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$VEGF_{121}b$, a new member of the $VEGF_{xxx}b$ family of VEGF-A splice isoforms, inhibits neovascularisation and tumour growth *in vivo*

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BACKGROUND: The key mediator of new vessel formation in cancer and other diseases is VEGF-A. VEGF-A exists as alternatively spliced isoforms - the pro-angiogenic $VEGF_{xxx}$ family generated by exon 8 proximal splicing, and a sister family, termed $VEGF_{xxx}b$, exemplified by $VEGF_{165}b$, generated by distal splicing of exon 8. However, it is unknown whether this anti-angiogenic property of $VEGF_{165}b$ is a general property of the $VEGF_{xxx}b$ family of isoforms.

METHODS: The mRNA and protein expression of $VEGF_{121}b$ was studied in human tissue. The effect of $VEGF_{121}b$ was analysed by saturation binding to VEGF receptors, endothelial migration, apoptosis, xenograft tumour growth, pre-retinal neovascularisation and imaging of biodistribution in tumour-bearing mice with radioactive $VEGF_{121}b$.

RESULTS: The existence of $VEGF_{121}b$ was confirmed in normal human tissues. $VEGF_{121}b$ binds both VEGF receptors with similar affinity as other VEGF isoforms, but inhibits endothelial cell migration and is cytoprotective to endothelial cells through VEGFR-2 activation. Administration of $VEGF_{121}b$ normalised retinal vasculature by reducing both angiogenesis and ischaemia. $VEGF_{121}b$ reduced the growth of xenografted human colon tumours in association with reduced microvascular density, and an intravenous bolus of $VEGF_{121}b$ is taken up into colon tumour xenografts.

CONCLUSION: Here we identify a second member of the family, $VEGF_{121}b$, with similar properties to those of $VEGF_{165}b$, and underline the importance of the six amino acids of exon 8b in the anti-angiogenic activity of the $VEGF_{xxx}b$ isoforms.

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The advent of anti-neovascular therapies in cancer and eye disease has prompted a burgeoning interest in the mechanisms behind the initiation, development and refinement of new vessel formation. The growth of new vessels from those already established is termed angiogenesis and although a complex process, involving over 50 growth factors, cytokines, receptors and enzymes, is dominated, in most situations, by the 38 kDa cysteine-knot protein vascular endothelial growth factor (VEGF). Following the initial identification of VEGF (Senger *et al*, 1983) many splice isoforms were subsequently identified, eventually forming a family of mature peptides – the $VEGF_{xxx}$ family (see Figure 1) (Houck *et al*, 1991). The individual family members have different expression patterns (e.g., $VEGF_{183}$ expression is mostly restricted to the eye (Jingjing *et al*, 1999), $VEGF_{145}$ in the reproductive system (Charnock-Jones *et al*, 1993)); unique characteristics with respect to heparin and receptor-binding properties (resulting from inclusion/exclusion of particular exon segments) and are named by amino acid size. Despite this heterogeneity they are all considered pro-angiogenic, pro-permeability vasodilators. The evidence for these properties for individual splice isoform recombinant proteins *in vivo*, however, varies from conclusive ($VEGF_{165}$) (Leung *et al*, 1989), through mixed ($VEGF_{121}$) (Keyt *et al*, 1996; Zhang *et al*, 2000) and scanty ($VEGF_{145}$) (Poltorak *et al*, 1997) to absent ($VEGF_{206}$).

In addition, it has now become evident that even the presence of six splice isoforms is an over-simplification. The pro-angiogenic potential of conventional VEGF isoforms is thought to result from the C-terminus (Keyt *et al*, 1996), more specifically the six amino acids of exon 8a – CDKPRR (Cebe Suarez *et al*, 2006; Cebe-Suarez *et al*, 2008). A sister family of splice isoforms has now been identified (see Figure 1) in which the pro-angiogenic domain (exon 8a) is replaced by the six amino acids coded for by exon 8b – SLTRKD. These exon 8b isoforms ($VEGF_{xxx}b$) are exemplified by the only isoform so far extensively investigated, $VEGF_{165}b$. $VEGF_{165}b$ has radically different properties to that of the widely studied $VEGF_{165}$. We, and others have shown that $VEGF_{165}b$ is not pro-angiogenic *in vivo* (Woolard *et al*, 2004) and specifically and actively inhibits $VEGF_{165}$-mediated endothelial cell proliferation and migration *in vitro* (Bates *et al*, 2002; Rennel *et al*, 2008a, b), vasodilatation *ex vivo* (Bates *et al*, 2002) and *in vivo* angiogenesis assays such as rabbit corneal eye pocket, chicken chorioallantoic membrane, mesenteric and Matrigel implants (Woolard *et al*, 2004;

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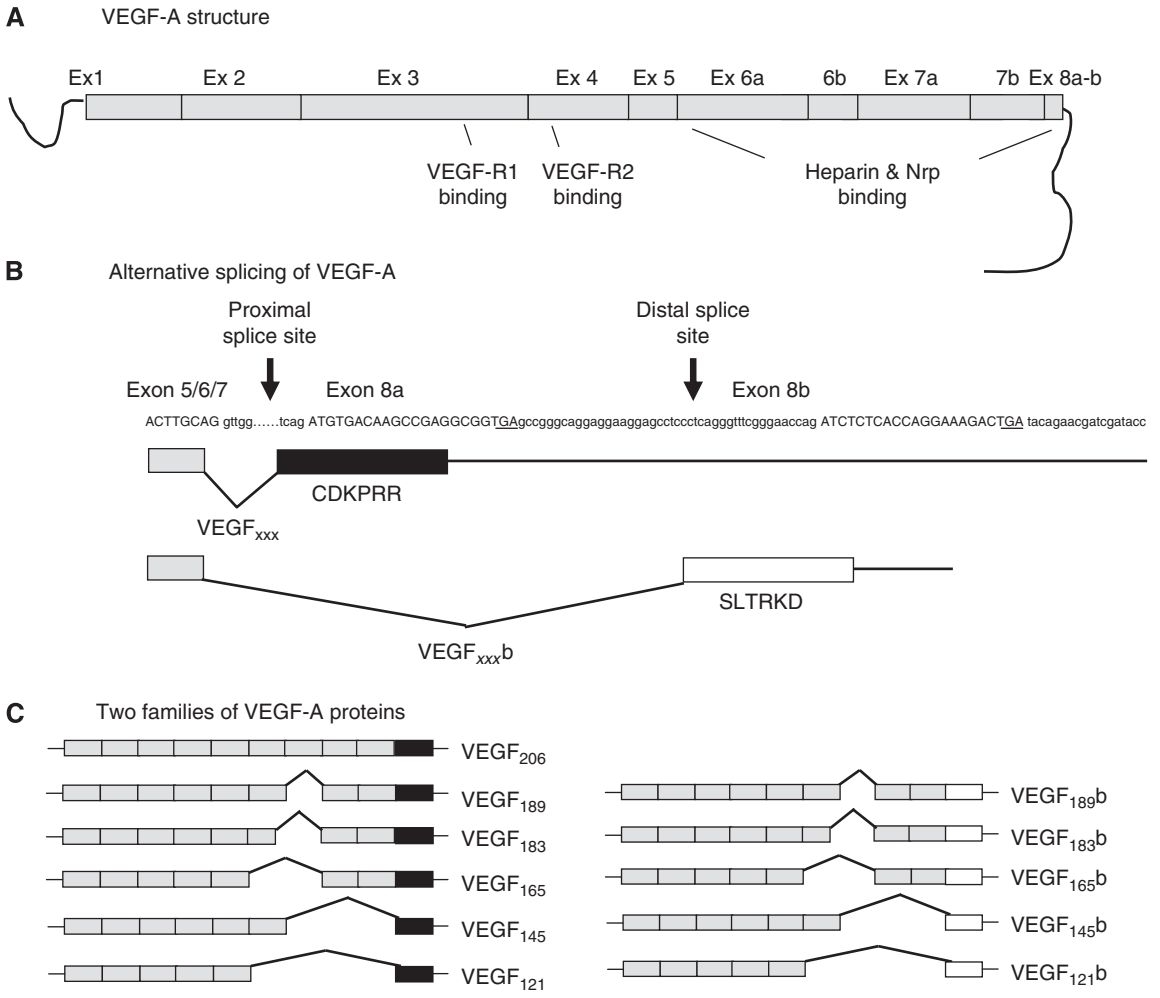


Figure 1 Alternative splicing of VEGF-A generating VEGF$_{xxx}$ and VEGF$_{xxx}$b isoforms. (**A**) VEGF-A contains eight exons with receptor binding found in exons 3 and 4, heparin and neuropilin binding in exons 6 and 7 and 8a. (**B**) Alternative splicing of the C-terminal end using proximal splice site generating VEGF$_{xxx}$ isoforms or distal splice site generating VEGF$_{xxx}$b isoforms. This splicing leads to an alterative last six amino acids (CDKPRR or SLTRKD). (**C**) The C-terminal splicing leads to the possibility of two sister families of VEGF-A isoforms; VEGF$_{xxx}$ and VEGF$_{xxx}$b, differing only in the C-terminal.

