Title: Vascular Endothelial Growth Factor (VEGF-A)_{165b} is protective and restores endothelial glycocalyx in diabetic nephropathy

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Abstract

Diabetic nephropathy is the leading cause of end-stage kidney disease in high-income countries, and a growing problem across the world. VEGF-A is thought to be a critical mediator of vascular dysfunction in diabetic nephropathy, and yet both VEGF-A knockout and overexpression of angiogenic VEGF-A isoforms worsen diabetic nephropathy. We examined the vasculoprotective effects of the VEGF-A isoform VEGF-A_{165b} in diabetic nephropathy. VEGF-A_{165b} was upregulated in diabetic individuals with well-preserved kidney function, but not in those with progressive disease. Reproducing this VEGF-A_{165b} upregulation in mouse podocytes in vivo prevented functional and histological abnormalities in diabetic nephropathy. Systemic VEGF-A_{165b} injections, before and after the onset of kidney disease, reduced features of diabetic nephropathy in models of both early and advanced nephropathy in type 1 diabetes and early nephropathy in type 2 diabetes. VEGF-A_{165b} normalised glomerular permeability through phosphorylation of VEGFR2 in glomerular endothelial cells, and reversed diabetes-induced damage to the glomerular endothelial glycocalyx. VEGF-A_{165b} also improved the permeability function of diabetic human glomeruli. These results show that VEGF-A_{165b} acts via the endothelium to protect blood vessels and ameliorate diabetic nephropathy.
**Introduction**

Diabetic nephropathy is the leading cause of end-stage kidney disease in the industrialised world\(^1\), diabetic retinopathy is the leading cause of blindness in the working-age population\(^2\), and diabetic neuropathy eventually affects up to 50% of patients with long-standing diabetes\(^3\). Microvascular dysfunction contributes to the development and progression of each of these complications, with abnormal vascular haemodynamics and disturbed vascular permeability leading to clinically important excessive vessel leak, such as macular oedema and albuminuria\(^4\).

In recent years, there has been increasing interest in identifying factors that are endogenously produced within the vasculature, are activated or inhibited in diabetes, and therefore could afford protection against diabetic vasculopathy\(^5\) and potentially be used alongside traditional treatments. These factors, such as activated protein C\(^6\) modify the response of the vasculature to the environment, thereby protecting endothelial and other vascular cells from the diabetic milieu. Vascular Endothelial Growth Factor-A (hereafter “VEGF-A”), a family of secreted glycoprotein isoforms, has been suggested as such an endogenous protective factor\(^5,7\). VEGF-A levels are dysregulated, and VEGF-A receptor pathways activated, in many dysfunctional vascular beds in diabetes\(^5\). In glomeruli, VEGF-A is predominantly produced by podocytes, and its actions on endothelial cells and podocytes have been described\(^8,9\). Targeted, inducible genetic deletion of all VEGF-A isoforms from podocytes causes glomerular disease in healthy animals\(^10\), and accelerated nephropathy in diabetic animals\(^7\). Total glomerular VEGF-A levels decrease as diabetic nephropathy progresses in man\(^11\), and anti-VEGF-A antibodies induce proteinuria in humans\(^10\). VEGF-A is therefore considered to provide an endogenous "protective" signal that prevents apoptosis of vascular wall cells and hence progression of diabetic nephropathy.

However, upregulating specific VEGF-A isoforms, such as VEGF-A\(_{165a}\), can also accelerate diabetic nephropathy\(^12\). VEGF-A\(_{165a}\) is a potent vasoactive agent, increasing vasodilation, vascular permeability and angiogenesis\(^13\), processes that can drive the adverse functional consequences of diabetic vasculopathy. An
equilibrium between sufficient (protective) and excessive (damaging) levels of VEGF-A, or an appropriately-timed VEGF-A response, may underly this paradox. An alternative explanation, however, is that these protective and deleterious VEGF-A actions in diabetes could be specific to VEGF-A isoforms, where some VEGF-A isoforms (e.g. VEGF-A\textsubscript{164a}) are detrimental, and others confer protection\textsuperscript{(14,15)}. An alternative VEGF-A isoform, VEGF-A\textsubscript{165b}, has recently been shown to confer benefit in other microvascular disease states\textsuperscript{(16-18)}. We therefore tested the hypothesis that VEGF-A\textsubscript{165b} could act as a protective factor in diabetic nephropathy.
Results

**VEGF-A\textsubscript{165b} is upregulated in humans with diabetic nephropathy and well-preserved kidney function**

In twelve patients with biopsy-confirmed diabetic glomerulosclerosis, expression of exon 5/7/8-containing VEGF-A isoform (i.e. VEGF-A\textsubscript{165a} + VEGF-A\textsubscript{165b}) mRNA was significantly reduced compared with five non-diabetic individuals (11.9±4.2 vs 80.0±42.3 pg/ug mRNA; p<0.05, unpaired t-test). When diabetic nephropathy was categorised as “early” with well preserved kidney function and low-grade proteinuria (creatinine 89±8 µmol/L; proteinuria 1.0±0.3g/L; n=5; supplementary table 1) or “late” with elevated creatinine and heavy proteinuria (serum creatinine 242±10 µmol/L; proteinuria 7.8±0.2g/L; n=7; supplementary table 1), clear differences in the expression of different VEGF isoforms were apparent (figure 1). Total VEGF-A\textsubscript{165a} + VEGF-A\textsubscript{165b} mRNA expression progressively declined from “early” to “late” diabetic nephropathy, predominantly due to decreased VEGF-A\textsubscript{165a} mRNA levels (figure 1A, B). However, whilst VEGF-A\textsubscript{165b} levels also decreased in “late” diabetic nephropathy (figure 1C), VEGF-A\textsubscript{165b} levels significantly increased ~6-fold in “early” diabetic nephropathy. Thus only those with “early” diabetic nephropathy exhibited an ~12-fold increase in VEGF-A\textsubscript{165b} as a proportion of total VEGF-A isoforms present in the kidney (figure 1D). Similar changes were observed in just the glomerular compartment (figure 1E) and at the protein level (figure 1F). On the basis of these observations, we hypothesised that increasing glomerular VEGF-A\textsubscript{165b} confers protection against diabetic nephropathy.

**Podocyte-specific VEGF-A\textsubscript{165b} overexpression protects against diabetic nephropathy.**

Streptozotocin (STZ)-induced diabetic wild-type and podocyte-specific VEGF-A\textsubscript{165b} – overexpressing mice (nephVEGF-A\textsubscript{165b}) (figure 2A) demonstrated equivalent hyperglycaemia (figure 2B). Albuminuria increased 10.8-fold in diabetic wild-type mice but only 2.2-fold in diabetic nephVEGF-A\textsubscript{165b} mice, a change that was not statistically significantly different from non-diabetic controls (figure 2C). The early diabetes-related rise in creatinine clearance in diabetic wild-type mice was also attenuated in diabetic nephVEGF-A\textsubscript{165b} mice (figure 2D). After 6 weeks of diabetes,
VEGF-A165b-overexpressing mice also demonstrated less glomerular hypertrophy (figure 2E,F), less mesangial matrix expansion (figure 2G,H) and less glomerular basement membrane thickening (figure 2I,J).

Given that podocyte-specific overexpression of murine VEGF-A164a (podVEGF-A164a) accelerates diabetic nephropathy(19), and that nephVEGF-A165b overexpression can block inducible podVEGF-A164a-overexpression permeability changes in healthy mice(20), we hypothesised that nephVEGF-A165b effects would be sufficiently robust to prevent the more aggressive form of nephropathy in diabetic podVEGF-A164a mice. Wild-type, single and double-transgenic nephVEGF-A165b/podVEGF-A164a mice (figure 3A) all demonstrated equal hyperglycaemia 5 weeks after streptozotocin. Albuminuria increased a further 3-fold above diabetic wild-type littermate values after 1 week of VEGF-A165a induction in diabetic podVEGF-A164a mice, an effect completely blocked by co-overexpression of VEGF-A165a with VEGF-A165b (figure 3B).

