ONLINE SUPPLEMENT

Gpr97 exacerbates acute kidney injury via mediating Sema3A signaling in mice

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Running title: Gpr97 and acute kidney injury

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Materials and Methods

Human renal biopsy samples: Renal biopsies had been performed as part of routine clinical diagnostic investigation and collected. The investigations were conducted in accordance with the principles of the Declaration of Helsinki and were approved by the Research Ethics Committee of Shandong University after informed consent was obtained from the patients. The samples of renal biopsies were obtained from Department of Pathology, Shandong University School of Medicine. None of these patients started dialysis therapy at the time of kidney biopsy. Control samples were obtained from the healthy kidney poles of individuals who underwent tumor nephrectomies without renal disease.

Mouse models of acute kidney injury: An established mouse model of renal ischemia/reperfusion injury (IRI) was performed as described previously1 . In brief, after Gpr97$^{-/-}$ mice and age-matched wild type (WT) mice were anesthetized with intraperitoneal pentobarbital (50 mg/kg body weight), a midline abdominal incision was made and bilateral renal pedicles were clipped for 30 minutes using microaneurysm clamps. At the end of the ischemic period, the vascular clamps were removed and the kidneys were observed for 5 minutes to document reflow. The incision was then closed and the animal was allowed to recover. During the ischemic period, body temperature was maintained between 36~37.5°C using a temperature-controlled heating system. After reperfusion at 24 h or 48 h, blood samples were obtained via cardiac puncture and anesthetized mice were perfused with phosphate buffered saline (PBS) at 150-160 mmHg via a butterfly 23G needle inserted into left ventricle to totally remove blood. One kidney was then harvested and the other kidney was further perfused with 4% paraformaldehyde for histopathological analysis2, 3 .

In addition, acute kidney injury (AKI) in mice was also induced by a single intraperitoneal injection of cisplatin at a dose of 30 mg/kg (Sigma, St. Louis, MO). At 3 d, 5 d and 10 d after injection, mice were sacrificed, and serum and kidney samples were collected for various analyses 4 .

Assessment of renal function: High-performance liquid chromatography (HPLC) was performed to measure serum creatinine using Agilent 1100 HPLC system (Agilent

Technologies, Santa Clara, CA). The procedure was performed as described previously5, 6 .

Histology examination: Formalin-fixed kidney sections were stained with hematoxylin and eosin (H&E). The percentage of tubules in the corticomedullary junction that displayed cellular necrosis and loss of brush border were counted and scored in a blinded manner as follows: 0, none; 1, 0-10%; 2, 11–25%; 3, 26–45%; 4, 46–75%; and 5, >75%. At least 10 high power fields (HPFs，X 200 magnification) per section for each sample were examined.

Microarray analysis： The microarray experiments were performed by Shanghai Biotechnology Corporation (Shanghai, China). Total RNA was isolated using TRIzol reagent (Invitrogen) from the kidney of mice with or without renal IRI. Agilent Whole Mouse Genome Oligo Microarray (4×44K) (Agilent Technologies, Santa Clara, CA, USA) was used for transcriptome analysis. Microarray data was normalized using GeneSpring GX software (Agilent Technologies) and genes were categorized into multiple biological pathways using David database (https://david.ncifcrf.gov/).

TUNEL assay: Cell death in the kidney after I/R was detected by TUNEL assay following the manufacturer's protocol (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol as described.

Cell culture and treatments: Immortalized rat proximal tubule epithelial cells (NRK-52E) cells (American Type Culture Collection, Manassas, VA) were cultured as described7 . Different approaches to mimic hypoxia conditions:

1. Chemical anoxia/recovery was induced by incubating NRK-52E cells in glucose-free medium with antimycin A (AA, 10 μM)/2-deoxyglucose (2-DG, 25 mM) for 60 min (anoxia) to inhibit aerobic and substrate-dependent ATP generation (ATP depletion) as described8, 9 . Then, the cells were cultured in glucose-replete complete growth medium for 6 h, 12 h, 24 h or 48 h (recovery), individually.

2. Incubation of NRK-52E cells in a hypoxic environment for 90min (0.1% O_2) followed by reoxygenation at different time points.

