EP4 knockdown alleviates glomerulosclerosis through Smad and MAPK pathways in mesangial cells

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Abstract. Prostaglandin E2 has exhibited pleiotropic effects in the regulation of glomerulosclerosis progression through its four receptors. The current study aimed to evaluate the effect of prostaglandin receptor EP4 on mesangial cell proliferation. *In vivo*, 5/6 nephrectomy was introduced into $EP4^{+/-}$ and wild-type (WT) mice. Clinical parameters were monitored post-surgery. At 8 weeks post-surgery, glomerular fibrosis-associated indicators were measured by immunohistochemical staining and trichrome staining. *In vitro*, mesangial cells in different groups (transfected with green fluorescent protein, AD-EF4 or AD-CRE) were exposed to transforming growth factor (TGF)-β1 for 24 h to detect the level of downstream signaling. Corresponding signaling inhibitors were also used to validate the signaling effects. Following surgery, $EP4^{+/-}$ mice presented a higher survival rate and normal urine volume compared with the WT group, and serum creatinine level and 24 h urine protein were lower in the EP4+/- mice. Furthermore, associated profibrotic indicators were identified to have decreased at 8 weeks post-surgery along with less tubule-interstitial fibrosis. *In vivo*, the inhibition of extracellular signal-regulated kinase and P38 phosphorylation alleviated the accumulation of mesangial matrix, and these signals were enhanced when EP4 was overexpressed. EP4 enhancement aggravated imbalanced mesangial cell proliferation stimulated by TGF-β1 and GS of mice treated with 5/6 nephrectomy through the Smad and mitogen-activated protein kinase pathways.

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Introduction

Proliferation of mesangial cells (MCs) and accumulation of extracellular matrix (ECM) are critical to glomerulosclerosis (GS), which serves as an end-stage event in chronic renal failure (1-3). Sclerotic glomeruli are characterized by loss of functional cells and the overexpression of non-functional matrix, commonly composed of type I collagen (COL-1) and fibronectin (FN) (4-6). These factors initially form in the mesangium, and the response may be enhanced by intraglomerular hypertension (7-9) or certain chemical cytokines (10-13). Intraglomerular hypertension, which is attributed to amplified cyclic stretch, causes mechanical damage to MCs (7-9). Certain chemical cytokines, including transforming growth factor (TGF)-β1, cause MCs to undergo a phenotype transformation to resemble fibroblasts, accompanied by the infiltration of inflammatory cells, causing glomerular scarring. This type of scarring eventually results in glomerular sclerosis (14). In patients with glomerulotubular imbalance, when glomerular filtration rate (GFR) declines to 10% of normal values, renal replacement therapy is required, as currently there is no specific treatment to reverse the progression.

Prostaglandin E2 (PGE2) has multiple biological effects in organ fibrosis. In the kidney, through its four receptors (EP1, EP2, EP3 and EP4), there is also a dual regulatory role of PGE2 (15-19). The present study focused on EP4, the role of which still remains controversial, particularly in the regulation of renal diseases, depending on the cell type and context. Our previous study identified that EP4 plays a maladaptive role in MC injury associated with chronic kidney disease (20-22). Consistent with these findings, inflammation and albuminuria was minimized through cyclooxygenase-2 (COX-2)/EP4 suppression in streptozotocin-induced diabetic mice (23,24). In addition, diabetes-induced COL-1 and connective tissue growth factor (CTGF) expression was markedly increased in EP4 agonist-treated mice (24,25). However, Vukicevic *et al* (25), argued that both EP2 and EP4 receptor agonists prevent the progression of chronic renal failure in unilateral ureteral obstruction models. EP2 and EP4, which both bind to G stimulatory (Gs) protein to increase cyclic adenosine 5'-monophosphate (cAMP) levels, are present in the medulla and glomerulus, respectively (26,27). It is reported that EP2 primarily increases the level of cAMP and delivers a

protective effect in the regulation of tissue fibrosis, but EP4 is not thought to have the same effect (28-30). Therefore, it was hypothesized that EP4 may be involved in a different signaling pathway.

TGF-β1, a fibrotic stimulus, is able to activate Smad proteins (Smad2 or Smad3) directly to cause renal fibrosis (31-34). Mitogen-activated protein kinase (MAPK) pathways, which comprise extracellular signal-regulated kinase (ERK), c-JUN N-terminal kinase (JNK) and p38 signaling, have been reported to play a role in inflammatory regulation (35,36), and also contribute to the amplification of transmitted signals to promote cellular processes, including proliferation and differentiation, either in the cytoplasm or nucleus (37,38). Thus, the current study aimed to investigate two areas: Firstly, whether there is a cross-talk effect between Smad3 and MAPKs in glomerulosclerosis; and secondly, whether the phosphorylation of MAPKs may be mediated by EP4 receptors.

In the present study, 5/6 nephrectomy was performed to examine the effect of EP4 on glomerulosclerosis. Primary MCs of different genotypes were also cultured to detect possible signaling pathways. Briefly, the aim of the current study was to investigate whether EP4 is required for mesangium fibrosis.

Materials and methods

Experimental animals. The experimental animals were provided by the Animal Experimentation Committee of Beijing University Health Science Center (Beijing, China). The mice were C57/BL6 type and bred in a specific-pathogen-free environment. The animal protocol in this study was approved by the Beijing University Animal Care and Use Committee. Wild-type (WT), EP4 heterozygotes ($EP4^{+/-}$) and $EP4^{flox/flox}$ (with a conditional knock-out EP4 gene sequence between LoxP sites flanking exon 2 of the EP4 gene) male mice aged 8-12 weeks were used for experiments. $EP4^{flox/flox}$ mice were transfected with Cre adenovirus to knock out EP4 receptors (23). All mice were kept in an air-conditioned environment (20˚C, 50% humidity) with a 12-h light/dark cycle and had free access to food and water prior to and following surgery. The mice were euthanized with 150 mg/kg pentobarbital by intraperitoneal injection.

5/6 nephrectomy. A total of 40 C57/BL6 mice weighing 25-35 g were included in the experiment, and were not limited in eating and drinking the night prior to surgery. The 5/6 nephrectomy was performed according to our previous study (39). The mice were divided into four groups (n=10 per group): 1, WT Control (CON) group; 2, WT 5/6 Nephrectomy (Nx) group; 3, $EP4^{+/-}$ CON group; 4, $EP4^{+/-}$ 5/6 Nx group.

Sample collection. Prior to sacrifice, WT CON, WT 5/6 Nx, $EP4^{+/-}$ CON and $EP4^{+/-}$ 5/6 Nx mice (n=10 in each group) were placed individually in metabolic cages for 24-h urine collection. Urine samples were centrifuged at 10,000 x g for 10 min at room temperature and the supernatants were stored at -20˚C until analysis. Prior to sacrifice, the mice were anaesthetized with 1% pentobarbital (40 mg/kg) via intraperitoneal injection for serum and kidney collection. Blood samples (~0.8 ml) were collected from the postcava in heparinized tubes and centrifuged at 5,000 x g for 15 min to obtain serum for the measurement of serum creatinine, urea nitrogen and urine protein. The kidneys were quickly removed and either frozen immediately in liquid nitrogen or fixed with 4% buffered formalin. Residual tissues and serum were preserved at -80˚C. Finally, animals were sacrificed as aforementioned.

Albumin and creatinine concentrations were determined using Exocell assays, according to the manufacturer's instructions (Exocell, Inc., Philadelphia, PA, USA).

