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Original Paper

The Effect of cAMP-PKA Activation on TGF-β1-Induced Profibrotic Signaling

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Key Words

Autosomal dominant polycystic kidney disease (ADPKD) • Chronic kidney disease (CKD) • cAMP-PKA signaling pathway • Transforming growth factor-β1 (TGF-β1) • Extracellular regulated protein kinase (ERK)

Abstract

Background: The cAMP-PKA signaling pathway and TGF-β1-dependent fibrosis pathways are of particular importance in ADPKD progression, but the cross-talk between these pathways remains unclear. Therefore, we used an MDCK-cell model and embryonic kidney-cyst model to study the regulat ory role of cAMP-PKA signaling in the TGF-β1 induced fibrotic process. ***Method and Results:*** *Pkd1*$^{flox/flox}$*; Ksp-Cre* and *Pkd1*$^{+/+}$*; Ksp-Cre* mice were used as an *in vivo* model. Increased kidney volume, renal cysts formation and up-regulation of thefibrosis-related proteins TGF-β1, connective tissue growth factor (CTGF), and fibronectin (FN) can be observed in *Pkd1*$^{flox/flox}$*; Ksp-Cre* mice. In an embryonic kidneys-cyst model, TGF-β11, FN and collagen type I were highly expressed. Western blotting revealed the obviously up-regulation of TGF-β1, CTGF, FN and collagen type I expression following forskolin treatment in MDCK cells. Selective PKA inhibition with H89 may partially reversed the above effects. Pretreatment with the TGF-β RI kinase inhibitor VI SB431542 suppressed the increased expression of CTGF, FN and collagen type I caused by forskolin. Our data also indicate that forskolin inhibited TGF-β-induced ERK1/2 phosphorylation and FN up-regulation. ERK inhibition useing PD98059 significantly inhibited the expression of CTGF, FN and collagen type I caused by TGF-β1. ***Conclusions:*** The cAMP-PKA signaling pathway can directly promote the production of TGF-β1 and/or TGF-β1-dependent fibrogenetic molecules in MDCK cells and embryonic kidney cysts, but when TGF-β1 and its downstream pathways were highly expressed in MDCK cells, cAMP-PKA had a significantly negative effect on TGF-β1 induced p-ERK1/2 and FN expression.

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Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is one of the most common life-threatening monogenic hereditary renal diseases and is mainly caused by mutations in two genes, *Pkd1* (85% of all ADPKD cases) and *Pkd2* (15% of cases), which encode polycystin-1 (PC-1) and polycystin-2 (PC-2) [1, 2]. With the progressive increase of bilateral renal cysts, 5-10% patients exhibit a decline in kidney function leading to chronic kidney disease (CKD) [3]. In addition, approximately 50% of polycystic kidney disease (PKD) patients develop end-stage renal disease (ESRD) in the 3rd and 4th decade of life within 5–10 years of the development of renal insufficiency. Dialysis treatment or renal transplantation usually become necessary when PKD has progressed to ESRD. Despite the severity and incidence of ADPKD, there unfortunately remains a lack of effective therapeutic methods to slow the progression from PKD to ESRD. Over the past decade, the proportion of patients hospitalized due to aggravated kidney failure increased significantly. Hence, the identification of specific drug targets todecrease or halt the progression of cyst development and thus slow the deterioration of renal functional and prevent the development of ESRD is of tremendous importance.

The principal pathological manifestations of PKD include 1) excessive cell proliferation and fluid secretion; 2) alterations in cell polarity; and 3) disordered extracellular matrix (ECM)composition and deposition. Pathological manifestations of ADPKD also include extra-renal cyst formation in the liver and pancreas, aneurysms and vascular abnormalities [4, 5]. The enlargement of renal cysts and abnormal changes in ECM ultimately cause renal insufficiency via extensive nephron loss and replacement of adjacent parenchyma with fibrosis. However, the mechanisms by which ECM accelerate the progression of PKD are still unclear.

Studies with animal models or patients suggest that the dysregulation of two major interacting second messengers, intracellular calcium and adenosine 3',5'-cyclic monophosphate (cAMP), are the most important driving factors in the pathogenesis of PKD. Previous evidence has demonstrated that cAMP-PKA signals play a central role in cyst genesis by driving both abnormal epithelial cell proliferation and cyst-filling fluid secretion. The cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel plays a major role in cAMP-PKA induced cyst-filling fluid secretion [6-9]. Renal cAMP concentrations are elevated in animal models of PKD [10]. Forskolin, a direct activator of adenylyl cyclase, stimulated cAMP-dependent cell proliferation and fluid secretion, and the cAMP analogue 8-Br-cAMP is commonly used to probe the mechanism of cAMP-PKA-dependent stimulation. Treatment of embryonic kidney explants from *Pkd1* mutant mice with 8-Br-cAMP results in tubular dilation [8]. The cAMP-PKA activated extracellular signal-regulated kinase 1/2 (ERK1/2) pathway has been implicated in the proliferative response of primary cells derived from polycystic renal tissue, but inhibits proliferation in cells derived from healthy human kidneys [11, 12]. The cAMP-PKA pathway can promote cell proliferation by stimulating the Ras/B-Raf/MEK1/ERK1/2 pathway in decreased intracellular calcium circumstances in ADPKD. Utilizing forskolin-treated Madin-Darby canine kidney (MDCK) cells, a classic *in vitro* model of renal cysts was created. MDCK cells form hollow cysts in a three-dimensional collagen matrix and tubulate in response to hepatocyte growth factor, which increases the levels of active ERK1/2.

At present, most studies focus on the role of cAMP-PKA signals on cell proliferation and fluid secretion in ADPKD. Although interstitial fibrosis plays an important role in progressive renal dysfunction in ADPKD and will lead to ESRD, the role of the cAMP-PKA pathway in interstitial lesions of ADPKD remains poorly understood.

In a number of *in vivo* models of PKD and in ADPKD patients, increased TGF-β expression has been observed in the kidney. Among the mediators influencing ECM composition, TGF-β is a crucial regulator of ECM neosynthesis, as it controls the expression of ECM network components such as collagens and FN. TGF-β-Smad signaling was shown to be up-regulated in ADPKD, suggesting that TGF-β-Smad signaling is associated with renal EMT and renal

fibrosis in ADPKD [13-15]. TGF-β can induce tissue fibrosis through the induction of various ECM proteins.

One study suggested that cAMP can promote TGF-β/-Smad3-mediated expression in breast cancer cells by up-regulating the expression of the TGF-β receptor TβRI [16]. Another study found that cAMP-PKA can inhibit the enhancement of collagen type I mRNA expression caused by TGF-β, which was reversed by pre-treatment with H89 in human peritoneal mesothelial cells (HPMC) [17]. These data suggest the involvement of the cAMP-PKA pathway in the TGF-βsignaling pathway.

Therefore, in this study we used the MDCK cell model, an embryonic kidney cyst model and a *Pkd1*-knockout mouse model to examine changes in TGF-β1-activated fibrosis related signaling. Meanwhile, we further explored the function of the cAMP-PKA pathway in ECM protein production as well as the cross-talk between the cAMP-PKA and TGF-β1 pathways. Studies of the pharmacologic modulation of these two pathways would help identify a novel role for cAMP-PKA in TGF-β1-dependent PKD renal interstitial lesions.

Materials and Methods

Ethics statement

This study was carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the Chinese Association for Laboratory Animal Science. All animal care and protocols were approved by the Animal Care Committee of Peking University Health Science Center. Every effort was made to minimize animal suffering.

