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Original Article

PAK4 enhances TGF-β1-induced epithelial-mesenchymal transition through activating β-catenin signaling pathway in renal tubular epithelial cells

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Abstract: Epithelial-mesenchymal transition (EMT) of renal tubular epithelial cells contributes to development and progression of renal interstitial fibrosis in CKD. p21-activated kinase 4 (PAK4) is a member of serine/threonine protein kinases but the role of PAK4 in renal fibrosis remains unknown. In this study, we investigated the effects of PAK4 on transforming growth factor-β1 (TGF-β1)-treated human renal tubular epithelial cells (HK-2 cells) and aimed to elucidate probable mechanisms for its fibrogenic effects. Our results revealed that PAK4 was highly expressed in TGF-β1-treated HK-2 cells. Overexpressing PAK4 could further decrease TGF-β1-induced E-cadherin expression and increase TGF-β1-induced fibronectin and vimentin expression in HK-2 cells. In addition, overexpressing PAK4 could promote the translocation of β-catenin from cell membranes into the nucleus in TGF-β1-treated HK-2 cells. These results indicate that PAK4 could enhance TGF-β1-induced EMT in renal tubular epithelial cells. Our findings indicate that PAK4 may promote renal interstitial fibrosis by activating β-catenin signaling pathway. Thus, we suggest that PAK4 might be a potential therapeutic target for ameliorating renal interstitial fibrosis.

Keywords: p21-activated kinase 4 (PAK4), transforming growth factor beta 1 (TGF-β1), epithelial-mesenchymal transition (EMT), beta-catenin, renal interstitial fibrosis (RIF)

Introduction

Chronic kidney disease (CKD) is a progressive irreversible process whose final outcome is end-stage renal disease (ESRD) [1, 2]. The fatality rate of CKD in children with renal disease is 9% and that of ESRD is extremely high [3]. There are various causes of CKD, with renal interstitial fibrosis as the most important pathological feature [4-7]. It is believed that the extent of renal interstitial fibrosis is closely related to prognosis of CKD. The pathogenesis of renal interstitial fibrosis is very complex and remains unclear. Therefore, we need to deeply study the mechanism of renal interstitial fibrosis and provide a theoretical basis for ameliorating progression of CKD. Recently, great efforts have been made to explore the mechanism of renal interstitial fibrosis.

EMT occurs in different contexts of embryonic development, tissue fibrosis, and tumorigenesis [8] and contributes to renal interstitial fibrosis [9-11]. EMT can be caused by massive mediators such as various cytokines and growth factors, among which TGF-β is considered the key fibrogenic growth factor [9]. TGF-β expression has been detected in damaged kidneys, both in patients suffering from kidney diseases and in animal models with renal injuries [12, 13]. Clinical studies have also found elevated TGF-β expression in urine of patients with kidney diseases and level of TGF-β has been positively related to severity of renal interstitial fibrosis [12, 13].

PAKs are a family of serine/threonine protein kinases that are evolutionarily conserved and act as key mediators for RHO family small GT-

Pase signaling. PAKs can phosphorylate massive substrates and affect a wide range of biological processes including cytoskeletal construction, cell mitosis, proliferation, motility, transformation, stress, inflammation, and gene expression [14]. PAKs consist of 6 members that are divided into two categories: type I PAKs (PAK1, PAK2 and PAK3) and type II PAKs (PAK4, PAK5, and PAK6), according to their structural characteristics and similarity [15]. PAK4 is the most representative of the type II PAKs. One study has shown that reducing PAK4 expression inhibited occurrence of EMT in prostate cancer cells and colon cancer cells [16]. PAK4 has been suggested to promote the EMT process of tumor cells. However, the effect of PAK4 on EMT of renal tubular epithelial cells, a potential mechanism of renal interstitial fibrosis, remains unknown.

In this study, we investigated the effect of PAK4 on EMT of renal tubular epithelial cells and illuminated the molecular mechanism. We found that PAK4 could induce EMT and enhance TGF-β1-induced EMT by activating β-catenin signaling pathway in renal tubular epithelial cells. These data indicate that PAK4 might play a crucial role in renal interstitial fibrosis through enhancing the EMT process, providing a therapeutic target for CKD.

Materials and methods

Cell culture

Normal human renal tubular epithelial cell line HK-2 was cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (Gibco, Los Angeles, CA, USA) containing 10% fetal bovine serum (FBS), incubated at 37°C in a humidified incubator with 5% CO_2.