**Systemic injections of VEGF-A165b protect against diabetic nephropathy.**

Systemic injections of recombinant human VEGF-A165b protein (rhVEGF-A165b) reduce angiogenesis-related cancer growth in mice without altering blood pressure, renal function or eliciting adverse effects(21). We therefore assessed the efficacy of systemic rhVEGF-A165b injections in STZ-induced diabetes in DBA2J mice before and after the onset of diabetic nephropathy (figure 4A). After 2 weeks of consistent hyperglycaemia, low-dose 0.04µg/kg rhVEGF-A165b injections were initiated and continued until an approved study endpoint occurred (significant weight loss). Equivalent blood glucose concentrations were observed in both treatment groups (figure 4B). rhVEGF-A165b injections consistently reduced urinary albumin:creatinine ratios in diabetic mice, and this effect persisted throughout the entire period of hyperglycaemia (figure 4C).

In a separate cohort of diabetic DBA2J mice, low (0.04µg/kg) or high dose (0.2µg/kg) systemic rhVEGF-A165b injections were initiated only after the onset of heavy albuminuria (12 wks post-STZ; figure 4D). Notably, albuminuria in these different cohort of mice receiving systemic injections was 5–20-fold greater than in the above groups. Again, vehicle- and rhVEGF-A165b-treated diabetic mice had equivalent blood
glucose concentrations. Urinary albumin:creatinine ratios continued to rise in vehicle-treated diabetic DBA2J mice (figure 4E). Despite having the highest urine albumin:creatinine ratios at the start of rhVEGF-A165b treatment, no increase in albuminuria was observed in mice receiving 0.04µg/kg rhVEGF-A165b injections, and there was a reduction in albuminuria in mice receiving 0.2µg/kg rhVEGF-A165b (figure 4E,F). The rhVEGF-A165b-induced improvements in albuminuria were significant at both treatment doses (figure 4F). rhVEGF-A165b injections also improved diabetes-induced glomerular basement membrane thickening (figure 4G,H) but not mesangial matrix expansion.

**Systemic injections of VEGF-A165b improve early albuminuria but not GFR in a type 2 diabetic nephropathy model.**

The global increase in diabetic nephropathy is predominantly due to type 2 diabetes. We therefore administered 8 weeks of systemic rhVEGF-A165b injections to obese db/db mice after the onset of hyperglycaemia but before the onset of albuminuria or decreased GFR (figure 5A). rhVEGF-A165b injections (0.2µg/kg) did not alter blood glucose or body weight (figure 5B), but significantly reduced albuminuria (p<0.05, figure 5C). Despite this improvement in albuminuria, however, rhVEGF-A165b injections did not prevent the diabetes-related decline in creatinine clearance (figure 5D) in these mice. As in the type 1 diabetes model, we also assessed the efficacy of rhVEGF-A165b injections started after the onset of both albuminuria and decreased creatinine clearance (figure 5E). No progression of albuminuria was noted in these db/db mice after this time (figure 5F), and rhVEGF-A165b injections initiated at this later timepoint did not modify the pattern of albuminuria (figure 5F), prevent further loss of GFR (figure 5G) or decrease mesangial matrix expansion (figure 5H).

**VEGF-A165b acts via VEGFR-2 to modify glomerular endothelium.**

Both podocytes and endothelial cells play important roles in the progression of diabetic nephropathy. Human podocytes exhibited significant apoptosis when cultured in high glucose-containing medium, a response partially rescued by rhVEGF-A165b (figure 6A). Human endothelial cells also demonstrated significant apoptosis in response to hyperglycaemia, and rhVEGF-A165b completely blocked the
hyperglycaemia-induced endothelial cell apoptosis (figure 6B,C). The pan-VEGFR tyrosine kinase inhibitor PTK787 (figure 6C) prevented the anti-apoptotic effects of rhVEGF-A\textsubscript{165b} on hyperglycaemic endothelial cells.

To determine the functional significance of this VEGF-A\textsubscript{165b}-VEGFR2 signalling, we measured glomerular water permeability in healthy and diabetic rat glomeruli in the presence of VEGF-receptor inhibitors (figure 6D). Single glomeruli harvested from diabetic rat kidneys had higher volume-corrected glomerular ultrafiltration coefficient ($L_P/A/V$) than glomeruli from non-diabetic rats, and this was normalised by blockade of all VEGF receptors with PTK787 but not just by blockade of VEGFR-2 with ZM323881, indicating that multiple VEGF receptors contribute to glomerular permeability defects in diabetes. In keeping with effects in the intact animal, this diabetes-induced glomerular permeability change was reversed by rhVEGF-A\textsubscript{165b}. This normalisation by rhVEGF-A\textsubscript{165b} was completely blocked by the pan-VEGF-receptor inhibitor PTK787, and also blocked by the selective VEGFR-2 inhibitor ZM323881, indicating a key role for VEGFR-2 in diabetic nephropathy in mediating these beneficial effects of VEGF-A\textsubscript{165b}.

Immunofluorescence studies demonstrated increased total and phosphorylated forms of VEGFR2 in the glomeruli of both neph\textsuperscript{h}VEGF-A\textsubscript{165b}-overexpressing mice (figure 6E,G), and more robustly in the glomeruli of streptozotocin-induced diabetic mice treated with systemic rhVEGF-A\textsubscript{165b} injections (figure 6F,H). These latter findings confirm that systemic injections of VEGF-A\textsubscript{165b} had direct effects on VEGF-signalling pathways within glomerular cells. In keeping with previous transgenic studies(9), and dominant anti-apoptotic effects of VEGF-A\textsubscript{165b} on endothelial cells, upregulated VEGFR2 in neph\textsuperscript{h}VEGF-A\textsubscript{165b}-overexpressing mice co-localised with PECAM-1 in glomerular endothelial cells (figure 7A,B), but not with nephrin (figure 7C,D). Thus the dominant actions of acute incubation, local overexpression and systemic treatment with VEGF-A\textsubscript{165b} appear to involve phosphorylation of VEGFR2 in the glomerular endothelium.

**VEGF-A\textsubscript{165b} restores glomerular endothelial glyocalyx in diabetic nephropathy.**

VEGF-A\textsubscript{165b}-induced VEGFR2 activation in the glomerular endothelium raises the
question of how glomerular endothelial modifications could restore normal glomerular function in diabetes. Electron micrographs (figure 8A) revealed decreased glomerular endothelial fenestration in STZ-injected diabetic vehicle-treated DBA2J mice, but this was not reversed by rhVEGF-A165b treatment (figure 8B). Direct imaging of the glomerular endothelial glycocalyx (figure 8A) demonstrated a 50% reduction in glycocalyx depth in vehicle-treated diabetic mice (p<0.05, figure 8C). In keeping with the observed improvements in albuminuria, rhVEGF-A165b injections restored glomerular endothelial glycocalyx depth in diabetic mice to levels observed in healthy non-diabetic control animals (figure 8C). Loss of endothelial glycocalyx in diabetes, and restoration by rhVEGF-A165b, was confirmed by confocal imaging of viable, unfixed glomeruli in STZ-injected diabetic rats (figure 9).

