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SRPK1 inhibition modulates VEGF splicing to reduce pathological neovascularisation in a rat model of Retinopathy of Prematurity

Melissa V.R. Gammons[1A], **Andrew. D. Dick**[1B], **Steven. J. Harper**[1A], and **David. O. Bates**[1A]

[1A]Microvascular Research Laboratories, School of Physiology and Pharmacology, University of Bristol, Bristol U.K.

[1B]School of Clinical Sciences and School of Cellular and Molecular Medicine, University of Bristol, Bristol U.K.

Abstract

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 neovascularisation 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 h of 50% oxygen alternating with 24 h of 10% oxygen, pups received intraocular injections of SRPIN340, vehicle, $VEGF_{165}$b, anti-VEGF antibody or saline. Wholemounts of retinas were prepared for isolectin immunohistochemistry, and pre-retinal or intravitreal neovascularisation (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 pro-angiogenic $VEGF_{165}$ without affecting $VEGF_{165}$b expression.

CONCLUSIONS—These results suggest that splicing regulation through selective downregulation of pro-angiogenic VEGF isoforms (via SRPK1 inhibition) or competitive inhibition of VEGF signalling by rh$VEGF_{165}$b has the potential to be an effective alternative to potential cyto– and neuro- toxic anti-VEGF agents in the treatment of pathological neovascularisation in the eye.

Introduction

Retinopathy of Prematurity (ROP) is a potentially blinding disease that alters the normal development of retinal blood vessels in premature infants leading to retinal neovascularisation (NV)[1, 2]. During the initial phase the hyperoxic post-natal environment[3] reduces growth factor production, induces vasoattenuation leading to a impaired retinal vascular development[4, 5]. Subsequently vasoproliferation and pre-retinal 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

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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 alternatively spliced to form two families of isoforms that differ in structure and function. $VEGF_{xxx}$ isoforms are noted for their pro-angiogenic activity[18-21] and $VEGF_{xxx}$b isoforms are conversely noted for their anti-angiogenic 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 anti-angiogenic 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 auxillary 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, pro-angiogenic 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 neovascularisation 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 are currently two widely accepted OIR models for the study of ROP, the murine 75% hyperoxia model developed by Lois Smith[34] and the alternating 50/10% oxygen rat model developed by John Penn[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 pre-retinal NV[37-40]. The neovascularisation 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 mechanistically linked to the progression of PRNV, is also increased at mRNA level during human ROP[42]. During this study we investigated the splicing of VEGF during OIR and tested the specific hypothesis that both rh$VEGF_{165}$b and SRPIN340 would have anti-angiogenic 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 HUVEC isolation were obtained with patient consent from St. Michaels Hospital, Bristol, UK. Cords were stored in 1x phosphate buffered saline (PBS) and 1% PenStrep (Invitrogen). 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). Primary human RPE isolations were performed on human donor globes obtained within 24 hours post-mortem from the Bristol Eye bank (Bristol Eye Hospital). 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 5ml Dulbecco's Modified Eagle Medium (DMEM):F12(1:1)+GlutaMax (Gibco) supplemented with 0.3mg/ml collagenase for 15 minutes at 37°C. Digested choroid-RPE sheets were added to 30ml media (DMEM:F12+GlutaMax) supplemented with 10% fetal bovine serum (FBS), 0.5% PenStrep (Invitrogen) and spun at 1500 rpm (251g) for 10 minutes to pellet cells. Supernatent was aspirated off, pellet resuspended in 4ml media supplemented with 25% FBS (Gibco) and transferred to a T25 flask (Greiner). Cells were grown in cell culture flasks (Greiner) and split at 80% confluence.

Migration Assay

A series of chemoattractant solutions were made in triplicate with 1nM $VEGF_{165}$ 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) was added at 0.12ug/ml to 0.3nM $VEGF_{165}b$. Full growth media and serum free media, were used as positive and negative controls, respectively. Chemoattractant solutions were added to 24-well plates (500µl/well) and HUVECs, previously serum starved for 15-16 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 40x 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. Five-ten percent of the cDNA was added to a reaction mixture containing: 2x PCR Master Mix (Promega), primers (1µM 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 Coorporation, Basingstoke) 30-35 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 0.5µg/ml ethidium bromide (BioRad) 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 ~112bp 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 $VEGF_{xxx}b$ enzyme-linked immunosorbent assay (ELISA)

