

Original Paper

Akt2 Mediates TGF- β 1-Induced Epithelial to Mesenchymal Transition by Deactivating GSK3 β /Snail Signaling Pathway in Renal Tubular Epithelial Cells

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Key WordsAkt2 • TGF- β 1 • Epithelial-mesenchymal transition (EMT) • Snail**Abstract**

Background: The epithelial-mesenchymal transition (EMT) induced by growth factors or cytokines, particularly transforming growth factor- β (TGF- β 1), plays an important role in kidney tubulointerstitial injury. However, signaling pathways mediating TGF- β 1-induced EMT are not precisely known. In this study, we examined the role of Akt2 on EMT. **Methods:** HK-2 cells were exposed to 10 ng/ml TGF- β 1 to establish a model of EMT. The expression of proteins were detected by western blot assay and Immunofluorescence. The levels of genes were tested by RT-PCR. **Results:** We found that treatment of HK-2 cells, a human proximal tubular cell line, with 10 ng/ml TGF- β 1 resulted in activation of phosphatidylinositol 3-kinase (PI3K)/Akt2 signaling as evidenced by increased p-PI3K, Akt2 and p-Akt (Ser 473) expression. Importantly, TGF- β 1 treatment decreased zona occludins 1 (ZO-1) and E-cadherin (epithelial markers) expression, increased fibronectin and vimentin (mesenchymal makers) expression, which were prevented by Ly294002 (the inhibitor of PI3K) or small interfering RNA (siAkt2), suggesting that Akt2 mediated TGF- β 1-induced EMT. Meanwhile, RNA and protein levels of Snail1, the key inducer of EMT, were significantly elevated in TGF- β 1-treated HK-2 cells. TGF- β 1 also induced inactivation of glycogen synthase kinase-3 β (GSK3 β), an endogenous inhibitor of Snail. Knockdown of Akt2 using siRNAs or the PI3K inhibitor Ly294002 inhibited TGF- β 1-induced phosphorylation of GSK3 β and expression of Snail1. **Conclusion:** These findings revealed that knockdown of Akt2 antagonized TGF- β 1-induced EMT by inhibiting GSK3 β /Snail signaling pathway.

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Introduction

Renal tubular epithelial cells injury has been recognized as a major process in the progression of renal disease. Injured renal tubular epithelial cells have been implicated in increasing kidney myofibroblast populations through the process of epithelial-mesenchymal transition (EMT) [1-3], ultimately leading to inevitable progressive chronic kidney disease (CKD) [4, 5]. The EMT defines a phenotypic conversion of primary epithelial cells into mesenchymal cells, characterized by loss of epithelial cells makers such as E-cadherin, ZO-1 and gain of mesenchymal cells makers such as vimentin and fibronectin [6]. Tubular EMT can be induced by TGF- β 1 [7-12], advanced-glycation end products [13, 14], and angiotensin II [15], but TGF- β 1 is probably a major inducer of EMT, because TGF- β 1 signaling is sufficient to induce EMT in epithelial cells [9, 11]. Whereas TGF- β 1 responses are initiated by the interaction of TGF- β 1 with cell surface receptors [16], the intracellular signaling pathway involved in EMT are complex.

At the molecular level, down regulation of E-cadherin expression can be achieved by transcriptional suppression mediated by members of the basic helix-loop-helix family, including Snail [17-19]. Furthermore, GSK3 β , as an active kinase of Snail, can bind to Snail and facilitate its proteasomal degradation [20, 21]. Accumulating evidence indicates that GSK3 β is important to maintain the epithelial architecture [22]. In addition, GSK3 β can be inactivated by Akt upon phosphorylation. The Akt/PKB family of kinases, a downstream effector of phosphatidylinositol 3-kinase (PI3K) pathway, plays an important role in regulating growth, proliferation, survival, metabolism, and other cellular activities [23-25]. Akt activity is induced by insulin, growth factors (such as EGF), and cytokines (such as TGF- β 1). In a normal rat tubular epithelial cell line NRK-52E, Kattla JJ et al., [26] showed that TGF- β 1 induced Akt phosphorylation and resulted in the transformation of epithelial cell into myofibroblast phenotype, indicating that Akt activity involved TGF- β 1-induced EMT. To date, there are three major isoforms of Akt: Akt1, -2, and -3. However, which isoform mediates TGF- β 1-induced EMT remains unclear.

Although the three Akt isoforms are structurally homologous, they have different features and functions. Akt1 and -2 are ubiquitously expressed whereas Akt3 has been reported to have specific tissue distribution [27]. And studies of Akt isoforms deficient mice highlight the non-redundant functions of Akt1 and -2. Akt1 $^{-/-}$ mice are with significant growth defects, and Akt2 $^{-/-}$ mice are unable to maintain normal glucose homeostasis [28, 29]. It is known that EMT may occur in tumor migration and progression. Emerging evidence indicates distinct functions of Akt isoforms in tumor cells. Akt2 amplification or mutations have been detected in breast, ovarian, and colon tumors [30, 31], whereas Akt1 amplification has been reported only in a single gastric cancer line [32]. So, Akt2 may exert isoform-specific effects on tumor progression. It is worth noting that Akt2 expression was required for EMT-like morphological changes induced by insulin-like growth factor-I (IGF-I), and Akt2 down-regulation reverted all aspects of IGF-IR-induced phenotypic changes in 3D acinar structures in breast epithelial cells [33]. Considering the known functional characteristics of Akt2, we hypothesized that Akt2 may play an important role in TGF- β 1-induced EMT.

Materials and Methods

Reagents

The human proximal tubular epithelial cells (HK-2 cells) were from Peking Union Medical College Cell Bank. Recombinant human TGF- β 1 was purchased from Sigma-Aldrich (St Louis, Mo, USA). The DMEM-F12 medium and fetal bovine serum (FBS), were supplied by Gibco (BRL Grand Island, NY, USA). Antibodies for Akt1, Akt2, Akt3, p-Akt (Ser473), p-Akt (Thr308), p-PI3K (phosphatidylinositol 3-kinase), GSK3 β (glycogen synthase kinase-3 β), p-GSK3 β , p-Smad3, Smad3, E-cadherin were purchased from Cell Signaling Technology (Beverly, MA). ZO-1 (zona occludins 1) and Snail1 were obtained from Abcam (Cambridge, UK). Vimentin,

fibronectin and protein A agarose were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The reverse transcription system was obtained from Promega (Madison, WI). SYBR Premix Ex TaqTMII was from Takara (Shiga, Japan).

Cell culture and Treatments

HK-2 cells were grown in DMEM-F12 medium supplemented with 10% fetal bovine serum (FBS), 2 mM 1-glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin at 37°C under an atmosphere of 5% CO₂ and 95% air. For TGF- β 1 treatments, HK-2 cells (2×10^5) cultured in six-well plates were made quiescent by culturing in medium containing 0.1% FBS for 24 h before treatment with 10 ng/ml TGF- β 1. Pharmacological inhibitor was added before TGF- β 1 as follows: 20 μ M Ly-294002 (PI3K inhibitor) for 30 min.