3. Different concentration of $CoCl_2$ treatment.

4. NRK-52E cells were treated with cisplatin (50 μM) for 4 h, 8 h, 12 h or 24 h.

5. Recombinant mouse protein Sema3A was used to treat NRK-52E cells. Recombinant mouse Sema3A-Fc purchased from R&D Systems was added 12 h before stimulation with the final concentration of 100 ng/ml in the medium as described.

6. Recombinant HuR-expressing adenoviral plasmid (ORF of human HuR in adenoviral vector pAd with C terminal Flag and His tag) constructed by Vigene Biosciences (Rockville, USA) was efficiently transfected into NRK-52E cells.

RNA extraction and real time RT-PCR: Total RNA was isolated from the kidney or cells using TRIzol reagent (Invitrogen) as described previously10 . The mRNA levels for target genes were analyzed by real-time quantitative RT-PCR using a Bio-Rad iCycler system (Bio-Rad, Hercules, CA). The specific primers for target genes in this study are listed in Table S1. Level of the housekeeping gene β-actin was used as an internal control for the normalization of RNA quantity and quality differences among the samples.

Detection of cytokines and chemokines: Chemokines and cytokines in the kidney tissue were measured by real time RT-PCR. Primers used in this study were summarized in Table S1.

Western blot analysis: Total tissue or cellular lysates preparation and Western blot analysis were performed as described previously11 . Antibodies used in this study were summarized in Table S2. To document the loading controls, the membrane was reprobed with a primary antibody against housekeeping protein GAPDH.

Immunofluorescence staining and confocal microscopy： Immunofluorescent staining was performed as described1, 12 using a LSM780 laser scanning confocal microscope (ZEISS, Germany) equipped with a Plan-Apochromat 63×/1.4 objective. To define the tubular segment-specificity of Gpr97 expression in the kidney, we utilized double immunostaining for Gpr97 (green) and various tubular markers (red) in the kidney. Segment-specific tubular markers were used based on previous studies1, 13 as follows: proximal tubule, aquaporin-1 (AQP1); distal tubule, calbindin D28K; and collecting duct, aquaporin-3 (AQP3).

Sema3A serum level: The measurement of sema3A serum level was conducted using a commercial ELISA kit (CUSABIO, Wuhan, China) according to the manufacturer's instructions. The serum samples were stored at -80°C until ELISA evaluation.

RNA electrophoretic mobility-shift assay (EMSA): EMSAs and supershift analyses with protein extracts were performed as described14 . In this experiment, recombinant HuR protein (ProteinTech Group) was used as control. The biotinylated RNA oligonucleotides were synthesized. The sequences of the RNA oligonucleotides used for EMSA in accordance with the pentameric ARE sequences were as follows:
Sema3A-ARE1,5'-AAGCUAUAAUUUAAAUGUACC-3';
Sema3A-ARE2,5'-UAUAAUUUAGUGAUUUAUAAU-3';
Sema3A-ARE3,5'-UGUUCUAUAUUUAUAGUUAUG-3'.

Recombinant HuR proteins obtained from ProteinTech Group (Chicago, IL) were used as a positive control to confirm the interaction between HuR and Sema3A-AREs.

RNA interference: Small interference RNAs (siRNAs) were synthesized by GeneParma (Shanghai, China). The DNA target sequence for
siRNA-Gpr97 was 5'-GCTACTTCCTTGTCACCTT-3';
siRNA-HuR was 5'-AAGAGGCAAUUACCAGUUUCA-3'
siRNA-Sema3A was 5'-GCAATGGAGCTTTCTACTA-3'.

The scrambled small RNA was confirmed as a nonsilencing double-stranded RNA and was used as control in the present study. In these experiments, siRNA transfection was performed using the Bio-Rad siLentFect lipid transfection kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions.

Caspase-3 activity assay: Caspase-3 activity was measured as described previously15 . Relative caspase-3 activity was calculated as a ratio of emission of treated cells to untreated cells.

