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Fluorofenidone suppresses epithelial-mesenchymal transition and the expression of connective tissue growth factor via inhibiting TGF-β/Smads signaling in human proximal tubular epithelial cells

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Objectives: The present study was designed to investigate the potential effects and mechanism of fluorofenidone (AKF-PD) on transforming growth factor β_1 (TGF-β_1)-induced tubular epithelial-mesenchymal transition (EMT) and the expression of connective tissue growth factor (CTGF) in human proximal tubular epithelial cells. **Methods:** HK-2 cells were pretreated with AKF-PD, pirfenidone (PFD), Losartan, and SB431542 (an inhibitor of TGF-β type I receptor). The pretreated HK-2 cells were subsequently co-treated with TGF-β_1 (5 ng/ml). The morphological changes of HK-2 cells were observed under an inverted microscope. Expression of α-SMA was detected by Western blot and immunofluorescence. The protein expression of ZO-1, fibronectin, CTGF, phosphorylated Smad2 (p-Smad2) and phosphorylated Smad3 (p-Smad3) were evaluated by Western blot. **Results:** Through down-regulation of p-Smad2 and p-Smad3 proteins, AKF-PD significantly inhibited protein expression of α-SMA, fibronectin, and CTGF. Meanwhile, the depressed ZO-1 expression and morphological changes induced by TGF-β_1 were attenuated by AKF-PD. **Conclusion:** AKF-PD acts as an anti-fibrotic agent through blocking TGF-β/Smads signaling and consequently inhibits TGF-β_1-induced EMT and CTGF expression in human proximal tubular epithelial cells.

1. Introduction

Chronic kidney disease (CKD) is increasingly recognized as a global public health problem (Levey et al. 2007). Renal fibrosis, consisting of glomerulosclerosis and tubulointerstitial fibrosis (TIF), is the final common manifestation of a wide variety of CKD (Schieppati et al. 2005). Irrespective of the underlying glomerular lesion, TIF characterized by the accumulation of myofibroblasts and extracellular matrix (ECM) (Eddy 1996) is a robust predictor of renal functional impairment (Nath 1992).

EMT, a key event in the pathogenesis of TIF (Tan et al. 2006), involves loss of epithelial tight junctions (Rastaldi et al. 2002), up-regulation of mesenchymal cell markers of α-SMA and fibronectin (Kang et al. 2004), disruption of tubular basement membrane and subsequent migration of transformed cells to the interstitium (Liu 2004). When renal fibrogenesis sets in, about 36% of new fibroblasts come from local EMT (Iwano et al. 2002). These findings reinforce the notion that fibrogenesis is a local epithelial event (Kalluri et al. 2003). Because TGF-β_1 signaling is sufficient to induce EMT in cultured epithelial cells (Bottinger et al. 2002), TGF-β_1 is probably the key inducer of EMT (Yang et al. 2001, 2002). In the meanwhile, study results indicated that CTGF plays a pivotal role in TGF-β_1 dependent tubulointerstitial fibrosis (Lan et al. 2003; Yokoi et al. 2004; Okada et al. 2005) and mainly transduces EMT signals to tubular epithelial cells (Lin et al. 2002; Zhang et al. 2004).

The development of novel anti-fibrotic drugs is of great significance in the treatment of CKD. Several studies suggested that EMT of the tubular epithelial cell could be reversed by anti-fibrotic agents (Yang et al. 2002, 2003; Zeisberg et al. 2003). It has been documented that pyridone agents, such as PFD, can attenuate and potentially reverse collagen deposition in many organs including kidney (Cho et al. 2007; RamachandraRao et al. 2009), lung (Hirano et al. 2006; Nakayama et al. 2008), liver (Di Sario et al. 2004) and heart (Nguyen et al. 2010; Van Erp et al. 2006). As a newly-developed pyridone agent with similar chemical structure to PFD, AFK-PD has potential broad-spectrum antifibrotic characteristics (Wang et al. 2009). Our recent data suggest that AKF-PD could attenuate collagen I and TGF-β_1 expression through a nicotinamide adenine dinucleotide phosphate oxidase-dependent way in NRK-52E cells (Peng et al. 2009); inhibit TGF-β_1 induced CTGF via MAPK pathways in mouse mesangial cells (Wang et al. 2009); and protect mice from lethal endotoxemia through the inhibition of TNF-α and IL-1β release (Tang et al. 2010). However, the therapeutic effects and mechanisms of AKF-PD on renal epithelial EMT remain unclear so far. The present study was designed to investigate the effects of AKF-PD on TGF-β_1-induced EMT, CTGF expression and the related TGF-β/Smads signaling in a human proximal tubular epithelial cell line of HK-2.