Pkd1/Ksp-Cre mouse model of ADPKD

Pkd1flox mice (from Yale PKD Center) and Ksp-Cre transgenic mice (from UT Southwestern O'Brien Center) were generated in a C57BL/6 background . Pkd1flox/+; Ksp-Cre mice were generated by cross-breeding Pkd1flox mice with Ksp-Cre transgenic mice. Ksp-Cre mice can express Cre recombinase under the control of the Ksp-cadherin promoter. Pkd1flox/flox; Ksp-Cre, Pkd1flox/+; Ksp-Cre and Pkd1+/+; Ksp-Cre mice were generated by cross-breeding Pkd1flox/+; Ksp-Cre with Pkd1flox/+; Ksp-Cre mice. Neonatal mice (age 1 day) were genotyped by genomic PCR. Pkd1flox/flox mice were used as an *in vivo* PKD model. Pkd1+/+; Ksp-Cre mice from the same litter were used as wild-type. Kidneys were collected at different ages (days1, 3, 5, and 7). Protocols were approved by the Peking University Health Center Committee on Animal Research.

Embryonic kidney cyst model

Mouse embryonic kidneys at embryonic day 13.5 (E13.5) were dissected and placed on transparent Falcon 0.4 mM diameter porous cell culture inserts as described previously [8]. The lower chambers were filled with a 1:1 mixture of DMEM/Ham's F-12 nutrient medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 10 mM HEPES, 5 mg/ml insulin, 5 mg/ml transferrin, 2.8 nM selenium, 25 ng/mlprostaglandin E, 32 pg/ml T3, 250 U/ml penicillin, 250 mg/ml streptomycin, and 100 mM 8-Br-cAMP. The medium was replaced every 12 h. Kidneys were photographed using a Nikon inverted microscope (Nikon TE 2000-S) equipped with a 310 objective lens, a 520 nm band-pass filter, and a high-resolution PixeLINK color CCD camera.

Materials

TGF-β1 was obtained from R&D Systems (Minneapolis, MN). The TGF-β RI kinase inhibitor VI SB431542 was obtained from Calbiochem (616461, Calbiochem). The selective PKA inhibitor H-89 was obtained from abcam (Cambridge, MA). Forskolin (F6886, Sigma), and 8-bromo (Br)-cAMP (B-5380, Sigma) were each dissolved in 100% DMSO to prepare 100 mM stock solutions and were stored at -20°C. The ERK1/2 inhibitor PD98059 (513000, Calbiochem) and the p38 inhibitor SB203580 (559389, Calbiochem) were purchased from Calbiochem. Dimethylsulfoxide (DMSO) was purchased from Sigma (St. Louis, MO). Antibodies against collagen type I and FN were purchased from Abcam (Cambridge, MA). Antibodies against TGF-β1, CTGF, phosphorylated and non-phosphorylated ERK, a-smooth muscle actin (a-SMA) and

β-actin were purchased from Santa Cruz Biotechnology (CA, USA). Antibodies against phosphorylated and non-phosphorylated p38, Smad3 and against phosphorylated Smad2 were purchased from Cell Signaling Technology (New England, MA). Antibodies against Smad2 were purchased from Invitrogen. Antibodies against E-cadherin were purchased from Bioworld. Goat anti-rabbit IgG, goat anti-mouse IgG and rabbit anti-goat IgG were purchased from Santa Cruz Biotechnology. BCA reagents were from Pierce (Rockford, IL). FBS was purchased from Gibco (Grand Island, NY).

Cell culture

Type I MDCK (Madin-Darby canine kidney) (ATCC no. CCL-34) cells were cultured in a humidified atmosphere with 5% CO2-95% air at 37°C in DMEM nutrient medium containing 10% fetal bovine serum (FBS; Gibco), 2 mM glutamine, 100U/ml penicillin and 100 mg/ml streptomycin.

Cells were cultured to 70% to 80% confluence and were then serum deprived for 24 h. Cells were pretreated with H89 at different concentrations (10, 20, 40 μM) or TGF-β RI kinase inhibitor VI, SB431542 for 1 h and then cultured with forskolin for 1 h or 24 h. Cells were cultured to 70% to 80% confluence, and then pretreated with mitogen-activated protein kinase (MAPK) inhibitors, PD98059 (Calbiochem) or SB-203580 (Calbiochem), at 40μM (diluted from a 20 mM stock solution in DMSO), PKA inhibitor H89 (abcam), at 20μM (diluted from a 20 mM stock solution in DMSO) and forskolin (Sigma), at 10μM (diluted from a 100 mM stock solution in DMSO) for 1 h, and then cells were cultured in TGF-β1 for 1 h (24 h). Cells were cultured to 70% to 80% confluence, and then cultured in TGF-β1 for 24 h.

Western blot analysis

Tissues or cells were homogenized in RIPA lysis buffer containing protease inhibitor cocktail (Roche). Mitochondrial and cytosolic proteins were isolated using the Mitochondria/Cytosol Fractionation Kit according to the manufacturer's protocol (Beyotime Inst Biotech). Total protein concentrations were measured by BCA assay (Pierce) and size separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Proteins were blotted to polyvinylidene difluoride membranes (Amersham Biosciences, Piscataway, NJ, USA). Blots were incubated with antibodies against FN, collagen type I (Abcam), TGF-β1, CTGF, p-ERK, ERK2, α-smooth muscle actin (α-SMA) and β-actin (Santa Cruz), p-p38, p38, p-Smad2, p-Smad3, Smad3 (Cell Signaling Technology), Smad2 (Invitrogen), E-cadherin (Bioworld). Goat anti-rabbit IgG, goat anti-mouse IgG and rabbit anti-goat IgG (Santa Cruz) were added and the blots were developed with ECL plus kit (Amersham Biosciences).

Immunofluorescence

MDCK cells plated on coverslips were treated with 10 ng/ml TGF-β1 for 48 h. After treatment, cells were rinsed with pre-warmed PBS (0.01 M) and fixed with 4% paraformaldehyde for 10 min and then rinsed three times with PBS and permeabilized with 0.3% Triton-100 for 15 min. Subsequently, the cells were blocked with 5% goat serum for 50 min. The cells were then incubated with primary antibody (1:200) against α-SMA (Santa Cruz Biotechnology (CA, USA)) at room temperature for 2 h, followed by secondary antibody (Alexa-fluor 488 goat-anti-mouse IgG, 1:200) for 1 h at room temperature. Phalloidin was used to visualize the actin microfilaments. Hoechst dye 33342 (1:1000; Leagene, Beijing, China) was used to label nuclei. Images were captured on a fluorescence microscope (DM5000B, Leica).

Statistical analyses

All results are expressed as the mean±SEM. For multiple comparisons, statistical analyses were performed using one-way ANOVA followed by Tukey's multiple comparison tests. P-values<0.05 were considered statistically significant.

Results

The up-regulation of TGF-β1, CTGF and FN in Pkd1 flox/flox; Ksp-Cre mice

Because interstitial fibrosis is very important in the progression of PKD, we clarified the change in interstitial fibrosis-related proteins in Pkd1flox/flox; Ksp-Cre mice, which

