Published in final edited form as:
Nephron Physiol. 2008 ; 110(4): p57–p67. doi:10.1159/000177614.

The Alternatively Spliced Anti-Angiogenic Family of VEGF Isoforms $VEGF_{xxx}$bin Human Kidney Development

Heather S. Bevan[a], **Nynke M.S. van den Akker**[c], **Yan Qiu**[a], **Japke A.E. Polman**[c], **Rebecca R. Foster**[a], **Justin Yem**[a], **Ali Nishikawa**[a], **Simon C. Satchell**[b], **Steven J. Harper**[a], **Adriana C. Gittenberger-de Groot**[c], and **David O. Bates**[a]

[a]Microvascular Research Laboratories, Department of Physiology, University of Bristol, Bristol, UK [b]Academic Renal Unit, Southmead Hospital, Bristol, UK [c]Department of Anatomy and Embryology, Leiden University Medical Center, Leiden,The Netherlands

Abstract

Background/Aim—Vascular endothelial growth factor (VEGF), required for renal development, is generated by alternative splicing of 8 exons to produce two families, pro-angiogenic $VEGF_{xxx}$, formed by proximal splicing in exon 8 (exon 8a), and anti-angiogenic $VEGF_{xxx}$b, generated by distal splicing in exon 8 (exon 8b). $VEGF_{165}$b, the first described exon 8b-containing isoform, antagonises $VEGF_{165}$ and is anti-angiogenic in vivo.

Methods—Using $VEGF_{xxx}$b-specific antibodies, we investigated its expression quantitatively and qualitatively in developing kidney, and measured the effect of $VEGF_{165}$b on renal endothelial and epithelial cells.

Results—$VEGF_{xxx}$b formed 45% of total VEGF protein in adult renal cortex, and $VEGF_{165}$b does not increase glomerular endothelial cell permeability, it inhibits migration, and is cytoprotective for podocytes. During renal development, $VEGF_{xxx}$b was expressed in the condensed vesicles of the metanephros, epithelial cells of the comma-shaped bodies, invading endothelial cells and epithelial cells of the S-shaped body, and in the immature podocytes. Expression reduced as the glomerulus matured.

Conclusion—These results show that the anti-angiogenic $VEGF_{xxx}$b isoforms are highly expressed in adult and developing renal cortex, and suggest that the $VEGF_{xxx}$b family plays a role in glomerular maturation and podocyte protection by regulating the pro-angiogenic pro-permeability properties of $VEGF_{xxx}$ isoforms.

Keywords

Kidney development; Vascular endothelial growth factor $_{165}$b; Angiogenesis

Introduction

Vascular endothelial growth factor A (VEGF-A) is a potent angiogenic factor that induces endothelial cell migration, proliferation, differentiation and regeneration [1]. In kidneys of embryos to adults, VEGF-A is expressed in presumptive and mature glomerular epithelial cells (podocytes) and tubular epithelial cells [2-7]. Normal glomerulogenesis requires the

Dr. David O. Bates, Microvascular Research Laboratories, School of Veterinary Sciences, University of Bristol, Southwell Street, Bristol BS2 8EJ (UK), Tel. +44 117 928 9818, Fax +44 117 928 8151, E-Mail Dave.Bates@bris.ac.uk.

coordinated induction of epithelial differentiation, endothelial invasion, and growth of tubular and vascular tissues. In mice, specific overexpression or deletion of the *VEGF-A* gene in podocytes results in glomerular dysfunction [8, 9]. A podocyte-specific cre-recombinase knockout of even a single gene copy leads to nephrotic syndrome, uraemia and death 9 weeks post-partum, whilst complete knockouts died a few hours post-partum [8]. In mice, glomerular overexpression of the most widely studied isoform of VEGF-A, $VEGF_{165}$ results in death a few days post-partum with renal haemorrhages [8]. In VEGF inhibition studies, murine pups treated at postnatal day 0 with VEGF-blocking antibodies exhibit marked glomerular abnormalities, with many glomeruli lacking capillary tufts [4]. Similarly, treatment of murine pups with mFlt (1-3)-IgG (a soluble VEGF receptor-1 chimeric protein) postnatally on day 1 and day 8, results in marked glomerular defects, including loss of endothelial cells, mesangial matrix accumulation and hypocellularity [10]. These results suggest that tight control of VEGF-A expression is required for normal glomerular development and well-being.

The close temporal and spatial association of VEGF-A expression (by podocytes) and its receptors (on glomerular endothelial cells, GEnc) suggests that VEGF-A plays a pivotal role in the maintenance of glomerular integrity through the existence of a paracrine loop [11], and dysregulation of glomerular VEGF-A expression has been implicated in a wide range of renal diseases in humans [11]. Moreover, VEGF-A acts as an autocrine growth factor on both proliferating and differentiating glomerular visceral epithelial cells (podocytes) [9], and this results in prolonged survival and resistance to apoptosis, associated with changes in intracellular calcium concentration [12].

Isoforms of VEGF-A, termed according to their amino acid number, are generated by the differential splicing of eight exons of the full-length pre-mRNA from a single *VEGF-A* gene. The differential splicing of exons 6 and 7 generates isoforms with differing heparin-binding affinities [13], whilst the differential splicing of exon 8 (the terminal exon) generates two families of isoforms, pro-angiogenic and anti-angiogenic, which differ by only six amino acids at their C-terminus [14]. The pro-angiogenic VEGF-A isoforms, i.e. $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$ (collectively termed $VEGF_{xxx}$, where xxx is the number of amino acids encoded), are formed by the selection of a proximal splice site in exon 8, termed exon 8a, which results in an open reading frame of 6 amino acids being translated. The anti-angiogenic VEGF-A isoforms are generated by the use of a more distal splice site in exon 8, termed exon 8b, resulting in an open reading frame of the same number of nucleotides as proximal (or pro-angiogenic) splice variants, but encoding a different amino acid sequence. Thus, the resulting proteins are of the same amino acid length as the conventional isoforms and are collectively termed $VEGF_{xxx}b$ [15]. The first anti-angiogenic isoform to be identified from human renal cortex was $VEGF_{165}b$ [14]. $VEGF_{165}b$ inhibits $VEGF_{165}$ and hypoxia-driven angiogenesis in vivo in rat, rabbit and mouse models of physiological and pathological angiogenesis [16, 17]. $VEGF_{165}b$ does result in weak and tardy signalling through MAPK in microvascular endothelial cells in vitro [18] and induces a rapid but transient puff of fluid extravasation upon first exposure in intact microvessels in vivo but does not stimulate a sustained change in water permeability of microvessels [19]. $VEGF_{165}b$ therefore does appear to have a stimulatory physiological role. $VEGF_{xxx}b$ at the protein level appears to be the dominant isoform in many adult tissues, such as ocular tissues, colon and pancreatic islets [15 and Bates, unpubl. data]. $VEGF_{xxx}b$ may therefore play a role in defining the physiological phenotype of the normal mature glomerulus (high permeability to water, low to protein in the absence of angiogenesis).

In most studies of VEGF-A in developing or mature glomerulus, a role of $VEGF_{165}$ or of other pro-angiogenic splice variants has been investigated or assumed. Previous studies have used antibodies that detect both families of VEGF-A isoforms (pan-VEGF antibodies) as

there were no antibodies or probes that distinguished between the $VEGF_{xxx}$ and $VEGF_{xxx}b$ families of isoforms. The only published report of $VEGF_{165}b$ expression in developing kidney does show a significant alteration in terminal exon splicing in Denys-Drash syndrome, underlying a potential role for $VEGF_{165}b$ in human glomerular developmental diseases [20]. Therefore, $VEGF_{xxx}b$ isoform expression during normal renal development and the roles it might play are unknown. In this study, we examined expression of $VEGF_{xxx}b$ in metanephric kidneys from human fetuses, and performed parallel in vitro experiments to understand the role of $VEGF_{xxx}b$ on cell types involved in glomerular function.

Materials and Methods

Tissue Source

Human adult renal cortex was collected from the normal pole of unilateral, unipolar renal carcinoma nephrectomy specimens with local ethics committee approval (Bristol). Three human female fetuses of 10 and 12 weeks' pregnancy were obtained with local ethics committee approval (Leiden).

