

# Prednisone inhibits the focal adhesion kinase/receptor activator of NF- $\kappa$ B ligand/mitogen-activated protein kinase signaling pathway in rats with adriamycin-induced nephropathy

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**Abstract.** The aim of the present study was to investigate the mechanisms underlying the effects of prednisone on adriamycin-induced nephritic rat kidney damage via the focal adhesion kinase (FAK)/receptor activator of nuclear factor- $\kappa$ B ligand (RANKL)/mitogen-activated protein kinase (MAPK) signaling pathway. An adriamycin-induced nephritic rat model was established to investigate these mechanisms. A total of 30 healthy male Sprague-Dawley rats were randomly assigned to the normal, model or prednisone group. Samples of urine were collected over the course of 24 h at days 7, 14, and 28, and renal cortex tissue samples were harvested at days 14, and 28 following nephritic rat model establishment. The total urinary protein content was measured by biuret colorimetry. Pathological changes in the kidney tissue samples were observed using an electron microscope. The mRNA expressions levels of FAK, RANKL, p38, extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and nephrin were then quantified by reverse transcription-quantitative polymerase chain reaction. In addition, the protein expressions levels of FAK, RANKL, p38, ERK, JNK, phosphorylated (p)-FAK, p-ERK, and p-JNK were quantified by western blotting. As compared with the normal group, the protein expression levels of FAK, RANKL, p-FAK, p38 and p-ERK in the model group were increased. In the prednisone group, the protein expression levels of p-ERK decreased, as compared with the normal group. In the prednisone group, the urinary protein levels, the protein expression levels of FAK, RANKL, p38, p-FAK, p-p38 and the mRNA expression levels of FAK, p38, RANKL, ERK, JNK decreased, as

compared with the model group. In the prednisone group, the mRNA and protein expression levels of nephrin and the serum expression levels of RANKL increased, the serum expression levels of osteoprotegerin (OPG) were decreased, as compared with the model group. No significant changes in the protein expression levels of JNK were observed among the groups. These results suggested that prednisone is able to protect podocytes from apoptosis, and reduce urinary protein levels by inhibiting the FAK/RANKL/MAPK signaling pathway in kidney tissue samples. Serum prednisone may induce osteoporosis via the OPG/RANK/RANKL signaling pathway.

## Introduction

Focal adhesion kinase (FAK) is expressed in podocytes, where it affects the  $\alpha$ -actin cytoskeleton thereby regulating cell adhesion and migration. FAK may be activated by glomerular injury, resulting in proteinuria and foot process fusion. In animal models, the elevated expression levels of FAK may lead to foot process fusion and increased levels of proteinuria, processes which were significantly reduced following knock down of FAK. Previous studies have demonstrated that the migration and activation of podocytes was significantly reduced in the absence of FAK (1,2). Activation of mitogen-activated protein kinase (MAPK) signaling pathway is associated with podocyte injury, foot process fusion and proteinuria (3). Furthermore, FAK affects podocyte structure via the MAPK signaling pathway (4). The MAPK family is composed of extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK) and p38 (5). Receptor activator of nuclear factor  $\kappa$ B (RANK) and its ligand (RANKL) are cytokines that are able to activate the nuclear factor  $\kappa$ B (NF- $\kappa$ B) or MAPK signaling pathways following binding (6). The MAPK and NF- $\kappa$ B signaling pathways regulate numerous biological cellular processes, including cell proliferation, transduction and apoptosis. Liu *et al* (7) demonstrated that RANKL inhibits the apoptosis of podocytes, and the expression levels of RANKL increased following podocyte injury. Prednisone is the preferred drug for the treatment of nephrotic syndrome; however, the

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**Key words:** prednisone, focal adhesion kinase, receptor activator of nuclear factor- $\kappa$ B ligand, mitogen-activated protein kinase

association between RANKL and kidney proteinuria remains to be elucidated.

The aim of the present study was to investigate the possible mechanisms underlying the therapeutic effects of prednisone, including the decreased protein levels in kidney tissue samples via the FAK/RANKL/MAPK signaling pathway in an adriamycin-induced nephritic rat model. The rats with adriamycin-induced nephropathy were treated with prednisone. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was used to quantify the mRNA expression levels of FAK, RANKL, p38, ERK, JNK and nephrin; electron microscopy was used to observe renal pathology; immunohistochemistry was used to detect the levels of nephrin in the kidney; and western blot analysis was used to quantify the protein expression levels of FAK, phosphorylated (p)-FAK, RANKL, p38, p-p38, ERK, p-ERK, JNK, p-JNK, and nephrin in the kidney tissue samples.