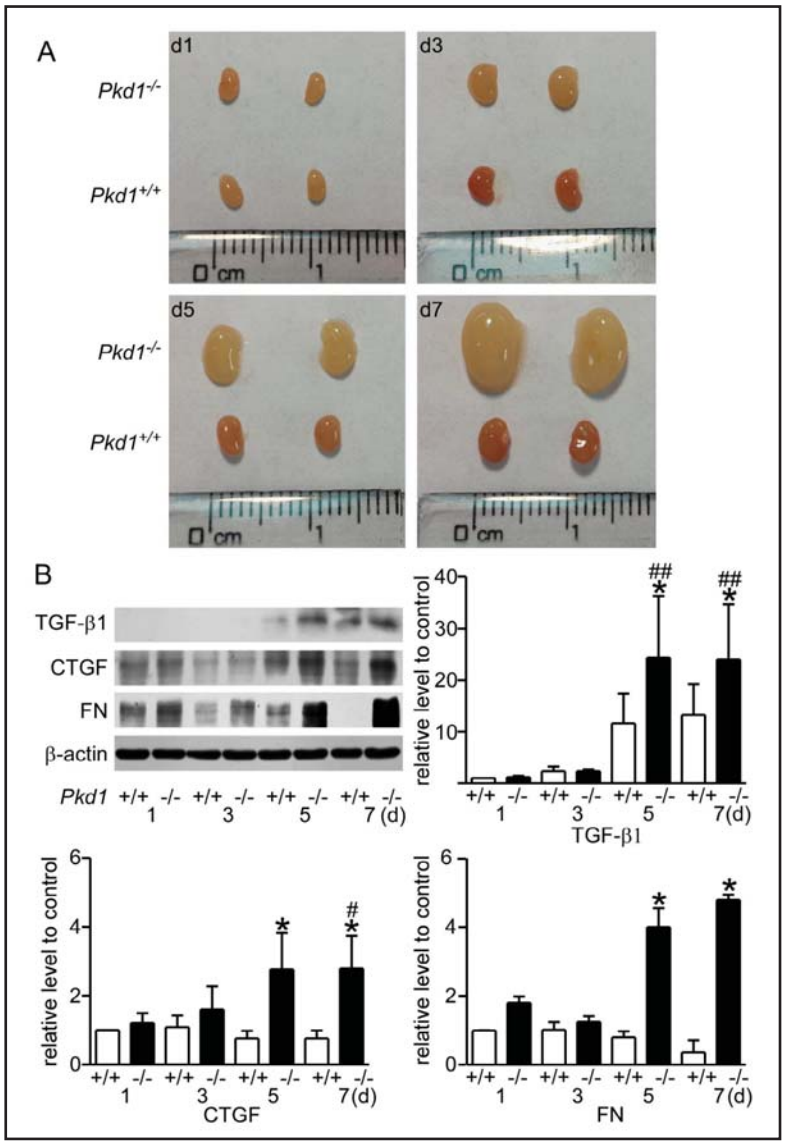

Fig. 1. TGF-β1, CTGF and FN were up-regulated in *Pkd1*$^{flox/flox}$*; Ksp-Cre* mice. Kidneys of *Pkd1*$^{flox/flox}$ mice and *Pkd1*$^{+/+}$*; Ksp-Cre* mice were collected at different ages (days1, 3, 5, and 7). Expression levels of TGF-β1, CTGF and FN were detected by Western blot analysis. Representative blotting (upper left) and quantification of protein levels (lower and upper right) are shown. Mean +/- SEM. n = 3; *P<0.05, **P<0.01, ***P<0.001 vs. wild-type mice. #P<0.05, ##P<0.01, ###P<0.001 vs. Pkd1$^{flox/flox}$;Ksp-Cre mice born on the first day.

were used as an *in vivo* model. The kidneys of wild-type and Pkd1flox/flox; Ksp-Cre mice were collected at different ages (days1, 3, 5, and 7). As shown in Fig. 1A, as PKD progressed, the kidney size of Pkd1flox/flox; Ksp-Cre mice became larger than the kidneys of wild-type mice from the beginning of the 3rd day. TGF-β1, CTGF and FN protein levels were higher in Pkd1flox/flox; Ksp-Cre mice than in wild type mice from the beginning of the 5th day (Fig. 1B). These results show that TGF-β1-dependent fibrosis related signaling participated in the early progression of PKD.

cAMP-PKA signaling up-regulated the expression of interstitial fibrosis- associated proteins in embryonic kidney cysts and MDCK cells

To explore the function of the cAMP-PKA pathway on interstitial fibrosis related signaling pathways in an embryonic kidney cysts model, kidneys cultured in specific circumstance were treated with or without 8-Br-cAMP for 6 days. On the 6th day, 8-Br-cAMP-treated embryonic kidneys showed significant cyst formation (Fig. 2A) as well as higher TGF-β1, FN, collagen type I protein expression than the control group (Fig. 2B). Then, we examined whether the cAMP-PKA signaling pathway modulates the expression of TGF-β1 and CTGF (a growth factor that can stimulate fibroblast proliferation and collagen deposition) in MDCK cells. In MDCK cells treated with forskolin (10 μM) for 24 h, we found that both proteins were

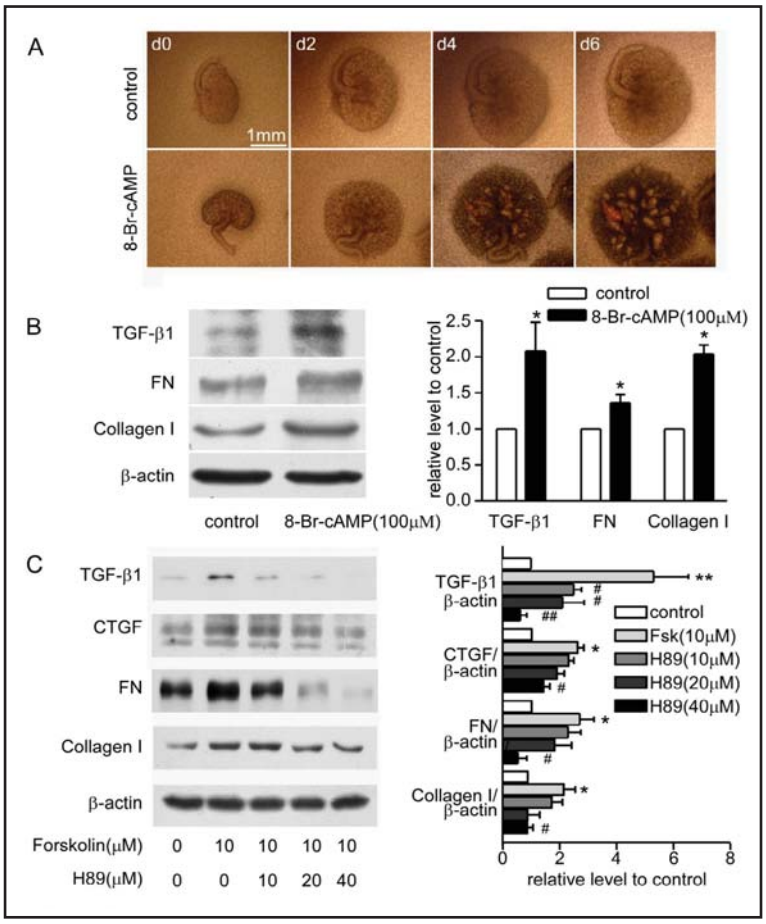

Fig. 2. cAMP-PKA signaling up-regulated the expression of TGF-β1, CTGF, FN and collagen type I. 8-Br-cAMP-treated embryonic kidneys on the 6th day and the control group (A). The expression of TGF-β1, FN and collagen type I were tested (B). Cells (C) were treated with forskolin (10μM) in the presence or absence of H89 (10, 20, 40 μM) for 24 h (H89 pretreatment for 1 h). Western blotting was performed to test the expression of TGF-β1, CTGF, FN and collagen type I. Representative blotting (left) and quantification of protein levels (right) are shown. Mean +/- SEM. n = 3; *P<0.05, **P<0.01, ***P<0.001 vs. control. #P<0.05, ##P<0.01, ###P<0.001 vs. Forskolin treated group.

stimulated. As shown in Fig. 2C, TGF-β1 was up-regulated by forskolin stimulation (10 μM), and this function can be reversed by H89 (10, 20, and 40 μM). Then, we examined the effect of forskolin (10 μM) on FN and collagen type I, and the results showed (Fig. 2C) that all of these proteins can be up-regulated by forskolin (10 μM), which can be reversed by H89 (10, 20, and 40 μM). These results showed that the activation of the cAMP-PKA signaling pathway can promote the expression of interstitial fibrosis-related proteins in both MDCK cells and in embryonic kidney cysts. Thus, cAMP-PKA signaling may be involved in promoting interstitial fibrogenesis in PKD.