Cebe Suarez *et al*, 2006; Kawamura *et al*, 2008). Furthermore, VEGF$_{165}$b not only inhibits physiological angiogenesis in developing mammary tissue in transgenic animals over-expressing VEGF$_{165}$b (Qiu *et al*, 2008) but also pathological angiogenesis in models of the proliferative ocular lesions of diabetes (retinopathy of pre-maturity) (Konopatskaya *et al*, 2006) and age-related macular degeneration (laser-induced choroidal neovascularisation, Hua *et al*, unpublished) and in six different tumour models *in vivo* (Woolard *et al*, 2004; Varey *et al*, 2008; Rennel *et al*, 2008a, b). The above anti-angiogenic properties occur when delivered as VEGF$_{165}$b-transfected cells (Woolard *et al*, 2004; Varey *et al*, 2008; Rennel *et al*, 2008a), VEGF$_{165}$b-adenoviral constructs (Woolard *et al*, 2004) or recombinant human protein (Konopatskaya *et al*, 2006; Rennel *et al*, 2008b).

Although a significant amount of evidence on the expression and function of the VEGF$_{165}$b isoform has already been published, there is very little evidence on other VEGF$_{xxx}$b family members, and their existence and activity is still uncertain. This study, therefore, investigates the properties of a second member of the VEGF$_{xxx}$b family – the peptide VEGF$_{121}$b, addressing the hypothesis that VEGF$_{121}$b will also show anti-angiogenic properties. We present descriptive expression data and functional data on cell migration, receptor binding and *in vivo* tumour and ocular angiogenesis *in vivo*.

MATERIALS AND METHODS

Tissue samples

Paired colon samples were from partial colon resection for carcinoma. Samples were obtained by taking biopsies of the fresh specimen from a non-necrotic central portion of the tumour and from a peripheral part of the macroscopically normal colon ($n = 15$). The mean patient age was 71.5 (58–80) years, with 62% males and Duke's staging as follows: 6.7% A, 46.7% B and 46.7% C. Informed consent for obtaining the tissue was gained in compliance with local ethics committee approval.

PCR on cDNA from human total RNA

Total RNA was extracted from human tissue using TRIZOL (Invitrogen, Carlsbad, CA, USA) (Chomczynski, 1993) and treated with DNase (Promega, Madison, WI, USA). Complementary DNA was made using oligo dT primers or 3′UTR primer (GCTCTA GAAGCAGGTGAGAGTAAGCG) as described earlier (Bates *et al*, 2002). PCR was used to identify presence of VEGF$_{121}$b, VEGF$_{165}$ and VEGF$_{165}$b using specific primers; VEGF$_{165}$/VEGF$_{165}$b exon 7 GGCAGCTTGAGTTAAACGAACG, 3′UTR ATGGATCCGTATCAG TCTTTCCTGG, VEGF$_{121}$b exon 5/8b GAAAAATCTCTCACCAGG

AAA, 3′UTR II CTGGATTAAGGACTGTTCTG, VEGF$_{121}$ exon 5/8a GAAAAATGTGACAAGCCGAG 3′UTR II CTGGATTAAGGACTGT TCTG, GAPDH forward TGATGACATCAAGAAGGTGGTGAAG, reverse TCCTTGGAGGCCATGTGGGCCAT and PCR products were separated and visualised using 4% agarose/ethidium bromide gel. Putative VEGF$_{121}$b PCR products were purified using QIAquick gel extraction (Qiagen, Crawley, UK) and inserted into pGEM vector and confirmed by sequencing of plasmid.

Production of recombinant protein

Recombinant human VEGF$_{121}$b (VEGF$_{121}$b) and VEGF$_{165}$b were produced in Chinese hamster ovary cells as glycosylated dimers by Cancer Research Technologies (London, UK) as described earlier (Rennel *et al*, 2008b). VEGF$_{165}$ and VEGF$_{121}$ were from R&D Systems, Minneapolis, MN, USA.

In vitro assays with human umbilical vein endothelial cells

Human umbilical vein endothelial cells (HUVEC) were extracted from umbilical cords from caesarean sections (St Michael's Hospital, Bristol, UK). HUVEC migration was performed as described earlier (Rennel *et al*, 2008b). Briefly, HUVECs were serum starved for 6 h and 100 000 serum-starved cells were plated into collagen-coated 8 μm inserts (Millipore, Billerica, MA, USA) placed in 24-well plates with 500 μl of chemoattractant in M200 medium with 0.1% v/v FCS and 0.2% w/v BSA and incubated overnight at 37°C to allow for migration. Migrating cells were stained in Mayer's haematoxylin. Migrating cells were counted (10 random fields per insert) and expressed as percentage of migrating cells in relation to the total number of seeded cells. Experiments were performed in triplicates. A peptide (Ac-SLTRKD) was generated and increasing concentrations (1 nM – 100 μM) peptide was combined with or without 1 nM VEGF$_{165}$ to analyse impact on HUVEC migration.

Cell cytotoxicity was performed using a lactate dehydrogenase assay (Promega); 13 000 cells were seeded in cell culture 96-well plates in triplicates or quadruplicates in 100 μl of full growth media (FGM) for 24 h. Recombinant VEGF protein was added in 100 μl media with 0.1% FCS for 48 h. Conditioned media and lysed cells were spun at 250 **g** for 15 min at 4°C followed by addition of kit components, incubated for 30 min and read at 490 nm. Receptor and kinase inhibitors were added at indicated concentrations with or without 1.4 nM VEGF$_{121}$b.

For direct counting, HUVECs were seeded in 24-well plates in triplicates in FGM 24 h followed by 48 h exposure to 1.4 nM growth factors or FGM. Cells were trypsinised and counted using a haemocytometer.

In vivo tumour model

LS174t human colon carcinoma cell lines (ECACC, Salisbury, UK) (Yuan *et al*, 1996; Lee *et al*, 2000) were grown in Minimum Essential Eagle's Medium supplemented with 10% v/v FCS, 2 mM L-glutamine and 1% non-essential amino acids (all from Sigma Aldrich, Gillingham, UK). Cells were transfected with 1 μg/well of pcDNA$_3$ empty vector or pcDNA$_3$-VEGF$_{121}$b, using lipofectamine plus (Invitrogen) as per manufacturer's instructions and selected using 500 μg ml^{-1} geneticin (Sigma Aldrich). Conditioned media and cell lysate were analysed by western blotting to confirm expression levels of VEGF isoforms. Two million cells were injected subcutaneously into the lumbar region of nude mice (six per group). Xenotransplanted tumours were measured by calliper every 2–3 days and tumour volume was calculated according to (length × width × [length + width]/2). Mice were culled by cervical dislocation and tumours were removed when the first tumour reached 16 mm in any direction all mice were killed in that group. Injections, measurements and analysis were all carried out with the investigators blinded to group.

Tumour vessel density was counted in 10 random fields in haematoxylin and eosin stained 6 μm sections frozen sections from tumours. Vessel presence was confirmed by staining by blocking in 5% v/v goat Ig for 30 min, 20 μg ml^{-1} biotinylated isolectin B4 (L-2140 Sigma-Aldrich) or 1 μg ml^{-1} Flk-1 (sc-6251, Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight, 2 μg ml^{-1} anti-mouse biotin antibody (Vector Laboratories, Burlingame, CA, USA) for 1 h followed by avidin biotinylated enzyme complex (Vector Laboratories) for 30 min followed by DAB substrate (Vector Laboratories). Sections were examined using a Nikon Eclipse E400 microscope and photos were captured using Nikon Eclipse Net software. To visualise apoptosis, tumour sections were stained with cleaved caspase 3 antibody (1 : 400 dilution, CatNo9661 Cell Signaling Technology, Danvers, MA, USA) and positive staining areas were circled and compared to total tumour area using Image J software.