Immunohistochemistry and ELISA

Sections were microwave-heated in 0.01 m_M citric buffer saturated sodium citrate pH buffer (pH 6.0) for either 12 min at 95°C ($VEGF_{xxx}b$), or for 7 min at 800 W followed by 9 min at 120 W (pan-VEGF staining). Sections were washed twice with PBS, incubated with 3% hydrogen peroxide solution for 20 min, washed again, blocked with 10% BSA (Sigma; A4378) in 0.05% Tween-PBS (TBS), and then with 1.5% normal horse serum (NHS, Vector Lab; S-2000) in TBS (1 h). Sections were incubated with 8 μg/ml primary antibody (MAB3045, R&D Systems; Sigma, I8765, or Santa Cruz, 7269) in TBS (pH 7.4) with 1% BSA, washed twice with TBS, blocked again, then incubated with secondary antibody (Vector Lab; BA2000, 1:200 dilution in NHS) in TBS for 1 h, washed twice, then incubated with Vectastain ABC solution (Vector Lab; PK4000) for 45 min.

Cytotoxicity, ELISA Flow Cytometry and Migration Assays

VEGF ELISA [21], cytotoxicity [12], apoptosis [22], and migration [23] were determined as previously described.

Culture of GEnC

GEnC derived from decapsulated glomeruli isolated from normal human kidney (according to the supplier's data sheet) were obtained at passage 2 from the Applied Cell Biology Research Institute (ACBRI, Kirkland, Wash., USA). Cells were cultured in endothelial growth medium 2 - microvascular (EGM2-MV; Cambrex, Wokingham, UK), made up from endothelial basal medium 2 (EBM2; Cambrex) and fetal calf serum (5%), antimicrobial agents and growth factors as supplied. Cells being prepared for, or being used in, experiments were cultured in EGM2-MV without VEGF.

Measurement of Trans-Endothelial Electrical Resistance

Trans-endothelial electrical resistance (TEER) is a measure of ion flux and is inversely related to the fractional area of pathways open to water and small molecules across a cell monolayer. Tissue culture inserts containing polycarbonate supports (0.4 μm pore size, Nalge Nunc International, Rochester, N.Y., USA) were seeded with GEnC at 100,000 cells/cm^2. Measurement of TEER of GEnC monolayers was performed using an Endohm 12 electrode chamber and EVOMx voltmeter (World Precision Instruments, Sarasota, Fla., USA) as previously described [24]. Medium was replaced with serum-free medium (EBM2).

Baseline TEER was measured after 1 h and the culture medium was again replaced, this time with SFM alone (control) or containing 1 nM $VEGF_{165}$ (R&D Systems) or 1 nM $VEGF_{165}b$. TEER was remeasured at 15, 30 and 60 min. Previous work has demonstrated a peak response to VEGF between 30 and 60 min in this assay.

Results

$VEGF_{xxx}b$ Expression in Adult Renal Cortex

To determine quantitatively the contribution of $VEGF_{xxx}b$ isoforms to the total VEGF expression in normal adult kidneys, $VEGF_{xxx}b$ and total VEGF were measured in protein extracted from freshly frozen renal cortex. Total protein was measured using the commercially available ELISA, and $VEGF_{xxx}b$ levels measured by a comparable ELISA but using a biotinylated detection antibody specific to the C terminus of $VEGF_{xxx}b$. Total VEGF concentrations in normal renal cortex averaged of 54.2 ± 14 ng/mg protein. $VEGF_{xxx}b$ concentrations averaged 25.8 ± 9.6 ng/mg (n = 3; fig. 1), or 45 ± 5% of the total VEGF. This was similar to the relative proportion of total VEGF that was $VEGF_{165}b$ measured in protein extracted from normal isolated glomeruli collected from human nephrectomy specimens (46.6 ± 18%, n = 3).

$VEGF_{xxx}b$ Staining in Adult Kidney

The antibody to $VEGF_{xxx}b$ used for immunohistochemistry is an affinity-purified mouse monoclonal IgG_1 antibody, Cat MAB3045, commercially available through R&D Systems, which has been characterised previously [15, 16, 25]. It binds recombinant $VEGF_{165}b$, and shows expression of $VEGF_{165}b$, $VEGF_{189}b$, $VEGF_{121}b$, $VEGF_{183}b$ and $VEGF_{145}b$, collectively termed $VEGF_{xxx}b$, but not $VEGF_{165}$. Western blotting has previously shown that all the proteins recognised by this antibody are also recognised by commercial antibodies raised against VEGF-A. This antibody does not recognise the $VEGF_{xxx}$ isoforms, but does recognise recombinant $VEGF_{165}b$ and $VEGF_{121}b$, conclusively demonstrating that this antibody is specific for $VEGF_{xxx}b$ [16]. $VEGF_{xxx}b$ staining was limited to a significant proportion of podocytes (fig. 2a), but present in parietal epithelial cells, macula densa and proximal and distal tubules of the renal cortex (fig. 2b). $VEGF_{xxx}b$ staining was also observed in the vasa recta, collecting ducts and ascending thin and thick loop of Henle (fig. 2b). In the epithelial cells of the ascending thick loop of Henle, strong intracellular staining was observed, whereas in the epithelial cells of the collecting ducts, staining was highly localised to the tips of the apical surface (short arrow) and to the basolateral cytoplasm (long arrow, fig. 2c). A similar trend was observed for pan-VEGF staining (fig. 2d-f). Staining was never seen when an isotype-matched IgG antibody was used as a control (fig. 2g-i) under the same conditions.

$VEGF_{xxx}b$ Staining in Developing Glomerulus

To investigate $VEGF_{165}b$ expression in the developing glomerulus, immunohistochemistry was carried out on sections of human fetal renal tissue. Immunohistochemical staining for $VEGF_{xxx}b$ of 10- and 12-week-old fetuses showed clear expression in the developing nephron that was noticeably stronger than the surrounding mesenchyme (fig. 3a, 10 weeks; fig. 3b, 12 weeks). Staining was very intense in all stages of nephrogenesis from the condensed vesicle stage onwards (fig. 4a-d). Staining with an antibody to all isoforms of VEGF confirmed that VEGF was located throughout the developing kidney (fig. 3c, 10 weeks; fig. 3d, 12 weeks). Interestingly, there were no areas of the kidney that stained for $VEGF_{xxx}b$ but did not stain for pan-VEGF. In contrast, there were a number of areas, including in the mesenchyme, where $VEGF_{xxx}b$ antibodies did not detect expression, but the pan-VEGF antibody did (fig. 4a vs. fig. 4e). No staining was seen using any isotype-matched affinity-purified mouse IgG (fig. 3e, 10 weeks; fig. 3f, 12 weeks).

In the condensed vesicle (fig. 4b), $VEGF_{xxx}b$ staining was greater than the surrounding mesenchyme (fig. 4a, b). The greatest intensity of staining could be seen during epithelialisation, and staining was highest in both the apical and basolateral parts of the primitive epithelial cell and weakest in the nuclear regions, indicating a cytoplasmic subcellular localisation. Pan-VEGF staining was also apparent in these regions in the condensed vesicle (fig. 4e, f). As development proceeded to comma- (fig. 4c, g) and S-shaped bodies (fig. 4d, h), $VEGF_{xxx}b$ staining (fig.4c, d) became even more restricted to the apical and basolateral parts of the primitive columnar epithelial cells of the developing nephron, as did pan-VEGF staining (fig. 4g, h). Of note was the more diffuse $VEGF_{xxx}b$ staining in the glomerular cleft (fig. 4d, h), the site at which endothelial cells will invade. This pattern of staining, observed in the primitive epithelial cells and glomerular cleft appeared to be more diffuse in the capillary loop stage of glomerulogenesis (fig. 5a), in contrast to pan-VEGF which appeared stronger (fig. 5a in comparison with 5b contrasts with 4d in comparison with 4h). As the glomerulus was formed (fig. 5c), $VEGF_{xxx}b$ staining appeared to diminish in the developing glomerular visceral epithelial (podocytes) and endothelial cells (fig. 5e), but there was still marked staining in the parietal epithelial cells lining Bowman's capsule, and cells of the maculae densa (fig. 5e). Pan-VEGF expression appeared to be maintained through glomerular maturation, and stained up more glomerular epithelial cells than $VEGF_{xxx}b$ (fig. 5d, f). In comparison, in the mature glomeruli, $VEGF_{xxx}b$ staining was limited to a subpopulation of podocytes (fig. 2a). This appeared to be true for pan-VEGF staining too, but the pan-VEGF antibody identified more podocytes than the $VEGF_{xxx}b$ antibody (fig. 2d).

$VEGF_{xxx}b$ Staining in Developing Tubules of the Primitive Renal Cortex

Throughout the developmental stages, examined $VEGF_{xxx}b$ staining was clearly seen in both the proximal and distal portions of the convoluted tubules (fig. 6a). More specifically, staining was seen both in the apical and basolateral parts of the primitive epithelial cells. In addition, staining was seen in all areas of tubule development in the renal cortex. Comparable pan-VEGF staining was seen in the tubules of the developing renal cortex (fig. 6b).

