Original Research

Effects of all-trans retinoic acid on signal pathway of cyclooxygenase-2 and Smad3&7 in transforming growth factor-β-stimulated glomerular mesangial cells

Jinyi Han[1,2], Li Zhang[1,3], Xiaolan Chen[1], Bin Yang[1,4], Naifeng Guo[1] and Yaping Fan[1]

[1]Department of Nephrology, Affiliated Hospital of Nantong University, Nantong 226001, PR China; [2]Department of Nephrology, The First people's Hospital of Changshu, Changshu 215500, PR China; [3]Department of Cardiology, The Second Affiliated Hospital of Nantong University, Nantong, 226001, PR China; [4]Department of Infection, Immunity and Inflammation, University of Leicester, Leicester General Hospital, University Hospitals of Leicester, Leicester LE5 4PW, UK

Corresponding author: Xiaolan Chen or Bin Yang. Email: xlchen8448@hotmail.com or by5@leicester.ac.uk

Abstract

All-trans retinoic acid (ATRA) has been used for the treatment of acute promyelocytic leukemia. It remains unclear, however, whether ATRA affects cyclooxygenase-2 (COX-2; an enzyme involved in prostaglandin production), PGE_2, and thromboxane A_2 (TXA_2) (metabolic products of COX-2) by a transforming growth factor-β/Smad-signaling pathway, which plays important roles in mesangial-cell proliferation and renal fibrosis. In this study, the mRNA and protein of Smad3, Smad7, and COX-2 were detected by reverse transcription-polymerase chain reaction and Western blot, respectively, in mesangial cells stimulated by transforming growth factor-β (TGF-β) and treated with ATRA at various concentrations and times. The protein level of PGE_2 and TXA_2 was also measured by enzyme-linked immunosorbent assay. The localization of Smad3 and Smand7 was observed by confocal microscope. Cell proliferation was detected by MTT assay, while apoptosis was determined using Hoechest staining. The expression of Smad3, Smad7, and COX-2 mRNA and protein was increased by exogenous TGF-β, but inhibited by pretreatment of ATRA, in dose and time-dependent manners. In addition, the expression of Smad3 and Smad7 was significantly reduced not only by staurosporine, an inhibitor of threonine/serine protein kinases as well as smad, but also by NS-398, an inhibitor of COX-2. PGE_2 and TXA_2 were raised by TGF-β, but also decreased by ATRA, staurosporine, and NS-398. Moreover, ATRA reversed the translocation of Smad3 and Smad7 induced by TGF-β. Compared with the control, TGF-β also significantly enhanced proliferation and inhibited apoptosis of mesangial cells. ATRA dose-dependently inhibited TGF-β-induced cell proliferation, but had no significant effect on apoptosis in rat mesangial cells. Therefore, ATRA repressed COX-2, PGE_2, and TXA_2 via the TGF-β/Smad-signaling pathway and inhibited mesangial-cell proliferation, which might subsequently prevent renal fibrosis.

Keywords: All-trans retinoic acid, transforming growth factor-β, Smad, mesangial cell, cyclooxygenase-2

***Experimental Biology and Medicine* 2014; 239: 272–283. DOI: 10.1177/1535370213519216**

Introduction

Transforming growth factor-β (TGF-β), a multifunctional cytokine, plays a crucial role in the development of renal fibrosis in various glomerular and tubulointerstitial injuries of kidney diseases.[1,2] Smad proteins are not only intracellular kinase substrates but also downstream signal transduction factors of TGF-β receptors.[3] After TGF-β binds to its receptor, Smad2 and Smad3 are phosphorylated and heteromerized with Smad4 and then translocated into the nucleus where they regulate transcriptional activity and modify cell proliferation, differentiation, migration, and apoptosis. Smad7 is the function-specific endogenous inhibitor of TGF-β, thereby inhibiting phosphorylation, blocking signal transduction, and regulating negative feedback.

Cyclooxygenase-2 (COX-2) is an important rate-limiting enzyme for catalyzing arachidonic acid into prostaglandin (PG), which is involved in the inflammation reaction in the pathogenesis of renal diseases.[4,5] Both glomerular macrophages and mesangial cells are the origins of COX-2. PGE_2, a leading member of the PG family, is widely distributed in various organ systems and plays a variety of biological roles such as regulating inflammation and immune response.[6,7] Thromboxane A_2 (TXA_2) is synthesized and released by platelet AA under the action of COX-2 and TXA_2 synthase. However, TXA_2 is unstable, rapidly

degrading to TXB_2. TXA_2 contracts renal vascular and glomerular mesangial cells, increases the peritubular vascular loop resistance, and decreases the renal cortical blood flow.[8,9] TXA_2 is also able to reduce the glomerular filtration area, increases the permeability of the glomerular filtration membrane, and induces proteinuria. Therefore, COX-2-derived PG, particularly PEG_2 and TXA_2, has important pathogenic effect on glomerular injury and renal function.

It has been shown that all-trans retinoic acid (ATRA) regulates the proliferation and differentiation of various renal inherent cells and inhibits glomerular proliferation, inflammation, and extracellular matrix (ECM) secretion in mesangial proliferative glomerulonephritis in rats.[10] However, the possible mechanism of renoprotection induced by ATRA has not been well defined. The main objective of this study is to investigate the effects of ATRA on the expression of COX-2 and its metabolites, PGE_2 and TXA_2, in mesangial cells stimulated by TGF-β with a focus on the TGF-β/Smad-signaling pathway, and this may provide a theoretical basis for applying ATRA in kidney disease.

Materials and methods

Cell culture

The rat mesangial-cell line was a gift from the Laboratory of Kidney Disease, Shanghai Changzheng Hospital. Cells were cultured in RPMI1640 medium (Gibco, NY, USA) and supplemented with 10% fetal bovine serum (HyClone, UT, USA), 2 mM L-glutamine, 100 U/mL penicillin G, and 100 U/mL streptomycin at 37°C under a humidified atmosphere of 95% air/5% CO_2. The fourth generation of mesangial cells was randomly divided into five groups: (a) the control group; (b) TGF-β (5 and 10 μg/L; Pepro Tech EC Ltd, London, UK) group; (c) 10 μg/L TGF-β + 5 nM staurosporine group; (d) 10 μg/L TGF-β + 10 μM NS-398 group; (e) the treatment groups: the mesangial cells were pretreated with ATRA (Sigma, MO, USA) at ultimate concentrations of 0.001, 0.1 and 10 μM for 24 h and then stimulated with 10 μg/L TGF-β for different periods of 4, 12, and 24 h. The concentrations of different reagents used in this study such as staurosporine or NS-398 were optimized in preliminary studies.

MTT assay

Cell proliferation ability was detected by MTT assay. Rat mesangial cells, 5×10^4 cells/well, were planted into 96-well plates and cultured under standard conditions until 60–70% confluence and then changed to serum-free RPMI 1640 medium for 24 h. The treatment of ATRA is the same as above, while 10 μg/L TGF-β was added to different wells for 0, 4, 12, 24, and 48 h, respectively. MTT (20 μL/well) was added and developed for 4 h, and DMSO (150 μL/well) was added finally. Optical densities of wells were examined at a wave length of 450 nm.

Staining of apoptotic cells

The cells were seeded onto coverslips in 24-well plates at a density of 5×10^4 cells/well and then were treated in the same way described above. Cells were fixed in freshly prepared 4% paraformaldehyde solution in PBS (PH 7.4) for 1 h at 4°C and washed with fresh PBS three times. The cells were then stained with the nuclear stain Hoechst 33258 (10 ng/L, Sigma, MO, USA) for 1 h, washed with PBS, and examined using a confocal microscope (Olympus, Tokyo, Japan).

Flow cytometry analysis

Propidium iodide (PI, 50 ng/L) was used for the determination of cell death. After exposure to different experimental conditions, the cells were trypsinized and incubated with PI for 20 min at 37°C. The samples were then detected using a flow cytometer (FACS, Becton-Dickinson, USA) and analyzed by MYCYCLE software.

RT–PCR analyses

To detect the expression of COX-2, Smad3, and Smad7 mRNA, a set of reverse transcription-polymerase chain reaction (RT–PCR) was performed. The primers of COX-2, Smad3, Smad7, and housekeeping gene β-actin were designed using the primer 5 software according to the published GenBank database (http://www.ncbi.nlm.nih.gov/) and synthesized by Sangon Biotech Co. Ltd (Shanghai, China). Gene-specific primers are listed in Table 1.

Total RNAs from rat mesangial cells were isolated using Trizol reagent and quantified. For cDNA synthesis using reverse transcription (RT), 1 μg total RNA from each sample was gently mixed with RT Master Mix containing 5 × RT buffer, dNTP mixture, RNA enzyme inhibitors, AMV reverse transcriptase, Oligo-dT, and RNase-free H_2O and incubated at 30°C for 10 min, 42°C for 45 min, and 95°C for 5 min. One microliter cDNA from the RT reaction was added to 19-μL PCR cocktail containing 1-μL sense primer, 1-μL antisense primer, 2 × Taq Master Mix, and 7 μL H_2O, which were then amplified under different conditions. PCR products were obtained after 35 (for COX-2, Smad3, and Smad7) or 28 (for β-actin) cycles of amplification with an annealing temperature of 55°C (for COX-2) and 57°C (for Smad3, Smad7, or β-actin). PCR products were analyzed by 1.5% agarose gel electrophoresis containing ethidium bromide and quantified digitally using Scion Image software.