One μg/ml pan-VEGF capture antibody (Duoset VEGF ELISA DY-293; R&D systems) was incubated overnight at room temperature. The plates were blocked (Superblock; Thermo Scientific) and serial dilutions of recombinant human (rh)$VEGF_{165}$ or rh$VEGF_{165}$b standards (ranging from 4ng/ml to 16.25pg/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 100μl/well of either biotinylated goat anti-human VEGF (0.1μg/ml; R & D systems) or mouse anti-human $VEGF_{165}$b (0.25μg/ml) for one further hour at 37°C. After washing, 100μl/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 colour change induced with substrate A and B (DY-999; R&D Systems) for 20mins under light protection. The reaction was stopped by addition of 100μl/well of 1M H_2SO_4 and the absorbance was read immediately in an ELISA plate reader (Dynex Technologies Opsys MR system plate reader) at 450nm with a control reading at 570nm. Revelation Quicklink 4.25 software was also 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). Thirty micrograms of total protein was resuspended in sample buffer, heated at 95°C for 5 min, and subjected to sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. Subsequently, proteins were electrotransferred for 2 hours at 4°C to polyvinylidene fluoride (PVDF) membranes. The membranes were then 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 Oxygen Induced Retinopathy (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 14-17 pups were exposed to alternating 24-hour cycles of hyperoxia (50% O_2) and hypoxia (10% O_2). Pups received intraocular injection of (i) 25ng SRPIN340 or (ii) vehicle (saline +0.05%DMSO) at p12, or (iii) 25ng $VEGF_{165}$b, (iv) 1μg anti-VEGF (G6-31) or (v) saline at p14. In a previous study of OIR mouse IgG (control for G6-31) was compared to saline

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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 flatmounted. 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 regulations for the use of animals in research.

Intraocular Injection

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

Quantification of pathology and statistical analysis

Flatmounted 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 was calculated and the two values expressed as a ratio. Statistical analysis was performed using GraphPad Prism software. Means are expressed ± standard error of the mean. Clock hours were analysed using a two-sample Mann-Whitney rank sum test (two-sided) as the data was integral. Other analyses used students paired t-test and one-way ANOVA with bonferroni post hoc unless otherwise stated.

Results

The 50/10 OIR insult induced PRNV and increased pro-angiogenic VEGF expression

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

in OIR samples compared to normoxic samples (Figure 1E; left panel). However, assessment of the $VEGF_{xxx}b$ isoforms (56/1; R&D) showed a reduction in the expression of certain isoforms. In addition to previously identified bands at 46kDa (MW of VEGF dimer = 46kDa), bands were observed at 23kDa, 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 33kDa ($VEGF_{121}b$), and 16kDa ($VEGF_{121}b$ monomer) - these bands were absent from OIR retinal protein (Figure 1E; right hand blot). Densitometry analysis revealed a significant upregulation of pro-angiogenic $VEGF_{165}$ but not $VEGF_{165}b$ (46kDa band; $p<0.05$, students 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 anti-angiogenic $rhVEGF_{165}b$ can be used to prevent VEGF-mediated cell migration. Here we have used pro-angiogenic and pro-migratory $rhVEGF_{165}$ (1nM) 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 Dunnetts post hoc) at 0.2, 0.3, 0.5 and 1nM $VEGF_{165}b$ concentrations (Figure 2A). When $VEGF_{165}$ and $VEGF_{165}b$ were in combined to reduce migration by 56% compared to $VEGF_{165}$ alone, pre-incubation 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; Figure 2B).

When tested in a mouse model of OIR, $rhVEGF_{165}b$ has been shown to be anti-angiogenic 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 25ng $rhVEGF_{165}b$, (Figure 3A), reduced PRNV in the eye following 50/10 OIR insult and was equivalent to anti-VEGF (G6-31; Roche) 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 with control eyes in the same pup, 4.1±0.21 ($p<0.001$, Mann Whitney rank sum test; n=20, power = 99.8%. Figure 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 ($r_s=0.71$, Spearmans Rank correlation coefficient) between 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 non-isoform specific pan VEGF inhibition (Figure 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 ($p>0.05$; Figure 3C), $rhVEGF_{165}b$ significantly reduced arterial tortuosity ($p<0.05$, unpaired t-test; Figure 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 23kDa even six days after its injection, suggesting its stability in the eye (Figure 3E).

SRPK1 inhibition modulates pro-angiogenic VEGF expression and reduces PRNV

The SRPK selective inhibitor, SRPIN340 dose-dependently and selectively reduced $VEGF_{165}$ expression, achieving maximal inhibition at 10μM (Figure 4A). Furthermore, treatment with 10μM 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 (figure 4Bi), which express low endogenous $VEGF_{xxx}b$ levels (figure 4Biii), and significantly reduced pro-angiogenic VEGF expression in primary RPE cells (figure 4Bii), which had significantly higher endogenous $VEGF_{xxx}b$ expression (Figure 4Biii).

SRPIN340 was subsequently 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 prior to the VEGF surge experienced on day 14[45, 46]. A total of 25ng SRPIN340 was injected intraocularly in a volume of 2.5μl; 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 with control eyes in the same pup ($p<0.05$, paired t-test; Figure 5A). This was confirmed by PRNV area analysis showing a 33.3±15.7% reduction in PRNV area compared with control eyes (Figure 5B). In addition we assessed the effect of SRPIN340 administration on avascular area (Figure 5C) and arterial tortuosity (Figure 5D). SRPIN340 had no effect on either vascularity or tortuosity ($p<0.05$; students 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 with control although $VEGF_{xxx}b$ isoform expression was unchanged (Figure 5E). A single dose of SRPIN340, was capable of significantly reducing pro-angiogenic 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 (1μl 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, flatmounted 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, both 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 (Suppl. Figure 1).