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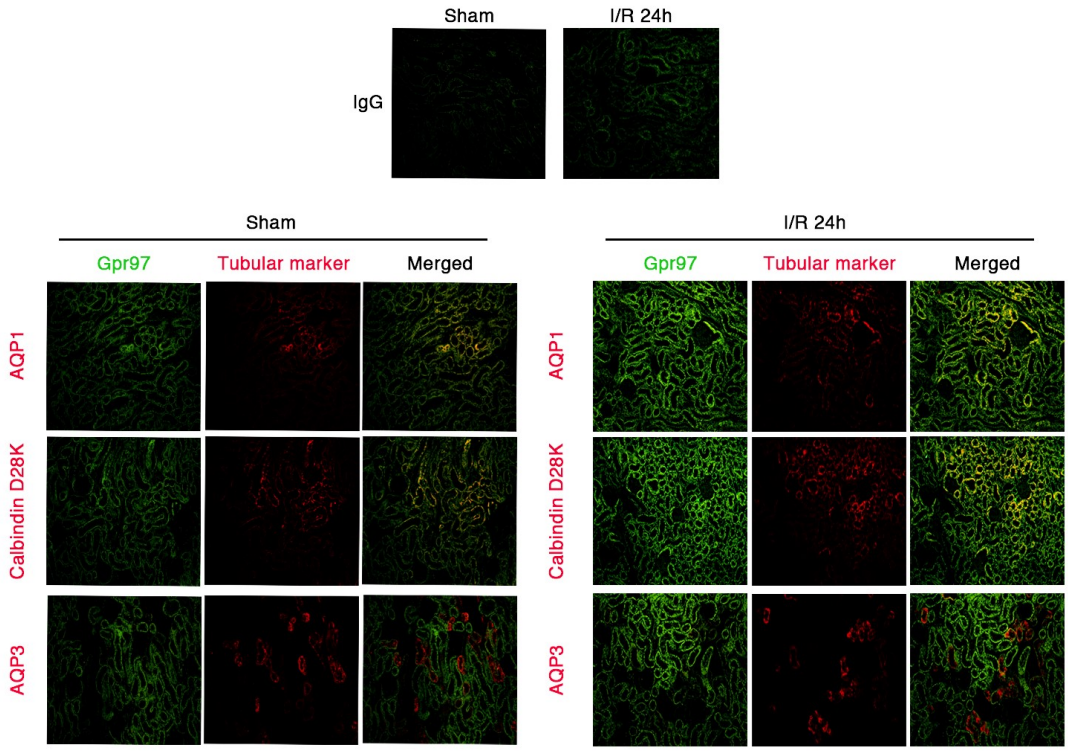

Figure S1. Co-immunofluorescence staining for Gpr97 and tubular segment specific markers in the kidney after renal I/R. Segment-specific tubular markers were used as follows: proximal tubule, aquaporin-1 (AQP1); distal tubule, calbindin D28K; and collecting duct, aquaporin-3 (AQP3). It was found that Gpr97 was mainly expressed in proximal tubules and distal tubules. (n=8).