Real-time PCR

Total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, USA) from HK-2 cells. Total RNA (1 μg) was used for synthesis of cDNA using PrimeScript™ RT Reagent Kit (TaKaRa, China). Sequences (5'-3') of the primers for Fibronectin were ACAACACCGAGGTGACTGAGAC (F) and GGACACAACGATGCTTCCTGAG (R), Vimentin were AGGCAAAGCAGGAGTCCACTGA (F) and ATCTGGGCGTTCCAGGGACTCAT (R), E-cadherin were GCCTCCTGAAAAGAGAGTGGAAG (F) and TGGCAGTGTCTCTCCAAATCCG (R), PAK4 were GATGATTCGGGACAACCTGCCA (F) and AGGAATGGGTGCTTCAGCAGCT (R), GAPDH were GTCTCCTCTGACTTCAACAGCG (F) and ACCACCCTGTTGCTGTAGCCAA (R). Real-Time PCR was performed using SYBR green mix (TaKaRa, China). Reactions were performed with a 7500 Fast Real-Time PCR System (Applied Biosystems, La Jolla, CA).

Western blot assay

HK-2 cells were lysed in RIPA lysis buffer supplemented with protease inhibitors (Roche, USA). Nuclear protein was extracted through a Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime, China). Total protein extracts were separated by 8% SDS-PAGE and then transferred to a PVDF membrane (Millipore, USA). The membranes were incubated with anti-E-cadherin (DB, 1:5000), anti-Fibronectin (Sigma, 1:5000), anti-Vimentin (Abcam, 1:1000), anti-PAK4 (Cell Signal, 1:1000), anti-Flag (Shang Hai Ruixing, 1:2000), anti-β-catenin (Abcam, 1:1000), anti-GAPDH (KangChen, China, as a loading control, 1:15000), and anti-Lamin B (Abcam, as a loading control, 1:1000) antibodies.

Transwell migration assay

Lentivirus-infected HK-2 cells (5×10^4 shPAK4 and NC) were respectively placed into the upper chambers of Transwell chambers (8 μm BioCoat Control Inserts, Corning Costar, USA). The lower chambers were filled with 600 μl DMEM/F12 medium added with 10% FBS. After incubation for 24 hours at 37°C, the cells were fixed by 4% paraformaldehyde and stained with 0.4% trypan blue. Cells in the upper chambers were removed with cotton swabs, gently, and counted (five random fields per well at 100X magnification) under a light microscope.

Lentiviral infection

PAK4-shRNA and control lentivirus were obtained from Shanghai Genechem Co. Ltd. PAK4-shRNA target sequence was 5'-CTTCATCAAGATTGGCGAG-3' and the control-shRNA(NC) sequence was 5'-UUCUCCGAACGUGUCACGUTT-3'. HK-2 cells were seeded into 12-well plates overnight, then infected with PAK4-shRNA and control-shRNA (NC), following manufacturer guidelines (GeneChem, China). 5 μg/mL puromycin (Sigma, USA) was added into the medium to select infected cells. Fluorescence microscopy was used to detect the lentiviral infection rate 5 days after infection.