Discussion

We have demonstrated selective upregulation of pro-angiogenic but not anti-angiogenic VEGF whilst 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 pre-retinal neovascularisation (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]. $VEGF_{xxx}b$ expression has not been previously investigated 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) will also bind to endogenous mouse IgG which also runs at ~46kDa. 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 both 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], pro-angiogenic VEGF isoforms are selectively upregulated. Alterations in the splicing of VEGF creating an 'angiogenic switch' have also been observed in pathological situations where angiogenesis promotes disease progression. Pro-angiogenic isoform expression is selectively upregulated in numerous human cancers[23, 24, 52] 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 this 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 this study also showed $rhVEGF_{165}b$ could be inhibited by pre-incubation with a $VEGF_{xxx}b$ specific antibody (Figure 2B). These are important controls 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 anti-angiogenic activity, and some angiogenic activity[25]. This is also the first evidence 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 anti-migratory 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 over-expressed 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 intra-ocular injection of 1μg G6-31 or 25ng rhVEGF$_{165}$b on day 14 significantly reduced the prevalence of PRNV on day 20 by clock hour analysis and by PRNV area analysis, previously described[61, 62](Figure 3A-B). 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 pro-angiogenic VEGF isoforms (Figure 4), but not anti-angiogenic isoforms is capable of reducing PRNV (Figure 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 non-specific effects, although initial toxicology tests showed gram quantities of SRPIN340 administered to animals resulted in no ill effect[33]. Other downstream targets of SRPIN340 are currently being investigated and it is possible that other angiogenic genes, similarly altered during OIR, may also be regulated by SRPK1. Modulation of SRPK1 activity may therefore 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, micro-vessel density and vessel tortuosity, and the latter has been linked to a poor prognosis in patients suffering from ROP[63]. In this study we observed that neither G6-31 nor rhVEGF$_{165}$b was capable of increasing normal retinal vascularisation (Figure 3C), but we did note a significant reduction in vessel tortuosity for rhVEGF$_{165}$b ($p<0.05$; students t-test; Figure 3D). SRPIN340 failed to affect either avascular area or vessel tortuosity (Figure 5C-D). Neutralising antibodies have previously 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 neutralising antibody in this model, but such an antibody has not yet become available.

PRNV has clearly 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 intra-ocular 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 and colleagues 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 non significant 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].

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 7-8 year period (Bhisitkul RB et al. IOVS 2012;53:ARVO E-Abstract 3679). Moreover, while VEGF-A has been shown for some time to be neuroprotective for retinal cells[15], it has recently 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 25ng rhVEGF$_{165}$b or SRPIN340 (Figure 6; Suppl. Figure 1). Although VEGF$_{xxx}$b isoforms are largely considered as being anti-angiogenic[22, 23], we have previously shown that rhVEGF$_{165}$b is also cytoprotective for both endothelial cells and epithelial cells including retinal pigmented epithelial cells[58]. Furthermore, we have recently demonstrated that VEGF$_{165}$b is neuroprotective for sensory neurons including retinal ganglion cells *in vivo*[81].

