

DETAILED METHODS

Human urine and kidney biopsies samples

Human urine and serum samples as well as human kidney specimens were obtained from diagnostic renal biopsies performed at the Nanfang Hospital, Southern Medical University. Some urine and serum samples were also collected from healthy volunteers without preexisting clinical conditions. After centrifugation to remove debris, the urinary samples were stored at -80°C. Paraffin-embedded human kidney biopsy sections were used for immunohistochemical staining. All the studies involving human samples were approved by the Ethic Committee on Human Subjects of the Nanfang Hospital, Southern Medical University.

TNC ELISA

Human Tenascin-C Assay Kit was purchased from the Immuno-Biological Laboratories (IBL) (#27767; IBL Company, Gunma, Japan). This assay employs the quantitative sandwich enzyme immunoassay technique, which can detect TNC high molecular weight variants including the FNIII-B domain. Human urinary and serum TNC were measured according to the assay procedures specified by the manufacturer. Urinary TNC levels were calculated after normalization with urinary creatinine and expressed as nanograms per 1 mg creatinine. Serum TNC levels were expressed as nanograms per milliliter.

Animal models

Male BALB/c mice weighing about 22-24g were obtained from the Southern Medical University Animal Center (Guangzhou, China). Unilateral IRI (UIRI) was performed as described previously.^{S1 S2} Briefly,

unilateral renal pedicles were clipped for 35 minutes in mice using microaneurysm clamps. During the ischemic period, body temperature was maintained between 37~38°C using a temperature-controlled heating system. At day 10 after IRI, the contralateral intact kidney was removed. Mice were sacrificed at 11 days post-IRI, and serum and kidney tissues collected for various analyses. All animal studies were approved by the Experimental Animal Committee at the Nanfang Hospital, Southern Medical University.

Knockdown of TNC *in vivo*

Knockdown of TNC expression *in vivo* was performed using shRNA-mediated approach, as recently reported.^{S3} Male BALB/c mice were divided into three groups (n=6 in each group): (i) sham-operated mice, (ii) UIRI mice injected with control shRNA, and (iii) UIRI mice injected with TNC-shRNA. Mice were subjected to UIRI as described above. Four days after IRI, mice were injected with either pLVX-shTNC or control plasmid (pLVX-control) *via* tail-vein injection. At day 10, the contralateral intact kidney was removed. Mice were sacrificed at 11 days post-IRI, and serum and kidney tissues were analyzed.

Inhibition of FAK *in vivo*

For assessing the therapeutic efficacy of FAK inhibition, mice were divided into three groups (n=6 in each group): (i) sham-operated mice, (ii) UIRI injected with vehicle, and (iii) UIRI injected with FAK inhibitor. Mice were daily injected intraperitoneally with vehicle (PBS) or FAK inhibitor PF573228 (#3239; TOCRIS bioscience, Bristol, UK) at 5 mg/kg body wt beginning from day 4 after surgery. At day 10,

the contralateral intact kidney was removed. Mice were sacrificed at 11 days post-IRI, and serum and kidney tissues were analyzed.

Cell culture and treatment

Human kidney proximal tubular epithelial cell line (HKC-8) was described previously.^{S4} Serum-starved HKC-8 cells were treated by human recombinant TNC protein (R&D Systems, Minneapolis, MN) at varying dosages in the serum-free medium for various periods of time as indicated. For some experiments, HKC-8 cells were pretreated with integrin $\alpha\beta6$ blocking antibody (10D5) (Ab77906; Abcam) or FAK inhibitor PF573228 (#3239; Tocris Bioscience) for 1 hour, followed by incubating with vehicle or TNC (50 ng/ml). The cells were then subjected to Western blot analyses.

Mouse primary proximal tubular epithelial cells

Mouse primary proximal tubular epithelial cells were isolated and cultured as previously described.^{S5} Briefly, the cortical part of mouse kidneys were minced, then digested in pre-warmed 0.75 mg/ml collagenase 4 for 40 min at 37°C, after which the mashed tissue was sieved in DMEM/F-12. The tubular tissues were centrifuged using 31% Percoll gradients for 10 minutes, resuspended and washed twice with DMEM/F-12. Tubules were finally suspended in DMEM/F-12 supplemented with 10% bovine calf serum, 50 U/ml penicillin and 50 mg/ml streptomycin. Cells were cultivated for 4–8 days until they reached 60%–80% confluency. Tubular epithelial cells were characterized by morphology, positive staining for E-cadherin and negative staining for vimentin, respectively. The culture medium was changed on day 2 and 5, and then every 3 days.