Table 1 Primers for amplification of rat COX-2, Smad3, Smad7, and β-actin

Primer	Primer	Sequence (5′ to 3′)	Products
β-Actin	Sense	TTTAATGTCACGCACGATTTC	150 bp
	Anti-sense	CCCATCTATGAGGGTTACGC	
COX-2	Sense	ACGCTTCTCCCTGAAACCTTAC	378 bp
	Anti-sense	TTGATGGTGGCTGTCTTGGTA	
Smad3	Sense	AGGGCTTTGAGGCTGTCTACC	364 bp
	Anti-sense	GTCCATCGCTGGCATCTTCTG	
Smad7	Sense	CTCAGGCATTCCTCGGAAG	148 bp
	Anti-sense	CCATCGGGTATCTGGAGTAAG	

Western blot analysis

The protein expression of COX-2, Smad3, and Smad7 was detected by Western blot. Rat mesangial cells were scraped off and lysed in ice-cold lysis buffer, and total proteins were quantified using a BCA protein assay kit (Pierce, Rockford, USA). The proteins were separated by 10% SDS–PAGE gel electrophoresis and then transferred to nitrocellulose membranes at 0.37 A for 1 h. The membranes were blocked at room temperature for 1 h in 5% (w/v) non–fat-dried milk and then incubated with anti-Smad3, anti-COX-2, and anti-Smad7 antibodies (Santa Cruz Biotechnology, Inc, CA, USA) at 4°C overnight. The membrane was washed, incubated with alkaline phosphatase-conjugated secondary antibody (Santa Cruz Biotechnology, Inc, CA, USA), and scanned by the Odyssey® Infrared Imaging System (LI-COR Inc, USA) for semi-quantitative analysis.

ELISA assay

The level of PGE_2 and TXA_2 in supernatants of mesangial cells was determined by enzyme-linked immunosorbent assay (ELISA). The cell-culture fluid collected at different hours (4, 12, and 24 h) was measured using ELISA kits (R & D Company Systems, Minneapolis, MN, USA). The methods were operated strictly according to the instruction of manufacture. Each detection was repeated three times, and the mean was taken for statistical analysis.

Immunofluorescence and confocal microscope examination

Cells were grown on glass slides and treated in the same way as above. After incubation, the cells were fixed with 4% formaldehyde for 30 min, permeabilized in 0.2% Triton X-100 for 15 min, blocked with 5% bovine serum albumin (BSA), and incubated with the primary antibody (anti-Smad3, anti-Smad7, Abcam, London, UK) overnight at 4°C. The next day, the slides were incubated with FITC-conjugated goat antimouse IgG (Santa Cruz Biotechnology) for 2 h at 37°C, stained with Hoechst stain for 5 min at room temperature, washed, and mounted with Tris–glycerol. The cells were examined using a confocal laser scanning microscope.

Statistical analysis

The results are expressed as mean ± standard error of the mean. Statistical differences in two or multiple groups were analyzed by *t*-test or one-way analysis of variance (ANOVA). All statistical analyses were carried out by SPSS 13.0 software (SPSS, Chicago, IL, USA). P values < 0.05 were considered to be statistically significant.

Results

Anti-proliferative effects of ATRA

The proliferative ability of cells was gradually increased by 10 μg/L TGF-β with prolonged incubation time and peaked at 24 h, but significantly decreased by ATRA treatment in a dose-dependent manner ($P < 0.05$).

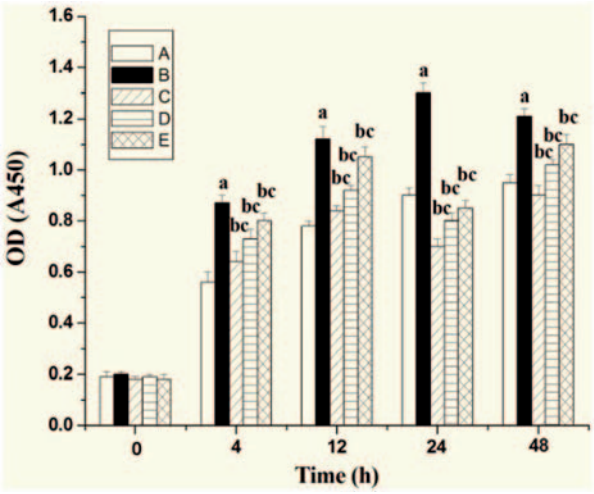

Figure 1 Growth-inhibitory effects of ATRA on mesangial cells at different time points (0, 4, 12, 24, and 48 h) determined by MTT assay. Data expressed as means ± SD are representative of three independent experiments. (a) Control; (b) 10 μg/L TGF-β; (c) 10 μM ATRA; (d) 0.1 μM ATRA; (e) 0.001 μM ATRA. $^{a}P < 0.05$ versus A; $^{b}P < 0.05$ versus B; $^{c}P < 0.05$ among C, D, and E

Based on these findings and previous reports, the time point of 24 h was chosen to further investigate the other effects of TGF-β and ATRA on mesangial cells (Figure 1).

Effects of ATRA on apoptosis in TGF-β-stimulated mesangial cells

Flow cytometry analysis showed that the rate of apoptotic cells, indicated by the sub-G1 peak in the representative pictures, in the control, 10 μg/L TGF-β, or 10 μM ATRA and TGF-β group, was 8.38, 2.56, and 3.01%, respectively. It was demonstrated that 10 μg/L TGF-β could inhibit apoptosis of mesangial cells ($P < 0.05$ versus control). In addition, ATRA had no effect on apoptosis of mesangial cells stimulated with TGF-β ($P > 0.05$ versus TGF-β alone, Figures 2 and 3).

Effects of ATRA on TGF-β-induced COX-2 mRNA expression

The level of COX-2 mRNA detected by RT–PCR was significantly increased by 5 μg/L TGF-β (track B) compared with that in the control group (track A; $P < 0.05$), which was further raised by 10 μg/L TGF-β (track C; $P < 0.05$). Treatment of staurosproine (track D) or NS-398 (track E) significantly inhibited the increase of COX-2 mRNA induced by 10 μg/L TGF-β ($P < 0.05$, respectively). In the cells treated with ATRA at different concentrations, the expression of COX-2 mRNA was consistently reduced at 4, 12, and 24 h compared with that of the cells treated by TGF-β (10 μg/L) alone. Compared with the expression of COX-2 mRNA at 4 h, the expression of COX-2 mRNA declined sharply at 12 h and then slowed down at 24 h (track F, G, H; $P < 0.05$, Figure 4).

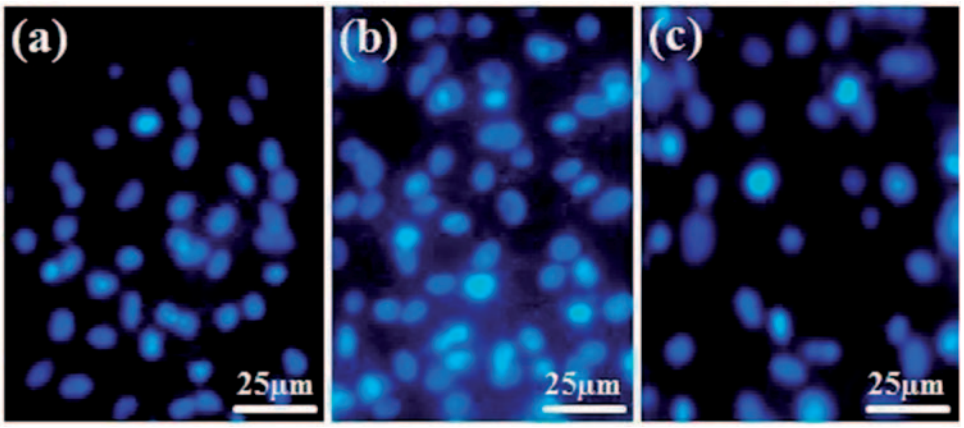

Figure 2 Mesangial cells were stained with Hoechst 33258. Cell morphology was observed by a fluorescence microscope. (a) Control; (b) 10 μg/L TGF-β; (c) 10 μM ATRA. $^{a}P < 0.05$ versus control; $^{b}P > 0.05$ versus TGF-β alone. (A color version of this figure is available in the online journal)