## Materials and methods

**Reagents and instruments.** The materials for the present study were purchased from the following suppliers: SYBR Premix Ex Taq<sup>TM</sup> II kit (cat. no. DRR820A; Takara Biotechnology Co., Ltd., Dalian, China); RNAiso Plus (cat. no. D9108A; Takara Biotechnology Co., Ltd.); ExScript<sup>TM</sup> RT Reagent kit (cat. no. DRR037A; Takara Biotechnology Co., Ltd.); rat glyceraldehyde 3-phosphate dehydrogenase GAPDH primer (cat. no. D379212; Takara Biotechnology Co., Ltd.); oxorubicin hydrochloride (cat. no. H31020675; Zhejiang Hisun Chemical Co., Ltd., Taizhou, China); RT-qPCR primers (Takara Biotechnology Co., Ltd.); RT-qPCR kits (Beyotime Institute of Biotechnology, Haimen, China); western blotting-associated reagents (Beyotime Institute of Biotechnology), including SDS-PAGE kit, radioimmunoprecipitation (RIPA) lysis buffer, BeyoECL Plus A, BeyoECL Plus B, nitrocellulose membranes (cat. no. FFN09), and phenylmethanesulfonyl fluoride (cat. no. ST506); secondary antibody dilution buffer (cat. no. P0023D; Beyotime Institute of Biotechnology); primary antibody dilution buffer (cat. no. P0023A; Beyotime Institute of Biotechnology); anti-GAPDH monoclonal antibody (cat. no. ab8245; Abcam, Cambridge, UK); anti-FAK (cat. no. 3285), p38 (cat. no. 6381), ERK (cat. no. 2265) and JNK (cat. no. 3630) total protein polyclonal antibodies (Bioworld Technology, Inc., St. Louis Park, MN, USA); anti-RANKL monoclonal antibody (cat. no. ab12125; Abcam); anti-nephrin monoclonal antibody (cat. no. 377246; Santa Cruz Biotechnology, Inc., Dallas, TX, USA); anti- $\beta$  actin monoclonal antibody (cat. no. ab8226; Abcam); PV-6001 Two-Step Detection kit (OriGene Technologies, Inc., Beijing, China); Polink-2 Plus<sup>®</sup> Polymer horseradish peroxidase (HRP) Detection system for rabbit primary antibody (GBI Labs, Bothell, WA, USA); osteoprotegerin (OPG) ELISA kit (Nanjing Senbeijia Biotechnology Co., Ltd., Nanjing, China); RANKL ELISA kit (Nanjing Senbeijia Biotechnology Co., Ltd.); 7500 Fast Real-Time PCR system (Applied Biosystems Life Technologies, Foster City, CA, USA); EL-311S enzyme standard instrument (BioTek Instruments, Inc., Winooski, VT, USA); western blotting kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA); gel image analyzer with Genesnap and Genetool (Syngene, Frederick, MD, USA).

**Model establishment and group assignment.** Experimental procedures were conducted in conformity with the institutional guidelines for the care and use of laboratory animals of the Fujian University of Traditional Chinese Medicine (Fujian, China), and conformed to the Laboratory Animal Management Regulations. A total of 30 healthy male Sprague-Dawley rats (age, 6–8 weeks; weight, 180–220 g) were obtained from the Medical Experimental Animal Center of Guangdong Province (certificate no. 70092411). Animals were fed on a standard laboratory diet and provided with *ad libitum* access to water. The medical experimental animals were housed in the environmental facilities of the Mouse Laboratory of Animal Experimental Center in the Fujian University of Traditional Chinese Medicine in a pathogen-free environment. All the animal experimental procedures were conducted in accordance with the management rules of the Fujian Province Medical Laboratory Animal Management Committee. Rats were kept at 23°C and 60% humidity in a 12 h light-dark cycle. The rats were randomly divided into the normal, model and prednisone groups (n=10 per group). The rats in the model group and prednisone groups were treated with a single intravenous injection of 6.5 mg/kg adriamycin into the tail (8). The normal rats were injected with an equal quantity of saline. A total of 7 days after treatment with adriamycin, samples of urine were collected over the course of 24 h, and the urinary protein levels were measured, indicating the successful establishment of a nephritic rat model. Following model establishment, the rats in the prednisone group were treated with a daily dose of 10 mg/kg/day prednisone (cat. no. H31020675; Shanghai Sine Pharmaceutical Laboratories Co., Ltd., Shanghai, China) by gastric gavage. The rats in the normal and model groups were treated daily with an equal amount of normal saline. At days 21 and 35 after model establishment, the rats were anesthetized by intraperitoneal injection with chloral hydrate (BIO BASIC Int., Markham, ON, Canada) at a dose of 0.3 ml/100 g body weight. A midline incision was made in the abdomen and blood samples were obtained from the aorta. The kidneys were removed immediately and weighed, and renal cortex tissue was removed using a small blade. The renal cortex tissue were stored in 10% formalin subsequent to pathological examination and immunohistochemical studies. The remaining renal tissues were immediately snap-frozen in liquid nitrogen and stored at -80°C for later analysis. Pre-experimental observations determined that 7 days after model establishment, the adriamycin-induced nephritic rats exhibited symptoms of proteinuria, and 21 and 35 days after model establishment proteinuria symptoms significantly increased ( $P<0.05$ ).

