Supplemental material

Experimental Procedures

Materials and chemicals were purchased from Sigma (Dorset, UK) and Starlab (Milton Keynes, UK) unless otherwise stated.

In vivo experiments in animal model of diabetes

Generation of transgenic mice and transgene induction: Mouse Angpt1 cDNA was obtained from the plasmid PEF-Angpt1,[1] and cloned in the plasmid pBIG/TetVector (GenBank #U89933)(Clontech, Saint-Germain-en-Laye, France)(pTRE bidirectional *LacZ/Angpt1*) and subsequently used for generation of transgenic animals in BL6-CBA fertilized zygotes. FVB/N mice homozygous for *podocin-rtTA* have been provided by Dr J Kopp (Bethesda, MD, USA).[2] Animals were kept according to the "Guidelines on the Use of Animals in Research". Animals were administered DOX (2mg/ml in drinking water with sucrose 5% wt/vol) or vehicle (sucrose only). Genotyping was conducted by PCR with the following set of primers: sense 5´-ACCTATAAAAATAGGCGTATCACGA-3´, antisense 5´-TGGCTGATTATGATCCTGCA-3' for "pTRE bidirectional LacZ/Angpt1" transgene, and as described for the *podocin-rtTA* transgene.[2] Control littermates were utilised for all determinations.

Diabetes Study: Diabetes was induced with streptozotocin (50 µg/g bw) for 5 days, in 5-week-old male mice, as recommended by the Animal Models of Diabetic Complications Consortium (http://www.AMDCC.org). Control mice were injected with citrate buffer only. After 2 weeks, diabetes was verified by blood glucose determination with the glucose oxidase method. Mice with a glycemia <22 mmol/l were not included in the study.

Clinical and biochemical characteristic: Glycaemia was recorded with glucose oxidase method (Ascensia Contour, Bayer). Twenty-four hours urine collections were carried out for albuminuria determination (Bethyl Laboratories, TX, USA). Plasma and urine creatinine were measured by isotope dilution electrospray mass spectrometry and creatinine clearance

determined. In the non-diabetic study blood pressure was determined with telemetry (Data Sciences International, St. Paul, MN) as described.[3] In the diabetic study systolic blood pressure was measured by non-invasive pletismography (Kent Scientific, CT, USA).[4]

RNA analysis: RNA was obtained from isolated glomeruli by Dynabead perfusion and gene expression of *Angpt1* and *Angpt2* analysed by real-time PCR as described.[5, 6] Expression of target sequences was normalized to the geometric mean of three housekeeping genes actin, hypoxanthine phosphoribosyl-transferase-I, and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase, as previously shown in experimental animal model of diabetes.[7, 8] Primer details available on request.

Western immunoblotting: Renal cortex was homogenised in RIPA buffer with phosphatases inhibitors and lysates separated by SDS-PAGE. Immunoreactive bands were visualized with chemiluminescence, quantified using densitometry and related to the house-keeping proteins β-actin or α-tubulin. Angpt1 and Angpt2 antibodies were from Alpha Diagnostic (San Antonio, TX, USA), VEGF-A, eNOS, VEGFR2, VEGFR2-P (Tyr^{951}), Tie-2 (C-terminus), Tie-2-P (Tyr^{1100}) from Santa Cruz Biotechnology (Santa Cruz, CA, USA), anti phospho eNOS Ser^{1177} from Cell Signalling Technology (Danvers, MA, USA), the anti-nephrin antibody was provided by Prof H. Holthöfer (Dublin University, Ireland) and the P-nephrin antibody by Prof L. Holzman (University of Pennsylvania, Philadelphia, PA, USA).[9]