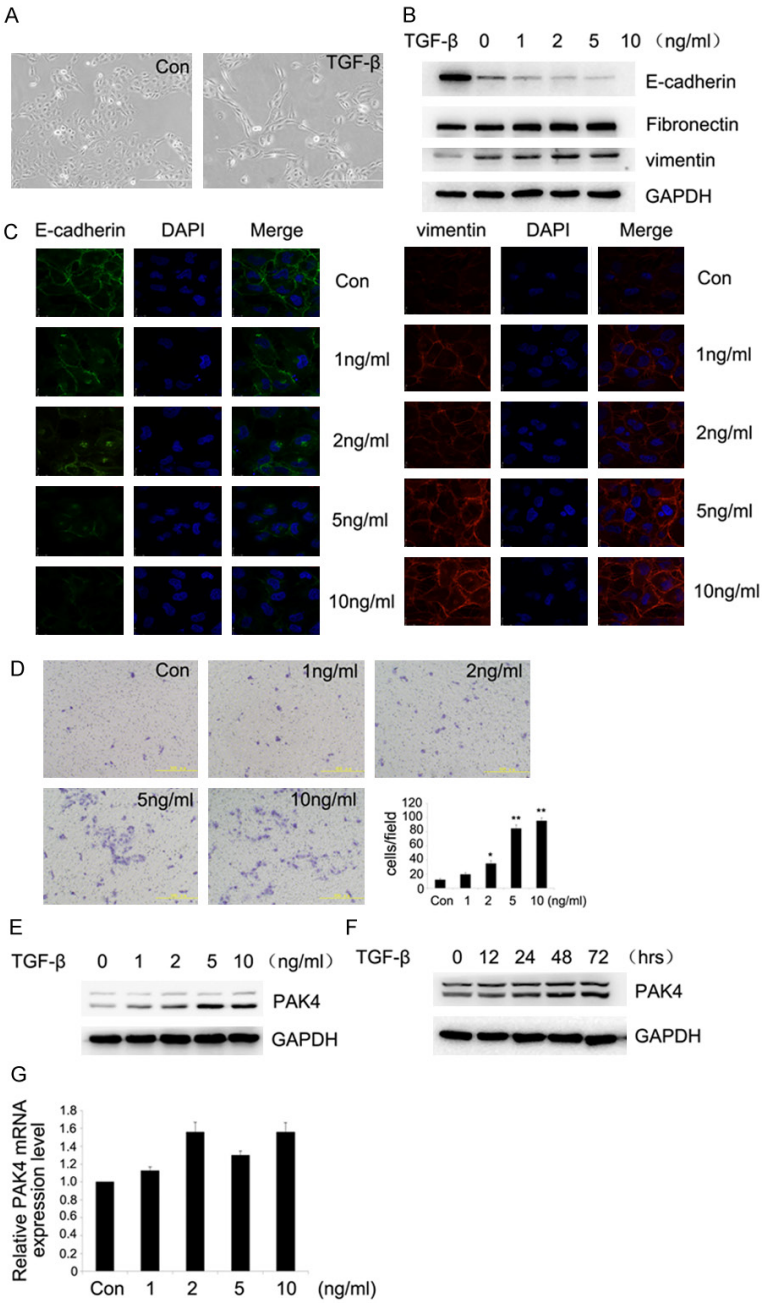

Figure 1. TGF-β1 induced morphological changes and EMT in HK-2 Cells and expression of PAK4 in TGF-β1-treated HK-2 C cells. (A) HK-2 cells were treated with 5 ng/mL TGF-β1 for 72 h. The morphological change was observed with inverted microscope (10 ×). (B) HK-2 cells were treated with 0, 1, 2, 5, 10 ng/ml TGF-β1 for 48 h. Western Blot assay was carried out using anti-E-cadherin, fibronectin, vimentin, GAPDH antibodies. (C) HK-2 cells were treated as (B) and analyzed by immunofluorescence using anti-E-cadherin, vimentin antibodies followed by Alexa Flour 488 (green) or 594 (red) antibody and nucleus was stained by DAPI (blue) (60 ×). (D) HK-2 cells were treated as (B). Effects of different concentration of TGF-β1 on the migration of cultured HK-2 cells were examined by Transwell migration assays. Results are representative of three independent experiments. Migrated cells were plotted as the average number per field of view. *P<0.05, **P<0.01. (E, F) HK-2 cells were treated with 0, 1, 2, 5, 10 ng/ml TGF-β1 for 48 h (E) and with 5 ng/mL TGF-β1 for 0, 12 h, 24 h, 48 h and 72 h (F). Protein expression was detected by Western Blot assay with anti-PAK4, GAPDH antibodies. (G) The same stimulation as (B). Real-Time PCR was performed to measure the mRNA of PAK4.

Immunofluorescence

HK-2 cells were fixed with methanol at room temperature for 20 minutes and blocked with normal goat serum for 30 minutes. The cells were incubated with primary antibody at 4°C overnight and with the secondary antibody conjugated with Alexa Fluor 488 (Green) or 594 (red) dye from Molecular Probes, after washing three times in PBT (PBS with 1‰ triton x-100). DNA dye DAPI (Molecular Probes) was used (blue). Confocal scanning analysis system was performed using a Leica laser confocal scanning microscope, in accordance with established methods, utilizing sequential laser excitation to minimize the possibility of fluorescent emission bleed-through.

Statistical analysis

Differences between the two groups were evaluated with one-way ANOVA and t-test. All data were analyzed using Statistical SPSS Version 17.0. *P* value <0.05 was considered statistically significant. *P* value <0.01 was considered remarkably statistically significant.