$VEGF_{xxx}b$ Staining in the Primitive Renal Medulla

$VEGF_{xxx}b$ staining appeared to be more specifically localised in the primitive epithelial cells of the developing nephron (fig. 6c), whereas pan-VEGF staining was intense and widespread throughout the renal medulla (fig. 6d), $VEGF_{xxx}b$ staining was seen in both the apical and basolateral sides, but not as intense in the central, perinuclear regions in the epithelial cells of the distal tubules extending into the medulla and of the collecting ducts (fig. 6c). Furthermore, it appeared that where the more distal portions of the convoluted tubules differentiate into their specialised transporting segments (the thin and thick loop of Henle) $VEGF_{xxx}b$ staining was less intense (fig. 6c). Weak $VEGF_{xxx}b$ staining was also observed in the endothelial cells of the vasa recta (fig. 6c).

The Effect of $VEGF_{165}b$ on Human Glomerular and Endothelial Cells in vitro

Alterations in expression may reflect changes in function in the embryo, in the adult and in disease. The role of $VEGF_{xxx}b$ in the developing human kidney is not known. Although $VEGF_{165}b$ has been shown to inhibit endothelial cell migration in response to $VEGF_{165}$, it is not known whether this inhibition can be balanced by controlling the expression level of VEGF isoforms. To determine whether $VEGF_{165}b$ could dose-dependently affect endothelial cells in vitro, the effect of $VEGF_{165}b$ on human endothelial cell migration was estimated. Figure 7a shows that $VEGF_{165}b$ dose-dependently inhibited HUVEC migration responses to $VEGF_{165}$, with an IC_{50} of 0.29 ± 0.03-fold excess (i.e. 40 ng/ml $VEGF_{165}$ was 50% inhibited by 11.4 ± 1.4 ng/ml $VEGF_{165}b$, n = 3). This is consistent with

downregulation of $VEGF_{165}b$ during the endothelial invasion phase of glomerular development. To determine whether $VEGF_{165}b$ might have positive benefits during glomerular development, we measured the effect of $VEGF_{165}b$ on podocyte cytotoxicity. $VEGF_{165}b$ dose-dependently decreased cytotoxicity of primary cultured podocytes (fig. 7b) with an EC_{50} of 107 ± 1.2 pM, showing that $VEGF_{165}b$ had a cytoprotective effect (n = 8). The LDH assay measures only the number of cells releasing a cytoplasmic protein and hence does not distinguish between apoptosis and necrosis. Interestingly, $VEGF_{165}b$ did not affect podocyte cell proliferation ($16.5 \pm 1.1 \times 10^3$ cpm/cell compared with $15.8 \pm 1.0 \times 10^3$ cpm/cell, n = 6), thus suggesting an anti-apoptotic effect on human podocytes. This was confirmed by flow cytometry using annexin V and propidium iodide staining (fig. 7c). Whereas serum starvation induced a significant proportion of the cells to undergo apoptosis (region A in fig. $7c_1$), this was inhibited by treatment with 0.3 nM $VEGF_{165}b$ (fig. $7c_2$). To determine whether $VEGF_{165}b$ could affect glomerular endothelial barrier function a trans-electrical endothelial resistance (TEER) in vitro assay of glomerular endothelial permeability was used. Although $VEGF_{165}$ significantly reduced glomerular endothelial TEER (indicating an increase in monolayer permeability), $VEGF_{165}b$ resulted in a significant increase in TEER, and $VEGF_{165}b$ inhibited the increase induced by $VEGF_{165}$ (fig. 7d, n = 4). Thus in contrast to $VEGF_{165}$, $VEGF_{165}b$ prevents endothelial cell migration and reduces monolayer permeability, while maintaining podocyte cell survival in vitro.

Discussion

The role of VEGF in renal function and development has been the subject of intense scrutiny since VEGF expression was demonstrated in the renal cortex and medulla by antibody staining, RT-PCR, in situ hybridisation and Northern blotting, in both normal and disease states. VEGF is highly expressed in the kidney - more so than nearly any other tissue, but very few studies have accounted for the $VEGF_{xxx}b$ variants that are anti-angiogenic [16, 20, 25]. mRNA encoding the $VEGF_{xxx}b$ splice variants were first described in normal renal cortex, and $VEGF_{165}b$ protein was first identified in human podocytes by isoform-specific siRNA [26]. The experiments described here, however, are the first to quantitate the contribution of $VEGF_{xxx}b$ to the total VEGF expressed. The finding that, in normal renal cortex, almost half of the VEGF found is $VEGF_{xxx}$ has significant implications for our interpretation of the many studies that have investigated VEGF expression in normal renal tissues and disease states [15]. The finding that $VEGF_{xxx}b$ isoformsw are a highly significant component of the total VEGF in renal tissues implies an as yet unknown physiological relevance.

Pan-VEGF and $VEGF_{xxx}b$ Staining Patterns Compared

VEGF-A, both mRNA and/or protein, of unknown isoform family has been detected in the presumptive and mature podocytes and primitive columnar epithelial cells of the developing nephron, in both rodent and human tissues [2, 4-7, 27, 28]. In this study, we sought to determine the presence and localisation of $VEGF_{xxx}b$ proteins in human metanephric kidneys and compare its spatiotemporal staining pattern to that detected by pan-VEGF antibodies. We detected $VEGF_{xxx}b$ in metanephric kidneys from 10- and 12-week fetuses using immunohistochemical staining. Our pan-VEGF staining of the metanephric kidney is in close agreement with previous studies; VEGF was detected in presumptive and mature podocytes and in the primitive columnar epithelial cells of the nephron [2, 4-7, 29].$VEGF_{xxx}b$ isoforms appear to be present in a subset of cells that express VEGF, as there were no areas in the metanephric kidney that stained for $VEGF_{xxx}b$ isoforms but not for pan-VEGF but some areas that stained positively for pan-VEGF but not for $VEGF_{xxx}b$. In the adult kidney, the presence of VEGF in the convoluted tubules is in contrast to in situ hybridisation studies, which show in adult tissues the primary source of renal VEGF

synthesis to be the podocyte [30], suggesting the possibility of glomerular-derived VEGF protein uptake by tubular cells.

Glomerulogenesis and $VEGF_{xxx}b$

Glomerulogenesis is dependent on reciprocally inductive interactions between renal endothelial cells and nephron epithelial cells, but although various genes [31-34] and growth factors [9, 11, 35-37] have been implicated at specific stages, the molecular regulators of the cell differentiation events are poorly understood. A dosage sensitivity to VEGF exists within the developing glomerulus [8], similar to that seen when VEGF expression was manipulated throughout the embryo [38, 39]. As $VEGF_{165}b$ has been shown to counteract some of the effects of $VEGF_{165}s$, and has a dose-dependent effect on podocyte survival, it is likely that dosage sensitivity of glomerulogenesis to $VEGF_{xxx}b$ may also be a critical component of normal renal cortex formation, and a recent study showing that transgenic mice over-expressing $VEGF_{165}b$ in mouse podocytes have reduced glomerular permeability characteristics supports this suggestion [40].

Previous Studies on VEGF

Apart from the original isolation of $VEGF_{165}b$ mRNA from renal cortex [14] and protein in the glomeruli [16], and the identification of $VEGF_{165}b$ mRNA and protein in differentiated, but not proliferating conditionally immortalised podocyte cell lines [26], previously used methodologies either did not detect $VEGF_{xxx}b$ isoforms (RT-PCR using primers in the proximal part of exon 8), or did not distinguish $VEGF_{xxx}b$ isoforms from $VEGF_{xxx}$ isoforms. The only study that has addressed this examined microdissected mRNA from fetal, child and adult glomeruli, and found that expression of $VEGF_{165}b$ mRNA was lower in the S- and C-shaped bodies than in adult or child glomeruli. The decreasing protein expression we see here from condensed vesicle through S- and comma-shaped bodies to immature glomeruli may therefore be a result of this endogenous downregulation at the mRNA level, temporally shifted slightly, as the VEGF protein is turned over more slowly than the mRNA. Schumacher et al. [20] also noted higher $VEGF_{165}b$ expression in the adult glomeruli compared with $VEGF_{165}$. Unfortunately, antibodies that specifically detect $VEGF_{xxx}$ isoforms are not yet available, but it appears likely that most of the VEGF staining in adult glomeruli is $VEGF_{165}b$. Interestingly, in that study, Schumacher et al. [20] demonstrate a complete loss of $VEGF_{165}b$ in Denys-Drash glomeruli, indicating a link to WT1, a finding recently confirmed by over-expression studies in vitro [41]. Podocyte-specific knockout of VEGF during development resulted in a lack of formation of glomeruli and renal failure immediately after birth followed by death within 6 h [8], presumably because endothelial cells fail to migrate into the glomerulus (as is evidenced by a lack of phenotypically discernable endothelial cells in the glomerulus), and thus aberrant microvessel formation and glomerular filtration. VEGF knockouts, however, also are $VEGF_{xxx}b$ knockouts, so it is not clear which part of the phenotype is dependent on $VEGF_{xxx}b$ knockout. Inhibitors of VEGF such as VEGF-TRAP [42], sFlt-1 [43], bevacizumab [44] and other monoclonal antibodies to VEGF, shown to affect renal function, are also likely to affect the $VEGF_{xxx}b$ isoforms. Therefore, it is not clear whether the results in studies previously carried out on the inhibitory role of VEGF in glomerular function were due to the pro-angiogenic isoforms, or the anti-angiogenic isoforms, or both.