Tie-2 receptor phosphorylation was also assessed in parallel studies conducted with Tie-2 receptor immunoprecipitation as previously described.[10] Tie-2 receptor immunoprecipitation was conducted in kidney cortex lysate homogenised in RIPA buffer containing phosphatase inhibitors. Approximately 1-2 mg of tissue sample was incubated with 5 µl of anti-mouse Tie-2 antisera (R&D anti-mouse Tie-2 antibody - AF762) overnight at 4 ^{o}C. Magnetic protein-G beads were then added for 1 hour at room temperature. A magnet was then utilised to isolate the protein-G/Ab/Tie-2 complex after few washes with PBS. Pellets were then heat-denaturated and run on SDS-PAGE gel; immunoblotting was performed with anti-phosphotyrosine 4G10 antisera (Millipore, Watford, UK)(1:1000), and bands visualised with chemiluminescence .

X-gal staining: X-gal histochemical detection was utilised for nuclear β-galactosidase activity as described.[3]

sVEGFR1 ELISA: sVEGFR1 level in mouse cortex lysates by ELISA (R&D Systems, Abingdon, UK). Results were corrected for protein concentration.

Immunohistochemistry: 10 micron frozen sections were cut from renal biopsies, fixed in 4% paraformaldehyde and stained with antibodies for PECAM-1 (Thermo Scientific Pierce) and Ki-67 (Abcam, Cambridge, UK), then labelled with fluorescent-conjugated secondary antibodies (Jackson Immuno-Research, Suffolk, UK) and 4',6-diamidino-2-phenylindole (DAPI) for nuclear staining. To visualise the endothelial glycocalyx FITC-conjugated wheat germ agglutinin was used. The numbers of total and endothelial proliferating cells and the % area of glomeruli stained with wheat germ agglutinin (for glycocalyx) were counted in 50 glomeruli per animal.

Electron microscopy: Quantitative glomerular ultrastructural analysis was performed with transmission electron microscopy techniques as previously described.[4] Tie-2 immunogold staining was conducted in renal cortex tissue fixed in 2% paraformaldehyde for 4 hours at 4 ^{o}C, and then dehydrated in ethanol and embedded in LRWhite resin. Ultrathin sections were then mounted on nickel grids and immersed, for 10 minutes at RT, initially in 80mM ammonium chloride in phosphate buffered saline (PBS), and then in PBS containing 0.2M glycine and 0.5% bovine serum albumin (BSA), for extra 10 minutes at RT. The grids were then incubated in 10% normal goat serum for 30 minutes at RT before being incubated overnight at 4 ^{o}C with primary antibody (rabbit polyclonal Tie2 sc9026, Santa Cruz, CA, USA) diluted 1:200 in PBS + 0.5% BSA. After washing in PBS + 0.5% BSA the grids were incubated with 10nm gold-conjugated goat anti-rabbit IgG diluted 1:20 in PBS + 0.5% BSA for one hour at RT. Grids that had not been incubated in primary antibody were used as negative controls. After washing, the grids were stained with 2% aqueous uranyl acetate and examined with a CM100 transmission electron microscopy.

In vitro experiments in cultured podocytes

Conditionally immortalised human podocytes have been obtained from Prof Moin Saleem.[11] Cells were cultured as previously described, and experiments conducted in fully differentiated cells at 37^0C after serum starvation (1% serum) for 6-8 hours.[11]

Podocytes were exposed to media containing different glucose concentrations (25mM glucose for "high glucose", and 5mM glucose supplemented with mannitol to ensure equivalent osmolality for "normal glucose") for 24 hours. Subsequently podocyte *ANGPT1 and ANGPT2* mRNA levels were assessed using techniques as previously described.[12] *GAPDH* was utilised as housekeeping gene as previously validated.[13, 14] Primers available on request.

Ex vivo experiments in human tissue

Glomerular Angpt1 and Angpt2 mRNA expression levels were obtained from the European Renal cDNA Bank-Kroener-Fresenius biopsy bank and analysed with microarray technology as described.[15] Biopsies were obtained from patients with diabetic nephropathy (6 patients with type-2 diabetes, one patients with type-1 diabetes, 5 patients with diabetes of unknown type; eGFR 52.8±30 ml/min mean±SEM), and from live donor kidney (as controls) after informed consent and with approval of the local ethics committees.

