Overexpression of BMP-7 reverses TGF-β1-induced epithelial-mesenchymal transition by attenuating the Wnt3/β-catenin and TGF-β1/Smad2/3 signaling pathways in HK-2 cells

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Abstract. Tubular epithelial cells undergoing epithelial-mesenchymal transition (EMT) is a crucial event in the progression of renal interstitial fibrosis (RIF). Bone morphogenetic protein-7 (BMP-7) has been reported to exhibit anti-fibrotic functions in various renal diseases. However, the function of BMP-7 in regulating EMT and the progression of RIF remains largely unknown. The aim of the present study was to examine the potential effect of BMP-7 on transforming growth factor β1 (TGF-β1)-induced EMT and the underlying mechanisms by which BMP-7 exerted its effects. Human renal proximal tubular epithelial cells (HK-2) were treated with TGF-β1 for various time periods and at various concentrations and lentiviral vectors were used to overexpress BMP-7. Cell Counting Kit-8 and Transwell assays were used to evaluate the viability and migration of HK-2 cells *in vitro*. EMT was estimated by assessing the changes in cell morphology and the expression of EMT markers. In addition, the activation of the Wnt3/β-catenin and TGF-β1/Smad2/3 signaling pathways were analyzed using western blotting. TGF-β1 induced EMT in a time- and dose-dependent manner in HK-2 cells. Treatment with TGF-β1 induced morphological changes, decreased cell viability and the expression of E-cadherin, increased cell migration and the expression of α-smooth muscle actin, fibroblast-specific protein 1, collagen I and vimentin, and activated the Wnt3/β-catenin and TGF-β1/Smad2/3 signaling pathways in HK-2 cells. However, BMP-7 overexpression notably reversed all these effects. These results suggest that BMP-7 effectively suppresses TGF-β1-induced EMT through the inhibition of the Wnt3/β-catenin and TGF-β1/Smad2/3 signaling pathways, highlighting a potential novel anti-RIF strategy.

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Key words: bone morphogenic protein-7, transforming growth factor β1, epithelial-mesenchymal transition, renal interstitial fibrosis, signaling pathways

Introduction

Renal interstitial fibrosis (RIF), which represents a universal pathway for all progressive kidney diseases, has long been associated with progressive renal function loss and end-stage renal disease (1,2). RIF is characterized by the excessive extracellular matrix component deposition in the tubular interstitium by activated fibroblasts (also referred to as myofibroblasts) (3,4). Activated fibroblasts often express α-smooth muscle actin (α-SMA), fibronectin, fibroblast-specific protein 1 (FSP-1) and collagen I (5,6). Changes in the expression levels of these proteins are often accompanied by the epithelial-mesenchymal transition (EMT), in which endothelial cells and tubular epithelial cells transform into a more mesenchymal-like phenotype (5,7). This transition is characterized by the loss of epithelial proteins including E-cadherin, cytokeratin and zonula occludens-1, and the upregulation of mesenchymal markers, including α-SMA, fibronectin, vimentin, FSP-1 and collagen I (8,9). During EMT in RIF, the EMT of tubular epithelial cells serves a key function (4,5), and transforming growth factor β1 (TGF-β1) is regarded as a central regulator of the process. TGF-β1 is able to initiate and support the progression of the entire EMT process (7,10).

Bone morphogenetic protein-7 (BMP-7) is a member of the TGF-β superfamily of proteins. Previous studies have revealed that in the mature kidney, BMP-7 exhibits protective and regenerative potential, and also serves a crucial function in suppressing the gradual development of RIF in a mouse model of unilateral urethral obstruction (11-13). Furthermore, it has been reported that the exogenous administration of BMP-7 or BMP-7 mimics may present a promising therapeutic option for serious diseases of the kidney (14,15). However, BMP-7 is freely soluble in water and has a short biological half-life span *in vivo*, which results in the maintenance of local concentrations being difficult (16). Lentiviral-based gene therapy systems offer prolonged gene expression (17), and may be ideal for gene therapy strategies. Therefore, the present study constructed lentiviral vectors that overexpress BMP-7 and evaluated the potential function and mechanism of BMP-7 in the progression of RIF. Furthermore, to the best of our knowledge, the effect of BMP-7 on the migration induced by TGF-β1 during EMT, a key event in RIF, has not yet been determined.

Previous studies have demonstrated that BMP-7 attenuates TGF-β-induced EMT in cholangiocarcinoma (18) and pulmonary fibrosis (19). However, the effect and mechanisms of BMP-7 on EMT during RIF remain yet to be elucidated. In the present study, it was hypothesized that BMP-7 may inhibit TGF-β1-induced EMT in renal tubule epithelial cells. To validate this hypothesis, lentiviral vectors were used to overexpress BMP-7 in human renal proximal tubular epithelial cells (HK-2). Cells were treated with TGF-β1 for various durations and concentrations of TGF-β1. Subsequently, the potential effects of BMP-7 on EMT and the potential underlying mechanisms of BMP-7 in HK-2 cells were determined.