Cell proliferation by flow cytometry analysis and direct counting

Trypsinised LS174t cells were fixed in 70% ethanol for 30 min at 4 °C followed by 100 μg ml^{-1} ribonuclease (Sigma Aldrich) for 5 min. Propidium iodine (PI, 50 μg ml^{-1}) was added to the cells and analysed by flow cytometer.

For healthy, apoptotic and necrotic cell determination a PI-annexin V-FITC kit was used (Bender MedSystems, Vienna, Austria). Cell culture media was centrifuged at 1500 r.p.m. to collect non-adherent cells, added to trypsined cells and diluted in binding buffer to approximately 300 000 cells/ml. Cells were stained with 25 μl annexin V-FITC for 10 min, washed, stained with 20 μg ml^{-1} PI and analysed by flow cytometer.

For direct counting, 135 000 LS174t cells/24 well was seeded out in triplicates in media supplemented with 0.1% FCS in the presence or absence of 1 nM VEGF$_{121}$b. Cells were trypsined and counted using a haemocytometer after 24 and 48 h.

In vivo imaging of ^{125}I-VEGF$_{165}$b biodistribution

Nude mice were injected with LS174t tumours on the right hindleg. ^{125}I-VEGF$_{121}$b was generated using Iodogen tubes (Pierce Biotechnology, Cramlington, UK) and purified with NAP-10 columns (GE Healthcare, Chalfont St Giles, UK). Analysis by thin layer chromatography showed >95% purity (^{125}I-VEGF$_{121}$b/total ^{125}I). Approximalty 3.2 MBq (70 μg protein) was injected into the tail vein when tumours were >10 mm in diameter. Anaesthesia was maintained by 2% halothane during X-ray and scanning (NanoSPECT/CT, Bioscan, Washington, DC, USA), after 40, 70, 120, 240 or 1440 min. For biodistribution studies, 0.10 MBq (3 μg) ^{125}I-VEGF$_{121}$b was injected into the tail vein and mice were culled at 120 or 240 min. Organs and tissues of interest were excised and assessed using a gamma counter (LKB Wallac 1282 Compugamma CS, Wallac, Perkin Elmer, MA, USA). Uptake was expressed as % injected dose/g tissue.

In vitro saturation binding of ^{125}I-VEGF$_{xxx}$b to Fc-VEGFR-1

One μg ml^{-1} human VEGFR-1-Fc chimaera or VEGFR-2-Fc (R&D Systems) was bound to Immulon II HB Flat 96-well plates (Thermo Labsystems, Basingstoke, UK) overnight followed by blocking with 3% w/v BSA for 2 h and binding of ^{125}I-rhVEGF$_{121}$b or ^{125}I-rhVEGF$_{165}$b for 4 h in 1% w/v BSA in triplicates. Plates were washed three times with PBS + 0.05% v/v Tween in between each step. To detach the complex 10% w/v SDS was added and counted in a gamma counter. Data were calculated as c.p.m. and expressed as % binding compared with maximum binding concentration.

SDS–PAGE and immunoblotting of tissue and cell lysate

Tissue lysates from transfected cells or human colon tissue were extracted using RIPA buffer (50 mM Tris, 150 mM NaCl, 1% v/v NP-40, 0.25% w/v Na-deoxycholate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg ml^{-1} of each of aprotinin, pepstatin and leupeptin) and homogenised using a polytron and spun at 12 000 r.p.m. for 10 min at 4°C. Lysates were separated on 15% SDS–PAGE, transferred to polyvinylidene fluoride membranes, blocked in 10% w/v dry milk, incubated with a mouse monoclonal anti-human VEGF$_{xxx}$b antibody (2 μg ml^{-1}, R&D Systems) for 1 h followed by horse radish peroxidase conjugated anti-mouse antibody (1/5000, Pierce Biotechnology) for 1 h. Membranes were developed using supersensitive West Femto Maximum Sensitive Substrate (Pierce Biotechnology). Densitometry analysis of western blots was performed using Image J. VEGF$_{121}$b is detected by immunoblotting using anti-VEGF antibodies (A-20 Santa Cruz or AF-293 R&D Systems) and the commercial VEGF$_{xxx}$b antibody (A56/1, R&D Systems, cat no. MAB3045). Commercial ELISA for VEGF$_{165}$b (R&D Systems) and panVEGF (Duoset R&D Systems) can pick up VEGF$_{121}$b but panVEGF ELISA underestimates the VEGF$_{121}$b levels (and VEGF$_{165}$b) by approximately 40% (Varey *et al*, 2008).

Oxygen-induced retinopathy mouse model

The oxygen-induced retinopathy (OIR) model was performed as described earlier (Smith *et al*, 1994; Konopatskaya *et al*, 2006) with minor modifications. Neonatal C57/Bl6 mice and nursing CD1 dams were exposed to 75% oxygen between P7 and P12. Return to room air induced hypoxia in the ischaemic areas. On P13, mice received either VEGF$_{121}$b (1 or 10 ng) or Hank's buffered solution in 1 μl intraocular injections using a Nanofil syringe fitted with a 35 gauge needle (WPI, Sarasota, FL, USA) into the left eye under isoflurane anaesthesia. On P17, both eyes were dissected, fixed in 4% paraformaldehyde for 4 h at 4°C and retinas were dissected. Retinas were permeabilised in PBS containing 0.5% Triton X-100 and 1% bovine serum albumin, stained with 20 μg ml^{-1} biotinylated isolectin B4 (Sigma Aldrich) in PBS pH 6.8, 1% Triton-X100, 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, followed by 20 μg ml^{-1} ALEXA 488-streptavidin (Molecular Probes, Eugene, OR, USA) and flat mounted in Vectashield (Vector Laboratories). Quantification of neovascular and ischaemic areas were performed in a blinded fashion using Photoshop CS3 along with Image J and expressed as percentage of total retinal area (= normal + ischaemic + neovascular).

Statistical analysis

Statistical analysis was performed using GraphPad Prism software (version 4.0cx). Data are given as means ± s.e.m. or means ± 95 % confidence interval when stated. One-way ANOVA was used to compare migration, cytoprotection, tumour growth and retinal vascularisation. *T*-test was used to compare tumour growth, apoptosis and microvascular density and paired *t*-test used for retinal areas.

RESULTS

Expression of VEGF$_{121}$b in human colon tissue and its reduction in colon carcinoma

Earlier, we reported a shift in expression of VEGF$_{xxx}$b isoforms in human malignant melanoma (Pritchard-Jones *et al*, 2007), prostate (Woolard *et al*, 2004; Rennel *et al*, 2008a) and colon carcinoma (Varey *et al*, 2008) leading to less anti-angiogenic VEGF$_{xxx}$b splice isoforms in cancer tissue. The ELISA used to quantify the levels of VEGF$_{xxx}$b isoforms does not distinguish between the individual members of the VEGF$_{xxx}$b isoform family. Earlier RT–PCR used to detect VEGF$_{xxx}$b isoforms (using exon 7 and 3′UTR primers) did not detect VEGF$_{121}$b (see Figure 2A) as the forward primer was in exon 7 (spliced out in VEGF$_{121}$b and VEGF$_{121}$). To identify VEGF$_{121}$b, mRNA primers were used that spanned the exon 5/exon 8b boundary. These primers detected VEGF$_{121}$b but not VEGF$_{165}$b or VEGF$_{165}$ (see Figure 2B). VEGF$_{121}$b was detected in normal tissue such as aorta, spleen, placenta and some normal and tumour colon tissue (see Figure 2B), which also expressed VEGF$_{165}$ and VEGF$_{165}$b (see Figure 2A) and retina and vitreous (Figure 2C). Presence of VEGF$_{121}$b was confirmed by sequencing of PCR products.

