CHANG ET AL. SUPPLEMENTAL MATERIAL

SUPPLEMENTAL METHODS

Blood and urine of patients

Fasting blood and spot urine samples were collected in the morning. Blood samples were taken in the sitting position after resting for more than 5 min. Plasma and urine were kept frozen in aliquots. Complete blood cell count and all biochemical analyses were performed in the Department of Laboratory Medicine, National Taiwan University Hospital. The urine ablumin-creatinine ratio was calculated by dividing the spot urinary albumin-creatinine concentration.

Enzyme-linked immunosorbent assay

Plasma Angpt1, Angpt2, VEGF-A, and soluble Tie-2 receptor (sTie-2) were measured in duplicate using commercial enzyme-linked immunosorbent assays (ELISA) (R&D Systems for human Angpt1, Angpt2, VEGF-A, sTie-2 and mouse VEGF-A, USCN Life Science Inc. for mouse Angpt1 and Angpt2). The sensitivities of Angpt1, Angpt2, VEGF-A, and sTie-2 assays were 1.36, 1.20, 1.61, and 1.00 pg/mL respectively. Intraassay coefficients of variation of Angpt1, Angpt2, VEGF-A, and sTie-2 were 2.1%, 1.3%, 1.6%, and 0.8%, respectively. Interassay coefficients of variance of Angpt1, Angpt2, VEGF-A, and sTie-2

were 1.8%, 2.0%, 2.1%, and 9.3%, respectively.

RT-PCR

Total RNA was extracted using RNeasy Mini Kit (Qiagen). Purity was determined by A260 to A280. cDNA was synthesized using oligo(dT) and random primers. Quantitative PCR (Q-PCR) was performed using methods described previously.[1] The specific primer pairs used are listed in Supplementary Table 2.

Western blot analysis

Total cellular protein extracted using RIPA buffer was subjected to Western blot analysis using methods described previously.[2] The following primary antibodies were used to detect the specific protein: Angpt1 (Millipore, Billerica, MA), Angpt2 (Calbiochem), glyceraldehyde 3-phosphate dehydrogenase (Santa Cruz Biotechnology).

In situ hybridization

Angpt2 partial cDNA sequence amplified by primers used in RT-PCR was cloned into pGEM-T Easy vector (Promega, Madison, WI). Sense and anti-sense digoxigenin-labelled

RNA probe was generated according to the user's manual (Roche Diagnostics, Mannheim, Germany). Deparaffinized 4-μm section was hybridized with 5 ng RNA probe in the hybridization buffer containing 40% deionized formamide and then the signal was developed by adding substrate containing NBT/BCIP according to the in situ hybridization manual (Roche Diagnostics). Blue-purple color was the positive signal.

Adenovirus administration and detection of plasma transgene expression. Adult (8-12wk) Adult *Coll-GFP*^{Tg} mice received single i.v. tail vein injection of 1 X 10^9 pfu of the adenovirus encoding human Angpt2 (AdAngpt2) or empty adenovirus (AdCon).[3] Mice were sacrificed 12 days later (n=6, 3 male and 3 female, for each group). Plasma levels of Angpt2 were quantified using an Angpt2-specific ELISA (R&D Systems).

Blood pressure analysis in mice

Systolic blood pressures were measured in conscious mice using a computerized tail-cuff method (BP-2000; Visitech Systems, Apex, NC). Mice were acclimatized to the system before the initiation of studies. Systolic blood pressures were measured the day before surgery or euthanasia. Blood pressures were measured for five times to determine the mean levels.

Purification and culture of aortic endothelial cells

The thoracic aortas of adult male or female *Tie-2-GFP*^{Tg} mice were diced, incubated at 37ºC for 45 min with liberase (0.5 mg/ml, Roche Applied Science, Indianapolis, IN) and DNase (100U/ml, Roche Applied Science) in HBSS. After centrifugation, cells were resuspended in 5 ml of PBS/1% BSA, and filtered (40 μm). Aortic endothelial cells were purified by isolating CD31+;Tie-2+ cells using FACSAria cell sorting (BD Biosciences, San Jose, CA), then total RNA was isolated, or purified cells were cultured in DMEM/F12 medium with 10% FBS and 15 ng/mL endothelial cell growth supplement (Sigma, St. Louis, MO). The primary cultured endothelial cells used in this study were between passages 4 and 6. Recombinant Angpt2 (100, 200, and 500 ng/mL, R&D Systems) was used for experiments.