2. Investigations and results

2.1. AKF-PD blocked TGF-β_1-induced phenotypical transformation and morphological changes of HK-2 cells

The effects of AKF-PD on TGF-β_1-mediated induction of EMT were analyzed by western blot and immunofluorescence. Interestingly, α-SMA protein expression in HK-2 cells was at a low level and significantly elevated by TGF-β_1. AKF-PD, as well as the experimental control reagents of PFD, losartan and SB431542 significantly attenuated α-SMA expression boosted by TGF-β_1 (Fig. 1A). At the concentration of 2 mM, AKF-PD exhibited stronger α-SMA inhibition ability than PFD ($p < 0.05$). This result was independently confirmed by indirect immunofluorescence staining for α-SMA in HK-2 cells. As shown in Fig. 1C, TGF-β_1 induced *de novo* expression of α-SMA that was assembled into abundant α-SMA-positive microfilament fibers in the cytoplasm of HK-2 cells. Incubation with AKF-PD, as with SB431542, losartan and PFD, significantly decreased the α-SMA expression induced by TGF-β_1. Moreover, ZO-1 protein expression in HK-2 cells was at a high level and significantly reduced by TGF-β_1. AKF-PD, as well as the experimental control reagents of PFD, losartan and SB431542 significantly attenuated ZO-1 repression induced by TGF-β_1. AKF-PD (2 mM) was more effective than PFD (2 mM) ($p < 0.01$) and losartan (10 μM) ($p < 0.05$) in the prevention of TGF-β_1-induced ZO-1 downregulation (Fig. 1B).

To observe the effects of AKF-PD on TGF-β_1-induced EMT, we further monitored the morphological changes of cultured HK-2 cells treated with TGF-β_1 alone or in combined with AKF-PD or other anti-fibrotic agents used as the control groups in the study. HK-2 cells without any treatment showed typical epithelial cobblestone morphology. Treatment of TGF-β_1 alone caused the cells to appear elongated and adopt a spindle-like shape. Moreover, AKF-PD, SB431542, PFD, or losartan incubation in the presence of TGF-β_1 treatment partially blocked the TGF-β_1-induced changes in cellular morphology (Fig. 2).

2.2. AKF-PD restrained TGF-β_1-induced fibronectin expression in HK-2 cells

Besides detecting the effects of AKF-PD on TGF-β_1-induced EMT of HK-2 cells, we further examined the influences of AKF-PD on TGF-β_1-enhanced ECM protein expression of fibronectin. As shown in Fig. 3, TGF-β_1 treatment significantly promoted the expression levels of fibronectin protein in HK-2 cells. Interestingly, a treatment of AKF-PD, SB431542, PFD, or losartan significantly restrained TGF-β_1-enhanced fibronectin expression. Among different experimental groups, the attenua-