**Analysis of 24 h urinary protein levels.** The rats were placed in individual metabolic cages for 24 h during which time samples of urine were collected, and the 24 h urinary protein levels were measured by biuret colorimetry (Shanghai Yucan Biological Technology, Co., Ltd.) as described previously (9).

**Transmission electron microscopy.** The renal cortex samples were fixed using 3% glutaraldehyde, 0.22 mmol/l sucrose phosphate buffer (pH 7.2), post-fixed in 1% osmium tetroxide, progressively dehydrated in ethanol, and embedded in epoxy resin. The tissue samples were then examined for kidney

Table I. Primer sequences.

Gene	Primer sequences	Size (bp)
FAK	Forward 5'-GGACCTTACTGGCAACTGTGGA-3' Reverse 5'-CATACTGCTGCGCCAGCTTC-3'	138
RANKL	Forward 5'-GCAGCATCGCTCTGTTCCTGTA-3' Reverse 5'-GCATGAGTCAGGTAGTGCTTCTGTG-3'	164
p38MAPK	Forward 5'-TTACCGATGACCACGTTTCAGTTTC-3' Reverse 5'-AGCGAGGTTGCTGGGCTTTA-3'	107
ERK	Forward 5'-CTGGACCAGCTCAACCACATTC-3' Reverse 5'-ACTGTAGGTAGTTTCGGGCCTTCA-3'	97
JNK	Forward 5'-ACCACCAAAGATCCCTGACAA-3' Reverse 5'-TAGTTCGCTCCTCCAAATCCA-3'	104
Nephrin	Forward 5'-GACACCTGCATGATGAAGTGGAG-3' Reverse 5'-CAGGCCAGCGAAGGTCATAG-3'	102

FAK, focal adhesion kinase; RANKL, receptor activator of nuclear factor- $\kappa$ B ligand; p38, p38 mitogen-activated protein kinase; ERK, extra-cellular signal-regulated kinase; JNK, c-Jun N-terminal kinase.

pathology using a HU-12A transmission electron microscope (Hitachi, Ltd., Tokyo, Japan).

**Serum OPG and RANKL expression in each group.** A total of 21 and 35 days after the first adriamycin injection, 3 ml blood was collected from the abdominal aorta, and serum was obtained via centrifugation (1,478 x g at 4°C for 5 min). Serum RANKL and OPG levels were analyzed by ELISA. Nephrin expression was detected by immunohistochemical staining (10).

**RT-qPCR.** The rat renal cortex tissue samples were packed with tinfoil, frozen in liquid nitrogen, and preserved at -80°C until further experimentation. Total renal cortex RNA was extracted using TRIzol® Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA was reverse transcribed to cDNA using an ExScript™ RT kit and SYBR Premix Ex Taq II Reagent kit in order to conduct the fluorescence amplification. RT-qPCR was carried out on a Thermal Cycler Dice™ Real Time system (Takara Biotechnology Co., Ltd.) according to the manufacturer's instructions, and the target primers were synthesized by Takara Biotechnology Co., Ltd. (Table I). The PCR cycling conditions were as follows: 95°C for 10 min, followed by 40 cycles at 95°C for 30 sec, and 60°C for 60 sec. The mRNA levels were normalized to GAPDH. The mRNA expression levels in the normal group were used to calculate the relative mRNA levels in the other groups.

**Protein expression levels of FAK, RANKL, p38, ERK, and JNK, as determined by western blot analysis.** A total of 200 mg renal tissue samples, stored at -80°C, were obtained from each group. The renal tissue samples were lysed in 0.4 ml RIPA lysis buffer and homogenized. The homogenates were placed into a 1.5 ml EP tube and lysed on ice for 30 min. The homogenates were then centrifuged at 23,663 x g at 4°C for 60 min. The supernatants were collected from the EP tube (Axygen, Union City, CA, USA). Following extraction of total protein, the protein concentrations were measured using a Bicinchoninic Acid (BCA) Protein Concentration Assay kit (Beyotime

Institute of Biotechnology). The protein extracts were separated by 12% SDS-PAGE for 124 min at 80 V, and transferred onto nitrocellulose membranes for 1 h at 100 V. The nitrocellulose membranes were then blocked with 5% non-fat dry milk in Tris-buffered saline with Tween 20 (TBST; Beyotime Institute of Biotechnology) for 2 h at room temperature. The membranes were exposed to rat anti-FAK, anti-p-FAK, anti-p38, anti-p-p38, anti-RANKL, and anti-GAPDH (1:1,000), overnight at 4°C. The membranes were washed three times in TBST for 5-10 min. The membranes were then incubated with HRP-labeled goat anti-rabbit secondary antibody (1:1,000; Beyotime Institute of Biotechnology; cat. no. A0208), or goat anti-rat IgG (H+L) (1:1,000; Bioworld Technology, Inc., cat. no. BS10010). Following extensive washing in TBST, the bands were detected by chemiluminescence and analyzed by a gel image analyzer, and the relative density of each band was calculated and normalized to that of GAPDH (10).