Results

TGF-β1 induced morphological changes and EMT in HK-2 cells and PAK4 was involved in TGF-β1-induced EMT in HK-2 cells

Since TGF-β is recognized as the major factor causing EMT, regular cultivating HK-2 cells

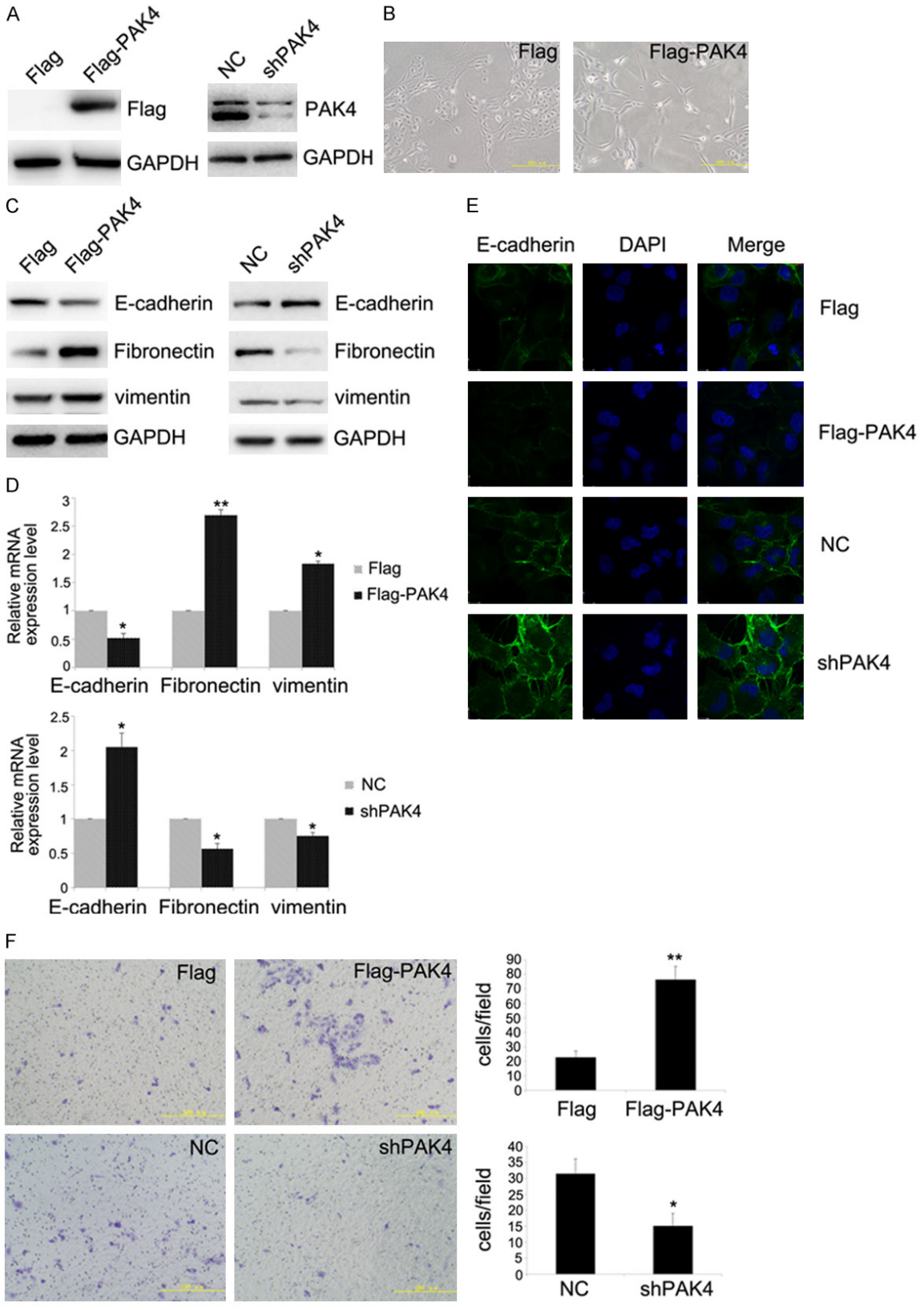

Figure 2. PAK4 induced morphological changes and EMT in HK-2 Cells. (A) Empty vector, Flag-PAK4, NC, and shPAK4 were stably expressed in HK-2 cells through lentivirus. (B) Empty vector and Flag-PAK4 stably expressed HK-2 cells were used for observing the morphological change. (C, D) Western Blot assay and real-time PCR were performed