Materials and methods

Reagents and antibodies. TGF-β1 was obtained from R&D Systems, Inc. (Minneapolis, MN, USA). Lipofectamine® 3000 Transfection Reagent (cat. no. L3000015) was purchased from Thermo Fisher Scientific, Inc. Anti-E-cadherin (cat. no. ab76055), anti-α-SMA (cat. no. ab5694), anti-FSP-1 (cat. no. ab41532), anti-collagen I (cat. no. ab34710), anti-vimentin (cat. no. ab92547), anti-Wnt3/3a (cat. no. ab172612) and anti-BMP-7 (cat. no. ab56023) antibodies were purchased from Abcam (Cambridge, UK). Anti-phospho-Smad2 (cat. no. 3108), anti-phospho-Smad3 (cat. no. 9520), anti-Smad2 (cat. no. 3122), anti-Smad3 (cat. no. 9513), anti-glycogen synthase kinase 3β (GSK-3β; cat. no. 12456), anti-phospho-GSK-3β (cat. no. 5558), anti-phospho-β-Catenin (Ser33/37/Thr41) (cat. no. 9561), anti-Non-phospho (Active) β-Catenin (Ser33/37/Thr41) (cat. no. 8814), anti-rabbit horseradish peroxidase (HRP)-linked secondary (cat. no. 7074) or anti-mouse HRP-linked secondary (cat. no. 7076) antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Anti-β-actin (cat. no. sc-8432) antibody was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA).

Cell culture and treatment. The cell lines HK-2 (cat. no. CRL-2190™) and HEK 293T (cat. no. CRL-11268™) were purchased from the American Type Culture Collection (Manassas, VA, USA). HK-2 cells were maintained in Dulbecco's modified Eagle's medium/F12 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) at 37˚C with 5% CO_2 in a humidified incubator. HEK 293T cells were grown in DMEM media (Gibco; Thermo Fisher Scientific, Inc.) under the same conditions. For inducing EMT, cells were starved without serum for 12 h and subsequently treated with different concentrations of TGF-β1 (0, 2, 5 and 10 ng/ml) at 37˚C for 0, 24 and 48 h as described previously (20).

Lentiviral vectors for BMP-7 overexpression. pGCL-green fluorescent protein (GFP)-lentivirus carrying a full-length human BMP-7 cDNA sequence (LV-BMP-7) and a non-targeting sequence (LV-Control) were synthesized by Shanghai GeneChem Co., Ltd. Lentivirus packaging and infection were performed as described previously (21). One day before transfection, 1.2×10^7 HEK 293T cells were plated at 90-95% confluence (in 15-cm dishes). At the time of transfection, the packaging HEK 293T cells were cotransfected with pGC-LV (20 µg), plasmid pHelper 1.0 (15 µg) and plasmid pHelper 2.0 (10 µg) using Lipofectamine® 3000 (Thermo Fisher Scientific, Inc.). The titer of the recombinant lentiviral vector was 3×10^8 TU/ml. The recombinant lentivirus was stored at -80˚C. For lentiviral infection, add recombinant lentivirus (4 µl) at 3×10^8 TU/ml to each well (2×10^5 cells per well in 6-well plates). Polybrene (Shanghai GeneChem Co., Ltd.) was added to a final concentration of 5 µg/ml. Following incubation at 37˚C for 24 h, the cell culture medium was then replaced with DMEM and 10% (vol/vol) FBS. Cells were incubated at 37˚C for another 48 h and then the stable overexpression of BMP-7 in HK-2 cells was confirmed using reverse transcription-quantitative (RT-q)PCR and western blotting.

RT-qPCR. Total RNA from HK-2 cells was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), and cDNA was synthesized using a M-MLV kit (Takara Bio, Inc., Otsu, Japan) according to the manufacturer's protocol. Specific primers for α-SMA, collagen I, FSP-1, vimentin, E-cadherin, BMP-7 and GAPDH are listed in Table I. qPCR was performed using a SYBR-Green Real-Time PCR assay kit (Takara Bio, Inc.) on a CFX96 Touch Sequence Detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The thermocycling conditions were as follows: Initial denaturation at 95˚C for 10 min, followed by 35 amplification cycles at 95˚C for 15 sec and 60˚C for 1 min. GAPDH was used as the endogenous control for normalization, and the expression was analyzed using the $2^{-\Delta\Delta Cq}$ method (22).

Western blotting. Western blotting was performed as described previously (23,24). Total protein was isolated from HK-2 cells using NE-PER™ Nuclear and Cytoplasmic Extraction reagents (Thermo Fisher Scientific, Inc.; cat. no. 78833), and the protein concentration was examined using a Pierce bicinchoninic acid assay (Pierce; Thermo Fisher Scientific, Inc.; cat. no. 23227). Equal quantities of protein (20 µg) were loaded on a 10-12% SDS-gel and resolved using SDS-PAGE. Resolved proteins were transferred to a 0.22 µm polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA). Subsequently, membranes were blocked using 5% non-fat milk for 1 h at room temperature and incubated overnight at 4˚C with anti-E-cadherin (1:1,000), anti-α-SMA (1:1,000), anti-FSP-1 (1:1,000), anti-collagen I (1:1,000), anti-vimentin (1:1,000), anti-Wnt3/3a (1:500), anti-BMP-7 (1:1,000),

Table I. Primers used for reverse transcription-quantitative PCR.