Histology and immunohistochemistry. Kidneys were fixed with 10% neutral buffered formalin and processed for histology or immunostaining using standard techniques. Histological sections (5-μm thick) were prepared and stained with anti-FN (Abcam, Cambridge, MA, USA), anti-COI-I, anti-COX-2, anti-EP4, anti-CTGF (Bio-Rad Laboratories, Inc., Hercules, CA, USA), anti-phosphorylated (p)-Smad3, anti-p-p38 and anti-ERK (Cayman Chemical Company, Ann Arbor, MI, USA) overnight at 4˚C, and incubated at 37˚C for 1 h. Then Secondary antibodies were added. For staining quantification (FN, COI-I, COX-2, and CTGF), Six golmeruli per mouse from each group and 3 mice per group were randomly and blindly selected and analyzed. The percentage of the positively stained area was measured by using ImageJ sofware. The degree of fibrosis was quantified in trichrome sections by assessing the surface area of the cortical area (avoiding great vessels and glomeruli) as a ratio of total surface area at x400 magnification. All measurements and quantification were performed in a random blinded manner using an Olympus BX50 microscope (Olympus Corporation, Tokyo, Japan), a Micropublisher 3.3 RTV camera (Q-Imaging, Surrey, BC, Canada), and NIS Elements Imaging software (Nikon Instruments, Inc., Melville, NY, USA).

Culture of primary MCs. MC culture was performed according to the protocol in our previous study (21). The primary mice MCs at passages 8 to 10 were used, and were cultured at 37˚C in a humidified incubator containing 5% CO_2, with the addition of 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Then, WT cells were divided into the following groups: 1, WT + AD-GFP group; 2) WT + AD-GFP+ TGF-β1 group; 3, WT + AD-EP4 group; 4, WT + AD-EP4 + TGF-β1 group. $EP4^{flox/flox}$ cells were also divided into four groups: 1, $EP4^{flox/flox}$ + AD-GFP group; 2) $EP4^{flox/flox}$ + AD-GFP + TGF-β1 group; 3, $EP4^{flox/flox}$ + AD-CRE group; 4, $EP4^{flox/flox}$ + AD-CRE + TGF-β1 group. Prior to the experiments, the cells were incubated in serum-free medium for 24 h. The optimum dose and reaction time of TGF-β1 (10 ng/ml, 24 h) was based on a previous study (14). Each individual assay was repeated at least three times with different cell preparations.

Adenoviral constructs and infection of cultured mouse MCs. AD-CRE and AD-EP4 were generated by the Shanghai GenePharma Co., Ltd., (Shanghai, China). Linearized recombinant adenoviral plasmid was transfected into AD-293 cells to obtain a primary viral stock, which was amplified and purified. For optimization of infection conditions, differentiated mouse MCs were infected with AD-CRE at a multiplicity of infection (MOI) of 5 or AD-EP4 at an MOI of 10 for 72 h.

Western blot analysis. Cell lysis buffer was added to the wells and the plate was placed on ice for 30 min. Then, cells

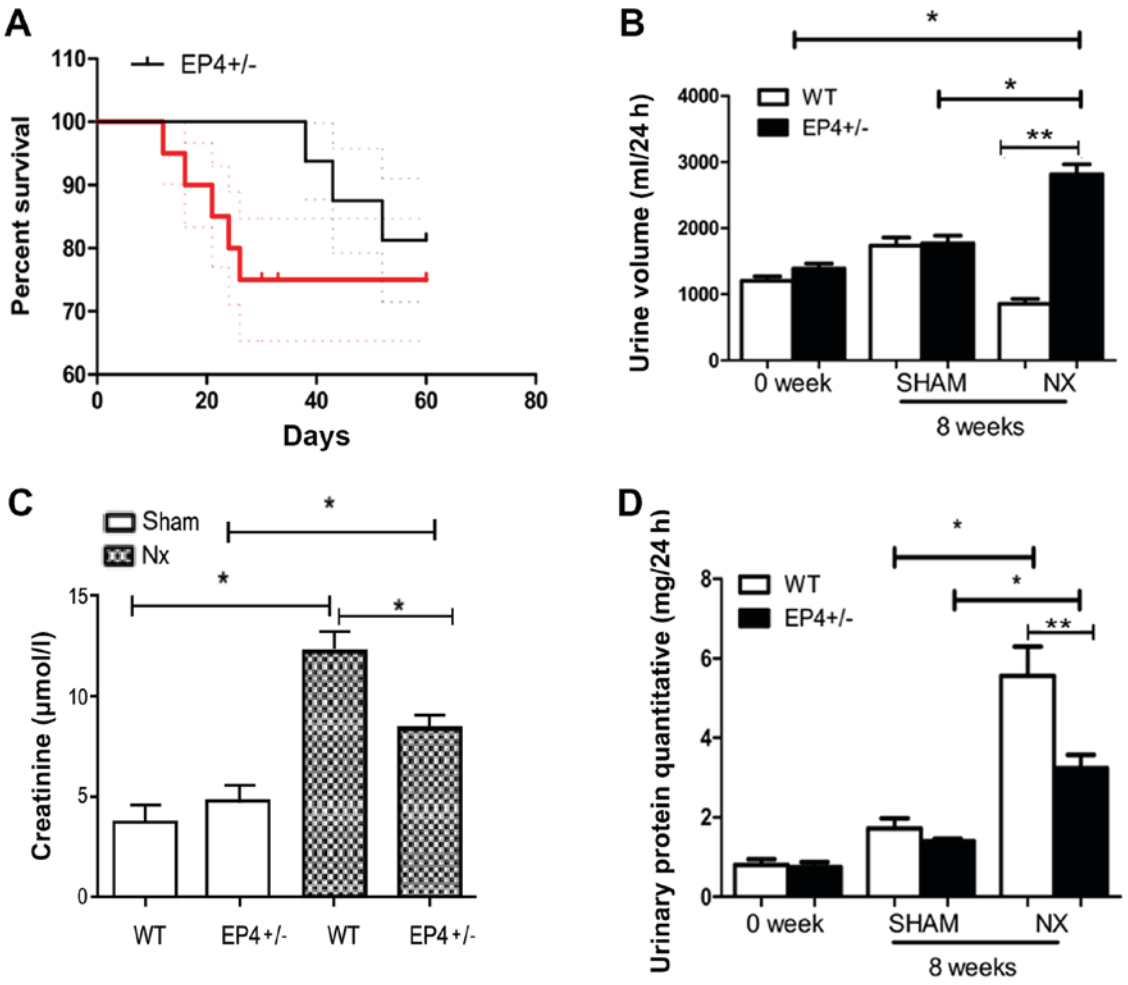

Figure 1. Evaluation of renal indexes in 5/6 Nx mice. (A) At 8 weeks post-surgery, EP4$^{+/-}$ mouse survival rate was significantly higher compared with WT mice. (B) Urine volume of the WT-Nx group decreased significantly compared with the WT-Sham group, but it was increased in EP4$^{+/-}$-NX mice, compared with the EP4$^{+/-}$-Sham group. (C) Serum creatinine of EP4$^{+/-}$ mice over the time course post-surgery was significantly decreased compared with WT mice. (D) 24-h urine protein of 5/6 Nx mice increased compared with the Sham group, but the increase was more notable in WT mice compared with EP4$^{+/-}$ mice. *P<0.05, **P<0.01. Nx, nephrectomy; WT, wild-type.

treated as described above were scraped, and cell lysate was removed to 1.5 ml EP tubes and centrifuged for 15 min (4˚C, 12,000 rpm). Protein concentration in the supernatant was determined by BCA assay (Pierce; Thermo Fisher Scientific, Inc.). Samples were diluted in the loading buffer and boiled for 5 min. Then, 130 mg of each sample was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a PVDF membrane for 2 h. The membrane was blocked at room temperature for 1 h in 5% (w/v) non-fat dry milk. The PVDF membrane was then incubated with primary antibodies (rabbit anti-p-Smad3, rabbit anti-Smad3, rabbit anti-p-p38, rabbit anti-p38, rabbit anti-p-ERK, rabbit anti-ERK, mouse anti-p-JNK and mouse anti-JNK; 1:1,000; Cayman Chemical Company) at 4˚C overnight.