Possible Functions of Renal $VEGF_{xxx}b$ Expression

$VEGF_{165}b$ inhibits $VEGF_{165}$-mediated endothelial cell proliferation and migration in vitro and vasodilatation in isolated arteries ex vivo [14, 16], $VEGF_{165}$-mediated physiological angiogenesis in the mesentery and the eye [16], the chicken chorioallantoic membrane and the dorsal skin chamber in mice [18], pathological VEGF-mediated angiogenesis in tumour models [16], and hypoxia-driven retinal angiogenesis in the eye in vivo [17]. $VEGF_{165}b$ has

Europe PMC Funders Author Manuscripts

been shown to have both dominant negative and partial agonist activity on endothelial-mediated signalling [16, 18], potentially explaining its ability to both inhibit migration and protect against cytotoxicity. In contrast, no effect of $VEGF_{165}b$ was seen on glomerular endothelial monolayer integrity in vitro (fig. 7c). In the developing kidney, $VEGF_{xxx}$ isoforms are thought to mediate endothelial cell survival and migration, microvascular permeability [9] and perhaps epithelial cell survival [12]. The results shown here are consistent with the concept that the $VEGF_{xxx}b$ isoforms also support epithelial cell survival, without increased permeability, and are downregulated during endothelial cell migration presumably to allow invasion into the glomerular cleft.

Eremina et al. [8] have shown that unrestricted expression of $VEGF_{165}$ during development is significantly detrimental, which taken together with these results suggest a balance of pro-angiogenic/anti-angiogenic VEGF-A is required for normal development and function [8, 9, 31, 45, 46]. Expression of $VEGF_{xxx}b$ isoforms and crucially the control of distal and proximal 3′-end splicing control during kidney development may therefore play a significant role in the modulation of $VEGF_{xxx}$-driven responses. $VEGF_{xxx}b$ may play a modulatory role in the developing kidney. For example, factors must limit and halt the endothelial cell invasion into the glomerular cleft at the primitive glomerulus and subsequent stages of glomerular development. To address this hypothesis, further investigation is required including conditional transgenic knock-out and over-expressing models that are designed to take account of both sides of the VEGF-A biology - angiogenesis and permeability - and perhaps, more importantly, their inhibition.

Acknowledgments

This work was supported by the Wellcome Trust (69029) and the British Heart Foundation (BB2000003).

References

1. Carmeliet P. Angiogenesis in health and disease. Nat Med. 2003; 9:653–660. [PubMed: 12778163]
2. Robert B, Zhao X, Abrahamson DR. Coexpression of neuropilin-1, Flk1, and VEGF (164) in developing and mature mouse kidney glomeruli. Am J Physiol Renal Physiol. 2000; 279:F275–F282. [PubMed: 10919846]
3. Robert B, St John PL, Hyink DP, Abrahamson DR. Evidence that embryonic kidney cells expressing flk-1 are intrinsic, vasculogenic angioblasts. Am J Physiol. 1996; 271:F744–F753. [PubMed: 8853438]
4. Kitamoto Y, Tokunaga H, Tomita K. Vascular endothelial growth factor is an essential molecule for mouse kidney development: glomerulogenesis and nephrogenesis. J Clin Invest. 1997; 99:2351–2357. [PubMed: 9153276]
5. Kaipainen A, Korhonen J, Pajusola K, Aprelikova O, Persico MG, Terman BI, Alitalo K. The related FLT4, FLT1, and KDR receptor tyrosine kinases show distinct expression patterns in human fetal endothelial cells. J Exp Med. 1993; 178:2077–2088. [PubMed: 8245783]
6. Dumont DJ, Fong GH, Puri MC, Gradwohl G, Alitalo K, Breitman ML. Vascularization of the mouse embryo: a study of flk-1, tek, tie, and vascular endothelial growth factor expression during development. Dev Dyn. 1995; 203:80–92. [PubMed: 7647376]
7. Breier G, Albrecht U, Sterrer S, Risau W. Expression of vascular endothelial growth factor during embryonic angiogenesis and endothelial cell differentiation. Development. 1992; 114:521–532. [PubMed: 1592003]
8. Eremina V, Sood M, Haigh J, Nagy A, Lajoie G, Ferrara N, Gerber HP, Kikkawa Y, Miner JH, Quaggin SE. Glomerular-specific alterations of VEGF-A expression lead to distinct congenital and acquired renal diseases. J Clin Invest. 2003; 111:707–716. [PubMed: 12618525]
9. Eremina V, Quaggin SE. The role of VEGF-A in glomerular development and function. Curr Opin Nephrol Hypertens. 2004; 13:9–15. [PubMed: 15090854]

10. Gerber HP, Hillan KJ, Ryan AM, Kowalski J, Keller GA, Rangell L, Wright BD, Radtke F, Aguet M, Ferrara N. VEGF is required for growth and survival in neonatal mice. Development. 1999; 126:1149–1159. [PubMed: 10021335]
11. Schrijvers BF, Flyvbjerg A, De Vriese AS. The role of vascular endothelial growth factor (VEGF) in renal pathophysiology. Kidney Int. 2004; 65:2003–2017. [PubMed: 15149314]
12. Foster RR, Hole R, Anderson K, Satchell SC, Coward RJ, Mathieson PW, Gillatt DA, Saleem MA, Bates DO, Harper SJ. Functional evidence that vascular endothelial growth factor may act as an autocrine factor on human podocytes. Am J Physiol Renal Physiol. 2003; 284:F1263–F1273. [PubMed: 12620928]
13. Houck KA, Ferrara N, Winer J, Cachianes G, Li B, Leung DW. The vascular endothelial growth factor family: identification of a fourth molecular species and characterization of alternative splicing of RNA. Mol Endocrinol. 1991; 5:1806–1814. [PubMed: 1791831]
14. Bates DO, Cui TG, Doughty JM, Winkler M, Sugiono M, Shields JD, Peat D, Gillatt D, Harper SJ. VEGF165b, an inhibitory splice variant of vascular endothelial growth factor, is down-regulated in renal cell carcinoma. Cancer Res. 2002; 62:4123–4131. [PubMed: 12124351]
15. Perrin RM, Konopatskaya O, Qiu Y, Harper S, Bates DO, Churchill AJ. Diabetic retinopathy is associated with a switch in splicing from anti- to pro-angiogenic isoforms of vascular endothelial growth factor. Diabetologia. 2005; 48:2422–2427. [PubMed: 16193288]
16. Woolard J, Wang WY, Bevan HS, Qiu Y, Morbidelli L, Pritchard-Jones RO, Cui TG, Sugiono M, Waine E, Perrin R, Foster R, Digby-Bell J, Shields JD, Whittles CE, Mushens RE, Gillatt DA, Ziche M, Harper SJ, Bates DO. VEGF165b, an inhibitory vascular endothelial growth factor splice variant: mechanism of action, in vivo effect on angiogenesis and endogenous protein expression. Cancer Res. 2004; 64:7822–7835. [PubMed: 15520188]
17. Konopatskaya O, Churchill AJ, Harper SJ, Bates DO, Gardiner TA. $VEGF_{165}b$, an endogenous C-terminal splice variant of VEGF, inhibits retinal neovascularisation in mice. Mol Vis. 2006; 12:626–632. [PubMed: 16735996]
18. Cebe Suarez S, Pieren M, Cariolato L, Arn S, Hoffmann U, Bogucki A, Manlius C, Wood J, Ballmer-Hofer K. A VEGF-A splice variant defective for heparan sulfate and neuropilin-1 binding shows attenuated signaling through VEGFR-2. Cell Mol Life Sci. 2006; 63:2067–2077. [PubMed: 16909199]
19. Glass CA, Harper SJ, Bates DO. The anti-angiogenic VEGF isoform VEGF165b transiently increases hydraulic conductivity, probably through VEGF receptor 1 in vivo. J Physiol. 2006; 572:243–257. [PubMed: 16423853]
20. Schumacher V, Jeruschke S, Eitner F, Becker J, Pitschke G, Ince C, Miner JH, Leuschner I, Engers R, Everding AS, Bulla M, Royer-Pokora B. Impaired glomerular maturation and lack of VEGF165b in Denys-Drash syndrome. J Am Soc Nephrol. 2007; 18:719–729. [PubMed: 17267748]
21. Varey AH, Rennel ES, Qiu Y, Bevan HS, Perrin RM, Raffy S, Dixon AR, Paraskeva C, Zaccheo O, Hassan AB, Harper SJ, Bates DO. $VEGF_{165}b$, an antiangiogenic VEGF-A isoform, binds and inhibits bevacizumab treatment in experimental colorectal carcinoma: balance of pro- and antiangiogenic VEGF-A isoforms has implications for therapy. Br J Cancer. 2008; 98:1366–1379. [PubMed: 18349829]
22. Foster RR, Saleem MA, Mathieson PW, Bates DO, Harper SJ. Vascular endothelial growth factor and nephrin interact and reduce apoptosis in human podocytes. Am J Physiol Renal Physiol. 2005; 288:F48–F57. [PubMed: 15339792]
23. Rennel ES, H-Zadeh MA, Wheatley E, Schüler Y, Kelly SP, Cebe Suarez S, Ballmer-Hofer K, Stewart L, Bates DO, Harper SJ. Recombinant human $VEGF_{165}b$ protein is an effective anti-cancer agent in mice. Eur J Cancer. 2008; 44:1883–1894. [PubMed: 18657413]
24. Satchell SC, Anderson KL, Mathieson PW. Angiopoietin 1 and vascular endothelial growth factor modulate human glomerular endothelial cell barrier properties. J Am Soc Nephrol. 2004; 15:566–574. [PubMed: 14978158]
25. Bates DO, Macmillan PP, Manjaly JG, Qiu Y, Hudson SJ, Bevan HS, Hunter AJ, Soothill PW, Read M, Donaldson LF, Harper SJ. The endogenous anti-angiogenic family of splice variants of VEGF, VEGFxxxb, are down-regulated in pre-eclamptic placentae at term. Clin Sci (Lond). 2006; 110:575–585. [PubMed: 16451124]