Measurement of VEGF levels in an earlier study on human colon carcinoma showed that the angiogenic VEGF isoforms were upregulated compared with matched colon tissue whereas the levels of anti-angiogenic VEGF$_{xxx}$b isoforms remained unchanged. Western blot on that tissue showed two bands consistent with expression of VEGF$_{121}$b and VEGF$_{165}$b (Varey *et al*, 2008). Quantification of the western blot analysis of paired human colon tissues from tumour and adjacent normal colon tissue indicates a downregulation of VEGF$_{121}$b in tumour tissue compared to VEGF$_{165}$b (see Figure 2D, $P<0.01$ paired *t*-test, $n=14$ pairs).

VEGF$_{121}$b is secreted, inhibits migration and reduces apoptosis in endothelial cells

To determine whether VEGF$_{121}$b can be secreted by colon cells, VEGF$_{121}$b recombinant cDNA was transfected into colon carcinoma cells. Western blotting of the lysate and the media showed that VEGF$_{121}$b was found pre-dominantly in the media, indicating that it is freely secreted (see Figure 3A), in contrast to cells that were not transfected with the VEGF$_{121}$b plasmid, in which no VEGF$_{121}$b was seen in the media or the lysate. VEGF$_{121}$b binding to VEGFR-1 was analysed by incubating increasing concentrations of radiolabelled VEGF$_{121}$b with immobilised VEGFR-1 (see Figure 3B). The saturation-binding kinetics of VEGF$_{121}$b (see Figure 3B, $\log_{10}EC_{50}=-9.9\pm0.04$) was similar to that for VEGF$_{165}$b measured at the same time ($EC_{50}=-9.65\pm0.04$, Figure 3C).

We have shown earlier that VEGF$_{165}$b conditioned media (Bates *et al*, 2002; Rennel *et al*, 2008a) or recombinant human VEGF$_{165}$b (Woolard *et al*, 2004; Rennel *et al*, 2008b) inhibits VEGF$_{165}$-induced migration of endothelial cells. Migration of HUVEC was inhibited by the addition of VEGF$_{121}$b either when stimulated with VEGF$_{165}$ (see Figure 3D, VEGF$_{165}$ *vs* any treatment $P<0.05$) or VEGF$_{121}$ (see Figure 3E, VEGF$_{121}$ *vs* any treatment $P<0.05$). The level of inhibition was similar to that observed with VEGF$_{165}$b (see Figure 3D) and no additional significant reduction in migration was observed with the combination of VEGF$_{121}$b and VEGF$_{165}$b (see Figure 3D). Addition of up to 100 μM peptide of the last six amino acids had no effect on VEGF$_{165}$-induced HUVEC migration (data not shown) indicating that the 3D structure and or the full length of the VEGF$_{xxx}$b protein is needed for inhibition.

VEGF$_{165}$ is known to protect endothelial cells from apoptosis and incubation of HUVECs with recombinant VEGF$_{121}$b was equally cytoprotective (see Figure 3F, no addition *vs* any treatment $P<0.01$). This resulted in a small increase in cell number induced by treatment with VEGF$_{121}$b (see Figure 3G). To determine the mechanism of this cytoprotective effect of VEGF$_{121}$b, HUVECs were treated with inhibitors of receptor tyrosine kinases and signalling kinases (see Figure 3H). Inhibition of VEGFR-1 and VEGFR-2 by 100 nM PTK787 resulted in an inhibition of the cytoprotective effect of VEGF$_{121}$b, as did incubation with the VEGFR-2-specific antagonist ZM323881 (10 nM). The cytoprotective effect was blocked by inhibitors of PI3Kinase (LY294002) and MEK1/2 (PD98059), but not by inhibitors of p38 MAPK (SB203580, see Figure 3H).

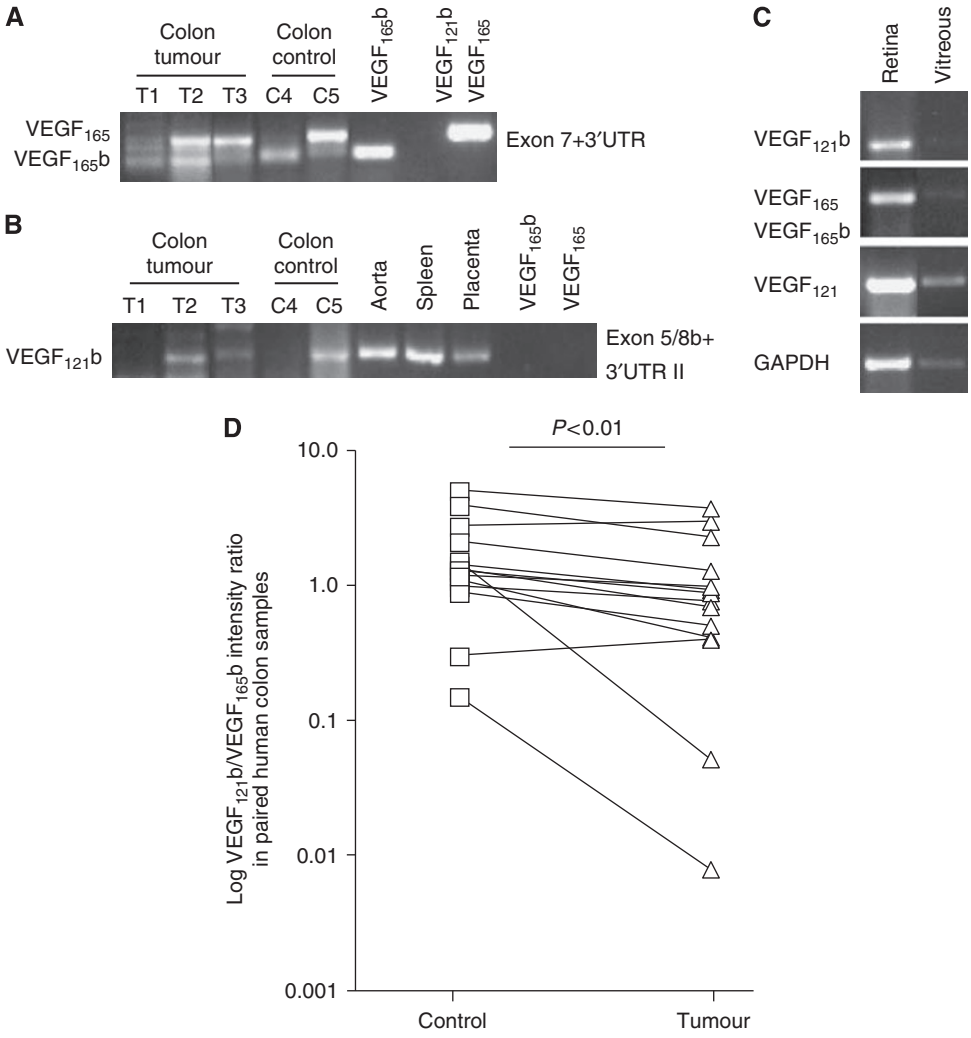


Figure 2 VEGF$_{121}$b is expressed in human tissue and the expression is reduced in human colon tumours. (**A–C**) PCR on cDNA from different human tissues using primers to detect VEGF$_{121}$b, VEGF$_{165}$b and VEGF$_{165}$. VEGF$_{165}$ and VEGF$_{165}$b are detected in colon cDNA both in tumour and adjacent control colon (**A**). VEGF$_{121}$b is detected in colon tissue, aorta, spleen and placenta using VEGF$_{121}$b-specific primers (**B**). VEGF$_{121}$, $_{165}$, $_{121}$b and $_{165}$b can be found in the retina and to a lesser extent in the human vitreous (**C**). (**D**) Densitometry analysis of VEGF$_{121}$b and VEGF$_{165}$b protein expression in matched human control colon samples and colon tumours analysed by western blot using a VEGF$_{xxx}$b-specific antibody ($n=14$ pairs, $P=0.0067$ paired *t*-test).