Conclusion

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 vascularisation 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 pro-angiogenic VEGF isoforms is sufficient to significantly reduce PRNV in this model. More potent SRPK inhibitors or targeting other factors involved in the splicing of VEGF may therefore 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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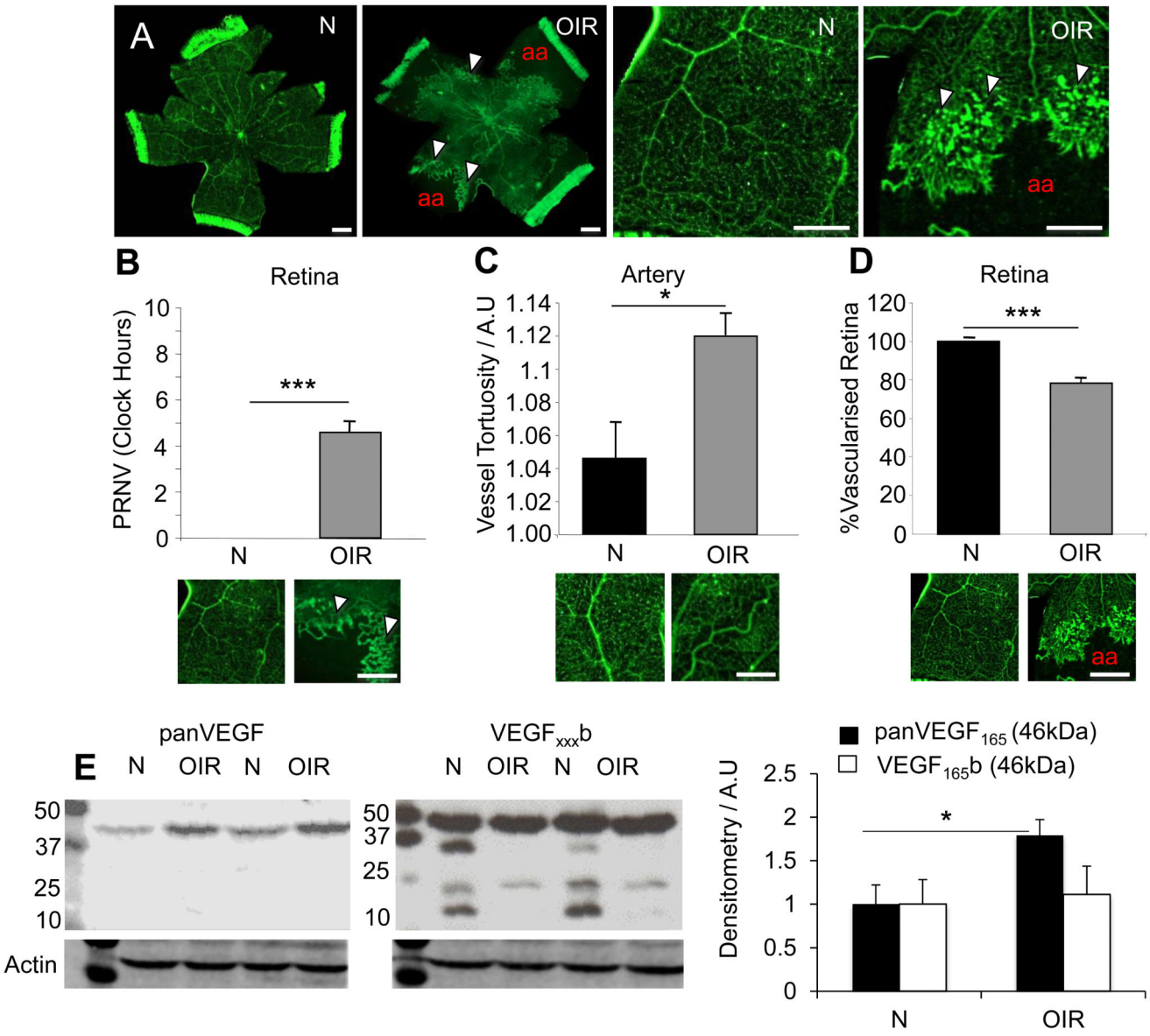

Figure 1. The 50/10 OIR insult induces pathologies reminiscent of human ROP

Litters of 12-14 Sprague Dawley pups were raised with their dams in either normoxia or under conditions of fluctuating oxygen levels between 50% and 10% every 24 hours, for the first 14 days of life (N = Normoxia raised. OIR = 50/10 OIR raised; White arrowheads indicate PRNV; aa – avascular area). A) Examples of whole flatmounted 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 with normoxic raised pups ($p<0.001$, unpaired t-test). In addition protein was extracted from P20 rats raised either in normoxia (N) or in altered oxygen levels between 50-10% (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 = 1mm.

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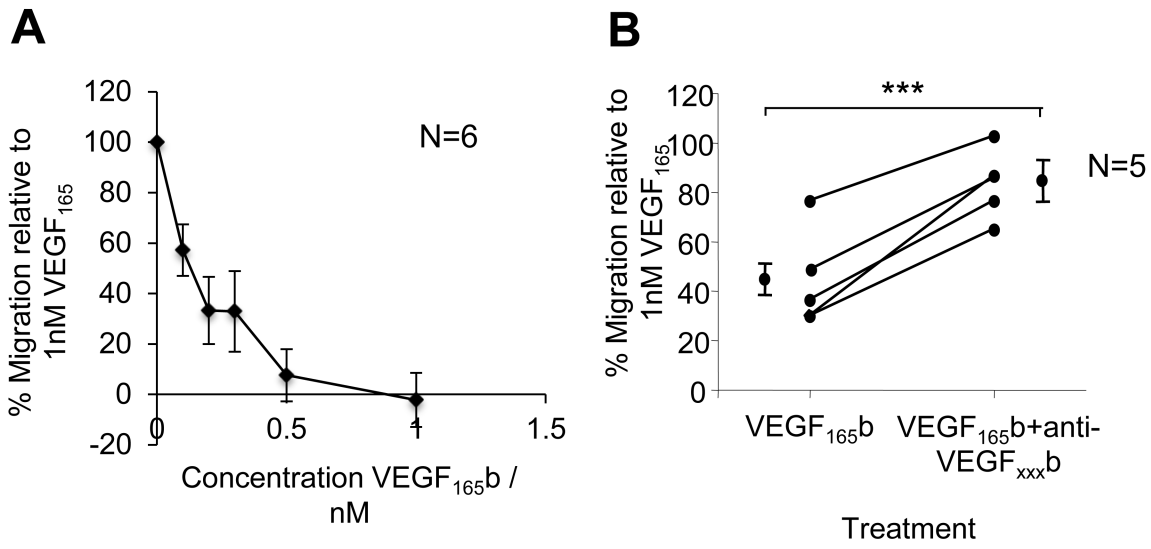