to examine the EMT markers in the Empty vector and Flag-PAK4, or NC and shPAK4 stably expressed HK-2 cells. *P<0.05, **P<0.01. (E) The Empty vector, Flag-PAK4, NC, and shPAK4 stably expressed HK-2 cells were used to detected E-cadherin with immunofluorescence by Alexa Flour 488 (green) antibody and nucleus was stained by DAPI (blue) (60 ×). (F) The same cells as (E) were used to observe the migration activity by Transwell migration assays. Results are representative of three independent experiments. Migrated cells were plotted as the average number of cells per field of view. *P<0.05, **P<0.01.

were treated with 5 ng/mL TGF-β1 for 72 hours. Morphological changes were observed by phase contrast microscope. Compared with normal cultured HK-2 cells, TGF-β1-treated HK-2 cells were remodeled, the morphology was found to be spindle-shaped with increased intercellular space instead of a polygonal shape (**Figure 1A**). HK-2 cells were incubated with TGF-β1 (0, 1, 2, 5 and 10 ng/mL) for 48 hours. The results of Western Blot assay showed that TGF-β1 downregulated protein expression of E-cadherin and upregulated protein expression of fibronectin and vimentin in a dose dependent manner (**Figure 1B**). Furthermore, protein expression was detected by immunofluorescence. Results showed that E-cadherin presented a continuously linear distribution on cell membranes while Vimentin was few in normal HK-2 cells. With increasing concentrations of TGF-β1, protein expression of E-cadherin decreased while that of Vimentin increased in a dose dependent manner (**Figure 1C**). Transwell migration assay was performed and our results revealed that HK-2 cell migration ability was obviously enhanced along with increasing concentrations of TGF-β1 (**Figure 1D**). All of these results suggest that TGF-β1 could, indeed, induce EMT in HK-2 cells. Further Western Blot assay showed that with the increase of TGF-β1 concentration and incubating time, protein expression levels of PAK4 gradually increased (**Figure 1E**, **1F**). PAK4 mRNA expression changed without statistical significance, however, after treatment with TGF-β1 (**Figure 1G**).

PAK4 induced morphological changes and EMT in HK-2 cells

In order to detect the effects of PAK4 on EMT in renal tubular epithelial cells, we successfully constructed stable infected HK-2 cells with overexpressing PAK4 and silencing PAK4 using lentivirus. Western Blot assay detected that Flag-PAK4 protein expression was efficiently upregulated compared to that in Flag vacant vector group. Protein expression of PAK4 in shPAK4 group was significantly lower than in NC group (**Figure 2A**). Compared with Flag vacant vector group, HK-2 cells with overexpressing PAK4 appeared as a spindle shape with increased intercellular space (**Figure 2B**). The results of Western blot and real-time PCR revealed that overexpressing PAK4 downregulated protein and mRNA expression of E-cadherin and upregulated protein and mRNA expression of fibronectin and vimentin. However, silencing PAK4 had the exact opposite results (**Figure 2C**, **2D**). Moreover, immunofluorescence assay and Transwell assay were used to further verify effects. Immunofluorescence results revealed that overexpressing PAK4 reduced E-cadherin expression while silencing PAK4 increased E-cadherin expression, remarkably (**Figure 2E**). Under an inverted microscope, much more cells migrated to the lower layer of microporous membrane, showing that overexpressing PAK4 increased cell migration ability and silencing PAK4 reduced cell migration ability (**Figure 2F**).

PAK4 enhanced TGF-β1-induced EMT process in HK-2 cells

In previous results, we found that PAK4 was involved in TGF-β1- induced EMT in HK-2 cells. In the following study, we further explored the role of PAK4 in this process. Stably infected HK-2 cells that overexpress and silence PAK4 were still used. Western blot assay showed that overexpression of PAK4 further reduced protein expression of E-cadherin and increased that of fibronectin and vimentin induced by TGF-β1. Protein expression of E-cadherin induced by TGF-β1 was not obviously changed but protein expression of fibronectin and vimentin decreased in silencing PAK4 group (**Figure 3A**). Real-time PCR results revealed that overexpression of PAK4 further reduced mRNA expression of E-cadherin and increased mRNA expression of fibronectin and vimentin induced by TGF-β1. Decreased mRNA expression of E-cadherin and increased expression of fibronectin and vimentin induced by TGF-β1 were significantly reversed by silencing PAK4 (**Figure**

