Overexpression of VEGF₁₆₅b in Podocytes Reduces Glomerular Permeability

Yan Qiu,* Joanne Ferguson,* Sebastian Oltean,* Chris R. Neal,* Amit Kaura,* Heather Bevan,† Emma Wood,* Leslie M. Sage,* Silvia Lanati,* Dawid G. Nowak,* Andy H.J. Salmon,*‡ David Bates,* and Steve J. Harper*

*Microvascular Research Laboratories, Department Physiology and Pharmacology, Bristol Heart Institute, Preclinical Veterinary School, Bristol, United Kingdom; and †Academic Renal Unit, Department of Clinical Science, North Bristol, University of Bristol, Southmead Hospital, Bristol, United Kingdom

ABSTRACT

The observation that therapeutic agents targeting vascular endothelial growth factor-A (VEGF-A) associate with renal toxicity suggests that VEGF plays a role in the maintenance of the glomerular filtration barrier. Alternative mRNA splicing produces the VEGF₁₆₅b family, which consists of antiangiogenic peptides that reduce permeability and inhibit tumor growth; the contribution of these peptides to normal glomerular function is unknown. Here, we established and characterized heterozygous and homozygous transgenic mice that overexpress VEGF₁₆₅b specifically in podocytes. We confirmed excess production of glomerular VEGF₁₆₅b by reverse transcriptase–PCR, immunohistochemistry, and ELISA in both heterozygous and homozygous animals. Macroscopically, the mice seemed normal up to 18 months of age, unlike the phenotype of transgenic podocyte-specific VEGF₁₆₄-overexpressing mice. Animals overexpressing VEGF₁₆₅b, however, had a significantly reduced normalized glomerular ultrafiltration fraction with accompanying changes in ultrastructure of the glomerular filtration barrier on the vascular side of the glomerular basement membrane. These data highlight the contrasting properties of VEGF splice variants and their impact on glomerular function and phenotype.


The glomerular filtration barrier (GFB) is a unique multilayered structure demonstrating a striking dichotomy in its ability to restrict the extravasation of molecules of various sizes, shapes, and charges. Poorly permeable to large, lipid-insoluble, or anionic molecules, the GFB is highly permeable to water and small water-soluble agents. In glomerular disease, this strict segregation is impaired or lost, resulting in albumin in the urine. The mechanisms underlying proteinuria have been widely investigated, both because of its link to glomerular disease (heavy proteinuria tends to be associated with more severe glomerular lesions) and because, even at modest levels, proteinuria is now categorized as a major risk factor for vascular disease,¹ even among the general population.²,³

Glomerular permselectivity remains poorly understood; however, although the controlling mechanisms of the normal glomerular phenotype are probably highly complex, in simple terms, they are likely to depend on two general factors: (1) Physical structure (e.g., foot processes, slit diaphragms and related proteins, fenestrae, glomerular basement membrane [GBM], glycocalyx, subpodocyte space [SPS]) and (2) the function of cell types that contribute to the barrier, through either physical change (e.g., podocyte move-
ment, contraction, effacement) or growth factor expression/secretion (e.g., VEGF-A, angiopoietin-1, VEGF-C)—that is, podocyte-derived agents that are known to influence permeability in other microvascular beds and the receptors for which reside on the glomerular endothelial cells (GECs)\(^4\) and sometimes on the podocytes themselves.\(^5\) The specific role of podocyte-derived VEGF remains contentious; however, its angiogenic/permeability potency ensures continuing interest, even in the context of a VEGF glomerular literature that is replete with apparent contradictions and, on initial inspection, unexplainable observations.

For example,

1. Constitutive transgenic podocyte-specific VEGF\(_{164}\) overexpression leads to proteinuria, collapsing nephropathy, uremia, and death 5 days after birth;\(^6\) however, podocyte VEGF-A glomerular reduction (heterozygous inactivation) similarly demonstrates nephrotic syndrome, uremia, and death at 2 to 5 weeks in the context of glomerular endotheliosis.\(^6\)

2. In mature glomeruli, induced transgenic podocyte-specific VEGF\(_{164}\) overexpression results in increased water permeability-area product (LpA/Vi) and protein-creatinine ratio at 7 days after induction;\(^7\) however, systemic inhibition of VEGF with Avastin in humans also causes proteinuria\(^8\) and occasionally renal failure.\(^9\)

3. Anti-VEGF antibody administration reduces proteinuria in an animal model of diabetic nephropathy\(^10\) but induces proteinuria in normal animals,\(^11\) and VEGF administration in some nondiabetic animal models ameliorates glomerular injury.\(^12\)

Many of these carefully conducted studies are irreconcilable if VEGF is regarded only as a proangiogenic, propermeability vaso-dilator acting solely on endothelial cells. Two key changes in our understanding of VEGF have forced a radical reevaluation of VEGF biology. The first is the identification of the antiangiogenic VEGF\(_{a}\) family of peptides (VEGF\(_{XXb}\)). Replacement of 8a by exon 8b in the VEGF\(_{XXb}\) family produces peptides that are antiangiogenic, inhibit permeability chronically, and reduce rather than promote tumor growth.\(^13,15–20\) Alternative splicing of ex-
ons 6 and 7 produces multiple isoforms within each family with differing heparin-binding properties. The dominant member in each family contains 165 amino acids (Figure 1).