Figure 2. Recombinant human $VEGF_{165}b$ blocks $VEGF_{165}$ mediated cell migration

A) HUVECs were left to migrate towards chemoattractant 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) Pre-incubation with 0.12ng/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 % migration relative to $VEGF_{165}$ alone (***, $p<0.001$, unpaired t-test).

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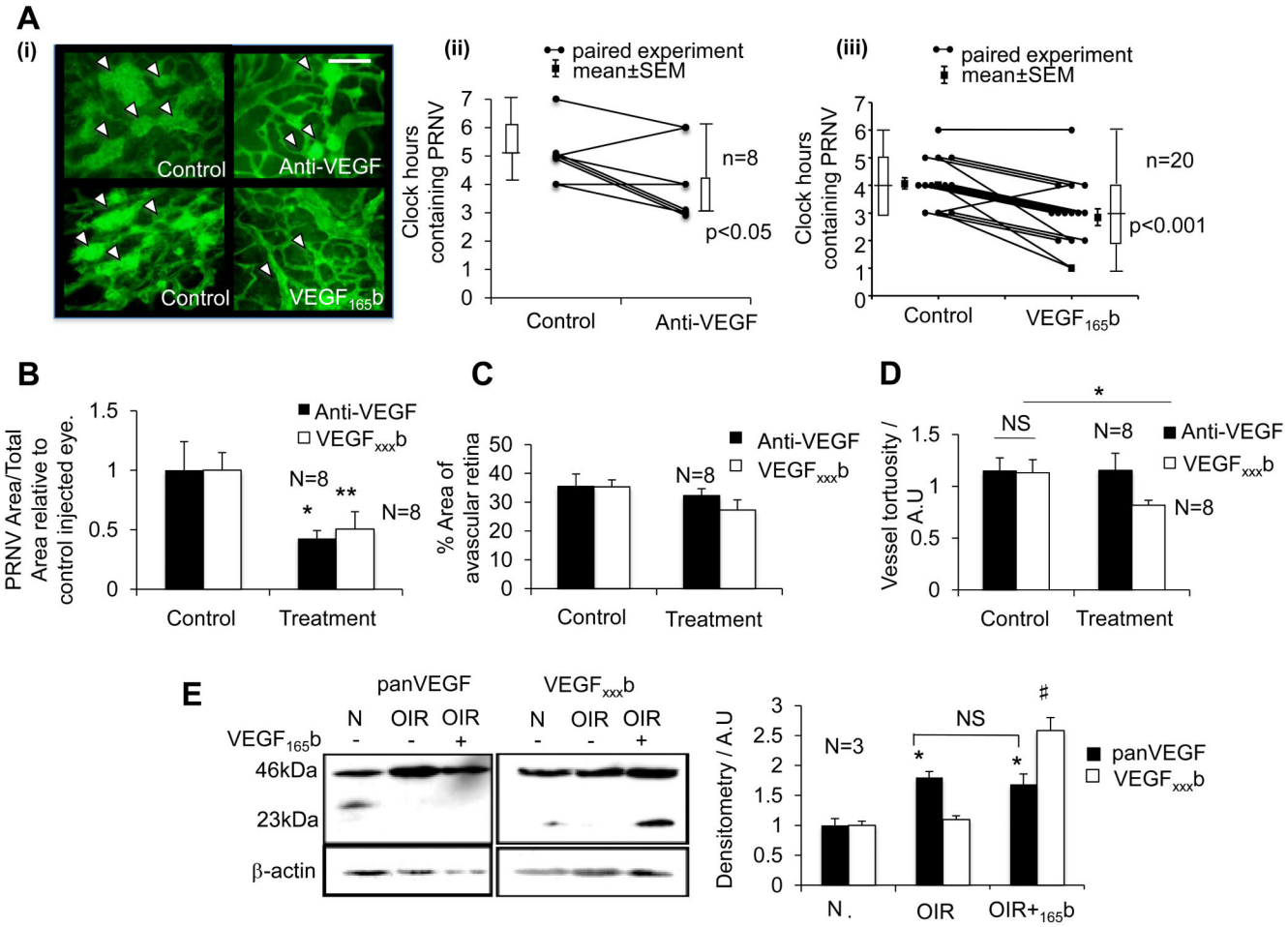

Figure 3. Recombinant human VEGF$_{165}$b significantly reduces PRNV and vessel tortuosity in the rat 50/10 OIR model

Litters of 12-14 Sprague Dawley pups were raised with their dams under conditions of fluctuating oxygen levels between 50% and 10% every 24 hours, for the first 14 days of life. At P14 pups received IO injections of either anti-VEGF antibody, G6-31 (1μg) or rhVEGF$_{165}$b (25ng) in the ipsilateral eye and control injections in the contralateral eye. A) (i) Examples of control and treated microvasculature shown (White arrowheads indicate PRNV) (ii) G6-31 ($p<0.05$; n=8) and (iii) 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 signficant 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).