Western blot analysis

Total cells were homogenized in lysis buffer (25 mM HEPES (pH 7.5), 150 mM NaCl, 1% Igepal CA-630, 10 mM MgCl₂, 1 mM EDTA, 10% glycerol, 10 mg/ml aprotinin, 10 mg/ml leupeptin, 100 mM phenylmethylsulfonylfluoride, 25 mM NaF, 1 mM Na₃VO₄ and centrifuged at 14,000 g for 30 min. Supernatant was recovered and proteins were quantified using the BCA protein assay kit. Lysates (30 μ g per lane for cells) were loaded onto 10% SDS-polyacrylamide gel electrophoresis (PAGE), and the proteins were transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked for 1.5 h at room temperature (RT) in fresh blocking buffer (0.1% Tween20 in Tris-buffered saline (TBS-T) containing 5% fat-free milk) and then incubated with the primary antibodies anti-p-PI3K, anti-p-Akt(Ser473), anti-p-Akt(Thr308), anti-Akt1, anti-Akt2, anti-Akt3, anti-p-GSK3 β , anti-t-GSK3 β , anti-E-cadherin, anti-fibronectin, anti-vimentin, anti-Snail1, anti-Smad3, anti-p-Smad3 or GAPDH antibodies (1:1000 dilution) overnight at 4°C, then secondary antibodies (1:5000 dilution, horseradish peroxidase-conjugated goat anti-rabbit IgG) for 1.5 h at RT. Membranes were washed three times with TBS-T and the signals were visualized and analyzed using Odyssey image system (Li-COR, Lincoln, NE, USA). Each experiment was repeated at least three times. The density of specific bands was measured and normalized to that of GAPDH.

RT-PCR

Total RNA and then cDNA were prepared from cultured cells using TRIzol reagent and RT-PCR kits. The primers were: E-cadherin, sense 5'-GCCGAGCCCTGCCACCCTG-3', antisense 5'-CTTTCTGTAGGTGGAGTCCC-3'. N-cadherin, sense 5'-GGAATCCCGCCTATGAGTGG-3', antisense 5'-CGTCTAGCCGTCTGATTCCC-3'. Snail1, sense 5'-AAGATGCACATCCGAAGCCA-3', antisense 5'-CAAAAACCCACGACAGACAGG-3'. GAPDH was used for normalization, and the primers were as follows: sense 5'-GCGAGACCCCACTAACATCA-3', antisense 5'-GAGTTGGGATAGGGCTCTCTT-3'. Real-time PCR was performed in a 96-well optical reaction plate using SYBR Premix Ex TaqTMII.

Gene knockdown

Small interfering RNAs (siRNA) against human Akt2 subunit mRNA (NM_001243028), human Akt1 subunit mRNA (NM_001014432) and human GSK3 β subunit mRNA (NM_001146156.1) were synthesized by GenePharma Co., Ltd (Beijing, China). The siRNA of Akt2 (siAkt2), siRNA of Akt1, siRNA of GSK3 β and random non-coding RNA (siNC) were transfected into HK-2 cells using Lipofectamine 2000, according to the manufacturer's instruction (Invitrogen, USA). siAkt2, siAkt1, siGSK3 β or siRNA-NC (50 nM) was incubated with the cells for 6 h for transfection. Efficiency of genetic silencing by siRNA was evaluated by western blot.

Immunofluorescence staining

The procedures were performed as previously described [26] with minor modification. Briefly, cells were fixed in 4% paraformaldehyde in PBS for 10 min at room temperature (RT). Cells were permeabilized in PBS containing 0.1% Triton X-100 and blocked by incubating in 5% bovine serum albumin for 30 min. Fixed cells were washed with PBS and incubated for 60 min at RT or overnight at 4°C with primary antibodies anti-ZO-1 (1:500 dilution), anti-p-GSK3 β (1:500 dilution), anti-Snail (1:200 dilution) and anti-vimentin (1:500 dilution). After washing three times, the cells were stained with Fluor secondary antibodies (Invitrogene). Images were obtained using a deconvolution microscope with a Zeiss Plan Apochromat 63 \times , 1.4 NA objective lens.

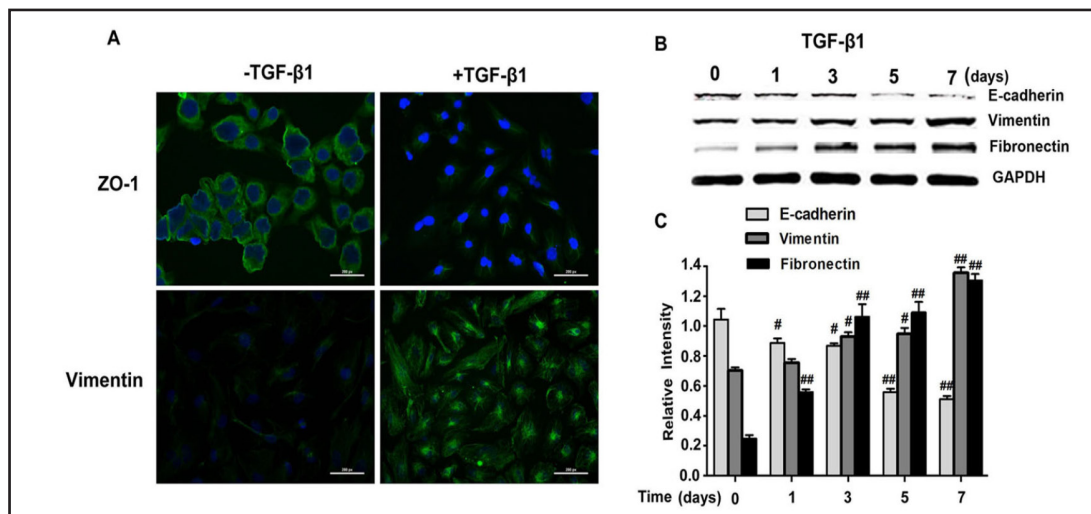


Fig. 1. Effects of TGF- β 1 on ZO-1, E-cadherin, vimentin, and fibronectin expression in HK-2 cells. (A) HK-2 cells were grown on glass coverslips in the presence of vehicle or 10 ng/ml TGF- β 1 for 5 days. Cells were fixed using 5% (wt/vol) paraformaldehyde and incubated with primary antibodies against zona occludins (ZO-1) to visualize cell junctions or vimentin (mesenchymal marker). $\times 400$ magnification. (B) Western blot analysis of extracts (30 μ g protein/lane) from HK-2 cells incubated in the presence or absence of 10 ng/ml TGF- β 1. Lysates were probed for E-cadherin, vimentin, fibronectin, or GAPDH overnight. (C) Densitometric analysis for the results in (B). GAPDH was used as internal loading control. Band intensities were calculated using Scion Image software. Values are expressed as a means \pm SEM (n=3). # P <0.05; ## P <0.01, compared with the control group.

Immunoprecipitation

After treatment of TGF- β 1, HK-2 cells were harvested in lysis buffer and Five hundred micrograms of cell lysate was incubated with 10 μ l of the p-Akt (Ser473) antibodies overnight at 4°C in a rotating. After overnight incubation, 50 μ l of protein A/G-agarose was added and the solution incubated for 6h at 4°C with rotation. The complexes were harvested by centrifugation, washed three times with RIPA buffer, and then dissociated from the beads by addition of 50 μ l of SDS sample loading buffer and heated for 5min at 95°C. The protein level of Akt1 and Akt2 were analyzed using western blot.