References

1. Procopio WN, Pelavin PI, Lee WM, Yeilding NM: Angiopoietin-1 and -2 coiled coil domains mediate distinct homo- oligomerization patterns, but fibrinogen-like domains mediate ligand activity. *J Biol Chem* 274**:** 30196-30201, 1999

2. Shigehara T, Zaragoza C, Kitiyakara C, Takahashi H, Lu H, Moeller M, Holzman LB, Kopp JB: Inducible podocyte-specific gene expression in transgenic mice. *J Am Soc Nephrol* 14**:** 1998-2003, 2003

3. Davis B, Dei CA, Long DA, White KE, Hayward A, Ku CH, Woolf AS, Bilous R, Viberti G, Gnudi L: Podocyte-specific expression of angiopoietin-2 causes proteinuria and apoptosis of glomerular endothelia. *J Am Soc Nephrol* 18**:** 2320-2329, 2007

4. Ku CH, White KE, Dei CA, Hayward A, Webster Z, Bilous R, Marshall S, Viberti G, Gnudi L: Inducible overexpression of sFlt-1 in podocytes ameliorates glomerulopathy in diabetic mice. *Diabetes* 57**:** 2824-2833, 2008

5. Kolatsi-Joannou M, Price KL, Winyard PJ, Long DA: Modified citrus pectin reduces galectin-3 expression and disease severity in experimental acute kidney injury. *PLoS One* 6**:** e18683, 2011

6. Long DA, Kolatsi-Joannou M, Price KL, Dessapt-Baradez C, Huang JL, Papakrivopoulou E, Hubank M, Korstanje R, Gnudi L, Woolf AS: Albuminuria is associated with too few glomeruli and too much testosterone. *Kidney Int*, 2013

7. Susztak K, Bottinger E, Novetsky A, Liang D, Zhu Y, Ciccone E, Wu D, Dunn S, McCue P, Sharma K: Molecular profiling of diabetic mouse kidney reveals novel genes linked to glomerular disease. *Diabetes* 53**:** 784-94, 2004

8. Biederman J, Yee J, Cortes P: Validation of internal control genes for gene expression analysis in diabetic glomerulosclerosis. *Kidney Int* 66**:** 2308-14, 2004

9. Verma R, Kovari I, Soofi A, Nihalani D, Patrie K, Holzman LB: Nephrin ectodomain engagement results in Src kinase activation, nephrin phosphorylation, Nck recruitment, and actin polymerization. *J Clin Invest* 116**:** 1346-59, 2006

10. Yuan HT, Khankin EV, Karumanchi SA, Parikh SM: Angiopoietin 2 is a partial agonist/antagonist of Tie2 signaling in the endothelium. *Mol Cell Biol* 29**:** 2011-2022, 2009

11. Saleem MA, O'Hare MJ, Reiser J, Coward RJ, Inward CD, Farren T, Xing CY, Ni L, Mathieson PW, Mundel P: A conditionally immortalized human podocyte cell line demonstrating nephrin and podocin expression. *J Am Soc Nephrol* 13**:** 630-638, 2002

12. Price KL, Long DA, Jina N, Liapis H, Hubank M, Woolf AS, Winyard PJ: Microarray interrogation of human metanephric mesenchymal cells highlights potentially important molecules in vivo. *Physiol Genomics* 28**:** 193-202, 2007

13. Lin CL, Wang FS, Hsu YC, Chen CN, Tseng MJ, Saleem MA, Chang PJ, Wang JY: Modulation of notch-1 signaling alleviates vascular endothelial growth factor-mediated diabetic nephropathy. *Diabetes* 59**:** 1915-25, 2010

