 5D). Neutralizing antibodies previously have been shown to decrease vessel tortuosity in this model,[38] but we observed no effect with G6-31 treatment. It would be interesting to determine the effect of a $VEGF_{165}$ specific neutralizing antibody in this model, but such an antibody has not yet become available.

PRNV clearly has been identified as the most damaging pathology of VEGF mediated ROP progression; the abnormal growth of vessels increases in line with the increase in severity of ROP,[64] and can lead to the formation of fibrous tissue and retinal detachment.[65] Current treatments for ROP include cryotherapy and peripheral diode laser photocoagulation. Studies have shown laser therapy to be the superior of the two[66,67]; however, laser therapy poses severe risks, including intraocular hemorrhage and cataract formation.[68,69] With the identification of VEGF as a critical factor in the progression of ROP,[70–72] off-label use of anti-VEGF inhibitors has been reported. Lee et al. reported regression of disease and a more rapid development of the peripheral retinal vascular bed after IVT bevacizumab injection combined with laser photocoagulation.[73] The BEAT-ROP trial tested anti-VEGF (intravitreal bevacizumab) therapy in premature babies suffering from ROP. This prospective randomized and controlled multicenter trial for zone I and zone II severe human ROP showed a significant benefit for zone I disease, suggesting an impressive benefit with anti-VEGF therapy.[74] Other studies have suggested treating the BEAT-ROP trial results with caution stating concerns over the safety (there was a nonsignificant increase in deaths in the bevacizumab group, and the trial was not large enough to demonstrate safety), data interpretation (time to endpoint was dependent on time to recurrence, which was greater for bevacizumab, and, therefore, recurrence may have been outside the endpoint, even though it occurred), post hoc determination of outcomes, and alteration of the primary endpoint,[75] as well as highlighting the failing of the trial to examine longer term ocular and systemic side effects.[76]

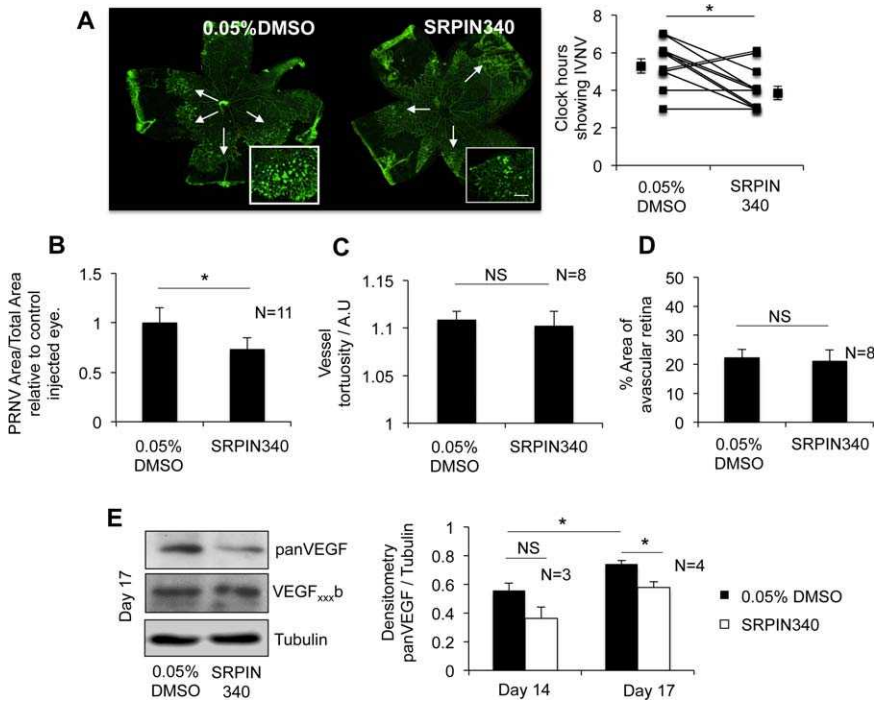

Figure 5. SRPIN340 significantly reduces PRNV and reduces proangiogenic VEGF in vivo. On day 12 of the OIR protocol, the rats were removed briefly from the chamber and given a 2.5 μL intraocular injection of 25 ng SRPIN340 and vehicle (saline + 0.05% DMSO) in the contralateral eye. (**A**) SRPIN340 treatment significantly reduced PRNV clock hours compared to control eyes ($P < 0.05$, Student's paired *t*-test), example flat mounts shown (*white arrows* indicate PRNV, *scale bar*: 500 μm) and (**B**) PRNV area relative to total retinal area ($P < 0.05$, Student's paired *t*-test). (**C**) Retinas treated with SRPIN340 showed no significant change in vessel tortuosity, or (**D**) the percentage of vascularized retina, compared to control eyes also subjected to the OIR paradigm. (**E**) Protein extracted from the retinae of these pups showed a reduction in VEGF in SRPIN340 eyes compared to control injected; however, no change was observed in $VEGF_{xxx}b$ expression.