The role of cAMP-PKA signaling in TGF-β1-induced Smad and non-Smad pathways

Because the TGF-β signaling pathway plays a very important role in fibrogenesis of progressive PKD and because the activation of Smad2 and Smad3 is major downstream event in TGF-β1 signaling, we first investigated the phosphorylation of Smad2 and Smad3 in MDCK cells. As shown in Fig. 3A, TGF-β1 (10 ng/mL) induced the rapid phosphorylation of Smad2 and Smad3, which began within 15 min, and peaked at 60 min. Then, we studied whether TGF-β1 could activate MAPK pathways in MDCK cells. As shown in Fig. 3A, ERK1/2 was activated within 15 min of TGF-β1(10 ng/mL) addition, peaking at 60 min, and the p38MAPK pathway was activated within 30 min of TGF-β1 adding, also peaking at 60 min and persisting for approximately 120 min (Fig. 3A). The results were expressed as the ratio between phosphorylated and nonphosphorylated Smad2, Smad3, ERK1/2, and p38. In summary, we found that Smad2, Smad3, ERK1/2, and p38 (Fig. 3A) were all activated by TGF-β1, which may be responsible for the enhanced CTGF, FN and collagen type I protein expression observed in MDCK cells.

The cAMP pathway has been shown to antagonize TGF-β1-elicited signaling in different cell types. Thus, we explored the effect of the cAMP-PKA pathway on TGF-β1inducedfibrosis-

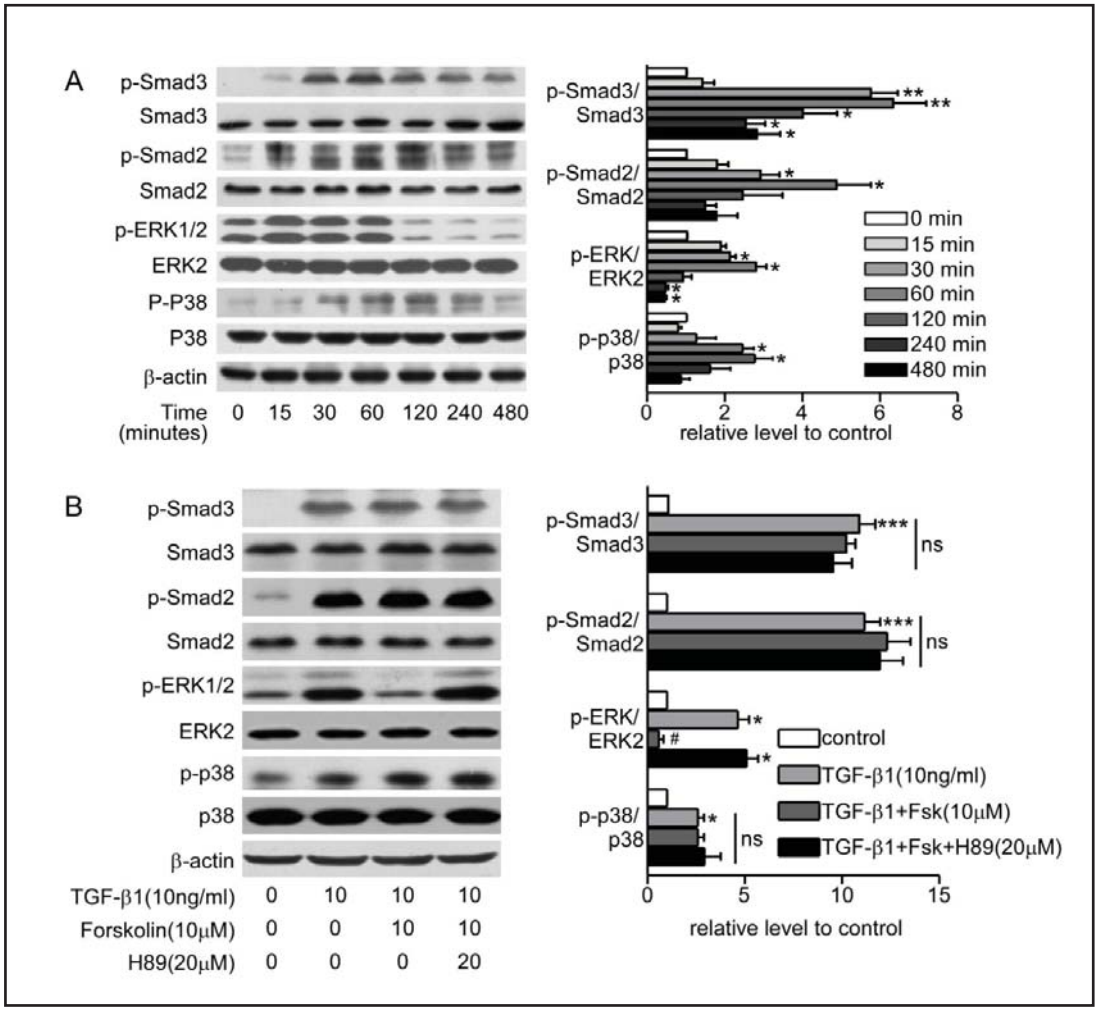

Fig. 3. The cAMP-PKA signaling pathway inhibited the phosphorylation of ERK1/2 induced by TGF-β1. Cells were treated with TGF-β1 (10 ng/mL) for the indicated time periods. Time course of Smad2 (A), Smad3 (A), ERK (A) and p38 (A) phosphorylation levels were detected by Western blot analysis. The function of forskolin on TGF-β-induced Smad2, Smad3, ERK1/2 and p38 activation in MDCK cells (B). Cells were treated with forskolin (10μM) in the presence or absence of H89 (20μM) for 1 h (H89 pretreatment for 1 h), then incubated with TGF-β1 (10 ng/mL) for an additional 1 h. Western blotting was performed to test the expression of phosphorylated and nonphosphorylated Smad2, Smad3, ERK1/2, p38 proteins. Representative blotting (left) and quantification of protein levels (right) are shown. Mean +/- SEM. n = 3; *P<0.05, **P<0.01, ***P<0.001 vs. control. #P<0.05, ##P<0.01, ###P<0.001 vs. TGF-β1 treated group.

related signaling molecules in MDCK cells. We first examined whether the cAMP-PKA signaling pathway modulates the phosphorylation of Smad2, Smad3, ERK1/2 and p38 caused by TGF-β1 in MDCK cells. As shown in Fig. 3B, the phosphorylation of Smad2, Smad3 and p38 by TGF-β1 was not significantly altered by forskolin (10 μM) treatment in either the presence or absence of H89 (20 μM). In contrast, forskolin (10μM) significantly suppressed ERK1/2 activation by TGF-β1 (10 ng/mL), which was reversed in the presence of H89 (20μM).

Inhibitory effect of cAMP-PKA signaling and ERK inhibition on TGF-β1 downstream targets

To explore the effect of cAMP-PKA signaling on TGF-β1 induced fibrotic processes, we next examined the levels of downstream fibrosis related proteins. Forskolin, the selective ERK1/2 inhibitor PD98059 (40 μM) and the selective p38 inhibitor SB203580 (40 μM) were used and CTGF, FN and collagen type I proteins expression inducted by TGF-β1 (10 ng/mL)