The second change in VEGF biology has been that, despite its nomenclature, VEGF is not endothelial cell specific but also has effects on nonendothelial cells. VEGF165, for example, is neuroprotective, and both VEGF165 and VEGF165b have human podocyte cytoprotective properties.

In the context of established proangiogenic, propermeability properties of VEGF165 (and the murine equivalent VEGF164), the characterized phenotype of constitutive podocyte–specific VEGF164 transgenic overexpressing animals, and recent in vitro data suggesting that VEGF165b reduces VEGF165-induced human endothelial monolayer permeability, in addition to being antiangiogenic in vivo, here we describe the derivation of heterozygous and homozygous transgenic animals that constitutively overexpress VEGF165b in podocytes to address the hypothesis that sustained expression of exon 8b containing VEGF peptides will produce a distinct phenotype from transgenic animals overexpressing exon 8a–containing peptides.

**RESULTS**

**Generation of pNeph-VEGF165b Construct**

VEGF165b-cDNA was cloned into an expression vector under the control of the Nephrin promoter (Figure 2Ai). To assess transfection and construct functionality, we transfected human conditionally immortalized podocytes with the construct, transfection and construct functionality, we transfected human conditionally immortalized podocytes with the construct, and assessed VEGF165b expression in the cell supernatant at 48 hours. Significantly more VEGF165b was seen in transfected podocytes compared with control vector or untransfected cells (Figure 2Aii). Potential founder lines were identified by PCR (Figure 2B) and Southern blot analysis (Figure 2C) of pups born from injected embryos. No difference in isolated glomerular functional phenotype (LpA/Vi) was seen between potential founder lines 1, 5, and 6 (Figure 2D), but line 1 was used for subsequent heterozygous and homozygous transgenic breeding studies.

**VEGF165b Expression in Renal Cortex of pNeph-VEGF165b Heterozygous and Homozygous Transgenic Mice**

Exon 8b–specific reverse transcriptase–PCR for the transgene (P < 0.05, χ² test for trend; n = 3 per group; Figure 3A), immunohistochemistry (Figure 3B) using an anti-human VEGF antibody, and an exon 8b–specific ELISA (Figure 3C) on protein extracted from renal cortex (P < 0.01, ANOVA) all demonstrated a gradient of expression from wild-type (WT) littermate controls through heterozygous to homozygous animals.

**Functional Phenotype: Podocyte-Specific VEGF165b Overexpression Reduces Glomerular Water Permeability**

To determine whether isolated glomerular permeability to water was altered by podocyte VEGF165b overexpression, we investigated normalized glomerular K̇(LpA/Vi) using the glomerular swelling assay modified by Salmon from Savin et al. A marked difference was seen in LpA/Vi among the three groups (Figure 4) from 1.95 ± 0.16 nl/min per mmHg in WT to 1.43 ± 0.10 nl/min per mmHg in homozygous to 0.67 ± 0.07 nl/min per mmHg in homozygous mice (Table 1). To determine whether this reduction was attenuated by exogenous VEGF165b, we measured LpA/Vi from glomeruli from transgenic mice, then exposed the same glomeruli to 1 nM VEGF antibody, and an exon 8b–specific ELISA (Figure 3C) on protein extracted from renal cortex from transgenic and WT mice using VEGFxxxb-specific ELISA (R&D Systems). P < 0.01 (ANOVA).

![Figure 3. pNeph-VEGF165b heterozygous and homozygous transgenic mice overexpress VEGF165b in the renal visceral glomerular epithelial cells (podocytes). (A) VEGF165b expression is determined in the renal glomerulus (exon 8b–specific reverse transcriptase–PCR of renal cortex for transgene, P < 0.05, χ² test for trend; n = 3 per group; Ai and Aii). (B) Immunohistochemistry using anti-human VEGF (A20 Santa Cruz) ×1000 under oil, demonstrating transgenic VEGF expression in the podocytes. (C) ELISA of protein extracted from renal cortex from transgenic and WT mice using VEGFxxxb-specific ELISA (R&D Systems). P < 0.01 (ANOVA).](image-url)
in VEGF\textsubscript{xxx} isoforms (data not shown). Plasma creatinine, urea levels, and GFR (306.70 ± 57.52 μl/min, n = 4 WT controls versus 344.10 ± 41.80 μl/min, n = 4 heterozygotes) were not significantly different. Urinary protein-creatinine ratio of urine collected using metabolic cages showed lower values in the homozygous animals (Table 1) but did not reach significance. Body weight of animals and blood glucose levels were also unchanged in the transgenic mice. To assess whether exogenous administration of recombinant human VEGF\textsubscript{165b} (rhVEGF\textsubscript{165b}) could reproduce the reduction in \(L_{P,A/V_i}\) we incubated WT glomeruli with increasing dosages of rhVEGF\textsubscript{165b}. Exogenous rhVEGF\textsubscript{165b} significantly reduced \(K_f\) in a dosage-dependent manner (Figure 5A). Figure 5B summarizes the characteristically distinct permeability changes induced in \(L_{P,A/V_i}\) elicited by VEGF\textsubscript{165} (increase) and VEGF\textsubscript{165b} (decrease).