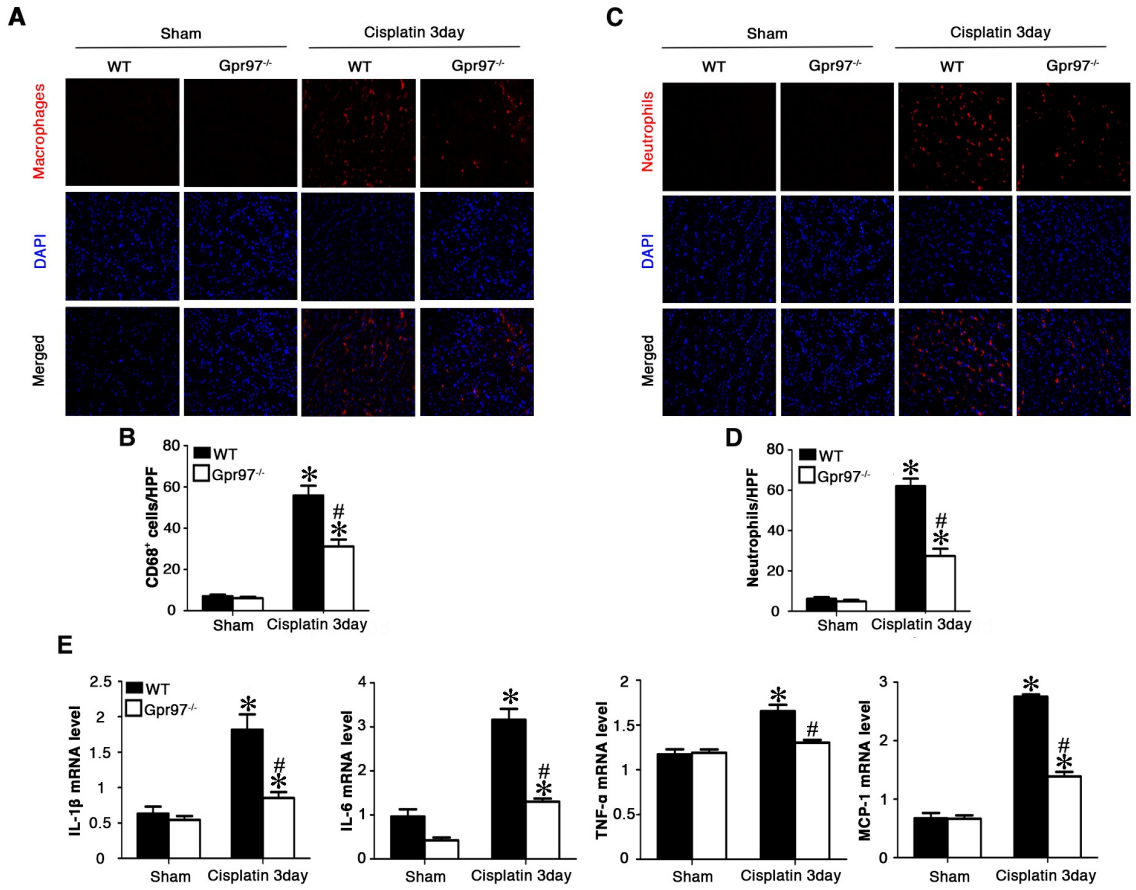

Figure S2. Gpr97 deficiency ameliorated macrophage and neutrophil infiltration in mice with AKI induced by cisplatin. (A) Representative sections of kidney stained for macrophages from different groups of mice. **(B)** Data analysis of macrophage infiltrates in the kidney (numbers/HPF). **(C)** Representative sections of kidney stained for neutrophils from different groups of mice. **(D)** Data analysis of neutrophil infiltrates in the kidney (numbers/HPF). **(E)** The levels of pro-inflammatory mediators including interleukin-1β (IL-1β), IL-6, tumor necrosis factor-α (TNF-α) and monocyte chemoattractant protein-1 (MCP-1) measured by mRNA analysis. *P<0.05 vs. sham-operated WT mice, #P<0.05 vs. ischemic WT mice at the same experimental conditions (n=8).