Gene	Primers
α-SMA	
Forward	5'-CCGAGATCTCACCGACTACC-3'
Reverse	5'-TCCAGAGCGACATAGCACAG-3'
FSP-1	
Forward	5'-ACCTCTCTGTTCAGCACTTCC-3'
Reverse	5'-GAACTTGTCACCCTCGTTGC-3'
Collagen I	
Forward	5'-ACATGCCGAGACTTGAGACTCA-3'
Reverse	5'-GCATCCATAGTACATCCTTGGTTAGG-3'
E-cadherin	
Forward	5'-CACACTGATGGTGAGGGTACAAGG-3'
Reverse	5'-GGGCTTCAGGAACACATACATGG-3'
Vimentin	
Forward	5'-GTTTCCCCTAAACCGCTAGG-3'
Reverse	5'-AGCGAGAGTGGCAGAGGA-3'
BMP-7	
Forward	5'-TGGCAGCATCCAATGAACAAGATCC-3'
Reverse	5'-TTCCTTTCGCACAGACACCAATGTG-3'
GAPDH	
Forward	5'-ACAAGATGGTGAAGGTCGGTG-3'
Reverse	5'-AGAAGGCAGCCCTGGTAACC-3'

α-SMA, α-smooth muscle actin; FSP-1, fibroblast-specific protein 1; BMP-7, bone morphogenetic protein-7.

anti-phospho-Smad2 (1:500), anti-phospho-Smad3 (1:500), anti-phospho-GSK-3β (1:1,000), anti-phospho-β-Catenin (1:1,000), anti-Smad2 (1:1,000), anti-Smad3 (1:1,000), anti-GSK-3β (1,000), anti-Non-phospho (Active) β-Catenin (1:1,000) and anti-β-actin (1:2,000). Subsequent to incubation with the primary antibodies, membranes were washed with 0.1% tris-buffered saline with 0.5% Tween-20 for 3 times, and the membranes were incubated at room temperature for 1 h with the anti-rabbit or anti-mouse HRP-linked secondary antibodies (1:2,000). Signals were visualized using an enhanced chemiluminescence detection system (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Densitometry analysis was performed using Quantity One version 4.62 (Bio-Rad Laboratories, Inc.).

Cell Counting Kit-8 and cell morphology assays. A total of 3x10^3 HK-2 cells were plated in 96-well plates and, after a 24 h incubation period, cells were treated with different concentrations of TGF-β1 (0, 2, 5 and 10 ng/ml) at 37˚C for 72 h. Subsequently, 10 μl CCK8 solution (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was added to each well and incubated at 37˚C for a further 2 h, and cell viability was determined by measuring the absorbance at 450 nm on a spectrophotometer. Cell morphological changes were observed by phase-contrast light microscopy (magnification x100; Olympus Corporation).

Migration assay. HK-2 cells transfected with LV-BMP-7 and control untransfected cells were pre-treated with 10 ng/ml TGF-β1 at 37˚C for 48 h. Cells were suspended in 200 μl serum-free medium. Cell suspensions containing 10 ng/ml TGF-β1 were added to the upper chamber of a Transwell insert (8 μm pores; Corning, Inc.) at a density of 1x10^5 cells/ml (200 μl per chamber). The lower chamber contained culture medium (600 μl per chamber) supplemented with 10% FBS which was used as a chemoattractant. Cells were incubated at 37˚C for 24 h. Cells on the upper surfaces of the chambers were gently scraped off with cotton swabs and the cells which had migrated to the lower surface of the chamber were fixed with 100% methanol at room temperature for 20 min, stained with 0.1% crystal violet at room temperature for 5 min, and the number of cells in 10 random fields of view were counted per well using a light microscope (magnification x100; Olympus Corporation).

Statistical analysis. GraphPad Prism version 8 (GraphPad Software Inc., La Jolla, CA, USA) was used for all statistical analyses. Data are presented as the mean ± standard error of the mean. A one-way or two-way analysis of variance or an unpaired Student's t-test were used to compare differences between groups and the LSD test served as the post hoc test for multiple comparisons. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Transfection of LV-BMP-7 increases BMP-7 mRNA and protein expressions levels in HK-2 cells. To assess the potential effect of BMP-7 on EMT in RIF, lentiviral vectors encoding the BMP-7 gene were used to infect HK-2 cells. Infection efficiencies were visualized by fluorescence microscopy (magnification, x100). To evaluate the efficiency of transfection, RT-qPCR and western blotting were performed. Subsequent to viral infection, >90% of the cells were GFP-positive, suggesting a high infection efficiency (Fig. 1). Additionally, as presented in Fig. 2, BMP-7 mRNA (Fig. 2A) and protein (Fig. 2B) expression levels in the HK-2 infected cells were significantly increased compared with the LV-Control and normal control cells (all $P<0.001$).