**Ultrastructural Phenotype:Podocyte-Specific VEGF\textsubscript{165b} Overexpression Reduces Fenestral Size and Density**

Up to 21 months, the transgenic mice had normal behavior, growth rate, and feeding and no urinary sediment. Histologic assessment with light microscopy also revealed no obvious abnormality (Figure 6A); however, serial transmission electron microscopy (EM) studies revealed that typical glomerular endothelial open fenestrations were difficult to identify in transgenic mice (Figure 6B). Ultrastructural measurements revealed no change in SPS coverage, foot process width, and GBM thickness in SPS-covered areas (Table 1); however, the GBM in areas of the GFB devoid of SPS coverage were significantly thinner in WT controls (196 ± 6 nm) versus homozygous animals (240 ± 14 nm; \(P < 0.01\)). In addition, fenestration density was reduced in transgenic animals (Figure 6C, Table 1). Moreover, as expected, the vast majority of fenestrations in WT littermate controls did not demonstrate fenestral diaphragms. In contrast, many of the fenestrations in the transgenic animals contained electron-dense material (Figure 6D) that contrasted with the conventional (but rare) distinct diaphragms (approximately 2%\textsuperscript{24}) seen in normal glomerular endothelium \textit{in vivo} (Figure 6E). Because, it was not possible to define whether these fenestrations contained atypical diaphragms, excess glycocalyx, glycocalyx-like material, or “sieve plugs”\textsuperscript{25,26} or was simply a reflection of smaller fenestrations with more frequent (relatively) sectioning through the attenuated edge of fenestrations, we termed these “closed” fenestrations. We therefore performed further EM characterization using 40-nm sections from paired samples fixed and processed in parallel from homozygous animals and littermate controls, and, because two filtration areas of the GFB (with and without covering SPS\textsuperscript{27–29}) have been identified, our analysis differentiated between these areas. Random measurements (in excess of 200 from multiple animals) were made using a Photoshop grid (Figure 7).

On the urinary side of the GBM, the podocyte foot process slit diaphragm width and density were not significantly different (Table 1). In contrast, on the vascular side, there was a significant increase in the proportion of “closed” fenestrations (Figure 7, A and C). Reduced fenestration number was preserved in both SPS- and non–SPS-covered areas of the GFB (Figure 7B). Furthermore, although the open fenestrations were of a similar size in WT controls and homozygous animals (Figure 7D), the closed fenestrations were significantly narrower (Figure 7E).

Although a detailed study of the nature of the closed fenestrations was not possible, we attempted to clarify whether the overexpression of VEGF\textsubscript{165b} had influenced plasmalemma vesicle protein 1 (PV-1) expression. We were unsuccessful using the established antibodies for immunogold studies and therefore studied PV-1 expression by Western blotting in conditionally immortalized GECs. These studies did not show any significant change in PV-1 expression at the protein level in GECs exposed to VEGF\textsubscript{165b}.

**DISCUSSION**

The traditional view of the GFB as a trilayered filter has evolved significantly\textsuperscript{30} with the identification of previously overlooked ultrastructural\textsuperscript{27–29} and biochemical (glycocalyx)\textsuperscript{31} features that contribute additional resistance to flow and with the real-
Table 1. Transgenic parameters characterized

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT Littermate Controls</th>
<th>Heterozygous Animals</th>
<th>Homozygous Animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glomerular volume (µl)</td>
<td>0.98 ± 0.16 (n = 8)</td>
<td>0.73 ± 0.07 (n = 18)</td>
<td>1.56 ± 0.11 (n = 36)</td>
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<tr>
<td>LpA</td>
<td>1.93 ± 0.32 (n = 8)</td>
<td>1.00 ± 0.12 (n = 18)</td>
<td>1.06 ± 0.16 (n = 23)</td>
</tr>
<tr>
<td>LpA/Vi</td>
<td>1.95 ± 0.17 (n = 8)</td>
<td>1.44 ± 0.11 (n = 18)</td>
<td>0.67 ± 0.07 (n = 23)</td>
</tr>
<tr>
<td>uPCR (mg/mmol)</td>
<td>20.16 ± 2.55 (n = 8)</td>
<td>20.74 ± 2.80 (n = 10)</td>
<td>14.80 ± 2.78 (n = 3)</td>
</tr>
<tr>
<td>Plasma creatinine (µmol/L)</td>
<td>3.40 ± 1.21 (n = 5)</td>
<td>4.50 ± 1.20 (n = 6)</td>
<td>6.00 ± 1.53 (n = 3)</td>
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<tr>
<td>Plasma urea (mmol/L)</td>
<td>8.60 ± 0.10 (n = 10)</td>
<td>9.32 ± 0.50 (n = 6)</td>
<td>11.07 ± 0.90 (n = 3)</td>
</tr>
<tr>
<td>GFR (µl/min)</td>
<td>306.70 ± 57.50 (n = 4)</td>
<td>344.10 ± 41.80 (n = 4)</td>
<td></td>
</tr>
<tr>
<td>Fenestration density (per µm GBM)</td>
<td>3.10 ± 0.10</td>
<td>0.64 ± 0.30</td>
<td></td>
</tr>
<tr>
<td>% SPS coverage</td>
<td>55 ± 5</td>
<td>ND</td>
<td>41 ± 10</td>
</tr>
<tr>
<td>Foot process width (nm)</td>
<td>400 ± 40</td>
<td>ND</td>
<td>460 ± 70</td>
</tr>
<tr>
<td>GBM thickness under SPS (nm)</td>
<td>248 ± 10</td>
<td>ND</td>
<td>252 ± 9</td>
</tr>
<tr>
<td>GBM thickness uncovered</td>
<td>196 ± 6</td>
<td>ND</td>
<td>240 ± 14*</td>
</tr>
</tbody>
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ND, not done; uPCR, urinary protein-creatinine ratio.