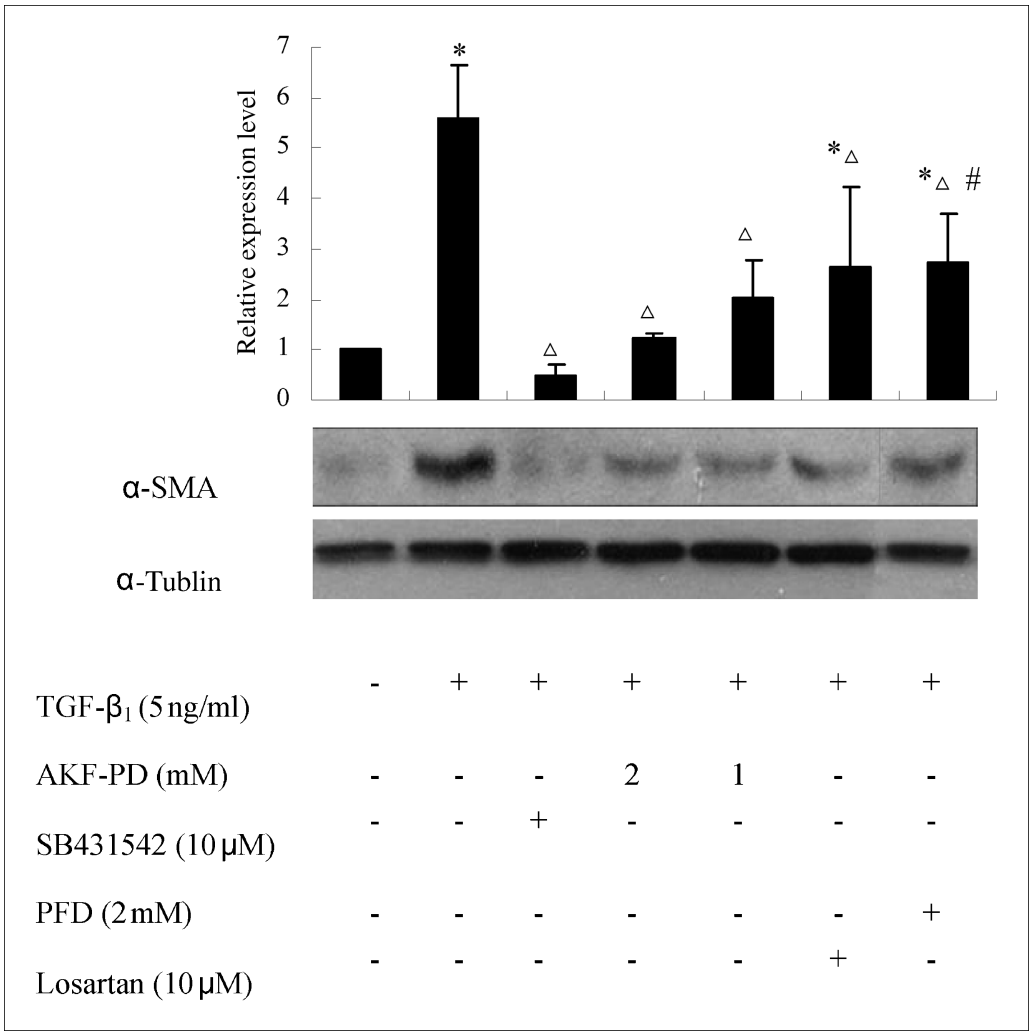

Fig. 1: AKF-PD inhibited TGF-β_1-induced EMT of HK-2 cells. The cells receiving no treatment were used as control, the other experimental groups were exposed to TGF-β_1, TGF-β_1 + SB431542 (10 μM), TGF-β_1 + AKF-PD (2 mM), TGF-β_1 + AKF-PD (1 mM), TGF-β_1 + losartan (10 μM), TGF-β_1 + PFD (2 mM) for 48 h. Equal protein loading was verified by α-tublin expression. Expressions of α-SMA (Figure 1A) and ZO-1 (Figure 1B) were tested. "*" and "Δ" represent $p < 0.05$, as compared with Control and TGF-β_1, respectively. "#" represents $p < 0.05$ as compared with TGF-β_1 + AKF-PD (2 mM). Immunofluorescence staining of α-SMA (Figure 1C): The cells receiving no treatment were used as control (A), the other experimental groups were exposed to TGF-β_1 (B), TGF-β_1 + SB431542 (10 μM) (C), TGF-β_1 + AKF-PD (2 mM) (D), TGF-β_1 + AKF-PD (1 mM) (E), TGF-β_1 + losartan (10 μM) (F), TGF-β_1 + PFD (2 mM) (G) for 48 h. Representative immunofluorescence pictures are shown. (Original magnification x 400).