BMP-7 overexpression alters expression of EMT-associated genes in HK-2 cells. The expression of EMT-associated biomarkers in infected and control HK-2 cells was determined. BMP-7 overexpression significantly increased the mRNA expression levels of E-cadherin, and significantly reduced the mRNA expression levels of α-SMA, collagen I, FSP-1 and vimentin compared with the LV-Control cells ($P<0.01$; Fig. 3A). Furthermore, western blotting exhibited similar changes in the protein expression of these proteins (Fig. 3B).

BMP-7 overexpression reverses the effects of TGF-β1. TGF-β1 has been demonstrated to be a strong promoter of EMT in renal tubular epithelial cells (25). Additionally, TGF-β1-induced EMT results in reduced cell proliferation (26). The effect of TGF-β1 on cell viability in HK-2 cells overexpressing BMP-7 was assessed. A CCK-8 assay was performed to assess cell viability in the HK-2 cells. Cell viability of HK-2 cells was not significantly affected by 5 ng/ml TGF-β1 (Fig. 4A). After 72 h, 10 ng/ml TGF-β1 significantly inhibited the viability rate of HK-2 cells (P=0.0003 vs. cells treated with 2 or 5 ng/ml TGF-β1; Fig. 4A), however, in the BMP-7 overexpressing cells, the viability rate was partially restored (P=0.0022 vs. cells treated with LV-Control+10 ng/ml TGF-β1; Fig. 4A).

To further investigate the potential function of BMP-7 in regulating the migratory capacity of HK-2 cells, Transwell assays were performed. As presented in Fig. 4B, 10 ng/ml TGF-β1 significantly increased the migratory capacity of HK-2 cells (P=0.0008 vs. cells infected with the LV-Control or LV-BMP-7) and overexpression of BMP-7 significantly reversed this effect (P=0.0262 vs. cells treated with LV-Control+10 ng/ml TGF-β1). These results demonstrate that BMP-7 overexpression reversed the suppression of viability and the increase in the migration induced by TGF-β1 in HK-2 cells.

BMP-7 overexpression inhibits TGF-β1-induced EMT in HK-2 cells. To investigate whether BMP-7 overexpression resulted in the suppression of TGF-β1-induced EMT, HK-2 cells were treated with 10 ng/ml TGF-β1 for 48 h and the morphological changes were observed in HK-2 cells. As presented in Fig. 5A, HK-2 cells, which traditionally exhibit a cobblestone-like morphology, exhibited a spindle-like morphology when treated with TGF-β1, and also exhibited reduced cell-cell adhesion. However, in cells overexpressing BMP-7, treatment with TGF-β1 did not result in morphological changes. Western blotting was performed to assess the expression levels of EMT-associated markers. As presented in Fig. 5B, 10 ng/ml TGF-β1 reduced BMP-7 expression in a time-dependent manner in the HK-2 cells. This reduction was accompanied by increased expression of the mesenchymal markers α-SMA, collagen I, FSP-1 and vimentin, and decreased the expression of E-cadherin, an epithelial marker. However, overexpressing BMP-7 in cells resulted in the upregulation of E-cadherin and downregulation of the mesenchymal markers α-SMA, collagen I, FSP-1 and vimentin compared with untransfected cells treated with TGF-β1.

To determine the effect of different concentrations of TGF-β1 on the expression of EMT markers, HK-2 cells were incubated with 0, 2, 5 and 10 ng/ml TGF-β1 for 48 h, and the expression of EMT markers were determined by western blotting. TGF-β1 substantially decreased the protein expression of the epithelial marker E-cadherin and increased the expression of the mesenchymal markers α-SMA, collagen I, FSP-1 and vimentin in a dose-dependent manner, with peak expression observed with 10 ng/ml TGF-β1 (Fig. 5C). Next, the effect of TGF-β1 on the BMP-7 overexpressing cells was determined with regards to the expression of EMT markers. Treatment with 10 ng/ml TGF-β1 did not result in a change in the expression of epithelial and mesenchymal markers in the overexpressing cells, with the cells possessing an epithelial expression profile (Fig. 5C). These data suggest that TGF-β1 induced EMT in a time- and dose-dependent manner in HK-2 cells, and that the overexpression of BMP-7 reversed the effects of TGF-β1.

BMP-7 overexpression antagonizes the TGF-β1-induced EMT of HK-2 cells by inhibiting Wnt3/β-catenin and TGF-β1/Smad2/3 signaling. To elucidate the mechanism by which BMP-7 treatment inhibited TGF-β1-induced EMT in HK-2 cells, the Wnt3/β-catenin and TGF-β1/Smad2/3 signaling pathways were assessed. As presented in Fig. 6A, TGF-β1 activated the Wnt3/β-catenin and TGF-β1/Smad2/3 signaling pathways in a time-dependent manner. However, in the BMP-7-overexpressing HK-2 cells, the activation of the two signaling pathways were notably reduced.