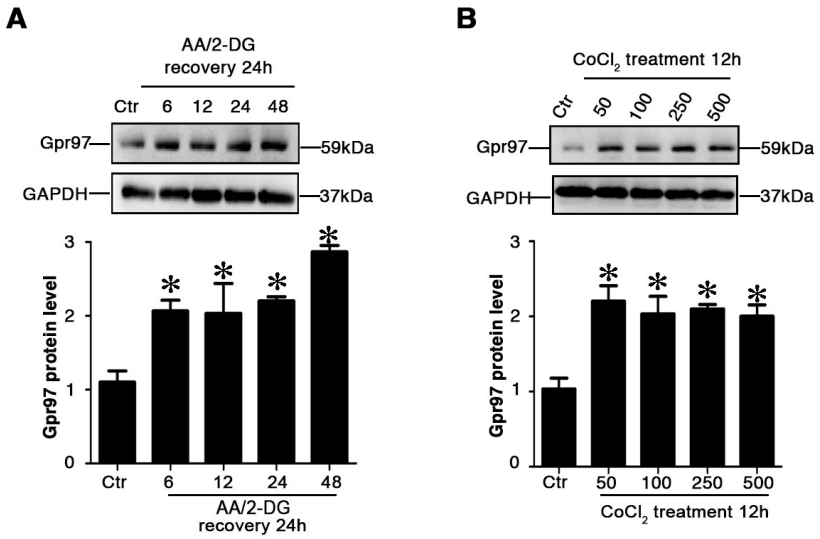

Figure S3. The levels of Gpr97 was increased in the NRK-52E cells under chemical anoxia/recovery condition. (A) Representative Western blot gel documents and summarized data showing the protein levels of Gpr97 in NRK-52E cells under chemical anoxia/recovery condition, which was induced by incubating cells in glucose-free medium with antimycin A (AA, 10 μM)/2-deoxyglucose (2-DG, 25 mM) for 60 min (anoxia) to inhibit aerobic and substrate-dependent ATP generation, and then in vitro reperfusion was achieved by incubating cells in glucose-replete complete growth medium for 6 h, 12 h, 24 h or 48 h (recovery), individually. **(B)** Representative Western blot gel documents and summarized data showing the protein levels of Gpr97 in NRK-52E cells with different $CoCl_2$ concentration treatment for 12 h.*$P<0.05$ vs. control of NRK-52E cells (n=6).

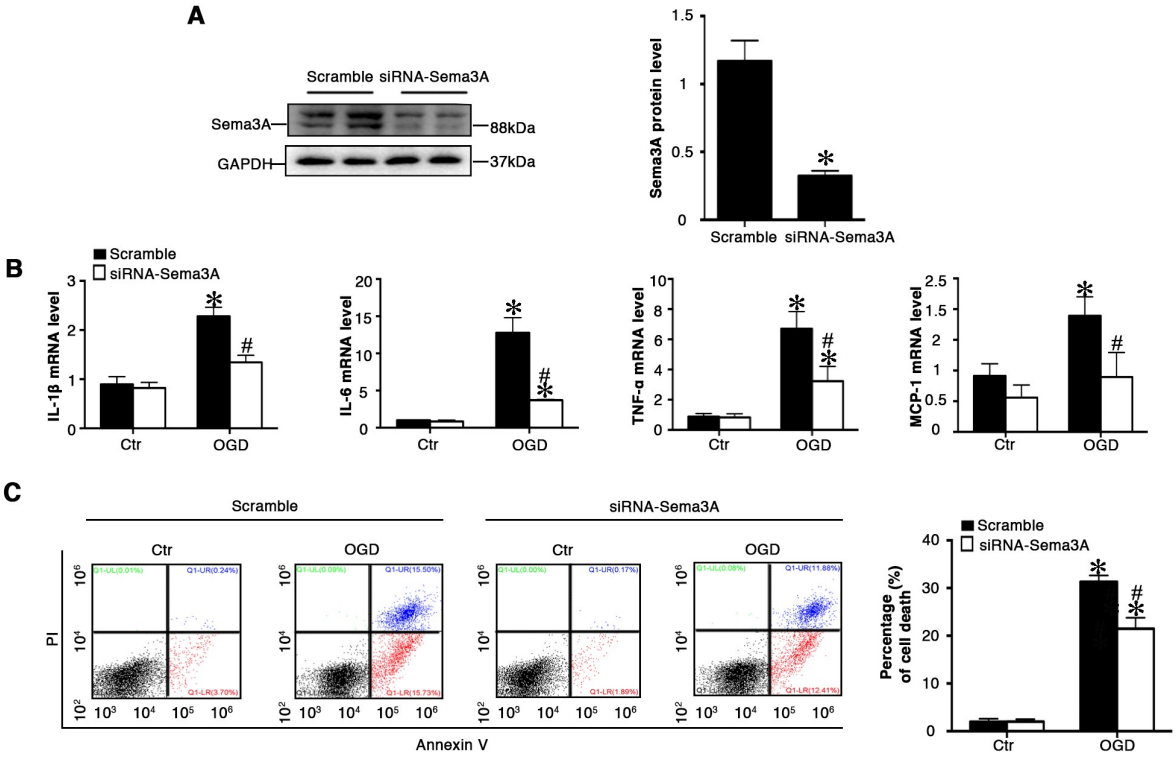

Figure S4. The effect of Sema3A knockdown on the hypoxia-induced production of pro-inflammatory mediators and apoptosis in NRK-52E cells. (A) Representative Western blot gel documents and summarized data showing the gene silencing efficiency of Sema3A by siRNA-Sema3A transfection. **(B)** The effect of Sema3A on the mRNA levels of pro-inflammatory mediators in NRK-52E cells under OGD condition (cultured in a hypoxic environment for 90 min (0.1% O2) followed by reoxygenation at 24 h). **(C)** Summarized data showing the amount of cell apoptosis determined by flow cytometric analysis in NRK-52E cells with different treatments. *P<0.05 vs. scramble of NRK-52E cells (n=6).