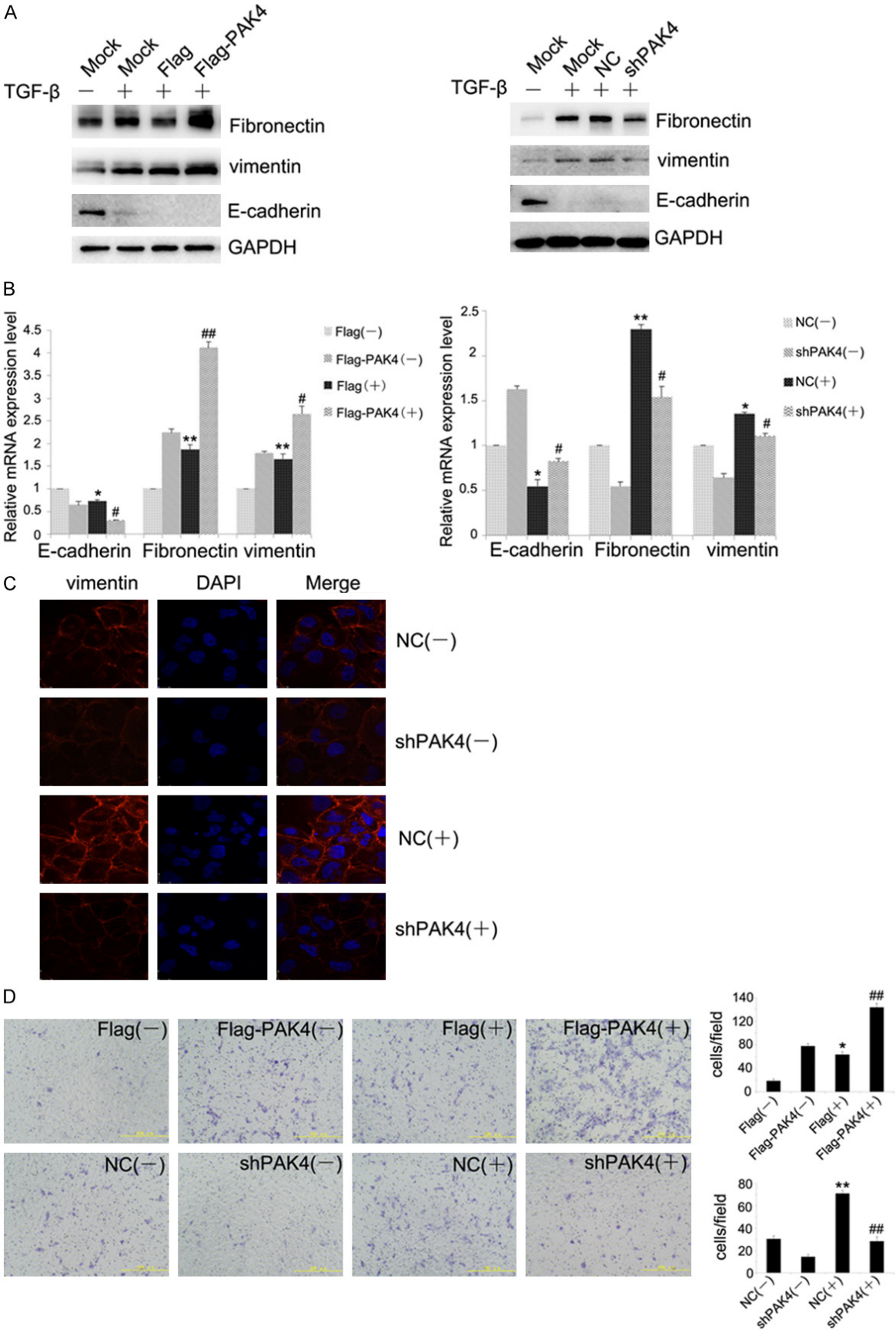
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Figure 3. PAK4 promotes TGF-β1-induced EMT process in HK-2 cells through activating β-catenin signaling pathway. (A) Western Blot assay was carried out to examine the EMT markers in a group of Mock/-, Mock/+, Empty vector/+, Flag-PAK4/+ HK-2 cells and another group of Mock/-, Mock/+, NC/+, shPAK4/+ HK-2 cells. (B) A group of Flag/-, Flag-PAK4/-, Flag/+, Flag-PAK4/+ HK-2 cells and another group of NC/-, shPAK4/-, NC/+, shPAK4/+ HK-2 cells were collected for measuring the EMT markers by Real-time PCR. *P<0.05, **P<0.01, #P<0.05, ##P<0.01. (C) NC/-, shPAK4/-, NC/+, shPAK4/+ HK-2 cells were used to detected vimentin with immunofluorescence by Alexa Flour 594 (red) antibody and nucleus was stained by DAPI (blue) (60 ×). (D) The same cells as (B) were cultured to observe the migration activity by Transwell migration assays. Results are representative of three independent experiments. Migrated cells were plotted as the average number of cells per field of view. *P<0.05, **P<0.01, #P<0.05, ##P<0.01.