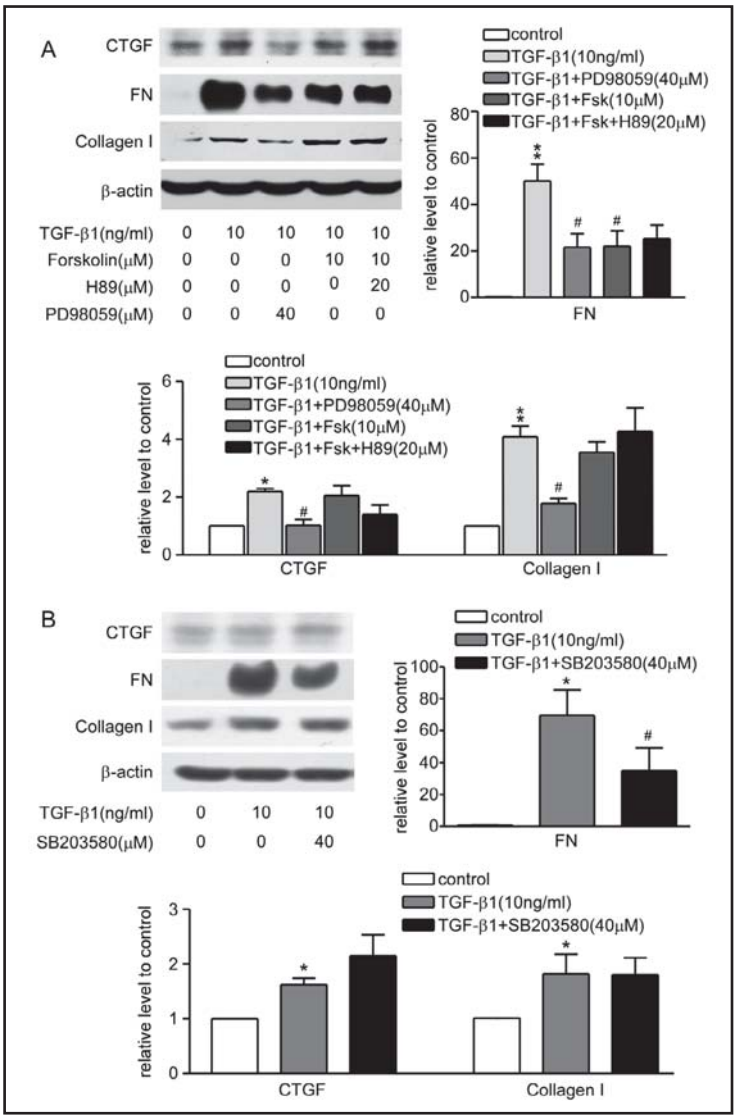

Fig. 4. The effect of cAMP-PKA and ERK/p38 inhibitor on the TGF-β1-induced expression of CTGF, FN and collagen type I. MDCK cells were pre-treated with forskolin in the presence or absence of H89 (20 μM) orselective kinase -inhibitors ERK1/2 PD98059 (40 μM) and p38 SB203580 (40 μM), for 1 h and then co-incubated with TGF-β1 (10 ng/mL) for 24 h. The expression levels of CTGF, FN and collagen type I were detected by Western blot analysis. Representative blotting (upper left) and quantification of protein levels (lower and upper right) are shown. Mean +/- SEM. n = 3; *P<0.05, **P<0.01, ***P<0.001 vs. control. #P<0.05, ##P<0.01, ###P<0.001 vs. TGF-β1 treated group.

was detected. PD98059 (40 μM) markedly inhibited the increased expression of CTGF, FN and collagen type I (Fig. 4A). The ability of TGF-β1 to stimulate CTGF and collagen type I expression was not affected by SB203580 (40 μM), and SB203580 only partially inhibited the expression of FN (Fig. 5B). Forskolin (Fig. 4A) only inhibited the increased expression of FN caused by TGF-β1, but this effect could not be reversed by pretreatment with H89 (20 μM). This effect may reveal a complex role for the cAMP-PKA signaling in TGF-β-mediated fibrotic processes in MDCK cells. These results (Fig. 3, 4) indicated that ERK1/2, but not the Smad2, Smad3 and p38 pathways is mainly involved in the inhibitory effect of the cAMP-PKA signaling on TGF-β-induced FN protein expression in MDCK cells.

Participation of the TGF-β and ERK pathway in cAMP-PKA-induced fibrotic process

To investigate whether TGF-β and its receptors are required for the cAMP-PKA-induced production of fibrosis-related proteins, MDCK cells were pretreated with 5 μM of the TGF-β RI kinase inhibitor VI SB431542 prior to forskolin stimulation. As shown in Fig. 5A, SB431542 inhibited the increased expression of CTGF, FN and collagen type I caused by forskolin. This finding suggested that cAMP-PKA signaling may promote the production of fibrosis-related molecules by stimulating the TGF-β pathway.

Given previous indication that PKA may promote the production of CTGF by directly activating Smad3, we then tested the effect of forskolin on the phosphorylation of Smad2

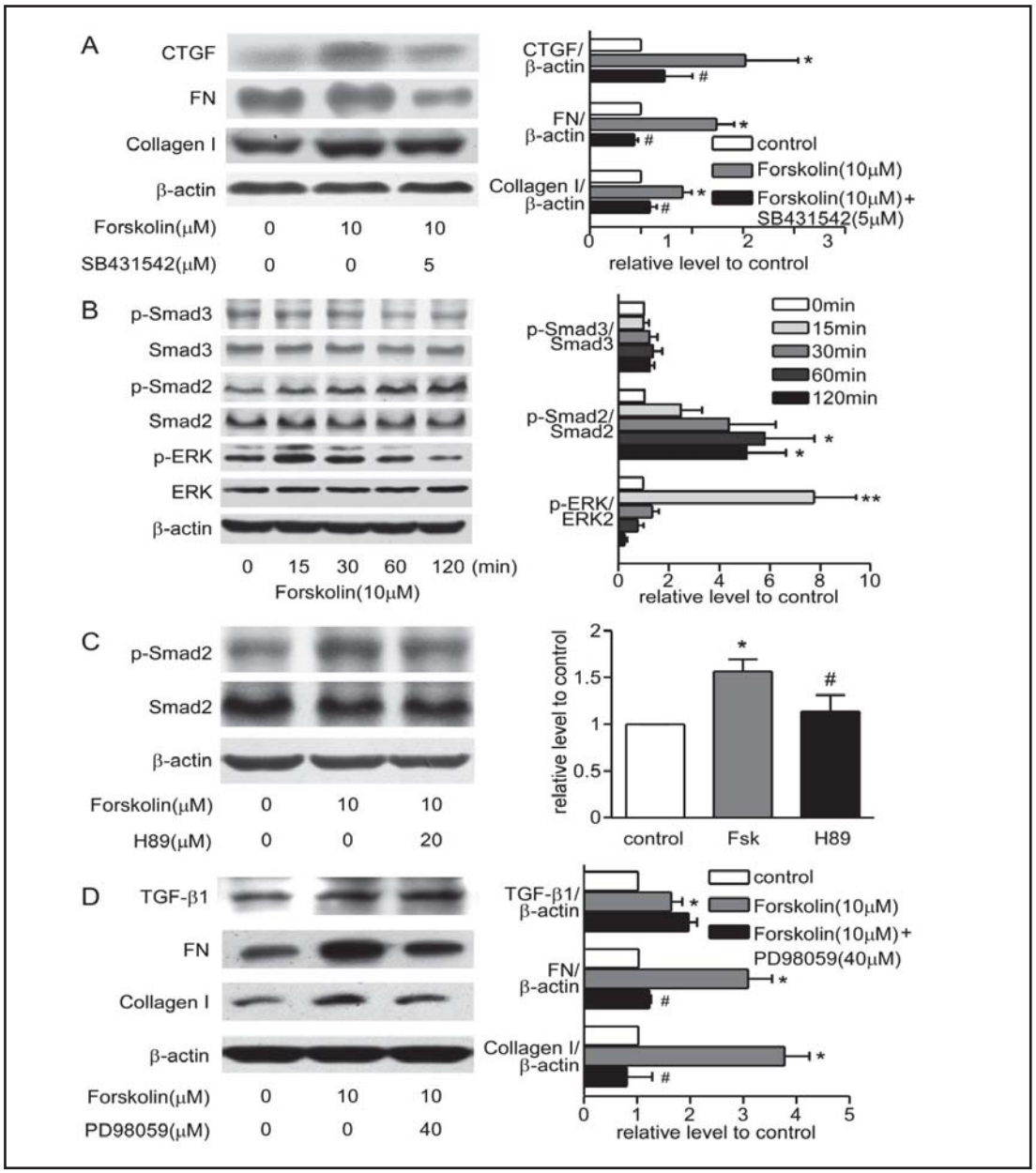

Fig. 5. Blockade of the ERK and TGF-β pathways can suppress the production of FN and collagen type I caused by forskolin. MDCK cells (A, D) were treated with forskolin (10μM) in the presence or absence of PD98059 (40μM) or SB431542 (5μM), Western blotting was performed to determine the expression of TGF-β1, FN and collagen type I. MDCK cells (B) were treated with forskolin (10 μM) for the indicated time periods (15, 30, 60, and 120 min). Smad2, Smad3, ERK1/2 (B) phosphorylation levels were detected. MDCK cells (C) were treated with forskolin (10μM) in the presence or absence of H89 (20 μM) for 1 h (H89 pre-treatment for 30 min). The Smad2 phosphorylation level was detected. Representative blotting (left) and quantification of protein levels (right) are shown. Mean +/- SEM. n = 3; *P<0.05, **P<0.01, ***P<0.001 vs. control. #P<0.05, ##P<0.01, ###P<0.001 vs. Forskolin treated group.