**Immunohistochemical analysis.** Immunohistochemical staining was conducted in a two-step method: The paraffin was removed from the sections using xylene and rehydrated in graded ethanol as follows: 100% ethanol for 5 min twice, 95% ethanol for 5 min twice, 90% for 5 min and 80% for 5 min. For antigen retrieval, the sections were placed in a microwave following treatment with blocking goat serum (Pingshui Biotechnology Co.) for 45 min, the sections were incubated overnight at 4°C with primary antibodies targeting nephrin. The sections were then incubated with the appropriate Streptavidin-HRP (Beyotime Institute of Biotechnology; cat. no. A0303) secondary antibodies for 30 min at 37°C. The sections were stained with diaminobenzidine, and counterstained with hematoxylin (Shanghai YANYU Information Technology Co. Ltd., Shanghai, China). The sections were subsequently observed under a microscope (EM-208; Philips, Amsterdam, Holland), and the positive integral optical density was calculated.

**Statistical analysis.** Data were presented as the mean  $\pm$  standard deviation. Groups of data were tested for normality using



Table II. Urinary protein levels over the course of 24 h.

Time	Group (n=10)		
	Normal (mg)	Model (mg)	Prednisone (mg)
Day 7	3.19±1.88	10.94±6.91 <sup>b</sup>	18.27±16.65 <sup>b</sup>
Day 21	5.59±1.67	40.98±15.37 <sup>c</sup>	19.96±5.32 <sup>b,d</sup>
Day 35	4.58±2.22	61.04±10.55 <sup>a,c</sup>	12.49±7.35 <sup>a,e</sup>

Results are presented as the mean ± standard deviation. <sup>a</sup>P<0.05, vs. the normal group at day 21; <sup>b</sup>P<0.05 and <sup>c</sup>P<0.01, vs. the normal group; <sup>d</sup>P<0.05 and <sup>e</sup>P<0.01, vs. the normal group.

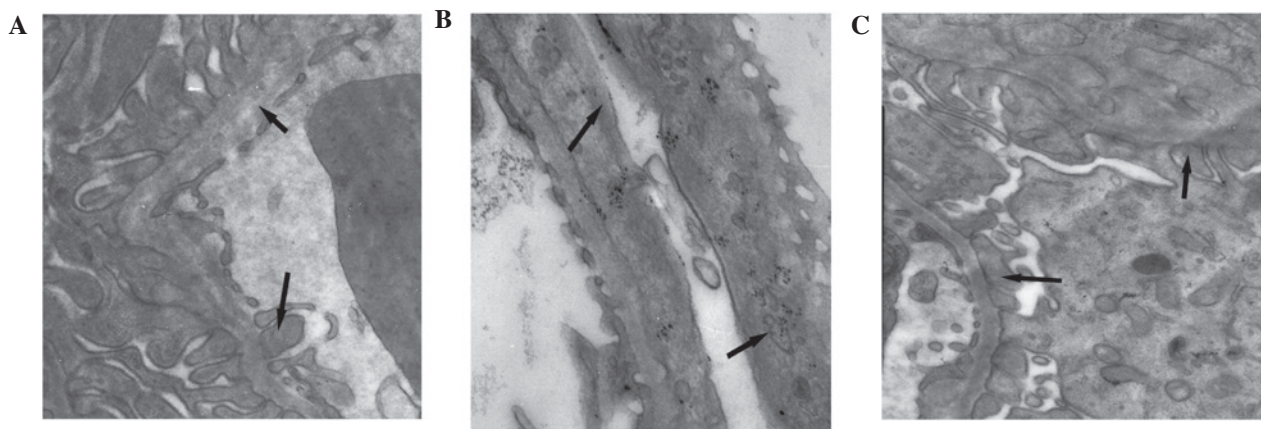


Figure 1. Kidney tissue samples observed under electron microscopy (magnification, x10,000). (A) Normal, (B) model and (C) prednisone groups.

a Shapiro-Wilk test, and tested for variance homogeneity. The results were analyzed using a one-way analysis of variance, and a least significant difference analysis method was used for multiple comparisons.  $P<0.05$  was considered to indicate a statistically significant result.

## Results

**Urinary protein levels.** As compared with the normal group, the 24 h urinary protein levels were significantly increased in the model and prednisone groups ( $P<0.05$ ). As compared with the model group, the urinary protein levels in the prednisone group were significantly decreased at day 21 ( $P<0.05$ ) and further decreased at day 35 ( $P<0.01$ ). As compared with the urinary protein levels at day 21, the urinary protein levels in the model group were significantly increased at day 35, and those of the prednisone group significantly decreased at day 35 ( $P<0.05$ ; Table II).