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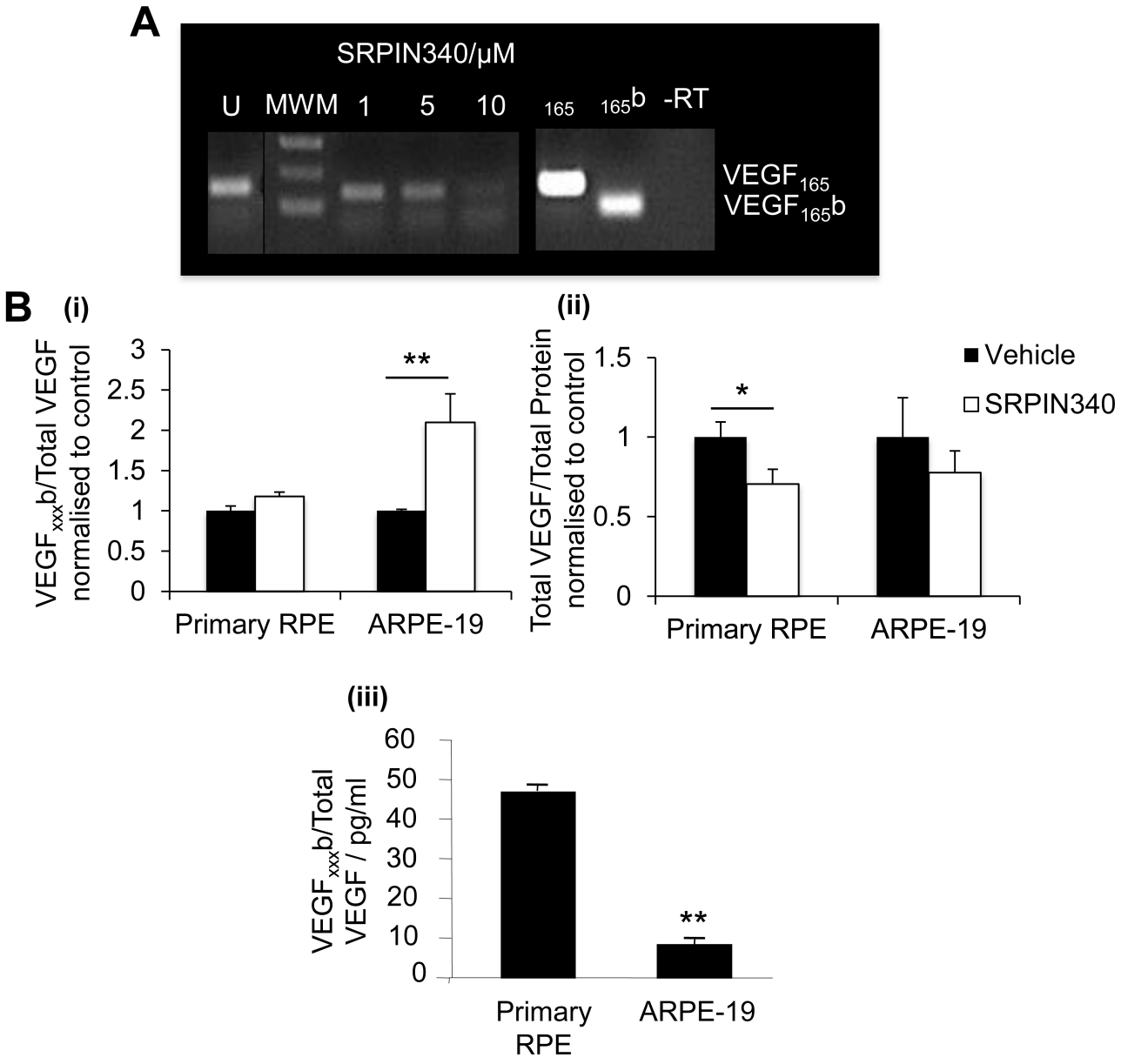

