

Prostaglandin E₂ EP1 receptor enhances TGF- β 1-induced mesangial cell injury

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Received April 20, 2014; Accepted October 15, 2014

DOI: 10.3892/ijmm.2014.1979

Abstract. Increasing evidence indicates that transforming growth factor- β 1 (TGF- β 1) is a pivotal mediator in the pathogenesis of renal fibrosis. Mesangial cells (MCs) are important for glomerular function under both physiological and pathological conditions. Studies have found that the expression level of prostaglandin E₂ (PGE₂) in MCs increases under high glucose conditions, that PGE₂ affects the proliferation and hypertrophy of MCs mainly through the EP1 pathway, and that the proliferation of MCs and the accumulation of extracellular matrix are the main events leading to glomerular fibrosis. In this study, we investigated the effects and mechanisms of action of the EP1 receptor, which is induced by transforming growth factor (TGF)- β 1, on the proliferation of mouse MCs, the accumulation of extracellular matrix and the expression of PGE₂ synthase. Primary mouse glomerular MCs were isolated from EP1 receptor-deficient mice (EP1^{-/-} mice, in which the EP1 receptor was knocked down) and wild-type (WT) mice (WT MCs). In our preliminary experiments, we found that cell proliferation, as well as the mRNA and protein expression of cyclin D1, proliferating cell nuclear antigen (PCNA), fibronectin (FN), collagen I (ColI), membrane-associated PGE₂ synthase-1 (mPGES-1) and cyclooxygenase-2 (COX-2) in the WT MCs were significantly increased following treatment with 10 ng/ml TGF- β 1 for 24 h. Compared with the WT MCs, following the knockdown of the EP1 gene, the TGF- β 1-induced MC injury was markedly suppressed. The aforementioned changes were notably enhanced following treatment with the EP1 agonist, 17-phenyl trinor PGE₂ ethyl amide. Additionally,

TGF- β 1 induced extracellular signal-regulated kinase (ERK) phosphorylation. We found that the TGF- β 1-induced ERK phosphorylation was alleviated by EP1 knockdown and promoted by EP1 expression. These results suggest that the EP1 receptor plays a role in the proliferation of mouse MCs, in the accumulation of extracellular matrix and in the expression of mPGES-1 induced by TGF- β 1. Its mechanisms of action are possibly related to the reinforcement of ERK phosphorylation.

Introduction

Renal fibrosis is the pathological response of kidneys to chronic impairment, which is both a common pathological basis for the progressive course of chronic kidney disease and an essential process from chronic kidney disease to the terminal stage of renal failure. Progressive glomerulosclerosis is a common pathological characteristic of a number of chronic glomerular diseases (1). In general, the quantity, form and position of glomerular mesangial cells (GMCs) are relatively stable and their ability to produce ground substance is relatively weak. However, when mesangial cells (MCs) are stimulated by damaging factors and hazardous substances and are thus revitalized and proliferated, these cells secrete a large amount of ground substance. In addition to the reduced degradation, glomerular sclerosis will eventually develop.

Transforming growth factor- β 1 (TGF- β 1) is a well-recognized factor contributing to inflammation and fibrosis. It has demonstrated that TGF- β is likely to be the core factor that leads to glomerular sclerosis. Over the past decades, studies have revealed various molecular pathways involved in the development and occurrence of renal fibrosis, which aids in the deeper understanding of the pathogenesis of diseases and provides novel strategies for effective prevention and treatment (2-6).

Prostaglandin E₂ (PGE₂) is the metabolite of arachidonic acid, which exists in the body. Membrane-associated PGE₂ synthase-1 (mPGES-1) in combination with cyclooxygenase-2 (COX-2) is the key enzyme for the compound of PGE₂ (7). The vital role of PGE₂ has been demonstrated in a number of pathophysiological processes, such as blood pressure regulation, inflammatory response, immunoreaction and energy metabolism (8). Increasing evidence indicates that PGE₂ also participates in the regulation of the contraction of vascular

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Key words: prostaglandin E₂ receptor EP1 subtype, gene defect, mesangial cell, transforming growth factor- β 1, cell proliferation, extracellular signal-regulated kinase

smooth muscle, glomerular filtration, the release of renin and water and salt transportation in kidney tubules (9). The biological function of PGE₂ is exerted by 4 types of membrane receptors, including EP1, EP2, EP3 and EP4 (10), which are coded by different genes with different signal transduction mechanisms (11). Upon activation, EP1 increases the concentration of Ca²⁺ in the intracytoplasm. EP2 and EP4 couple with activated G proteins (Gs) to increase the level of cyclic AMP (cAMP) in cells. On the contrary, EP3 is able to reduce the cAMP level (12). Previous studies have demonstrated that the EP1 receptor is involved in and regulates a number of physiological and pathological processes, such as the mediation of stress response (13,14), the promotion of chemical carcinogen generation (15) and the mediation of inflammation, fever and pain (16). Moreover, Makino *et al* (17) found strong hybridization signals of EP1 mRNA in the glomerulus and kidney tubules in the renal cortex through hybridization *in situ*, while in the study by Qian *et al* (18), it was demonstrated that PGE₂ mediates MC proliferation and cell cycle arrest through the EP1 receptor. All the abovementioned findings suggest that the function of the EP1 receptor is related to the damage to MCs and the production of glomerulosclerosis.

At present, the function of the EP1 receptor in the pathogenesis of renal fibrosis and chronic kidney diseases is not well understood. Therefore, in this study, we cultured primary GMCs isolated from wild-type (WT) mice and mice in which the EP1 gene was knocked down. We specifically activated EP1 through EP1 receptor stimulation, treating the MCs for 24 h with TGF- β 1. We aimed to determine the effects of EP1 receptor, which is induced by TGF- β 1, on the proliferation of mouse MCs, the accumulation of extracellular matrix and the expression of prostaglandin synthase. In addition, we examined the changes occurring in the extracellular signal-regulated kinase (ERK) signaling pathway, in order to elucidate the possible mechanisms involved.