Subsequent to stimulation by TGF-β1 (10 ng/ml) for 24 h, the medium of some dishes was removed and 1 mM PD98059, 10 mM P38ML3404 or 2 mM SP600125 was added for 30 min to block ERK, P38 or JNK signaling, respectively. The control cells were treated with vehicle (DMSO), then the PVDF membrane was incubated with primary antibodies (mouse anti-FN, mouse anti-COI-I, rabbit anti-COX2, mouse anti-CTGF 1:1,000; Rockland Immunochemicals, Inc., Pottstown, PA, USA) at 4˚C overnight. The membrane was washed with Tris-buffered saline with Tween, incubated with DyLight 800-labeled antibody to mouse IgG (1:5,000) or rabbit IgG for 2 h, and the membrane was scanned using the Bio-Rad Imaging System (Bio-Rad Laboratories, Inc.) for semi-quantitative analysis. EIF5 was used as a loading control.

Statistical analysis. All data are expressed as the mean ± standard error of the mean. Data were analyzed by Student's t-test (paired groups) or one-way analysis of variance, followed by Bonferroni's post-hoc test for multiple comparisons. Statistics were performed using GraphPad Pro (GraphPad, San Diego, CA, USA) P<0.05 was considered to indicate a statistically significant difference.

Results

EP4 knockout confers protection in response to 5/6 nephrectomy. To assess the protective effect of EP4 knockout in response to 5/6 nephrectomy, the survival rate of EP4$^{+/-}$ and WT mice was compared (Fig. 1). There was a significant

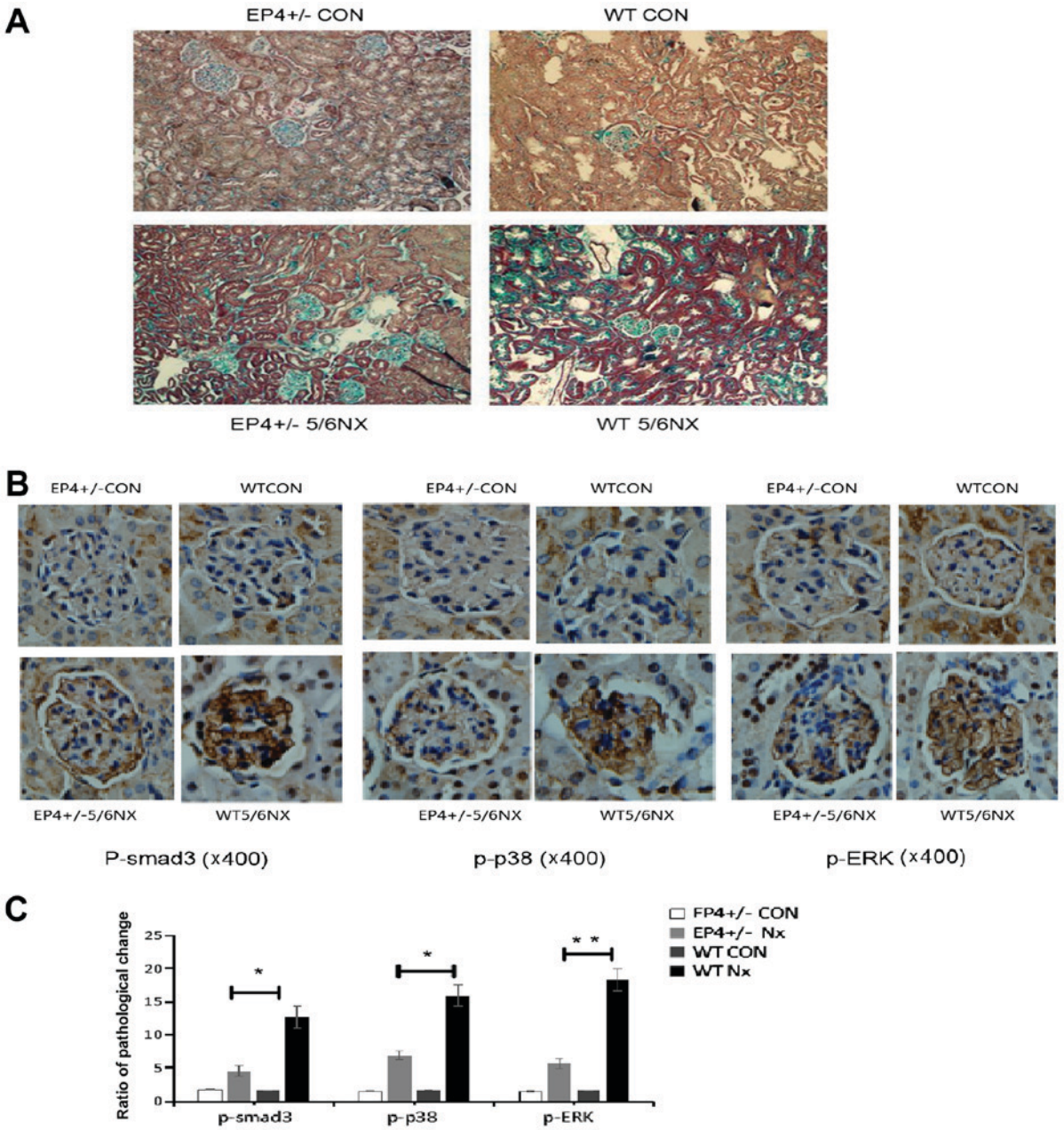

Figure 2. Renal fibrosis in mice following 5/6 nephrectomy. (A) Masson staining was used to visualize collagen deposition. Original magnification, x200. Immunohistochemistry was used to visualize the expression of cytokines in the signaling pathways at 8 weeks post-surgery. (B and C) The expression of p-Smad3, p-ERK and p-p38 was increased in 5/6 nephrectomy mice. Original magnification, x400. *P<0.05, **P<0.01.

difference in the survival rate between EP4$^{+/-}$ and WT mice (Fig. 1A). The WT group was severely polyuric, excreting almost three-fold the volume excreted by the litter-matched EP4$^{+/-}$ mice at 8 weeks post-surgery (Fig. 1B). Serum creatinine levels were lower in EP4$^{+/-}$ mice compared with WT mice at 8 weeks post-surgery (Fig. 1C). Furthermore, mice in both groups exhibited massive proteinuria at 8 weeks post-surgery. Compared with EP4$^{+/-}$ mice, WT mice presented two-fold proteinuria (Fig. 1D).

EP4 knockout reduces GS following 5/6 nephrectomy. To determine whether EP4 knockout reduces GS induced by 5/6 nephrectomy, Masson trichrome staining was used to detect the level of GS. GS following nephrectomy was obviously evident in the WT group at 8 weeks post-surgery, compared with a less obvious change in the EP4$^{+/-}$ group (Fig. 2A). The expression of p-Smad3, p-ERK and p-p38 was markedly increased in the 5/6 nephrectomy group compared with the EP4+/- group (Fig. 2B and C). A decrease in COX-2, FN, COI-I and CTGF was detected in EP4+/- mice compared with WT mice. These indicators were markedly increased at 8 weeks subsequent to 5/6 nephrectomy, particularly in the WT group (Fig. 3A and B).

