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Fluorofenidone inhibits TGF-β1 induced CTGF via MAPK pathways in mouse mesangial cells

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Objectives: The development of novel antifibrotic agent candidates for the treatment of diabetic nephropathy. The present study was designed to investigate the potential mechanism of fluorofenidone involving the downregulation of CTGF expression induced by TGF-β1 and the related signaling pathway in mouse mesangial cells (MMCs). *Methods:* Mouse mesangial cells were applied to explore the involvement of MAPK in TGF-β1 signal pathway to CTGF, and the regulation of fluorofenidone. The activation of three major members of MAPK, including ERK1/2, P38 and JNK was detected by Western blot; the expression of CTGF was investigated by real time PCR and Western blot. *Results:* Fluorofenidone significantly reduced the phosphorylation of ERK1/2, P38 and JNK induced by TGF-β1. Fluorofenidone, PD98059 and SB203580 could partially inhibit TGF-β1-induced expression of CTGF in mouse mesangial cells, however, JNK inhibitor II had no effect. *Conclusions:* The antifibrotic effects of fluorofenidone are suggested to be mediated by its actions through inhibition of MAPK activation and consequent reduction of CTGF expression.

1. Introduction

Diabetic nephropathy, a major complication of diabetes, has been admitted as leading cause of end-stage renal failure in many countries. The main features of early diabetic renal changes are glomerular hyperfiltration, glomerular and renal hypertrophy, increased urinary albumin excretion (UAE), increased basement membrane thickness (BMT), and mesangial expansion with the accumulation of extracellular matrix (ECM) proteins such as collagen, fibronectin, and laminin. Advanced diabetic nephropathy is characterized by glomerulosclerosis and interstitial fibrosis, which will result in renal dysfunction. However, current therapeutic approaches to the reversal of progressive renal fibrosis are under discussion (Nahas 1997; Liu 2006).

A series of cytokines and growth factors have been documented to play vital roles in the pathogenesis of diabetic nephropathy. Prominent among these is transforming growth factor β1 (TGF-β1). Its unique ability to stimulate ECM accumulation contributes to the major influence on the development of glomerulosclerosis and interstitial fibrosis (Wolf 2006). However, mice homozygous for a TGF-β1 gene deletion showed that a deficiency of TGF-β1 has the potential to up-regulate inflammatory activity leading to animal death (Kopp 1996). Concerning the important biological role of TGF-β1, a more specific inhibition of TGF-β1 downstream signals may provide the benefit of biological safety in anti-fibrosis treatment. As the major downstream mediator of the profibrotic activity of TGF-β1, connective tissue growth factor (CTGF) is suggested a potential therapeutic target without affecting the biological activity and functions of TGF-β1 (Sakharova 2001).

Although Smads proteins generally appear to be the major mediators for TGF-β1-induced CTGF expression, a growing body of information is emerging with regard to the additional important intracellular signaling pathways of TGF-β1. The signals of TGF-β1 can be transduced either in Smads-independent manner or through the cross talk with Smads signaling pathways. Mitogen-activated protein kinase (MAPK), which is another important signaling pathway, is also found to be activated by TGF-β1 in a number of various cells (Chin 1999). MAPK is an evolutionarily conserved family of enzymes that form a highly integrated network required to achieve specialized cell functions controlling cell differentiation, proliferation and death by transducing extracellular signals into cellular responses (Kyriakis and Avruch 2001; Cuschieri and Maier 2005). Three important members of MAPK family are extracellular signal-regulated kinase (ERK), p38, and c-Jun N-terminal kinase (JNK). They are of great importance for the development and progression of diabetic nephropathy (Kikkawa 2003).