Purification and culture of aortic vascular smooth muscle cells

The thoracic aortas from male CD1 mice were diced, incubated at 37ºC for 45 min with liberase (0.5 mg/ml, Roche Applied Science) and DNase (100U/ml, Roche Applied Science) in HBSS. The digested tissue was strained through 100 μm cell strainer. After centrifugation, cells were resuspended in 5 ml of PBS/1% BSA, and filtered (40 μm). Aortic VSMCs were purified by isolating PDGFRβ+;CD31– cells using FACSAria cell sorting (BD Biosciences),

then the purified cells were cultured in DMEM/F12 medium with 10% FBS. VSMCs were characterized on the basis of the presence of αSMA staining.[4] The primary cultured VSMCs used in this study were between passages 4 and 6. Recombinant TGF-β1 (5 ng/mL) and Angpt2 (100, 200, and 500 ng/mL) (R&D Systems) were used in experiments. Similar results were seen when using Angpt2 of different concentrations.

REFERENCES

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3. Ahmed A, Fujisawa T, Niu XL, Ahmad S, Al-Ani B, Chudasama K, Abbas A, Potluri R, Bhandari V, Findley CM, Lam GK, Huang J, Hewett PW, Cudmore M, Kontos CD: Angiopoietin-2 confers atheroprotection in apoE-/- mice by inhibiting LDL oxidation via