26. Cui TG, Foster RR, Saleem M, Mathieson PW, Gillatt DA, Bates DO, Harper SJ. Differentiated human podocytes endogenously express an inhibitory isoform of vascular endothelial growth factor (VEGF165b) mRNA and protein. Am J Physiol Renal Physiol. 2004; 286:F767–F773. [PubMed: 14644752]
27. Simon M, Rockl W, Hornig C, Grone EF, Theis H, Weich HA, Fuchs E, Yayon A, Grone HJ. Receptors of vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) in fetal and adult human kidney: localization and [125I]VEGF binding sites. J Am Soc Nephrol. 1998; 9:1032–1044. [PubMed: 9621286]
28. Tufro A, Norwood VF, Carey RM, Gomez RA. Vascular endothelial growth factor induces nephrogenesis and vasculogenesis. J Am Soc Nephrol. 1999; 10:2125–2134. [PubMed: 10505689]
29. Simon M, Grone HJ, Johren O, Kullmer J, Plate KH, Risau W, Fuchs E. Expression of vascular endothelial growth factor and its receptors in human renal ontogenesis and in adult kidney. Am J Physiol. 1995; 268:F240–F250. [PubMed: 7864162]
30. Bailey E, Bottomley MJ, Westwell S, Pringle JH, Furness PN, Feehally J, Brenchley PE, Harper SJ. Vascular endothelial growth factor mRNA expression in minimal change, membranous, and diabetic nephropathy demonstrated by non-isotopic in situ hybridisation. J Clin Pathol. 1999; 52:735–738. [PubMed: 10674029]
31. Sadl V, Jin F, Yu J, Cui S, Holmyard D, Quaggin S, Barsh G, Cordes S. The mouse Kreisler (Krml1/MafB) segmentation gene is required for differentiation of glomerular visceral epithelial cells. Dev Biol. 2002; 249:16–29. [PubMed: 12217315]
32. Loughna S, Hardman P, Landels E, Jussila L, Alitalo K, Woolf AS. A molecular and genetic analysis of renal glomerular capillary development. Angiogenesis. 1997; 1:84–101. [PubMed: 14517396]
33. Takemoto M, He L, Norlin J, Patrakka J, Xiao Z, Petrova T, Bondjers C, Asp J, Wallgard E, Sun Y, Samuelsson T, Mostad P, Lundin S, Miura N, Sado Y, Alitalo K, Quaggin SE, Tryggvason K, Betsholtz C. Large-scale identification of genes implicated in kidney glomerulus development and function. EMBO J. 2006; 25:1160–1174. [PubMed: 16498405]
34. Wong MA, Cui S, Quaggin SE. Identification and characterization of a glomerular-specific promoter from the human nephrin gene. Am J Physiol Renal Physiol. 2000; 279:F1027–F1032. [PubMed: 11097620]
35. Robert B, Abrahamson DR. Control of glomerular capillary development by growth factor/receptor kinases. Pediatr Nephrol. 2001; 16:294–301. [PubMed: 11322380]
36. Dolan V, Hensey C, Brady HR. Diabetic nephropathy: renal development gone awry? Pediatr Nephrol. 2003; 18:75–84. [PubMed: 12579392]
37. Bernhardt WM, Schmitt R, Rosenberger C, Munchenhagen PM, Grone HJ, Frei U, Warnecke C, Bachmann S, Wiesener MS, Willam C, Eckardt KU. Expression of hypoxia-inducible transcription factors in developing human and rat kidneys. Kidney Int. 2006; 69:114–122. [PubMed: 16374431]
38. Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, Fahrig M, Vandenhoeck A, Harpal K, Eberhardt C, Declercq C, Pawling J, Moons L, Collen D, Risau W, Nagy A. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature. 1996; 380:435–439. [PubMed: 8602241]
39. Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, O'Shea KS, Powell-Braxton L, Hillan KJ, Moore MW. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature. 1996; 380:439–442. [PubMed: 8602242]
40. Ferguson J, Qiu Y, Sage LM, Neal CR, Bates DO, Harper SJ, Salmon AH. Ultrafiltration co-efficient in isolated intact glomeruli from podocyte specific $VEGF_{165}B$ over-expressing transgenic mice. Microcirculation. 2008 in press.
41. Amin EM, Nowak DG, Saleem M, Bates D, Ladomery M. Relationship between WT1 and vascular endothelia l growth factor (VEGF) splicing. Microcirculation. 2008 in press.
42. Holash J, Davis S, Papadopoulos N, Croll SD, Ho L, Russell M, Boland P, Leidich R, Hylton D, Burova E, Ioffe E, Huang T, Radziejewski C, Bailey K, Fandl JP, Daly T, Wiegand SJ, Yancopoulos GD, Rudge JS. VEGF-Trap: a VEGF blocker with potent antitumor effects. Proc Natl Acad Sci USA. 2002; 99:11393–11398. [PubMed: 12177445]

43. Kendall RL, Wang G, Thomas KA. Identification of a natural soluble form of the vascular endothelial growth factor receptor, FLT-1, and its heterodimerization with KDR. Biochem Biophys Res Commun. 1996; 226:324–328. [PubMed: 8806634]
44. Presta LG, Chen H, O'Connor SJ, Chisholm V, Meng YG, Krummen L, Winkler M, Ferrara N. Humanization of an anti-vascular endothelial growth factor monoclonal antibody for the therapy of solid tumors and other disorders. Cancer Res. 1997; 57:4593–4599. [PubMed: 9377574]
45. Belteki G, Haigh J, Kabacs N, Haigh K, Sison K, Costantini F, Whitsett J, Quaggin SE, Nagy A. Conditional and inducible transgene expression in mice through the combinatorial use of Cre-mediated recombination and tetracycline induction. Nucleic Acids Res. 2005; 33:e51. [PubMed: 15784609]
46. Quaggin SE. Transcriptional regulation of podocyte specification and differentiation. Microsc Res Tech. 2002; 57:208–211. [PubMed: 12012385]