*P < 0.05.

which elicit paracrine alterations counterintuitively in the adjacent but nevertheless “upstream” glomerular endothelium. Mathematical models indicate that such molecules can diffuse upstream from adjacent podocytes and elicit similar properties. The dose of VEGF-A is required to maintain the GEC phenotype and multiple roles of glomerular VEGF and multiple VEGF isoforms with widely contrasting properties are required to support the notion that an optimal “dose of glomerular VEGF” may have inhibited both families. The podocyte-VEGF-A overexpressing mice developed uremia and collapsing nephropathy, a glomerular lesion typical of HIV nephropathy.41 Mice died at day 5. This contrasts sharply with the podocyte-VEGF165b phenotype of modestly reduced permeability to water and urinary protein loss in animals with a normal life expectancy. Future experiments on crosses between
these two lines to assess the effectiveness of VEGF\textsubscript{xxx}b in ameliorating the phenotype of the animals in the study by Eremina \textit{et al.}\textsuperscript{6} may be informative, as would isoform-specific knockouts.

Intriguingly, the heterozygote podocyte-VEGF knockout and the anticipated sequestration of VEGF (all isoforms) by soluble VEGF-R1 (sFlt) overexpression\textsuperscript{42} demonstrate a loss of fenestrations\textsuperscript{43}; in contrast, VEGF\textsubscript{165} induces endothelial fenestrations \textit{ex vivo}.\textsuperscript{43} The findings we present here suggest the qualitative balance of VEGF may be important for the establishment and maintenance of fenestrations and the phenotype thereof \textit{in vivo}. We previously showed that at least some of the VEGF expression at the S-shape stage of glomerular development is VEGF\textsubscript{xxx}b.\textsuperscript{20} Of note, in rodent development at least, the earliest fenestrations have diaphragms\textsuperscript{24} that may reform in association with injury.\textsuperscript{24} The lack of an effect on PV-1 suggests that the podocyte-VEGF\textsubscript{165}b phenotype may not simply relate to changes in the expression of this endothelial glycoprotein, but, clearly, further studies including immunogold may clarify the situation.

Exon 8b-specific isoforms predominate in many human tissues\textsuperscript{44} and contribute approximately half of the VEGF in the normal human kidney.\textsuperscript{16,20} The contrasting properties of the two VEGF isoform families is striking. VEGF\textsubscript{165}b has been shown in receptor-binding studies, in \textit{in vitro} endothelial proliferation and migration assays, \textit{ex vivo} isolated resistance vessel myograph studies, and in \textit{in vivo} neovascular and tumor growth models to inhibit the actions of VEGF\textsubscript{165}.\textsuperscript{13,19,45,46} Thus, parenterally administered rhVEGF\textsubscript{165}b (intraperitoneal and subcutaneous) halts colonic carcinoma tumor growth in nude mice.\textsuperscript{17} We have also shown that transgenic mice overexpressing VEGF\textsubscript{165}b in mammary tissue have inhibited physiologic angiogenesis.\textsuperscript{13} VEGF\textsubscript{165}b inhibits both angiogenesis and vasodilatation through inhibition of VEGF\textsubscript{165}-mediated full activation of VEGF-R2.\textsuperscript{47} The description here of a glomerular phenotype “at odds” with that of podocyte-specific VEGF\textsubscript{164} overexpression, in that VEGF\textsubscript{165}b overexpression in the podocytes does not result in overt glomerular pathology, adds further weight to the argument that exon 8a and exon 8b containing VEGF isoforms have opposing properties in terms of microvessel permeability in both systemic and highly specialized microvessels.

Although permeability of the isolated glomeruli was reduced in our transgenic animals, GFR was unchanged. This seems counterintuitive but may simply reflect that in the isolated glomerular technique, it is the properties of the barrier \textit{per se} that are studied, in the absence of confounding factors known to influence single-nephron GFR such as intraglomerular pressure and flow. Direct comparisons are therefore inappropriate.