Materials and methods

Animals and cell culture. We used male mice, 8-12 weeks old, with a C57BL/6 background. The use of the animals was approved by the Animal Experimentation Committee of Peking University Health Science Center, Beijing, China. The EP1 receptor-deficient (EP1^{-/-}) mice were generated as previously described (19), and the lack of functional EP1 receptor was confirmed by quantitative reverse transcription-polymerase chain reaction (RT-qPCR) and western blot analysis. Primary mice GMCs were cultured. The isolation of GMCs was performed according to the method described in the study by Takemoto *et al* (20). In brief, kidneys from 8- to 12-week-old male C57BL/6 mice were obtained. The glomeruli were purified from minced renal cortex tissue and the glomeruli suspension was digested for 40 min at 37°C with type I collagenase. The glomeruli were then collected through 70 and 40 μ m stainless steel sieves. The digested samples were then centrifuged at 1,000 rpm for 5 min, and the precipitate was resuspended in growth medium [Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum (FBS) (Gibco, Invitrogen, Carlsbad, CA, USA)]. The cells were cultured at 37°C in a humidified incubator containing 5% CO₂. Cells at passages 8 to 10 were used. Subsequently, the primary mouse GMCs were subjected to different treatments and

were divided into 5 different groups as follows: i) WT group, ii) WT + TGF- β 1 group, iii) EP1^{-/-} group, iv) EP1^{-/-} + TGF- β 1 group and v) WT + TGF- β 1 + EP1 agonist group. Prior to the experiments, the cells were incubated without FBS for 12 h. Each individual experiment was repeated at least 3 times with different cell preparations.

Reagents. The PGE₂ EIA kit was obtained from Cayman Chemical (Ann Arbor, MI, USA). 17-Phenyl trinor PGE₂ ethyl amide, a selective agonist of EP1 was obtained from Sigma-Aldrich (St. Louis, MO, USA). EP1 antibody was also from Cayman Chemical. The following antibodies were also used (all mouse antibodies): ERK (sc-135900; Santa Cruz Biotechnology, Santa Cruz, CA, USA), phosphorylated ERK (p-ERK, sc-16982; Santa Cruz Biotechnology), cyclin D1 (#2922) and proliferating cell nuclear antigen (PCNA, #2586; both from Cell Signaling Technology, Danvers, MA, USA), fibronectin (FN, sc-135900; Santa Cruz Biotechnology), collagen I (ColI, ab6308; Abcam, Cambridge, MA, USA), COX-2 (#12282; Cell Signaling Technology) and mPGES-1 (ab62049; Abcam). Other reagents were from Sigma-Aldrich unless otherwise indicated.

RNA extraction and RT-qPCR. Total RNA from the cells in the different groups was isolated using TRIzol reagent (Life Technologies, Carlsbad, CA, USA). Total RNA was reverse transcribed into cDNA using the TaqMan Reverse Transcription Reagents kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Quantitative (real-time) PCR was performed with the use of iCycler with the SYBR-Green I probe (Bio-Rad, Hercules, CA, USA). Each sample was analyzed in triplicate and normalized to β -actin. The FN PCR protocol was 95°C 5 min \rightarrow (95°C 30 sec \rightarrow 56°C 30 sec \rightarrow 72°C 30 sec) \times 35 \rightarrow 72°C 10 min. The ColI, COX-2, mPGES-1, cyclin D1, PCNA, β -actin PCR protocol was 95°C 5 min \rightarrow (95°C 30 sec \rightarrow 57°C 30 sec \rightarrow 72°C 30 sec) \times 35 \rightarrow 72°C 10 min. The primer sequences are presented in Table I. PCR products were validated by electrophoresis on a 2% agarose gel.

Western blot analysis. Following the addition of immunoprecipitation cell lysis buffer, the cells were incubated on ice for 30 min. Following treatment as described above, the cell lysate was removed to 1.5 ml EP tubes and spun for 15 min. The supernatant was used for the experiment. Protein concentrations were determined using the BCA assay kit (Pierce, Rockford, IL, USA). Samples were diluted in loading buffer, and then boiled for 5 min. Subsequently, 130 μ g protein of each sample were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred onto a nitrocellulose membrane at 0.37 A for 1 h. Following transfer, the blots were blocked at room temperature for 1 h in 5% (w/v) non-fat dried milk. This was followed by incubation with primary antibodies (mouse anti-FN, mouse anti-ColI, mouse anti-COX-2, mouse anti-mPGES-1, mouse anti-cyclin D1, mouse anti-PCNA and mouse anti-ERK, 1:1,000) at 4°C overnight. The membrane was washed with Tris-buffered saline with Tween-20 (TBST), incubated with DyLight 800-labeled antibody to mouse IgG (1:5,000) for 2 h, and the membrane was then scanned using the Odyssey Infrared Imaging System for semi-quantitative analysis.

Table I. Primers used for RT-qPCR.

Gene name	Chain	Sequence (5'→3')	Product size (bp)
β -actin	FP	TTTAATTTACGCACGATTTC	150
	RP	CCCATCTATGAGGGTTACGC	
EP1	FP	TAACGATGGTCACGCGATGG	291
	RP	ATGCAGTAGTGGGCTTAGGG	
COX-2	FP	AGAAGGAAATGGCTGCAGAA	194
	RP	GCTCGGCTTCCAGTATTGAG	
mPGES-1	FP	CGCGGTGGCTGTCATCA	205
	RP	AGGGTTGGGTCCCAGGAAT	
FN	FP	AATGGAAAAGGGGAATGGAC	244
	RP	CTCGGTTGTCCTTCTTGCTC	
ColI	FP	GAGCGGAGAGTACTGGATCG	142
	RP	GTTCGGGCTGATGTACCAGT	
Cyclin D1	FP	GCGTACCCTGACACCAATCT	219
	RP	GAACCGGTCCAGGTAGTTCA	
PCNA	FP	GACACATACCGCTGCGATCG	307
	RP	TCACCACAGCATCTCCAATAT	

PCR, polymerase chain reaction; COX-2, cyclooxygenase-2; mPGES-1, membrane-associated PGE₂ synthase-1; FN, fibronectin; ColI, collagen I; PCNA, proliferating cell nuclear antigen; FP, forward primer; RP, reverse primer.