Western blot analyses

Protein expression was analyzed by Western blot analysis as described previously.^{S6} The primary and secondary antibodies are summarized in Supplemental Table S2.

Quantitative real-time PCR

Total RNA was isolated using the TRIzol RNA isolation system (Life Technologies, Grand Island, NY). First-strand cDNA synthesis was performed by using 1 µg of RNA in 20 µl of reaction buffer using AMV-RT and random primers at 42°C for 60 min. Quantitative Real-time PCR (qPCR) was performed on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) as described previously.^{S7} The mRNA levels of different genes were calculated after normalization with β-actin. The sequences of primer pairs are presented as Supplemental Table S3.

Histology, immunohistochemical and immunofluorescence staining

Paraffin-embedded mouse kidney sections (3 mm thickness) were prepared by a routine procedure. The sections were stained with Masson's trichrome staining (MTS) reagents. Immunohistochemical staining was performed as described previously.^{S8} Kidney cryosections were fixed with 3.7% paraformaldehyde for 15 minutes, and then immersed in 0.2% Triton X-100 for 10 minutes at room temperature. After blocking with 10% donkey serum for 1 hour, slides were immunostained with the specified antibodies. These slides were then stained with cyanine Cy2- or Cy3-conjugated secondary antibodies. Antibodies used are summarized in Supplemental Table S2.

Preparation of fibroblast-derived ECM scaffold

Serum-starved NRK-49F cells were stimulated by human recombinant Shh protein at 50 ng/ml for 3 days. Decellularization was carried out with EGTA (#E3889; Sigma-Aldrich) (5×10^{-4} M; pH 7.4) in calcium-free PBS, followed by shaking at 4°C for 1 hour for 3-4 times until all cells were removed from their underlying matrix. The fibroblast-derived ECM scaffold was washed with PBS and then stored at 4°C until further usage.

Preparation of kidney tissue scaffold

Kidney tissue scaffolds (KTS) were prepared according to an established protocol with modification.^{S8} Briefly, at 11 days after IRI, groups of mice were sacrificed, and kidney perfused *in situ* using PBS to remove the blood. Each kidney was cut into 3-4 slices of same thickness along the sagittal plane. The kidney slices were then immersed in 100 ml of ultrapure water, followed by shaking for 30 min for three times. The slices were then incubated with 100 ml of 0.02% trypsin/0.05% EDTA solution by shaking for 1 hour at 37°C. After rinsed with water for three times, the slices were transferred to a flask with 100 ml of 3% Triton X-100 solution, followed by shaking for 12 hours. After brief washes, the kidney slices were immersed in 100 ml of 4% deoxycholic acid solution on a shaker for 5 hours. KTS were thoroughly rinsed with ultrapure water to remove residual surfactant, and then stored in ultrapure water at 4°C for further usage.

Cell culture on KTS

KTS was washed with sterile PBS for three times, and then transferred into 24-well culture plates.

HKC-8 cells were digested with 0.25% trypsin, and then cell suspension about 5×10^4 cells in 100 μ l was seeded into each kidney scaffold slice. Three hours later, another 900 μ l culture medium was added to each well, followed by incubating at 37°C in 5% CO₂. Two days after initial seeding, cell lysates were subjected to Western blot analyses.

Co-immunoprecipitation

The interaction of TNC with integrin α ν β 6 in mice was determined by co-immunoprecipitation. Three groups of mice were used: (i) sham-operated mice, (ii) UIRI mice injected with control shRNA, and (iii) UIRI mice injected with TNC-shRNA. Kidney homogenates from various groups were immunoprecipitated overnight at 4°C with anti-TNC antibody and protein A/G plus agarose (sc-2003; Santa Cruz Biotechnology). The precipitated complexes were washed with lysis buffer and boiled for 5 minutes in SDS sample buffer, followed by immunoblotting with anti-integrin α ν , anti-integrin β 6, or anti-TNC antibodies, respectively. In the reciprocal experiments, kidney homogenates were immunoprecipitated with anti-integrin α ν β 6 antibody, followed by immunoblotting with anti-TNC antibody. As a negative control, the potential interaction between TNC and integrin α 9 was also assessed by co-immunoprecipitation. Antibodies used are summarized in Supplemental Table S2.