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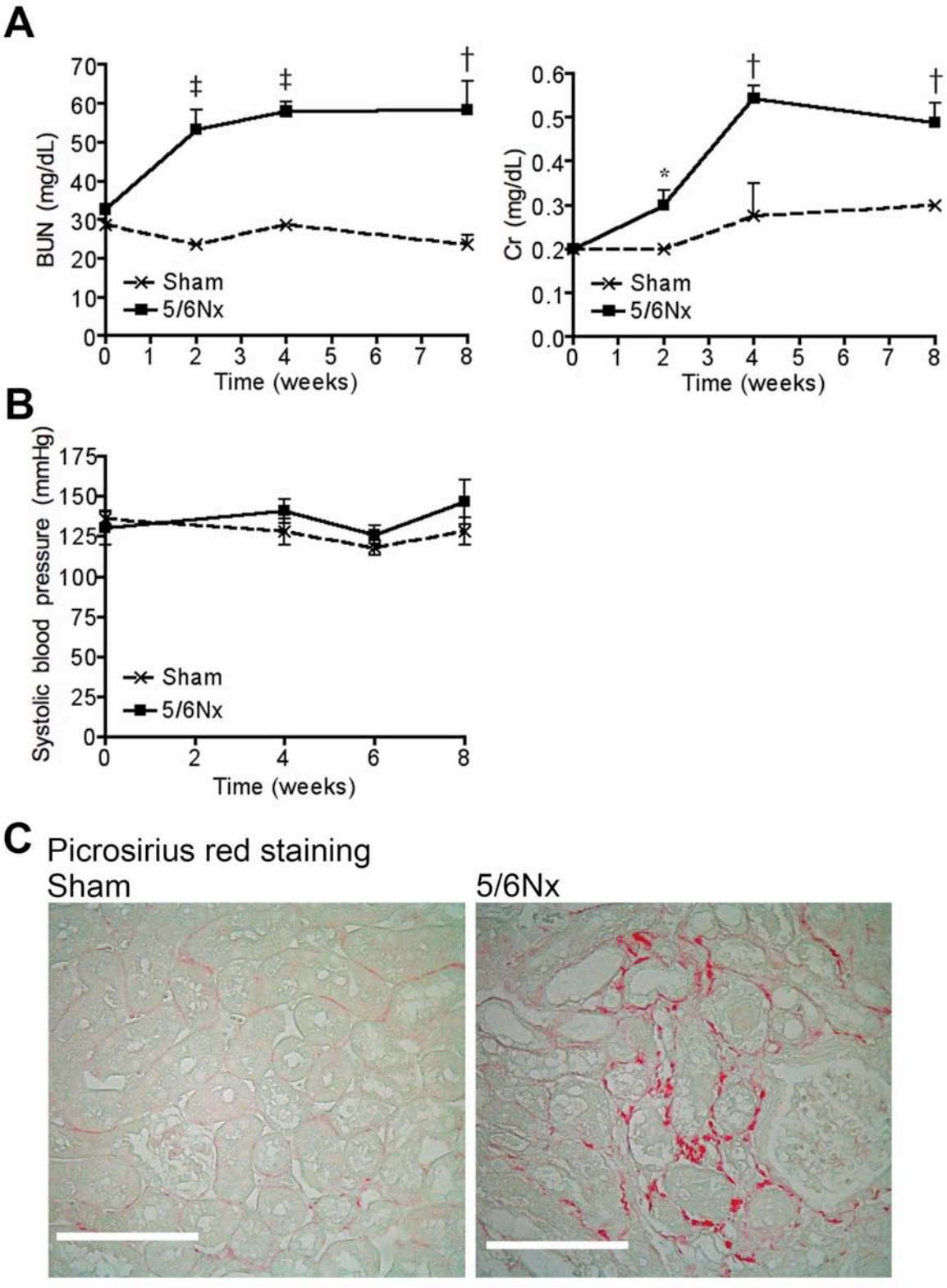
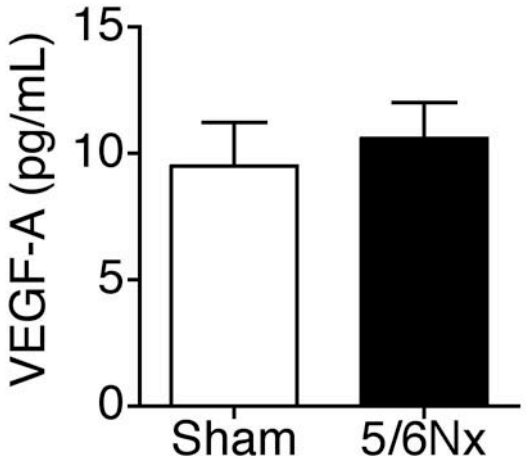
4. Chen YM, Wu KD, Tsai TJ, Hsieh BS: Pentoxifylline inhibits PDGF-induced proliferation of and TGF-beta-stimulated collagen synthesis by vascular smooth muscle cells. *J Mol Cell Cardiol* 31**:** 773-83, 1999

SUPPLEMENTAL FIGURES



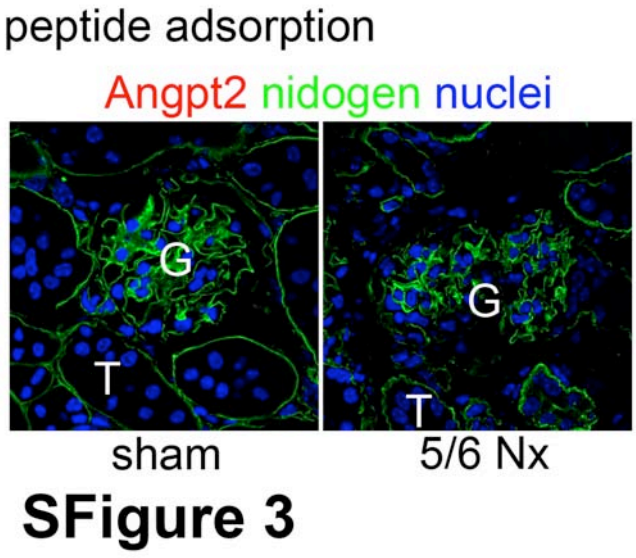
SFigure 1

Supplemental Figure 1. Chronic kidney disease was induced in mice after 5/6 subtotal nephrectomy (5/6Nx). (A) Plasma levels of blood urea nitrogen (BUN) and creatinine (Cr) in both sham and 5/6Nx. (B) Tail-cuff measured blood pressure in both sham and 5/6Nx. *P<0.05, †P<0.01, ‡P<0.001 versus sham at each time point. N=15/time point. (C) Representative images of picrosirius red-stained kidney sections for interstitial fibrillar collagen (red). Scale bar, 100 μm.