Statistical analysis

All data are representative of experiments done in triplicate and are expressed as the mean \pm SEM. Differences between groups were analyzed by one-way analysis of variance (ANOVA) using SPSS 13.0 software. Statistically significant was defined as P <0.05.

Results

TGF- β 1 induces a mesenchymal phenotype in tubular epithelial cells

Human proximal tubular epithelial cells (HK-2 cells) were used to examine TGF- β 1-induced EMT. As previously reported for other renal tubular epithelial cells [34-36], immunofluorescence analysis showed that decreased expression of ZO-1 (epithelial marker) and increased expression of vimentin (mesenchymal marker) were observed in cells treated with 10 ng/ml TGF- β 1 at 5 day (Fig. 1A). Western blot analysis revealed that treatment of HK-2 cells with 10 ng/ml TGF- β 1 for different times significantly decreased the level of E-cadherin protein at 5 day and 7 day (Fig. 1B). Densitometric analysis revealed that the protein levels of E-cadherin were decreased 45 and 50%, respectively, at 5 day and 7 day (Fig. 1C). TGF- β 1 also enhanced the protein levels of vimentin and fibronectin

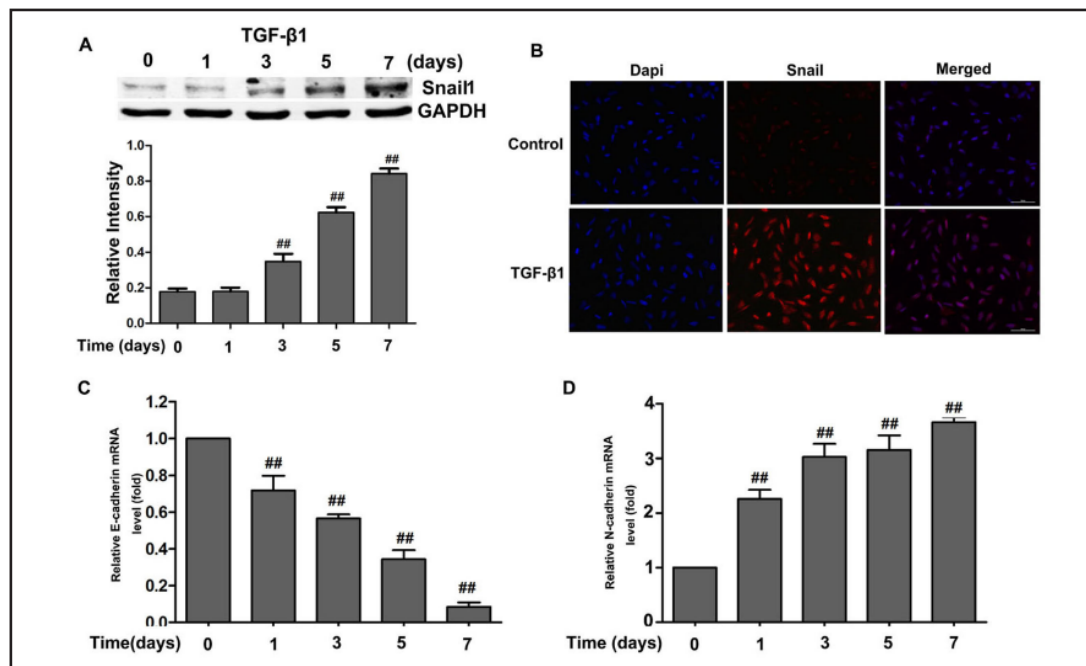


Fig. 2. TGF- β 1 induced a significant increase in protein level of Snail1 in HK-2 cells. HK-2 cells were treated with 10 ng/ml TGF- β 1, and total RNA and protein samples were collected at various times after TGF- β 1 treatment. (A) Time course for the effects of TGF- β 1 on the expression of Snail1 protein in HK-2 cells. (B) Immunofluorescence staining for Snail (red) and DAPI (blue) in HK-2 cells at 5 day treatment with TGF- β 1. $\times 400$ magnification. (C) and (D) RT-PCR analysis of E-cadherin mRNA and N-cadherin mRNA in the cells collected from 1~7day after TGF- β 1 treatment. Values are expressed as a means \pm SEM (n=3). ^{##} $P < 0.01$, compared with the control group.

in a time-dependent manner (Fig. 1B). These data suggest that TGF- β 1 induces HK-2 cells transdifferentiation to mesenchymal-like phenotype.

TGF- β 1 increases the protein level of Snail in tubular epithelial cells

Snail, a zinc finger-type transcription factor, is the most prominent EMT transcriptional regulator and contributes to EMT mainly by acting as an E-cadherin repressor [17, 18]. We used RT-PCR to examine the gene expression of Snail1 in HK-2 cells (the data not shown). To analyze the effect of TGF- β 1 on Snail1 protein expression, HK-2 cells were exposed to 10 ng/ml TGF- β 1 for different times. Western blot analysis showed that TGF- β 1 enhanced the protein level of Snail1 (Fig. 2A). Within 1~7day after exposure to TGF- β 1, there was a sustained increase in expression of Snail1, which peaked at 7day. Which were further confirmed by immunofluorescence analysis (Fig. 2B). We next examined the effect of TGF- β 1 on the gene expression of E-cadherin and N-cadherin. RT-PCR analysis showed a time-dependent reduction in the message level of E-cadherin and a time-dependent upregulation in the message level of N-cadherin in the TGF- β 1-treated HK-2 cells (Fig. 2C and D). Our data showed a concomitant change in the levels of Snail1 protein and E-cadherin mRNA after TGF- β 1 treatment, indicating that there is an association between repression of E-cadherin mRNA and activation of Snail1 during TGF- β 1-induced EMT in HK-2 cells.

GSK3 β phosphorylation mediated Snail1 expression induced by TGF- β 1 in tubular epithelial cells

GSK3 β , as a downstream target of PI3K/Akt, is necessary for the maintenance of epithelial architecture [22]. So, we examined expression of GSK3 β in TGF- β 1-treated HK-2 cells. As shown in Fig. 3, phosphorylation of GSK3 β (S9) was significantly increased in