Fluorofenidone (1-(3-fluorophenyl)-5-methyl-2-(1*H*)-pyridine) is a novel pyridone agent. Many studies have reported that pyridone agents, such as pirfenidone, can attenuate fibrosis in many organs including pulmonary (Nakayama 2008), cardiac (Van 2006), renal (Cho 2007)

and hepatic fibrosis (Di 2004). The antifibrotic activity of pirfenidone, which inhibits TGF-β1-induced p38 activation/activity in fibroblasts, is mediated at least partly through the inhibition of p38 MAPK signal transduction pathway (Ozes et al. 2006). Moreover, the pyridone agent has been shown the protection against renal fibrosis in streptozotocin-induced diabetic rats (Miric et al. 2001). Furthermore, it also demonstrated the effect of reducing CTGF expression in rat renal fibroblast (Hewitson et al. 2001). As a newly developed pyridone agent with potential broad-spectrum anti-fibrotic characteristics, fluorofenidone has a chemical structure similar to pirfenidone (Cao et al. 2006). The structure difference between fluorofenidone and pirfenidone is that the hydro- at the meta-position of the benzene ring in pirfenidone is replaced by fluoro- in fluorofenidone. As compared to hydrogen, fluorine has a smaller atomic radius and larger electronegativity. These properties of fluorine will increase the stability and physiological activity of fluorine compounds. Moreover, fluorine compounds also have a high hydrophobicity, which can promote their absorption and transmission speed *in vivo*. With similar toxicity between fluorofenidone and pirfenidone in the preliminary experiments (data not included here), we assumed that fluorofenidone with special physicochemical properties may have stronger anti-fibrosis effects than pirfenidone. However, fluorofenidone exerted the same abilities of anti-oxidation and anti-fibrosis as pirfenidone in a previous study (published elsewhere). Fluorofenidone can obviously inhibit the proliferation of rat renal fibroblasts, which may be effective for renal interstitial fibrosis prevention and treatment (Tao et al. 2004). However, its therapeutic effects and mechanism on renal fibrosis remain unclear so far. The present study is designed to investigate the mechanism of the downregulation of TGF-β1-induced CTGF expression by fluorofenidone and the related signaling pathway in mouse mesangial cells (MMCs).

2. Investigations and results

2.1. TGF-β1 activated MAPK in MMCs

As shown in Fig. 1, TGF-β1 (1 ng/ml) did not alter the protein expression levels of total ERK1/2, P38, and JNK from 15 min to 120 min after TGF-β1 treatment in MMCs. However, TGF-β1 significantly increased the phosphorylation of these three major MAPK members at the time point of 15 min.

2.2. Fluorofenidone inhibited TGF-β1-induced MAPK in MMCs

MMCs were pretreated with fluorofenidone (0.5 mM, 1 mM, 2 mM, 4 mM) 1 h before the stimulation of TGF-β1 (1 ng/ml) for 15 min. MAPK activation was detected by Western blot for p-MAPK signaling molecules. The protein expression of three major MAPK members, which were ERK1/2, P38 and JNK, remained unchanged after TGF-β1 treatment. Interestingly, fluorofenidone inhibited the phosphorylation of respective MAPK members induced by TGF-β1 in a dose-dependent manner. Due to the significant inhibition effect of fluorofenidone at the concentration of 2 mM on MAPK phosphorylation, a 2 mM concentration of fluorofenidone was chosen for subsequent experiments (Fig. 2). The inhibition effect of fluorofenidone (2 mM) on MAPK phosphorylation induced by TGF-β1 was further proved by the treatment of PD98059