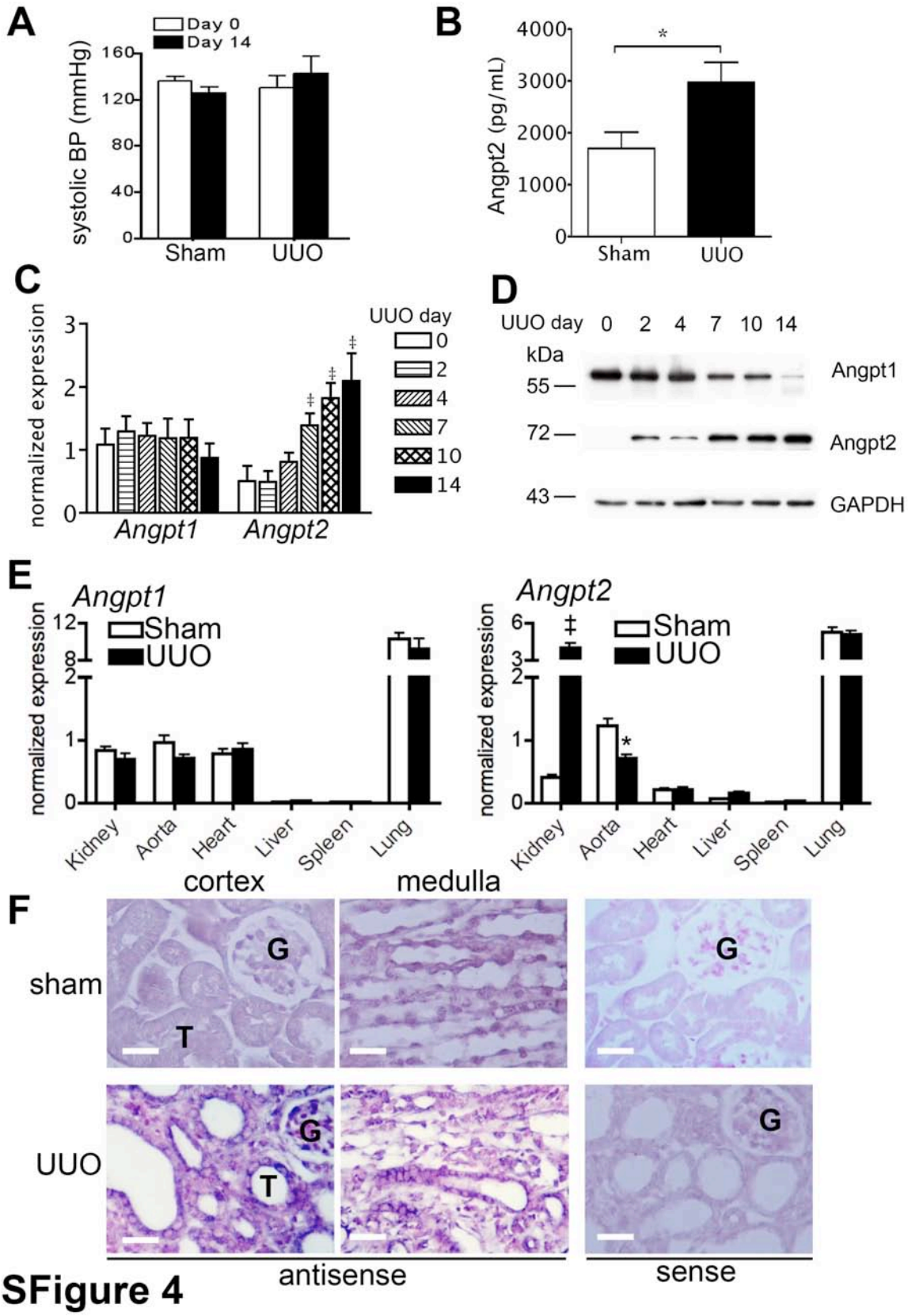


SFigure 2

Supplemental Figure 2. Plasma levels of VEGF-A 8 weeks after 5/6Nx. N=10/time point.