To determine the effect of different concentrations of TGF-β1 on the activation of the Wnt3/β-catenin and TGF-β1/Smad2/3 signaling pathways, HK-2 cells were incubated with various concentrations of TGF-β1 for 48 h, and the protein expression of members of the Wnt3/β-catenin and TGF-β1/Smad2/3 signaling pathways were determined. The expression of Wnt3/3a, active β-catenin, phospho-Smad2 and phospho-Smad3 were increased substantially, in addition to the expression of phospho-β-catenin and phospho-GSK-3β being decreased, whereas this effect was reversed in the BMP-7-overexpressing cells after 72 h of treatment (Fig. 6B). Altogether, these results demonstrate that BMP-7 overexpression notably attenuated the TGF-β1-induced activation of Wnt3/β-catenin and TGF-β1/Smad2/3 signaling pathways in HK-2 cells.

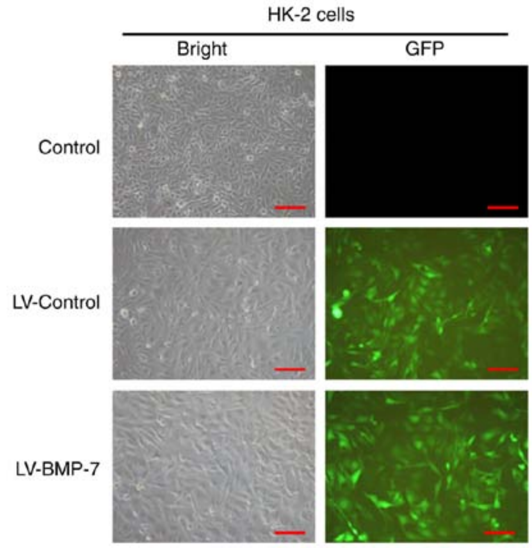

Figure 1. Lentiviral infection efficiency of LV-Control and LV-BMP-7. HK-2 cells were transfected with LV-Control or LV-BMP-7 for 72 h, and the infection efficiency was observed by a fluorescent microscope. Left, bright field; right, GFP. GFP expression (right panels) revealed that >90% of the cells had been successfully infected with lentiviruses. Magnification, x100. Scale bar, 100 μm. BMP-7, bone morphogenetic protein-7; LV-Control, pGCL-GFP-lentivirus carrying a non-targeting sequence; LV-BMP-7, pGCL-GFP-lentivirus carrying full-length human BMP-7 cDNA sequence; HK-2, human renal proximal tubular epithelial cells; GFP, green fluorescent protein.

Discussion

The present study demonstrated that BMP-7 overexpression suppressed TGF-β1-induced EMT in HK-2 cells. BMP-7 overexpressing exhibited a notably higher expression of E-cadherin and treatment with TGF-β1 did not affect E-cadherin expression. Expression of the mesenchymal markers was substantially lower in the BMP-7 overexpressing cells compared with the control cells treated with TGF-β1. Additionally, changes in the morphology induced by TGF-β1 were not observed in the BMP-7 overexpressing

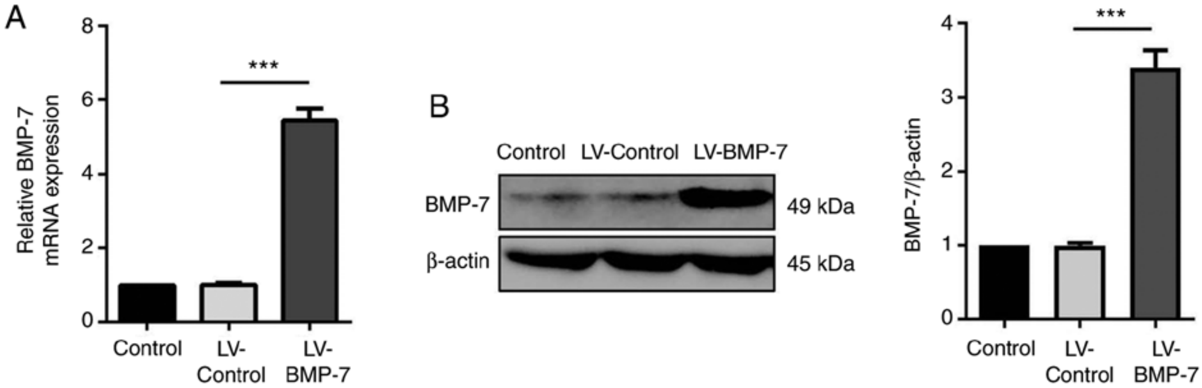

Figure 2. LV-BMP-7 increases BMP-7 mRNA and protein expression levels in transfected HK-2 cells. (A) mRNA expression levels of BMP-7 in normal control cells, and LV-Control or LV-BMP-7 infected cells. GAPDH was used as the control gene. (B) Western blotting of BMP-7 protein expression levels in normal control, and LV-Control or LV-BMP-7 infected cells. β-actin gene was used as the loading control. ***P<0.001 vs. LV-Control group. BMP-7, bone morphogenetic protein-7; LV-Control, pGCL-GFP-lentivirus carrying a non-targeting sequence; LV-BMP-7, pGCL-GFP-lentivirus carrying full-length human BMP-7 cDNA sequence; HK-2, human renal proximal tubular epithelial cells.