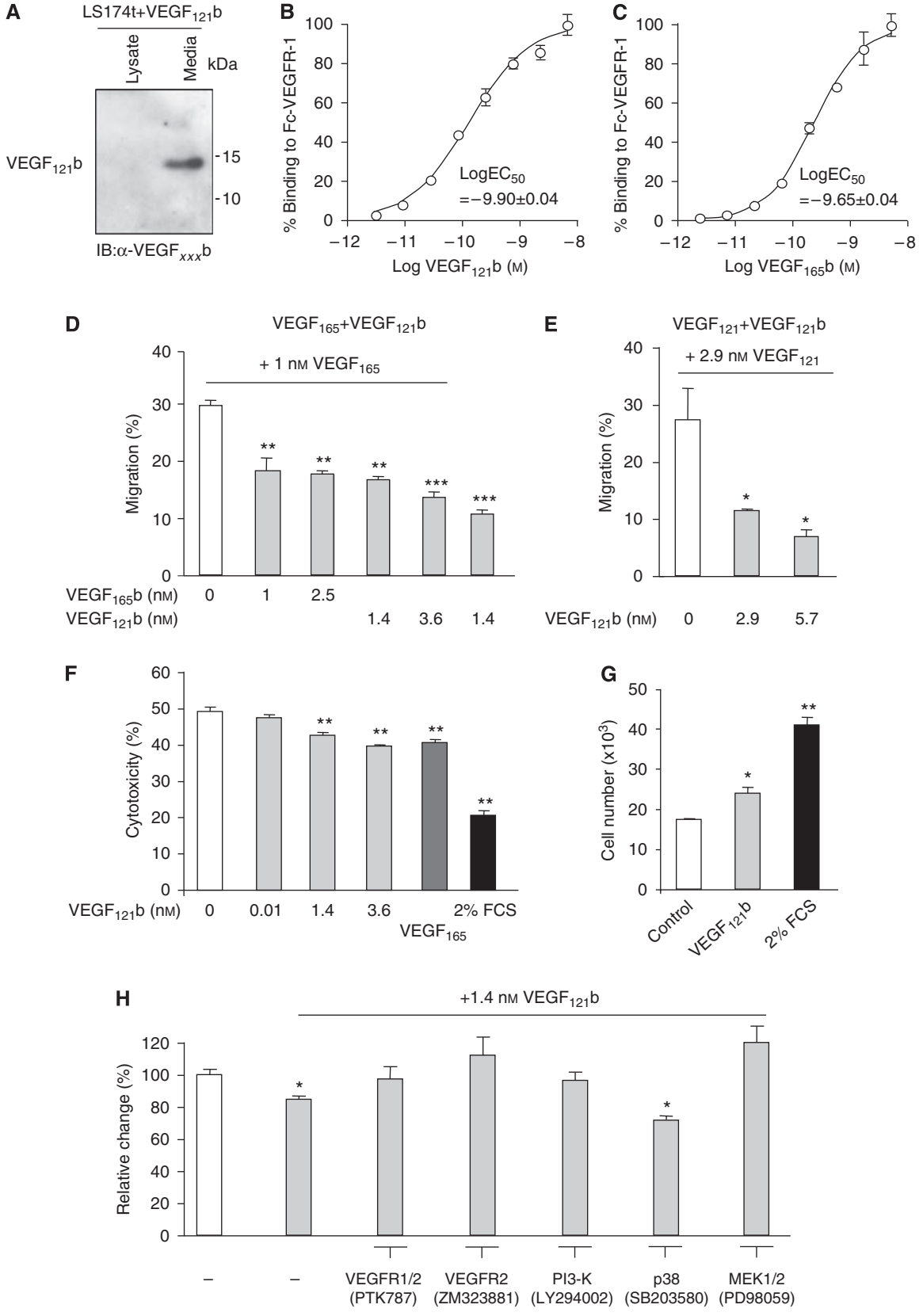
Over-expression of VEGF$_{121}$b reduces tumour growth through a direct effect on endothelial cells

We have shown earlier that over-expression of VEGF$_{165}$b reduces tumour growth in five different tumour types (Woolard *et al*, 2004; Varey *et al*, 2008; Rennel *et al*, 2008a), but VEGF$_{165}$b does not directly affect proliferation or apoptosis of LS174t cells (Varey *et al*, 2008). To verify whether VEGF$_{121}$b could inhibit tumour growth as well, LS174t colon carcinoma tumour cells were transfected with plasmid containing VEGF$_{121}$b or cloning vector alone (control). Expression levels were confirmed by immunoblot on lysate and conditioned media showing that VEGF$_{121}$b was secreted and found in the media (see Figure 3A). Cells were injected into the back of nude mice and tumour growth was monitored over time. VEGF$_{121}$b reduced tumour growth compared with empty vector ($290 \pm 66\,mm^3$ *vs* $925 \pm 268\,mm^3$ after 14 days, $P<0.05$, unpaired *t*-test Welch correction, see Figure 4A). VEGF$_{121}$b tumours were smaller and excised tumours were less haemorrhagic than those with empty vector (see Figure 4A inserted images). Immunohistochemical staining was performed to visualise blood vessel distribution (see Figure 4B for representative VEGFR-2 staining). Analysis of the vessel density in 10 random fields in each tumour showed a decreased number of vessels in VEGF$_{121}$b tumours (control *vs* VEGF$_{121}$b, 3.5 ± 0.6 *vs* 1.7 ± 0.1, $P<0.05$ unpaired *t*-test $n=6$ tumours per treatment 10 fields analysed per tumour, Figure 4B). VEGFR-2 staining of tumour sections showed expression of VEGFR-2 in the tumour vessels and not in the general tumour mass (see Figure 4B inserted images) indicating an effect of VEGF$_{121}$b on the endothelium rather than an anti-proliferative effect on the tumour cells. Earlier analysis has shown absence of VEGFR-1 and VEGFR-2 expression in LS174t cells (Rennel *et al*, 2008b). To determine whether VEGF$_{121}$b expression induced apoptosis in the tumour cells, tumours were stained for cleaved caspase 3. There was no change in caspase staining in control *vs* VEGF$_{121}$b-expressing tumours (see Figure 4C).

Transfected LS174t colon cells were analysed by flow cytometry and VEGF$_{121}$b had no effect on proliferation (see Figure 4D control *vs* VEGF$_{121}$b, S/G$_2$-M 21 ± 2.3 *vs* 27 ± 4.4, $P=0.36$ unpaired *t*-test). In addition, addition of recombinant VEGF$_{121}$b had no effect on tumour cell number (see Figure 4F). These data indicate that VEGF$_{121}$b reduced tumour growth by reducing the tumour-driven angiogenesis, rather than a direct effect on the tumour cells themselves.

Biodistribution of intravenous VEGF$_{121}$b

The results above indicate that VEGF$_{121}$b could be a potential therapeutic agent in angiogenic diseases, if the uptake into neovascular tissues was sufficient. To determine how intravenously (i.v.) injected recombinant VEGF$_{121}$b is distributed *in vivo*, ^{125}I-VEGF$_{121}$b was injected i.v. into tumour-bearing mice and imaged using high-resolution single photon emission computed