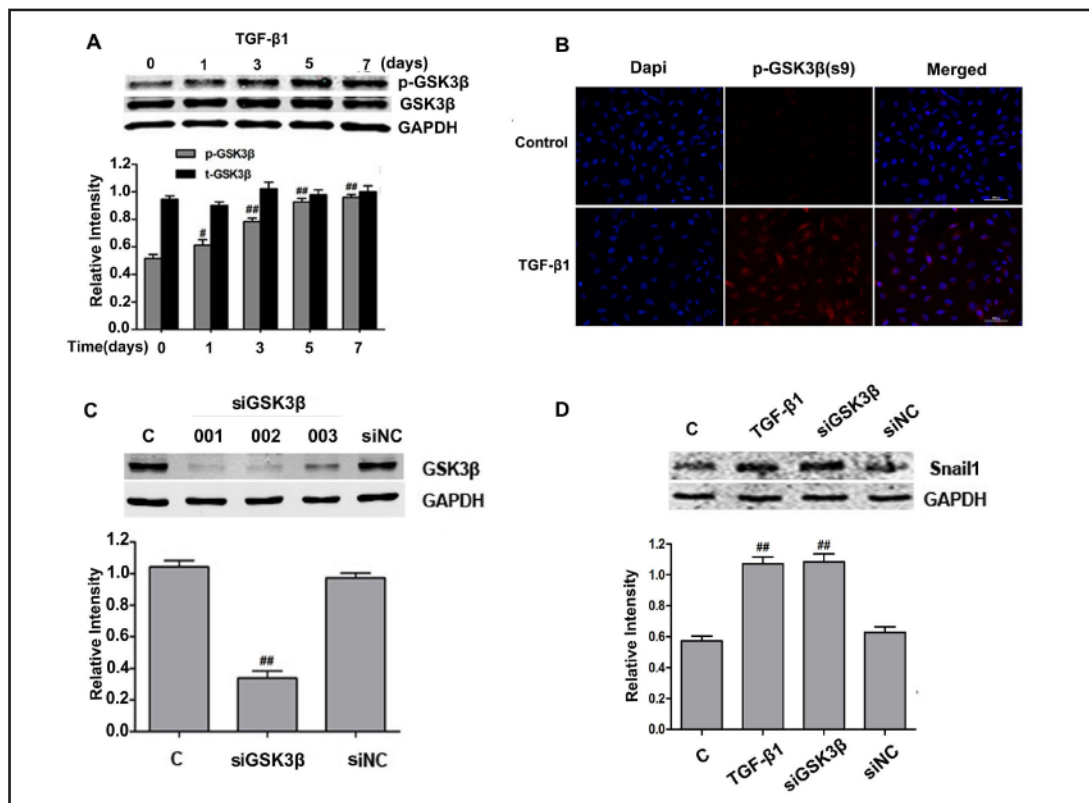


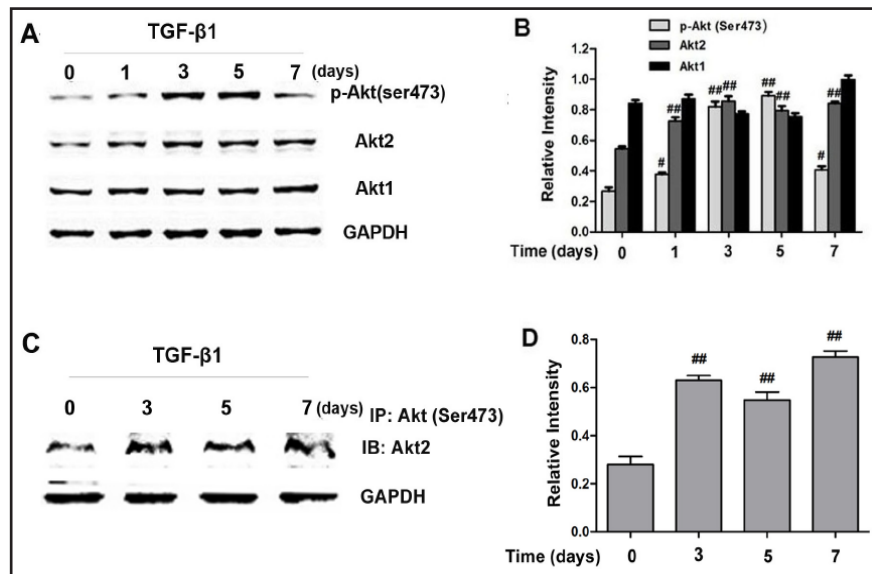
Fig. 3. Inactivation of GSK3 β mediated expression of Snail1 protein induced by TGF- β 1 in HK-2 cells. (A) Time course for the effects of TGF- β 1 on the expression of p-GSK3 β protein in HK-2 cells. (B) Immunofluorescence staining for p-GSK3 β (red) and DAPI (blue) in HK-2 cells at 5 day treatment with TGF- β 1. $\times 400$ magnification. (C) and (D) HK-2 cells were co-cultured with small interfering RNA (siGSK3 β) or random non-coding RNA (si-NC) at 50 nM for 6 h. Expressions of GSK3 β and Snail1 were detected by western blot assay. GAPDH was used as internal loading control. Band intensities were calculated using Scion Image software. Values are expressed as a means \pm SEM ($n=3$). $^{\#}P<0.05$; $^{\#\#}P<0.01$, compared with the control group.

TGF- β 1-treated HK-2 cells in a time dependent manner. However, the levels of total GSK3 β remained unchanged (Fig. 3A). Increased phosphorylation of GSK3 β was further confirmed by immunofluorescence analysis (Fig. 3B). As GSK3 β is an active kinase of Snail [20], we further examined the role of GSK3 β in Snail expression. HK-2 cells were co-cultured with small interfering RNA (siGSK3 β) at 50 nmol/L for 6 h (Fig. 3C), we found that siGSK3 β increased expression of Snail in HK-2 cells (Fig. 3D). The above data suggest that GSK3 β deactivation mediates the expression of Snail1 protein induced by TGF- β 1 in HK-2 cells.

TGF- β 1 activates PI3K/Akt2 signaling pathway in tubular epithelial cells

PI3K/Akt signaling has been suggested to play a role in TGF- β 1 induced EMT [26]. In agreement with the previous studies, we found that treatment of HK-2 cells with 10 ng/ml TGF- β 1 induced expression of phosphorylated (p)Akt (Ser473) at specific times (i.e. 1day, 3day, 5day, 7day after exposure to TGF- β 1) (Fig. 4A), but not (p) Akt (Thr308) (the data not shown). TGF- β 1 also induced expression of phosphorylated (p) PI3K for different times (Fig. 5A and B), inhibition of PI3K with Ly294002 prevented the expression of p-Akt (Ser473) (Fig. 5C and D), suggesting that TGF- β 1 triggers PI3K/Akt activation in HK-2 cells. However, Akt/PKB including three major isoforms: Akt1, -2, and -3. And it is known that Akt1 and Akt2 but not Akt3 mRNA are expressed in the kidney [37], we determined their protein levels in TGF- β 1-treated HK-2 cells. Western blot analysis showed that Akt2 expression was significantly increased in TGF- β 1-treated HK-2 cells in a time dependent manner. Within 1~7day after

Fig. 4. TGF- β 1 activated Akt2 signaling in HK-2 cells. (A) Time course for the effects of TGF- β 1 on the expression of p-Akt (Ser473), Akt2 and Akt1. (B) Denstometric analysis for the results in (A). (C) Western blot analysis of Akt2 in HK-2 cells following immunoprecipitation (IP) with immobilized Akt (Ser473)-phospho-specific antibodies.