In regard to microvessel permeability, studies using the Landis-Michel micro-occlusion technique, in cannulated single capillaries, demonstrated that an rhVEGF\textsubscript{165}b bolus increases microvascular permeability to water for a few seconds only (rapidly returning to normal), mediated by VEGF-R1\textsuperscript{35}; however, there is no physiologic correlate to this, and in the same study, no long-term change in water permeability was seen in response to VEGF\textsubscript{165}b, in contrast to that seen with VEGF\textsubscript{165}.\textsuperscript{33} VEGF\textsubscript{165}b also inhibits VEGF\textsubscript{165}-mediated reduction in transendothelial monolayer resistance (increased permeability) \textit{in vitro}.\textsuperscript{20}

We have demonstrated no difference in glomerular capillary surface area within transgenic compared with WT animals, suggesting VEGF\textsubscript{165}b overexpression does not adversely affect glomerular development. Indeed, VEGF\textsubscript{165}b is expressed early in human kidney embryonic development,\textsuperscript{20} although at a lower mRNA level than in the adult.\textsuperscript{48} Although an effect on glomerular development might be postulated, nephrin (and therefore transgene) expression occurs relatively late in kidney development in rodents (day 18 of 21 in rats)\textsuperscript{49}—that is, at the capillary loop stage.\textsuperscript{50} In addition, it cannot be assumed that the differential permeability properties of VEGF\textsubscript{165} and VEGF\textsubscript{165}b in vessels are
maintained in the embryo. Even if this were the case, it is also unknown how their putative conflicting properties on embryonic endothelial cells may interact with the similarity of their cytoprotective effects on glomerular epithelial cells.\textsuperscript{20}

The finding that exon 8a containing conventional isoforms (e.g., VEGF\textsubscript{165}) tend to predominate in de-differentiated human conditionally immortalized podocytes that lack exon 8b–containing isoforms, the latter being present in differentiated podocytes, prompted Schumacher \textit{et al.}\textsuperscript{48} to suggest that podocyte maturation (GECs and hence the GBM) may depend on isoform family ratio. The study by Schumacher \textit{et al.} of VEGF isoform expression in Denys-Drash syndrome (DDS; glomerular dysgenesis, male pseudohemaphroditism, FSGS, and uremia) showed that DDS podocytes produce ample proangiogenic, propermeability VEGF\textsubscript{165} but completely lack the antiangiogenic, anti-permeability form VEGF\textsubscript{165b}. The factors that influence splicing between the VEGF-A families are emerging,\textsuperscript{51} and because at least 74\% of human genes demonstrate alternative mRNA splicing,\textsuperscript{52} it is perhaps no surprise that many podocyte-derived products display this property (e.g., the transcription factor WT-1, which has been implicated in Wilms’ tumors and DDS\textsuperscript{53}). WT-1 has four major isoforms. The zinc finger regions of WT-1 bind both DNA and RNA, and although WT-1 targets are unknown, mutations in WT-1 in humans lead to mesangial sclerosis and well-characterized glomerular lesions.\textsuperscript{53,54} It has been suggested that WT-1 might regulate factors that affect vascular development, such as VEGF.\textsuperscript{55} WT-1 may therefore influence the splicing of VEGF in podocytes and the associated glomerular phenotype. We have now shown that VEGF\textsubscript{165b} is down-regulated in DDS podocytes because WT1 acts as a repressor for a splice factor kinase named SRPK1, which stimulates VEGF proximal splicing through phosphorylation and activation by nuclear localization of the splice factor ASF/SF2 (Amin \textit{et al.}, submitted). Altered VEGF isoform balance has been potentially linked to other forms of glomerular lesion (e.g., in a transgenic model in which the Hippel-Lindau gene was deleted\textsuperscript{39}), leading to increased HIF-1α subunits, increased Cxcr4 expression, and crescentic glomerulonephritis. In this model, the podocytes were functionally responding to the signaling pathways activated in hypoxia, which are known to increase VEGF\textsubscript{xxx} expression but have no effect on VEGF\textsubscript{xxxb} production.\textsuperscript{18}

In summary, here we show that VEGF\textsubscript{165b} overexpression results in physiologically healthy renal function and normal light microscopy but with reduced glomerular permeability to water associated with ultrastructural fenestral changes and contrasts with podocyte-specific VEGF\textsubscript{164} overexpression.\textsuperscript{6} The correlation of an ultrastructural change and an associated change in physiology (permeability to water) lends credence to the view that fractional fenestral area is a significant determinant of glomerular filtration\textsuperscript{24} and that fenestral density size and phenotype may be a dynamic process that responds to single-nephron or whole-organ demands.

**CONCISE METHODS**

**Animal Maintenance**

All transgenic lines were generated on C57BL6 × CBA/CA background. Animal care/procedures were conducted within UK Home-Office protocols/guidelines. For permeability experiments, F\textsubscript{2} to F\textsubscript{4} generation male transgenic mice and WT littermate controls were used.
Construction of pNephrin-VEGF₁₆₅b
PCDNA3-VEGF₁₆₅b was cloned as described previously,¹³ and a plasmid was generated with the mouse nephrin promoter upstream of VEGF₁₆₅b-cDNA and a poly-A signal (Figure 2Ai). The nephrin promoter (courtesy of Prof. S Quaggin) DNA fragment was inserted with rapid DNA ligation kit (Roche Applied Science) into the linearized pCDNA3-VEGF₁₆₅b, from which the cytomegalovirus promoter had been deleted and correctly oriented colonies selected.

Podocyte Transfection
Human conditionally immortalized visceral glomerular epithelial cells previously characterized⁵⁵ (donated by Prof. Moin Saleem) were transfected with equal amounts of pNephrin-VEGF₁₆₅b and empty vector 5'-Neprhin-pKO. Expression of VEGF₁₆₅b in the supernatant of control, mock-transfected, and pNephrin-VEGF₁₆₅b-transfected podocytes were analyzed by VEGF₁₆₅b family-specific ELISA (DY304E; R&D Systems; Figure 2Aii).