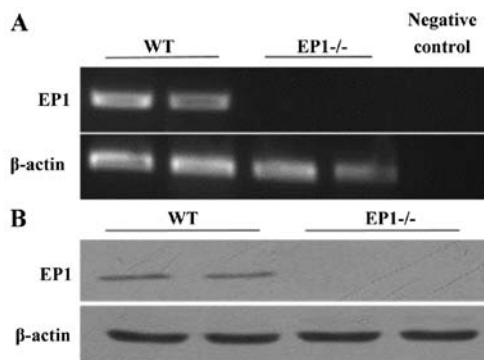


Figure 1. (A) RT-qPCR for the analysis of the mRNA expression of EP1. Negative control, mRNA expression in wild-type (WT) mouse with no reverse transcription. (B) Western blot analysis of the protein expression of EP1; β -actin was used as an internal reference.

PGE₂ ELISA analysis. Cell supernatant PGE₂ levels were measured with an Alpha screen PGE₂ Assay kit (Perkin Elmer, Massachusetts, SA, USA). The cells in the WT and EP1^{-/-} groups were either treated with or without TGF- β 1 for 24 h. For PGE₂ determination, supernatant was collected and PGE₂ levels were measured according to the supplier's instructions.

MTT assay. The cell proliferation ability was detected by MTT assay. Briefly, MCs (1x10³/well) were seeded in 96-well culture plates and incubated in DMEM containing 20% FBS under standard conditions until 40-50% confluence, then the medium was changed to serum-free DMEM for 12 h. The supernatant was removed, 10% FBS DMEM was added to the cells in group 1 (WT group) and group 3 (EP1^{-/-} group), and 10% FBS DMEM containing 10 ng/ml TGF- β 1 was added to the cells in group 2 (WT + TGF- β 1 group) and group 4 (EP1^{-/-} + TGF- β 1

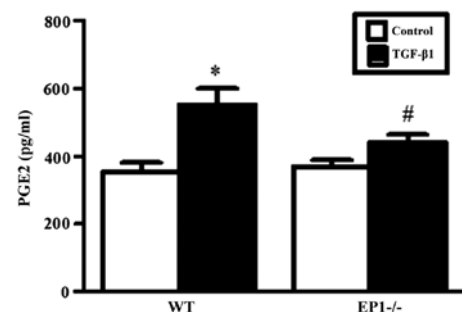


Figure 2. ELISA for the determination of the concentration of prostaglandin E₂ supernatant of wild-type (WT), EP1^{-/-} mouse MCs treated with or without transforming growth factor- β 1 (TGF- β 1). *P<0.05 vs. control group; #P<0.05 vs. WT + TGF- β 1 group.

group) for 24 h. Following treatment, 10 μ l MTT solution were added to each well, and the cells were continuously incubated for 1 h. The optical densities (OD) of the wells were examined at a wavelength of 450 nm (each group had 6 wells).

Statistical analysis. All values are expressed as the means \pm standard error of the mean (SEM). Data were analyzed using the Student's t-test (paired groups) or two-way ANOVA, followed by Bonferroni's post hoc test (multigroup comparisons). A value of P<0.05 was considered to indicate a statistically significant difference.

Results

Identification of mice with deficiency of EP1 gene. Total RNA and total protein was extracted from the kidneys of the WT mice and (EP1^{-/-}) mice in which the EP1 gene had been