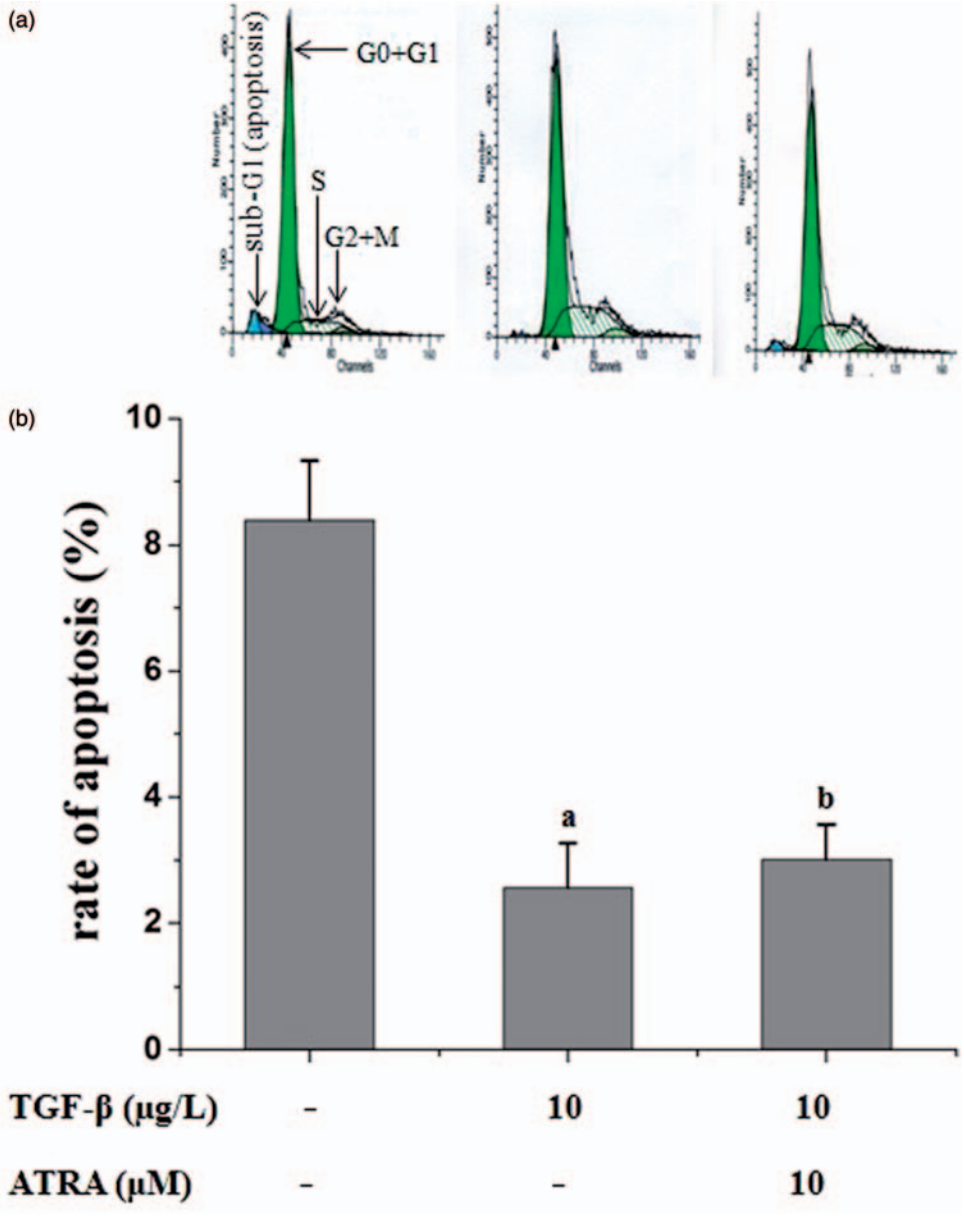

Figure 3 Effects of ATRA on apoptosis of TGF-β-stimulated mesangial cells was examined by flow cytometry. (a) The sub-G1 peak that indicates apoptosis was marked in the representative histograms. (b) The rate of apoptosis was shown in the bar graphs. All results were representative of three independent experiments. $^{a}P < 0.05$ versus control; $^{b}P > 0.05$ versus TGF-β alone. (A color version of this figure is available in the online journal)

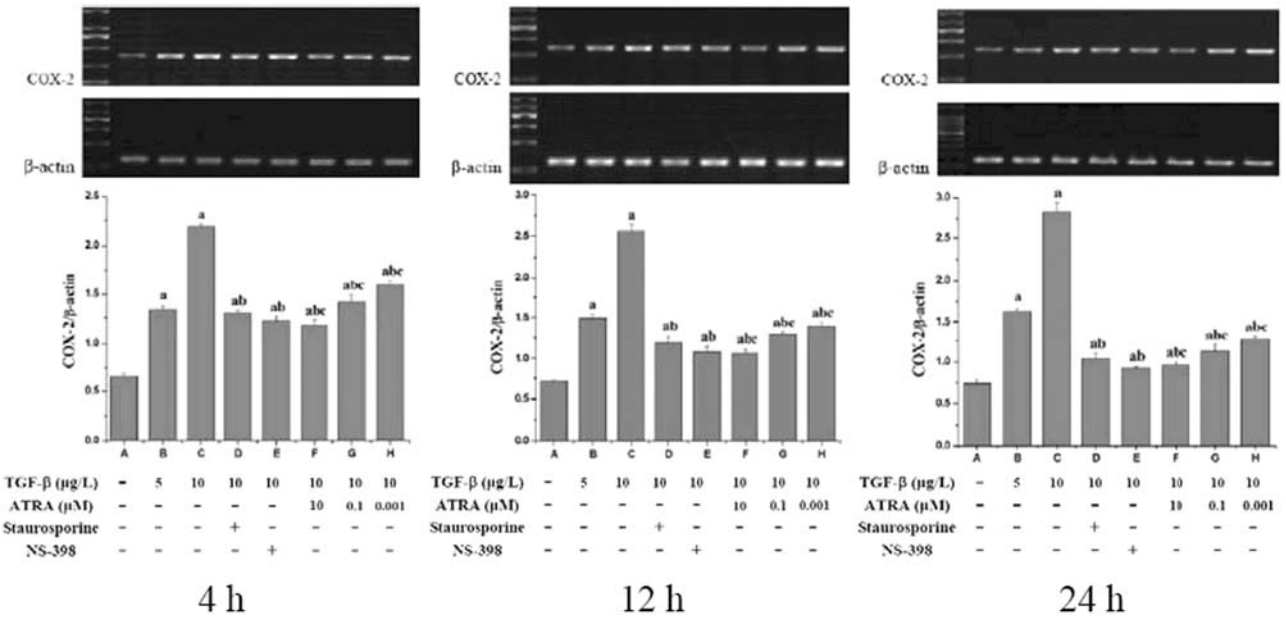

Figure 4 Effects of ATRA on COX-2 mRNA expression detected by RT–PCR in mesangial cells. The mesangial cells were treated by 5 and 10 μg/L TGF-β, as well as staurosporine, NS-398, or ATRA at 0.001, 0.1, or 10 μM for 4, 12, and 24 h. The mean results of three separate experiments were shown in the bar graph. $^{a}P < 0.05$ versus A; $^{b}P < 0.05$ versus C; $^{c}P < 0.05$ among F, G, and H

Effects of ATRA on TGF-β-induced Smad3 mRNA expression

The change trend of Smad3 mRNA detected by RT–PCR was consistent with that of COX-2 mRNA. The expression of Smad3 mRNA was dose-dependently increased by 5 and 10 μg/L (track A, B, C; $P < 0.05$). However, the increased Smad3 mRNA by 10 μg/L TGF-β was significantly blocked by staurosproine and NS-398 (track D, E; $P < 0.05$). ATRA similarly inhibited the increase of TGF-β-induced Smad3 mRNA in a time and concentration-dependent manner. Furthermore, the expression of Smad3 mRNA in the cells treated with ATRA was decreased slightly at 12 h, but intensely at 24 h (track F, G, H; $P < 0.05$, Figure 5).

Effects of ATRA on TGF-β-induced Smad7 mRNA expression

The level of Smad7 mRNA detected by RT–PCR was also significantly increased by 5 and 10 μg/L TGF-β (track A, B, C; $P < 0.05$). The raised Smad7 mRNA by 10 μg/L TGF-β was inhibited by staurosporine, NS-398 (track D, E; $P < 0.05$). ATRA similarly downregulated the increase of TGF-β-induced Smad7 mRNA in a time and concentration-dependent manner, with a sharp decrease at 12 h and almost leveled out at 24 h (track F, G, H; $P < 0.05$, Figure 6).

COX-2 protein expression

Western blot results showed that the expression of COX-2 protein was significantly increased by TGF-β compared with that in the control group (track A, B, C; $P < 0.05$). The staurosproine and NS-398 group partially blocked TGF-β-stimulated COX-2 protein expression (track D, E; $P < 0.05$). After the treatment of ATRA at different concentrations, the COX-2 protein expression was decreased in time and concentration-dependent manners, with a good trend of gradual reduction at 4, 12 and 24 h (track F, G, H; $P < 0.05$, Figure 7).

Smad3 protein expression

Western blot results showed that after TGF-β stimulation, Smad3 protein expression was significantly increased, compared with the control group (track A, B, C; $P < 0.05$). The staurosproine and NS-398 group can partially block TGF-β-stimulated Smad3 protein expression and reached a statistical significance (track D, E; $P < 0.05$). After treatments with different concentrations of ATRA, the Smad3 protein expression in mesangial cells was decreased in a time and concentration-dependent manner. As shown in Figure 5, the Smad3 protein expression was decreased gradually from 4 to 12 h and more intensely at 24 h (track F, G, H; $P < 0.05$, Figure 8).

Smad7 protein expression

Western blot results showed that after TGF-β stimulation, Smad7 protein expression was significantly increased, as compared with the control group ($P < 0.05$). Staurosproine and NS-398 group partially blocked TGF-β-stimulated Smad7 expression in mesangial cells, which was statistically significant ($P < 0.05$). After treatments of different concentrations of ATRA, Smad7 expression was decreased in mesangial cells, in a time and concentration-dependent manner, slightly at 12 h and intensely at 24 h (track F, G, H; $P < 0.05$, Figure 9).