Generation of Transgenic Mice
The DNA fragment of murine pNephrin-VEGF₁₆₅b-pA for microinjection was generated with HindIII and HaelII digests of pNephrin-VEGF₁₆₅b and gel-purified using QIAEX II DNA Extraction kit (Qiagen) before final purification with elutip minicolumns (Schleicher & Schuell biosciences). Microinjection of purified DNA into embryos was carried out by B&K Universal Ltd. Successfully injected embryos were cultured overnight and transplanted into oviducts of pseudopregnant mice. After weaning, tail-biopsy genomic DNA extracts were screened for the transgene via PCR (Figure 2B) and Southern blotting (Figure 2C). Animals were bred to homozygosity using a standard breeding/genotyping program. For generation of homozygous animals, siblings (from founder line 1) were crossed, and subsequent pups of the F1 generation, themselves crossed with WT mice, were also genotyped. For homozygous animals, all subsequent pups from at least three litters and a minimum of 20 pups were required to carry the transgene and all pups from subsequent litters. Numbers of animals for functional phenotype analysis were determined by the numbers required to demonstrate statistical analysis (from previous data showing that to demonstrate a significant difference in glomerular LpA/Vi of 25%, the use of a minimum of three animals and five numbers required to demonstrate statistical analysis (from previous data showing that to demonstrate a significant difference in glomerular LpA/Vi of 25%, the use of a minimum of three animals and five glomeruli is required), restrictions of UK home office license, and ethical review board.

Polymerase Chain Reaction
Primer pair (forward 5'-TCAGCCGAGCTACTGCACATC-3' and reverse 5'-GTGCTGGCCTTGGTGGATT-3') gave a PCR product of 208 bp to detect transgene specifically. Internal control primers for mouse β-globin (band 253 bp; forward 5'-AGTCTCAAGCGATGAGTG-3' and reverse 5'-AGCCCTTCTAGCATGCT-3') were included in amplification. Each reaction contained 2 μl of 10× buffer, 0.2 mM dNTPs, 1.5 mM MgCl₂, 500 nM forward and reverse primers, 0.5 U of Taq polymerase (Abgene), 0.5 μl of gDNA, and water to 20 μl. PCR was initiated at 94°C for 4 minutes, then 35 cycles of denaturation at 94°C for 30 seconds, annealing at 62°C for 30 seconds, extension at 72°C for 30 seconds, and a final extension at 72°C for 10 minutes.

Southern Blotting
Ten to 15 μg of tail gDNA was digested with EcoRI restriction enzyme. DNA was separated on 0.8% Agarose gel, denatured, and capillary-transferred to Hybond N⁺ membrane (Amersham). DNA was fixed with baking at 80°C for 2 hours. Membranes were probed with an alkaline phosphatase–labeled DNA fragment, exactly the same as the one used for microinjection. Probe preparation and transgene detection followed the manufacturer’s guideline of Gene Images Alkphos Direct Labeling and Detection System (Amersham).

Reverse Transcriptase–PCR
Briefly, total RNA was isolated with Trizol (Invitrogen) extraction and DNase I (Invitrogen) digested per the manufacturer’s instructions to prevent gDNA contamination. One microgram of DNase-treated RNA was reverse-transcribed into cDNA with AMV reverse transcriptase using the method of the manufacturer (Promega). Both cDNA and RNA treated with DNase I were subjected to PCR with forward primer 5'-ACAGATGCCAGCGGTGTA-3' and reverse primer 5'-ACAAGATGCCATGCGACGTGA-3'. PCR amplification was initiated at 94°C for 4 minutes, 35 cycles at 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 30 seconds, followed by final extension at 72°C for 10 minutes. A band at 199 bp indicated VEGF₁₆₅b transgene expression.

ELISA of VEGF₁₆₅b
Tissue protein lysate was prepared from mouse kidney tissue in RIPA buffer. For cultured podocytes, conditioned medium from cells with or without transfection was used. Protein concentration was determined by Bio-Rad assay (Bio-Rad), and the amount of VEGF₁₆₅b was determined by ELISA as described previously with a specific detection antibody against VEGF₁₆₅b isoforms.

Briefly, 0.08 μg of goat anti-VEGF polyclonal IgG (AF293-NA; R&D Systems) diluted in 1× PBS (pH 7.4) was adsorbed onto each well of a 96-well plate (Immunol 2HB; Thermo Life Sciences, Basingstoke, UK) overnight at room temperature. The plate was washed three times between each step with 1× PBS-Tween (0.05%). After blocking with 100 μl of 5% BSA in PBS for 1 hour at 37°C, 100 μl of recombinant human VEGF₁₆₅b (R&D Systems) diluted in 1% BSA in PBS (ranging from 62.5 pg/ml to 4.0 ng/ml) or protein samples was added to each well. After incubation for 1 hour at 37°C with shaking and three washes, 100 μl of mouse monoclonal anti-VEGF₁₆₅b biotinylated IgG (clone 264610/1; R&D Systems) at 0.4 μg/ml was added to each well, and the plate was left for 1 hour at 37°C with shaking. A total of 100 μl of streptavidin–horse-radish peroxidase (R&D Systems) at 1:200 dilution in 1% BSA in PBS was added; the plate was left at room temperature for 20 minutes, and 100 μl/well O-phenylenediamine dihydrochloride solution (substrate reagent pack DY-999; R&D Systems) was added. The plate was protected from light and incubated for 20 minutes at room temperature. The reaction was stopped with 50 μl/well 1 M H₂SO₄, and absorbance was read immediately in the Opsys MR 96-well plate reader (Dynex Technologies, Chantilly, VA) at 492 nm, with control reading at 460 nm.