14. Makino H, Miyamoto Y, Sawai K, Mori K, Mukoyama M, Nakao K, Yoshimasa Y, Suga S: Altered gene expression related to glomerulogenesis and podocyte structure in early diabetic nephropathy of db/db mice and its restoration by pioglitazone. *Diabetes* 55**:** 2747-56, 2006

15. Berthier CC, Zhang H, Schin M, Henger A, Nelson RG, Yee B, Boucherot A, Neusser MA, Cohen CD, Carter-Su C, Argetsinger LS, Rastaldi MP, Brosius FC, Kretzler M: Enhanced expression of Janus kinase-signal transducer and activator of transcription pathway members in human diabetic nephropathy. *Diabetes* 58**:** 469-77, 2009

	Pod/+ **VEH**	*Pod/Angpt1* **VEH**	*Pod/+* **DOX**	*Pod/Angpt1* **DOX**
Fed glycaemia (mM)	8.3±0.8	7.5±0.4	5.5±0.3	6.2±0.6
BW (g)	28.6±1.0	29.5±0.5	28.8±0.6	27.6±0.7
Kidney weight /BW (mg/g)	6.7±0.3	6.4±0.3	6.3±0.1	6.1±0.1
Creatinine Clearance (µl/min·g bw)	9.5±2	12.6±1.3	11.5±1.9	12.6±3.2
SBP/DBP (mmHg)	138±0.6/112±1	134±1.4/104±6	136±2.5/110±1	136±4/107±7
VvMes (arbitrary units)	0.14±0.01	0.18±0.04	0.15±0.03	0.17±0.04
GBM (nm)	160±19	189±24	167±16	189±28
Podocyte foot processes width (nm)	344±51	303±28	314±17	285±31
Podocyte density (number/10^5 μm^3)	75±12	70±13	83±13	88±26

Table 1: **Clinical, biochemical, and glomerular ultrastructural characteristics of non-diabetic Angpt1 overexpressing transgenic mice and controls.**

Data are shown for control (non-diabetic) *Pod/+* and *Pod+/Angpt1* transgenic mice after 10 weeks treatment with vehicle (VEH) or doxycycline (DOX). No differences in any variable were observed between groups. Abbreviations: glomerular basement membrane (GBM), systolic blood pressure (SBP), diastolic blood pressure (DBP), body weight (BW), mesangial volume fraction (VvMes)(n=5-12/group).