Level of PGE2 protein

The level of PGE_2 protein detected by ELISA was significantly increased by 5 and 10 μg/L TGF-β dose-dependently

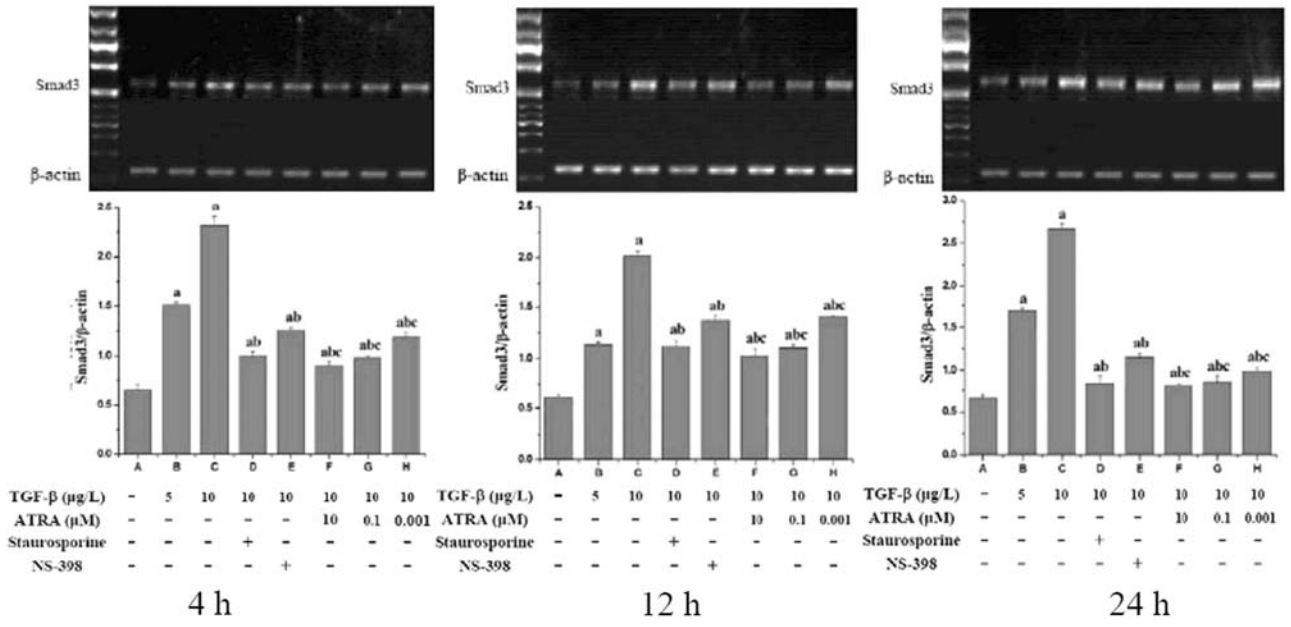

Figure 5 Effects of ATRA on Smad3 mRNA expression detected by RT–PCR in mesangial cells. The mesangial cells treated by 5 and 10 μg/L TGF-β, as well as staurosporine, NS-398, or ATRA at 0.001, 0.1, or 10 μM for 4, 12, and 24 h. The mean results of three separate experiments were shown in the bar graph. $^{a}P < 0.05$ versus A; $^{b}P < 0.05$ versus C; $^{c}P < 0.05$ among F, G, and H

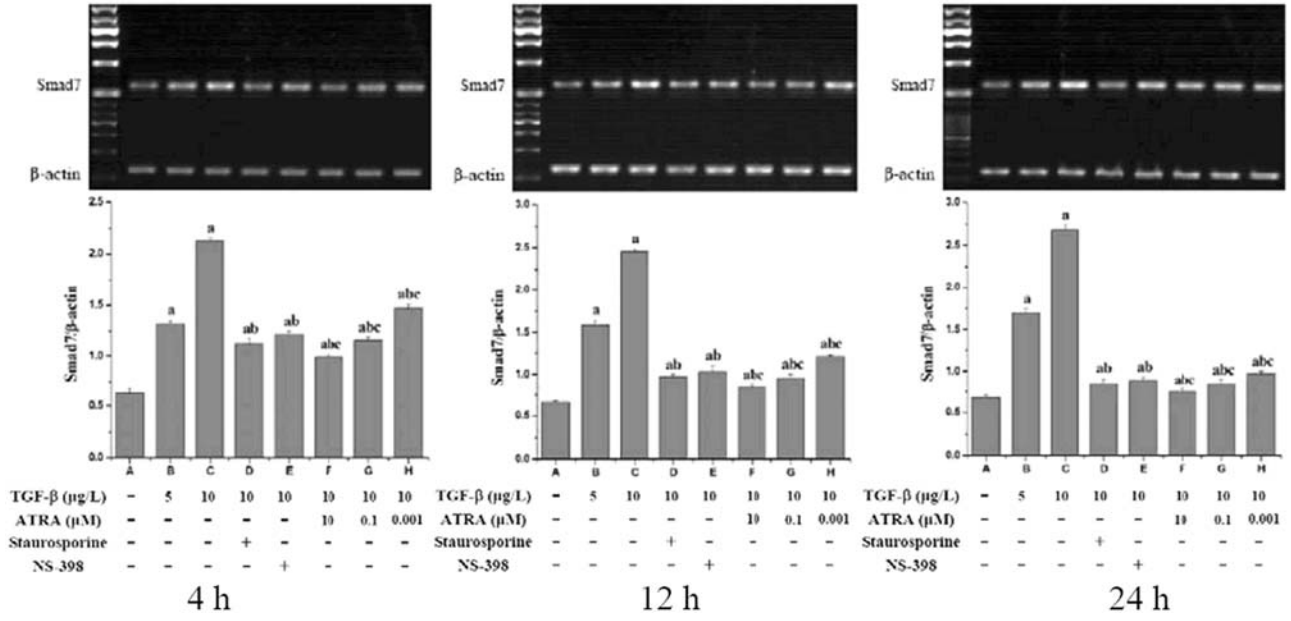

Figure 6 Effects of ATRA on Smad7 mRNA expression detected by RT–PCR in mesangial cells. The mesangial cells treated by 5 and 10 μg/L TGF-β, as well as staurosporine, NS-398, or ATRA at 0.001, 0.1, or 10 μM for 4, 12, and 24 h. The mean results of three separate experiments were shown in the bar graph. $^{a}P < 0.05$ versus A; $^{b}P < 0.05$ versus C; $^{c}P < 0.05$ among F, G, and H

as compared with that in the control group ($P < 0.05$). Staurosproine and NS-398 significantly inhibited the increase of PGE_2 induced by 10 μg/L TGF-β ($P < 0.05$). After the treatments of different concentrations of ATRA, PGE_2 was decreased in time and concentration-dependent manners in mesangial cells ($P < 0.05$, Figure 10).

Level of TXA2 protein

The change patter of TXA_2 protein detected by ELISA was similar to that of PGE_2. Briefly, the level of TXA_2 was significantly increased by TGF-β dose-dependently ($P < 0.05$). The increase induced by 10 μg/L TGF-β was inhibited by NS-398 and staurosproine ($P < 0.05$) as well as different concentrations of ATRA in concentration and time-dependent manners ($P < 0.05$, Figure 11).

Effects of ATRA on the subcelluar localization of Smad3 and Smad7

Confocal microscope examination showed that Smad3 localized in the cytoplasm, while the majority of Smad7

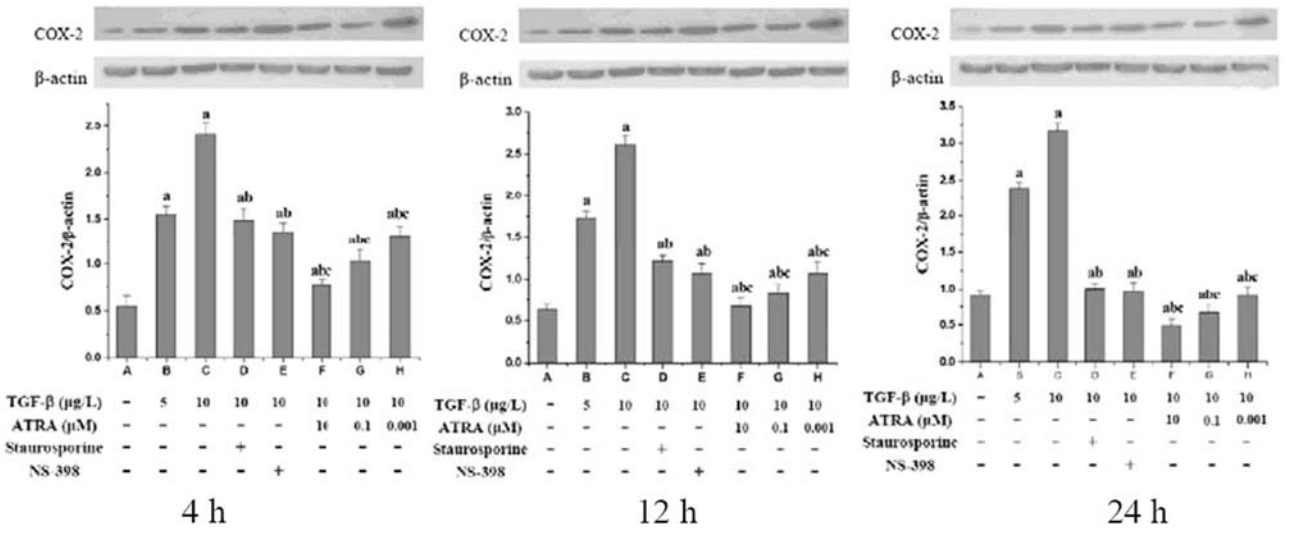

Figure 7 Effects of ATRA on COX-2 protein expression were detected using Western blot in mesangial cells. The mesangial cells treated by 5 and 10 μg/L TGF-β, as well as staurosporine, NS-398, or ATRA at 0.001, 0.1, or 10 μM for 4, 12, and 24 h. The mean results of three separate experiments were shown in the bar graph. $^{a}P < 0.05$ versus A; $^{b}P < 0.05$ versus C; $^{c}P < 0.05$ among F, G, and H