Europe PMC Funders Author Manuscripts

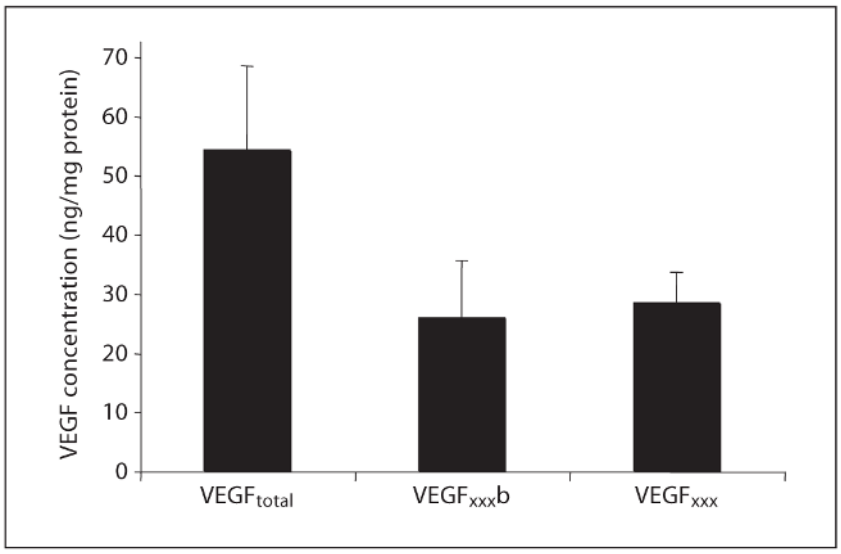

Fig. 1.
Expression of VEGF and its two families of isoforms in normal human renal cortex. $VEGF_{xxx}b$ isoforms comprise over 45% of total VEGF in adult human renal cortical tissues.

Europe PMC Funders Author Manuscripts

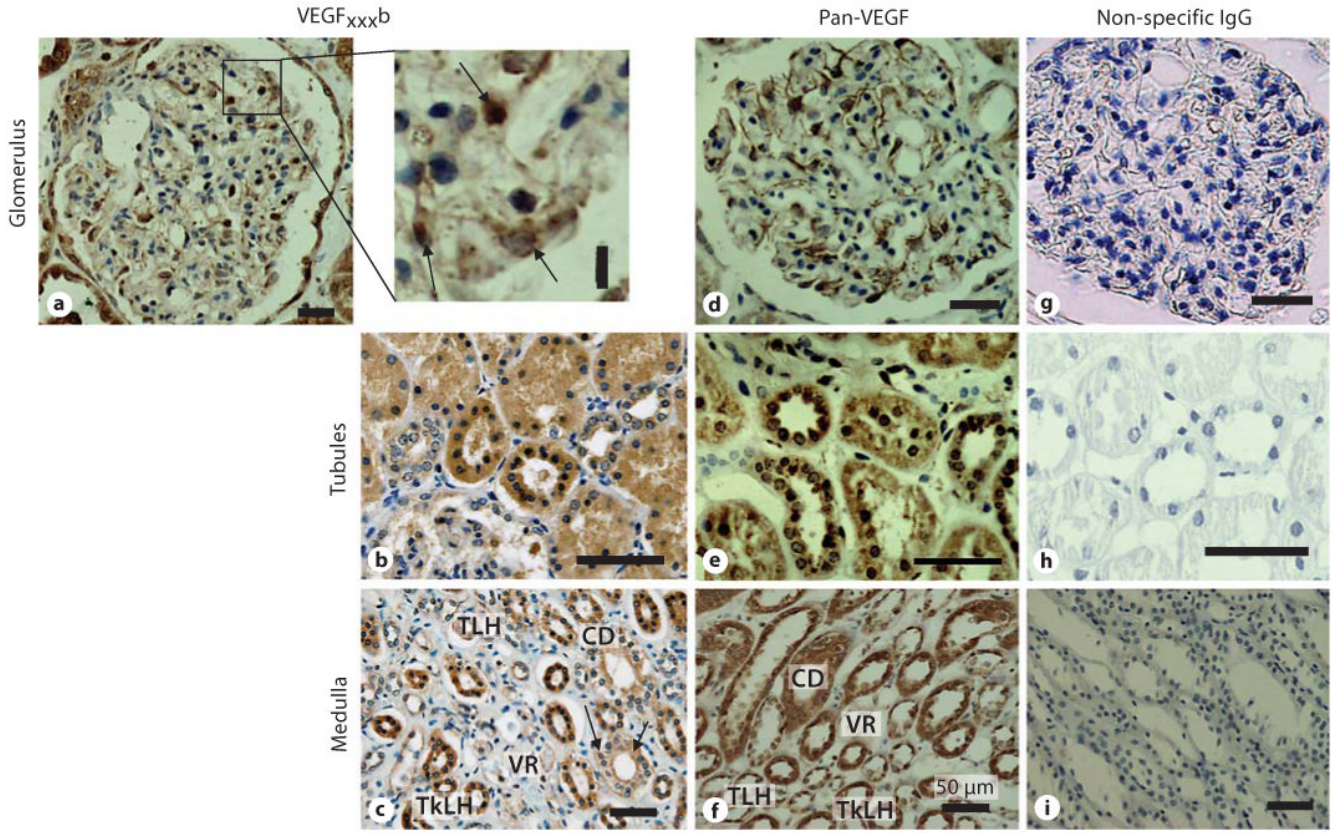

Fig. 2.

Immunohistochemical staining of VEGF$_{xxx}$b and pan-VEGF in the adult kidney. VEGF$_{xxx}$b staining in the podocytes of the glomerulus (**a**, arrows), proximal and distal tubules of the renal cortex (**b**) and in the ascending thick and thin loop of Henle in the renal medulla (**c**) was clearly seen. Sections treated with a pan-VEGF antibody (**d-f**) show a comparable staining pattern. Matched mouse IgG controls were negative (**g-i**). Scale bars = 30 μm, except inset in **a** =10 μm, and **f**. TLH = Thin loop of Henle; TkLH = thick loop of Henle; CD = collecting ducts; VR = vasa recta.

Europe PMC Funders Author Manuscripts

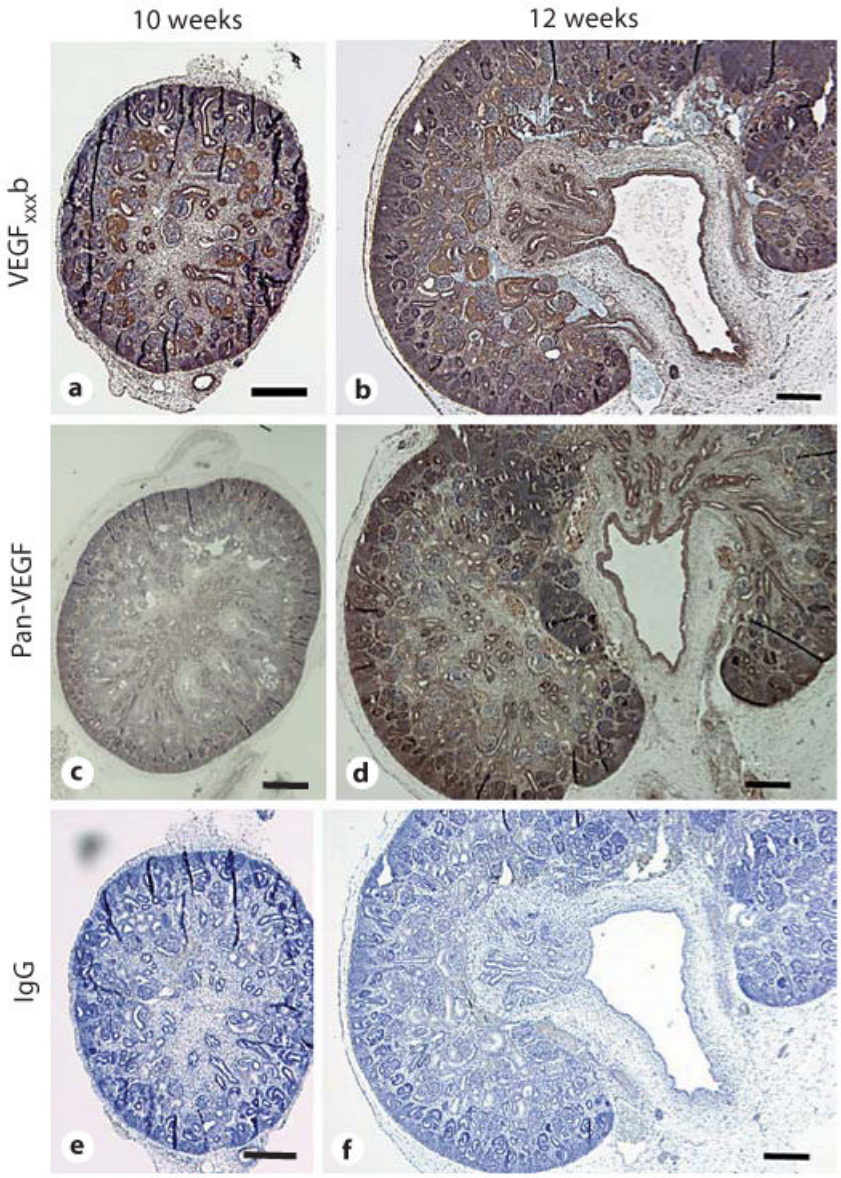

Fig. 3.