Glomerular Permeability
The normalized glomerular ultrafiltration coefficient (Lp/Vp) of isolated intact whole glomeruli was calculated using an onometric technique described in detail by Salmon et al.²⁵
Glomerular Isolation and Solutions
Mice aged between 8 and 10 months were killed by cervical dislocation, and kidneys were removed. Glomeruli were isolated in mammalian ringer solution containing 1% BSA using conventional techniques. The glomerular harvest retained by the 75-μm mesh sieve was kept on ice to preserve morphology. During isolation, the concentration of plasma proteins within the glomerular capillaries equilibrates with the surrounding solution. Perfusate containing either dilute BSA (1%) or concentrated BSA (8%) was made in mammalian ringer solution and adjusted to pH 7.45 ± 0.02.

Apparatus
Micropipettes were pulled from glass capillary tubes (outer diameter 1.2 mm; Clark Electromedical Instruments, Reading, UK). The 13-μm aperture tip was fitted within a rectangular cross-section glass microslide (inner diameter 400 μm × 4 mm; Camlab, Cambridge, UK). The microslide was visualized over the ×10 objective using an inverted microscope (Leica DM IL HC Fluor). A monochrome video camera (Hitachi KP-M3AP) was attached to the top of the microscope to permit recording of individual glomeruli loaded into the system. The video camera was connected through a digital timer (FOR.A VT33) to a video cassette recorder (Panasonic AG7350) and monochrome monitor (Sony SSM-125CE). Perfusates were held in elevated heated reservoirs connected to the microslide via tubing. A rapid-response remote tap (075P3; Bio-Chem Valve) controlled the choice of perfusate exciting the microslide. The fluid within the system was maintained at 37°C using a separate system of tubes and heating coils connected to a heated water bath.

Glomerular Volume Change
Glomeruli that were free of Bowman’s capsule and arteriolar or tubular fragments were chosen for study. All glomerular observations were performed within 3 hours of nephrectomy. After a period of equilibration in flowing dilute perfusate (2 minutes), the rapid remote tap was switched, allowing the concentrated BSA to enter the microslide.

Analysis of Glomerular Volumetric Change
Perfusate switches were recorded on videotape, and sequences were reviewed off-line using Apple imovies (Apple USA) and an analog-to-digital converter (ADVC-300; Canopus). All measurements were done by an operator who was blind to the genotype or treatment. A sequence of images straddling the time point at which perfusate switch occurred was created. The glomerular image in each was replicated in Adobe Photoshop CS3 (Adobe Systems), and the area (Aμm²) was calculated using ImageJ (National Institutes of Health). Glomerular volume was derived from area measurements by substituting glomerular image area (A) into the formula

\[ V = \frac{4}{3} \pi r^3 \]

where \( r \) is glomerular radius, to reveal

\[ V = \frac{4}{3} A (\pi / \pi) / 10^{-6} \]

Glomerular volume (nl) was plotted against time since the first appearance of the Schlieren phenomenon, marking the arrival of the new oncompressive perfusate. Two regression lines were then applied to these points. The slope of the first was set as 0 and applied to points before the solution switch when glomerular volume was stable. The two lines were calculated to meet at their break point. This point was defined as the time point at which glomerular volume begins to decline. The second line was applied to points covering a period of at least 0.04 second and no more than 0.1 second from the break point. Within these confines, the points at which the applied regression line had the greatest slope were chosen.

Calculation of \( L_pA \)
The slope of the second regression line describes the greatest initial rate of glomerular volume change and can therefore be equated to the term \( J_v \) in the Starling equation:

\[ J_v/A = L_p \left[ (P_c - P_i) - \sigma (P_e - P_i) \right] \]

The net hydrostatic pressures acting across an isolated glomerulus can be assumed to be negligible. Previous work suggested the reflection coefficient of an isolated glomerulus is not significantly different from 1.56 The Starling equation can therefore be rearranged to show that

\[ L_p A = J_v/\Delta \Pi \]

in nl/min per mmHg, where \( \Delta \Pi \) is the difference between capillary and interstitial oncotic pressure.