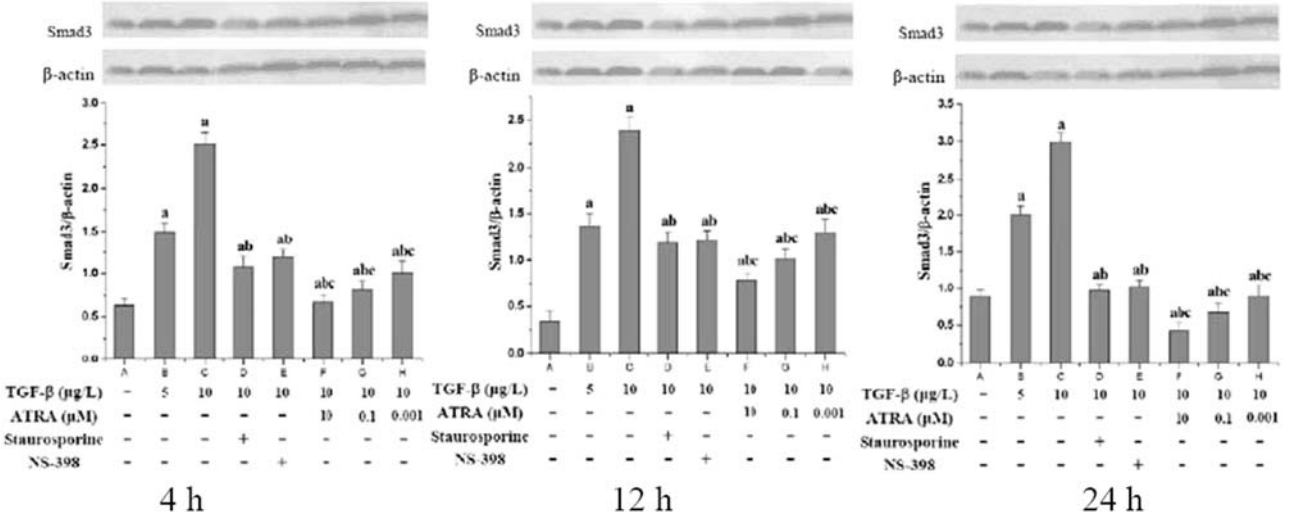

Figure 8 Effects of ATRA on Smad3 protein expression were detected using Western blot in mesangial cells. The mesangial cells treated by 5 and 10 μg/L TGF-β, as well as staurosporine, NS-398, or ATRA at 0.001, 0.1, or 10 μM for 4, 12, and 24 h. The mean results of three separate experiments were shown in the bar graph. $^{a}P < 0.05$ versus A; $^{b}P < 0.05$ versus C; $^{c}P < 0.05$ among F, G, and H

localized in the nucleus in the control group. When mesangial cells were stimulated with 10 μg/L TGF-β, the fluorescence intensity of both Smad3 and Smad7 was significantly higher than that in the control group ($P < 0.05$). However, Smad3 mainly localized in the nucleus, but Smad7 could be found in both nucleus and cytoplasm. In contrast, ATRA treatments inhibited the translocation of Smad3 from the cytoplasm to the nucleus and also inhibited the translocation of Smad7 from the nucleus to the cytoplasm, as the fluorescence intensity was significantly decreased compared with that in the TGF-β group ($P < 0.05$, Figures 12 and 13(a) and (b)).

Discussion

The effects of ATRA on COX-2, PGE_2, TXA_2, proliferation, and apoptosis in mesangial cells stimulated by TGF-β were observed in this study, with a focus on the TGF-β/Smad-signaling pathway. The expressions of Smad3, Smad7, and COX-2 mRNA and protein were significantly increased by TGF-β dose and time-dependently, but were decreased by staurosporine and NS-398. More interestingly, after pretreatment of ATRA at different concentrations and times, the expression of Smad3, Smad7, and COX-2 was also significantly reduced. In addition, PGE_2 and TXA_2, the metabolic products of COX-2, were enhanced by TGF-β, but reduced by staurosporine and NS-398, as well as ATRA. Furthermore, the translocation of Smad3 and Smad7 induced by TGF-β was significantly revised by ATRA treatment, while the proliferation of mesangial cells induced by TGF-β was also inhibited by ATRA, but apoptosis was not affected. Therefore, ATRA-repressed TGF-β induced the expression of COX-2, PGE_2, and TXA_2 through TGF-β/

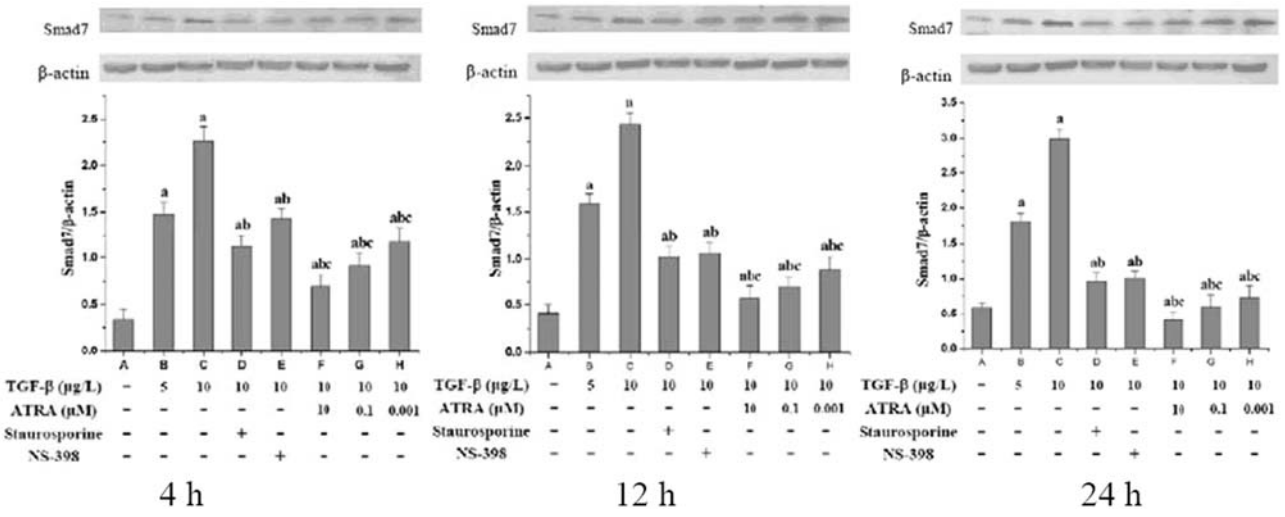

Figure 9 Effects of ATRA on Smad7 protein expression were detected using Western blot in mesangial cells. The mesangial cells treated by 5 and 10 μg/L TGF-β, as well as staurosporine, NS-398, or ATRA at 0.001, 0.1, or 10 μM for 4, 12, and 24 h. The mean results of three separate experiments were shown in the bar graph. $^{a}P < 0.05$ versus A; $^{b}P < 0.05$ versus C; $^{c}P < 0.05$ among F, G, and H

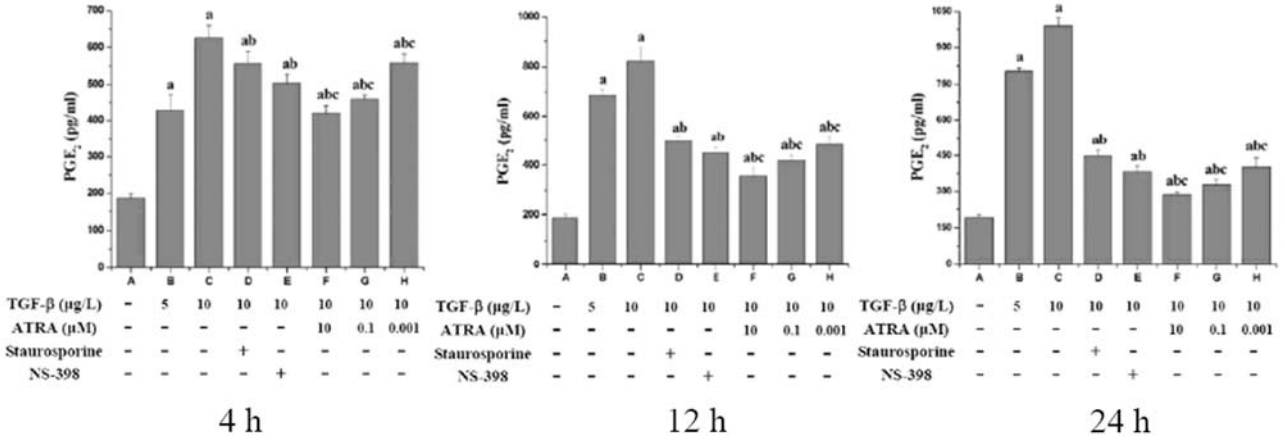

Figure 10 PGE_2 protein detected by ELISA in the supernatant of cultured mesangial cells. The mesangial cells were treated by 5 and 10 μg/L TGF-β, as well as staurosporine, NS-398, or ATRA at 0.001, 0.1, or 10 μM for 4, 12, and 24 h. The mean results of three separate experiments were shown in the bar graph. $^{a}P < 0.05$ versus A; $^{b}P < 0.05$ versus C; $^{c}P < 0.05$ among F, G, and H