Statistical analyses

All data examined were expressed as mean \pm SEM. Statistical analyses of the data were performed using SPSS 13.0 (SPSS Inc, Chicago, IL). Comparisons between groups were made by t test, or using

one-way ANOVA followed by the Student-Newman-Kuels test or Dunnett's T3 test. Spearman (nonparametric) correlation analysis was used to assess the relationship between urinary TNC and other variables. $P < 0.05$ was considered significant.

REFERENCES

- S1. Zhou D, Fu H, Zhang L, et al. Tubule-derived Wnts are required for fibroblast activation and kidney fibrosis. *J Am Soc Nephrol*. 2017;28:2322-2336.
- S2. Skrypnik NI, Harris RC and de Caestecker MP. Ischemia-reperfusion model of acute kidney injury and post injury fibrosis in mice. *J Vis Exp*. 2013;doi: 10.3791/50495.
- S3. Chen S, Fu H, Wu S, et al. Tenascin-C protects against acute kidney injury by recruiting Wnt ligands. *Kidney Int*. 2019;95:62-74.
- S4. Zhou D, Tian Y, Sun L, et al. Matrix metalloproteinase-7 is a urinary biomarker and pathogenic mediator of kidney fibrosis. *J Am Soc Nephrol*. 2017;28:598-611.
- S5. Bernhardt A, Fehr A, Brandt S, et al. Inflammatory cell infiltration and resolution of kidney inflammation is orchestrated by the cold-shock protein Y-box binding protein-1. *Kidney Int*. 2017;92:1157-1177.
- S6. Zhou L, Chen X, Lu M, et al. Wnt/beta-catenin links oxidative stress to podocyte injury and proteinuria. *Kidney Int*. 2019;95:830-845.
- S7. Zhao Y, Wang C, Hong X, et al. Wnt/beta-catenin signaling mediates both heart and kidney injury in type 2 cardiorenal syndrome. *Kidney Int*. 2019;95:815-829.
- S8. Fu H, Tian Y, Zhou L, et al. Tenascin-C is a major component of the fibrogenic niche in kidney fibrosis. *J Am Soc Nephrol*. 2017;28:785-801.

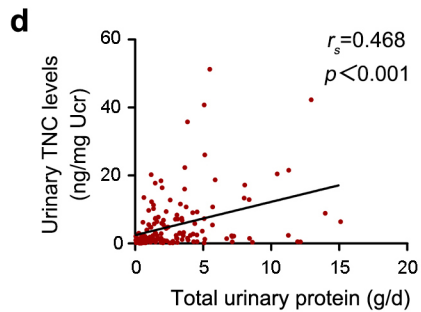
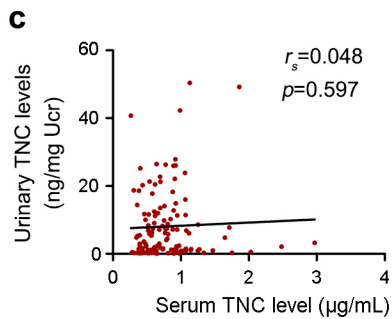
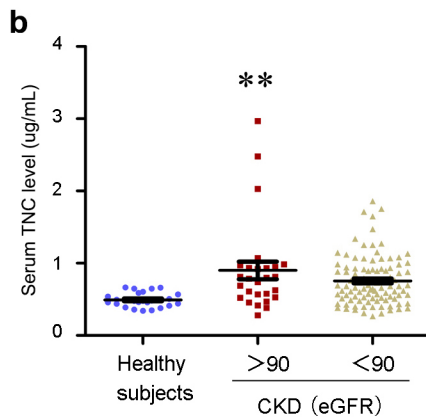
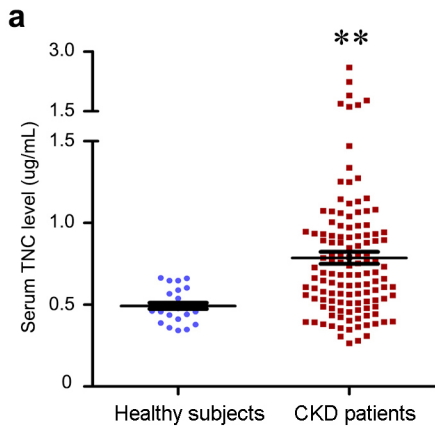