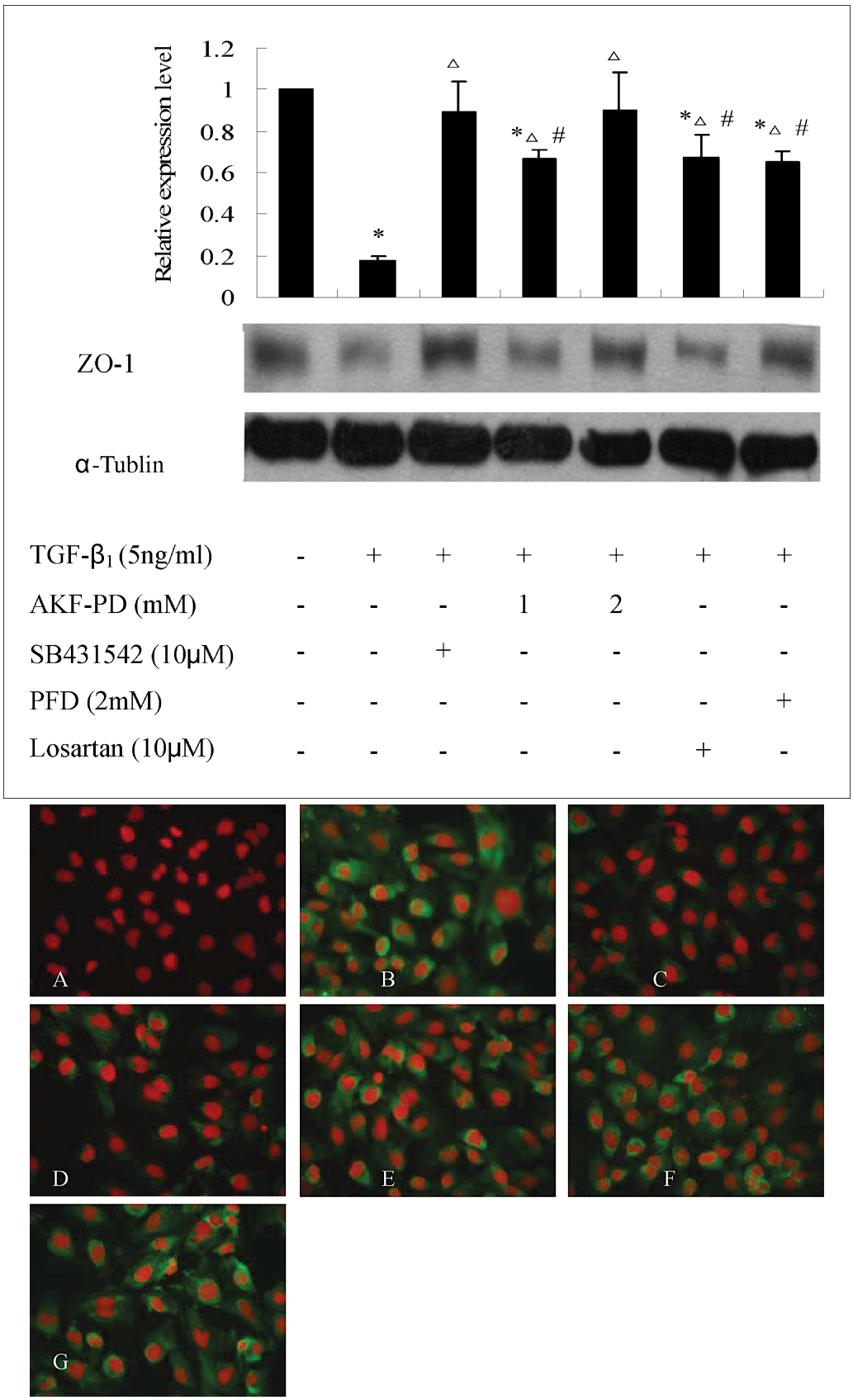

Fig. 1: (*Continued*).

tion effect of AKF-PD (2 mM) on TGF-β_1-induced fibronectin protein expression was stronger than that of PFD (2 mM) ($p < 0.01$) and losartan (10 μM) ($p < 0.01$) respectively. However, there was not a statistical difference of fibronectin attenuation between the groups of AKF-PD and SB431542 ($p > 0.05$).

2.3. AKF-PD inhibited TGF-β_1-induced protein expression of CTGF in HK-2 cells

CTGF is an important profibrotic protein related to renal fibrosis. As shown in Fig. 4, an exposure to TGF-β_1 for 48 h dramatically increased the protein level of CTGF in HK-2 cells. However,