Overview of $VEGF_{xxx}b$ and pan-VEGF immunohistochemical staining of the metanephric kidney. $VEGF_{xxx}b$ (**a**, **b**) and pan-VEGF (**c**, **d**) staining was observed in specific developmental regions of the metanephric kidney of 10-week (**a**, **c**) and 12-week (**b**, **d**) fetuses. Matched mouse IgG controls were negative (**e**, **f**). Scale bars = 600 μm.

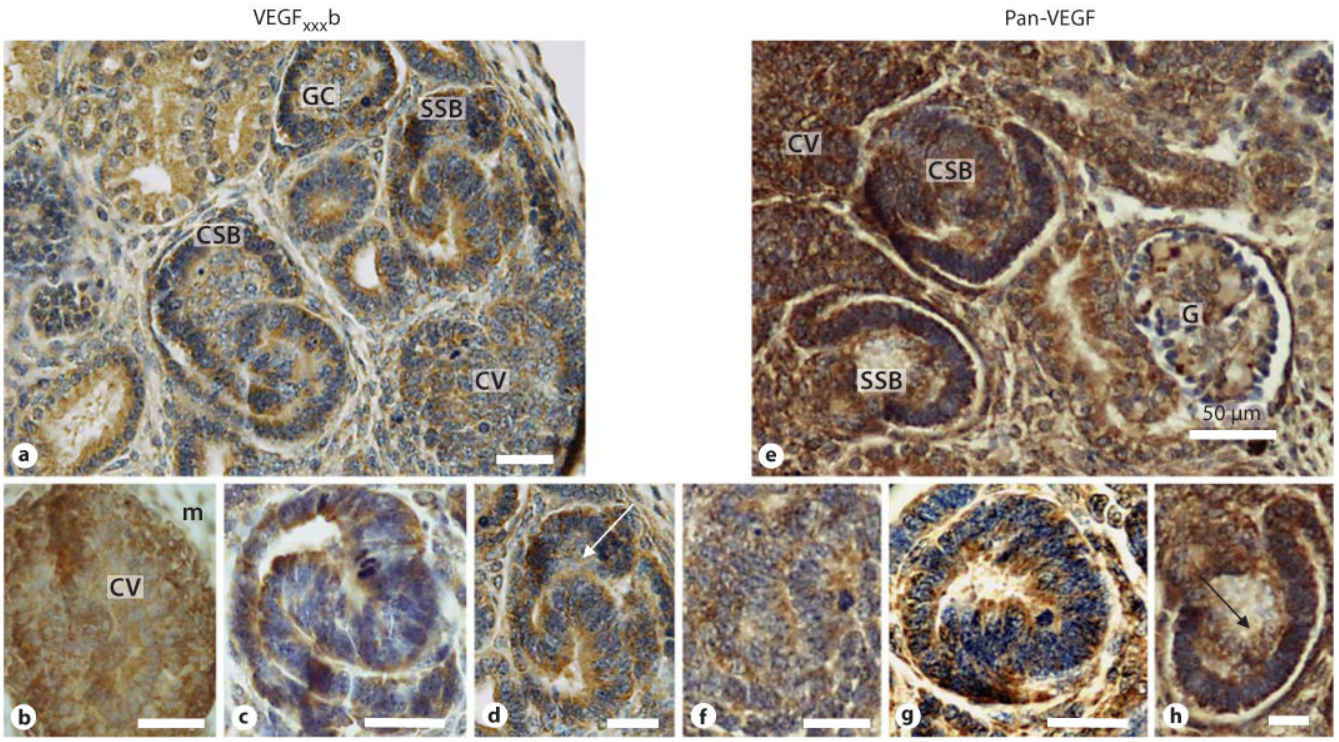

Fig. 4.

VEGF$_{xxx}$b and pan-VEGF immunohistochemical staining during the early stages of nephrogenesis. Intracellular VEGF$_{xxx}$b staining (**a-d**) and pan-VEGF staining (**e-h**) was observed during glomerular development. VEGF$_{xxx}$b (**a**) and pan-VEGF (**e**) staining was observed at the various stages of nephrogenesis, with a specific staining pattern. For example, in the condensed vesicle (**b**, **f**) the staining was more polarised as the mesenchymal cells gained epithelial characteristics. In comma-shaped (**c**, **g**) and S-shaped (**d**, **h**) bodies, VEGF$_{xxx}$b (**c**, **d**) and pan-VEGF (**g**, **h**) staining was localised to the primitive epithelial cells, especially on the apical side, and to the glomerular cleft. Scale bars = 30 μm, except **e**. m = Mesenchyme; GC = glomerular cleft; CV = condensed vesicle; CSB = comma-shaped body; SSB = S-shaped body; G = glomeruli. Arrows point to diffuse staining of the glomerular cleft.

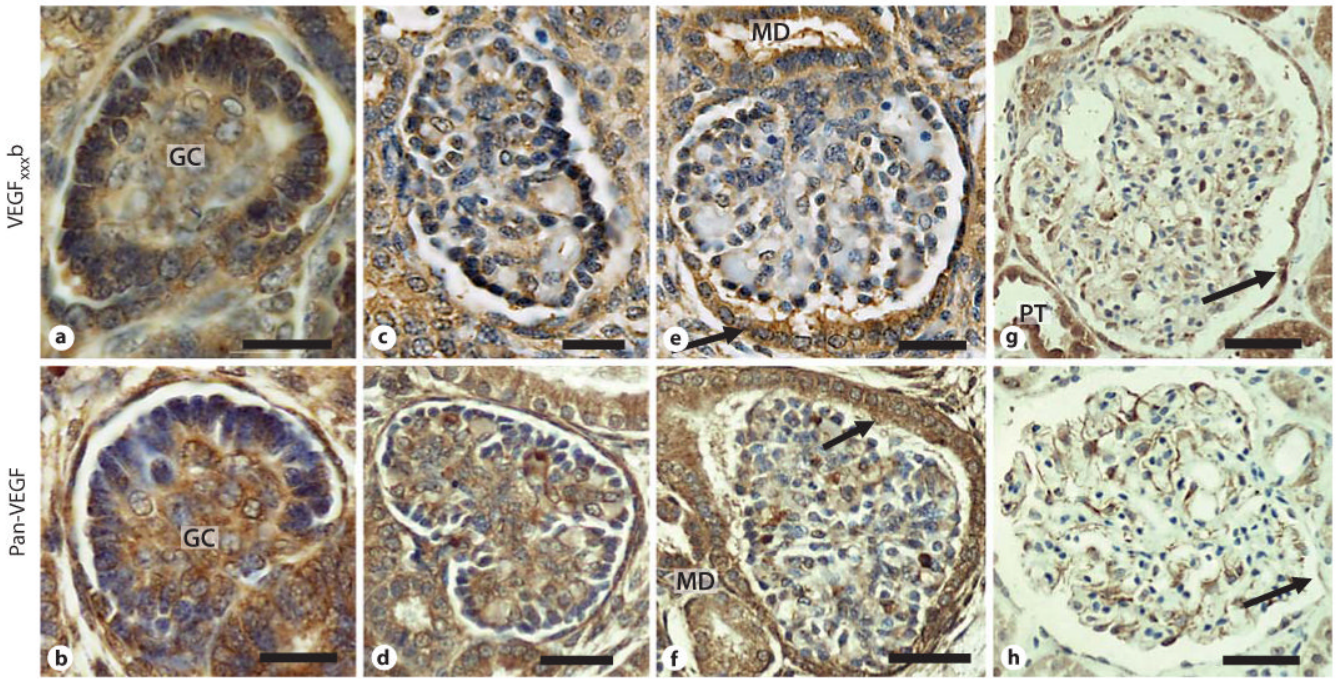

Fig. 5.