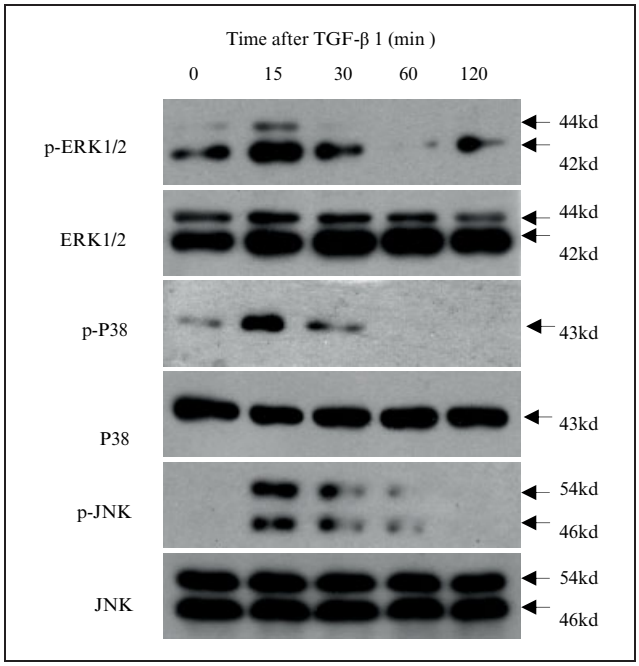

Fig. 1: Time-course changes of p-MAPK in MMCs. The gels represent the Western blot results of p-ERK1/2, p-P38 and p-JNK protein expression at different times after treatment of TGF-β1 respectively. T-ERK1/2, t-P38 and t-JNK are used as the internal loading control respectively

(25 μM), or SB203580 (10 μM), or JNK inhibitor II (10 μM of) for 1 h followed by TGF-β1 (1 ng/ml) stimulation for 15 min. The phosphorylation of ERK1/2, P38, and JNK, which were all blocked by fluorofenidone, was inhibited by PD98059 (Fig. 3, $p < 0.05$), SB203580 (Fig. 4, $p < 0.05$), and JNK inhibitor II (Fig. 5, $p < 0.05$).

2.3. Fluorofenidone inhibited TGF-β1-induced CTGF expression via MAPK pathway in MMCs

MMCs were pretreated with fluorofenidone, PD98059, SB203580 and JNK inhibitor II for 1 h followed by TGF-

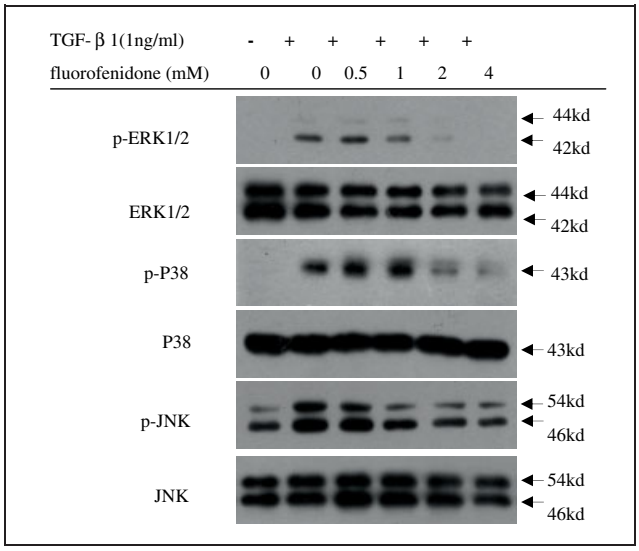

Fig. 2: In dose-dependence, fluorofenidone inhibits TGF-β1-induced MAPK in MMCs. The gels represent effects of different concentrations of fluorofenidone on TGF-β1-induced protein expression of p-ERK1/2, p-P38 and p-JNK in MMCs. T-ERK1/2, t-P38 and t-JNK are used as the internal loading control respectively