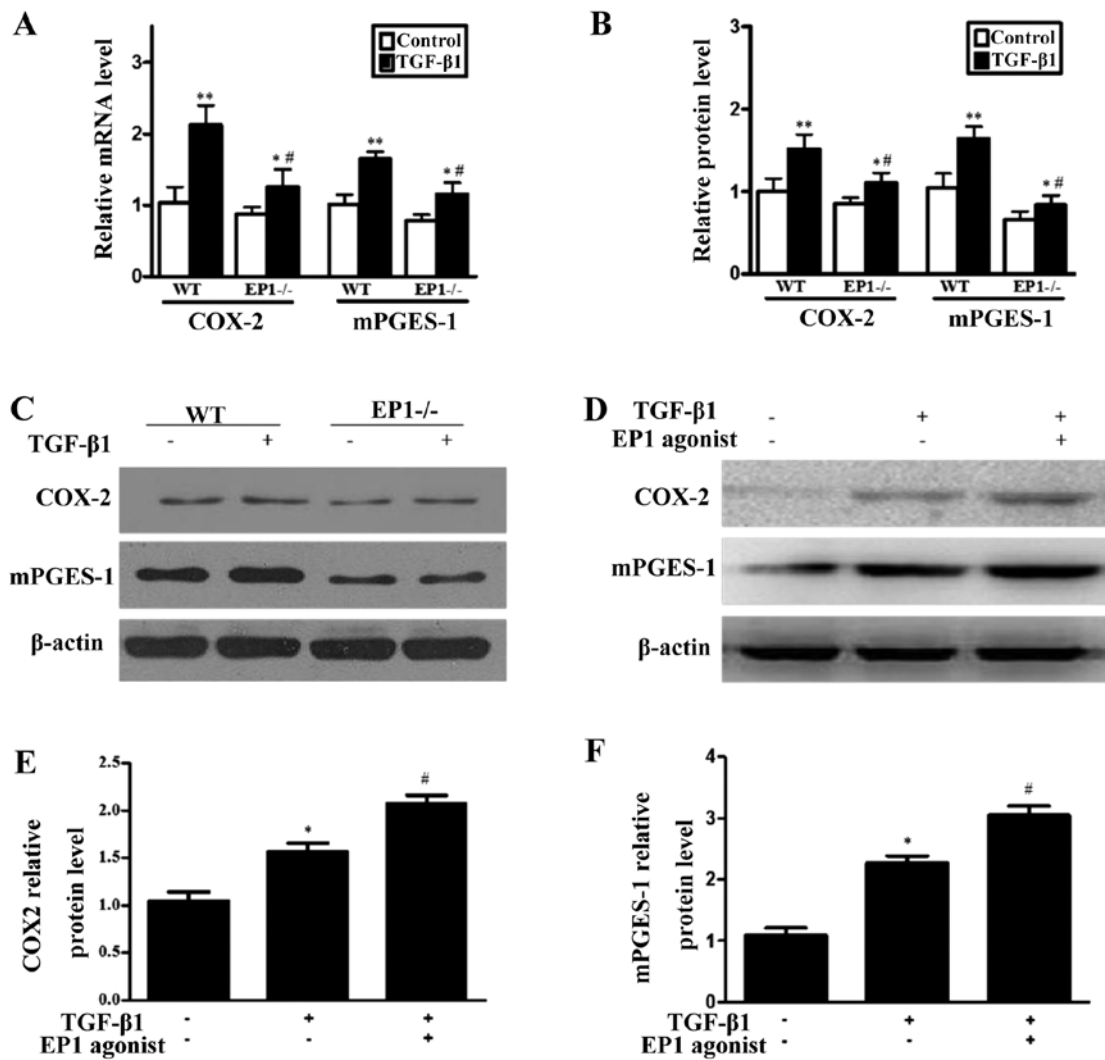


Figure 3. (A) Wild-type (WT) mesangial cells and EP1-deficient (EP1^{-/-}) mesangial cells treated with 10 ng/ml transforming growth factor- β 1 (TGF- β 1) for 24 h. The changes in the expression of cyclooxygenase-2 (COX-2) and membrane-associated PGE₂ synthase-1 (mPGES-1) were determined at the mRNA level; * P <0.05, ** P <0.01 compared to control group; # P <0.05 compared to WT + TGF- β 1 group; differences were statistically significant. (B-C) WT and EP1^{-/-} mesangial cells (MCs) treated with 10 ng/ml TGF- β 1 for 24 h. The changes in the expression of COX-2 and mPGES-1 were determined at the protein level; * P <0.05 and ** P <0.01 vs. control group; # P <0.05 vs. WT + TGF- β 1 group. (D-F) In the WT MCs, the effect of the EP1 receptor agonist, 17-phenyl trinor prostaglandin E₂ ethyl amide, on the protein expression level of mPGES-1 induced by TGF- β 1 was examined. TGF- β 1 (10 ng/ml) was used to stimulate cells in each group for 24 h and the cells were treated with or without EP1 agonist (10 μ M). Western blot analysis was used to determine the protein expression of COX-2 and mPGES-1. * P <0.05 vs. WT group; # P <0.05 vs. WT + TGF- β 1 group.

knocked down. RT-qPCR and western blot analysis were then performed to identify and confirm the generation of EP1^{-/-} mice (Fig. 1). The results revealed that EP1 expression in the WT mice was positive, while it was negative in the mice in which the EP1 gene had been knocked down.

Effect of EP1 receptor on PGE₂ content in MCs treated with TGF- β 1. Based on our pre-experimental results, the optimal treatment time and most effective concentration of TGF- β 1 for MCs was 24 h and 10 ng/ml, respectively (data not shown). As shown by ELISA, treatment of the MCs in the WT and EP1^{-/-} groups with 10 ng/ml TGF- β 1 for 24 h markedly increased the content of PGE₂ (P <0.05) compared with the relative control untreated groups. However, compared with the WT + TGF- β 1 group, the content of PGE₂ was lower in the EP1^{-/-} + TGF- β 1 group (P <0.05) (Fig. 2).

Effect of EP1 receptor on mRNA and protein expression of COX-2 and mPGES-1 in MCs treated with TGF- β 1. The results from RT-qPCR and western blot analysis revealed that following treatment of the MCs in the WT and EP1^{-/-} group with 10 ng/ml TGF- β 1 for 24 h, the mRNA and protein expression of COX-2 and mPGES-1 markedly increased compared with the relative untreated control groups and the differences were statistically significant (P <0.05); however, the mRNA and protein expression of COX-2 and mPGES-1 was lower in the EP1^{-/-} + TGF- β 1 group compared with the WT + TGF- β 1 group (P <0.05) (Fig. 3A-C). Following treatment of the TGF- β 1-treated MCs with 10 μ M of the EP1 receptor specific agonist, 17-phenyl trinor PGE₂ ethyl amide, the protein expression of COX-2 and mPGES-1 increased (P <0.05) (Fig. 3D-F). These results demonstrated that after the WT MCs were treated with TGF- β 1, the expression of COX-2 and mPGES-1 markedly