Expression of EP4 protein infected with AD-EP4 in WT mouse MCs and with AD-CRE in EP4$^{Flox/Flox}$ mouse MCs. To confirm that AD-EP4 had been successfully transfected in WT mouse MCs, and AD-CRE had been successfully transfected in EP4$^{Flox/Flox}$ mouse MCs (LoxP sequences were introduced at both ends of the EP4 gene, allowing CRE recombinant enzyme

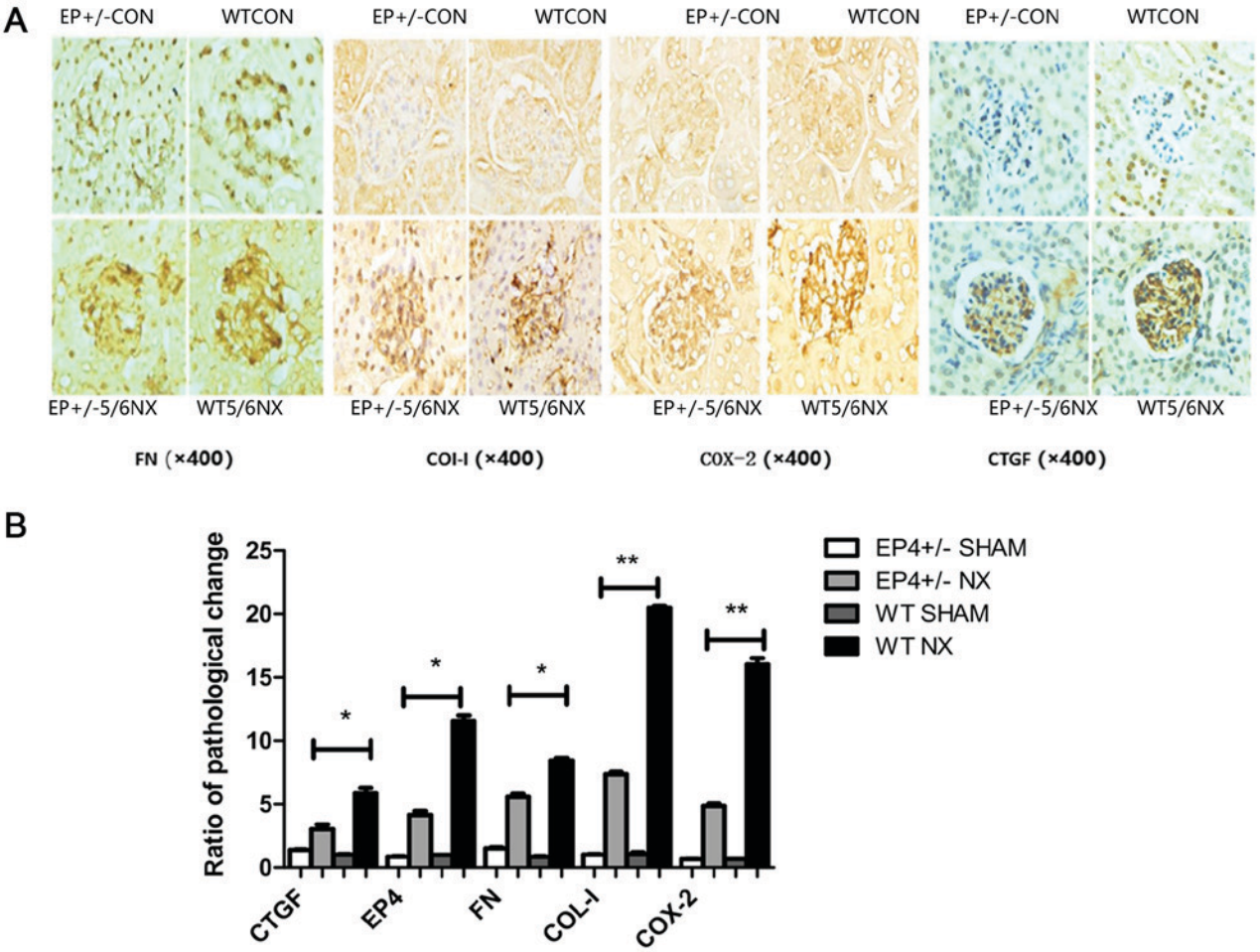

Figure 3. Expression of fibrogenic cytokines in mice following 5/6 nephrectomy. (A) Immunohistochemistry was used to visualize the expression of fibrogenic cytokines at 8 weeks post-surgery. (B) The expression of FN, COI-I, COX-2 and CTGF was increased in 5/6 nephrectomy mice. Original magnification, x400. *P<0.05, **P<0.01.

to remove the EP4 gene), western blotting was performed to evaluate EP4 protein expression. Expression of EP4 protein is increased following infection with AD-EP4 in WT mouse MCs, compared with the AD-GFP group. It is opposite following infection with AD-CRE in EP4Flox/Flox mouse MCs (Fig. 4A and B).

Expression of EP4 protein is increased following infection with AD-EP4 in WT mouse MCs induced by TGF-β1. Following infection with AD-EP4 (MOI=10) in WT mice MCs treated with 10 ng/ml TGF-β1 for 12 h, the expression of EP4 protein markedly increased compared with the AD-GFP group (control group, gene recombinant adenovirus with green fluorescent protein; Fig. 5A and B).

Expression of EP4 protein is decreased following infection with AD-CRE in EP4$^{Flox/Flox}$ mouse MCs induced by TGF-β1. AD-CRE had been transfected in EP4$^{Flox/Flox}$ mouse MCs treated with 10 ng/ml TGF-β1 for 12 h western blotting was performed to evaluate EP4 protein expression. Following infection with AD-CRE (MOI=10), the expression of EP4 protein decreased markedly compared with the AD-GFP groups (control group, gene recombinant adenovirus with green fluorescent protein; Fig. 5C and D).

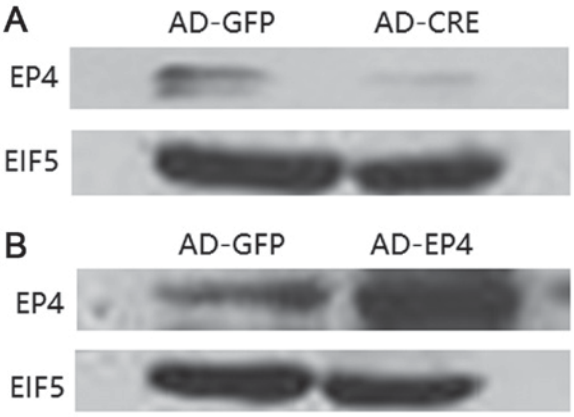

Figure 4. Following infection with AD-EP4 and AD-CRE, western blotting was used to evaluate the of EP4 protein. (A) There was almost no EP4 expression in MCs infected with AD-CRE at an MOI of 5 for 72 h. Lane 1 is AD-GFP group, lane 2 is AD-CRE group. (B) The expression of EP4 in MCs infected with AD-EP4 at an MOI of 10 for 72 h increased markedly. Lane 1 is AD-GFP groups, lane 2 is AD-EP4 groups. EIF5 was used as a loading control.