and Smad3. As shown in Fig. 5B, Smad2 was activated within 15 min of forskolin (10 μM) addition, peaking at 60 min, and persisting for approximately 120 min. However, Smad3 was not activated by forskolin within 120 min. These activation of Smad2was reversed in the presence of H89 (Fig. 5C).

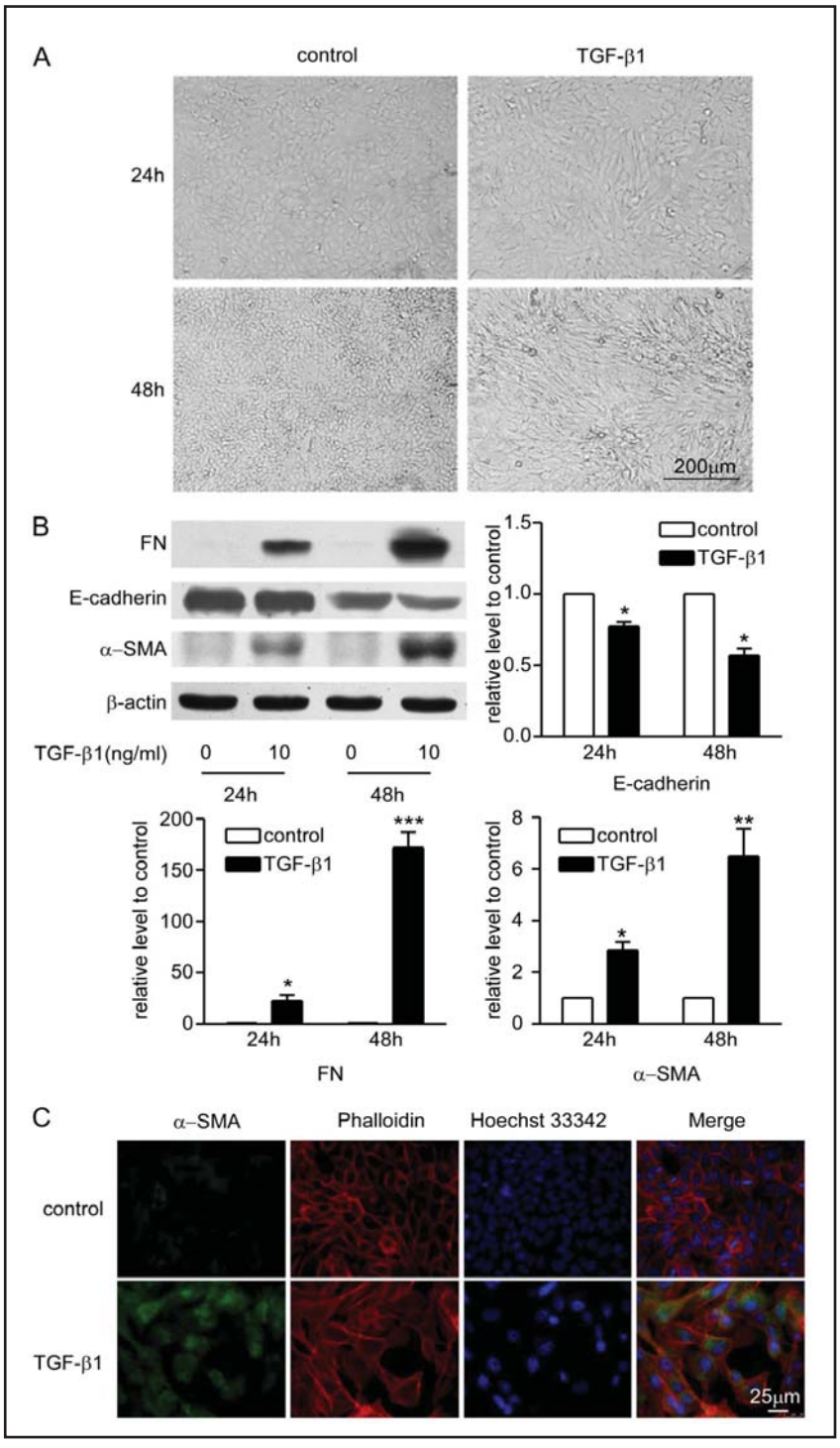

Fig. 6. TGF-β1 induced EMT in MDCK cells. MDCK cells were grown in 6-well plates until 60% confluence, serum deprived for 24 h, and then treated with vehicle or TGF-β1 (10 ng/ml) for 24 or 48 h. A microscope was used to take pictures of morphological changes between control and TGF-β1-treated MDCK cells at 24 h or 48 h (A). FN, α-SMA and E-cadherin were detected by Western blot analysis (B). Phalloidin and α-SMA immunofluorescence in TGF-β1 (10 ng/ml)-treated MDCK cells (C). Representative blotting (upper left) and quantification of protein levels (lower and upper right) are shown. Mean +/- SEM. n = 3; *P<0.05, **P<0.01, ***P<0.001 vs. control.

ERK1/2 was activated at 15 min after forskolin (10 μM) addition, and returned to baseline by 30 min (Fig. 5B). The results in Fig. 5D showed that TGF-β1 production stimulated by 10 μM forskolin was not reversed by ERK inhibition with PD98059, but the increased expression of FN and collagen type I caused by forskolin could be reversed by PD98059. This result suggested that TGF-β1 did not originate from ERK1/2-dependent paracrine pathway. Taken together, these results may indicate that cAMP-PKA signaling can promote the production of fibrosis-related molecules by directly stimulating the ERK1/2 and TGF-β pathways.

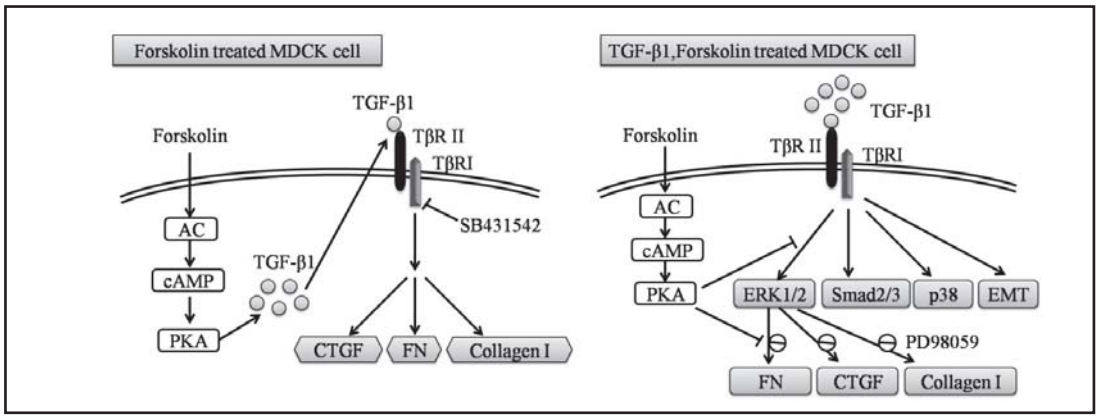

Fig. 7. Proposed mechanism of the regulatory function of the cAMP-PKA signaling pathway in the TGF-β1-induced fibrotic process. In forskolin-treated MDCK cells, the cAMP-PKA signaling pathway directly promotes the production of TGF-β1 and TGF-β1-dependent fibrogenetic molecules (CTGF, FN and collagen type I), which can be inhibited by the TGF-β RI kinase inhibitor VI SB431542. In TGF-β1 (10 ng/ml)-treated MDCK cells, TGF-β1 and its downstream pathways are highly expressed, and cAMP-PKA can negatively regulate TGF-β1-induced ERK1/2 phosphorylation and FN protein expression. TGF-β1-induced fibrosis-related protein expression can be inhibited by the ERK inhibitor PD98059.