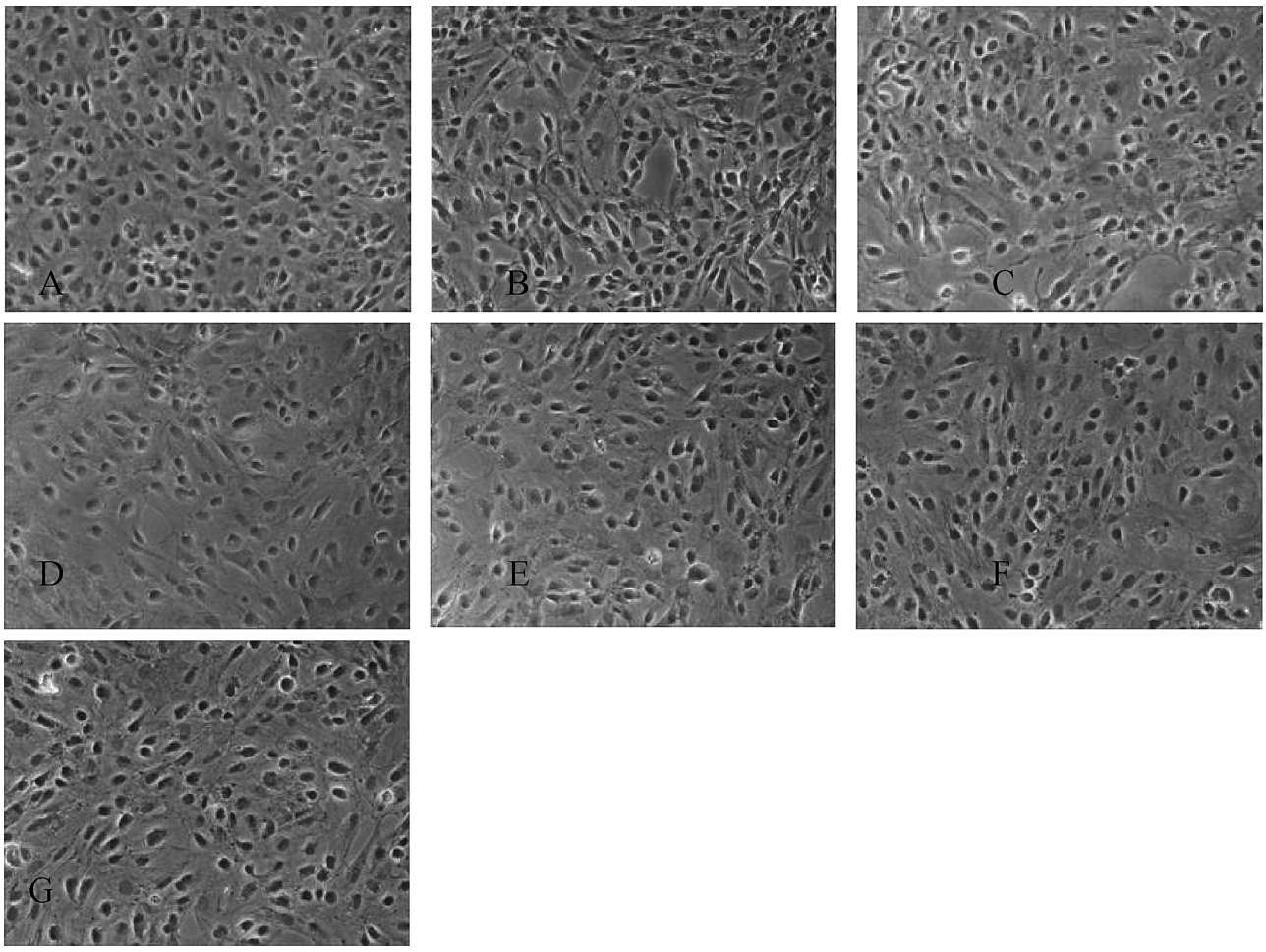

Fig. 2: AKF-PD prevented TGF-β_1-induced EMT-like morphologic changes of HK-2 cells. The cells receiving no treatment were used as control (A), the other experimental groups were exposed to TGF-β_1 (B), TGF-β_1 + SB431542 (10 μM) (C), TGF-β_1 + AKF-PD (2 mM) (D), TGF-β_1 + AKF-PD (1 mM) (E), TGF-β_1 + losartan (10 μM) (F), TGF-β_1 + PFD (2 mM) (G) for 48 h. Representative pictures are shown. (Original magnification x 100).

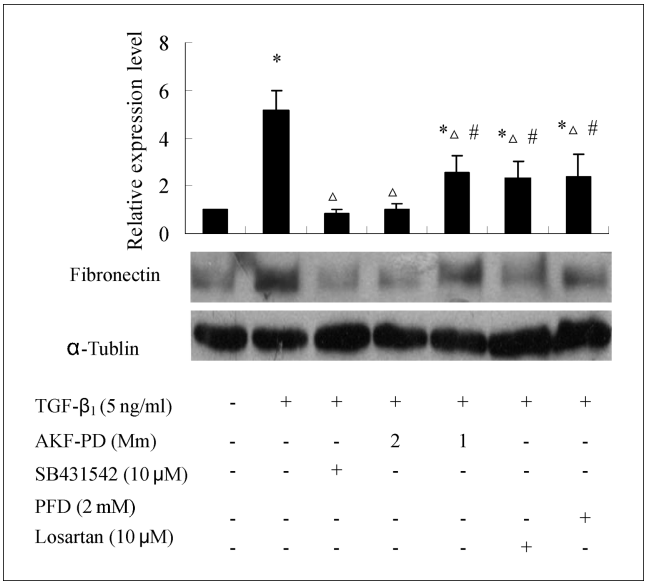

Fig. 3: AKF-PD prevented fibronectin expression triggered by TGF-β_1 in HK-2 cells. The cells receiving no treatment were used as control, the other experimental groups were exposed to TGF-β_1, TGF-β_1 + SB431542 (10 μM), TGF-β_1 + AKF-PD (2 mM), TGF-β_1 + AKF-PD (1 mM), TGF-β_1 + losartan (10 μM), TGF-β_1 + PFD (2 mM) for 48 h. Equal protein loading was verified by α-tublin expression. "*" and "Δ" represent $p < 0.05$, as compared with Control and TGF-β_1, respectively. "#" represents $p < 0.05$ as compared with TGF-β_1 + AKF-PD (2 mM).

a pre-treatment with AKF-PD (1 mM or 2 mM), SB431542 (10 μM), PFD (2 mM), or losartan (10 μM) could significantly block TGF-β_1-induced CTGF expression respectively. With regard to the inhibition abilities of different experimental reagents on CTGF expression enhanced by TGF-β_1 in HK-2 cells, there was no statistical difference between each two treatment groups of AKF-PD, PFD, and losartan ($p > 0.05$).

2.4. AKF-PD interfered with TGF-β_1-induced activation of Smad proteins

Both Smad2 and Smad3 proteins are the key transcription factors of TGF-β_1. From 15 min to 60 min after TGF-β_1 treatment (5 ng/ml), the expression levels of total Smad-2/3 proteins remained unchanged (Fig. 5A). However, TGF-β_1 significantly enhanced the phosphorylation of Smad2 (p-Smad2) and Smad3 (p-Smad3) as early as 15 min. Moreover, the protein levels of p-Smad2 and p-Smad3 peaked at 30 min and sustained to 60 min after TGF-β_1 treatment ($p < 0.05$, respectively). Interestingly, a pretreatment of AKF-PD (2 mM) or SB431542 (10 μM) for 24 h blocked TGF-β_1-induced protein expression of p-Smad2 and p-Smad3 significantly. SB431542 exerted stronger inhibition effect on TGF-β_1-induced p-Smad2 and p-Smad3 than AKF-PD (2 mM) (Fig. 5B).