Figure 4. SRPK1 inhibition modulates pro-angiogenic VEGF expression *in vitro*

A) Primary RPE cells were treated with varying concentration of SRPIN340 (1, 5, 10μM) for 24 hours. RT-PCR was performed with primers spanning VEGF exon 7b and 8b. SRPIN340 switched the expression of VEGF isoforms to favour $VEGF_{165}b$ dose-dependently from 1-10μM achieving significance at 5μM ($p<0.05$) and 10μM ($p<0.01$) (One-way ANOVA, Dunnetts post hoc). B) VEGF protein levels in primary RPE and ARPE-19 were assessed by ELISA, (i) SRPIN340 significantly increased the expression of $VEGF_{xxx}b$/Total VEGF in ARPE-19 cells ($p<0.01$), and (ii) significantly reduced pro-angiogenic VEGF in primary RPE cells ($p<0.05$; students t-test). (iii) $VEGF_{xxx}b$ expression was greater in primary RPE cells compared to ARPE-19 ($p<0.01$),

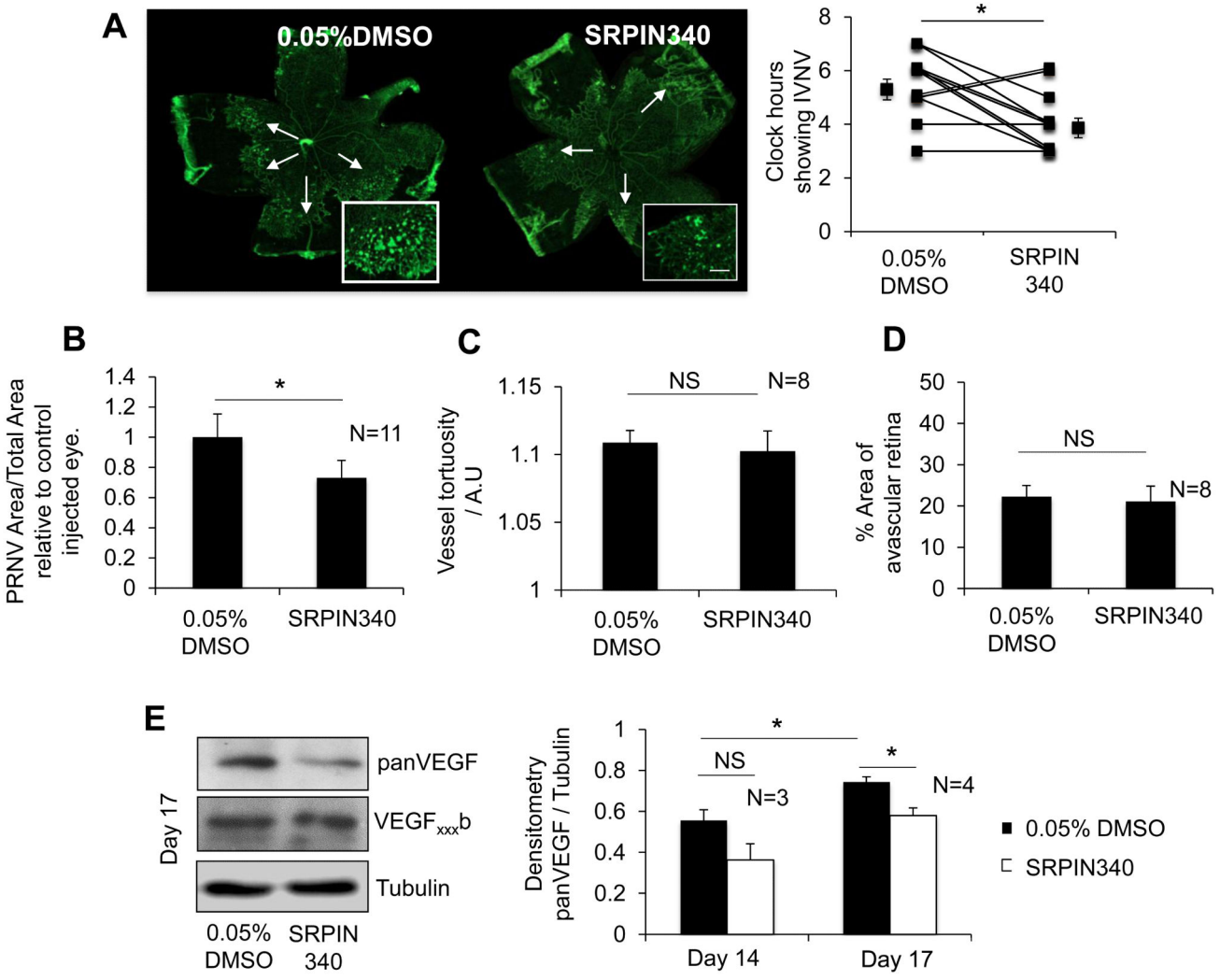

Figure 5. SRPIN340 significantly reduces PRNV and reduces pro-angiogenic VEGF *in vivo*

On day 12 of the OIR protocol the rats were briefly removed from the chamber and given a 2.5μl IO injection of 25ng SRPIN340 and vehicle (Saline +0.05% DMSO) in the contralateral eye. A) SRPIN340 treatment significantly reduced PRNV clock hours compared to control eyes ($p<0.05$, students paired t-test), example flat mounts shown (White arrows indicate PRNV, scale bar = 500μm) and B) PRNV area relative to total retinal area ($p<0.05$, students paired t-test). C) Retinas treated with SRPIN340 showed no significant change in vessel tortuosity, or the % 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 with control injected, however no change was observed in $VEGF_{xxx}b$ expression.