(D) Denstometric analysis for the results in (C). GAPDH was used as internal loading control. Band intensities were calculated using Scion Image software. Values are expressed as a means \pm SEM (n=3). # P <0.05; ## P <0.01, compared with the control group.

exposure to TGF- β 1, there was a sustained increase in expression of Akt2, which peaked at 3day (coinciding with increased Akt (Ser473) phosphorylation). However, TGF- β 1 treatment did not induce significant changes in expression of Akt1 in the indicated times (Fig. 4A and B). Simultaneously, we examined the expression of Akt3 after TGF- β 1 treatment (the data not shown). The concomitant increase in Akt2 protein levels and total Akt (Ser473) phosphorylation during TGF- β 1 treatment suggest that Akt2 may be the main isoform which is activated in TGF- β 1-treated HK-2 cells. We tested this by immunoprecipitating p-Akt (Ser473) and subsequently detecting Akt2 or Akt1 levels by western blot. After TGF- β 1 treatment, increases in Ser473-phosphorylated Akt2 were observed (Fig. 4B), but not Akt1 (the data not shown). The above findings suggest that PI3K/Akt2 signaling pathway was activated in TGF- β 1-treated HK-2 cells.

Furthermore, we also examined the canonical signaling pathway of TGF- β 1, Smad2/3, the downstream effector of TGF- β 1, was significantly activated by TGF- β 1 treatment in HK-2 cells (Fig. 6A). However, TGF- β 1 increased Smad3 phosphorylation was not diminished in the presence of inhibitor of PI3K (Fig. 6A), suggesting that TGF- β 1-stimulated phosphorylation of Smad3 occurs independently of the activity of this kinase pathway in HK-2 cells.

GSK3 β /Snail1 is downstream signals for TGF- β 1-induced Akt2 expression in tubular epithelial cells

To investigate whether PI3K/Akt2 regulates GSK3 β /Snail signaling in TGF- β 1 treated HK-2 cells. Firstly, cells were preconditioned with Ly294002 (the inhibitor of PI3K), we found that Ly294002 attenuated expression of p-GSK3 β and Snail1 protein (Fig. 5E and F) induced by TGF- β 1. Next, we used RNA interference of specifically down-regulate the expression of Akt2 or Akt1. Cells were co-cultured with siAkt2 and siAkt1, we found that siAkt2 down-regulated the expression of Akt2 at least 60% (Fig. 7A and B), but did not affect the expression of Akt1 (Fig. 7C and D), suggesting that siAkt2 inhibition is specific. However, siAkt1 down-regulated the expression of Akt1 at least 60% (Fig. 8A), we also found that siAkt1 down-regulated the expression of Akt2 at least 30% (Fig. 8B), suggesting that down-regulated Akt1 expression may affect Akt2 expression. Importantly, we observed that siAkt2 attenuated the expression of p-GSK3 β induced by TGF- β 1 at least 58% and the expression of Snail1 induced by TGF- β 1 at least 15%, respectively (Fig. 7E and F). Next, we examined the

Fig. 5. PI3K (phosphatidylinositol 3-kinase) regulated GSK3 β /Snail signaling in TGF- β 1 treated HK-2 cells. (A) Time course for the effects of TGF- β 1 on the expression of p-PI3K. (B) Denstometric analysis for the results in (A). (C) HK-2 cells were preconditioned with 20 μ M PI3K inhibitor Ly-294002 or DMSO for 30 min before exposure of cells to 10 ng/ml TGF- β 1 for 3 day. Western blot analysis of p-Akt (Ser473). (D) Denstometric analysis for the results in (C). (E) Western blot analysis of p-GSK3 β and Snail1 expression. (F) Denstometric analysis for the results in (E). GAPDH was used as internal loading control. Band intensities were calculated using Scion Image software. Values are expressed as a means \pm SEM (n=3). $^{##}P<0.01$, compared with the control group. $^{**}P<0.01$, compared with the TGF- β 1 treated group.

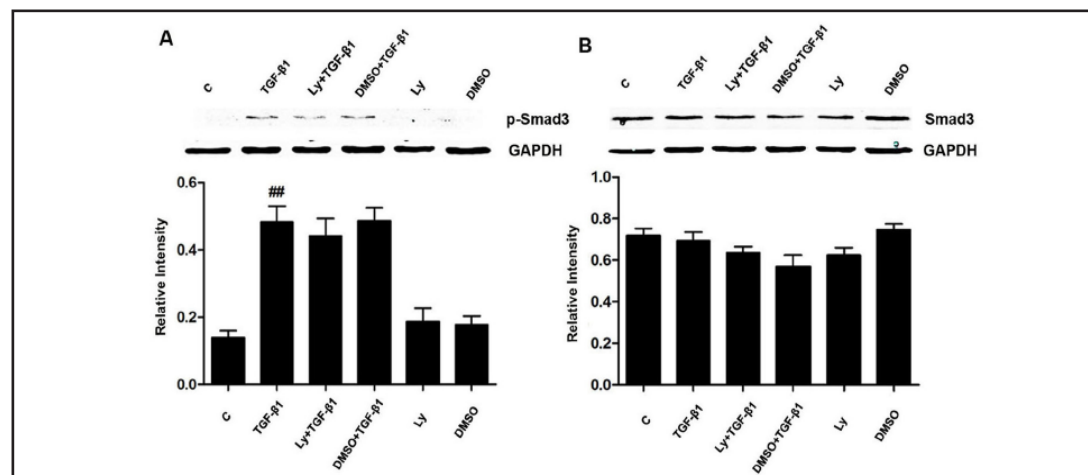
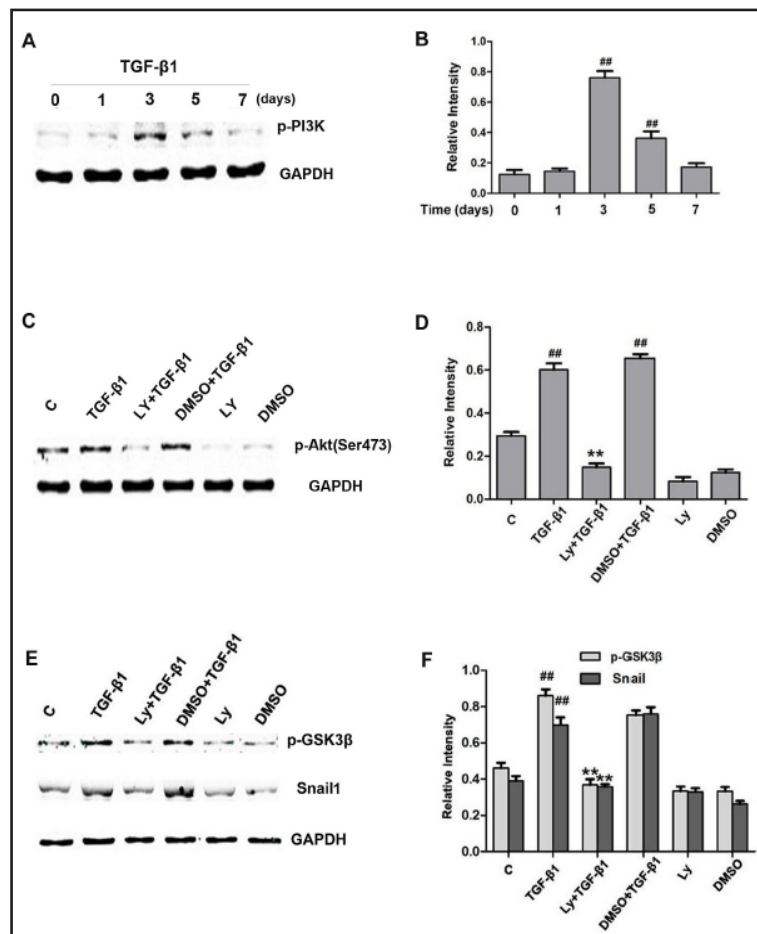