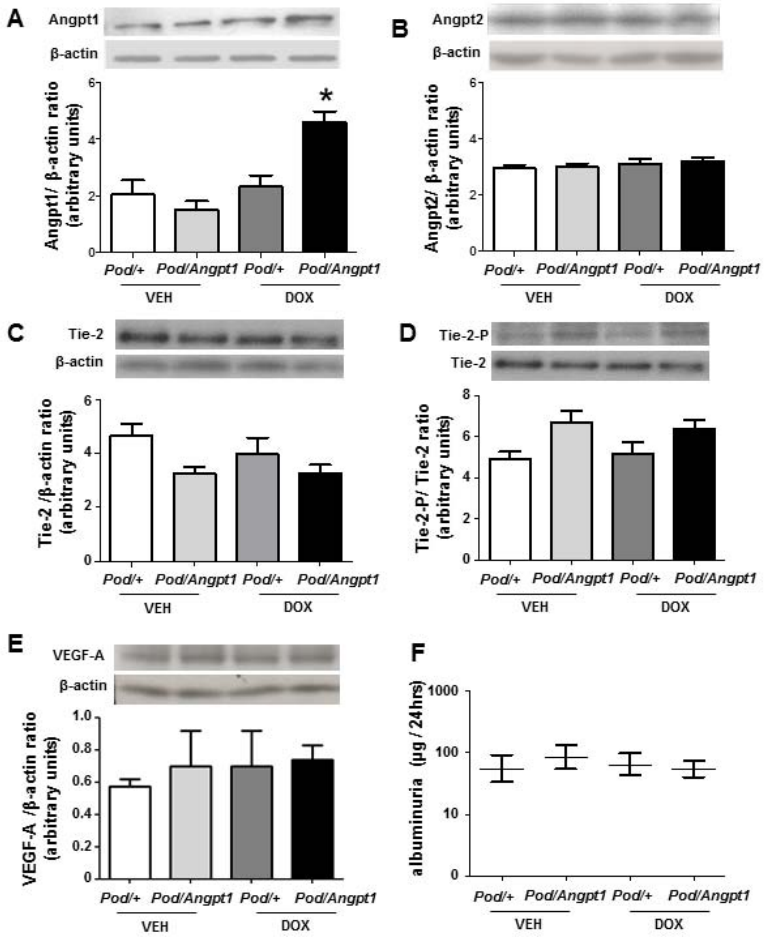

Fig. 1: Podocyte-specific Angpt1 overexpression does not modulate Tie-2 activation and VEGF-A levels.

Representative immunoblots and quantitative analysis for Angpt1 (**A**), Angpt2 (**B**), Tie-2 (**C**), phosphorylated Tie-2 (Tie-2-P)/total Tie-2 ratio (**D**), and VEGF-A (**E**) protein expression levels in kidney cortex lysates, in control (non-diabetic) *Pod/Angpt1* and *Pod/+* mice after 10 weeks VEH or DOX administration. Graphs for Angpt1/2 and Tie-2 are shown as a ratio with the 'housekeeping' protein β-actin (for Angpt1, *Pod/Angpt1* DOX *vs Pod/Angpt1* VEH, *Pod/+* VEH, and *Pod/+* DOX, *p<0.01, n=6-7/group). No change in albuminuria (**F**) was observed in male mice between the groups of animal studied (for albuminuria male and female mice were studied separately as per observed sex difference in albuminuria levels.[6] Similar results were observed for females, not shown).

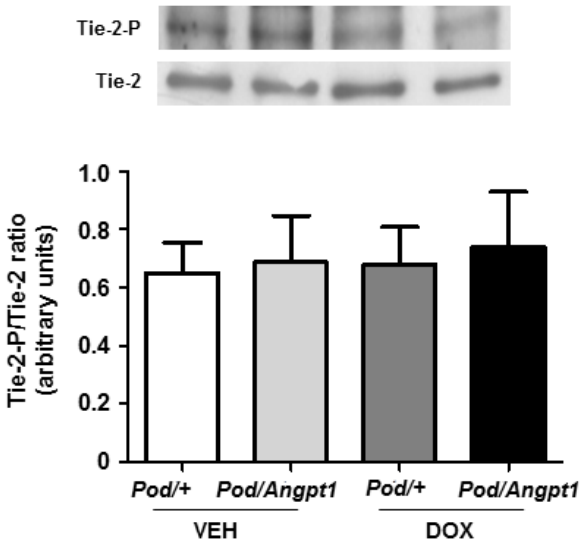

Fig. 2: Analysis of Tie-2 phosphorylation with Tie-2 immunoprecipitation experiments in podocyte-specific Angpt1 overexpressing mice.

Tie-2 receptor phosphorylation studies after Tie-2 receptor immunoprecipitation was conducted in kidney cortex lysate of non-diabetic mice overexpressing Angpt1 and their controls. No difference was observed in Tie-2 phosphorylation between Pod/Angpt1 DOX mice and controls (n=3-4/group).