**Pathomorphology of the tissue samples of each group, as determined by electron microscopy.** The foot processes in the normal group were clearly defined, with no observed fusion or microvilli degeneration. The podocytes of the model group exhibited swelling, and the foot processes were abnormally broadened and exhibited diffused fusion. In the prednisone group, the number of glomerular lesions decreased, and only partial foot process fusion was observed, as compared with the model group (Fig. 1).

**mRNA expression levels of FAK, RANKL, p38, ERK, and JNK.** At day 21, the mRNA expression levels of FAK were significantly higher in the model group, as compared with those of the normal group ( $P<0.01$ ). The mRNA expression levels of RANKL and ERK were significantly higher in the model group, as compared with those of the normal group ( $P<0.05$ ). The mRNA expression levels of JNK were significantly decreased in the prednisone group, as compared with those of the normal group ( $P<0.05$ ). The mRNA expression levels of p38 were significantly lower in the prednisone group, as compared with those of the model group ( $P<0.01$ ), and the mRNA expression levels of RANKL and ERK were significantly lower in the prednisone group, as compared with those of the model group ( $P<0.05$ ). At day 35, the mRNA expression levels of FAK in the model group were significantly higher, compared to the mRNA expression levels on day 21 ( $P<0.05$ ), and significantly higher, as compared with the prednisone group ( $P<0.01$ ). As compared with the normal group, a significant decrease was observed in the mRNA expression levels of p38 in the model and prednisone groups ( $P<0.01$ ). The mRNA expression levels of RANKL in the model group were significantly increased, as compared with the normal ( $P<0.05$ ) and prednisone groups ( $P<0.01$ ). At day 35, the mRNA expression levels of ERK in the prednisone group were significantly decreased ( $P<0.05$ ), as compared with normal group. No statistically significant differences were observed in the mRNA expression levels of FAK, p38, and RANKL at day 21, as compared with day 35 (Table III).

Table III. mRNA expression levels of FAK, RANKL, p38, ERK, JNK, and nephrin.

mRNA	Time	Group (n=5)		
		Normal	Model	Prednisone
FAK	Day 21	1.00±0.00	1.76±0.48 <sup>a</sup>	1.34±0.50
	Day 35	1.00±0.00	2.25±0.08 <sup>a,c</sup>	1.00±0.03 <sup>d</sup>
RANKL	Day 21	1.00±0.00	2.49±0.30 <sup>b</sup>	0.58±0.11 <sup>c</sup>
	Day 35	1.00±0.00	1.56±0.55 <sup>b</sup>	0.63±0.25 <sup>d</sup>
p38	Day 21	1.00±0.00	0.99±0.07	0.68±0.08 <sup>b,d</sup>
	Day 35	1.00±0.00	0.71±0.11 <sup>a</sup>	0.73±0.04 <sup>a</sup>
ERK	Day 21	1.00±0.00	1.17±0.06 <sup>b</sup>	0.91±0.30 <sup>c</sup>
	Day 35	1.00±0.00	1.23±0.01 <sup>b</sup>	0.77±0.05 <sup>c</sup>
JNK	Day 21	1.00±0.00	0.85±0.20	0.71±0.01 <sup>b</sup>
	Day 35	1.00±0.00	0.85±0.21	0.83±0.40
Nephrin	Day 21	1.00±0.00	0.39±0.11 <sup>a</sup>	0.59±0.10 <sup>a,d</sup>
	Day 35	1.00±0.00	0.38±0.08 <sup>a</sup>	0.54±0.01 <sup>a,c</sup>

Results are presented as the mean ± standard deviation. <sup>a</sup>P<0.01 and <sup>b</sup>P<0.05, vs. the normal group; <sup>c</sup>P<0.05, vs. the model group; <sup>d</sup>P<0.01, vs. the model group; <sup>e</sup>P<0.05, vs. day 21. FAK, focal adhesion kinase; RANKL, receptor activator of nuclear factor-κB ligand; p38, p38 mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase.

Table IV. Serum protein expression levels of OPG and RANKL.

Protein	Time	Group (n=10)		
		Normal	Model	Prednisone
OPG	Day 21	1.21±0.43	1.28±0.45	0.71±0.17 <sup>b</sup>
	Day 35	1.33±0.59	1.24±0.49	0.65±0.22 <sup>b</sup>
RANKL	Day 21	19.22±1.02	21.27±2.63	52.65±3.58 <sup>c</sup>
	Day 35	18.74±3.03	21.58±2.18	66.90±5.55 <sup>a,c</sup>

Results are presented as the mean ± standard deviation <sup>a</sup>P<0.05, vs. day 21; <sup>b</sup>P<0.05 and <sup>c</sup>P<0.01, vs. the model group. OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor-κB ligand.