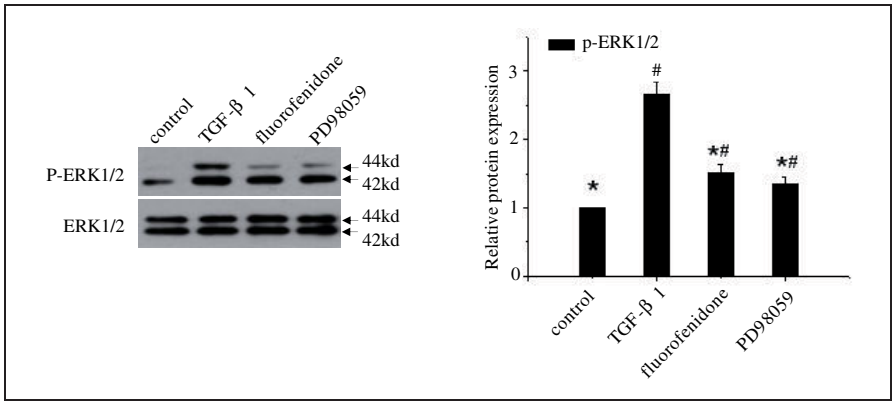

Fig. 3:
Effect of fluorofenidone on protein level of p-ERK1/2 in MMCs. The gels represent the Western blot results of p-ERK1/2 protein expression in the groups of TGF-β1+fluorofenirofenidone, TGF-β1+PD98059, TGF-β1, control respectively. TERK1/2 is used as the internal loading control. The columnar section is the optical density (OD) of the gels tested by Western blot. "#" and "*" represent $p < 0.05$, as respectively compared with control group and TGF-β1 group, respectively

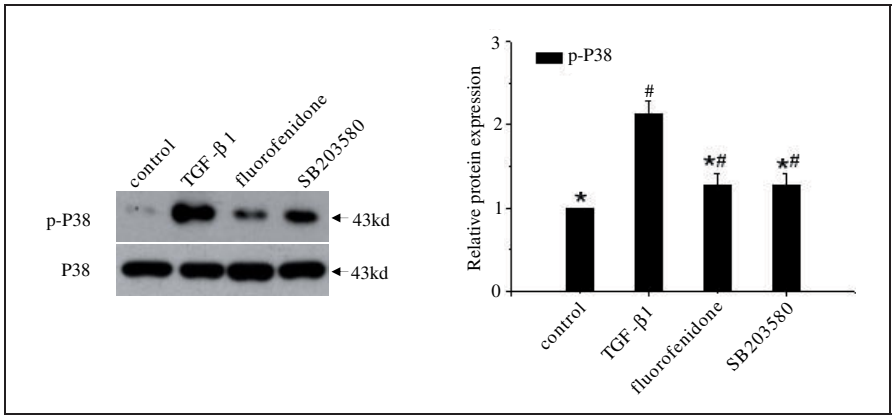

Fig. 4:
Effect of fluorofenidone on protein level of p-P38 in MMCs. The gels represent the Western blot results of p-P38 protein expression in the groups of TGF-β1+fluorofenidone, TGF-β1+SB203580, TGF-β1, control respectively. T-P38 is used as the internal loading control. The columnar section is the optical density (OD) of the gels tested by Western blot. "#" and "*" represent $p < 0.05$, as respectively compared with control group and TGF-β1 group, respectively

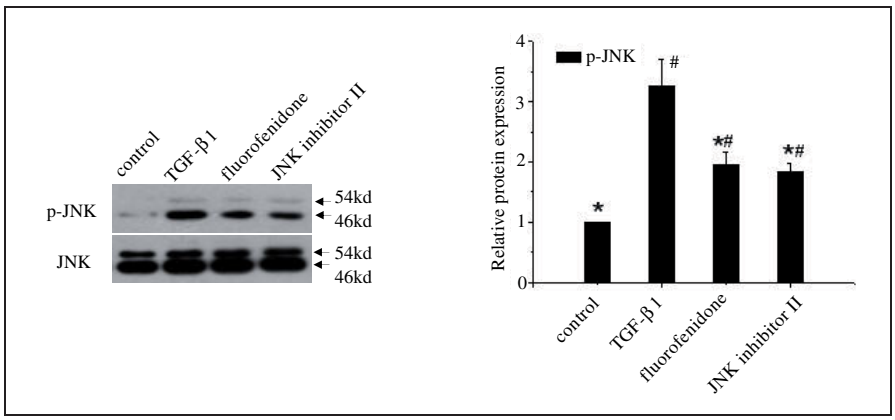

Fig. 5:
Effect of fluorofenidone on protein level of p-JNK in MMCs. The gels represent the Western blot results of p-JNK protein expression in the groups of TGF-β1+fluorofenidone, TGF-β1+JNK inhibitor II, TGF-β1, control respectively. T-JNK is used as the internal loading control. The columnar section is the optical density (OD) of the gels tested by Western blot. "#" and "*" represent $p < 0.05$, as respectively compared with control group and TGF-β1 group, respectively