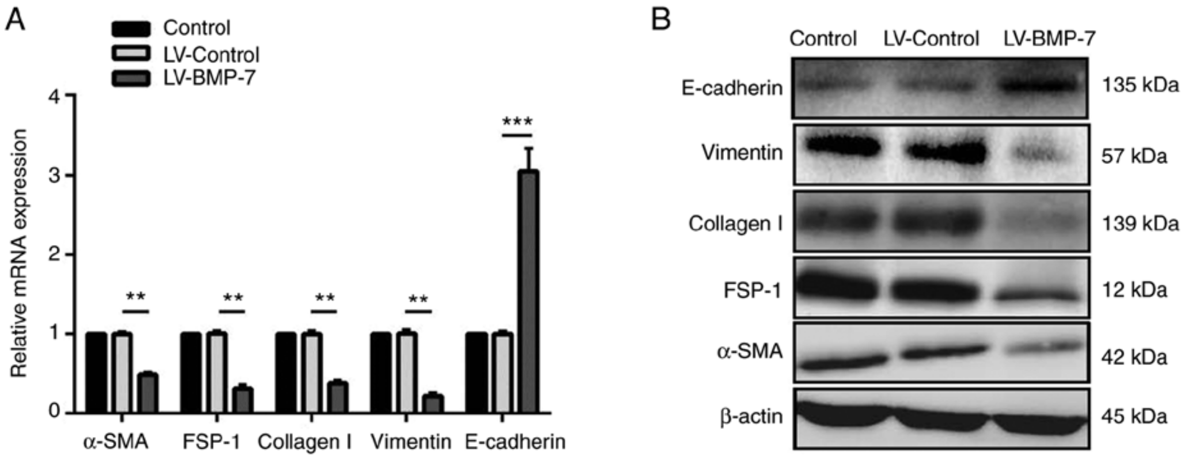

Figure 3. BMP-7 overexpression alters the expression of EMT-associated genes in HK-2 cells. (A) BMP-7 overexpression significantly reduced the mRNA expression of α-SMA, FSP-1, collagen I and vimentin, and significantly increased E-cadherin expression levels. Data are normalized to GAPDH. **P<0.01 and ***P<0.001 vs. LV-Control group. (B) Western blotting revealed that the protein expression of α-SMA, FSP-1, collagen I and vimentin was substantially decreased in the BMP-7 overexpressing cells, whereas the expression of E-cadherin was increased. BMP-7, bone morphogenetic protein-7; LV-Control, pGCL-green fluorescent protein-lentivirus carrying a non-targeting sequence; LV-BMP-7, pGCL-green fluorescent protein-lentivirus carrying full-length human BMP-7 cDNA sequence; HK-2, human renal proximal tubular epithelial cells; α-SMA, α-smooth muscle actin; FSP-1, fibroblast-specific protein 1; EMT, epithelial-to-mesenchymal transition.

cells. Furthermore, the mechanism by which BMP-7 overexpression prevented TGF-β1-induced EMT was associated with inhibition of the Wnt3/β-catenin and TGF-β1/Smad2/3 signaling pathways.

RIF is considered as the end outcome common to all end-stage renal diseases. Previous studies have demonstrated that tubular epithelial cells undergoing EMT is a crucial event in the progression of RIF (5,27). Therefore, blocking EMT may be an effective treatment method for preventing the progression of RIF. Studies have revealed that TGF-β1 is crucially involved in the pathogenesis of RIF and is associated with progressive kidney diseases (6,25). TGF-β1, a primary inducer of EMT, has been demonstrated to be necessary and sufficient for initiating and supporting the entire EMT process (4,28). Therefore, inhibiting TGF-β1-mediated signaling may be a promising therapeutic option for treating patients with RIF. Previously, BMP-7 has been demonstrated to antagonize TGF-β1-mediated fibrosis through the suppression of EMT in fibroses of a number of different organs, including the lung and liver (29-32). However, the effects of BMP-7 on EMT in RIF have not yet been determined previously.

A universal feature of EMT is the loss of expression of epithelial markers and gain of expression of mesenchymal markers (33,34). Considering that TGF-β1 is a critical mediator which contributes to EMT in RIF, the present study specifically examined whether BMP-7 overexpression reversed TGF-β1-induced EMT. Lentiviral vectors were used to overexpress BMP-7 in HK-2 cells to examine its effects on cell morphology, cell viability, migration and changes in the expression of EMT markers (α-SMA, collagen I, FSP-1, vimentin and E-cadherin), and the effect of TGF-β1 stimulation in BMP-7 expressing cells. Treatment with 10 ng/ml TGF-β1 for 48 h resulted in untransfected HK-2 cells exhibiting a mesenchymal phenotype. Furthermore, TGF-β1 treatment induced the inhibition of cell viability and resulted in increased migration in HK-2 cells. These alterations were