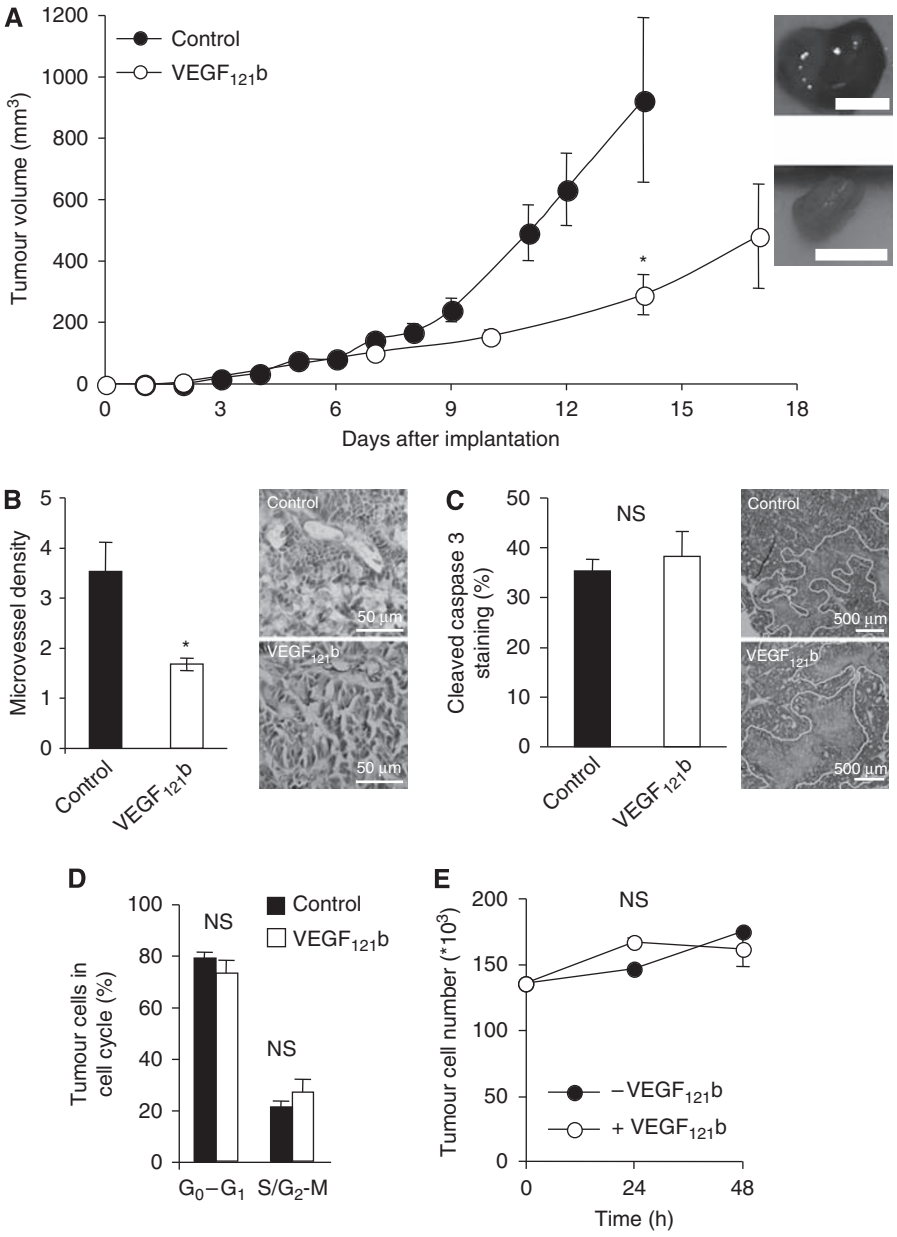


Figure 4 VEGF$_{121}$b reduces tumour growth in nude mice bearing colon carcinoma tumours by reducing tumour vessel ingrowth. (**A**) LS174t human colon carcinoma cells were transfected with pcDNA$_3$-VEGF$_{121}$b or empty pcDNA3 plasmid and injected subcutaneously into nude mice and tumour growth was monitored over time. Over-expression of VEGF$_{121}$b resulted in a reduced tumour growth and contained less blood compared with control cells (inserted images). Scale bar = 10 mm. (**B**) Immunohistochemistry staining of tumour sections for VEGFR-2 visualise microvessels (inserted images). Quantification of microvascular density showed significantly fewer blood vessels per unit area than control tumours. Each point represents the mean of 10 random analysed fields and six tumours per treatment were examined (*$P<0.05$ unpaired *t*-test). (**C**) Cleaved caspase 3 staining of tumour sections (inserted images) showed no significant difference in apoptosis. (**D**, **E**) VEGF$_{121}$b has no effect on LS174t colon carcinoma growth *in vitro*. Over-expression of VEGF$_{121}$b in LS174t colon carcinoma cells had no effect on cell proliferation analysed by FACS (**D**). Addition of 1 nM VEGF$_{121}$b had no effect of cell number analysed by direct counting of cells over 48 h (**E**).

Figure 3 VEGF$_{121}$b inhibits migration of endothelial cells and inhibits serum starved induced cell death in endothelial cells through classical VEGFR-induced pathways. (**A**) VEGF$_{121}$b is found in the media on transfected cancer cells. (**B**–**C**) VEGF$_{121}$b has similar saturation binding kinetics to VEGFR-1 as VEGF$_{165}$b. (**D**–**E**) VEGF$_{121}$b inhibits VEGF$_{165}$ (**D**) or VEGF$_{121}$ (**E**) induced migration of HUVEC. Cells were allowed to migrate through 8 μm pore inserts with increasing concentrations of VEGF$_{121}$b (0–6 nM) or VEGF$_{165}$b (0–2.5 nM) in the presence of 1 nM VEGF$_{165}$ or 2.9 nM VEGF$_{121}$. Data plotted as percentage migration compared to total number of seeded cells (**F**) VEGF$_{121}$b is cytoprotective to a similar degree as VEGF$_{165}$ in HUVEC. Release of lactate dehydrogenase as a measurement of cell viability was monitored after 48 h of serum starvation in the presence of increasing amounts of VEGF$_{121}$b (0–3.6 nM), 1 nM VEGF$_{165}$ or full growth media (FGM) with serum and growth factors. (**G**) Similar cytoprotection of VEGF$_{121}$b was observed in serum starved HUVEC as cell number was not reduced by the same amount after 48 h of cell starvation. (**H**) VEGF$_{121}$b cytoprotection is mediated via VEGFR-1 and -2, P13-K, MEK1/2. Inhibition of VEGFR1/2=200 nM PTK787, VEGFR-2=10 nM ZM323881, P13- K=15 μM LY294002, p38=10 μM SB203580 and MEK1/2=15 μM PD98059. (One-way ANOVA, Dunnett's *post hoc* test, 0 nM or control *vs* addition, *$P<0.05$, **$P<0.01$, ***$P<0.001$).

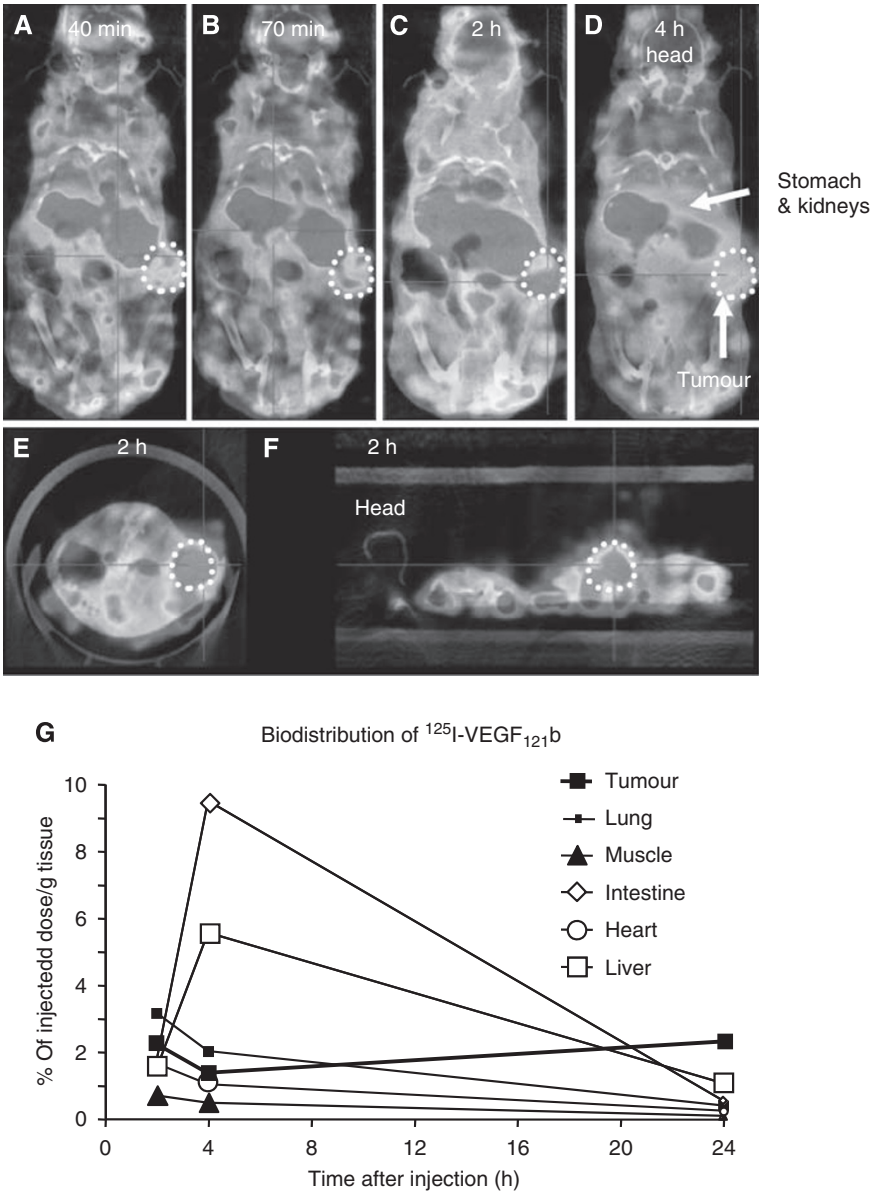


Figure 5 *In vivo* distribution of ^{125}I-VEGF$_{121}$b in tumour-bearing mice. Tumour-bearing mice received an intravenous injection of ^{125}I-VEGF$_{121}$b and 3D imaged using NanoSPECT/CT. (**A**–**D**) Time course for biodistribution of ^{125}I-VEGF$_{121}$b after tail vein injection using transverse sections. (**E**) Coronal. (**F**) Para-sagittal through the centre of the tumour. The tumour is circled and arrows indicate different organs. (**G**) Quantification uptake into different organs and tissues over time. Data expressed as % in tissue relative to the total injected dose, per gram of tissue.