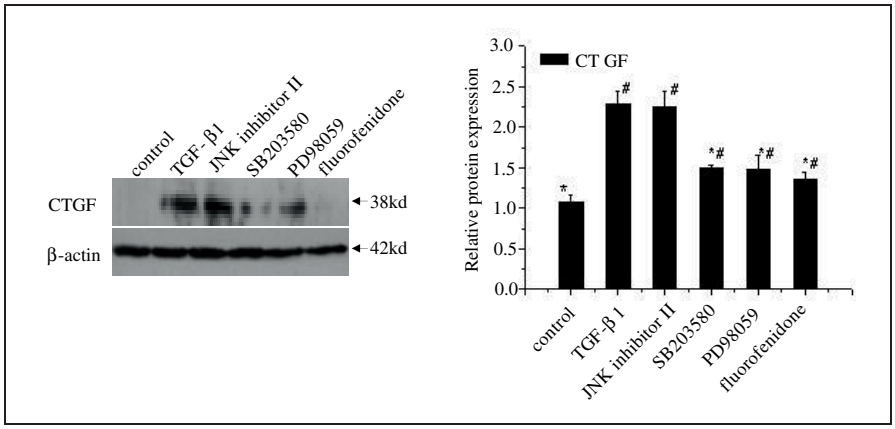

Fig. 6:
Effect of fluorofenidone on protein expression level of CTGF in MMCs. The gels represent the Western blot results of CTGF protein expression in the groups of TGF-β1, TGF-β1+PD98059, TGF-β1+SB203580, TGF-β1+JNK inhibitor II, TGF-β1+fluorofenidone, control respectively. Beta-actin are used as the internal loading control respectively. The columnar section is the optical density (OD) of the gels tested by Western blot. "#" and "*" represent $p < 0.05$, as respectively compared with control group and TGF-β1 group, respectively

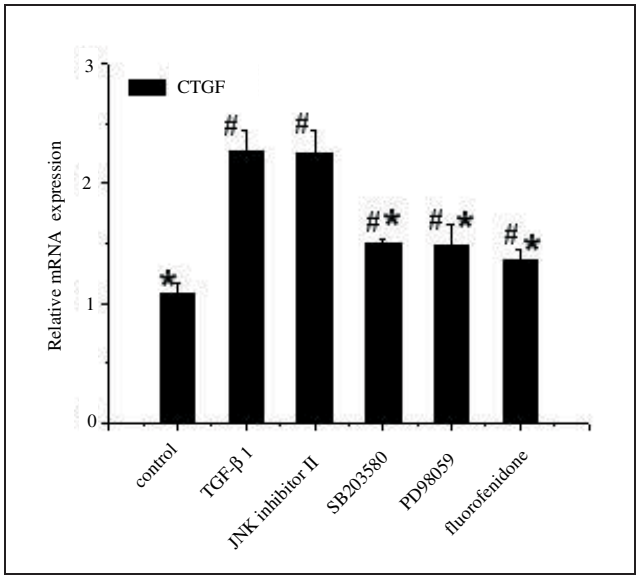

Fig. 7: Effect of fluorofenidone on relative expression level of CTGF mRNA in MMCs. The panel represents the mRNA expression of CTGF induced by TGF-β1 in the groups of TGF-β1, TGF-β1+PD98059, TGF-β1+SB203580, TGF-β1+JNK inhibitor II, TGF-β1+fluorofenidone, control respectively. "#" and "*" represent $p < 0.05$, as respectively compared with control group and TGF-β1 group, respectively

β1 (1 ng/ml) stimulation for 24 h. As compared with the control without any treatment, TGF-β1 significantly induced mRNA and protein expression of CTGF in MMCs. The induction of CTGF expression by TGF-β1 was significantly blocked by PD98059 (Erk inhibitor), SB203580 (P38 inhibitor), and fluorofenidone (Figs. 6, 7, $p < 0.05$, respectively). However, the pretreatment of JNK inhibitor II did not change CTGF expression enhanced by TGF-β1.