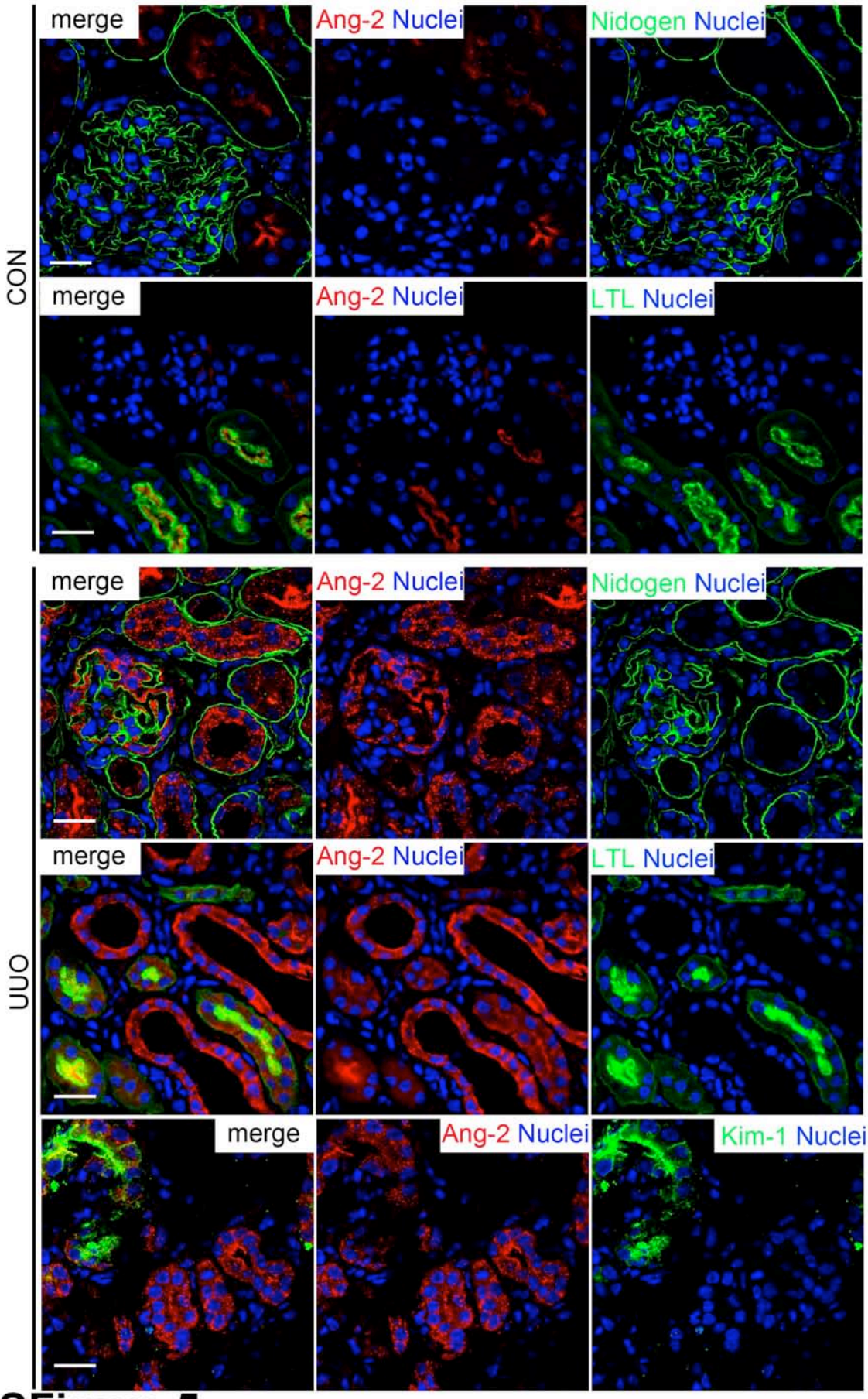


Supplemental Figure 3. The specificity of Angpt2 staining was confirmed by recombinant peptide adsorption. G and T denoted glomerulus and tubule respectively.