*Serum protein expression levels of OPG and RANKL.* As compared with the normal group, the expression levels of RANKL in the prednisone group were significantly higher at day 21 and day 35 (P<0.01 and P<0.05, respectively). In addition, at day 21 the expression levels of RANKL were significantly higher in the prednisone group, as compared with the model group (P<0.01). At day 35, the expression level of RANKL was significantly higher in the prednisone group, as compared with the model group (P<0.01). Conversely, at day 21 and day 35, the expression levels of OPG were significantly lower in the prednisone group, as compared with the model group (P<0.05; Table IV).

*Expression levels of FAK, p-FAK, p38, RANKL, p-p38, ERK, p-ERK, JNK and p-JNK in the kidney tissue samples of each group.* As compared with the normal group, the protein expression levels of FAK, p38 and ERK, and their phosphorylated counterparts were significantly higher in the model group (P<0.01). The protein expression levels of FAK,

Table V. Protein expression levels of nephrin.

Groups (n=3)	Integral optical density	
	Day 21	Day 35
Normal	135.69±14.83	132.66±6.72
Model	35.21±4.87 <sup>a</sup>	40.57±4.20 <sup>a</sup>
Prednisone	82.81±10.03 <sup>b</sup>	78.90±6.83 <sup>b</sup>

Results are presented as the mean ± standard deviation. <sup>a</sup>P<0.01, vs. the normal group; <sup>b</sup>P<0.01, vs. the model group.

p-FAK, p-ERK and p-p38 were significantly decreased in the prednisone group (P<0.01). As compared with the model group, the expression levels of p-ERK in prednisone group were decreased (P<0.05) at day 21 and significantly decreased at day 35 (P<0.01). As compared with the normal group, at