Figure S1

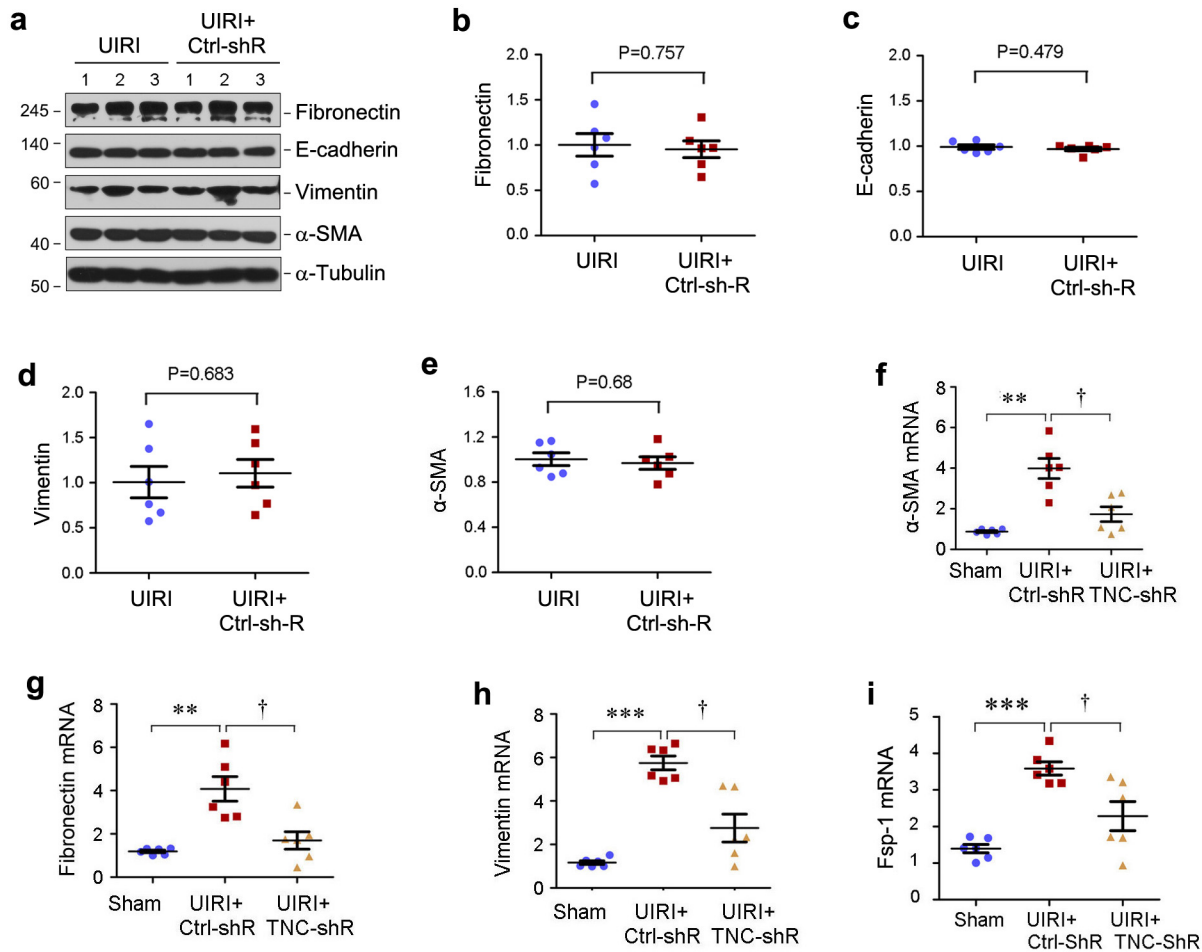


Figure S2

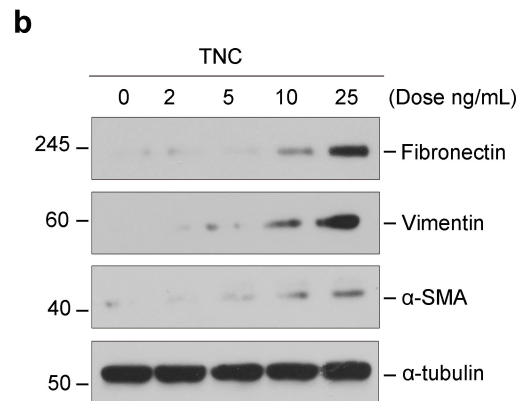
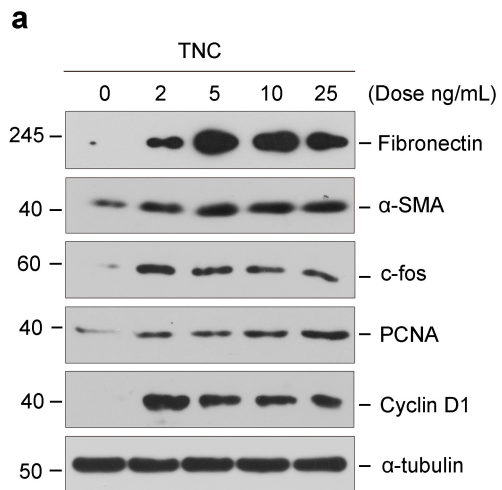