TGF-β1 induced EMT in MDCK cells

To probe the EMT changes, we used two independent parameters: cell morphology and the expression of the EMT markers α-SMA, FN and E-cadherin. We first monitored the morphological changes in MDCK cells treated with vehicle or TGF-β1. Under basal conditions, MDCK cells exhibited typical epithelial-like morphology (Fig. 6A), whereas TGF-β1 (10 ng/ml) treatment for 24 h or 48 h induced a significant conversion to spindle-like morphology (Fig. 6A). FN is a multifunctional, extracellular matrix glycoprotein and α-SMA is an actin isoform specific to myofibroblasts; the expression of these proteins undergoes characteristic changes during EMT. FN and α-SMA (Fig. 6B) protein levels in MDCK cells were markedly induced by 24 and 48-h treatments with TGF-β1 compared with the basal state (10 ng/ml). E-cadherin, a classic epithelial cell marker, is a membrane-bound protein involved in cell-cell interactions in intact renal tubular epithelial cells. Incubation with TGF-β1(10 ng/ml) for 24 or 48 h reducedE-cadherin expression compared with the control group (Fig. 6B). MDCK cells with TGF-β1 (10ng/ml) for 48h and immunofluorescence for α-SMA and Phalloidin were detected. α-SMA protein expression was also markedly induced, and cell morphology was markedly altered (Fig. 6C). These results show that TGF-β1 can induce EMT in MDCK cells and plays important role in ADPKD fibrotic processes.

Discussion

The pathogenesis of ADPKD consists of two important stages [3]. In the initial stage, numerous fluid-filled epithelial cysts arise from different nephron segments. In the progressive stage, these cysts gradually increase in number and size over time, accompanied by interstitial fibrosis. Pkd1 mutations lead to an earlier age of onset for ESRD than do Pkd2 mutations [18]. Cyst-lining epithelial cells exhibit increased nuclear staining for phosphorylated Smad2 and increased expression of TGF-β target genes such as collagen type I [19]. Microarray [20] studies of human ADPKD kidneys revealed that TGF-β and its downstream effectors are highly expressed in cystic epithelium. Liu et al. [21] demonstrate the deletion of the Pkd1 gene can increase the reactivity of phosphorylated smad2/3 and CTGF in response to TGF-β, thus contributing to the downstream genes expression of TGF-β downstream genes. These findings suggest that the loss of PC-1 plays a role in augmenting TGF-β signaling.

Pkd1 kidney-specific knockout mice (Pkd1flox/flox; Ksp-Cre genotype) rapidly develop PKD with many renal epithelium-specific cysts, and die soon after birth. In the present study, we demonstrated that TGF-β1 and its downstream effectors, CTGF and FN, were more highly expressed in the kidneys of Pkd1flox/flox; Ksp-Cre mice than in matched wild-type mice on the 5th day and the 7th day (Fig. 1). This result revealed the participation of the TGF-β1-mediated fibrosis related signaling pathway in PKD progression. Furthermore, we also utilized an embryonic kidney cyst model, which is widely used as at tissue model in PKD correlative investigations, because the activation of the cAMP-PKA pathway by 8-Br-cAMP can obviously promote the formation and growth of cysts derived from embryonic day 13.5 in specific cultures. We found that TGF-β1, FN and collagen type I are up-regulated by 8-Br-cAMP on the 6th day (Fig. 2B). TGF-β1 can stimulate the accumulation of ECM by enhancing the synthesis of various ECM macromolecules [22, 23]. Renal fibrosis, particularly tubulointerstitial fibrosis, is the principle process and common final outcome of almost all progressive CKD. It is a relatively uniform response involving glomerulosclerosis, tubulointerstitial fibrosis and changes in renal vasculature. Of these, tubulointerstitial fibrosis has become the most consistent predictor of an irreversible loss of renal function and progression to ESRD. As noted in earlier studies [24-26], matrix factors and autocrine factors play important roles in interstitial fibrosis and expanded cysts replace normal kidney tissue and paralyze normal kidney function. In ADPKD, reducted PC-1 or PC-2 expression is thought to cause incomplete cellular differentiation and the aberrant expression of proteins, including ECM molecules. ADPKD renal cysts originate from the epithelia of the nephrons and collecting ducts.

Forskolin can promote MDCK cell cyst formation and growth in 3D suspension cultures; therefore, MDCK cells are widely used as the cell model in PKD correlative investigations, including signaling pathways analysis. Thus, to clarify the role of cAMP-PKA, we also used MDCK cells to examine the cross-talk between the cAMP-PKA and TGF-β1-activated fibrosis -related signaling pathways. Firstly, we identified the cAMP-PKA activation by forskolin, a direct activator of adenylyl cyclase, stimulates TGF-β1 up-regulation and ECM production (CTGF, FN and collagen type I). All of these changes, shown in Fig. 2A, can be reversed in a dose-dependent manner by PKA specific inhibition with H89. According to these results, we can conclude that in normal MDCK cells and embryonic kidney cysts, cAMP-PKA signaling pathway directly promotes the production of TGF-β1 and TGF-β-dependent fibrogenetic molecules. The cAMP-PKA signaling pathway may play a role in promoting fibrosis processes in early stages of ADPKD.

In previous studies, antagonistic interactions have been reported between the TGF-β-induced fibrogenic response and cAMP-PKA pathways. In addition, dibutyryl-cAMP (DBcAMP) can suppress TGF-β downstream signaling by mediating the ERK1/2 and p38 MAPK pathways in human peritoneal mesothelial cells [15]. Another previous study [27] demonstrates that the PKA activators DB-cAMP and forskolin can efficiently inhibit TGF-β1-induced α-SMA expression in MDCK cells. Thus, we used TGF-β1-treated MDCK cells to study the action of the cAMP-PKA pathway in this process. TGF-β1 rapidly activates the phosphorylation of Smad2, Smad3, ERK1/2 and p38 MAPK (Fig. 3) and induces the production of downstream FN, collagen type I and CTGF proteins following long-term incubation (Fig. 4). These results show that a combination of Smad and non-Smad pathways is likely to serve as the central mediator of TGF-β signaling receptors to the nucleus in MDCK cells. Forskolin clearly inhibited the TGF-β1-induced phosphorylation of ERK1/2, whichwas reversed by H89, but had no effect on the phosphorylation of Smad2, Smad3 and p38 MAPK caused by TGF-β1. Previously, the ERK signaling pathway was shown to be involved in TGF-β-induced CTGF expression in hepatic stellate cells [28]. These data indicate that the ERK1/2 pathway may play a very important role in the antagonistic interactions between TGF-β1-mediated fibrogenic-response signals and the cAMP-PKA pathway in our investigation. Importantly, theresults in Fig. 4A also provided evidence that selective ERK1/2 inhibition with PD98059 can significantly inhibit the levels of CTGF, FN and collagen type I induced by TGF-β1. cAMP-PKA signaling antagonized TGF-β1-mediated fibrotic processes mainly by