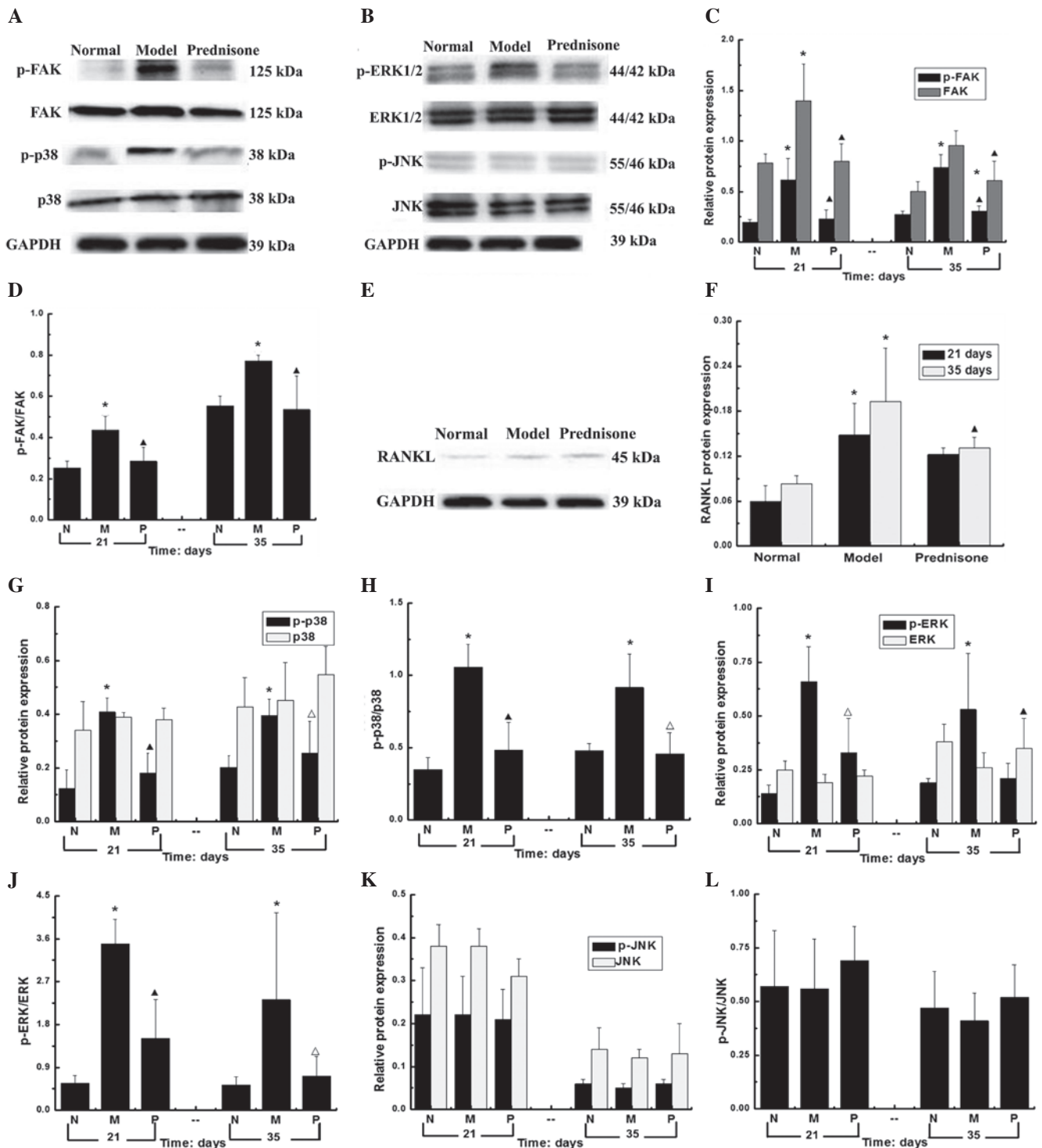


Figure 2. Protein expression levels of FAK, p-FAK, p38, p-p38, ERK, p-ERK, JNK, p-JNK, and RANKL at days 21 and 35. (A) The expression levels of FAK, p-FAK, p38 and p-p38; (B) the expression levels of ERK, p-ERK, JNK and p-JNK; (C) the relative protein expression levels of FAK and p-FAK; (D) The protein expression levels of FAK/p-FAK; (E) The protein expression levels of RANKL; (F) The protein expression levels of RANKL; (G) The relative protein expression levels of p38 and p-p38; (H) The protein expression levels of p-p38/p38; (I) The relative protein expression levels of ERK and p-ERK; (J) The protein expression levels of p-ERK/ERK; (K) The relative protein expression levels of JNK and p-JNK; (L) The protein expression levels of p-JNK/JNK. \* $P < 0.01$ , vs. the normal group; \* $P < 0.01$ , vs. the model group; and  $\Delta P < 0.05$ , vs. the model group. No statistically significant changes were observed in the protein expression levels of JNK and p-JNK. N, normal group; M, model group; P, prednisone group; p, phosphorylated; FAK, focal adhesion kinase; RANKL, receptor activator of nuclear factor- $\kappa$ B ligand; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase.

days 21 and 35, the protein expression levels of RANKL were significantly increased in the prednisone group ( $P < 0.01$ ).

However, no statistically changes in the protein expression levels of JNK and p-JNK were observed (Fig. 2).

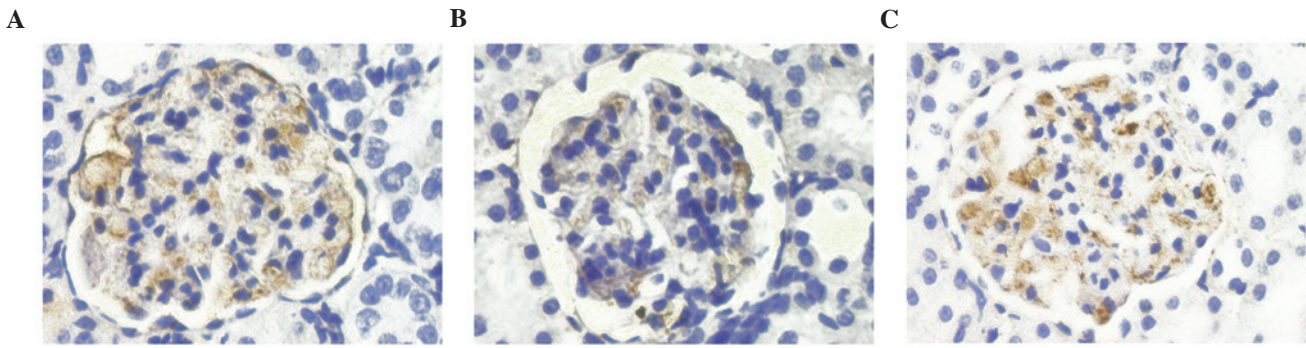


Figure 3. Nephlin protein levels as determined by immunohistochemistry. (A) Normal, (B) model and (C) prednisone group. Magnification, x10,000.

**Protein expression levels of nephrin, as determined by immunohistochemistry.** On day 21, the nephrin proteins of the model group exhibited uneven distribution, as compared with the normal group, suggesting the presence of focal enhancements. In addition, the protein expression levels of nephrin were significantly decreased ( $P < 0.01$ ). On day 35, the protein expression levels of nephrin in the model group were significantly lower, as compared with those of the normal group ( $P < 0.01$ ). As compared with the model group, the protein expression level of nephrin in the prednisone group increased ( $P < 0.01$ ; Table V; Fig. 3).