**VEGF-A165b improves human diabetic glomerular function.**

Animal models of diabetic nephropathy exhibit substantial differences from human disease. The functional benefits of VEGF-A165b observed in multiple diabetic animal models were therefore tested on diabetic human glomeruli as well. In humans, rats and mice, LpA/Vi was significantly higher in glomeruli harvested from diabetic kidneys (all p<0.05 vs non-diabetic controls, figure 10A). In addition, either long-term podocyte-specific overexpression of hVEGF-A165b (mice) or acute incubation with rhVEGF-A165b (rats and humans) reversed this diabetes-related increase in glomerular permeability (all p<0.05 vs vehicle-incubated diabetic glomeruli), and restored glomerular permeability to levels observed in non-diabetic controls (figure 10A). In paired measurements of diabetic human glomeruli permeability before and after exposure to rhVEGF-A165b, incubation with rhVEGF-A165b also reduced glomerular LpA/Vi to normal values (figure 10B).
Discussion

We show here that increasing VEGF-A\textsubscript{165b} levels effectively improves a number of functional and histological features of diabetic nephropathy. It can do so locally (through podocyte-specific overexpression) and systemically (via repeated intraperitoneal injections). It can do so in the context of additional upregulation of VEGF-A\textsubscript{164a} and in nephropathy complicating models of both type 1 and and type 2 diabetes, and it can do so both before and after the onset of albuminuria. We further demonstrate that VEGF-A\textsubscript{165b} acts on glomerular permeability through VEGFR2 in glomerular endothelial cells, and reverses diabetes-induced damage to the glomerular endothelial glycocalyx. We also demonstrate changes in the balance of VEGF-A\textsubscript{xxx}a/VEGF-A\textsubscript{xxx}b isoforms in human diabetic nephropathy, that glomerular permeability is increased in diabetic human glomeruli, and that the direct effects of VEGF-A\textsubscript{165b} on the glomerular capillary wall observed in animal models of diabetic nephropathy are reproduced at a functional level in diabetic human glomeruli. Thus it appears that VEGF-A\textsubscript{165b} can provide the endogenous protective actions of VEGF-A in diabetic nephropathy, without driving the deleterious consequences that appear to be mediated via VEGF-A\textsubscript{165a} in diabetic nephropathy.

Despite improved control of systemic parameters in diabetes such as hyperglycaemia, hypertension and hyperlipidaemia, diabetic nephropathy remains the leading cause of end-stage kidney disease across the developed world and no specific treatment for diabetic nephropathy is available\cite{1}. Recent developments have focussed on harnessing endogenous vasculoprotective factors that could provide additional and complementary benefits for patients with diabetes\cite{5}. VEGF-A has long been considered a critical component of the vascular complications of diabetes. VEGF-A is generated as two families of isoforms according to alternative splicing of the terminal exon, exon 8\cite{22}. The canonical, proangiogenic isoforms such as VEGF-A\textsubscript{165a} (VEGF-A\textsubscript{164a} in the mouse) are matched by a family of antagonistic isoforms (e.g. VEGF-A\textsubscript{165b}, generically termed VEGF-A\textsubscript{xxx}b where xxx is the number of amino acids they encode). These VEGF-A\textsubscript{xxx}b isoforms match the pro-survival and anti-apoptotic effects of angiogenic VEGF-A isoforms, but do not drive angiogenesis, do not cause sustained increases in permeability, do not cause vasodilatation and can prevent the angiogenic and pro-permeability effects of VEGF-
VEGF-A_{164a}(20,23). VEGF-A_{164a} is not an endogenous renoprotective factor in diabetes, since overexpressing VEGF-A_{164a} in diabetic mice accelerates nephropathy(19). Our studies demonstrate that the VEGF-A_{165b} isoform confers benefit in diabetic nephropathy. This clarifies the previous paradox that both VEGF overexpression and depletion are detrimental in diabetic nephropathy. It is therefore not just a timely and appropriate level of total VEGF-A that regulates glomerular and kidney function in diabetic nephropathy, but an appropriate balance of isoforms, and specifically sufficient levels of the protective VEGF-A_{165b} isoform.

This demonstration that VEGF-A_{165b} is a protective isoform in kidney disease is consistent with a series of reports that non-specific inhibition of all VEGF isoforms, for example with anti-VEGF antibodies, results in proteinuria and overt renal disease(10). Such strategies will deplete both VEGF-A isoform families, thereby removing the protective actions of VEGF-A_{165b} from the glomerular capillary wall and eliciting renal dysfunction and disease. Comparable isoform-specific benefits of VEGF-A_{165b} have also been reported in systemic sclerosis(17) and Denys-Drash syndrome(18), ocular disease(16,24) and cancer(25). Agents capable of manipulating the balance of VEGF-A isoforms could therefore offer a therapeutic strategy in a number of important human diseases, including diabetic nephropathy(26). We show here that diabetic patients with well preserved kidney function have increased VEGF-A_{165b} levels relative to VEGF-A_{165a}. Transgenic podocyte-specific VEGF-A_{165b} overexpression is the most analogous experimental model of this human situation, and it is noteworthy that the local upregulation of VEGF-A_{165b} was the most effective approach in preventing multiple physiological and histological changes of diabetic nephropathy. We hypothesise that patients capable of upregulating VEGF-A_{165b} relative to VEGF-A_{165a}, via the modifiable machinery that regulates alternative splicing of the VEGF-A gene(23, 26-29), may experience protection from progressive nephropathy in diabetes. How to manipulate this alternative splicing mechanism for therapeutic benefit requires further exploration.

VEGF-R2 mediates the major actions of VEGF-A family members, is indispensable
for normal glomerular function(9), and mediates the beneficial effects of VEGF-A165b observed here. VEGF-A165b and VEGF-A165a bind VEGF-R2 with equal affinity, but elicit different tyrosine residue phosphorylation patterns, different VEGF-
R2/neuropilin-1 heterodimerisation patterns(30,31), and different downstream signalling pathways(30), resulting in different migration, proliferation and cytoprotection behaviours in endothelial (and other) cell types(22,24,31). The observation that selective VEGF-R2 blockade in diabetic animals worsened albuminuria and endothelial cell apoptosis(32) is consistent with blocking beneficial endogenous VEGF-A165b signalling in rodents. Despite appropriate positive controls, however, the coding sequence of the rodent homologue of VEGF-A165b has not yet been identified(33,34). In addition, the coding sequences for exon 8 of VEGF-A165a (exon 8a) and VEGF-A165b (exon 8b) are overlapping, hitherto preventing generation of a VEGF-A165b knockout mouse model.