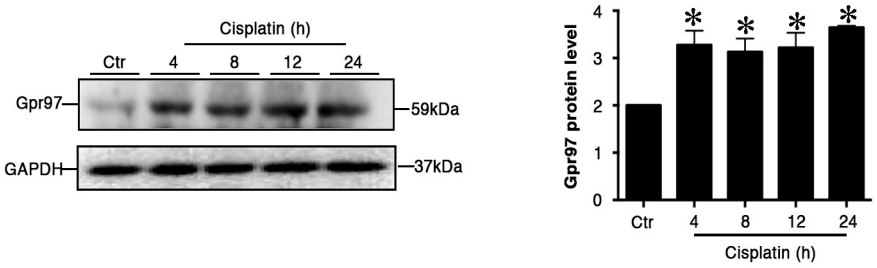

Figure S5. The upregulation of Gpr97 in cisplatin-treated NRK-52E cells. Representative Western blot gel documents and summarized data showing the protein levels of Gpr97 in NRK-52E cells treated with cisplatin (50 μM) for 4h,8h,12h or 24h. *P<0.05 vs. control of NRK-52E cells treated with cisplatin (n=6).

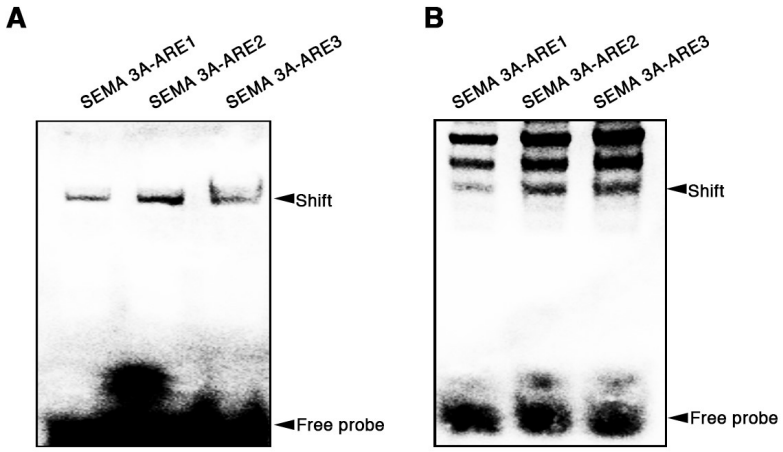

Figure S6. **HuR interacted with the 3'-UTR of Sema3A mRNA in HK-2 cells and the whole kidney homogenates from mice. (A)** The cytoplasmic extracts from HK-2 cells were prepared for RNA-EMSA analysis. Complexes were formed when Sema3A-ARE1, SemaA-ARE2 and Sema3A-ARE3 were applied for the binding reaction. **(B)** The cytoplasmic extracts from the mouse kidney were prepared for RNA-EMSA analysis. Complexes were formed when Sema3A-ARE1, SemaA-ARE2 and Sema3A-ARE3 were applied for the binding reaction.