## Discussion

FAK is a non-receptor tyrosine kinase that localizes to focal adhesions in adherent cells, and binds with the cytoplasmic tails of  $\beta 1$  integrins (11). A previous study demonstrated that phosphorylation of FAK regulates podocyte actin cytoskeletal formation and cell adhesion via the Ras/MAPK and phosphoinositide-3-kinase signaling pathways (12). Podocyte structure is regulated via the MAPK signaling pathway, composed of p38, ERK and JNK (5). Yang *et al* (13) demonstrated that the activation of ERK and p38/MAPK is able to decrease the expression levels of nephrin and podocin in podocytes. Therefore, the present study hypothesized that foot process fusion and proteinuria may be regulated by the FAK/MAPK signaling pathway. Podocyte migration and activity were significantly decreased in the absence of FAK, and glomerular injury occurred following FAK activation. In animal models, the levels of proteinuria was significantly reduced following FAK knockout (14). The present study demonstrated that adriamycin increased both the mRNA and protein expression levels of FAK, p38, ERK and their phosphorylated proteins in kidney tissue samples; increased the mRNA and protein expression levels of RANKL; increased the levels of proteinuria; and decreased the expression levels of nephrin in the model group. In addition, foot process fusion was observed under light microscopy. Following treatment with prednisone, the mRNA and protein expression levels of FAK, p38, ERK and their phosphorylated proteins decreased in the kidney tissue samples; the mRNA and protein expression levels of RANKL decreased; and the expression levels of nephrin increased. In addition, the podocyte lesions were less severe and proteinuria was markedly reduced in the prednisone group, as compared with the model group. Following treatment with prednisone, the expression levels of

RANKL significantly decreased. However, the role of RANKL in kidney tissues remains to be elucidated.

A recent study demonstrated that RANKL and its receptor RANK are cytokines (15). RANK and RANKL are not only involved in lymphocyte development and lymphoid organ formation; they are expressed in embryonic kidneys, myofibroblasts, vascular endothelial cells, and participate in the development of autoimmune diseases (16-19). A previous study reported that RANKL mRNA and protein expression was detected in the renal glomeruli, convoluted tubules, and parenchyma of developing fetal kidneys, whereas RANKL was not detected in adult kidneys (20). The same study reported that RANKL was moderately expressed in renal glomeruli, renal tubules and renal interstitia in rat embryos, and was moderately to highly expressed in neonatal rat renal tubules. However, lower expression levels of RANKL were present in the renal tissue samples of adult rats. Liu *et al* (7) demonstrated that the expression levels of RANKL in the kidneys of rats with puromycin aminonucleoside nephrosis were significantly increased, as compared with the control. In addition, RANKL was able to activate the endoplasmic reticulum  $\text{Ca}^{2+}$ /ATPase, and was important for the response to podocyte injury *in vitro*. RANKL expression reduced intracellular calcium levels, and eventually led to the KCa current suppression, thus reducing podocyte apoptosis. RANKL and RANK activate the transcription factor NF- $\kappa$ B and MAPK signaling pathways (21). In addition, a previous study demonstrated that prednisone inhibited NF- $\kappa$ B activation (22). In the present study the protein expression and phosphorylation levels of FAK, p38, ERK protein were significantly increased in the model group, as well as the mRNA and protein expression levels of RANKL, and the levels of proteinuria. After 21 and 35 days of prednisone treatment, the protein expression and phosphorylation levels of FAK, p38, and ERK were significantly reduced, as well as the protein expression levels of RANKL; the expression levels of nephrin were significantly increased, and the levels of proteinuria were markedly reduced. The protein expression levels of RANK were not detectable using various concentrations of antibodies. The results suggested that RANKL exerts protective effects on podocytes, and is able to reduce proteinuria. Following proteinuria reduction, the expression levels of RANKL decreased. These results suggested that the FAK/RANKL/MAPK and FAK/RANKL/NF- $\kappa$ B signaling pathways may be present in kidney tissues, and prednisone may reduce proteinuria by inhibiting the FAK/RANKL/MAPK and/or the FAK/RANKL/NF- $\kappa$ B signaling pathway.



In the serum of adriamycin-induced nephrotic rats, OPG and RANKL expression was unchanged in the model group, as compared with the normal group. The expression levels of OPG decreased significantly and those of RANKL increased significantly following treatment with prednisone. OPG acts as an osteoblast decoy receptor, whereas RANKL is the primary factor inducing osteoclast (OC) differentiation (8). OPG specifically competes with RANKL, and binds to RANK (the activation receptor of NF- $\kappa$ B) to suppress OC activity (8). Bucay *et al* (23) and Hofbauer *et al* (24) reported that OPG, RANK and RANKL were associated with osteoporosis and the occurrence of vascular calcification. The present study demonstrated that their expression levels of OPG were markedly reduced and those of RANKL were markedly increased in the prednisone group. These results suggested that prednisone may activate the OPG/RANK/RANKL signaling pathway in murine serum, thereby inducing abnormal bone metabolism, and the observed upregulation of RANKL expression may be due to the secretion of bone tissue in the blood.

In conclusion, the results showed that prednisone is able to reduce proteinuria by inhibiting the FAK/RANKL/MAPK and FAK/RANKL/NF- $\kappa$ B signaling pathways in kidney tissue samples, and RANKL has a role in transduction. In our future studies of the RANKL signaling pathway, we aim to observe the pathology of the kidney and proteinuria in the rats following knocking out the RANKL gene. These studies may result in a new direction for the treatment of kidney diseases.

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