Previous debates concerning VEGF blockade,[77] which can be damaging to cells and tissues,[78] have shown that perturbation of normal VEGF can prevent normal retinal function.[79] Administration of anti-VEGF therapies in age-related macular degeneration was shown during the Seven-up study to result in retinal atrophy in almost all (98%) of patients followed over the 7 to 8-year period.[80] Moreover, while VEGF-A has been shown for some time to be neuroprotective for retinal cells,[15] it recently has been shown that pan VEGF-A blockade exacerbated retinal ganglion cell death in animal models of glaucoma.[14] We tested G6-31, $rhVEGF_{165}b$, and SRPIN340 during development, and observed retarded growth of the retinal vasculature following anti-VEGF treatment, but not by a single dose of 25 ng $rhVEGF_{165}b$ or SRPIN340 (Fig. 6, Supplementary Fig. S1). Although $VEGF_{xxx}b$ isoforms are considered largely as being antiangiogenic,[22,23] we have shown previously that $rhVEGF_{165}b$ also is cytoprotective for endothelial cells and epithelial cells, including retinal pigmented epithelial cells.[58] Furthermore, we recently have demonstrated that $VEGF_{165}b$ is neuroprotective for sensory neurons, including retinal ganglion cells in vivo.[81]

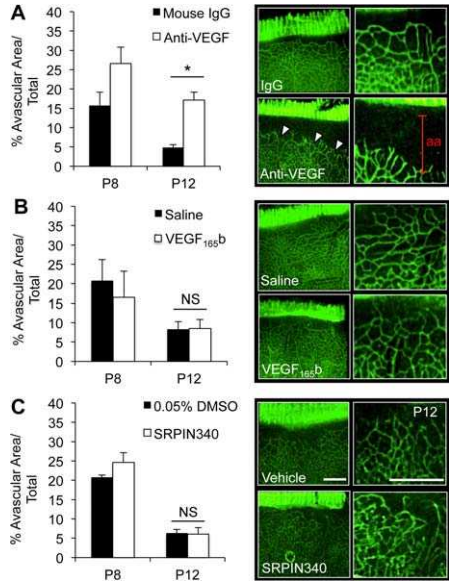

Figure 6. SRPIN340 and $rhVEGF_{165}b$ do not influence normal retinal development. A total of 18 P5 SD rat pups was injected intraocularly (1 μL) with either (**A**) 1 μg G6-31, (**B**) 10 ng $rhVEGF_{165}b$, or (**C**) 10 ng SRPIN340 in one eye, and 1 μg Mouse IgG, saline, or 0.05% DMSO, respectively, in the contralateral control injected eye. Pups were culled at P8 ($n = 3$ each group) and at P12 ($n = 3$). Flat mounted retinae were stained for isolectin IB4 imaged and avascular area quantified using image J. Both $rhVEGF_{165}b$ and SRPIN340 did not affect normal retinal vascularization, whereas anti-VEGF G6-31 (Hoffmann-La Roche) significantly reduced vascularization compared to control eyes at P12 ($P < 0.05$, Student's paired *t*-test).

Conclusions

Here, we have shown that cytoprotective $rhVEGF_{165}b$ administration is capable of reducing pathological PRNV in rats without the need for total VEGF blockade. Unlike anti-VEGF therapy, this treatment reduced arterial tortuosity and maintained the development of the normal retinal vasculature. Like anti-VEGF, $rhVEGF_{165}b$ failed to promote peripheral vascularization during OIR, although some reports suggest VEGF may not be the key mediator of this process. The SRPK1 inhibitor,

SRPIN340, mechanistically demonstrated that selectively reducing proangiogenic VEGF isoforms is sufficient to reduce PRNV significantly in this model. Therefore, more potent SRPK inhibitors or targeting other factors involved in the splicing of VEGF may be worth exploring as novel strategies for identifying potential ROP therapeutics. Recombinant human $VEGF_{165}b$ could be an alternative, potentially less damaging therapy to laser photocoagulation, or even anti-VEGF IVT injections in premature babies suffering from ROP. It will be important to determine whether $rhVEGF_{165}b$ treatment leads to long-term complications, toxicities, and systemic side effects, which have been associated with anti-VEGF therapy.

Acknowledgments

The authors thank John S. Penn (Vanderbilt University) and Xiaolin Gu (Alcon) for key advice and information on setting up the 50/10 OIR model and PRNV analysis.

Supported by Fight for Sight, Skin Cancer Research Foundation, the British Heart Foundation, Richard Bright VEGF Research Trust, The British Microcirculation Society and NERC, and by the MRC G10002073 (SJH).

Disclosure: **M.V.R. Gammons**, P; **A.D. Dick**, None; **S.J. Harper**, P; **D.O. Bates**, Roche (F), P

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