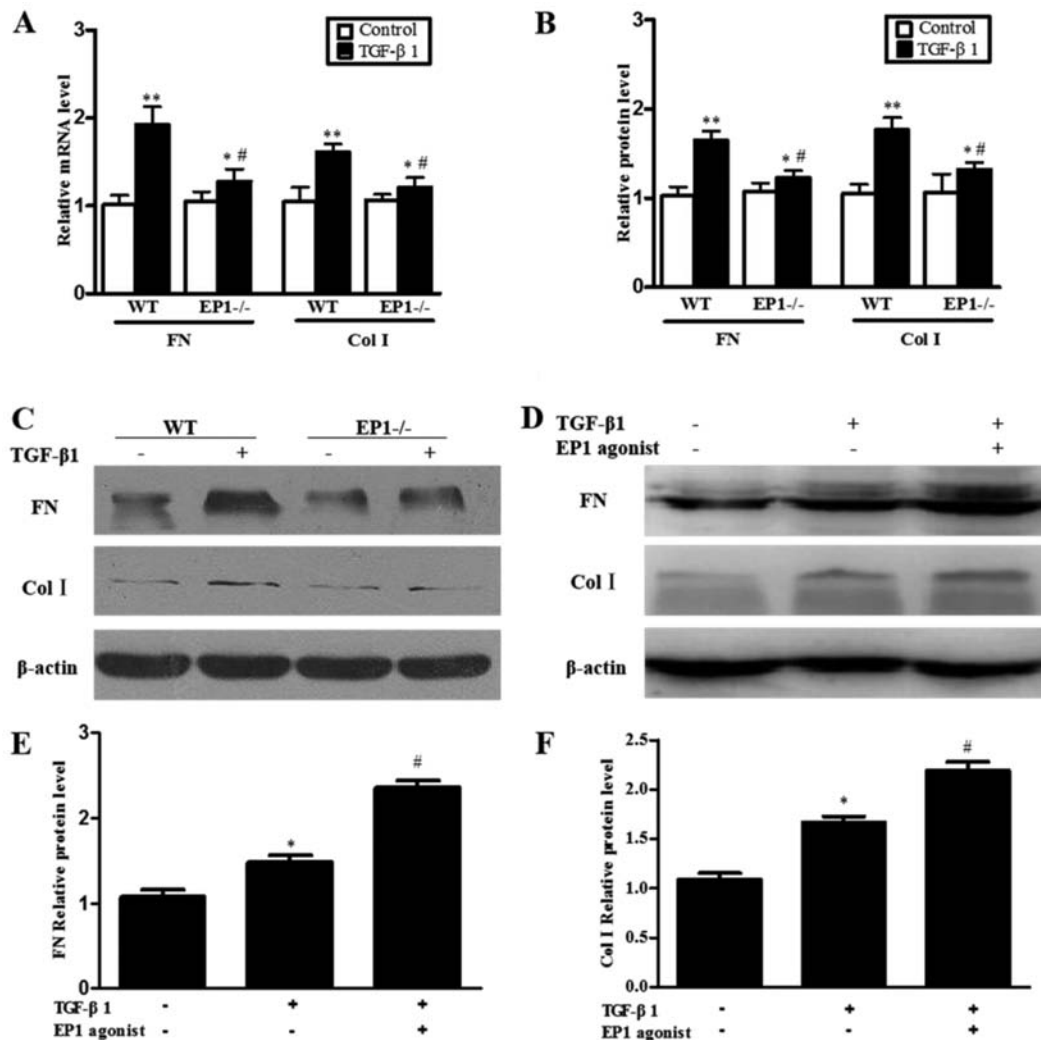


Figure 4. (A) Wild-type (WT) mesangial cells and EP1-deficient (EP1^{-/-}) mesangial cells treated with 10 ng/ml transforming growth factor-β1 (TGF-β1) for 24 h. The changes in the expression of fibronectin (FN) and collagen I (ColI) were determined at the mRNA level; (B-C) WT mesangial cells and EP1^{-/-} mesangial cells were treated with 10 ng/ml TGF-β1 and the changes in the expression of FN and ColI were determined at the protein level. *P<0.05 and **P<0.01 vs. control group; #P<0.05 vs. WT + TGF-β1 group. (D-F) In WT mesangial cells (MCs), the effect of the EP1 receptor specific agonist, 17-phenyl trinor prostaglandin E₂ ethyl amide, on the protein expression level of extracellular matrix induced by TGF-β1 was examined. TGF-β1 (10 ng/ml) was used to stimulate cells in each group for 24 h and the cells were treated with or without EP1 agonist (10 μM). Western blot analysis was used to determine the protein level of FN and ColI. *P<0.05 vs. WT group; #P<0.05 vs. WT + TGF-β1 group.

increased; the deficiency of the EP1 gene reduced the increase in the expression of mPGES-1 induced by TGF-β1, while the activation of the EP1 receptor promoted the increase in the expression of mPGES-1 in the MCs induced by TGF-β1.

Effect of EP1 receptor on the accumulation of extracellular matrix in MCs treated with TGF-β1. The results of RT-qPCR and western blot analysis revealed that following treatment of the WT and EP1^{-/-} MCs with 10 ng/ml TGF-β1 for 24 h, the mRNA and protein expression of FN and ColI markedly increased compared with the relative untreated control groups and the differences were statistically significant (P<0.05); however, the mRNA and protein expression of FN and ColI in the EP1^{-/-} + TGF-β1 group was distinctly lower than that in the WT + TGF-β1 group (P<0.05) (Fig. 4A-C). In order to further confirm that the EP1 receptor plays a role in the regulation of the accumulation of extracellular matrix in MCs, we examined the effects of TGF-β1 on the accumulation of

extracellular matrix in MCs following the specific activation of the EP1 receptor with the EP1 agonist, 17-phenyl trinor PGE₂ ethyl amide. The results revealed that the activation of the EP1 receptor increased the expression of FN and ColI in the cultured WT MCs treated with TGF-β1 and 10 μM 17-phenyl trinor PGE₂ ethyl amide (Fig. 4D-F). These results suggest that the EP1 receptor promotes the accumulation of extracellular matrix induced by TGF-β1.