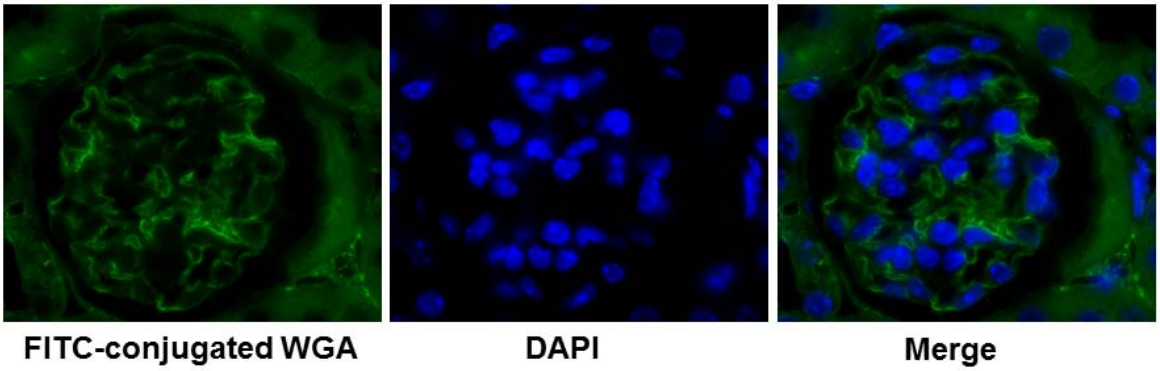

Fig.3: Representative image of glomerular glycocalyx stained with FITC-conjugated wheat germ agglutinin (WGA).

Assessment of glomerular glycocalyx using FITC-conjugated WGA immunohistochemistry was performed in non-diabetic *Pod/+* and diabetic *Pod/+* and *Pod/Angpt1* mice. No differences were observed between groups (n=3/group, 50 glomeruli analysed/animal).

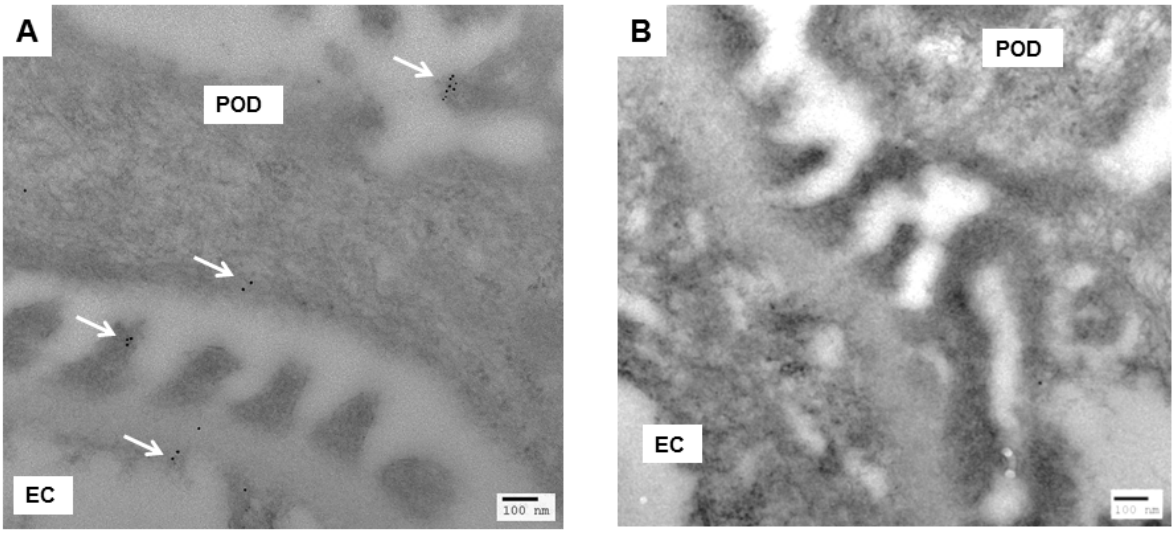

Fig. 4: Tie-2 receptor is expressed in podocytes and glomerular endothelial cells.

Immunogold staining for Tie-2 receptor in glomerular cells (A): clear staining is seen in podocytes (POD) and endothelial cells (EC). (B) Tie-2 Ab + blocking peptide: negative control.