Supplemental Figure 4. Angpt2 was increased in plasma and injured renal tubular epithelial

cells in mice after unilateral ureteral obstruction (UUO). (A) Tail-cuff measured blood pressure after UUO. (B) Plasma levels of Angpt2 14 days after UUO. $^{*}P < 0.05$ versus sham. (C) Q-PCR for the transcripts of *Angpt1* and *Angpt2* in UUO kidneys. Expression levels were normalized by *GAPDH*. $^{‡}P < 0.001$ versus UUO day 0. (D) Western blot of whole kidney lysates for Angpt1 and Angpt2 in UUO at different time points. (E) Q-PCR for transcripts of *Angpt1* and *Angpt2* in different organs 14 days after UUO. $^{‡}P < 0.001$, $^{*}P < 0.05$ versus sham. N = 5/time point. (F) In situ hybridization (ISH) showing increased transcripts of *Angpt2* gene in injured renal tubular epithelial cells (T) and interstitial cells after UUO surgery compared with normal kidney (sham). (G, glomeruli). Right panel showed the specificity of ISH using sense RNA probe. Scale bar, 25μm.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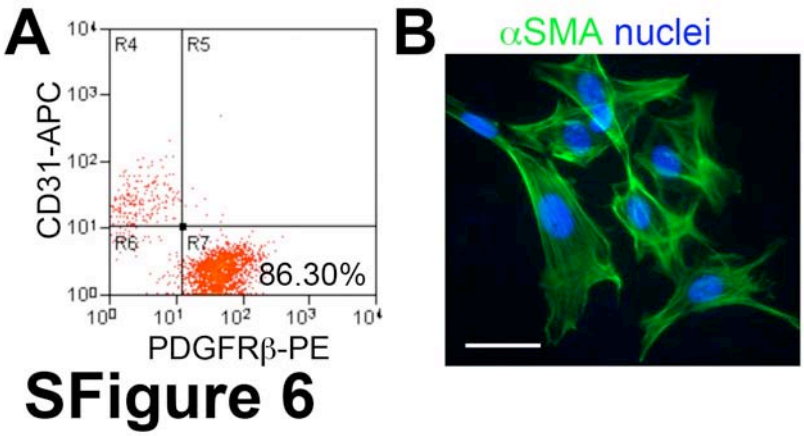
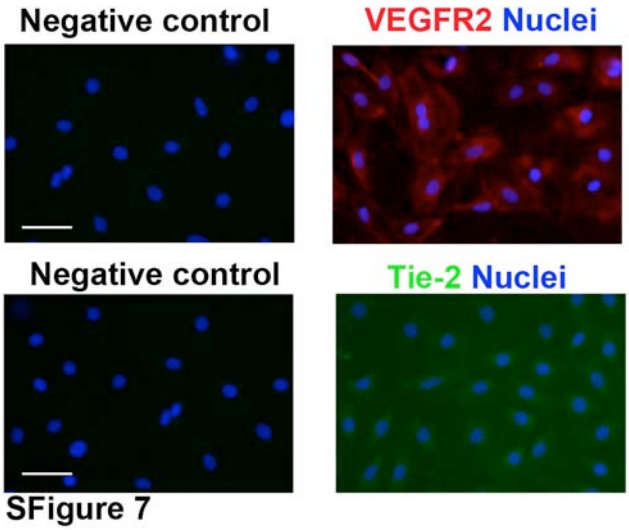
SFigure 5

Supplemental Figure 5. Angpt2 was increased in injured renal tubular epithelial cells and glomeruli after UUO. Confocal microscopic images showing that Angpt2 was expressed in injured tubular epithelial cells and glomeruli after UUO surgery. Scale bar, 25μm.