Fig. 6. TGF- β 1-stimulated Smad3 phosphorylation in HK-2 cells is insensitive to PI3K inhibition. (A) and (B) HK-2 cells were treated with DMSO, 20 μ M Ly-294002 for 30 min before treatment with 10 ng/ml TGF- β 1. Protein extracts (30 μ g) were probed using antibodies raised against pSmad3, total Smad3, or GAPDH as loading control. Values are representative of 3 experiments carried out in duplicate. Band intensities were calculated using Scion Image software. Data are means \pm SEM (n=3). $^{##}P<0.01$, compared with the control group.

effect of siAkt1 on the expression of p-GSK3 β and Snail1, as shown in Fig. 8C and D, siAkt1 attenuated the expression of p-GSK3 β induced by TGF- β 1 at least 30%. However, siAkt1

Fig. 7. PI3K (phosphatidylinositol 3-kinase) /Akt2 signaling mediated inactivation of GSK3 β and upregulation of Snail1 induced by TGF- β 1 in HK-2 cells. (A) and (C) HK-2 cells were co-cultured with small interfering RNA (si-AKT2) at 50 nM for 6 h prior to 10 ng/ml TGF- β 1 treatment for 3 day, expression of Akt2, and Akt1 were detected by western blot assay. (B) and (D) Denstometric analysis for the results in (A) and (C). (E) Western blot analysis of p-GSK3 β and Snail1 expression. (F) Denstometric analysis for the results in (E). GAPDH was used as internal loading control. Band intensities were calculated using Scion Image software. Values are expressed as a means \pm SEM (n=3). # P <0.05; ## P <0.01, compared with the control group; * P <0.05; ** P <0.01, compared with the TGF- β 1 treated group.

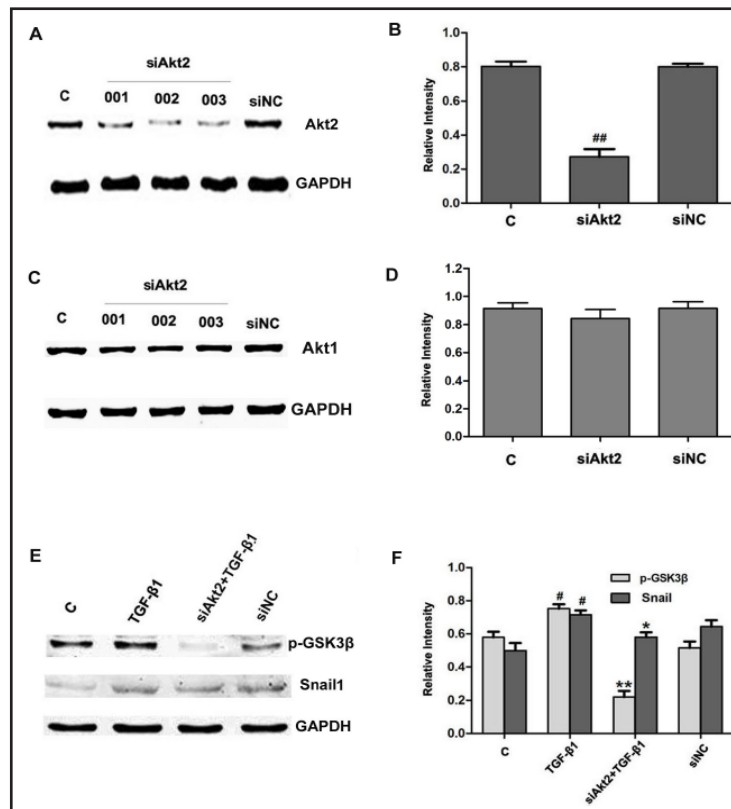
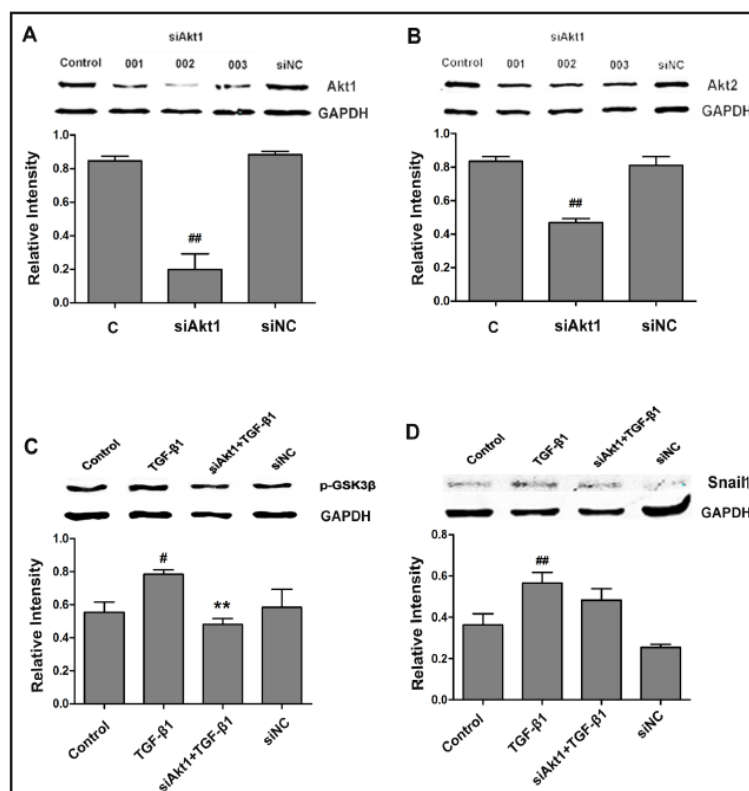


Fig. 8. Effects of siAkt1 on the expression of p-GSK3 β and Snail1 induced by TGF- β 1 in HK-2 cells. (A) and (B) HK-2 cells were co-cultured with small interfering RNA (si-AKT1) at 50 nM for 6 h prior to 10 ng/ml TGF- β 1 treatment for 3 day, expression of Akt1 and Akt2 were detected by western blot assay. (C) and (D) Western blot analysis of p-GSK3 β and Snail1 expression. GAPDH was used as internal loading control. Band intensities were calculated using Scion Image software. Values are expressed as a means \pm SEM (n=3). # P <0.05; ## P <0.01, compared with the control group; ** P <0.01, compared with the TGF- β 1 treated group.



did not attenuate the expression of Snail1 induced by TGF- β 1, we supposed these effects of siAkt1 may be due to its effect on Akt2 expression. All the above findings indicating us that GSK3 β /Snail1 are downstream signals for TGF- β 1-induced Akt2 expression in HK-2 cells.