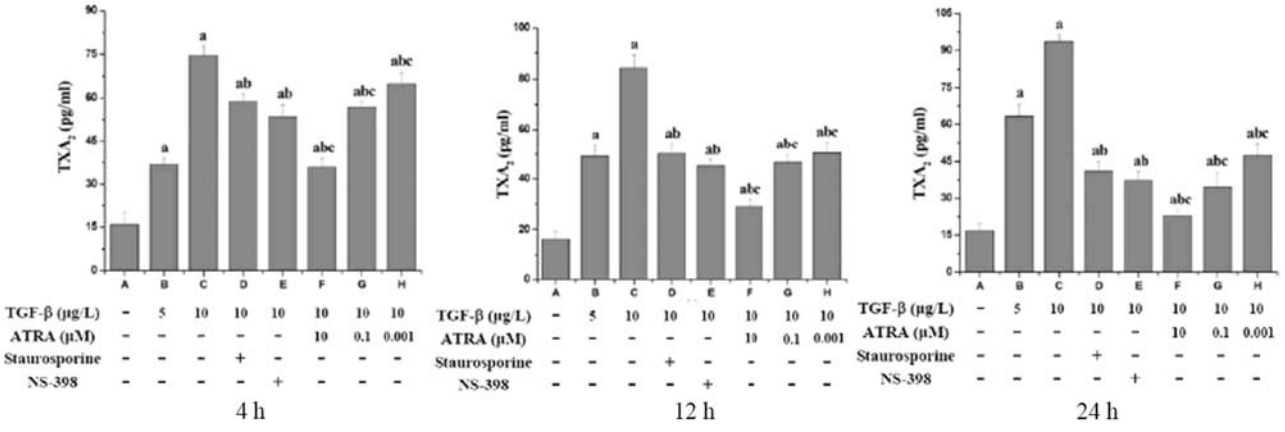

Figure 11 TXA_2 protein detected by ELISA in the supernatant of cultured mesangial cells. The mesangial cells were treated by 5 and 10 μg/L TGF-β, as well as staurosporine, NS-398, or ATRA at 0.001, 0.1, or 10 μM for 4, 12, and 24 h. The mean results of three separate experiments were shown in the bar graph. $^{a}P < 0.05$ versus A; $^{b}P < 0.05$ versus C; $^{c}P < 0.05$ among F, G, and H

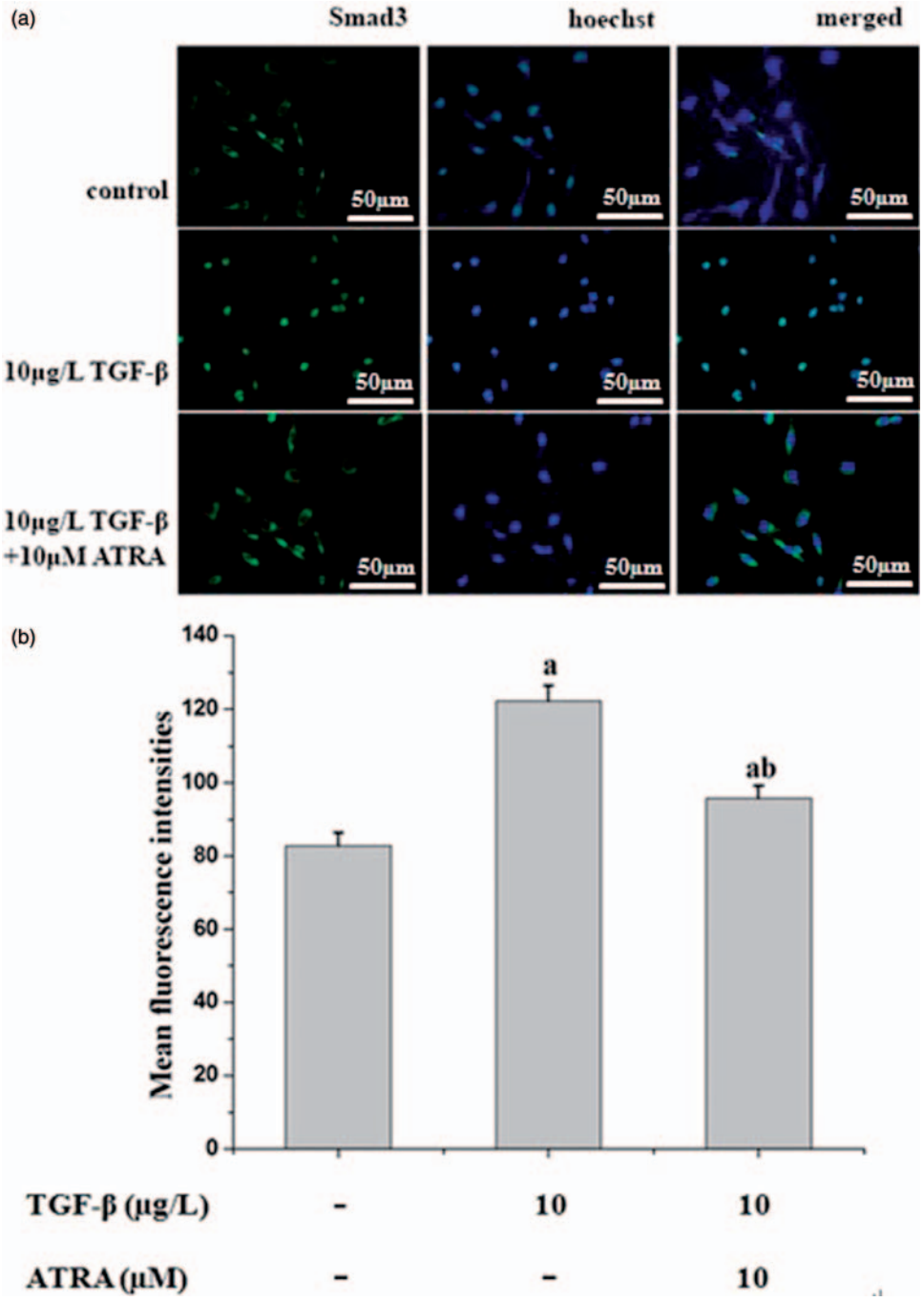

Figure 12 Confocal microscope analysing the effects of ATRA on the subcellular localization of Smad3 in TGF-β-stimulated mesangial cells. (a) Smad3 was mainly localized in the cytoplasm of the control cells. After the stimulation of 10 μg/L TGF-β, not only the fluorescence intensity of Smad3 was increased, but also Smad3 was translocalized in the nucleus. In contrast, ATRA treatments inhibited the translocation of Smad3 from the cytoplasm to the nucleus. (b) The quantitative analysis of Smad3 level was shown in the bar graph. $^{a}P < 0.05$ versus control; $^{b}P < 0.05$ versus TGF-β alone. (A color version of this figure is available in the online journal)

Smad-signaling pathway, which suppressed mesangial-cell proliferation and might subsequently prevent renal fibrosis.

A growing number of studies have recently shown the renoprotective role of retinoic acid (RA) as a new class of drug.[10–13] RA has wide biological functions such as directly or indirectly regulating a variety of growth factors, cytokines, and kinases, among which TGF, CTGF, activator protein-1, interleukin-6, and tumor necrosis factors are mainly associated with chronic renal fibrosis, but also in inflammatory pathways in long-term renal ischemia-reperfusion injury.[14–17] However, there were only limited data for the effect and mechanism of ATRA in the treatment of renal disorders. In this study, ATRA decreased the expression of COX-2, Smad3, and Smad7 at mRNA and protein level in time and dose-dependence manners in mesangial cells stimulated by TGF-β. These results indicate that ATRA inhibited COX-2 expression induced by TGF-β might be through TGF-β/Smad-signaling pathway in mesangial cells.