Importantly, systemic injections of VEGF-A165b in the type 1 diabetes model also decreased heavy albuminuria and improved some of the histological features of diabetic nephropathy, even when treatment was started after the onset of nephropathy as might be required in clinical practice. The importance of defects in glomerular permeability in animal models of early albuminuric diabetic nephropathy is contested(35,36). These studies confirm that at least some facets of glomerular structure (endothelial glycocalyx) and permeability (ultrafiltration coefficient) are abnormal in early albuminuric diabetic nephropathy, and that reversing these glomerular defects through VEGF-A165b administration improves glomerular structure/function, albuminuria and histology. This emphasizes the important link between changes in glomerular function and albuminuria in early diabetic nephropathy, without excluding the potential importance of diabetes-induced changes in other parts of the nephron(35-37). In the type 2 diabetes db/db mouse model, VEGF-A165b injections reduced early albuminuria, but did not preserve GFR nor modify outcomes when initiated after the onset of nephropathy. Whether greater VEGF-A165b efficacy in type 1 diabetes models represents differences in disease timecourse(4), pharmacokinetics or validity of the animal model(38, 39) is not clear. In this type 2 diabetes model, however, albuminuria was relatively low in all animals, and after 3 months regressed rather than progressed with time.
VEGFR2 activated by VEGF-A_{165b} was localised to the endothelium. Decreased glomerular endothelial fenestrations, as observed here, has also been reported in diabetic humans, and the possibility of an associated reduction in water permeability was considered (40). In fact, the opposite is the case – water permeability is increased in diabetic human glomeruli. Moreover, VEGF-A_{165b} restored normal water permeability and decreased albuminuria but did not modify fenestral density, suggesting that an alternative endothelial structure may be physiologically important, and amenable to regeneration diabetic nephropathy. The endothelial glycocalyx is a key regulator of the amount of water and solutes that can cross vessel walls throughout the microcirculation, as well as regulating leukocyte adhesion and endothelial responses to mechanical stimuli (41). The techniques employed here confirm (42) that glomerular endothelial glycocalyx can be imaged directly in functioning glomeruli and coupled with physiological measurements, complementing techniques involving tissue fixation and indirect estimates of glycocalyx depth by exclusion. The observed decrease in glomerular endothelial glycocalyx depth using two different methods is consistent with albuminuria and increased water permeability in these two animal models of diabetic nephropathy, and matches glomerular permeability defects (fig 10) and systemic endothelial glycocalyx defects (43,44) in diabetic humans. In addition, both acute and chronic restoration of the endothelial glycocalyx by VEGF-A_{165b} are also compatible with the observed improvements in both water permeability and albuminuria. The timecourse of acute VEGF-A_{165b}-induced changes in glomerular structure and function match the timecourse of VEGF-induced changes in glomerular permeability (15,45,46), and both growth factor-induced (47) and pharmacological (48) restoration of endothelial glycocalyx, and indicate that turnover and restoration of endothelial glycocalyx can be rapid. Importantly, this is also the first demonstration that long-term restoration of the endothelial glycocalyx is both feasible and associated with improved long-term outcomes. Since the endothelial glycocalyx regulates mechanotransduction, leukocyte adhesion and nitric oxide bioavailability (49), all of which are abnormal in diabetic microvessels, restoring the endothelial glycocalyx may improve multiple vascular abnormalities in diabetes. Identifying the causal nature of the relationship
provides an important direction for future work that may have implications for vasculoprotection in a broad range of renal and other pathologies.

In summary, we show here that VEGF-A\textsubscript{165b} is upregulated in diabetic patients whose kidney function is well preserved, and that when VEGF-A\textsubscript{165b} is administered through a variety of routes, confers functional and histological benefit in a series of animal models of diabetic nephropathy. VEGF-A\textsubscript{165b} also effectively restores the endothelial glycocalyx in diabetic nephropathy, and improves the permeability function of glomeruli from diabetic humans. VEGF-A\textsubscript{165b} therefore appears to exert important protective actions in diabetic nephropathy.
Methods

All experiments were conducted in accordance with UK legislation and local ethical committee approval. Studies on human kidney tissue were approved by National and Local Research Ethics Committees (Institutional Ethical Committee, Leiden University Medical Centre, The Netherlands; South West – Central Bristol NHS REC, UK; East Midlands – Leicester NHS REC, UK), and conducted in accordance with the Declaration of Helsinki. Human renal cortex samples were harvested from kidneys offered for transplantation but technically unsuitable for implantation from donors without diabetes and those with early diabetic nephropathy, and for the late nephropathy group from renal biopsy specimens harvested for clinical reasons. Single glomeruli were harvested by differential sieving for molecular and physiology studies. Animal studies were approved by University of Bristol research ethics committee, and conducted in accordance with UK legislation.

Isoform-specific RT-qPCR

Total RNA (RNA) was isolated from human kidney tissues with Trizol (Invitrogen) and cleaned up with RNeasy Micro Kit (Qiagen) as per the manufacturer’s suggestion. 0.5 to 1 μg of RNA was reverse transcribed into cDNA with QuantiTect Reverse Transcription Kit (Qiagen). cDNA was subjected to quantitative PCR (qPCR) with forward primer 5’- GAGCAAGACAAGAAAATCCC -3’ that spans exon 5 and 7 and reverse primer 5’- CCTCGGCTTGTCACATCTG -3’ spanning exon 7 and 8a to amplify VEGF-A_{165a}, and with forward primer 5’- GAGCAAGACAAGAAAATCCCTGACATCTG -3’ and reverse primer 5’- GTGAGAGATCTGCAAGTACG -3’ spanning exon 7 and 8b to amplify VEGF-A_{165b}. Each reaction contained 10 μl of SYBR green master (from Qiagen), 400 nM forward and reverse primers, 2 μl plasmid DNA or sample cDNA, and water to 20 μl. qPCR amplification was initiated with 95°C for 15 mins, 45 cycles of 95°C for 30 secs, 60°C for 30 secs and 72°C for 30 secs, followed by final extension at 72°C for 10 mins. For VEGF-A_{165a} qPCR, both standards (10^{-2} to 10^{-6} ng of pcDNA3-VEGF-A_{165a}) and samples were repeated in duplicate. Amplification of known amounts of plasmid pcDNA3-VEGF-A_{165a} DNA rendered the formation of a standards curve of DNA amount (x-axis) against Ct values (y-axis). The amount of VEGF-A_{165a} mRNA expression was extrapolated by comparing
sample Ct value with the standards curve from plasmid DNA amplification. Interference for VEGF-A\textsubscript{165a} qPCR from VEGF-A\textsubscript{165b} was calculated from Ct values obtained from the amplification of VEGF-A\textsubscript{165a} signal from known concentrations of plasmid pcDNA3-VEGF-A\textsubscript{165b} (10\textsuperscript{-2} to 10\textsuperscript{-4} ng). The interference was less than 1/1000. For VEGF-A\textsubscript{165b} qPCR, a standard curve was formed from amplification of known amounts of plasmid pcDNA3-VEGF-A\textsubscript{165b} DNA (10\textsuperscript{-2} to 10\textsuperscript{-6} ng). The amount of VEGF-A\textsubscript{165b} in samples was extrapolated by comparing sample Ct value with the standards curve from plasmid DNA amplification. Interference for VEGF-A\textsubscript{165b} qPCR from VEGF-A\textsubscript{165} was calculated from Ct values obtained from the amplification of VEGF-A\textsubscript{165b} signal from known concentrations of plasmid pcDNA3-VEGF-A\textsubscript{165} (10\textsuperscript{-2} to 10\textsuperscript{-4} ng). The interference was less than 1/3000.

*Enzyme-linked immunosorbent assay (ELISA) of pan-VEGF & VEGF-A\textsubscript{165b}*  
Protein was isolated from human kidney tissues after being homogenised in RIPA buffer (Sigma) and quantified with Bio-rad assay (Bio-rad). Pan-VEGF-A & VEGF-A\textsubscript{165b} detection were followed with the manufacture’s suggestion (Duoset human VEGF-A, Cat. No. DY293, R&D, & Duoset human VEGF-A\textsubscript{165b} Cat. No. DY3045, R&D). Briefly, 0.08 µg of capture antibody diluted in 1x PBS (pH 7.4) was adsorbed onto each well of a 96-well plate (Immuron 2HB, Thermo Life Sciences, Basingstoke, UK) overnight at room temperature. The plate was washed three times between each step with 1xPBS-Tween (0.05%). After blocking with 100 µl of 1%BSA in PBS for 1 h at 37°C, 100 µl of recombinant human VEGF-A\textsubscript{165a} for VEGF-A ELISA, or recombinant human VEGF-A\textsubscript{165b} for VEGF-A\textsubscript{165b} ELISA, diluted in 1%BSA in PBS (ranging from 62.5 pg/ml to 4 ng/ml) or protein samples were added to each well. After incubation for 1 h at 37°C with shaking and three washes, 100 µl of detection antibody at 0.05 µg/ml was added to each well, and the plate left for 1 h at 37°C with shaking. 100 µl of streptavidin-HRP (R&D Systems) at 1:200 dilution in 1%BSA in PBS was added, the plate left at room temperature for 20 mins and 100 µl/well O-phenylenediamine dihydrochloride solution (Substrate reagent pack DY-999; R&D Systems) added, protected from light and incubated for 20 mins at room temperature. The reaction was stopped with 50 µl/well 1 M H\textsubscript{2}SO\textsubscript{4} , and absorbance read immediately in the Opsys MR 96 well plate reader at 492 nm, with control reading at 460nm.
**Animal studies**

Transgenic mice were obtained from in-house colonies. Adult male mice were used for all mouse studies, and wild-type littermate controls used for comparison. Details of the transgenic strategies for the *neph*\(^h\)VEGF-A\(_{165b}\) and *pod-rTta:TetO-\(^m\)VEGF-A\(_{164a}\) mice are provided in detail elsewhere (15, 20). *pod-rTta:TetO-\(^m\)VEGF-A\(_{164a}\) mice were generated by crossing *podocin*-\(rTtA\) and *TetO-VEGF-A\(_{164a}\) mice supplied by Prof Susan Quaggin with permission from Jeffrey Kopp (NIDDK, NIH, Bethesda) and Jeff Whitsett (Children’s Hospital Medical Center, Cincinnatti, OH).