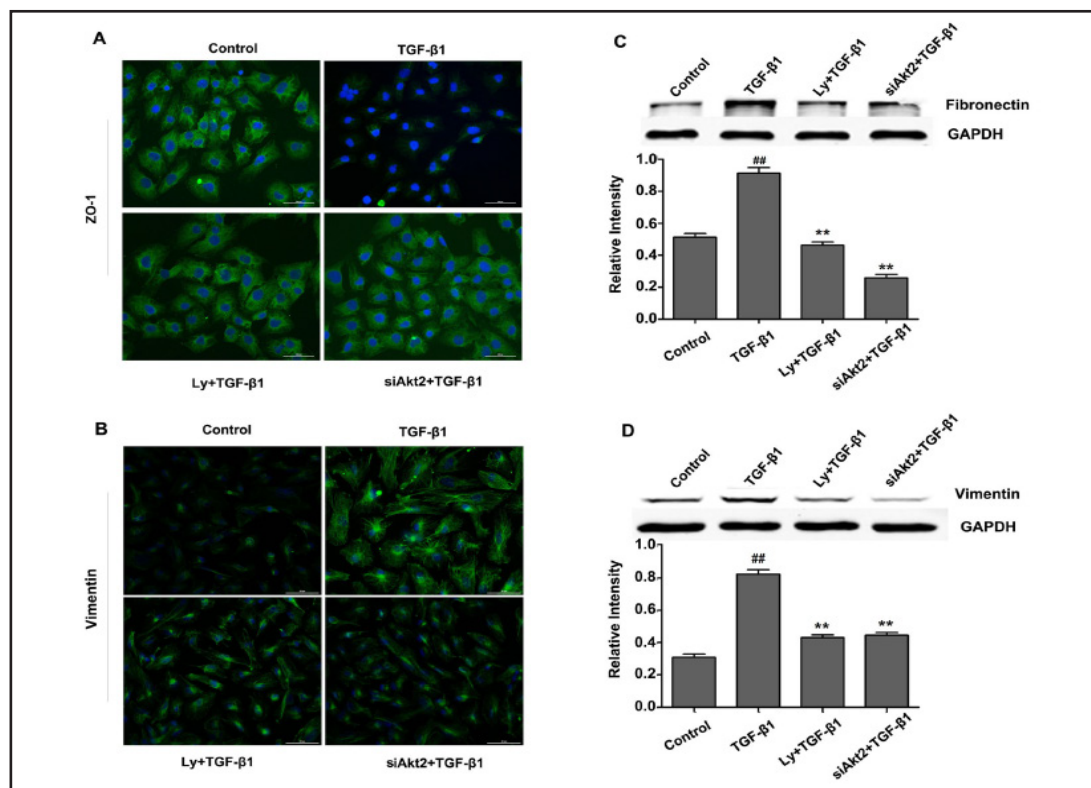


Fig. 9. TGF- β 1-mediated activation of PI3K/Akt2 signaling is important for EMT in HK-2 cells. HK-2 cells were pretreated with Ly294002 (PI3K inhibitor) 20 μ M for 30 min or Co-cultured with small interfering RNA (siAkt2) at 50 nmol/L for 6 h before treatment with 10 ng/ml TGF- β 1 for 5 day. (A) Cells were stained for epithelial marker ZO-1. (B) Cells were stained for mesenchymal marker vimentin. (C) and (D) Protein extracts (30 μ g protein/lane) were probed using antibodies against fibronectin and vimentin. GAPDH was used as internal loading control. Band intensities were calculated using Scion Image software. Values are expressed as a means \pm SEM (n=3). [#] P <0.01 compared with the control group; ^{**} P <0.01 compared with the TGF- β 1 treated group.

PI3K/Akt2 signaling involves TGF- β 1-induced EMT in tubular epithelial cells

To further confirm the role of PI3K/Akt2 signaling in TGF- β 1-induced EMT. We used Ly-294002 to precondition cells before TGF- β 1 treatment or knockdown Akt2 and Akt1 with siRNAs. Immunofluorescence analysis showed that Ly-294002 or siAkt2 abrogated the TGF β 1-induced decrease in E-cadherin protein (epithelial maker) (Fig. 9A). Ly-294002 or siAkt2 also blocked the increase in vimentin protein induced by TGF β 1 (Fig. 9B), which were further confirmed by western blot analysis (Fig. 9D). Similarly, western blot analysis showed that Ly-294002 or siAkt2 prevented the increase in fibronectin protein in HK-2 cells (Fig. 9C). We also examined the effect of siAkt1 on the expression of vimentin and fibronectin, we detected that siAkt1 attenuated the expression of vimentin and fibronectin, but these effects are less than siAkt2 (Fig. 10A and B). These data suggest that PI3K/Akt2 signaling is important for TGF- β 1-induced EMT in HK-2 cells.

Discussion

Excessive deposition of extracellular matrix (ECM) in the tubulointerstitium is closely associated with progressive decline in renal function in renal diseases [4, 38]. Myofibroblasts have been recognized as the major effectors involved in excessive deposition of ECM under

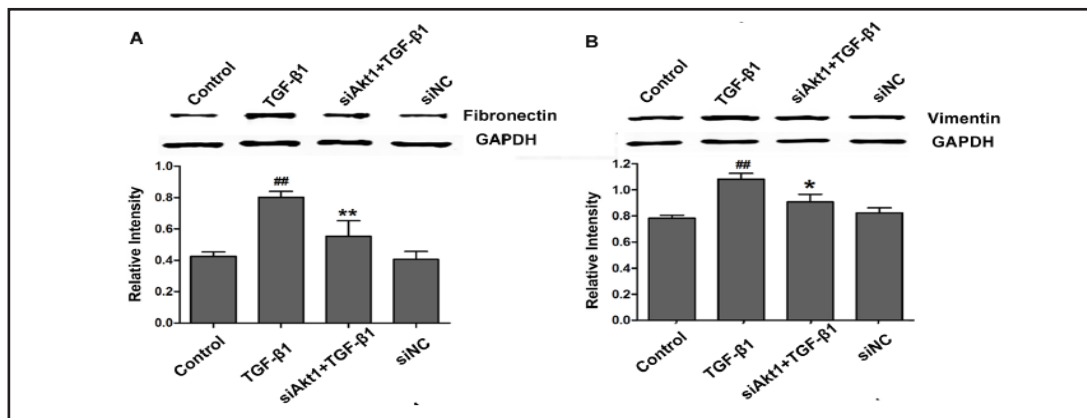
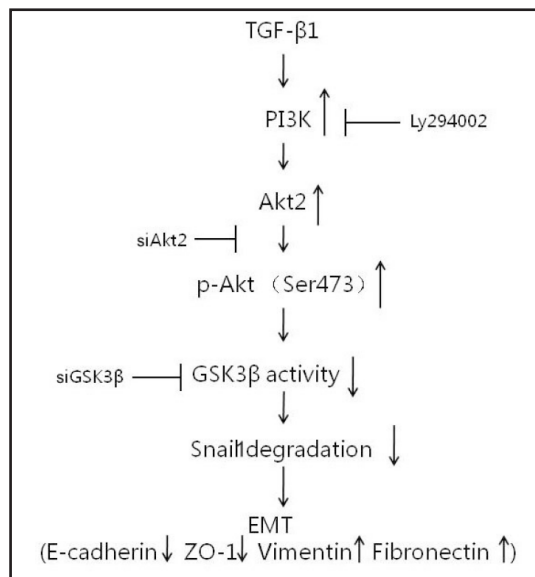


Fig. 10. Effects of siAkt1 on the expression of fibronectin and vimentin induced by TGF- β 1 in HK-2 cells. (A) and (B) HK-2 cells were co-cultured with small interfering RNA (siAKT1) at 50 nM for 6 h prior to 10 ng/ml TGF- β 1 treatment for 3 day, expression of fibronectin, and vimentin were detected by western blot assay. GAPDH was used as internal loading control. Band intensities were calculated using Scion Image software. Values are expressed as a means \pm SEM (n=3). ## P <0.01, compared with the control group; * P <0.05, ** P <0.01 compared with the TGF- β 1 treated group.