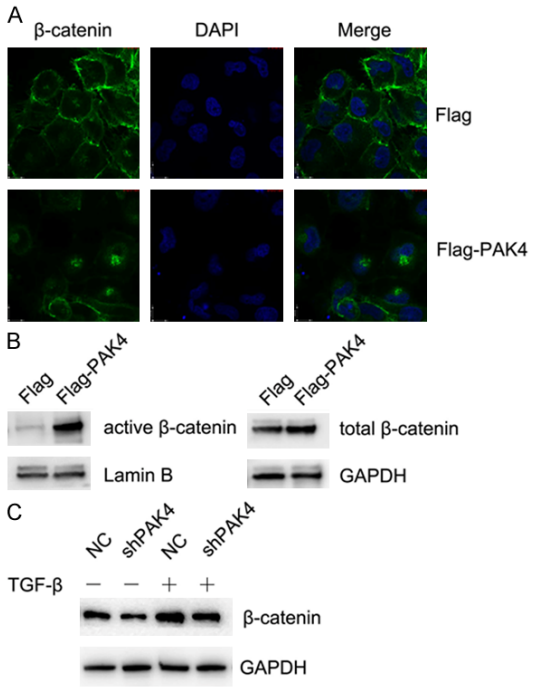

Figure 4. PAK4 promotes the TGF-β1-induced EMT process in HK-2 cells through activating β-catenin signaling pathway. (A) Empty vector and Flag-PAK4 stably expressed HK-2 cells were used to observe the translocation of β-catenin with immunofluorescence by Alexa Flour 488 (green) antibody and nucleus was stained by DAPI (blue) (60 ×). (B) Western Blot assay was employed to measure the active and total β-catenin expression separately in Empty vector and Flag-PAK4 stably expressedHK-2 cells. (C) NC/-, shPAK4/-, NC/+, shPAK4/+ HK-2 cells were collected for detecting the β-catenin by Western Blot assay using anti-β-catenin, GAPDH antibodies.

3B). In addition, immunofluorescence assay results presented that silencing PAK4 attenuated protein expression of vimentin induced by TGF-β1 (**Figure 3C**). Alterations of cell migration ability were also tested by Transwell migration assay. Overexpression of PAK4 could increase the number of cells migrating to the lower layer of microporous membranes, illustrating that overexpressing PAK4 could efficiently increase the migration ability of HK-2 cells (**Figure 3D**). All of the above data suggests that PAK4 enhances TGF-β1-induced EMT in HK-2 cells.

PAK4 enhanced TGF-β1-induced EMT process in HK-2 cells through activating β-catenin signaling pathway

Research has pointed out that activation of Wnt/β-catenin signaling pathways contributes to renal interstitial fibrosis [17]. Therefore, we further investigated whether β-catenin participates in the process of promoting TGF-β1-induced EMT by PAK4. Immunofluorescence assay was used to observe the changes of β-catenin. The images of confocal laser microscopy showed that overexpressing PAK4 made β-catenin proteins translocate from cell membranes into the nucleus in TGF-β1-treated HK-2 cells (**Figure 4A**). Total protein and nuclear protein of β-catenin in HK-cells were extracted, respectively, using a protein extraction kit. We found that overexpressing PAK4 could remarkably increase active β-catenin protein expression and total β-catenin protein expression increased a little bit more (**Figure 4B**). Western Blot results demonstrated that silencing PAK4 caused the reduction of β-catenin in HK-2 cells induced by TGF-β1 (**Figure 4C**). Thus, we suggest that PAK4 enhances TGF-β1-induced EMT process in HK-2 cells through activating β-catenin signaling pathway.