EP4 knockout reduces Smad3, ERK and P38 phosphorylation in MCs induced by TGF-β1. Smad signaling is generally

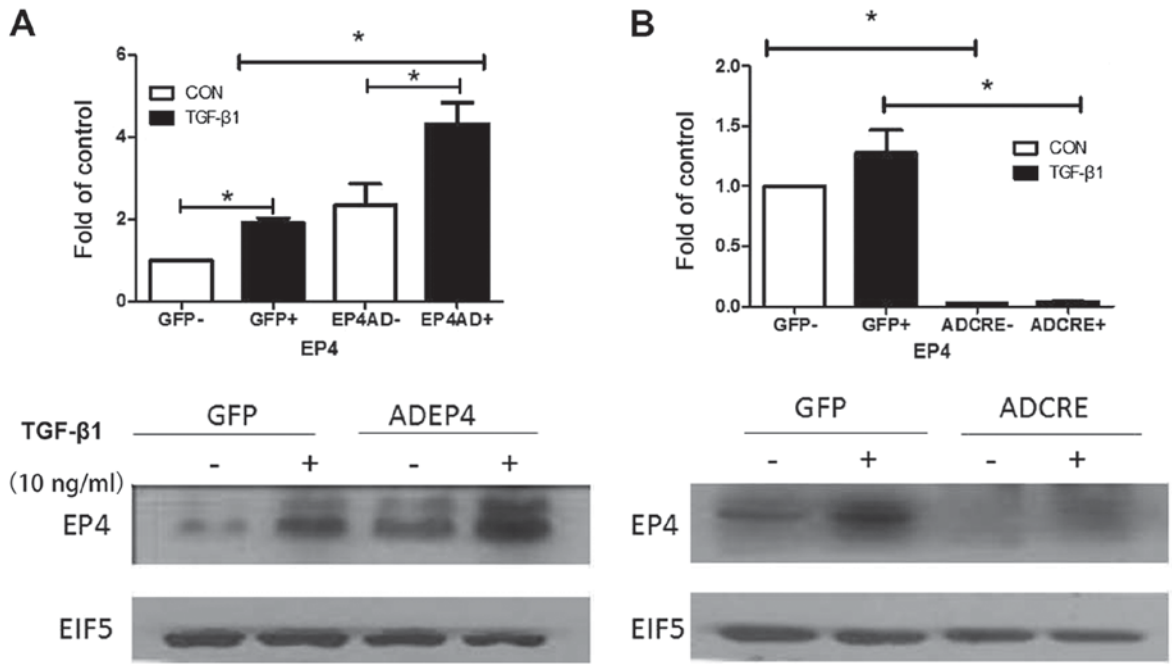

Figure 5. Following treatment with 10 ng/ml TGF-β1 for 12 h, western blotting was used to evaluate the expression of EP4 protein. (A) The expression of EP4 in MCs infected with AD-EP4 at an MOI of 10 for 72 h increased markedly. Lane 1 and 2 are AD-GFP groups, lane 3 and 4 are AD-EP4 groups. (B) There was almost no EP4 expression in MCs infected with AD-CRE at an MOI of 5 for 72 h. Lane 1 and 2 are AD-GFP groups, lane 3 and 4 are AD-CRE groups. *P<0.05, **P<0.01. EIF5 was used as a loading control. MCs, mesangial cells.

considered to be the classical mechanism for fibrosis in physiological change induced by TGF-β1 (32). To confirm the fibrosis effect in MCs induced by TGF-β1, the level of Smad3 was detected and it was identified that TGF-β1 effectively enhanced the phosphorylation level of Smad3 in both MC genotypes. Compared with GFP-treated WT cells, AD-EF4 transfection increased the level of phosphorylation, while AD-CRE administered in $EP4^{flox/flox}$ MCs downregulated the level of p-Smad3 (Fig. 6A and B).

Furthermore, previous research has indicated that there is cross-talk between Smad and MAPK signaling (32). In addition, although both EP2 and EP4 couple with Gs protein to upregulate the level of cAMP, it is EP2 that primarily increases cAMP level (25). Therefore, the current study aimed to reveal the possible mechanism of the maladaptive role of EP4 in MC injury. It was identified that there was a marked increase in phosphorylation of ERK and P38 when MCs were stimulated by TGF-β1, while JNK signaling did not exhibit phosphorylation. The phosphorylation of ERK and P38 was markedly enhanced with the introduction of AD-EP4, and markedly reduced with the introduction of AD-CRE, compared with the respective GFP-treated cells. However, there was no significant change in the level of p-JNK (Fig. 6C and D).

To confirm the critical role of MAPK signaling in the regulation of MC injury, three corresponding signaling inhibitors against ERK (PD98059), P38 (ML3404) and JNK (SP600125) were used to block their effects. The expression of COI-I and COX-2 was inhibited ~2-fold by PD98059 in comparison with the control group. The level of CTGF was primarily reduced by ML3404. FN expression was reduced upon administration of PD98059 and ML3404.SP600125 did not exhibit any effects (Fig. 7).

Discussion

PGE2 is widely secreted and plays a significant role in pathological processes. Our previous research indicated that conditional deletion of EP4 from MCs conferred partial protection from glomerular damage, and such protection against mesangial ECM accumulation primarily involves the COX2-PGE2-EP4 system (22). However, these findings contrast with those of Vukicevic *et al* (25). The current findings strongly indicate that EP4 plays a role in the injury of MCs and development of glomerular sclerosis. Therefore, the EP4 receptor may exert pleiotropic effects on kidney injury, depending on the specific tissue or cell type. In the present study, subtotal-nephrectomy (5/6) was also performed to further confirm the effect of EP4. This model showed that mesangial cell hyperplasia appeared from the second week, and this becomed more serious after 8 week. We analyzed the potential signaling pathway of such damage.

As $EP4^{-/-}$ mice inevitably suffer from neonatal patent ductus arteriosus (19), heterozygotic mice were used to establish the model in the current study. Partial EP4 silencing was efficient in the current research, as the level of cAMP decreased significantly compared with WT mice (27). The knockdown of EP4 resulted in higher survival rate and suitable regulation of urine osmotic pressure, suggesting the receptor's possible maladaptive effect in the balance between glomerular infiltration and tubular re-absorption. This imbalance also led to secondary hypertension, as indicated by renal artery stenosis (RAS), and it interacted with mesangium expansion in the progression of glomerular sclerosis. It has been reported that the stimulation of EP4 receptors could exacerbate glomeruli sclerosis associated with enhanced glomerular capillary pressure (40). Hartono demonstrated that db/db mice with RAS develop