suppressing this pathway, despite the fact that cAMP elevation stimulated ERK1/2 activation its own (as shown in Fig. 5B). On the other hand, selective p38 inhibition by SB203580 only partly down-regulate FN expression. Previous studies have confirmed that ERK1/2 activity plays an important role in cyst proliferation in PKD [12, 29] and ERK inhibition slowed cyst growth in a PKD mouse model [30]. All of these findings emphasize the importance of the ERK1/2 pathway in ADPKD. Our results revealed that the ERK1/2 pathway also plays a pivotal role in non-Smad pathways downstream of TGF-β in fibrotic processes in PKD. This result is consistent with a previous report in which cAMP-elevating agents inhibited the fibrotic effects of TGF-β in cardiac fibroblasts largely by inhibiting ERK1/2 phosphorylation [31]. Although the ERK inhibitor PD98059 significantly inhibited the up-regulation of FN and collagen type I caused by TGF-β1, it could not completely inhibit the increased expression of these proteins. FN and collagen type I protein levels were still higher than in controls, likely due to the importance of the TGF-β/Smad2/3 pathway. TGF-β1 can induce EMT via the Smad2/3, ERK1/2 and GSK3β/Snail signaling pathway[32]. The Samd-family members have been identified as major intracellular mediators of TGF-β signaling [33]. TβRI and Smad3 are major players in TGF-β induced EMT [34], TGF-β1 can induce EMT via the TGF-β1/Smad2/3 signaling pathway in alveolar epithelial cells[35]. Both the TGF-β/Smad2/3 pathway and the TGF-β/Ras/Raf/MEK1/ERK1/2 pathway are critical in ADPKD fibrotic-process. In TGF-β1-treated MDCK cells, TGF-β1 and TGF-β-dependent fibrogenetic molecules were highly expressed. Under such circumstances, cAMP-PKA negatively regulated TGF-β1-induced ERK1/2 phosphorylation and FN expression. In the progressive stage of ADPKD, fibrosis is obvious, TGF-β1 and TGF-β-dependent fibrogenetic molecules are highly expressed, and cAMP-PKA signaling pathway may play a role in inhibiting this process.

It was recently shown that TGF-β-dependent CTGF expression involves Smad3 and Ras/MEK/ERK signaling [36-39]. The repression of basal CTGF expression also resulted in the down-regulation of the ECM proteins FN and collagen type I. Although previous studies show that the elevation of cAMP/PKA levels negatively regulated CTGF expression [40, 41], we do not know the exact mechanisms by which forskolin suppressed the synthesis of only one of these ECM proteins in MDCK cells in response to TGF-β1 stimulation in the present study. Because the activation of Smad3 has been specifically linked to CTGF expression in response to TGF-β, we think the main reason may be related to forskolin being unable to negatively regulate the phosphorylation of Smad3. Other possible causes might involve thecAMP-PKA-enhanced production of CTGF through the direct activation of Smad3 or other matrix regulatory enzymes. However, the data in Fig. 5 show that forskolin does not induce Smad3 phosphorylation. In addition, it is possible that forskolin does not inhibit TGF-β1-induced Smad2 and Smad3 phosphorylation whether because the TGF-β1 concentration is too high to be inhibited. TGF-β1 (at a concentration of 5ng/ml) can up-regulate Smad2 and Smad3 phosphorylation, but still cannot be inhibited by forskolin (data not shown).

TGF-β super-family members are multifunctional cytokines. TGF-β first binds to the type II receptor on the cell membraneand then triggers the interaction between TGF-β receptor II (TβRII) and TGF-β receptor I (TβRI), leading to the activation of the TβRI kinase [42, 43]. As a consequence, the downstream mediators Smad2 and Smad3 are activated. Activated Smad2/3 forms a heteromeric complex with Smad4 and translocates to the nucleus to induce the transcription of target genes. TGF-β1 induced EMT can promote the fibrosis process, and it also can increase cancer cell motility in the progression of epithelial ovarian cancer [44]. In our study, both the ERK inhibitor PD98059 and the TGF-β RI kinase inhibitor VI SB431542 inhibited the overexpression of FN and collagen type I proteins induced by forskolin (Fig. 5). These results indicate that forskolin can promote the fibrotic process by directly stimulating the TGF-β and ERK pathways.

A recent study suggested that cAMP can promote TGF-β/-Smad3-mediated expression in breast cancer cells by upregulating the expression of the TGF-β receptor TβRI, and a 3 h or 6 h duration of forskolin treatment is sufficient to significantly raise TβRI expression indicating that TβRI levels rose early in response to forskolin [16]. These imply that in MDCK cells, forskolin-promoted TGF-β/ Smad2-mediated expression may also by regulating

TβRI expression. Another study showed that Smad2 siRNA can significantly reduce collagen type I and FN synthesis in TGF-β1-stimulated ADPKD cyst lining epithelial cells [45]. The direct effect of forskolin on the TGF-β and Smad2 pathways may explain why forskolin cannot inhibit TGF-β1-induced CTGF and collagen type I expression. However this is just our hypothesis and still needs further research. Fig. 5 also showed that PD98059 did not inhibit the expression of TGF-β1 induced by forskolin. This result may indicate that TGF-β1 didn't come from ERK induced paracrine synthesis. TGF-β1 is a protein stimulus for its own activation [46], and autocrine synthesis of TGF-β1 may play an important role in the cellular response to injury.

Although the degree to which EMT contributes to kidney fibrosis remains a matter of intense debate, increasing evidence suggests that EMT is one of the mechanisms contributing to the pathogenesis of tubulointerstitial fibrosis. As shown in a recent study [47], TGF-β can induce EMT of human tubular epithelial cells, and interstitial myofibroblasts originated from tubular epithelial cells via EMT. Myofibroblast activation played a critical role in renal interstitial fibrogenesis. EMT can occur in tubular epithelial cells, and these cells are able to produce ECM proteins contributing to interstitial fibrogenesis [47-49]. In addition, gene profiling of human ADPKD showed that 26 of the 87 specifically regulated genes were typical for smooth muscle [25], suggesting that EMT is a pathogenetic factor in ADPKD. With regard to TGF-β action, it appears that EMTs are crucial morphogenetic responses to TGF-β1 in renal epithelial cells during fibrotic diseases [50-52], and the EMT also depends on ERK activity. Therefore, we used TGF-β1 (10 ng/ml) to stimulate MDCK cells and demonstrated that TGF-β1 can induce EMT in these cells (Fig. 6). However, forskolin (10 ng/ml) failed to significantly inhibit the up-regulation of α-SMA and the down-regulation of E-cadherin (data not shown). A previous study showed that TGF-β-Smad signaling was up-regulated in ADPKD, and it is highly likely that the enlarged cyst stretched tubular cells that would secrete the TGF-β, and that increased TGF-β can mediate EMT and interstitial fibrosis in ADPKD[13].

In addition to fibrogenesis, TGF-β also controls proliferation, differentiation, vascular calcification [53] and other functions in most cells. We also found transforming growth factor beta regulator 1 (TBRG1), a growth inhibitory protein induced in cells undergoing arrest in response to DNA damage and TGF-β1 [54], was more highly expressed in Pkd1flox/flox; Ksp-Cre mice than in matched wild type mice on the 1st day after birth in our proteomics study (data not shown). Further studies are needed toclarify the complex cross-talk between the cAMP-PKA and TGF-β-activated pathways in PKD.

Conclusion

In conclusion, cAMP-PKA played the role of a double-edged sword in ADPKD interstitial fibrosis processes. On the one hand, cAMP-PKA signaling pathway directly promoted the production of TGF-β and/or TGF-β-dependent fibrogenetic molecules in an MDCK cell model and an embryonic kidney cysts model. However, when TGF-β1 and its downstream pathways were overexpressed in MDCK cells, cAMP-PKA negatively regulated TGF-β-1induced ERK1/2 phosphorylation and FN expression. These results offer novel insights into the development of new therapeutic strategies for retarding ADPKD.

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Disclosure Statement

The authors declare no conflict of interest.

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