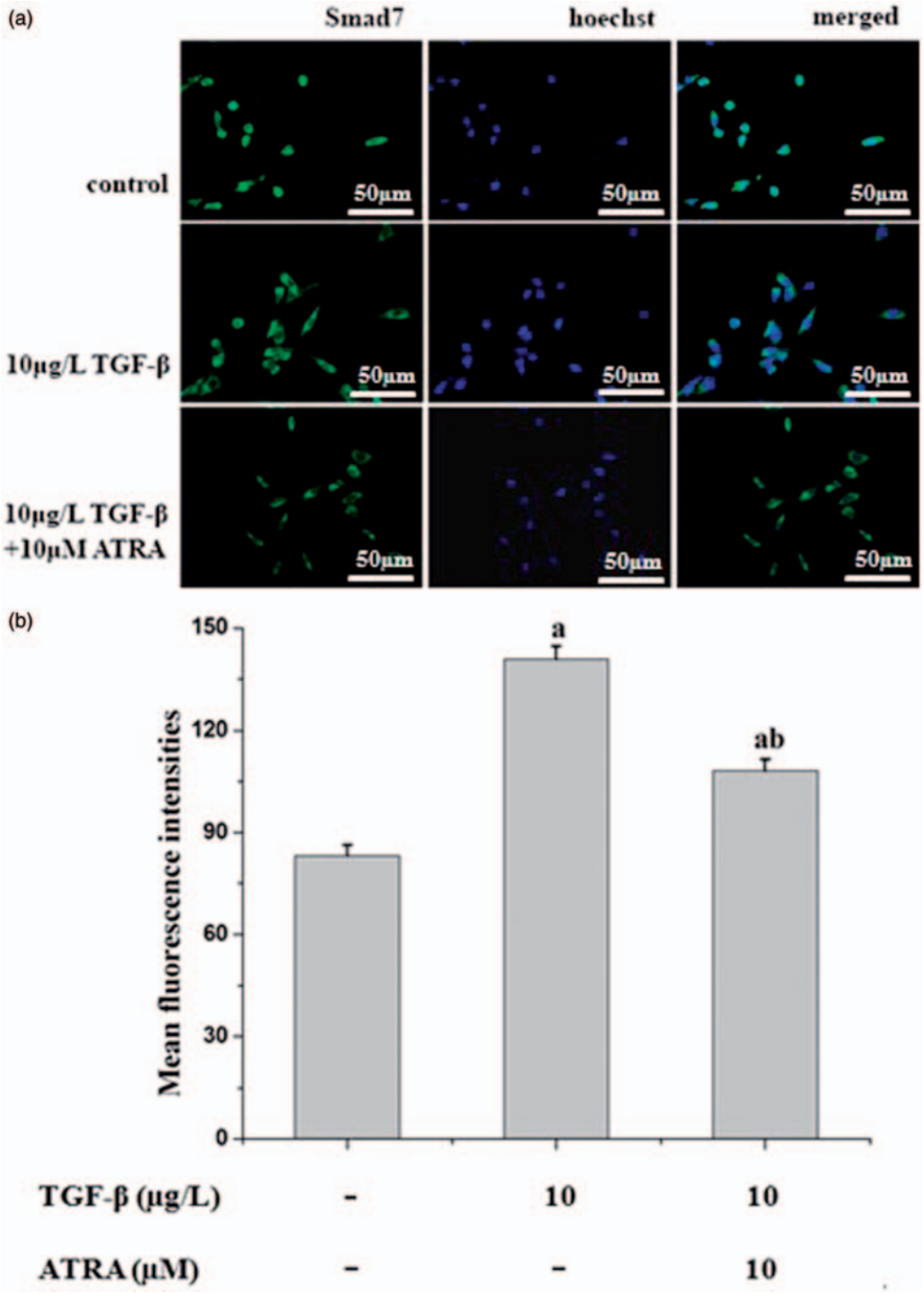

Figure 13 Confocal microscope analysing the effects of ATRA on the subcellular localization of Smad7 in TGF-β-stimulated mesangial cells. (a) The majority of Smad7 was localized in the nucleus in the control group. When mesangial cells were stimulated with 10 μg/L TGF-β, Smad7 was visible in both nucleus and cytoplasm; and its fluorescence intensity was also significantly higher than that in the control group. ATRA treatments inhibited the translocation of Smad7 from the nucleus to the cytoplasm, as the fluorescence intensity was significantly decreased compared with that in the TGF-β group. (b) The quantitative analysis of Smad7 level was shown in the bar graph. $^{a}P < 0.05$ versus control; $^{b}P < 0.05$ versus TGF-β alone. (A color version of this figure is available in the online journal)

TGF-β, a multifunction ambidirectional dominance growth factor, has strong effects on inducing fibrosis. TGF-β and a variety of other cytokines, autocrined by mesangial cells and epithelial cells, promote cell division and proliferation, ECM synthesis, and secretion and then eventually lead to glomerular sclerosis.[18] Kidney is also one of the most active organs containing rich COX-1 and COX-2 to synthesize PG. The expression of COX-2, as well as immunoreactivity, was increased in some residual glomerular visceral epithelial cells and mesangial cells; therefore, COX-2 also plays an important role in the development of many renal diseases.

TGF-β also promotes the activation and/or expression of phospholipase A2 in mesangial cells, and then the membrane phospholipid is hydrolysed, which releases arachidonic acid, necessary substrate of COX-2, and produces prostaglandin that eventually involves in renal inflammatory responses.[19] In order to observe the effect of TGF-β on

COX-2, mesangial cells were stimulated by 5 and 10 μg/L TGF-β for 4, 12, and 24 h, respectively. The expression of COX-2 at both mRNA and protein level was increased dose and time-dependently. Therefore, TGF-β induced the increase of COX-2 not only at transcription level, but also at translation level. In addition, TGF-β may also activate the initiating factors of COX-2,[20] which could stabilize COX-2 and prolong its half-life.

Moreover, the expression of PGE_2 and TXA_2, downstream metabolic products of COX-2, was also increased by TGF-β in mesangial cells. The expression of PGE_2 and TXA_2 was rapidly increased under the stimulation of exogenous TGF-β at different concentrations and reached a peak at 24 h. The reasonable statistical differences were also revealed in the TGF-β-treated groups compared with the control group; and in the groups treated TGF-β at different times. These results indicate that the expression of PGE_2 and TXA_2 in renal mesangial cells stimulated by TGF-β was consistent with that of COX-2.

According to the effect of Smad proteins on the signal transduction of TGF-β family, it can be divided into three categories: first, the control type of receptor Smads, including Smad1, 2, 3, 5, and Smad8; second, common-mediated Smads, namely Smad4; third, inhibitory Smads, including Smad6 and Smad7.[21] In this study, the level of Smad3 mRNA and protein was significantly increased in the mesangial cells treated by TGF-β, because Smad3 is the direct bearers of TGF-β intracellular-signaling transmission and activation. After Smad3 is phosphorlated and activated, Smad3 is translocated into the nucleus, with the participation of Smad2. The expression of Smad7 mRNA and protein was also increased in TGF-β-treated mesangial cells. This is consistent with the report from Park *et al.*[22] Smad7 is a specific endogenous inhibitor of TGF-β, whose role is to inhibit the process of phosphorylation and prevent the signal transduction of TGF-β. In addition, the translocation of Smad3 and Smad7 induced by TGF-β was reversed by ATRA treatment, which further indicated that ATRA-modulated mesangial cells stimulated by TGF-β was via TGF-β/Smad-signaling pathway.

To confirm whether the increased expression of COX-2 in TGF-β stimulated mesangial cells is through the Smad-signaling pathway, an inhibitor of threonine/serine protein kinases, as well as Smad, staurosporine, was applied. It was observed that not only the expression of Smad, but also COX-2 was inhibited. The result suggests that staurosporine inhibited TGF-β/Smad-signaling pathway that subsequently affected its downstream biological events such as the expression of COX-2. The possible mechanism may be that staurosporine prevents TGF-β-dependent formation of Smad2/4 complex and inhibits Smad phosphorylation and translocation into the nucleus. Staurosporine may also inhibit TGF-β-signaling pathway by directly binding activated TGF-β factors, reducing the number of TGF-β receptors on the membrane, and affecting the acetylation of transcription factors. When TGF-β was combined with Smad, the activation of COX-2 promoter factor was consequently reduced, and the expression of COX-2 was then decreased.

Additionally, after NS-398, a selective COX-2 inhibitor was added, of course, the expression of COX-2, as well as PGE_2 and TXA_2, was inhibited. Interestingly, the level of Smad was also significantly decreased by NS-398. It has been reported that NS-398 suppressed PGE_2 synthesis and induced collagen expression in hepatic stellate cells. The mechanism may be that NS-398 affected the phosphorylation and nuclear translocation of Smads induced by TGF-β, blocked the Smad accumulation in cells, and then blocked the activation of TGF-β responding genes.[23] Therefore, we speculated that COX-2 may be one of the target genes regulated by TGF-β via Smad-signaling pathway.

Renal fibrosis is characterized by glomerulosclerosis, tubular atrophy, and ECM deposition, the final pathological change of renal injury induced by many different causes. Mesangial cells are the major cells to produce ECM, which are most associated with the pathological process of glomerulosclerosis. Studies have shown that TGF-β promoted glomerulosclerosis by inducing secretion and accumulation of ECM. In the present study, TGF-β as astimulator induced mesangial-cell proliferation, which was dose-dependently inhibited by ATRA. These results indicate a beneficial effect of ATRA that may be applied in treating mesangial proliferative glomerulonephristis and preventing glomerulosclerosis.

TGF-β has also been shown to be a potent modulator of apoptosis in a variety of cell types, including human cholangiocarcinoma cells,[24] podocytes,[25] and hepatocellular carcinoma cell lines.[26] However, substantial evidence suggests that TGF-β also have the ability to inhibit apoptosis in many cell types, such as murine macrophages,[27] lung mesenchymal cells,[28] murine neurons,[29] and fibroblast cells.[30] It has been reported that TGF-β inhibits mouse mesangial-cell apoptosis via induction of autophagy.[31] In our study, TGF-β inhibited apoptosis of mesangial cells, which was consistent with the previous reports. In addition, ATRA can induce a variety of cell apoptosis, including normal human mammary epithelial cells[32] and vascular smooth muscle cells,[33] but the reports about the effect of ATRA on mesangial-cell apoptosis are rare. The result from this study showed that ATRA had no significant effect on the apoptosis of mesangial cells detected by flow cytometry, which was consistent with the previous studies.[34,35]

In conclusion, ATRA inhibited the expression of COX-2, PGE_2, and TXA_2 through TGF-β/Smad-signaling pathway in TGF-β-stimulated mesangial cells and suppressed mesangial-cell proliferation, which might subsequently prevent renal fibrosis. The direct effects of ATRA on mesangial function and renal fibrosis need to be further investigated in both *in vitro* and *in vivo* models.

Author contributions: All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript; JH, NG and YF conducted the experiments; JH, LZ and NG analyzed samples and data; JH and LZ wrote the paper; XC and BY participated in research design and support. JH and LZ contributed equally to this work.