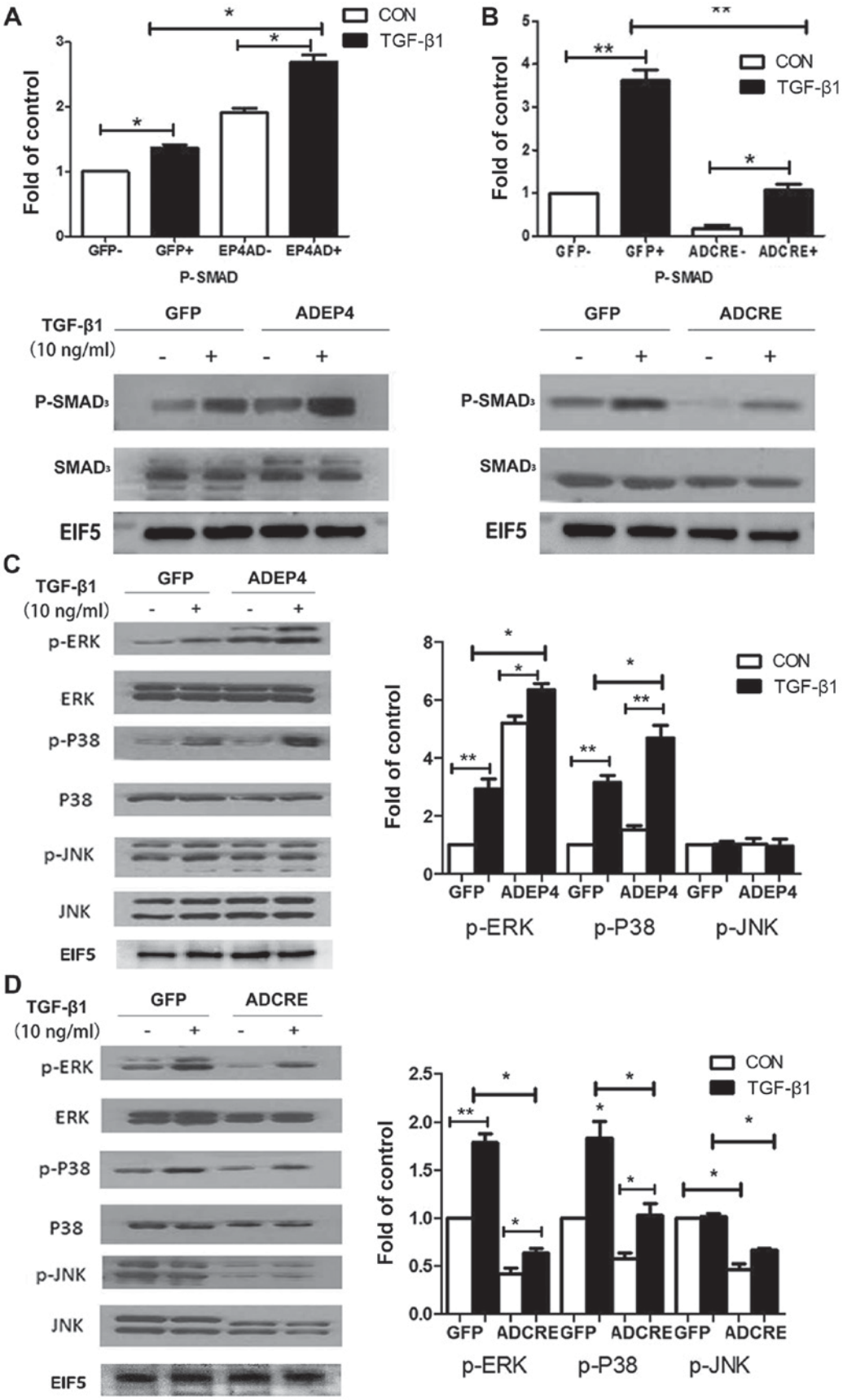

Figure 6. Following treatment with 10 ng/ml TGF-β1 for 5 min, western blotting was used to evaluate the expression of p-Smad3 protein. (A and B) The expression of p-Smad3 in the AD-EP4 group increased markedly, and it was decreased in the AD-CRE group. (C) Following treatment with 10 ng/ml TGF-β1 for 5 min, western blotting was used to evaluate the expression of p-ERK, p-P38 and JNK protein in MCs infected with AD-EP4 and AD-GFP. The expression of p-ERK and p-P38 increased compared with the control group. This trend was more evident in the AD-EP4 group. There was no obvious change in the expression of JNK. (D) Following treatment with 10 ng/ml TGF-β1 for 5 min, western blotting was used to evaluate the expression of p-ERK, p-P38 and JNK protein in MCs infected with AD-GFP and AD-CRE. The expression of p-ERK and p-P38 decreased, compared with the control group. This trend was more evident in the AD-CRE group. There was no obvious change in the expression of JNK. $^{*}P<0.05$, $^{**}P<0.01$. EIF5 was used as a loading control. MCs, mesangial cells.

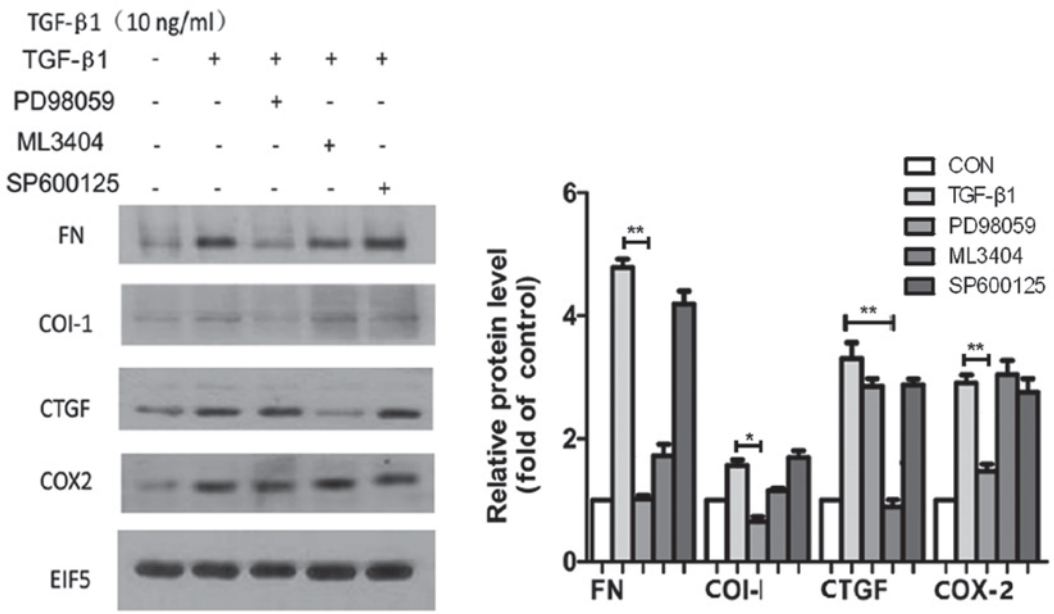

Figure 7. Following treatment with 10 ng/ml TGF-β1 for 24 h, the expression of FN, COI-I, COX-2 and CTGF protein increased in MCs of WT mice, compared with the control group. Inhibitors of ERK, p38 MAPK and JNK (PD98059, ML3404 and SP600125, respectively) were added to MCs for 30 min prior to treatment with 10 ng/ml TGF-β1 for 24 h. The expression of FN and COI-I decreased following treatment with PD98059, and the expression of CTGF decreased following treatment with ML3404. However, no obvious differences were observed following treatment with SP600125. *P<0.05, **P<0.01. MCs, mesangial cells; WT, wild type.

diffuse mesangial sclerosis (41). These reports are consistent with the current findings.

Recent studies have reported that Gq-dependent signaling induces COX-2 expression in podocytes, and its upregulation is associated with PGE2 synthesis (42). Our previous study confirmed the synergistic effect of COX-2 in MCs injury induced by TGF-β1 (20).

In the present study, the level of COX-2 in intravital glomeruli was measured, and pathological changes were evaluated to indicate the maladaptive role of COX-2 in 5/6 nephrectomy. The results indicated that glomerular COX-2 induction coupled with enhanced PGE2 synthesis required the activation of EP4. Although it has been indicated that the administration of EP4 agonist intravenously may assist in preserving renal blood flow (18), to the best of our knowledge, the expression of EP4 in resident glomeruli has not been studied in detail previously. In the current study, EP4 level was highest in WT 5/6 nephrectomy mice, which indicated a specific role of EP4 in PGE2/COX-2-associated injury.