Role of EP1 receptor in the proliferation of MCs treated with TGF-β1. The results of MTT assay revealed that when comparing the WT and EP1^{-/-} MCs treated with 10 ng/ml TGF-β1 for 24 h with each relative untreated control group, there was a marked increase in cell proliferation and the differences were statistically significant (P<0.05); however, when comparing the EP1^{-/-} + TGF-β1 group with the WT + TGF-β1 group, cell proliferation was markedly decreased in the EP1^{-/-} + TGF-β1 group, with statistically significant differences

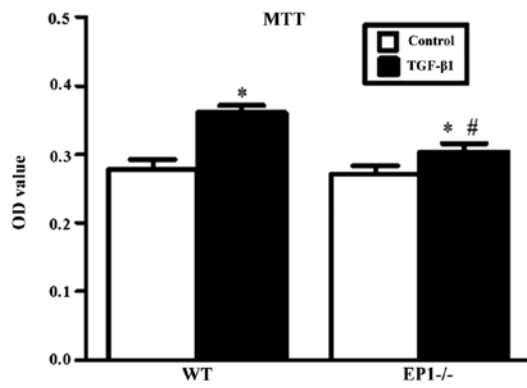


Figure 5. MTT assay was used to examine cell proliferation in each group after wild-type (WT) and EP1^{-/-} mesangial cells (MCs) were treated with 10 ng/ml transforming growth factor- β 1 (TGF- β 1) for 24 h. *P<0.05 vs. control group; #P<0.05 vs. WT + TGF- β 1 group.

(P<0.05) (Fig. 5). These results suggest that following the knockdown of the EP1 gene, the proliferation of the MCs which was induced by TGF- β 1 decreased.

Effect of EP1 receptor on the expression of cyclin D1 and PCNA in MCs treated with TGF- β 1. In order to further elucidate the mechanisms of action of the EP1 receptor in the regulation of MC proliferation, we performed RT-qPCR and western blot analysis to determine the expression levels of cyclin D1 and PCNA in the MCs treated with TGF- β 1. The results revealed that compared to the WT + TGF- β 1 group, the mRNA and protein expression of cyclin D1 in the EP1^{-/-} + TGF- β 1 group was markedly decreased (P<0.05); there were no statistically significant differences in PCNA expression between these 2 groups (Fig. 6). The results also indicated that the deficiency of the EP1 receptor suppressed the expression of cyclin D1. Moreover, in the WT MCs treated with TGF- β 1, the expression of cyclin D1 increased following the addition of the EP1 agonist, 17-phenyl trinor PGE₂ ethyl amide (P<0.05) (Fig. 7). These results suggest that the deficiency of the EP1 gene induces cell cycle arrest by suppressing the expression of cyclin D1 instead of PCNA and therefore suppressing the proliferation of MCs induced by TGF- β 1.

Effect of EP1 receptor on ERK phosphorylation in MCs treated with TGF- β 1. Considering the vital role of ERK in activating cell proliferation and the accumulation of the extracellular matrix (21), we further examined the effects of the deficiency of the EP1 gene and EP1 receptor activation on the phosphorylation of ERK mediated by TGF- β 1. The results revealed that the knockdown of the EP1 receptor suppressed the phosphorylation of ERK which was induced by TGF- β 1 (Fig. 8). However, the activation of the EP1 activator markedly increased the phosphorylation of ERK which was induced by TGF- β 1 (Fig. 9). Therefore, our results suggest that EP1 promotes the proliferation of MCs and the accumulation of extracellular matrix by enhancing ERK phosphorylation.

Discussion

With the in-depth investigation of the prostaglandin system, the effects of PGE₂ receptor have gained widespread attention. It has been demonstrated that 4 types of EP receptors are

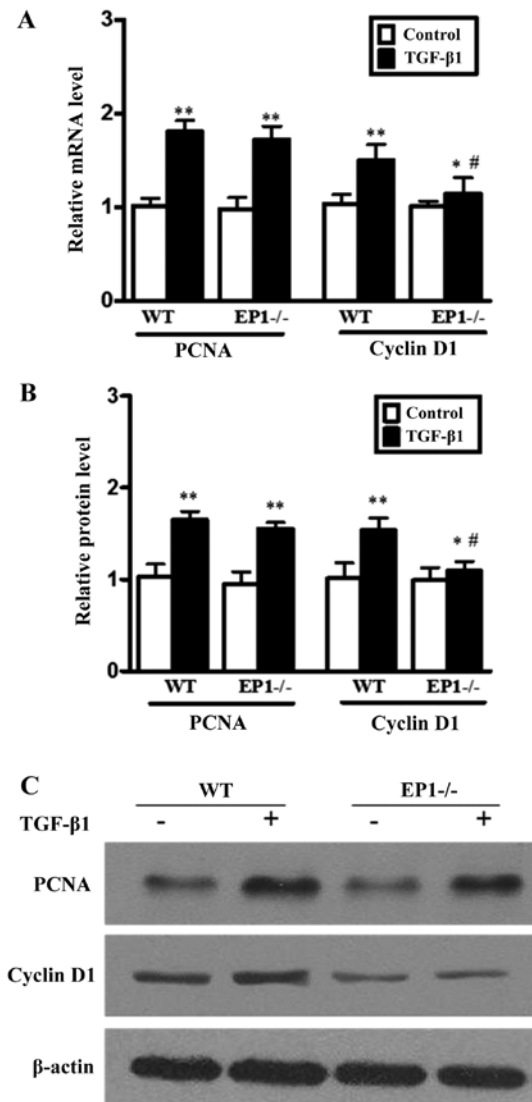


Figure 6. (A) Changes in the expression of cyclin D1 and PCNA at the mRNA level following treatment of wild-type (WT) and EP1-deficient (EP1^{-/-}) mesangial cells (MCs) with transforming growth factor- β 1 (TGF- β 1). (B-C) The changes in the expression of cyclin D1 and PCNA at the protein level following of WT and EP1^{-/-} MCs with TGF- β 1. *P<0.05 and **P<0.01 vs. control group; #P<0.05 vs. WT + TGF- β 1 group.