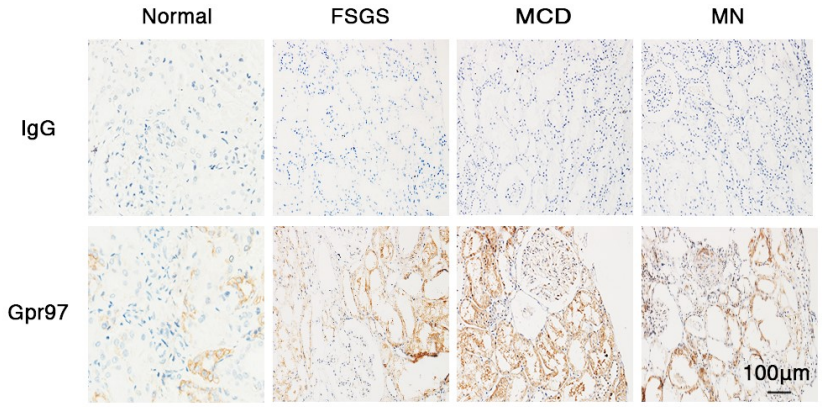

Figure S7. **The expression of Gpr97 in renal biopsies from patients with chronic kidney disease such as focal segmental glomerulosclerosis (FSGS), minimal change disease (MCD), and membranous lomerulonephritis (MN).** We also detected the expression of Gpr97 in renal biopsies from patients with other different forms of chronic kidney disease such as focal segmental glomerulosclerosis (n=6), membranous glomerulonephritis (n=6), and minimal change disease (n=7). The levels of Gpr97 were also markedly increased in these patients compared with control subjects, indicating that Gpr97 may be a common predictor of renal injury.

Table S1. Primer pairs of target genes used for real time RT-PCR in this study

Genes	Accession No.	Forward	Reverse
MUS GPR97	NM_173036.3	CACCTTCGACTTGAATGACACTGCTC	TGCTGATGTTCTGGATCAATGCCTT
MUS SEMA3A	NM_001243072.1	GTTGTAGACCGGGTGGATGC	TCGGAGCAGTGAGTCAGTGG
MUS TNF-α	NM_001278601.1	GAAAAGCAAGCAGCCAACCA	CGGATCATGCTTTCTGTGCTC
MUS IL-1β	XM_006498795.1	CTGCAGCTGGAGAGTGTGG	GGGGAACTCTGCAGACTCAA
MUS IL-6	NM_031168.1	AGTTGCCTTCTTGGGACTGA	TCCACGATTTCCCAGAGAAC
MUS MCP-1	NM_011333.3	ACCTGCTGCTACTCATTCAC	TTGAGGTGGTTGTGGAAAAG
MUS B-ACTIN	NM_007393.3	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT
RAT IL-1β	NM_031512.2	CCAAGCCCTTGACTTGGGCTGTC	TGGGTCCTCATCCTGGAAGCTCC
RAT IL-6	NM_012589.2	CCGGAGAGGAGACTTCACAGAG	CAGTGCATCATCGCTGTTCATAC
RAT MCP-1	NM_031530.1	CTGGGCCTGTTGTTCACAGTTGC	CTTTGGGACACCTGCTGCTGGTG
RAT TNF-α	NM_012675.3	CCACCACGCTCTTCTGTCTA	TTTGCTACGACGTGGGCTAC
RAT B-ACTIN	NM_031144.3	GACAGGATGCAGAAGGAGATTAT	TGATCCACATCTGCTGGAAGGT
RAT TLR4	NM_005104.4	GGGGCAACCGCTGGGAGAGA	AACCAGCGGAGGCCGTGAGA

Table S2. Antibodies used in this study

Primary antibodies	Host	Dilution and supplier	Application
Gpr97	Rabbit	1:1000;Abcam,Cambridge,MA,USA	WB,IHC,IF
AQP1	Goat	1:100;Santa Cruz, Dallas, TX	IF
Calbindin D28K	Mouse	1:100;Santa Cruz, Dallas, TX	IF
AQP3	Goat	1:100;Santa Cruz, Dallas, TX	IF
Sema3A	Rabbit	1:1000; Affinity Biosciences, OH	WB,IHC
HuR	Rabbit	1:1000; ProteinTech Group, Chicago, IL	WB, IF
Survivin	Rabbit	1:1000; ProteinTech Group, Chicago, IL	WB
p-Stat3	Rabbit	1:1000; Affinity Biosciences, OH	WB
Neutrophil	Rat	1:100; AbD Serotec, Oxford, UK	IF
CD68	Rat	1:100; AbD Serotec, Oxiford, UK	IF
GAPDH	Mouse	1:6000; ProteinTech Group, Chicago, IL	WB