3. Discussion

Tubulointerstitial fibrosis (TIF), a common pathological feature of CKD, is characterized by gradual loss of the tubular epithe-

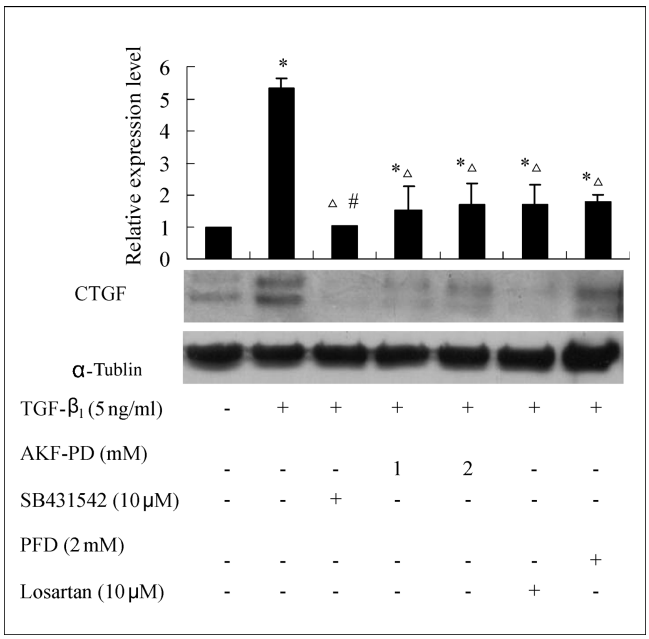

Fig. 4: AKF-PD prevented CTGF expression stimulated by TGF-β_1. HK-2 cells without any treatment were used as the control, the others experimental groups were exposed to TGF-β_1, TGF-β_1 + SB431542 (10 μM), TGF-β_1 + AKF-PD (2 mM), TGF-β_1 + AKF-PD (1 mM), TGF-β_1 + losartan (10 μM), TGF-β_1 + PFD (2 mM) for 48 h. Equal proteins loading was verified by α-tublin expression. "*" and "Δ" represent $p < 0.05$ as compared with the groups of Control and TGF-β_1, respectively. "#" represents $p < 0.05$ as compared with TGF-β_1 + AKF-PD (2 mM).