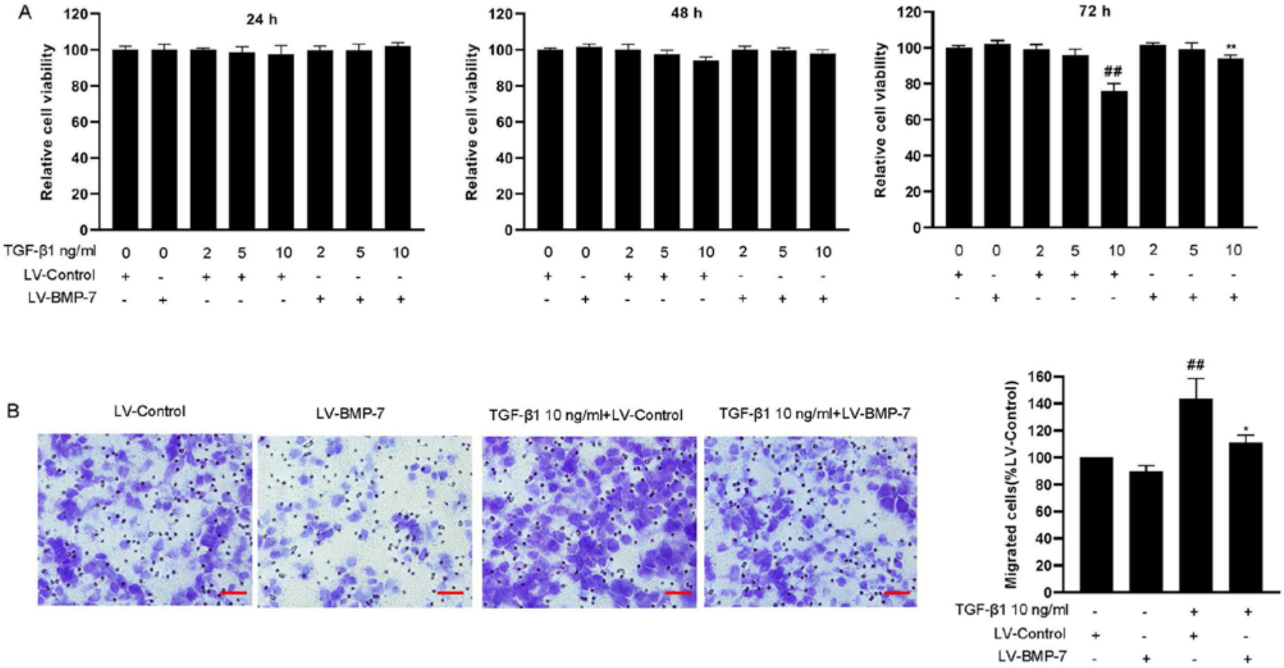

Figure 4. BMP-7 overexpression reverses the TGF-β1-induced suppression of viability and increase in migration in HK-2 cells. (A) Viability of HK-2 cells subsequent to treatment with TGF-β1 for various durations and concentrations. Viability was determined using a Cell Counting Kit-8 assay. After 72 h, BMP-7 overexpression restored 10 ng/ml TGF-β1-induced suppression of viability. $^{\#\#}$P<0.01 vs. 2 or 5 ng/ml TGF-β1; **P<0.01 vs. LV-Control+10 ng/ml TGF-β1. (B) Transwell assays revealed that BMP-7 overexpression significantly reversed the TGF-β1-induced increase in migration in HK-2 cells. Scale bar, 100 μm. $^{\#\#}$P<0.01 vs. LV-Control or LV-BMP-7; *P<0.05 vs. LV-Control+10 ng/ml TGF-β1. BMP-7, bone morphogenetic protein-7; HK-2, human renal proximal tubular epithelial cells; TGF-β1, transforming growth factor β1; LV-Control, pGCL-green fluorescent protein-lentivirus carrying a non-targeting sequence; LV-BMP-7, pGCL-green fluorescent protein-lentivirus carrying full-length human BMP-7 cDNA sequence.