Glomerular Volume and Glomerular Capillary Surface Area
The average glomerular volume in each animal was determined using the method described by Pagutlanan et al.57 Glomerular images were replicated in Adobe Photoshop CS3 (Adobe Systems), and the area (Aμm²) was calculated using ImageJ (National Institutes of Health). In each animal, glomerular area was measured in at least 30 profiles from at least three different sections. Glomerular volume was calculated from the area measurements using the formula

\[ V = \beta (k (A_{GC})^{1/2})^{1/2} \]

where \( \beta \) is the shape coefficient for spheres (the idealized shape of glomeruli) and is equal to 1.38 and \( k \) is the size distribution coefficient and is equal to 1.15,58

The surface area of the glomerular capillary wall was determined from the surface density, using resin-embedded toluidine blue-stained kidney sections, as described previously.57,59 High-magnification light micrographs of whole glomeruli were opened in Photoshop, and a 10-mm² grid was superimposed. The number of points (corners of grid squares) falling inside the glomerular profile (V grid) and the intercepts of the grid lines with the capillary wall (I grid) were counted. From these measurements, the surface density (Sf) was calculated using the equation

\[ S_f = 2 \cdot \frac{\sum I_{grid}}{K \cdot \sum P_{EP}} \]

where \( k \) is the real length of the grid line segments. For each animal, Sf was determined in between three and six glomerular profiles. The average surface area of the glomerular capillary wall expressed in mi-
crometers squared was then calculated as the product of the surface density and mean glomerular volume for each animal.

**VEGF_{165b} Experiments**

In a separate group of experiments, glomeruli from WT C57/Blk6 mice were exposed to rhVEGF_{165b} (PhiloGene). After isolation, glomeruli were incubated at 37°C in 1% BSA solution or 1% BSA solution containing either 40 pM of VEGF_{165b} or 1 nM VEGF_{165b}. Glomeruli from each solution were then individually loaded into the microslide, and the ultrafiltration coefficient was calculated as described already.

**Phenotype and Histologic Analysis**

A separate group of animals aged between 8 and 10 months were used to collect tissue, plasma, and urine for phenotypic and histologic analysis. Animals were individually housed in metabolic cages for up to 12 hours to obtain a urine sample. They were anesthetized using 5% isoflurane, and a blood sample was taken by direct cardiac puncture. Mice were then killed by cervical dislocation. The kidneys were removed, divided, and preserved by immersion in 4% paraformaldehyde, 2.5% gluteraldehyde, or liquid nitrogen.

**Immunohistochemistry**

Kidney samples from WT, heterozygous, and homozygous mice were formalin-fixed and embedded in paraffin. Five-micrometer sections were mounted onto gelatin/poly-l-lysine-coated glass slides. The sections were dried onto the slides in a 37°C incubator overnight. Sections were incubated with freshly prepared 3% hydrogen peroxide (BDH, Poole, UK) diluted in 1 vol/vol normal goat serum for 1 hour in a humid chamber at room temperature. Sections were washed twice with 0.05% vol/vol PBS-Tween, 5 minutes per wash, then incubated with a prepared avidin-biotinylated enzyme complete kit (Vector Laboratories) for 45 minutes in a humid chamber at room temperature. Again, the sections were washed twice with 0.05% vol/vol PBS-Tween, 5 minutes each time, followed by incubation with 3,3’-diaminobenzidine substrate (Vector Laboratories) to yield a brown-colored product. The reaction was stopped by washing twice with deionized water for 5 minutes. Sections were counterstained with Mayer’s hematoxylin (BDH) for 5 minutes, then differentiated in water. Sections were dehydrated by passing through increasing concentrations of ethanol (70, 90, and 100% vol/vol) for at least 2 minutes each, cleared in xylene for at least 10 minutes, and permanently mounted in DPX mountant for histology. Staining was examined with a Nikon Eclipse E-400 microscope; images were captured using a DCN-100 digital imaging system (Nikon Instruments).

**EM Analysis**

Kidney fixation procedures were adapted and modified from Hayat. Portions of kidney from each mouse were rapidly excised and sliced in a pool of glutaraldehyde fixative (2.5% glutaraldehyde in 0.1 M cacodylate buffer [pH 7.3, 4 to 8°C]). Cubes (0.5 to 1.0-mm diameter) of kidney cortex were further fixed at 4°C with glutaraldehyde fixative. After a minimum of 3 hours of fixation, the tissues were left in fresh fixative overnight, then washed in cacodylate buffer postfixed for 1 hour in osmium (1% osmium tetroxide in 0.1 M cacodylate buffer [pH 7.3, 4°C]). Tissues were washed in cacodylate buffer and then distilled water before ethanol dehydration, infiltration, and embedding in Araldite resin (Agar Scientific, Stansted, UK). Glomeruli were identified from 0.5 μm of toluidine blue-stained survey sections. Glomeruli were cut at 70 to 100 nm thick for EM observation. Analysis was conducted on digital electron micrographs (taken at ×890 and ×2900). Measurements were made of percentage of coverage of the GBF by the SPS, thickness of the GBM, height of SPS, foot process width (or separation between slit diaphragms), and separation between endothelial fenestrations and width of fenestrations. Linear measurements from electron micrographs were made at random points using a Photoshop grid. To clarify changes in fenestrations, 40-nm sections were used.

**Glomerular Filtration Rate**

GFR was determined in anesthetized 9-month-old heterozygous and age-matched littermate controls using a single bolus injection of FITC-Inulin.

**Statistical Analysis**

Data are means ± SE. P < 0.05 was regarded as significant. Methods of statistical analysis are included in relevant figure legend.

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REFERENCES


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