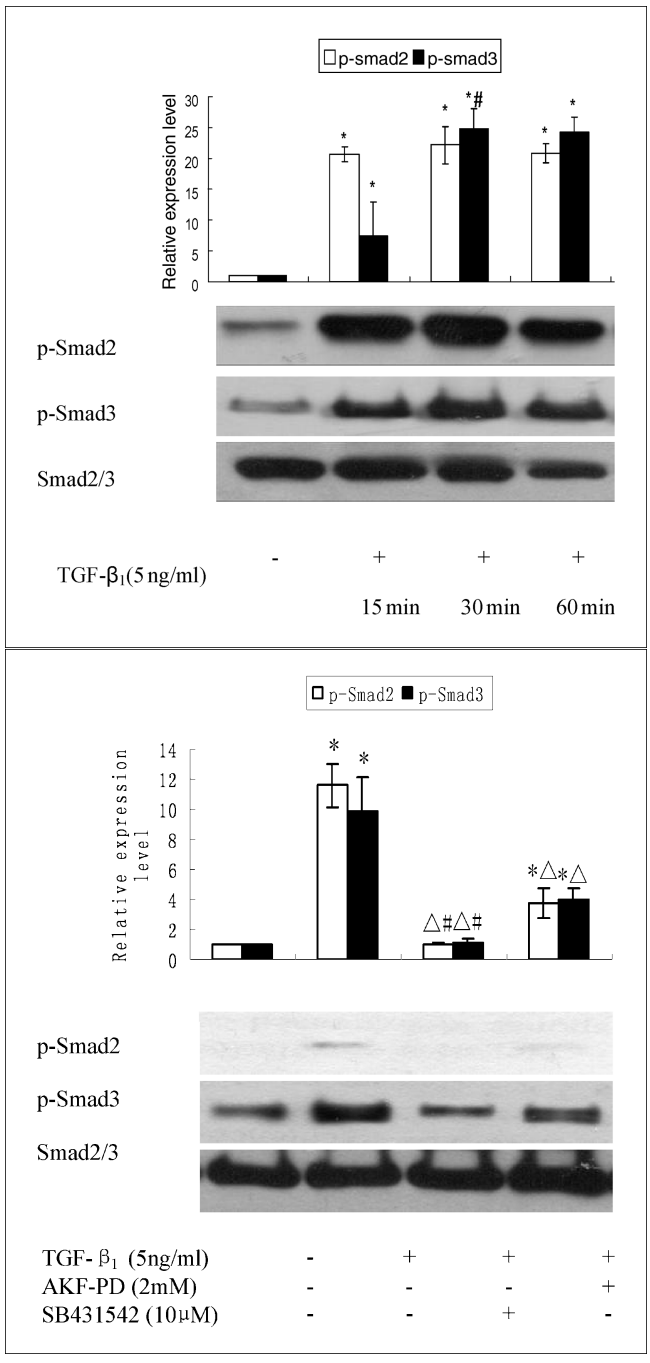

Fig. 5: AKF-PD attenuated expression of p-Smad2 and p-Smad3 in TGF-β_1 stimulated HK-2 cells. In Fig. 5A, the gels represent the Western blot results of p-Smad2 and p-Smad3 protein expression at different times after treatment of TGF-β_1 respectively. Total Smad2/3 is used as the internal loading control respectively. "*" represent $p < 0.05$ as compared with Control. "#" represents $p < 0.05$ as compared with TGF-β_1 15 min. In Fig. 5B, the gels represent effects of different concentrations of fluorofenidone on TGF-β_1-induced protein expression of p-Smad2 and p-Smad3 in HK-2 cells. Smad2/3 is used as the internal loading control. "*"and "Δ" represent $p < 0.05$ as compared with control and TGF-β_1, respectively. "#" represents $p < 0.05$ as compared with TGF-β_1 + AKF-PD (2 mM) at the same time point.

lium, concomitant with progressive accumulation of fibroblasts and α-SMA-positive myofibroblasts (Eddy 2000). Recent studies suggest that EMT is one of the key mechanisms underlying renal fibrosis (Kalluri et al. 2003; Liu 2004). The prevention or inhibition of EMT could be one therapeutic option that potentially inhibits renal fibrosis (Park et al. 2007).

AKF-PD is a novel pyridone agent which exerted significant antifibrotic effect and anti-inflammatory effect in our previous studies (Tang et al. 2010; Peng et al. 2009; Wang et al. 2009). PFD has a hydrogen bond at the meta-position of the benzene ring, while AKF-PD has a fluorine bond. 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* (Peng et al. 2009; Wang et al. 2009). With similar toxicity between AKF-PD and PFD in the preliminary experiments (data not included here), we assumed that AKF-PD with special physicochemical properties may have stronger anti-fibrosis effects than PFD. As demonstrated in the present study, AKF-PD is more effective than PFD in the attenuation of EMT stimulated by TGF-β_1 in HK-2 cells.

CTGF, which facilitates the binding of TGF-β_1 to its receptor (Abreu et al. 2002), plays a pivotal role in TGF-β_1 dependent TIF (Lan et al. 2003; Yokoi et al. 2004; Okada et al. 2005). A therapy of antisense oligodeoxynucleotide against CTGF was proved to treat kidney disease, so that the agents targeting CTGF should be promising to ameliorate TIF (Yokoi et al. 2004; Okada et al. 2005). In consistent with other findings on mesangial cells (Wang et al. 2009), we demonstrated that AKF-PD significantly attenuated TGF-β_1-induced CTGF expression in HK-2 cells. The inhibition of CTGF expression in renal cells by AKF-PD may contribute to retarding renal fibrosis.

Several cellular growth factors, including TGF-β, orchestrate the EMT of various epithelial tissues in response to injury and play a key role in the development of renal fibrosis (Bottinger et al. 2002; Moustakas et al. 2002; Ten Dijke et al. 2002). TGF-β type I and type II transmembrane receptor serine/threonine kinases transduce downstream signals of TGF-β_1 via Smad2 and Smad3 proteins. Smad2/3 proteins act as the transcriptional regulators of TGF-β_1-targeting downstream genes, including those being essential for apoptosis, differentiation, and proliferation (Massague et al. 2000; Ten Dijke et al. 2002). Our data indicate that AKF-PD, as effective as SB431542, prevents

EMT induced by TGF-β_1 in proximal tubular epithelial cells. As compared to a TGF-β type I receptor inhibitor of SB431542, AKF-PD exerts less inhibition activities on the phosphorylation of Smad2 and Smad3. AKF-PD might restrain EMT stimulated by TGF-β_1 in HK-2 through other potential TGF-β signaling pathways, such as phosphatidylinositol 3-kinase (Bakin et al. 2000), Rho-A (Bhowmick et al. 2001), p38MAPK (Bhowmick et al. 2001), and/or β-catenin (Dai et al. 2003; Li et al. 2003). On the other hand, the involvement of several nuclear transcriptional factors (e.g., zinc-finger factors Snail and Slug, 2-handed zinc-finger factors ZEB1 and SIP1, and basic helixloop-helix factors Twist and E12/E47) in TGF-β_1-regulated EMT process has recently been documented (Peinado et al. 2007; Thuault et al. 2008; Horiguchi et al. 2009). Further studies are therefore needed to understand the underlying mechanism of the AKF-PD in attenuating EMT in addition to TGF-β/Smads signaling pathway.