3. Discussion

The mesangial cell occupies a central position in the renal glomerulus. It has characteristics of a modified smooth muscle cell, but is also capable of a number of other functions. Among these are the generation of prostaglandins (PGs) and mediators of inflammation (Buschhausen et al. 2001); production and breakdown of basement membrane and other bio-matrix material (Ahn 2004); synthesis of cytokines (Cernuda-Morollón et al. 2002); and uptake of macromolecules, including immune complexes (Novak et al. 2002). TGF-β1 is closely associated with mesangial cell fibrogenesis (Schnaper et al. 2003), and it has been reported to be expressed in human glomeruli and be associated with increased mesangial matrix in diabetic nephropathy (Weigert et al. 2000a; Ceol et al. 2000b). Meanwhile, the effects of TGF-β on fibroblast proliferation and ECM deposition are mediated by CTGF (Okada et al. 2005; Ikawa et al. 2008). More recent studies have shown that CTGF expression is strongly up-regulated in diabetic nephropathy and other progressive renal diseases (Qi et al. 2005). Moreover, the involvement of MAPK in TGF-β signaling has been identified in various cells (Chin 1999). The present study further provides the evidences that TGF-β1 stimulates the activation of all three major members of MAPK pathway including ERK, p38 MAPK, and JNK in MMCs. The decreased TGF-β1-induced CTGF expression by only the blockade of ERK with PD98059 and p38 MAPK with SB203580 indicates that the regulation of TGF-β1 on CTGF expression is through ERK and p38 pathways, but not via JNK. Our results are consistent with the finding that PD98059 and SB203580 can downregulate TGF-β1-induced increase of CTGF promoter activity in mouse NIH/3T3 cells (Zhao 2004). With not only the promoter region of the CTGF gene but also the substrates of MAPK, the transcription factor of AP-1 may play important roles in CTGF expression regulated by TGF-β1 (Moritani et al. 2003; Ouyang et al. 2008). Additionally, the cross-talk between MAPK and Smad pathways may also contribute to TGF-β1-regulated CTGF expression, because ERK and p38 MAPK pathway can directly interact with activated Smad proteins (Xiao et al. 2002).

As a newly developed pyridone agent, fluorofenidone exerts its inhibition effects on TGF-β1-induced MAPK activation and CTGF expression in MMCs for the first time in our study. Moreover, our data indicate that TGF-β1-induced CTGF expression is blocked by fluorofenidone through its inhibition on ERK and P38 phosphorylation. Since the JNK signaling pathway is a cell stress activated pathway involved in the regulation of cell proliferation and apoptosis (Hommes et al. 2003), the inhibition of fluorofenidone exerting on JNK activation may have other kind of functions on the biological behaviors of MMCs.

In summary, fluorofenidone inhibited TGF-β1-induced CTGF expression in MMCs. The downregulation of fluorofenidone on CTGF expression induced by TGF-β1 are suggested to be through the blockage of activation of both ERK and P38 by fluorofenidone. Fluorofenidone is supposed to be a new antifibrotic agent for preventing kidney diseases leading to renal fibrosis.