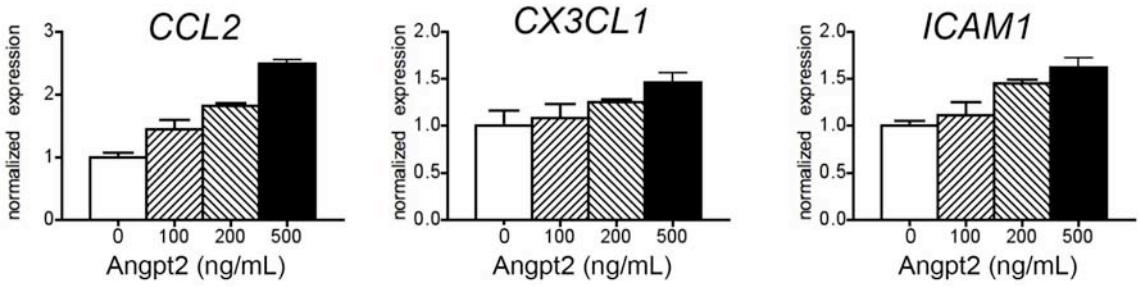


Supplemental Figure 6. Primary culture of aortic vascular smooth muscle cells (VSMCs). (A) Aortic VSMCs were purified by sorting PDGFRβ+;CD31– cells from single cell preparation of thoracic aortas. (B) Immunofluorescence image showed that more than 90% cells were positive for αSMA staining (B). Scale bar, 25 μm.



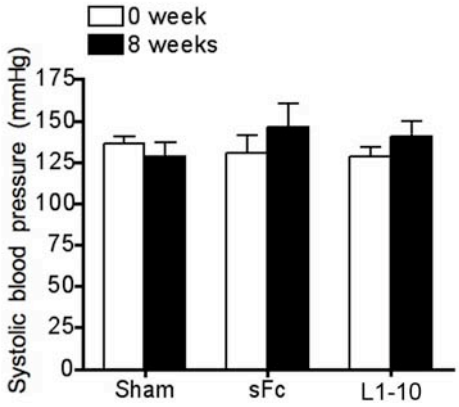
SFigure 7

Supplemental Figure 7. Primary culture of aortic endothelial cells expressed endothelial cell receptors. Immunofluorescence images showing vascular endothelial growth factor receptor-2 (VEGFR2) and Tie-2 expression in cultured aortic endothelial cells. Scale bar, 25 μm.



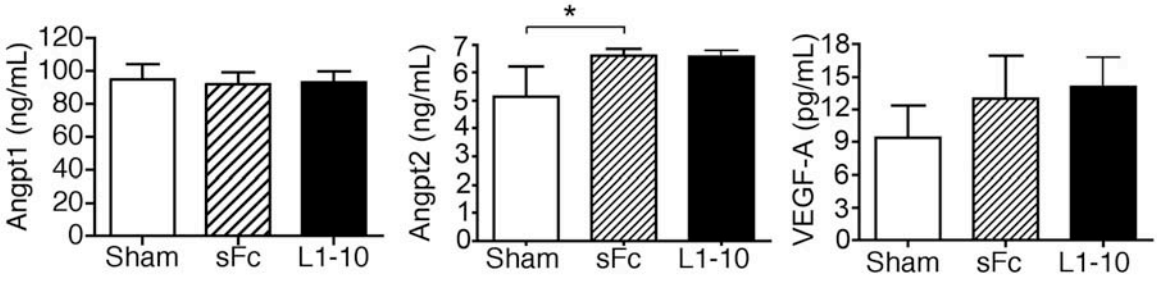
SFigure 8

Supplemental Figure 8. Q-PCR for the transcripts of *CCL2*, *CX3CL1*, and *ICAM1* in primary cultured aortic endothelial cells stimulated with recombinant Angpt2 of different concentrations for 24 h as indicated.