Smads mediate the signal transduction from TGF-β1. Smad3 is well known as a key regulator of fibrosis in many organ systems, and this is supported by the findings that mice lacking Smad3 are protected against fibrosis in multiple disease models (32-34). MAPK signaling is able to regulate fibrosis either independently or through cross-talk with Smad pathways (36-38). It has been reported that both TGF-β1/Smad and ERK/MAPK signaling act as pro-fibrotic pathways accelerated by blood glucose fluctuation (43-45). In the present study, the regulatory effect of MAPKs in MC injury induced by TGF-β1 was evaluated. The effect was also validated via the introduction of MAPK pathway inhibitors. The results indicated that ECM accumulation was primarily mediated by Smad3, ERK/MAPK and P38 signaling. Subsequently, to confirm the role of EP4 in MC damage, AD-CRE and AD-EP4 were introduced. These two compounds have been well documented in our previous research (20-22). The level of phosphorylated Smad3, ERK and P38, but not JNK, were obviously enhanced and reduced in MCs transfected with AD-EP4 and AD-CRE, respectively.

In conclusion, EP4 enhancement could accelerate MC damage induced by TGF-β1 with increasing ECM protein synthesis. ERK, P38 and TGF-β1/Smad3 signaling appear to play a key role in the injury. If the two pathways interact with each other, it is our next work.The maladaptive effect of EP4 is also manifested *in vivo*, with glomerulo-tubular imbalance, higher reno-hypertension and glomerular sclerosis. The current findings on EP4 identify a novel potential target for treatment in order to delay the progression of glomerulopathy.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

YJC and TYP performed the experiments, participated in collecting data and drafted the manuscript. NFG and BCW performed statistical analysis and participated in study design. XLC designed the experiments. JHW performed the animal experiments. XLC and JHW helped to draft the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The animal protocol in this study was approved by the Beijing University Animal Care and Use Committee (Beijing, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Hu C, Sun L, Xiao L, Han Y, Fu X, Xiong X, Xu X, Liu Y, Yang S, Liu F and Kanwar YS: Insights into the mechanisms involved in the expression and regulation of extracellular matrix proteins in diabetic nephropathy. Curr Med Chem 22: 2858-2870, 2015.
2. Guo F, Wang Q, Zhou Y, Wu L, Ma X, Liu F, Huang F and Qin G: Lentiviral vector-mediated FoxO1 overexpression inhibits extracellular matrix protein secretion under high glucose conditions in mesangial cells. J Cell Biochem 117: 74-83, 2016.
3. Peng FF, Xiao ZL, Chen HM, Chen Y, Zhou J, Yu H and Zhang BF: Parathyroid hormone inhibits TGF-β/Smad signaling and extracellular matrix proteins upregulation in rat mesangial cells. Biochem Biophys Res Commun 478: 1093-1098, 2016.
4. Chen FQ, Wang QY, Wei GZ, Ma XY, Ma DW, Deng WW and Sun WB: Effects of mycophenolate mofetil on the expression of monocyte chemoattractant protein-1 and fibronectin in high glucose cultured human mesangial cells. Genet Mol Res 13: 3154-3161, 2014.
5. Huang P, Zhang Y, Jiang T and Zhang N: Effects of p38 MAPK signaling pathway and aldose reductase on transforming growth factor-β1 induced expression of fibronectin in cultured human mesangial cells. Zhonghua Bing Li Xue Za Zhi 44: 778-782, 2015 (Iin Chinese).
6. Zhu L, Qi XY, Aoudjit L, Mouawad F, Baldwin C, Nattel S and Takano T: Nuclear factor of activated T cells mediates RhoA-induced fibronectin upregulation in glomerular podocytes. Am J Physiol Renal Physiol 304: F849-F862, 2013.
7. Ohara N, Hanyu O, Hirayama S, Nakagawa O, Aizawa Y, Ito S and Sone H: Hypertension increases urinary excretion of immunoglobulin G, ceruloplasmin and transferrin in normoalbuminuric patients with type 2 diabetes mellitus. J Hypertens 32: 432-438, 2014.
8. Zhang Y, Thai K, Kepecs DM and Gilbert RE: Sodium-Glucose linked cotransporter-2 inhibition does not attenuate disease progression in the rat remnant kidney model of chronic kidney disease. PLoS One 11: e0144640, 2016.
9. Guan T, Gao B, Chen G, Chen X, Janssen M, Uttarwar L, Ingram AJ and Krepinsky JC: Colchicine attenuates renal injury in a model of hypertensive chronic kidney disease. Am J Physiol Renal Physiol 305: F1466-F1476, 2013.
10. Har R, Scholey JW, Daneman D, Mahmud FH, Dekker R, Lai V, Elia Y, Fritzler ML, Sochett EB, Reich HN and Cherney DZ: The effect of renal hyperfiltration on urinary inflammatory cytokines/chemokines in patients with uncomplicated type 1 diabetes mellitus. Diabetologia 56: 1166-1173, 2013.
11. Izzedine H, Escudier B, Lhomme C, Pautier P, Rouvier P, Gueutin V, Baumelou A, Derosa L, Bahleda R, Hollebecque A, *et al*: Kidney diseases associated with anti-vascular endothelial growth factor (VEGF): An 8-year observational study at a single center. Medicine (Baltimore) 93: 333-339, 2014.
12. Batal I, De Serres SA, Mfarrej BG, Grafals M, Pinkus GS, Kalra A, Weins A, Bijol V, Rennke HG, Guleria I, *et al*: Glomerular inflammation correlates with endothelial injury and with IL-6 and IL-1β secretion in the peripheral blood. Transplantation 97: 1034-1042, 2014.
13. Zelveian PA and Dgerian LG: The main pathophysiological mechanisms of kidney injury in obstructive sleep apnea syndrome. Ter Arkh 86: 100-105, 2014 (In Russian).
14. Zhang S, Zhang M, Huang H, Zhou S, Du Y, Yi X and Luo J: High glucose-induced Matrilin-2 expression in mouse mesangial cells was mediated by transforming growth factor beta 1 (TGF-β1). Biochem Biophys Res Commun 474: 303-308, 2016.
15. Akaba T, Komiya K, Suzaki I, Kozaki Y, Tamaoki J and Rubin BK: Activating prostaglandin E2 receptor subtype EP4 increases secreted mucin from airway goblet cells. Pulm Pharmacol Ther 48: 117-123, 2018.
16. Jin Y, Smith C, Hu L, Coutant DE, Whitehurst K, Phipps K, McNearney TA, Yang X, Ackermann B, Pottanat T and Landschulz W: LY3127760, a selective prostaglandin E4 (EP4) receptor antagonist and celecoxib: A comparison of pharmacological profiles. Clin Transl Sci 11: 46-53, 2018.
17. Fujioka H, Funabashi T and Akema T: Prostaglandin E2 modulates presynaptic regulation of GnRH neurons via EP4 receptors in accordance with estrogen milieu. Neuroscience 360: 139-145, 2017.
18. Thieme K, Majumder S, Brijmohan AS, Batchu SN, Bowskill BB, Alghamdi TA, Advani SL, Kabir MG, Liu Y and Advani A: EP4 inhibition attenuates the development of diabetic and non-diabetic experimental kidney disease. Sci Rep 7: 3442, 2017.
19. Hong YA, Yang KJ, Jung SY, Park KC, Choi H, Oh JM, Lee SJ, Chang YK, Park CW, Yang CW, *et al*: Paricalcitol pretreatment attenuates renal ischemia-reperfusion injury via prostaglandin E2 receptor EP4 pathway. Oxid Med Cell Longev 2017: 5031926, 2017.
20. Xi PP, Xu YY, Chen XL, Fan YP and Wu JH: Role of the prostaglandin E2 receptor agonists in TGF-β1-induced mesangial cell damage. Biosci Rep 36: pii: e00383, 2016.
21. Chen X, Jiang D, Wang J, Chen X, Xu X, Xi P, Fan Y, Zhang X and Guan Y: Prostaglandin E2 EP1 receptor enhances TGF-β1-induced mesangial cell injury. Int J Mol Med 35: 285-293, 2015.
22. Yang GX, Xu YY, Fan YP, Wang J, Chen XL, Zhang YD and Wu JH: A maladaptive role for EP4 receptors in mouse mesangial cells. PLoS One 9: e104091, 2014.
23. Mohamed R, Jayakumar C, Ranganathan PV, Ganapathy V and Ramesh G: Kidney proximal tubular epithelial-specific overexpression of netrin-1 suppresses inflammation and albuminuria through suppression of COX-2-mediated PGE2 production in streptozotocin-induced diabetic mice. Am J Pathol 181: 1991-2002, 2012.
24. Mohamed R, Jayakumar C and Ramesh G: Chronic administration of EP4-selective agonist exacerbates albuminuria and fibrosis of the kidney in streptozotocin-induced diabetic mice through IL-6. Lab Invest 93: 933-945, 2013.
25. Vukicevic S, Simic P, Borovecki F, Grgurevic L, Rogic D, Orlic I, Grasser WA, Thompson DD and Paralkar VM: Role of EP2 and EP4 receptor-selective agonists of prostaglandin E(2) in acute and chronic kidney failure. Kidney Int 70: 1099-1106, 2006.
26. Olesen ET, Moeller HB, Assentoft M, MacAulay N and Fenton RA: The vasopressin type 2 receptor and prostaglandin receptors EP2 and EP4 can increase aquaporin-2 plasma membrane targeting through a cAMP-independent pathway. Am J Physiol Renal Physiol 311: F935-F944, 2016.
27. Chang HH, Young SH, Sinnett-Smith J, Chou CE, Moro A, Hertzer KM, Hines OJ, Rozengurt E and Eibl G: Prostaglandin E2 activates the mTORC1 pathway through an EP4/cAMP/PKA- and EP1/Ca2+-mediated mechanism in the human pancreatic carcinoma cell line PANC-1. Am J Physiol Cell Physiol 309: C639-C649, 2015.
28. Rumzhum NN and Ammit AJ: Prostaglandin E2 induces expression of MAPK phosphatase 1 (MKP-1) in airway smooth muscle cells. Eur J Pharmacol 782: 1-5, 2016.
29. Liu C, Zhu P, Wang W, Li W, Shu Q, Chen ZJ, Myatt L and Sun K: Inhibition of lysyl oxidase by prostaglandin E2 via EP2/EP4 receptors in human amnion fibroblasts: Implications for parturition. Mol Cell Endocrinol 424: 118-127, 2016.
30. Shishikura K, Horiuchi T, Sakata N, Trinh DA, Shirakawa R, Kimura T, Asada Y and Horiuchi H: Prostaglandin E2 inhibits neutrophil extracellular trap formation through production of cyclic AMP. Br J Pharmacol 173: 319-331, 2016.