4. Experimental

4.1. Materials

MMCs were purchased from American Type Culture Collection (Rockville, MD). Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS), and Trizol Reagent were purchased from Invitrogen (Carlsbad, CA). PD98059 (a specific inhibitor of ERK1/2 MAPK), SB203580 (a specific inhibitor of p38 MAPK), and JNK inhibitor II (a specific inhibitor of JNK MAPK) were purchased from Calbiochem (Darmstadt, Germany). The respective antibody against phospho-P38 (p-P38) MAPK, p-ERK1/2, p-JNK, and total specific MAPK (t-MAPK) was purchased from Cell Signaling Technology (Danvers, MA). HRP-conjugated secondary antibodies and other chemicals were from Sigma-Aldrich Ltd (St. Louis, MO) unless otherwise indicated. Fluorofenidone (Lot No.070501) were synthesized by Pharmaceutical School of Central South University.

4.2. Cell culture and treatment

MMCs were cultured in DMEM (containing 5.5 mM glucose) supplemented with 10% FBS, 110 mg/L sodium pyruvate, 10 mM HEPES, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Before treatment, 80%–85% confluent cells were incubated with serum-free media for 6 h to arrest and synchronize the cell growth. Cells were pretreated with PD98059 (25 μM), SB203580 (10 μM), and JNK inhibitor II (10 μM) in the absence or presence of 2 mM fluorofenidone for 1 h. The pretreated cells were subsequently treated with recombinant TGF-β_1 at the concentration of 1 ng/ml (R & D Systems, Minneapolis, MN).

4.3. Western blot

The cell lysate containing 20 μg of total protein was separated on 10% sodium dodecyl sulfate-polyacrylamide (SDS-polyacrylamide) gel under reducing conditions, and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, USA). The transferring quality of each gel was monitored by Ponceau-S staining of the membrane. Nonspecific antibody binding was blocked by a pre-incubation of the membranes in 1X Tris-buffered-saline (TBS) containing 5% skim milk for 1 h at room temperature. Membranes were then incubated overnight at 4 °C with primary antibodies against p-ERK1/2, p-JNK, p-P38, or CTGF (Abcam, Cambridge, England) at 1 : 1000 dilutions in 1X TBS containing 2% skim milk. After washing, they were incubated with HRP-conjugated secondary antibodies at 1 : 1000 dilutions for 1 h at room temperature. Bands were visualized by

employing the enhanced chemiluminescence kit (Amersham, Piscataway, NJ) per the manufacturer's manual (Shen et al. 2005). For equal loading test, the blotted membranes were stripped and re-probed with the primary antibody against t-MAPK or β-actin, respectively. The autoradiographic films were scanned densitometrically and quantitated using Quantity One System (Bio-Rad, Hercules, CA).

4.4. Quantitative real-time PCR

Total RNA was isolated from MMCs by Trizol reagent according to the manufacturer's instruction. First-strand cDNAs were synthesized from 2 μg of total RNA in 20 μl reaction using reverse transcriptase (Fermentas, Hanover, MD). Real-time PCR was performed in an Applied Biosystems 7900HR Fast Real-Time PCR System using a SYBR green PCR reagent kit (SYBR Premix Ex Taq, Takara, Japan). The mRNA expression levels of interested genes were determined based on the formula $2^{-\Delta\Delta CT}$, where ΔCt is the value from the threshold cycle (Ct) value of the treated sample subtracting the Ct value of the untreated or zero time-point control sample. The relative amount of the target mRNA was normalized to GAPDH mRNA.

The specific primers for CTGF and GAPDH, which synthesized by Bio Basic Inc. (Shanghai, China), were designed from their GenBank sequences as follows: CTGF (Forward: 5′-ACCCGAGTTACCAATGACAATACC-3′, Reverse: 5′-CCGCAGAACT- TAGCCCTGTATG-3′), GAPDH (Forward: 5′-AAATGGTGAAGGTCGGTGTG-3′; Reverse: TGAAGGGGTCGTTGATGG).

4.5. Statistical analysis

All results were expressed as mean ± SE. Using StatView (version 5.0) software (SAS Institute Inc. Cary, NC), one-way ANOVA analysis was employed to test the differences between the experimental groups. The differences with p values below 0.05 were considered to be significant.

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