Overexpression of human VEGF-A\(_{165b}\) in podocytes (*neph*\(^h\)VEGF-A\(_{165b}\)) was constitutive. Overexpression of murine VEGF-A\(_{164a}\) (*\(^m\)VEGF-A\(_{164a}\)) in podocytes in *pod-rTta:TetO-\(^m\)VEGF-A\(_{164a}\) mice was induced by adding doxycycline (2mg/ml) in light-protected drinking water containing 5%w/v sucrose that was changed twice per week.

*neph*\(^h\)VEGF-A\(_{165b}\) were generated on a C57Bl6 background, and diabetes induction with a moderate-dose streptozotocin regime (100 mg/kg for 3 consecutive days) was therefore required to elicit nephropathy in the littermate controls. After crossing *neph*\(^h\)VEGF-A\(_{165b}\) mice with *pod-rTta:TetO-\(^m\)VEGF-A\(_{164a}\) mice (mixed strain background), the low-dose AMDCC streptozotocin protocol (50 mg/kg for 5 consecutive days) was used. DBA2J mice were also treated with the low-dose AMDCC streptozotocin protocol (50) to induce diabetes and associated nephropathy. *db/db* mice (BKS.Cg-Lepr\(^{db}\)/Lepr\(^{db}\)/OlaHsd) were purchased from Harlan Laboratories U.K., and fed a standard rodent diet. Diabetes was induced in rats with a single intravenous injection of streptozotocin (45mg/kg) (51). All animals were fasted for 4-6 hours prior to administration of streptozotocin. Recombinant human VEGF-A\(_{165b}\) (*\(^m\)VEGF-A\(_{165b}\)) for intraperitoneal injection was generated as previously described (30), and was administered after confirmation of two consecutive readings of hyperglycaemia (>16 mmol).

Body weight and blood glucose (ACCU-CHEK Aviva, Roche) were monitored weekly starting 4 weeks after streptozotocin administration in mice, 1 week after
administration of streptozotocin in rats, and starting from 6 weeks of age in db/db mice. Urine samples were collected by overnight housing in metabolic cages. Urinary albumin was quantified with albumin ELISA (Bethyl Laboratories, Inc.), and creatinine using an enzymatic spectrophotometric assay (Konelab T-Series 981845; Thermo Fisher Scientific). Urinary albumin:creatinine ratio was calculated as the quotient of albumin concentration and creatinine concentration. Creatinine clearance was calculated from the volume of urine produced in a timed collection, creatinine concentration in the same sample and creatinine concentration in blood harvested at culling. GFR was determined with FITC-Inulin injection in conscious mice prior to culling, in accordance with the AMDCC protocol (52).

Kidney cortex samples harvested immediately post-mortem were flash frozen, and 5- to 10μm sections cut using a rotary microtome. Sections were mounted onto glass slides and stained with haematoxylin and eosin using standard techniques. Images were captured using a DCN-100 digital imaging system (Nikon Instruments, Surrey, UK). Glomerular area was calculated from the glomerular outline. Separate sections were stained with 0.5% periodic acid, Schiff reagent and Mayer’s haematoxylin. The proportion of the glomerulus positive for PAS stain was determined according to AMDCC protocols using Adobe Photoshop software.

For standard transmission electron microscopy animals were flushed by transcardiac perfusion with Ringer solution and then fixed with 2.5% glutaraldehyde (± 1% alcian blue for endothelial glycocalyx labeling), and small pieces (0.5- to 1-mm diameter) of kidney cortex studies were rapidly excised and fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (4°C), washed in 0.1 M cacodylate buffer, postfixed in 1% osmium tetroxide and washed in distilled water. Tissues were ethanol-dehydrated and embedded in an Araldite (Agar Scientific). 50- to 100-nm thick sections were stained with 3% (aqueous) uranyl acetate and Reynolds’ lead citrate solution. Digital micrographs were taken on either a Phillips 100CS microscope or Tecnai T12 microscope at x940, x1250, and x6,200. Images of the glomerular capillary wall were blinded, and detailed measurements taken randomly using a pre-defined algorithm using Adobe Photoshop. A fixed digital grid was superimposed over the electron micrograph, and glycocalyx depth was measured at points of intersection between these fixed gridlines and the image of the glomerular capillary wall. In total, 1,696
randomised measurements of glycocalyx depth in 179 glomerular capillaries from 9 animals across the 3 experimental groups were made using this algorithm by a blinded observer, and all analyses were completed prior to unblinding. Endothelial fenestrae were also counted in the same images, and fenestral density was calculated as the percentage of GBM filtration surface covered by endothelial fenestrae.

Kidney samples for immunofluorescence studies were formalin-fixed and embedded in wax. 5μm sections were mounted onto glass slides and left to dry overnight at 37°C. Sections were then de-waxed and re-hydrated before staining using anti-VEGFR-2 1:50 (Cell Signaling) and anti-P-VEGFR-2 (Y-951) 10μg/ml (Santa Cruz). Primary antibodies were left to incubate at 4°C overnight before incubating with the appropriate fluorescent secondary. Before imaging, the sections were also stained for Hoechst, and then viewed on the fluorescent microscope. The area of staining for VEGFR-2/p-VEGFR-2 was normalized to glomerular area to calculate the percentage glomerular area covered by staining. For co-localisation studies, fresh-frozen 5μm sections were embedded in O.C.T. compound. Sections were fixed in 4% PFA before co-staining with anti-VEGFR-2 1:50 (Cell Signaling) and either anti-nephrin 10μg/ml (2B Scientific/Acris) or anti-pecam-1 10μg/ml (Abcam). Z-stack confocal microscopy imaging was used to assess co-localisation.

For confocal imaging of the glomerular endothelial glycocalyx in functioning glomeruli avoiding fixation artefact, kidneys were perfused in vivo with R18 (cell membrane label) and alexa-488-labelled wheat germ agglutinin lectin (labels N-acetyl glucosamine (a component of heparan sulfate and hyaluronan glycosaminoglycans) and N-acetyl neuraminic acid (a major sialic acid present on the endothelial cell surface) glycosaminoglycans on the endothelial cell surface). Glomeruli were isolated by differential sieving, incubated in vehicle or 1nM rhVEGF-A165b to match glomerular physiology measurements, and imaged with confocal microscopy. Glomerular endothelial glycocalyx depth was measured as the distance between peak fluorescent signal from the cell membrane over the endothelial cell body, and from the overlying glomerular endothelial glycocalyx; measurements were taken over the endothelial cell body to eliminate interference from labelled glycosaminoglycans within the glomerular basement membrane beneath attenuated and fenestrated endothelial areas elsewhere in the glomerular capillary wall.
**Cell culture studies**

Caspase 3 activity was measured in conditionally immortalised human podocytes (53) using the CaspACE assay kit (Promega) according the manufacturer’s indications. Briefly, cells were lysed and incubated with the Asp-Glu-Val-Asp-p-Nitroaniline (DEVD-pNA) colorimetric substrate which, upon cleavage by caspase 3, results in free pNA. Free pNA produces a yellow colour monitored at 405nm and proportional to the caspase activity. Absorbion at 405nm is normalized on the amount of total protein in samples.

Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cords obtained with consent under ethical approval, and cultured (up to passage 6) until they reached 80% confluence. HUVEC were then treated with either normal EBM2 media (5mM glucose; Lonza) or with media that contained an additional 30mM glucose for hyperglycaemic conditions. Osmotic control was performed replacing 30mM glucose with 30mM mannitol. Drug groups used combinations of the following Vehicle (PBS; n=4 per group), 2.5nM VEGF-A_{165b} (n=4 per group) and/or 200nM PTK787 (Vandetanib: (54)). Number of activated caspase III cells were totalled from 5 random field of views per well. These were incubated for 24hrs and were processed for polyclonal rabbit activated caspase III (apoptotic cell death marker; (1 in 500 New England Biolabs) staining and hoescht. Negative controls were performed; no primary antibody or cleaved caspase III blocking peptide (New England Biolabs).

**Glomerular permeability assay**

Glomeruli were harvested from mouse, rat or human cortical renal tissue by differential sieving and the glomerular ultrafiltration coefficient (\(L_{PA}\): hydraulic conductivity-area product) measured using a validated oncometric assay as previously described (45). In brief, individual glomeruli were mounted on a holding pipette within a flow-controlled observation chamber by gentle aspiration. The perifusate surrounding the glomerulus was rapidly exchanged from incubating solution (1% bovine serum albumin (BSA) in HEPES Ringer) to 8% BSA-HEPES Ringer, thereby increasing the oncotic pressure outside the glomerular capillary wall.
relative to the glomerular capillary lumen. The consequent flux of water out of the glomerular capillaries results in a rapid reduction in glomerular volume, which was recorded continuously on video-camera and analysed off-line using Fiji software (55) with in-house macros (developed by K Arkill). Linear regression analysis was used to calculate the initial (<0.1s) rate of reduction of glomerular volume ($J_V$) in response to the applied oncotic pressure gradient ($\Delta\Pi$), the quotient of which describes glomerular ultrafiltration coefficient ($L_P A$). For incubation studies, glomeruli were incubated for 1 hour with appropriate agents: 1nM of rhVEGF-A165b (30); 10μM ZM 323881 (Calbiochem, CA, USA); 100nM PTK 787 (Novartis Pharma, Basel, Switzerland). Human glomeruli are significantly larger than rodent glomeruli (p<0.05, supp figure 1A) and therefore present a greater surface area ($A$) for fluid exchange across the glomerular capillary wall. Glomeruli harvested from healthy humans had substantially higher ultrafiltration coefficient ($L_P A$) values than rats and mice (p<0.05, supp figure 1B). When corrected for glomerular volume ($V_i$), human and rat glomeruli had identical normalised ultrafiltration coefficient values ($L_P A/V_i$, supp figure 1C), but had significantly lower $L_P A/V_i$ than mouse glomeruli (both p<0.05, supp figure 1C).
Acknowledgements

We would like to thank kidney donors and their families for consenting to research on donated organs that were not suitable for transplantation, and to transplant coordinators in Bristol and other centres for enabling these studies. This work was supported by grants from the Medical Research Council (G0802829 fellowship to AHJS; G10002073, GR0600920 projects to SJH, DOB, AHJS), Kidney Research UK (RP18/2010 to AHJS, DOB, SJH; ST5/2012 to AHJS, RRF, SCS; RP45/2013 to AHJS, RRF, SCS, SJH), British Heart Foundation (FS/05/114/19959 studentship to SJH and AHJS, project PG08/022/21636 to DOB, SJH and AHJS, PG/08/059/25335 to CRN and KA), Wellcome Trust (project 079736 to DOB and SJH), Diabetes UK (RJ5522 to LFD and DOB), BBSRC project grant BB/J007293/1 (SO and DOB), Swiss National Science Foundation (Grant 31003A-130463), Oncosuisse (Grant OC2 01200-08-2007), NOVARTIS Stiftung für medizinischbiologische Forschung (Grant 10C61) to KBH, and Richard Bright VEGF Research Trust (to MS, AR, and AHJS).
References


Figure legends

Figure 1. VEGF-A_{165b} is upregulated in humans with early diabetic nephropathy and well-preserved kidney function.

A. mRNA expression of exon 5/7/8 containing VEGF-A isoforms (i.e. those coding for 165 amino-acid proteins) determined by RT-qPCR relative to total RNA extracted in whole renal cortical tissue from kidneys unsuitable for transplantation from deceased donors without diabetes (“none”), donors with early diabetic nephropathy with well-preserved kidney function (“early”), and from kidney biopsy specimens from patients with diabetes and advanced nephropathy (“late”). B. VEGF-A_{165a} mRNA expression (note logarithmic scale). C. VEGF-A_{165b} expression (note logarithmic scale). D. VEGF-A Isoform expression ratio calculated as VEGF-A_{165b} mRNA per unit total mRNA divided by VEGF-A_{165a} mRNA per unit total mRNA. E. Ratio of VEGF-A_{165a} to VEGF-A_{165b} mRNA in control and early diabetic samples in which it was possible to analyse mRNA from single glomeruli isolated by differential sieving. F: Isoform-specific VEGF-A_{165b} protein determined by ELISA as a proportion of total VEGF-A in whole kidney cortical tissue in kidney donors with diabetes and early nephropathy (“early”) and those without diabetes (“none”). Multiple comparisons: one-way ANOVA. Two-group comparisons: unpaired t-test. *p<0.05, **p<0.01, ***p<0.005, throughout.
Figure 2. Podocyte-specific overexpression of VEGF-A\textsubscript{165b} ameliorates streptozotocin-induced diabetic nephropathy.

A. Transgenic mice constitutively overexpressing human VEGF-A\textsubscript{165b} in podocytes under control of the nephrin promoter (blue; *neph\textsuperscript{1}\textsuperscript{VEGF-A\textsubscript{165b}}) received injections of streptozotocin (STZ) or vehicle at 12 weeks of age. Plasma glucose (*), urine albumin:creatinine ratio (C) and creatinine clearance (D) measured in non-diabetic wild-type, diabetic wild-type, non-diabetic transgenic and diabetic transgenic mice 6 weeks after diabetes induction. n=5-10 mice per group. *p<0.05, one-way ANOVA.

E,F: Glomerular area assessed in haematoxylin and eosin stained kidney sections from 21-55 glomeruli from 3 mice per group. Scale bars: 20\mu m. *p<0.05, one-way ANOVA. G,H: Periodic acid schiff stained kidney sections from each group of mice. Mesangial matrix expansion (arrows) measured as a proportion of total glomerular area in 45-60 glomeruli from 3-4 mice per group. Scale bars: 20\mu m. *p<0.05, one-way ANOVA.

I: Low- and high-magnification electron micrographs (scale bars: 1\mu m and 250nm respectively) of glomerular capillaries and the glomerular capillary wall (GCW) from each group of mice. CL: capillary lumen. rbc: red blood cell. US: urinary space. pfp: podocyte foot process. gbm: glomerular basement membrane. ec: endothelial cell. J: Glomerular basement membrane (GBM) width calculated from 20-60 measurements in 8-16 glomerular capillaries per group. *p<0.05, one-way ANOVA.
Figure 3. Podocyte-specific overexpression of VEGF-A_{165b} reduces albuminuria in the dual insult of VEGF-A_{164a} overexpression and streptozotocin-induced diabetic nephropathy.