31. Wang HW, Shi L, Xu YP, Qin XY and Wang QZ: Oxymatrine inhibits renal fibrosis of obstructive nephropathy by downregulating the TGF-β1-Smad3 pathway. Ren Fail 38: 945-951, 2016.
32. Hong F, Wu N, Ge Y, Zhou Y, Shen T, Qiang Q, Zhang Q, Chen M, Wang Y, Wang L and Hong J: Nanosized titanium dioxide resulted in the activation of TGF-β/Smads/p38MAPK pathway in renal inflammation and fibration of mice. J Biomed Mater Res A 104: 1452-1461, 2016.
33. Wu W, Huang YR, Wan YG, Yang HM, Mao ZM, Yang JJ, Shi G and Sun W: Effects and mechanisms of UCG ameliorating renal interstitial fibrosis by regulating TGF-β1/SnoN/Smads signaling pathway in renal failure rats. Zhongguo Zhong Yao Za Zhi 41: 2291-2297, 2016 (In Chinese).
34. Loboda A, Sobczak M Jozkowicz A and Dulak J: TGF-β1/Smads and miR-21 in renal fibrosis and inflammation. Mediators Inflamm 2016: 8319283, 2016.
35. Liu J, Wang B, Huang P, Wang H, Xu K, Wang X, Xu L and Guo Z: Microcystin-LR promotes cell proliferation in the mice liver by activating Akt and p38/ERK/JNK cascades. Chemosphere 163: 14-21, 2016.
36. Sun Y, Zhang L, Zhang M, Li R, Li Y, Hu X, Wang S and Bao Z: Characterization of three mitogen-activated protein kinases (MAPK) genes reveals involvement of ERK and JNK, not p38 in defense against bacterial infection in Yesso scallop Patinopecten yessoensis. Fish Shellfish Immunol 54: 507-515, 2016.
37. Costa AP, Lopes MW, Rieger DK, Barbosa SG, Goncalves FM, Xikota JC, Walz R and Leal RB: Differential activation of mitogen-activated protein kinases, ERK 1/2, p38(MAPK) and JNK p54/p46 during postnatal development of rat hippocampus. Neurochem Res 41: 1160-1169, 2016.
38. Munoz L, Yeung YT and Grewal T: Oncogenic Ras modulates p38 MAPK-mediated inflammatory cytokine production in glioblastoma cells. Cancer Biol Ther 17: 355-363, 2016.
39. Li X, Xue C, Wang L, Tang D, Huang J, Zhao Y, Chen Y, Zhao D, Shi Q, Wang Y and Shu B: Osteoprotective effects of osthole in a mouse model of 5/6 nephrectomy through inhibiting osteoclast formation. Mol Med Rep 14: 3769-3776, 2016.
40. Stitt-Cavanagh EM, Faour WH, Takami K, Carter A, Vanderhyden B, Guan Y, Schneider A, Breyer MD and Kennedy CR: A maladaptive role for EP4 receptors in podocytes. J Am Soc Nephrol 21: 1678-1690, 2010.
41. Hartono SP, Knudsen BE, Lerman LO, Textor SC and Grande JP: Combined effect of hyperfiltration and renin angiotensin system activation on development of chronic kidney disease in diabetic db/db mice. BMC Nephrol 15: 58, 2014.
42. Wang L, Fields TA, Pazmino K, Dai Q, Burchette JL, Howell DN, Coffman TM and Spurney RF: Activation of Galpha q-coupled signaling pathways in glomerular podocytes promotes renal injury. J Am Soc Nephrol 16: 3611-3622, 2005.
43. Xiao K, Cao S, Jiao L, Song Z, Lu J and Hu C: TGF-β1 protects intestinal integrity and influences Smads and MAPK signal pathways in IPEC-J2 after TNF-α challenge. Innate Immun 23: 276-284, 2017.
44. Li J, Zhao Z, Liu J, Huang N, Long D, Wang J, Li X and Liu Y: MEK/ERK and p38 MAPK regulate chondrogenesis of rat bone marrow mesenchymal stem cells through delicate interaction with TGF-beta1/Smads pathway. Cell Prolif 43: 333-343, 2010.
45. Guo B, Inoki K, Isono M, Mori H, Kanasaki K, Sugimoto T, Akiba S, Sato T, Yang B, Kikkawa R, *et al*: MAPK/AP-1-dependent regulation of PAI-1 gene expression by TGF-beta in rat mesangial cells. Kidney Int 68: 972-984, 2005.