ACKNOWLEDGEMENTS

This work is supported by the National Natural Science Foundation of China (81170656) and Jiangsu Basic Research Program (BK2008185).

REFERENCES

1. Klahr S, Morrissey JJ. The role of vasoactive compounds, growth factors and cytokines in the progression of renal disease. *Kidney Int* 2000;**75**(Suppl): S7–S14
2. Shihab FS, Yamamoto T, Nast CC, Cohen AH, Noble NA, Gold LI, Border WA. Transforming growth factor-beta and matrix protein expression in acute and chronic rejection of human renal allografts. *J Am Soc Nephrol* 1995;**6**:286–94
3. Wrana JL, Attlsano L. The Smad pathway. *Gytokine Growth Factor Rev* 2000;**11**(1–2): 5–13
4. Sun LK, Beck-Schimmer B, Oertli B. Hyaluronan-induced cyclooxygenase-2 in patients with active nephritis. *Kidney Int* 2001;**59**:190–6
5. Cheng HF, Wang CJ, Moeckel GW, Zhang MZ, McKanna JA, Harris RC. Cyclooxygenase-2 inhibitor blocks expression of mediators of renal injury in a model of diabetes and hypertension. *Kidney Int* 2002;**62**:929–39
6. Harris SG, Padilla J, Koumas L, Ray D, Phipps RP. Prostaglandins as modulators of immunity. *Trends Immunol* 2002;**23**:144–50
7. Poole S, Lorenzetti BB, Cunha JM, Cunha FQ, Ferreira SH. Bradykinin B1 and B2 receptors, tumour necrosis factor alpha and inflammatory hyperalgesia. *Br J Pharmacol* 1999;**126**:649–56
8. Nataraj C, Thomas DW, Tilley SL, Nguyen MT, Mannon R, Koller BH, Coffman TM. Receptors for prostaglandin E_2 that regulate cellular immune responses in the mouse. *J Clin Invest* 2001;**8**:1229–35
9. Lopez-Parra M, Claira J, Planaguma A, Titos E, Masferrer JL, Woerner BM, Koki AT, Jimenez W, Altuna R, Arroyo V, Rivera F, Rodes J. Cyclooxygenase-1 derived prostaglandins are involved in the maintenance of renal function in rats with cirrhosis and ascites. *Br J Pharmacol* 2002;**135**:891–900
10. Nozaki Y, Yamagata T, Yoo BS, Sugiyama M, Ikoma S, Kinoshita K, Funauchi M, Kanamaru A. The beneficial effects of treatment with all-trans-retinoic acid plus corticosteroid on autoimmune nephritis in NZB/WF mice. *Clin Exp Immunol* 2005;**139**:74–83
11. Oseto S, Moriyama T, Kawada N, Nagatoya K, Takeji M, Ando A, Yamamoto T, Imai E, Hori M. Therapeutic effect of all-trans retinoic acid on rats with anti-GBM antibody glomerulo- nephritis. *Kidney Int* 2003;**64**:1241–52
12. Zhang J, Pippin JW, Vaughan MR, Krofft RD, Taniguchi Y, Romagnani P, Nelson PJ, Liu ZH, Shankland SJ. Retinoids augment the expression of podocyte proteins by glomerular parietal epithelial cells in experimental glomerular disease. *Nephron Exp Nephrol* 2012;**121**(1–2): e23–37
13. Kinoshita K, Funauchi M. Therapeutic effect of retinoic acid in lupus nephritis. *Nihon Rinsho Meneki Gakkai Kaishi* 2012;**35**:1–7
14. Delgadillo D, Barbier O, Sierra G, Reyes JL. Retinoic acid improves recovery after nephrectomy and decreases renal TGF-β1 expression. Gender-related effects. *Fundam Clin Pharmacol*, doi:10.1111/fcp.12013
15. Ye Y, Dan Z. All-trans retinoic acid diminishes collagen production in a hepatic stellate cell line via suppression of active protein-1 and c-Jun N-terminal kinase signal. *J Huazhong Univ Sci Technol Med Sci* 2010;**30**:726–33
16. Shima Y, Iwano M, Yoshizaki K, Tanaka T, Kawase I, Nishimoto N. All-trans-retinoic acid inhibits the development of mesangial proliferative glomerulonephritis in interleukin-6 transgenic mice. *Nephron Exp Nephrol* 2005;**100**:e54–62
17. Nizamutdinova IT, Guleria RS, Singh AB, Kendall JA Jr, Baker KM, Pan J. Retinoic acid protects cardiomyocytes from high glucose-induced apoptosis through inhibition of NF-κB signaling pathway. *J Cell Physiol* 2013;**228**:380–92
18. Izabella ZA, Pawluczyk IZ, Harris KP. Cytokine interactions promote synergistic fibronectin accumulation by mesangial cells. *Kidney Int* 1998;**54**:62–70
19. Schalkwijk CG, de Vet E, Pfeilschifter J, van den Bosch H. Interleukin-1 beta and transforming growth factor-beta 2 enhance cytosolic high-molecular-mass phospholipase A2 activity and induce prostaglandin E2 formation in rat mesangial cells. *Eur J Biochem* 1992;**219**:169–76
20. Harding P, Balasubramanian L, Swegan J, Stevens A, Glass WF II. Transforming growth factor beta regulates cyclooxygenase-2 in glomerular mesangial cells. *Kidney Int* 2006;**69**:1578–85
21. Mishra L, Shetty K, Tang Y, Stuart A, Byers SW. The role of TGF-beta and Wnt signaling in gastrointestinal stem cells and cancer. *Oncogene* 2005;**24**: 5775–89
22. Park IS, Kiyomoto H, Abboud SL, Abboud HE. Expression of transforming growth factor-β and type IV collagen in early streptozotocin-induced diabetes. *Diabetes* 1997; **46**:473–80
23. Hui AY, Dannenberg AJ, Sung JJ, Subbaramaiah K, Du B, Olinga P, Friedman SL. Prostaglandin E2 inhibits transforming growth factor beta 1-mediated induction of collagen alpha 1_I in hepatic stellate cells. *J Hepatol* 2004;**41**:251–8
24. Lin Y, Zhang B, Liang H, Lu Y, Ai X, Zhang B, Chen X. JNK inhibitor SP600125 enhances TGF-β-induced apoptosis of RBE humancholangiocarcinoma cells in a Smad-dependent manner. *Mol Med Rep;* doi: 10.3892/mmr.2013.1711
25. Shi S, Yu L, Zhang T, Qi H, Xavier S, Ju W, Bottinger E. Smad2-dependent downregulation of miR-30 is required for TGF-β-induced apoptosis in podocytes. *PLoS One* 2013;**8**:e75572
26. Dzieran J, Fabian J, Feng T, Coulouarn C, IIkavets I, Kyselova A, Breuhahn K, Dooley S, Meindl-Beinker NM. Comparative analysis of TGF-β/Smad signaling dependent cytostasis in human hepatocellular carcinoma cell lines. *PLoS One* 2013;**8**:e72252
27. Chin BY, Petrache I, Choi AM, Choi ME. Transforming growth factor beta1 rescues serum deprivation-induced apoptosis via the mitogen-activated protein kinase (MAPK) pathway in macrophages. *J Biol Chem* 1999;**274**:11362–8
28. Horowitz JC, Lee DY, Waghray M, Keshamouni VG, Thomas PE, Zhang H, Cui Z, Thannickal VJ. Activation of the pro-survival phosphatidylinositol 3-kinase/AKT pathway by transforming growth factor-beta1 in mesenchymal cells is mediated by p38 MAPK-dependent induction of an autocrine growth factor. *J Biol Chem* 2004;**279**:1359–67
29. Dhandapani KM, Hadman M, De Sevilla L, Wade MF, Mahesh VB, Brann DW. Astrocyte protection of neurons: role of transforming growth factor-beta signaling via a c-Jun-AP-1 protective pathway. *J Biol Chem* 2003;**278**:43329–39
30. Chen HH, Zhao S, Song JG. TGF-beta1 suppresses apoptosis via differential regulation of MAP kinases and ceramide production. *Cell Death Differ* 2003;**10**:516–27
31. Ding Y, Kim JK, Kim SI, Na HJ, Jun SY, Lee SJ, Choi ME. TGF-β1 protects against mesangial cell apoptosis via induction of autophagy. *J Biol Chem* 2010;**285**:37909–19
32. Sumantran VN, Zhang R, Lee DS, Wicha MS. Differential regulation of apoptosis in normal versus transformed mammary epithelium by lutein and retinoic acid. *Cancer Epidemiol Biomarkers Prev* 2000;**9**:257–63
33. Ou H, Haendeler J, Aebly MR, *et al*. Retinoic acid-induced tissue transglutaminase and apoptosis in vascular smooth muscle cells. *Circ Res* 2000;**87**:881–7
34. Moreno-Manzano V, Ishikawa Y, Lucio-Cazana J, Kitamura M. Suppression of apoptosis by all-trans-retinoic acid: dual intervention in the c-Jun n-terminal kinase-AP-1 pathway. *J Biol Chem* 1999;**274**:20251–8
35. Su B, Chen X, Zhong C, Guo N, He J, Fan Y. All-trans retinoic acid inhibits mesangial cell proliferation by up-regulating $p21^{Waf1/Cip1}$ and p^{27Kip1} and down-regulating Skp2. *J Nephrol* 2012;**25**:1031–40

(Received February 22, 2013, Accepted November 19, 2013)