A: Heterozygous mice overexpressing human VEGF-A_{165b} in podocytes (blue: *neph*^{h}VEGF-A_{165b}) were crossed with mice in which murine VEGF-A_{164a} (^{m}VEGF-A_{164a}) overexpression in podocytes could be induced with doxycycline: heterozygous *podocin*-rtTA:TetO:^{m}VEGF-A_{164a} mice (red: *pod-TetO*:^{m}VEGF-A_{164a}), to generate mice co-overexpressing ^{m}VEGF-A_{164a} and ^{h}VEGF-A_{165b} in podocytes. Offspring were treated with streptozotocin (STZ) at 12 weeks of age to induce diabetes in all groups. After 5 weeks of diabetes, all mice received doxycycline, inducing ^{m}VEGF-A_{164a} overexpression in the two groups carrying the *podocin*-rtTA:TetO:^{m}VEGF-A_{164a} transgene. After one week of doxycycline treatment, urinary albumin:creatinine ratio (uACR) was measured and compared with uACR obtained prior to doxycycline. B: Change in uACR after 7 days of doxycycline treatment in diabetic mice with no transgenes, diabetic mice overexpressing ^{h}VEGF-A_{165b} alone, diabetic mice overexpressing ^{m}VEGF-A_{164a} alone, and diabetic mice co-overexpressing ^{m}VEGF-A_{164a} and ^{h}VEGF-A_{165b}. Dashed line represents no change in albuminuria during the 1 week doxycycline treatment period. n=3-6 mice per group. *p<0.05, one-way ANOVA.
Figure 4. Systemic treatment with VEGF-A\textsubscript{165b} blocks progression of albuminuria and glomerular basement membrane thickening in streptozotocin-induced diabetic nephropathy.

A: Diabetes was induced in wild-type DBA2J mice with streptozotocin injection (non-diabetic control mice received buffer injection alone). After 2 consecutive weeks of hyperglycaemia, mice received an 11 week course of bi-weekly intraperitoneal injections (blue arrows) of recombinant human VEGF-A\textsubscript{165b} (\(\text{rhVEGF-A}_{165b}\)) (n=16) or vehicle (n=16). B: Plasma glucose measured repeatedly in non-diabetic control, diabetic vehicle-treated, and 0.04\(\mu\)g/kg \(\text{rhVEGF-A}_{165b}\)-treated mice. *p<0.05 vs both diabetic groups, \(\text{n}^\ast\text{p}>0.05\) between diabetic groups, one-way ANOVA. C. Urine albumin-creatinine ratio (uACR) was measured repeatedly in diabetic DBA2J mice receiving injections of vehicle (n=4) or 0.04\(\mu\)g/kg \(\text{rhVEGF-A}_{165b}\) (n=4) for up to 11 weeks. *p<0.05, paired t-test. D. Diabetes was induced in wild-type DBA2J mice with streptozotocin injection, and vehicle- or \(\text{rhVEGF-A}_{165b}\)-treatment after the onset of albuminuria. E. uACR measured repeatedly in mice receiving bi-weekly injections of vehicle (n=10), 0.04\(\mu\)g/kg \(\text{rhVEGF-A}_{165b}\) (n=10) and 0.2\(\mu\)g/kg \(\text{rhVEGF-A}_{165b}\) (n=6) prior to organ harvest. F. Fold change in uACR (uACR at indicated timepoints, divided by uACR prior to initiating treatment (arrow)). Dashed line represents no change in albuminuria after starting injections. *p<0.05, two-way ANOVA. G: Low- and high-magnification electron micrographs (scale bars: 1\(\mu\)m and 500nm respectively) of glomerular capillaries and the glomerular capillary wall (GCW). CL: capillary lumen. rbc: red blood cell. US: urinary space. pfp: podocyte foot process. gbm: glomerular basement membrane. ec: endothelial cell. H: glomerular basement membrane (GBM) width measurements in indicated groups of diabetic mice. *p<0.05, one-way ANOVA.
Figure 5. Systemic treatment with VEGF-A\textsubscript{165b} blocks early albuminuria but not later features of diabetic nephropathy in a genetic model of type II diabetic nephropathy.

A: After the onset of hyperglycaemia at 6/7 weeks of age, db/db mice received bi-weekly intraperitoneal injections of recombinant human VEGF-A\textsubscript{165b} (rhVEGF-A\textsubscript{165b}) or vehicle (blue arrows) for 8 weeks. B: Blood glucose measurements in 6 lean and 20 db/db mice before and after receiving twice-weekly vehicle or rhVEGF-A\textsubscript{165b} (0.2µg/kg) injections. Dashed line indicates start of intraperitoneal injections. *p<0.05 compared with both groups of diabetic mice. ns p>0.05 between groups of diabetic mice. C: Urinary albumin:creatinine ratio (uACR) determined repeatedly during the 8 week treatment period, divided by uACR in the same animal prior to treatment (“baseline”) to calculate fold change in uACR. Dashed line represents no fold change in albuminuria since starting treatment. *p<0.05, paired t-test. D: Creatinine clearance measured at the end of the 8-week injection period. *p<0.05, ns p>0.05, one-way ANOVA. E: In a separate cohort of older mice, 5 weeks of bi-weekly intraperitoneal injections (blue arrows) of rhVEGF-A\textsubscript{165b} or vehicle were initiated in 14 week-old lean controls (n=5) and db/db mice (n=14) with long-standing hyperglycaemia, established albuminuria and decreased GFR. F: Serial measurement of uACR in these older vehicle- and rhVEGF-A\textsubscript{165b}–treated db/db mice. Dashed line indicates start of intraperitoneal injections. *p<0.05, paired t-test. G: At the end of the 5-week injection period, glomerular filtration rate (GFR) measurements in these older vehicle- and rhVEGF-A\textsubscript{165b}–treated db/db and lean mice. *p<0.05, ns p>0.05, one-way ANOVA. H: At the end of the 5-week injection period, mesangial matrix expansion (arrows) in periodic acid schiff stained kidney sections from each group of mice. Scale bar: 50µm.
Figure 6. VEGF-A165b reduces apoptosis, decreases glomerular permeability and acts via VEGFR2.

A: Apoptosis (caspase-3 activity) was determined in human podocytes in vitro exposed to normal (10mM) or high (30mM) cell culture glucose concentrations with or without recombinant human VEGF-A165b (rhVEGF-A165b). In triplicate. *p<0.05, one-way ANOVA. B: Caspase-3 staining performed on human microvascular endothelial cells exposed to normal (5mM) or high (30mM) cell culture glucose concentrations in the presence or absence of rhVEGF-A165b. C: Number of caspase-3 positive cells per high powered field (hpf) compared between conditions. In quadruplicate. ***p<0.001, ns p>0.05, one-way ANOVA. D: Glomerular water permeability (volume-corrected ultrafiltration coefficient: \( \text{L}_{\text{PA}}/\text{V}_i \)) measured in glomeruli isolated from vehicle-injected non-diabetic (open bar), and streptozotocin-injected diabetic rats (filled bars). Glomeruli were incubated for 1 hour in rhVEGF-A165b (1nM), VEGF-A receptor-2 antagonist ZM323881 (10µM), pan-VEGF-A receptor blocker PTK787 (100nM), saline, or combinations thereof prior to \( \text{L}_{\text{PA}}/\text{V}_i \) measurement. *p<0.05, ns p>0.05: compared with diabetic; #p<0.05, ns \( p>0.05 \): compared with diabetic+rhVEGF-A165b; one-way ANOVA. E,F: Total and phosphorylated VEGF-R2 in glomeruli of non-diabetic wild type and neph\( ^{\text{h}} \)VEGF-A165b mice (E), and in glomeruli from streptozotocin (STZ)-induced diabetic DBA2J mice treated with vehicle or rhVEGF-A165b (F). Controls without primary antibody are also shown. Scale bars: 20µm. VEGF-R2 and phosphorylated-VEGFR2 by area relative to the whole glomerulus quantified for non-diabetic neph\( ^{\text{h}} \)VEGF-A165b versus littermates (G) and rhVEGF-A165b-treated diabetic mice versus vehicle-treated diabetic mice (H). *p<0.05, one-way ANOVA.
Figure 7. VEGF-A\textsubscript{165}b stimulates VEGFR2 in glomerular endothelial cells in vivo.