Fig. 11. Summary of signaling pathways involved in TGF- β 1-induced EMT in renal tubular epithelial cells. PI3K/Akt2 signaling play an important role in TGF- β 1-induced EMT in HK-2 cells through GSK3 β /Snail1 signals.



pathological conditions. However, emerging evidence suggests that injured tubular epithelial cells play a pivotal role in tubulointerstitial fibrosis through the process of EMT [1-3]. Here, we demonstrate that TGF- β 1-induced PI3K/Akt2 signaling is required for EMT. Blockade of PI3K/Akt2 signaling reduced the TGF- β 1-induced deactivation of GSK3 β , expression of Snail, and induction of EMT.

EMT is defined by the loss of epithelial characteristics and the acquisition of mesenchymal phenotype [6]. Consistent with others [34-36], we found that TGF- β 1 downregulated expression of E-cadherin and ZO-1 in HK-2 cells, while increased expression of vimentin and fibronectin. Our data suggest that TGF- β 1 can induce a transition of HK-2 cells to mesenchymal-like phenotype. The molecular signaling underlying EMT remains incompletely understood. Multiple intracellular signaling pathways are involved in EMT regulation, including TGF- β 1/Smad, p38MAPK, integrin-linked kinase (ILK), and protein kinase B (PKB)/Akt [39-42]. A recent study showed that Akt activity played an important role in the TGF- β 1-induced EMT in NRK52E cells [26]. However, there are three Akt

isoforms, which are structurally homologous, they have different features and functions. Our data showed that the expression of p-PI3K, Akt2 and p-Akt (Ser473) were significantly increased in HK-2 cells after TGF- β 1 treatment, but not Akt1, and Akt2 was the main isoform of Ser473-phosphorylated Akt, suggesting that PI3K/Akt2 signaling may be activated in TGF- β 1-treated HK-2 cells. Importantly, inhibition of PI3K/Akt2 signaling pathway with Ly294002 or siAkt2, attenuated TGF- β 1-induced EMT-like changes in HK-2 cells, indicating that PI3K/Akt2 signaling, in part, mediated TGF- β 1-induced EMT in HK-2 cells. Along with our results, recent studies showed that Akt2 expression was required for EMT-like morphological changes induced by insulin-like growth factor-I (IGF-I) [33], and Akt2 has also been shown to be the isoform that mediates insulin receptor signaling in skeletal muscle and liver [28, 43]. Furthermore, inhibition of either PI3K or Akt activity caused a decrease in GSK3 β phosphorylation and attenuated TGF- β 1-induced EMT in rat kidney epithelial cells [26]. In addition, we detected TGF- β 1 triggered Smad3 phosphorylation in HK-2 cells, which independently of PI3K (Fig. 6). Our results are consistent with the ones reported by Jayesh J Kattla et al [26]. However, Runyan et al. [44] demonstrate that TGF- β -mediated Smad3 phosphorylation and activation is regulated by PI3K signaling in human mesangial cells. Thus cell type-specific signal transduction events downstream of TGF- β 1 may exist in different kidney cells.

GSK3 β , as the downstream of PI3K/Akt signaling, is also necessary for the maintenance of epithelial architecture [22]. In the present study, we showed that increased the phosphorylation of GSK3 β (Ser9), which was blocked by Ly294002 and siAkt2. Moreover, GSK3 β is constitutively active and can bind to and phosphorylate snail to facilitate its degradation in many cells [20]. Snail is a critical transcriptional regulator of E-cadherin [17-19], which binds to the E-boxes of the human E-cadherin promoter and repress E-cadherin expression. In our present study, we showed a marked increase in the protein and mRNA level of Snail1, and a concomitant decrease in the message level of E-cadherin and increase in the message level of N-cadherin after TGF- β 1 treatment in HK-2 cells, suggesting an association between repression of E-cadherin mRNA and expression of Snail during TGF- β 1-induced EMT. As Snail activity is regulated by GSK3 β -mediated phosphorylation [20], we examined the role of GSK3 β in the expression of Snail in TGF- β 1-treated HK-2 cells. Cells were co-cultured with small interfering RNA (siGSK3 β) prior to TGF- β 1 treatment, we found that siGSK3 β induced Snail1 expression in HK-2 cells, which was further confirmed that GSK3 β deactivation is important for Snail1 expression in TGF- β 1 treated cells. Our findings are consistent with those of the previous studies [12, 45] and comparable with the recent evidence that inactivation of GSK3 β mediates radiation-induced Snail1 expression in RLE-6TN cells [46]. In addition, inhibition of PI3K/Akt2 signaling using Ly294002 or siAkt2 before TGF- β 1 treatment prevented the TGF- β 1-induced modulation of GSK3 β , Snail, fibronectin, and vimentin protein levels in HK-2 cells. As we found the expression of Akt1 in HK-2 cells, so we also examined the effect of siAkt1 on the expression of GSK3 β , Snail, fibronectin, and vimentin protein levels in HK-2 cells. Firstly, we found that siAkt1 not only downregulated Akt1 expression (60%), but also affected Akt2 expression (30%), this result is similar with the study reported by Zhang Lan et al [47]. In this study, authors indicated that knockdown of Akt1 promotes Akt2 expression in H₂O₂-treated HLECs. Indicating us the effect of siAkt1 on the expression of Akt2 depend on the nature of the stimulus, the cell type, the duration of activation, and probably, most importantly, the activation of other signaling pathways. In addition, we found that siAkt1 attenuated the expression of p-GSK3 β , fibronectin and vimentin, but these effects are less than siAkt2, we supposed that these effects of siAkt1 may be due to its effect on Akt2 expression. As we found that siAkt2 attenuated the expression of Snail1 in HK-2 cells were only 15%, and siAkt1 did not attenuate Snail1 expression. Altogether, our data suggest that Akt2/GSK3 β /Snail signaling is important for TGF- β 1-induced EMT in HK-2 cells.

Based on our findings, we demonstrated a mechanism for TGF- β 1-induced EMT in HK-2 cells as shown in Figure 8, TGF- β 1, through activation of PI3K, leads to activation of Akt2. Activated Akt2 causes the phosphorylation of GSK3 β , resulting in the upregulation of Snail,

which represses the E-cadherin expression, ultimately leads to EMT in HK-2 cells. Taken together, our findings demonstrate that Akt2 mediates TGF- β 1-induced EMT through GSK3 β /Snail signaling. However, our current findings are observed *in vitro* conditions, thus, these *in vitro* findings need to be further confirmed using appropriate animal models to support the potential importance of Akt2/GSK3 β /Snail signaling in the TGF- β 1-induced EMT of renal tubular epithelial cells.

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Disclosure Statement

All authors declare that no conflicts of interest exist.

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