In summary, we demonstrated that the newly developed pyridone agent of AKF-PD attenuates TGF-β_1-induced EMT and CTGF expression in HK-2 cells. The anti-fibrotic effects of AKF-PD at least partly depend on its suppression abilities on Smad2 and Smad3 phosphorylation. AKF-PD has the potential to be a new antifibrotic agent to preventing renal fibrosis.

4. Experimental

4.1. Antibodies and reagents

HK-2 cells were purchased from American Type Culture Collection (ATCC, Rockville, MD, U.S.). Dulbecco's modified Eagle's medium: nutrient Ham's Mixture F-12 (DMEM/F12), fetal bovine serum (FBS) and penicillin/streptomycin were from Invitrogen (Carlsbad, CA, U.S.). SB431542, α-SMA, and α-tublin were purchased from Sigma-Aldrich Ltd (St. Louis, MO, U.S.). The antibody against fibronectin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.). Antibodies against CTGF, p-Smad3 and Smad2/3 were from Abcam (Cambridge, U.K.), and p-Smad2 was from Millipore (Billerica, MA, U.S.). Horseradish peroxide (HRP)-conjugated secondary antibodies for Western blot were from Jackson (West Grove, PA, U.S.), and secondary antibodies for immunohistochemistry were from GBI (Mukilteo, WA, U.S.). The enhanced chemiluminescence (ECL) kit for Western blot was from GE Healthcare (Buckinghamshire, U.K.). Recombinant human TGF-β_1 was purchased from Peprotech (Rocky Hill, NJ, U.S.). AKF-PD (Lot No.070501) and PFD (Lot No.070505) were synthesized by Sunshine Lake Pharma Co., Ltd. (Dongguan, GD, CN). Losartan (an ARB) was purchased from Merck (Whitehouse Station, NJ, U.S.).

4.2. Cell Culture and treatment

HK-2 cells were cultured in DMEM/F12 supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, CA, U.S.). The cells were cultured at 37 °C in a humidified 5% CO_2 atmosphere and 95% air. For protein detection of α-SMA, ZO-1, CTGF and fibronectin, 80–85% confluent cells were pretreated with drug interventions of AKF-PD (1 mM, 2 mM) PFD(2 mM), losartan(10 μM) or SB431542 (10 μM) for 1 h. TGF-β_1 (5 ng/mL) was subsequently added to the culture media containing different agents. The cells were treated for 48 h before protein extraction. For p-Smad2 and p-Smad3 analysis, HK-2 cells were pretreated with SB431542 (10 μM), AKF-PD (2 mM) for 24 h. The pretreated cells were subsequently treated with recombinant TGF-β_1 at a concentration of 5 ng/ml. The cells cultured in DMEM/F12 without any treatment were applied as the controls. Each individual experiment was replicated three times.

4.3. Western blot

The cell lysate containing 30 μg of total protein was separated on 8% or 10% sodium dodecyl sulfate-polyacrylamide (SDS-polyacrylamide) gel under reducing conditions, and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, U.S.). Nonspecific antibody binding was blocked by a pre-incubation of the membranes in 1 x Tris-buffered-saline (TBS) containing 5% skim milk for 1 h at room temperature. The membranes were then incubated overnight at 4 °C with primary antibodies against α-SMA (1:2500), fibronectin (1:400), ZO-1 (1:750), CTGF (1:2000), p-Smad2 (1:1000), p-Smad3 (1:2000) and smad2/3 (1:750) in 1 x TBS containing 5% bovine serum albumin (BSA). After washing, they were incubated with HRP-conjugated secondary antibodies at 1: 5000 dilutions for 1 h at room temperature. Bands were visualized by ECL and quantified using Glyko Bandscan 5.0 (Glyko, Novato, CA). Results are expressed as the percentage change in the mean band density as compared with the control values.

4.4. Immunofluorescence

For immunofluorescence analysis, HK-2 cells were cultured on sterile glass coverslips in 24-well plates and fixed with 3.7% paraformaldehyde in PBS for 30 min. After washing with PBS, cells were permeated with 0.3% Triton for 15 min and were incubated overnight with primary antibodies against α-SMA antibody (1:150) (Sigma, St. Louis, U.S.). After washing, they were incubated with secondary antibodies (Alexa Fluor 488, Jackson, PA, U.S.) for 1 h at room temperature. Finally, slides were counterstained with 4′, 6-diamidino-2-phenylindole. Negative controls were performed by replacing the primary antibody with 5% BSA in Tris-buffered saline Tween-20. Images were scanned using a fluorescent microscope (Leica 2000, NJ, U.S.).

4.5. Statistical Analyses

Data were expressed as means ± SE. Statistical analysis of data was performed with SPSS 16.0 software (SPSS Inc., IL, U.S.). Comparison among groups was made with one-way ANOVA. Multiple-comparison tests were applied only when a significant difference was determined by the ANOVA. $P < 0.05$ was considered statistically significant.

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