A. Sections of kidney tissue from wild type and \textit{neph}\textsuperscript{h}VEGF-A\textsubscript{165}b mice showing glomeruli co-stained for VEGF-R2 (green), and the endothelial cell marker PECAM-1 (red), and overlaid images showing colocalisation (yellow)  

B: High magnification of a single glomerular capillary loop showing VEGFR2 expression and endothelial cell PECAM-1 colocalise. 

C. Sections of kidney tissue from wild type and \textit{neph}\textsuperscript{h}VEGF-A\textsubscript{165}b mice showing glomeruli co-stained for VEGF-R2 (green), and the podocyte marker nephrin (red).  

D: High magnification of a single glomerular capillary loop showing that VEGF-R2 expression and podocyte nephrin do not colocalise.
Figure 8. VEGF-A$_{165b}$ restores the glomerular endothelial glycocalyx in early streptozotocin-induced diabetic nephropathy.

6 weeks after streptozotocin (or buffer) injections to induce diabetes (or normoglycaemia) in DBA2J mice, mice received bi-weekly intraperitoneal injections of vehicle or rhVEGF-A$_{165b}$ for 4 weeks. Animals were cardiac perfusion-fixed and renal cortex processed for transmission electron microscopy. A: Glomeruli were imaged at low magnification, the glomerular capillary wall at higher magnification, and endothelial cell surface at high magnification using transmission electron microscopy. us: urinary space. gc: glomerular capillary. fen: endothelial fenestra. e-glx: endothelial glycocalyx. ec: endothelial cell. gbm: glomerular basement membrane. pfp: podocyte foot process. B: Endothelial fenestral density and C: glycocalyx depth covering the glomerular endothelial cell surface quantified in EM images from vehicle-treated non-diabetic, vehicle-treated diabetic and rhVEGF-A$_{165b}$-treated diabetic animals. n=3 mice per group. *p<0.05, ns p>0.05, one-way ANOVA.
**Figure 9. VEGF-A165b restores glomerular endothelial glycocalyx acutely in diabetic rat glomeruli.**

Kidneys were perfused *in vivo* with cell membrane label (R18; red) and glycocalyx label (alexa-488-wheat germ agglutinin (WGA) lectin; green), and then glomeruli were isolated and treated with vehicle or VEGF-A165b for one hour (exactly as per physiology experiments) before imaging with confocal microscopy. 

*A*: whole glomerulus; scale bar 20μm.  
*B*: glomerular capillaries (gc) with endothelial cell (ec) bodies; scale bar 5μm.  
*C*: high-magnification image (scale bar 1μm) of a vehicle-treated glomerular capillary (GC) from a healthy animal, demonstrating glomerular endothelial glycocalyx (GLX) lining luminal surface of endothelial cell (ec) body. gcw: glomerular capillary wall.  
*D*: high-magnification image (scale bar 1μm) of a vehicle-treated glomerular capillary (GC) from a diabetic animal. Note absent glomerular endothelial glycocalyx (*absent* GLX) lining luminal surface of endothelial cell (ec) body.  
*E*: high-magnification image (scale bar 1μm) of a VEGF-A165b-treated glomerular capillary (GC) from a diabetic animal. Note restoration of glomerular endothelial glycocalyx (GLX).  
*F*: mean±sem glomerular endothelial glycocalyx depth in 5 glomeruli in each of 3 animals per group. *p<0.05, one-way ANOVA.*
Figure 109. VEGF-A\textsubscript{165b} normalises permeability of diabetic mouse, rat and human glomeruli.

A: Glomeruli were harvested from 6 diabetic (STZ-injected) wild-type, 4 non-diabetic wild-type, and 8 diabetic (STZ) neph\textsuperscript{h}VEGF-A\textsubscript{165b} mice (102 glomeruli), from 3 diabetic (STZ) and 3 non-diabetic rats (74 glomeruli), and from untransplantable kidneys from 3 non-diabetic and 3 diabetic human kidney donors (54 glomeruli). Glomerular water permeability (volume-corrected ultrafiltration coefficient: $L_P/A/V_i$) was measured in individual glomeruli following exposure to constitutively overexpressed $h$VEGF-A\textsubscript{165b} (mice) or 1 hour incubation in vehicle or $h$VEGF-A\textsubscript{165b} (rat & human glomeruli). *$p<0.05$, ns $p>0.05$, one-way ANOVA. B: Glomerular $L_P/A/V_i$ was measured twice in 3 diabetic human glomeruli, before (black) and after (blue) 1 hour incubation in $h$VEGF-A\textsubscript{165b}. *$p<0.05$, paired t-test.
Supplementary figure 1. Human glomerular ultrafiltration coefficient is high, on account of large surface area for fluid exchange.

A: Glomerular water permeability (ultrafiltration coefficient: $L_{\text{P}}A$) measured in individual glomeruli from healthy wild-type mice (67 glomeruli from 17 mice), healthy rats (134 glomeruli from 25 rats) and non-diabetic kidney donors (25 glomeruli from 3 donors). B: Glomerular volume determined in the same glomeruli. C: Volume-corrected ultrafiltration coefficient ($L_{\text{P}}A/V_i$) calculated as the quotient of glomerular ultrafiltration coefficient ($L_{\text{P}}A$) and glomerular volume ($V_i$) for individual glomeruli. *p<0.05, one-way ANOVA vs other two species, ns p>0.05, one-way ANOVA for comparisons as indicated.
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<td>-</td>
<td>Y</td>
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<tr>
<td>Early DN</td>
<td>91</td>
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<tr>
<td>Late DN</td>
<td>122</td>
<td>3.14</td>
<td>Mesangial expansion, nodular glomerulosclerosis, interstitial fibrosis, thickened GBM</td>
<td>Y</td>
<td>-</td>
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<tr>
<td>Late DN</td>
<td>350</td>
<td>&gt;10</td>
<td>Diffuse nodular mesangial matrix expansion, advanced sclerosis, interstitial scarring, thickened GBM</td>
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<tr>
<td>Late DN</td>
<td>144</td>
<td>11</td>
<td>Global sclerosis, Kimmelstiel-Wilson nodules, moderate tubular atrophy and interstitial fibrosis</td>
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<td>-</td>
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<tr>
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<td>Features of advanced diabetic nephropathy with moderate chronic damage</td>
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<tr>
<td>Late DN</td>
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<td>13</td>
<td>Global sclerosis, Kimmelstiel-Wilson nodules, marked chronic tubulointerstitial fibrosis</td>
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<td>-</td>
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<tr>
<td>Late DN</td>
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<td>2.4</td>
<td>Global sclerosis, nodular mesangial matrix expansion, widespread</td>
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<td>-</td>
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</tbody>
</table>
### Supplementary Table 1. Human kidney sample details.

* urinary protein excretion not routinely collected in kidneys offered for transplantation, and therefore quantification not available.
** physiology experiments preclude histology information from the same glomeruli
*** anonymised autopsy specimen: additional clinical details not available

<table>
<thead>
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<th>Late DN</th>
<th>269</th>
<th>5.4</th>
<th>Tubular atrophy and interstitial fibrosis, thickened GBM</th>
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Global mesangial sclerosis with nodularity, widespread tubular atrophy and interstitial fibrosis.