VEGF$_{xxx}$b immunohistochemical staining during glomerular maturation. Apical and basolateral VEGF$_{xxx}$b (**a**, **c**, **e**) and pan-VEGF (**b**, **d**, **f**) staining. **a**, **b** The presumptive podocytes and the glomerular cleft show diffuse staining in the capillary loop stage. As development of the glomerulus progresses and the presumptive podocytes mature, the intensity of the VEGF$_{xxx}$b staining decreases (**c**), whereas pan-VEGF staining is still intense (**d**) and VEGF$_{xxx}$b becomes more specific to a subpopulation of mature podocytes (**e**); pan-VEGF staining is more widespread in the podocytes at this stage (**f**). Scale bars = 50 μm. MD = Macula densa. Arrows point to parietal epithelial cells.

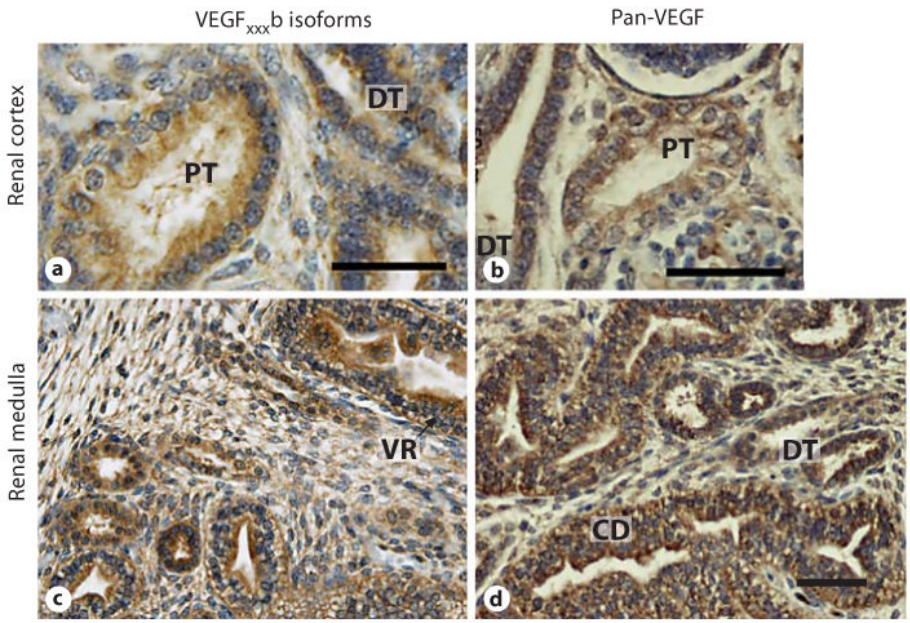

Fig. 6.

Immunohistochemical staining of VEGF$_{xxx}$b and pan-VEGF in the tubules of the developing renal cortex and medulla. **a**, **c** VEGF$_{xxx}$b staining was seen in the more proximal and distal regions of the convoluted tubules. Strong periluminal staining was observed in the proximal tubules and collecting ducts. **b**, **d** Similar immunohistochemical staining was observed for pan-VEGF. Scale bars = 30 μm. PT = Proximal tubule; DT = distal tubule.

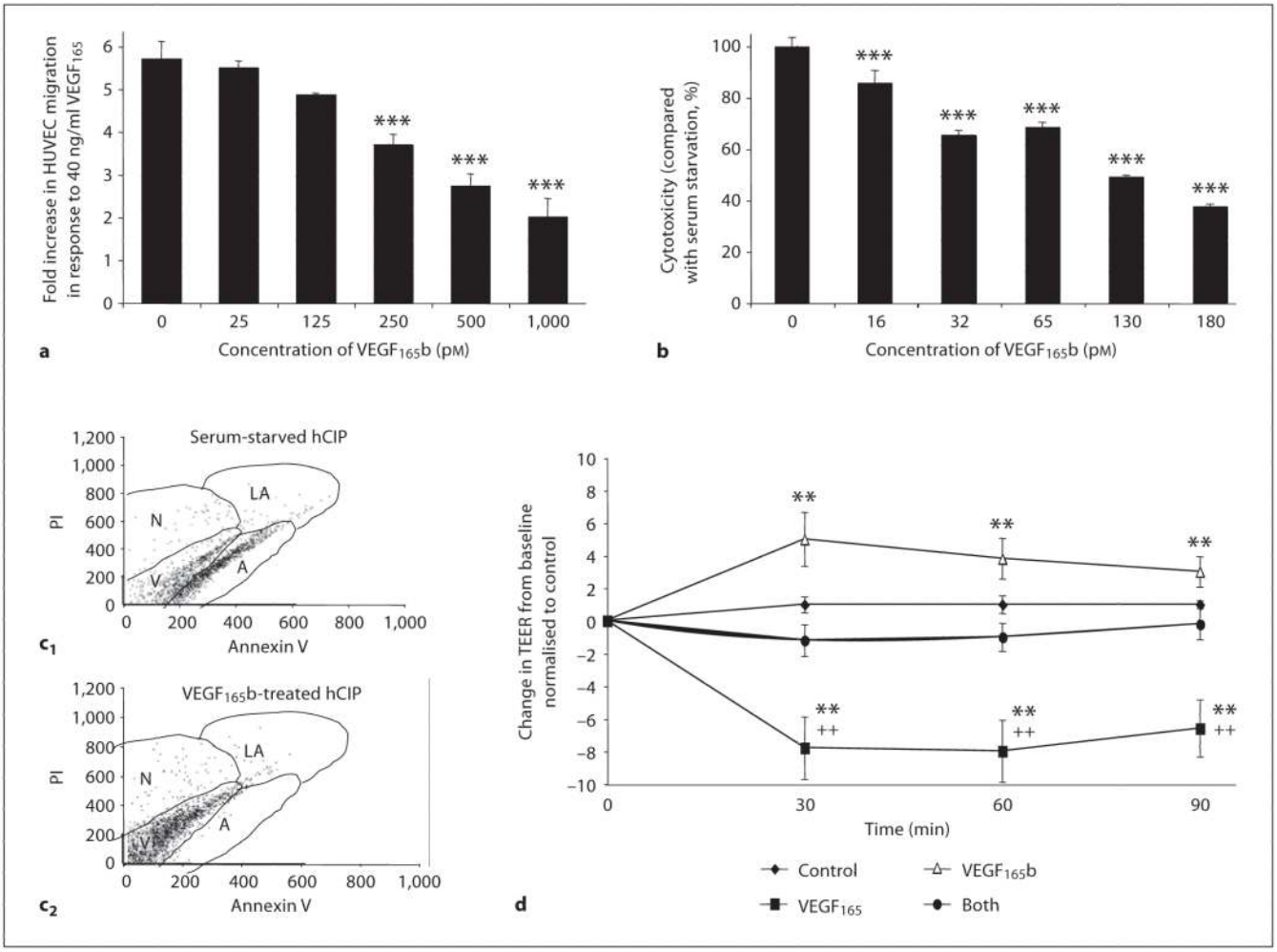

Fig. 7.

$VEGF_{165}b$ is cytoprotective for glomerular epithelial cells, and inhibits $VEGF_{165}$-mediated migration and increased permeability of endothelial cells. **a** $VEGF_{165}b$ dose-dependently inhibits endothelial cell migration induced by $VEGF_{165}$. *** $p < 0.001$, ANOVA. **b** $VEGF_{165}b$ dose-dependently prevents cell death in primary cultured podocytes. *** $p < 0.001$, ANOVA. **c** $VEGF_{165}b$ inhibits apoptosis induced by serum starvation. Flow cytometry of annexin V and propidium iodide-stained human conditionally immortalised podocytes (hCIP) treated with 48-hour serum starvation (SS; $\mathbf{c_1}$) or 1 nM $VEGF_{165}b$ and SS ($\mathbf{c_2}$). V = Viable population; N = necrotic; A = apoptotic; LA = late apoptotic. **d** $VEGF_{165}b$ reduces permeability of glomerular monolayers. $VEGF_{165}b$ increases glomerular transendothelial electrical resistance (TEER) in cultured monolayers, $VEGF_{165}$ reduces TEER (increases permeability), and $VEGF_{165}b$ inhibits the $VEGF_{165}$-mediated reduction. ** $p < 0.01$, compared with control; $^{++}$ $p < 0.01$ compared with both $VEGF_{165}b$ and $VEGF_{165}$; one-way ANOVA and Bonferroni post-hoc analysis.