tomography (NanoSPECT/CT). Radiolabelled VEGF$_{121}$b was distributed quickly through the mouse and images acquired at 40 min post injection (p.i.) showed accumulation in organs of metabolism and secretion. Thereafter, the overall signal gradually declined because of excretion/degradation of the radiolabelled protein. Figure 5A shows whole body images at different time p.i. Uptake of radioactivity can be seen in the tumour as well as abdominal tissues such as stomach and kidney. Coronal and sagittal sections also visualised uptake in the tumour at 2 h p.i. (see Figure 5E-F). Biodistribution of tissue and tumour indicated that approximately 2.5% of the total ^{125}I is found in the tumour at 2 h and stays at a similar level for up to 24 h (see Figure 5G). Intestine and liver showed high uptake with a maximum at 4 h. No obvious adverse haemodynamic effects were seen in the animals.

VEGF$_{121}$b rescues retinal vasculature by reducing neovascularisation and ischaemia

We have shown earlier that the anti-angiogenic VEGF$_{165}$b can inhibit retinal neovascularisation in an OIR mouse model when administered as a single intraocular injection (Konopatskaya *et al*, 2006). In this model retinal vaso-obliteration occurs in the central retina when pups are exposed to high oxygen (from P7 to P12) and when returned to room air the relative hypoxia induces neovascularisation with a peak reached at P17. Retinas were flat mounted and stained with isolectin to visualise vessels with neovascularisation occurring at the edge of the central ischaemic area (see Figure 6A). Blind quantification of the injected and uninjected contralateral retina, showed reduced neovascularisation after 1 or 10 ng VEGF$_{121}$b (see Figure 6B-C,

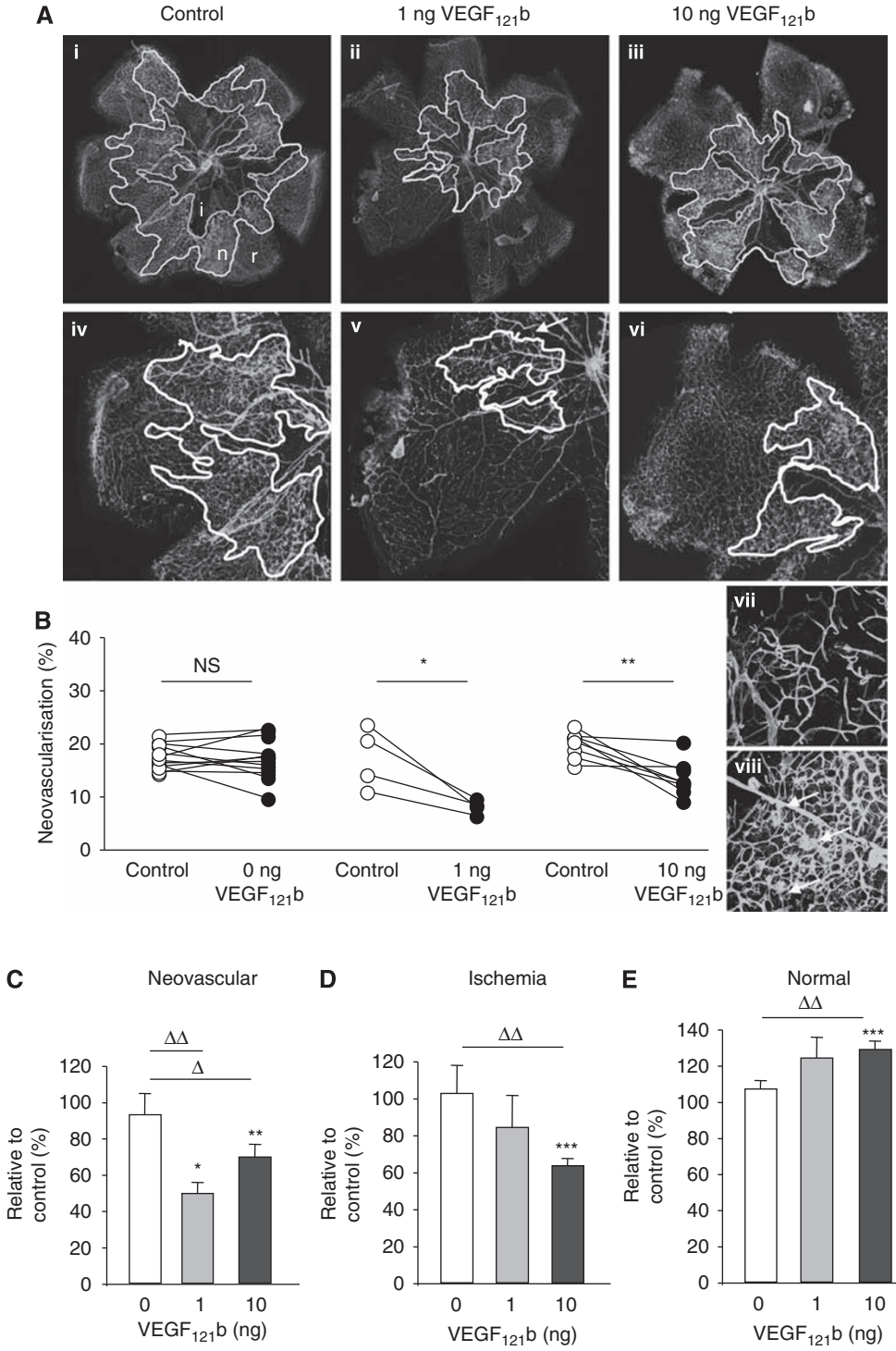


Figure 6 $VEGF_{121}b$ reduces hypoxia-induced retinal neovascularisation after single intraocular injection. The oxygen-induced retinopathy mouse model leads to neovascularisation induced by hypoxia. (**A**) Flat mounted mouse retinas with vessels visualised by isolectin staining. Ischaemic and neovascularised areas are circled (i, ischaemic, n, neovascular and r, normal retinal vessels). Normal retinal vessels (vii) are slender compared to neovascular vessels (viii). (**B**) Quantification of neovascularisation, ischaemic and normal vessel growth compared to matched uninjected control eye (paired *t*-test, $*P<0.05$, $**P<0.01$). (**C**–**E**). Distribution of ischaemic, neovascular and normal vessel growth in injected retinas relative to uninjected eye showed that $VEGF_{121}b$ reduced neovascularisation (**C**) and the ischaemic areas (**D**) leading to an increase in the revascularisation (**E**). (One-way ANOVA, Bonferroni *post hoc* test, $\Delta P<0.05$, $\Delta\Delta P<0.01$ compared to vehicle injected = 0 ng).

uninjected control *vs* 1 or 10 ng $VEGF_{121}b$ $P<0.05$ or control injection *vs* 1 or 10 ng $VEGF_{121}b$ $P<0.05$). Injection of 10 ng $VEGF_{121}b$ reduced the ischaemic area, and increased the area of normal vasculature (see Figure 6D-E, control injection *vs* 10 ng $VEGF_{121}b$ $P<0.05$ for ischaemic and normal vessel growth). The overall result was an increase in the proportion of normal vasculature in these retinae (see Figure 6E). Injection of control solution had no effect compared with contralateral eye (see Figure 6B uninjected control *vs* control injected eye, $P>0.05$, paired *t*-test).