SFigure 9

Supplemental Figure 9. Tail-cuff measured blood pressure in sham, 5/6Nx mice receiving soluble Fc fragment (sFc) or specific Angpt2 inhibitor L1-10. N=15/time point.



SFigure 10

Supplemental Figure 10. Plasma levels of Angpt1, Angpt2, and VEGF-A in sham, 5/6Nx mice receiving sFc or L1-10. N=15/time point. $^{*}P<0.05$.

SUPPLEMENTAL TABLES

Supplemental Table 1. Multivariate-adjusted regression analyses of plasma Angpt2 and PWV* in CKD stage 5

	PWV		
	Regression Coefficient β ($x10^{-5}$)	*P*	95% Confidence Interval ($x10^{-5}$)
Univariate			
Angpt2	2.03	<0.001	0.28–3.78
Multivariate			
Model 1	1.92	<0.001	1.21–2.63
Model 2	1.68	0.002	0.97–2.39
Model 3	1.41	0.014	0.66–2.16

*PWV was log transformed; Model 1, Angpt2+age; Model 2, Model 1+traditional risk (age, gender, hypertension, diabetes, smoking, dyslipidemia); Model 3: Model 2+nontraditional risk (Calcium phosphate product, medication including ACE inhibitor, ARB, statin, calcium channel blocker, β-blocker).

Supplemental Table 2. Primer sequences used in Q-PCR

Target	Primer	Sequence
Angpt1	Forward	5’-GGAACCGAGCCTACTCACAG-3’
	Reverse	5’-TTAGATTGGAAGGGCCACAG-3’
Angpt2	Forward	5’-CCGCTACGTGCTTAAGATCC-3’
	Reverse	5’-ATTGTCCGAATCCTTTGTGC-3’
TGFB1	Forward	5’-GGACTCTCCACCTGCAAGAC-3’
	Reverse	5’-GACTGGCGAGCCTTAGTTTG-3’
Col1a1	Forward	5’-GAGCGGAGAGTACTGGATCG-3’
	Reverse	5’-GTTCGGGCTGATGTACCAGT-3’
Col3a1	Forward	5’-ACCAAAAGGTGATGCTGGAC-3’
	Reverse	5’-GACCTCGTGCTCCAGTTAGC-3’
PDGF-B	Forward	5’-CCCACAGTGGCTTTTCATTT-3’
	Reverse	5’-GTGAACGTAGGGGAAGTGGA-3’
PDGF-C	Forward	5’-GTGCCAGGAAAGCAGACTTC-3’
	Reverse	5’-CACAGCATTGTTGAGCAGGT-3’
CCL2	Forward	5’-AGCACCAGCCAACTCTCACT-3’
	Reverse	5’-CGTTAACTGCATCTGGCTGA-3’
CCL5	Forward	5'-CCCTCACCATCATCCTCACT-3'
	Reverse	5'-CCTTCGAGTGACAAACACGA-3'
CX3CL1	Forward	5’-TCGGACTTTGTTGGTTCCTC-3’
	Reverse	5’-CAAAATGGCACAGACATTGG-3’
TNFα	Forward	5’-TAGCCAGGAGGGAGAACAGA-3’
	Reverse	5’-TTTTCTGGAGGGAGATGTGG-3’
CCR2	Forward	5’-ATTCTCCACACCCTGTTTCG-3’
	Reverse	5’-GATTCCTGGAAGGTGGTCAA-3’
CX3CR1	Forward	5’-GGAGACTGGAGCCAACAGAG-3’
	Reverse	5’-CCTGATCCAGGGAATGCTAA-3’
ICAM1	Forward	5’-AGCACCTCCCCACCTACTTT-3’
	Reverse	5’-AGCTTGCACGACCCTTCTAA-3’
VCAM1	Forward	5’-TACCAGCTCCCAAAATCCTG-3’
	Reverse	5’-TCTGCTAATTCCAGCCTCGT-3’
GAPDH	Forward	5’-CTGGAGAAACCTGCCAAGTA-3’
	Reverse	5’-AAGAGTGGGAGTTGCTGTTG-3’