Figure S3

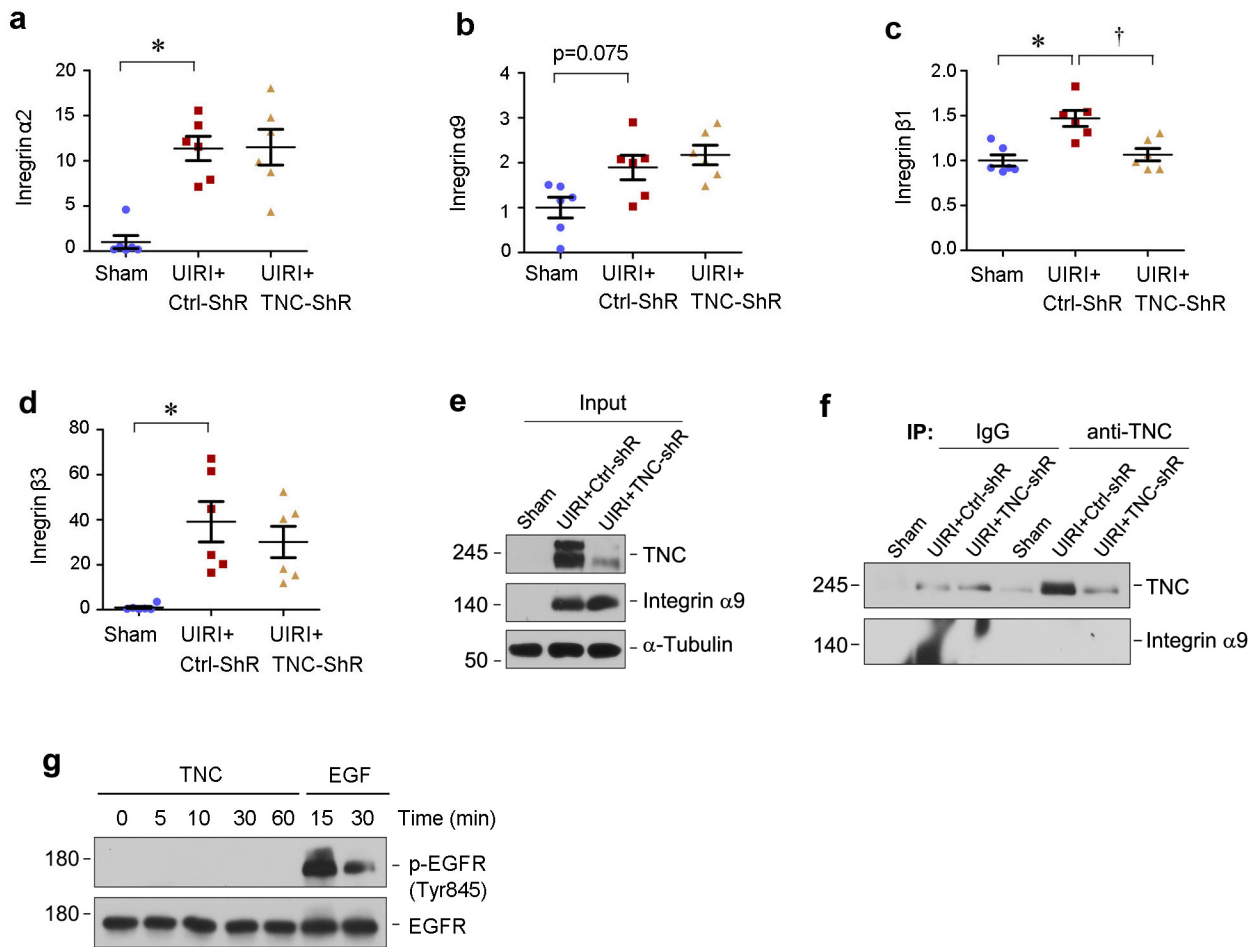


Figure S4

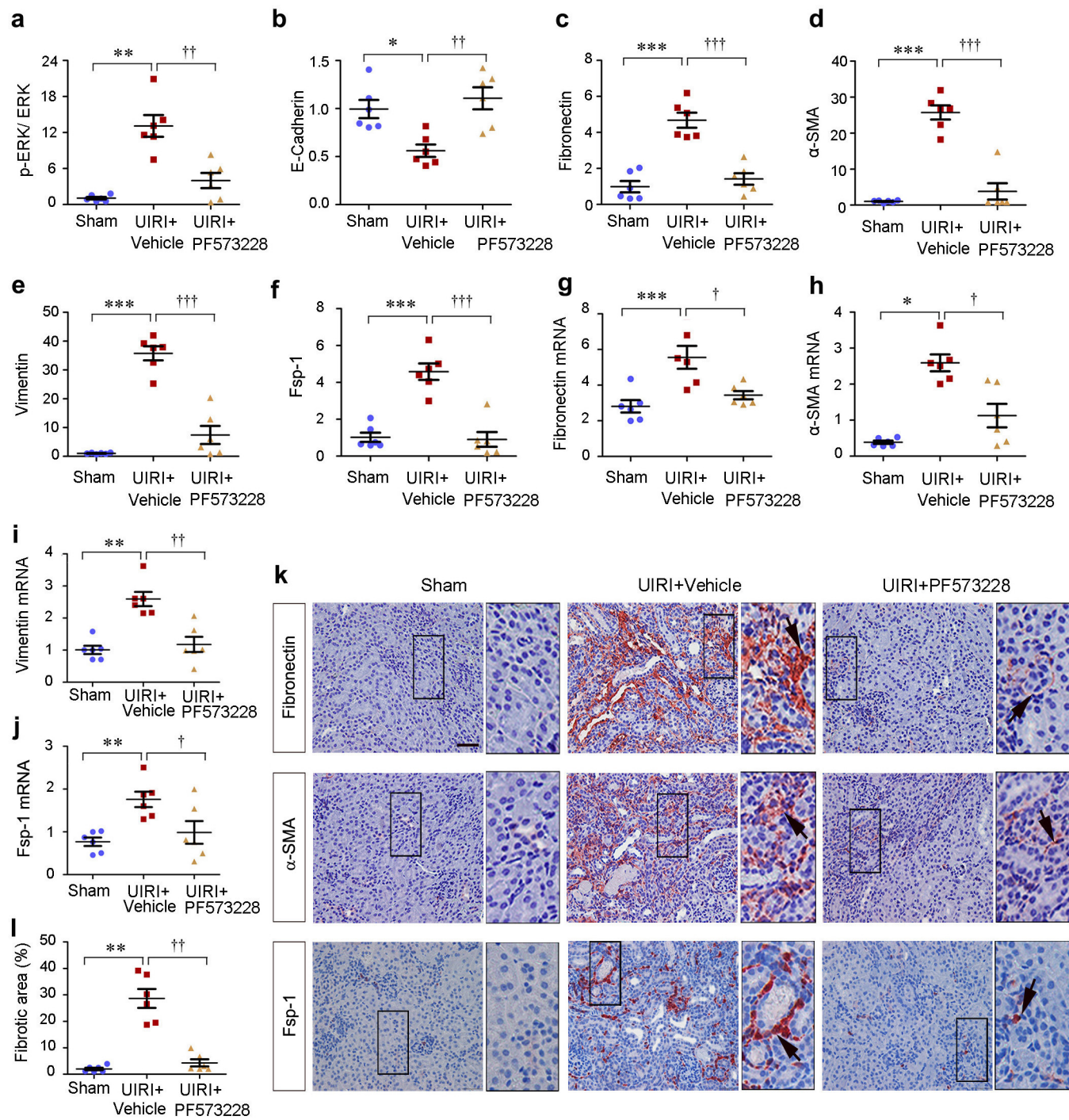


Figure S5

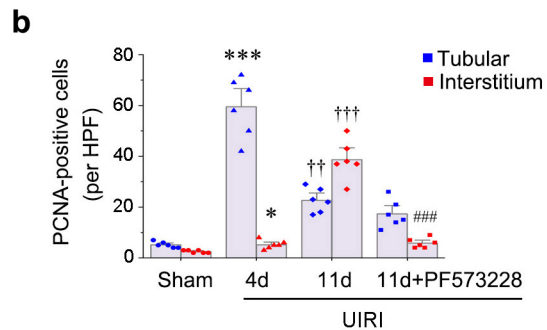
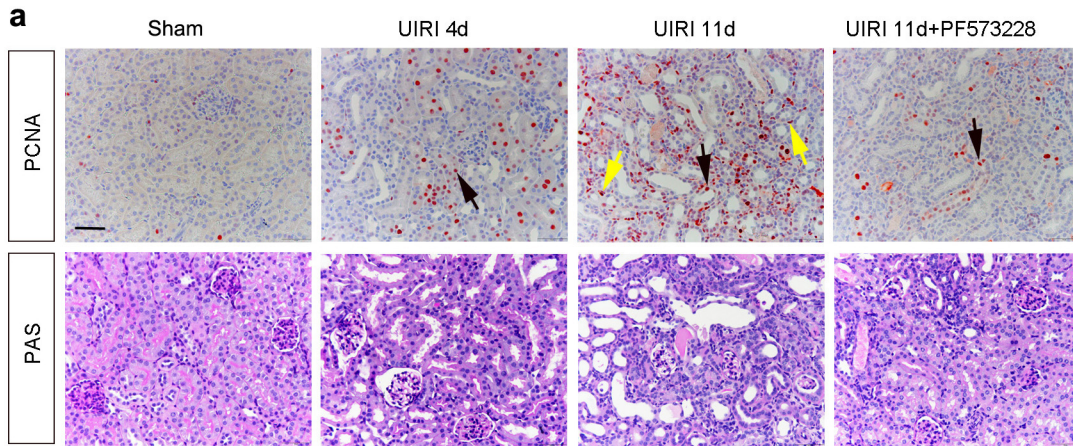


Figure S6

Supplementary Table S1. Demographic and clinical data of healthy subjects and CKD patients

Characteristics	Cohort	
	Normal subjects	CKD patients
Gender-No. (%)		
Male	13 (33)	122 (54)
Female	26 (67)	103 (46)
Age at entry-years		
Mean±SEM	36.5±1.5	44.7±1.1
Range	24-58	15-79
eGFR(ml/min/1.73m²)-No. (%)		
>90	39 (100)	79 (35)
<90	0 (0)	146 (65)
Pathological Diagnosis-No. (%)†		
MsPGN	0 (0)	39 (17)
MGB	0 (0)	21 (9)
MN	0 (0)	31 (14)
FSGS	0 (0)	23 (10)
LN	0 (0)	7 (3)
EPG	0 (0)	2 (1)
TMA	0 (0)	2 (1)
DN	0 (0)	8 (4)
Without pathological Diagnosis-No. (%)		
	39 (100)	92 (41)