distributed in the kidneys and blood vascular system (22). A number of studies have indicated that when acute and chronic renal damage occurs, the expression of the EP receptor undergoes marked changes (23-25). It has been reported that there exist obvious signals of EP1 hybridization *in situ* in the glomerulus mesentery. The proliferation of MCs which is induced under high glucose conditions is almost entirely suppressed by EP1 antagonist (17). Studies performing animal experiments have demonstrated that the selective antagonist of prostaglandin receptor EP1 can effectively prevent the development of diabetic nephropathy in mice induced by streptozotocin (STZ) (17) and reduce renal damage in mice caused by high blood pressure (26). Overall, the abovementioned data suggest that EP1 plays a role in multiple renal pathophysiological process. However, at present, to the best of our knowledge, there are no related reports on the effects of EP1 on chronic renal fibrosis.

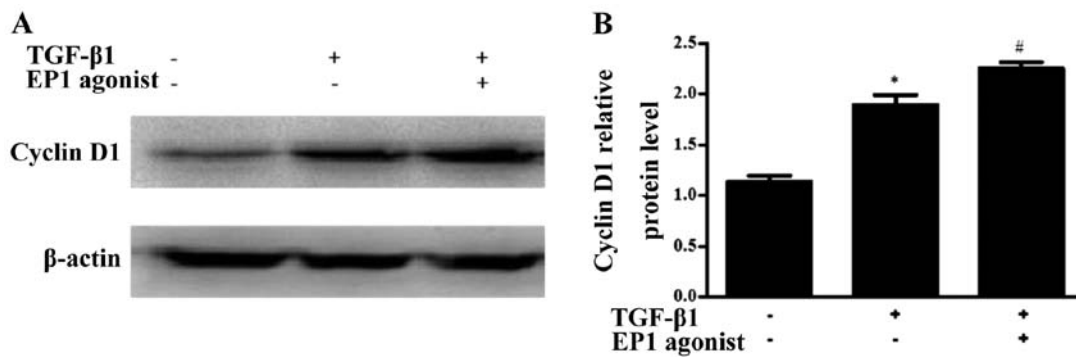


Figure 7. Effects of the EP1 receptor agonist, 17-phenyl trinor prostaglandin E₂ ethyl amide, on the protein expression of cyclin D1 induced by transforming growth factor-β1 (TGF-β1) in wild-type (WT) mesangial cells (MCs). TGF-β1 (10 ng/ml) was used to stimulate the cells in each group for 24 h and the cells were treated with or without EP1 agonist (10 μM). Western blot analysis was used to determine the protein level of cyclin D1. (A) Representative western blots. (B) Quantification of protein expression. *P<0.05 vs. WT group; #P<0.05 vs. WT + TGF-β1 group.

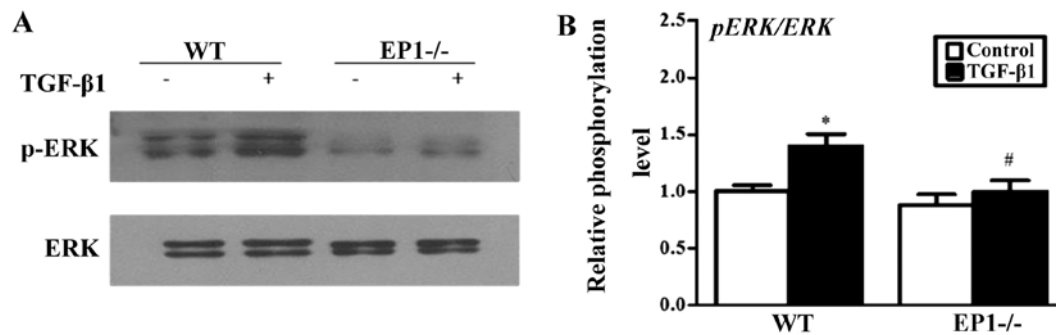


Figure 8. Wild-type (WT) and EP1^{-/-} mesangial cells (MCs) were treated with 10 ng/ml transforming growth factor-β1 (TGF-β1) for 24 h. Western blot analysis used to determine the level of phosphorylation of extracellular signal-regulated kinase (ERK). p-ERK, phosphorylated ERK; ERK, total ERK. (A) Representative western blots. (B) Quantification of protein expression. *P<0.05 vs. control group; #P<0.05 vs. WT + TGF-β1 group.

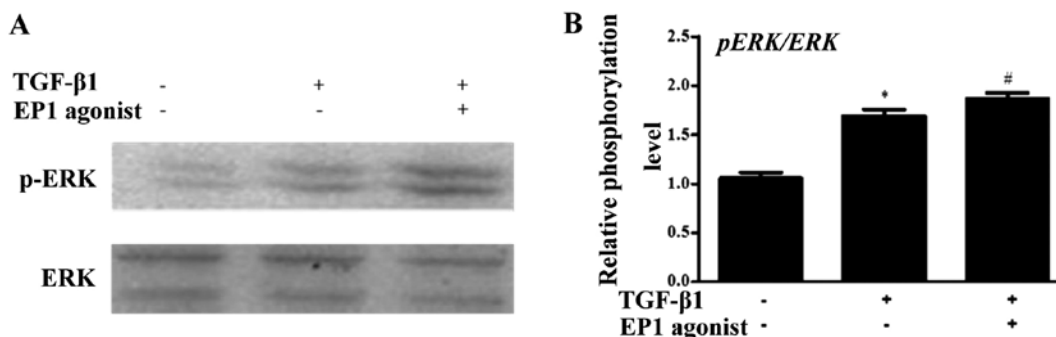


Figure 9. Effect of the EP1 receptor agonist, 17-phenyl trinor prostaglandin E₂ ethyl amide, on the phosphorylation of extracellular signal-regulated kinase (ERK) induced by transforming growth factor-β1 (TGF-β1) in wild-type (WT) mesangial cells (MCs). TGF-β1 (10 ng/ml) was used to stimulate the cells in each group for 24 h and the cells were treated with or without EP1 agonist (10 μM). Western blot analysis was used to determine the level of phosphorylation of ERK. (A) Representative western blots. (B) Quantification of protein expression. *P<0.05 vs. WT group; #P<0.05 vs. WT + TGF-β1 group.