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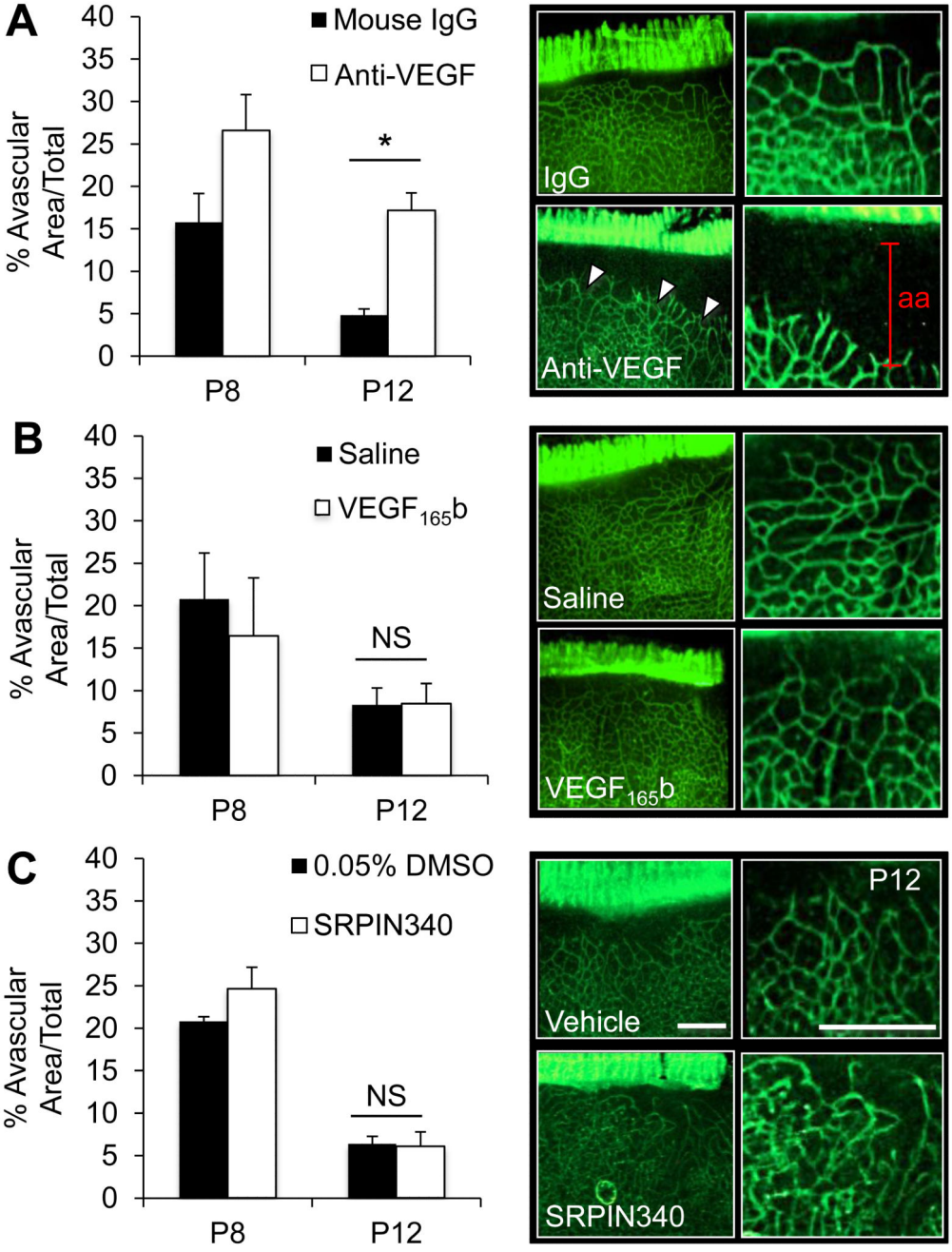

Figure 6. SRPIN340 and rhVEGF$_{165}$b do not influence normal retinal development

Eighteen P5 SD rat pups were injected IO (1μl) with either (A) 1μg G6-31, (B) 10ng rhVEGF$_{165}$b or (C) 10ng SRPIN340 in one eye and 1μg 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). Flatmounted 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 vascularisation whereas anti-VEGF G6-31 (Roche) significantly reduced vascularisation compared to control eyes at P12 ($P<0.05$, students paired t-test).

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