†MsPGN: mesangial proliferative glomerulonephritis; MGB: minor glomerular abnormality; MN: membrane nephritis; FSGS: focal segmental glomerulosclerosis; LN: lupus nephritis; EPG: endocapillary proliferative glomerulonephritis; TMA: thrombotic microangiopathy; DN: diabetic nephropathy.

Supplementary Table S2. The sources of antibodies used in this study

Antibodies	Catalogue number	Company	Location
Primary antibodies			
anti-TNC	ab108930	Abcam	Cambridge, MA
anti- α -SMA	A5228	Sigma-Aldrich	St. Louis, MO
anti-fibronectin	F3648	Sigma-Aldrich	St. Louis, MO
Anti-vimentin	#5741	Cell Signaling Technology	Danvers, MA
anti-E-cadherin	#3195	Cell Signaling Technology	Danvers, MA
anti- α -tubulin	RM2007	Ray Antibody Biotech	Peachtree Corners, GA
anti-Fsp-1	#07-2274	EMD Millipore	Burlington, MA
anti-p-FAK (Y925)	#3284	Cell Signaling Technology	Danvers, MA
anti-total-FAK	#3285	Cell Signaling Technology	Danvers, MA
anti-p-ERK1/2	#9101	Cell Signaling Technology	Danvers, MA
anti-total-ERK	#9102	Cell Signaling Technology	Danvers, MA
anti-integrin α v	Ab179475	Abcam	Cambridge, MA
anti-mouse integrin β 6	AF2389	R & D Systems	Minneapolis, MN
anti-human integrin β 6	AF4155	R & D Systems	Minneapolis, MN
anti-integrin α β 6(10D5)	Ab77906	Abcam	Cambridge, MA
anti-integrin α 2	Ab181548	Abcam	Cambridge, MA
anti-integrin α 9	Ab140599	Abcam	Cambridge, MA
anti-integrin β 1	Ab183666	Abcam	Cambridge, MA
anti-integrin β 3	Ab210515	Abcam	Cambridge, MA
anti-c-fos	#2250	Cell Signaling Technology	Danvers, MA
anti-PCNA	sc-56	Santa Cruz Biotechnology	Santa Cruz, CA
anti-cyclin D1	sc-753	Santa Cruz Biotechnology	Santa Cruz, CA
anti-GAPDH	RM2002	Ray Antibody Biotech	Peachtree Corners, GA
anti-p-EGFR(Tyr845)	#2231	Cell Signaling Technology	Danvers, MA
anti-EGFR	#2646	Cell Signaling Technology	Danvers, MA
Secondary antibodies			
Goat anti-mouse	BA1050	Boster Biological Technology	Wuhan, China
Goat anti-rabbit	BA1054	Boster Biological Technology	Wuhan, China
Rabbit anti-goat	BA1060	Boster Biological Technology	Wuhan, China
Rabbit anti-sheep	Ab6747	Abcam	Cambridge, MA

Supplementary Table S3. Nucleotide sequences of the primers used for qPCR

Mouse gene	Primer Sequence 5' to 3'	
	Forward	Reverse
<i>Acta2</i>	GAGGCACCACTGAACCCTAA	CATCTCCAGAGTCCAGCACA
<i>Fn1</i>	CGGTTATCTGACTCTGGCTTTAAG	TCTCCCTGACGATCCCACTTC
<i>Tnc</i>	CAGGAATCTCCGCCGTGTCT	GTGGCTTGCTGGCTCTTTGG
<i>Vim</i>	AGAGAGAGGAAGCCGAAAGC	GCTCCTGGATCTCTTCATCG
<i>S100a4</i>	AGCTACTGACCAGGGAGCTG	TCATTGTCCCTGTTGCTGTC
<i>Actb</i>	GAGCGCAAGTACTCTGTGTG	AACGCAGCTCAGTAACAGTC