The most important finding of this study is that PGE₂ is likely to activate EP1 receptor and thus exerts effects on the proliferation of MCs and the accumulation of extracellular matrix. Previous studies have indicated that although the development of renal fibrosis involves different mechanisms, all mechanisms involve the increase in cellular matrix compound or the increase in extracellular matrix caused by the decreased degradation, which are the essential factors resulting in glomerulosclerosis and renal interstitial fibrosis (27,28). Undoubtedly, inflammatory response plays an important role in the development of chronic kidney diseases. Moreover, during the

development of kidney disease, PGE₂, together with its 4 types of receptors, as well as other prostaglandin substances all participate in the regulation of the disease process throughout different stages of inflammation in a manner of conditional dependence. They participate in the regulation of the disease process in both inflammation and preventions (29,30). The biological functions of PGE₂ are exerted by the 4 types of receptors from EP1 to EP4. With the activation of one of these receptors, specific biological process occur through specific signaling pathways (11). Evidence indicates that the suppression of COX-2 and mPGES-1 protects cell proliferation (31-33).

Previous studies have demonstrated that selective COX-2 inhibitor attenuates the proliferation of MCs and suppresses the progression of renal fibrosis. These findings also suggest that PGE₂, which is the source of COX-2/mPGES-1 aggravates cell proliferation and the accumulation of extracellular matrix, thus taking part in the development of renal fibrosis. Among all enzymes involved in the production of PGE₂, COX-2 and mPGES-1 are constitutively expressed in cultured MCs (34,35). In this study, we found that after the MCs were stimulated with TGF- β 1, the expression of COX-2 increased, as well as that of mPGES-1. Therefore, the continuous and high-level expression of mPGES-1 plays an important role in the production of PGE₂ among damaged cells and the activation of EP1 receptor. In addition, following stimulation with TGF- β 1, the expression of COX-2/mPGES-1/PGE₂ and FN and ColI in the MCs lacking the EP1 gene decreased and cell proliferation decreased when compared to the WT MCs. However, the specific activation of the EP1 receptor enhanced the expression of PGE₂, as well as that of COX-2, mPGES-1, FN and ColI which was induced by TGF- β 1. Hence, our results suggest that following stimulation with TGF- β 1, the expression of COX-2/mPGES-1 in the MCs increases, which also increases the expression of PGE₂ and activates the EP1 receptor; on the contrary, the deficiency of the EP1 gene lowers the expression of COX-2/mPGES-1 and alleviates damage to MCs and renal fibration. Thus, EP1 may play an essential role in regulating the damage to MCs induced by TGF- β 1.

Cyclin D1 participates in the progression of the cell cycle from the G1 stage to the S stage. The suppression of its functions prevents cells entering the S stage. However, the overexpression of this gene may shorten the G1 stage and thus lead to incontrollable cell proliferation (36). In this study, we found that after the EP1 receptor is expressed, the expression level of cyclin D1 increases suggesting that EP1 is a type of receptor which promotes proliferation. This was supported by our results showing that in the MCs in which the EP1 gene was knocked down, cell proliferation and the expression of cyclin D1 induced by TGF- β 1 were suppressed.

ERK is a vital mediator in conducting signals from the cell surface to the cell nucleus. Different extracellular stimulations activate ERK, such as mitogens, growth factors, AngII and oxidative stress (37). The stimulated ERK molecule phosphorylates other substrates, such as cytoskeletal proteins and thus exerts biological effects directly, or transfers them to the nucleus and phosphorylates transcription factors, such as cAMP response element-binding protein (CREB) and nucleoprotein so as to regulate gene expression intracellularly. Previous studies have demonstrated that the activation of ERK plays an important role in the cell cycle and ECM accumulation (36,38-41). In our study, we found that the deficiency of the EP1 gene markedly suppresses the phosphorylation of ERK induced by TGF- β 1; however, following the activation of EP1, EP1 is able to strengthen the phosphorylation of ERK induced by TGF- β 1. This suggests that cell proliferation, cyclin D1 expression and the accumulation of extracellular matrix induced by TGF- β 1 may be partially related to ERK phosphorylation mediated by EP1.

Combined with our present experimental results, we suggest that the deficiency of the EP1 gene markedly suppresses the proliferation of MCs and the accumulation of extracellular matrix induced by TGF- β 1. The activation of PGE₂-EP1

plays an important role in inducing damage to MCs, which is related to cell cycle arrest caused by the reduction of cyclin D1 expression and the phosphorylation of ERK. In order to further confirm the abovementioned results, we used an EP1 receptor agonist (17-phenyl trinor PGE₂ ethyl amide) to specifically activate the EP1 receptor. Our results demonstrated that the activation of the EP1 receptor promoted the accumulation of MC extracellular matrix which was induced by TGF- β 1 and promoted the expression of cyclin D1 and the phosphorylation of ERK. These results suggest that the EP1 receptor may be regarded as a potential target for the treatment of renal fibrosis. However, further studies are required to confirm our results and determine whether this will provide a new strategy for the treatment of chronic kidney diseases.

Acknowledgements

This study was funded by grants from the National Natural Science Foundation of China (no. 81170656) and the Science Foundation of Nantong City, Jiangsu, China (no. HS2011021).

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