DISCUSSION

The existence of a second family of VEGF isoforms with overtly different properties from that of the conventional VEGF species required a radical re-evaluation of VEGF biology, not only academically – thousands of manuscripts have attempted to quantify 'VEGF' in body fluids or tissues using primers, probes or antibodies that would not distinguish these contrasting families; but also in terms of the pathogenesis of disease and the potential clinical importance of VEGF$_{xxx}$/VEGF$_{xxx}$b balance, because these families derive from the same gene. VEGF$_{165}$b is widely expressed (Bates *et al*, 2002, 2006; Cui *et al*, 2004; Perrin *et al*, 2005; Pritchard-Jones *et al*, 2007; Varey *et al*, 2008) and may form the pre-dominant isoform in many tissues (Perrin *et al*, 2005; Varey *et al*, 2008). The physiological role of these isoforms is only just now being elucidated in normal tissues, and it seems that they act as a brake on excess angiogenesis during conditions of controlled growth or VEGF$_{xxx}$ expression. Examples include development of the ovary, where the VEGF$_{xxx}$b levels are maintained to prevent angiogenesis into the developing germ bud and so prevent formation of a testicular artery (VEGF$_{xxx}$b antibodies are angiogenic in this model, Artac *et al*, in press), in the virgin mammary fat pad, where VEGF$_{xxx}$ levels are prevented from inducing angiogenesis until lacteal formation, when VEGF$_{xxx}$b levels fall (and prevention of this fall results in reduced milk production) (Qiu *et al*, 2008), and in the eye where a relative drop in VEGF$_{xxx}$b levels is accompanied with angiogenesis in the retina in diabetes (Perrin *et al*, 2005). Increased VEGF$_{xxx}$b levels also seem to be required for normal pregnancy, as a failure to upregulate VEGF$_{xxx}$b in first trimester pregnancies is associated with subsequent maternal–fetal pathology (Bills *et al*, 2009).

The development of an angiogenic milieu within a stable and non-angiogenic tissue, may depend to some degree on changes in gene (over) transcription or suppression but also on changes in mRNA splicing control. The control of splicing between the two VEGF isoform families has begun to be elucidated, and it has been shown that multiple stimulatory signals can differentially affect the choice of splice site in the terminal exon, including hypoxia (Varey *et al*, 2008), IGF, TNF and PDGF (Nowak *et al*, 2008), which all favour proximal splice site selection in exon 8, and hence angiogenic isoforms, and the latter three growth factors suppress distal splicing, whereas hypoxia seems not to affect it. TGF-β, on the other hand, seems to favour distally spliced isoforms (Nowak *et al*, 2008). This seems to be through mechanisms involving splice factors such as ASF/SF2 and SRp55. Additionally, alteration of splicing has been seen in human and animal models of disease including in Denys Drash Syndrome (Schumacher *et al*, 2007), kidney, prostate and colon cancer (Bates *et al*, 2002; Woolard *et al*, 2004; Varey *et al*, 2008; Rennel *et al*, 2008a), malignant melanoma (Pritchard-Jones *et al*, 2007) where the PSS is favoured and animal models of glaucoma where the DSS is favoured (Ergorul *et al*, 2008). However, there is very little known about regulation of splice site choice in the terminal exon from the exon 5 splice donor site, but the earlier studies on hypoxia indicate that this is unlikely to be a regulatory factor for VEGF$_{121}$b.

The most studied VEGF$_{xxx}$b family member – VEGF$_{165}$b – has been clearly shown not to be angiogenic. Other researchers have shown that the six amino acids of exon 8b are required for the anti-angiogenic activity of VEGF$_{165}$b because artificially generated VEGF$_{159}$ lacking both exons 8a and b, which is not angiogenic (Cebe Suarez *et al*, 2006). Exon 8a seems to be required for heparin and neuropilin-1 binding and full activation of the VEGFR-1 and -2, as it is likely to interfere with the correct folding and 3D structure of the protein, resulting in differential activation of VEGFR-2 tyrosine phosphorylation, and hence downstream signalling (Cebe Suarez *et al*, 2006; Cebe-Suarez *et al*, 2008; Kawamura *et al*, 2008). Here, we present for the first time new data suggesting that the presence of exon 8b in a different VEGF$_{xxx}$b family member endows it with similar properties to VEGF$_{165}$b: VEGF$_{121}$b inhibits experimental endothelial cell migration *in vitro* and new vessel formation *in vivo* in tumour and non-tumour-related angiogenesis. The VEGF$_{xxx}$b family members seem to share similar properties.

We have shown that VEGF$_{121}$b has similar bio-distribution after i.v. injection to VEGF$_{165}$b and a similar proportion of injected bolus reaching a single implanted tumour – with around 2% for VEGF$_{121}$b and 6% for VEGF$_{165}$b (Rennel *et al*, 2008b). Furthermore, we have shown in this study and in our earlier work (Woolard *et al*, 2004) that these two VEGF$_{xxx}$b family members bind the conventional tyrosine kinase VEGF receptors. VEGF$_{165}$b does not bind neuropilin-1 (Kawamura *et al*, 2008), and there is evidence that VEGF$_{121}$ also binds neuropilin poorly, suggesting that VEGF$_{121}$b would not bind it either. von Wronski *et al* (2007) have highlighted the likelihood that neuropilin-1 binds the residues coded for by exon 8a and replacement by 8b results in VEGF isoforms that do not seem to show neuropilin-1 binding (Cebe Suarez *et al*, 2006).

Although we have shown that VEGF$_{165}$b and VEGF$_{121}$b bind to the conventional VEGF receptors, further work will be required to investigate the mechanistic differences between VEGF$_{121}$b and its corresponding isoform VEGF$_{121}$. Studies into the mechanistic contrast between VEGF$_{165}$b and VEGF$_{165}$ are more advanced with increasing evidence now emerging that VEGF$_{165}$b, although binding to VEGFR-2 with equal affinity as VEGF$_{165}$, is not simply a classical competitive inhibitor. VEGF$_{165}$b does stimulate the phosphorylation of VEGFR-2 but qualitatively in a unique way. Although VEGF$_{165}$b does lead to the phosphorylation of tyrosine residues of the VEGF receptor, tryptic phosphopeptide mapping and the use of phospho-site-specific antibodies have shown that VEGF$_{165}$b is considerably poorer than VEGF$_{165}$ in inducing phosphorylation of the angiogenic positive regulatory site Y1052 (Y1054 in human sequence) in VEGFR-2 (Kawamura *et al*, 2008). This study also confirmed that the ability of different VEGF isoforms to induce angiogenesis correlated with their abilities to bind the VEGF co-receptor neuropilin-1 (Kawamura *et al*, 2008). Whether or not VEGF$_{121}$b activates VEGFR-2 in a similar way is yet to be investigated, as are the potential effects of simultaneous VEGF$_{121}$b and VEGF$_{165}$b administration of receptor activity, angiogenesis or tumour growth.

Another significant finding here is that VEGF$_{121}$b seems to act as a survival factor for human endothelial cells. This activity seems to be VEGFR dependent, and uses classical survival pathways such as the PI3 kinase and MEK/ERK pathways. This provides a potential explanation for the surprising finding that the ischaemic region in the retinal neovascular preparations was reduced by VEGF$_{121}$b administration. This is of particular interest as it suggests a vascular normalisation in the retina, leading to the possibility that VEGF$_{121}$b may be of use in ischaemic retinal diseases associated with angiogenesis such as diabetic retinopathy.

In summary, we provide new information about the existence, expression and activity of a second member of the anti-angiogenic VEGF$_{xxx}$b family. Our data confirm that VEGF$_{121}$b has similar inhibitory properties on the process of *in vivo* angiogenesis and the components of angiogenesis *in vitro* as does VEGF$_{165}$b, supporting the notion that the replacement of the terminal six amino acids with those coded for by exon 8b is of crucial importance in converting the dominant pro-angiogenic growth factor into a positively anti-angiogenic molecule that may have broad therapeutic potential. Further studies will be required to elucidate its mechanism of action of VEGF$_{121}$b and its potential clinical value along with its other family member VEGF$_{165}$b.

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Conflict of interest

Professor Bates and Dr Harper are co-inventors on the patent describing $VEGF_{165}b$ as a potential therapeutic in cancer. There are no other conflicts of interest.

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