Discussion

Renal interstitial fibrosis is a biological process managed by interaction between different cellular constituents and intricate networks of signaling pathways [18]. The pathogenesis of renal interstitial fibrosis is very complex and remains unclear. EMT in renal tubular epithelial cells plays a crucial role during the process of renal interstitial fibrosis [9-11]. EMT has been identified in tubular epithelial cells of human kidney biopsies with different kidney diseases

and has been correlated with the degree of diseases [19, 20]. TGF-β is regarded as the principle fibrogenic growth factor involved in the EMT process in a variety of diseases [21-24]. In our study, we confirmed that TGF-β1 could cause morphological changes and enhance migratory capacity of HK-2 cells. TGF-β1 could also induce EMT of HK-2 cells through downregulating E-cadherin expression while upregulating fibronectin and vimentin expression in a dose-dependent manner. The results of our study are similar to the results of Brockhausen et al. [21].

During the process of TGF-β1-induced EMT, we stumbled upon a situation in which PAK4 overexpressed in TGF-β1-treated HK-2 cells. Curiously, PAK4 mRNA expression changed, without statistical significance, after treatment with TGF-β1. This implies that regulation of PAK4 expression in TGF-β1-treated renal tubular epithelial cells might occur after translation and posttranslational modification. As one of the type II PAKs, PAK4 participates in massive biological processes. PAK4 plays a vital role in tumors, not only in tumorigenesis and progression, but also in invasion and metastasis [25-28]. So far, the effects of PAK4 on renal interstitial fibrosis have remained undefined. We detected that PAK4 could bring changes of EMT markers in HK-2 cells. Researches have demonstrated that PAK4 could take part in TGF-β causing biological effects in gastric cancer cells [28]. Thus, we speculated that PAK4 might be involved in TGF-β1-induced EMT in renal tubular epithelial cells. Our results revealed that overexpression of PAK4 further decreased E-cadherin protein expression and increased fibronectin and vimentin protein expression induced by TGF-β1 in HK-2 cells. These data suggest that PAK4 enhances the process of TGF-β1-induced EMT in renal tubular epithelial cells. Nonetheless, unnoticeable variance of E-cadherin protein expression induced by TGF-β1 was detected, after silencing PAK4. E-cadherin is a transmembrane glycoprotein, connecting epithelial cells together at adherent junctions, and loss of E-cadherin represents occurrence of EMT. Since TGF-β is regarded as the primary fibrogenic growth factor [29], we suspected that TGF-β was so powerful in reducing E-cadherin that silencing PAK4 could not reverse this effect. Therefore, further evidence from future studies is needed to investigate this phenomenon.

TGF-β-induced fibrosis is generally mediated by Smad-dependent and Smad-independent signaling pathways [30-32]. Wnt/β-catenin belongs to one of the Smad-independent signaling pathways. As a principal mediator of canonical Wnt signaling pathways, β-catenin plays a crucial role in governing organ development, tissue homoeostasis, and the pathologic process of diverse human disorders [33]. Activation of Wnt/β-catenin signaling pathways contributes to renal interstitial fibrosis [34]. We detected, in the immunofluorescence assay, that overexpressing PAK4 made the β-catenin protein translocate from cell membranes into the nucleus in TGF-β-treated HK-2 cells. Furthermore, results of Western Blot assay revealed that overexpressing PAK4 could remarkably increase active β-catenin protein expression. Once stimulated by upstream mediators, the β-catenin protein, which is free in the cytoplasm, was stabilized. Stable accumulation of β-catenin translocates into the nucleus and binds to T-cell factor (TCF)/lymphoid enhancer-binding factor (LEF) transcription factor family, then triggers the transcription of downstream target genes (such as c-myc, cyclin D1, etc.) [35]. Previous studies have presented similar results indicating that activation of β-catenin signaling induces tubular EMT and enhances the magnitude of EMT induced by TGF-β1 [36, 37]. Consistently, inhibiting Wnt/β-catenin signaling ameliorates kidney injuries and mitigates renal fibrotic lesions in chronic kidney disease [38]. β-catenin signaling could be a converging effector of several basic fibrotic signaling pathways. Therefore, inhibiting β-catenin transcription activity may be a promising method to attenuate renal interstitial fibrosis [17, 37].

In summary, our present study provides evidence of a novel role for PAK4 in TGF-β1-induced-EMT and suggests that it is a potential therapeutic target for ameliorating renal interstitial fibrosis of CKD.

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Disclosure of conflict of interest

None.

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