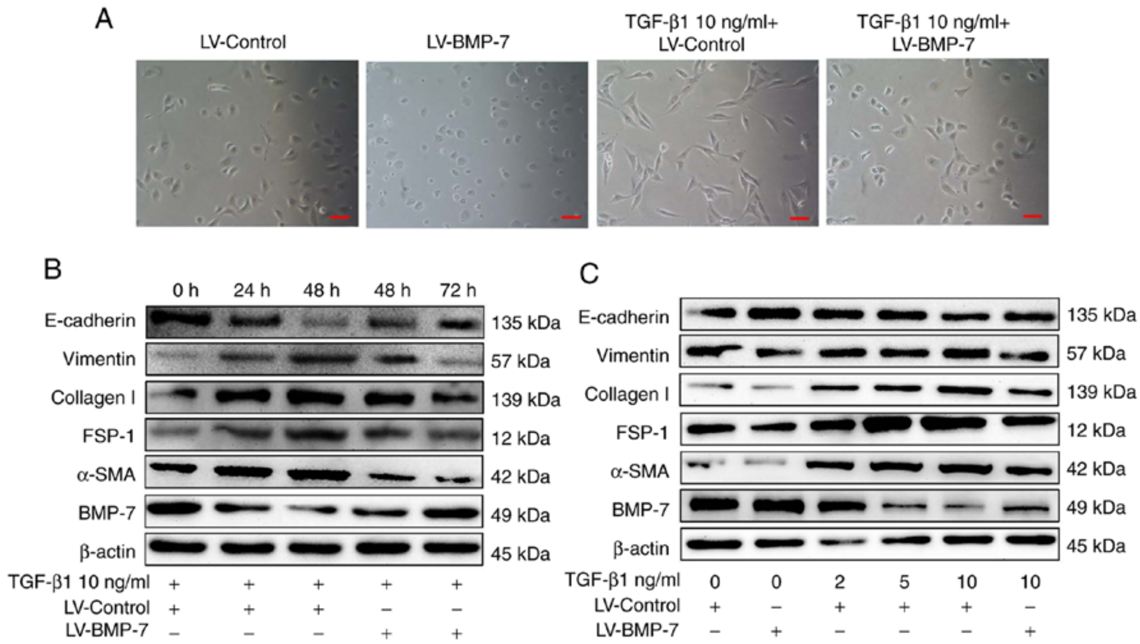

Figure 5. BMP-7 overexpression notably abrogates TGF-β1-induced epithelial-mesenchymal transition in HK-2 cells. (A) Representative images of morphological changes in HK-2 cells after 48 h. Scale bar, 100 μm. TGF-β1 treatment substantially increased expression of α-SMA, collagen I, FSP-1 and vimentin and decreased expression of E-cadherin in a (B) time- and (C) dose-dependent manner. BMP-7 overexpression notably suppressed the TGF-β1-induced decrease in E-cadherin expression and increase in α-SMA, collagen I, FSP-1 and vimentin expression. BMP-7, bone morphogenetic protein-7; HK-2, human renal proximal tubular epithelial cells; α-SMA, smooth muscle actin; FSP-1, fibroblast-specific protein 1; TGF-β1, transforming growth factor β1; LV-Control, pGCL-green fluorescent protein-lentivirus carrying a non-targeting sequence; LV-BMP-7, pGCL-green fluorescent protein-lentivirus carrying full-length human BMP-7 cDNA sequence.

accompanied with a notably increased expression of mesenchymal markers (α-SMA, collagen I, FSP-1 and vimentin), and a decreased expression of E-cadherin. These results are in agreement with previous studies (35-37). Interestingly, BMP-7

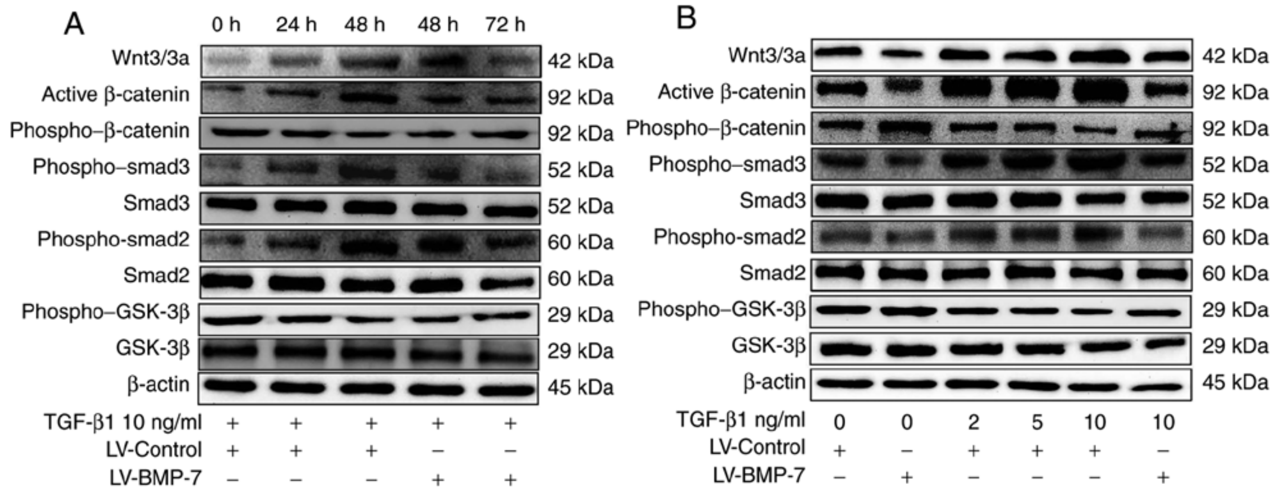

Figure 6. BMP-7 overexpression abrogates TGF-β1-induced epithelial mesenchymal transition in HK-2 cells by inhibiting the Wnt3/β-catenin and TGF-β1/Smad2/3 signaling pathways. (A) Wnt3/β-catenin and TGF-β1/Smad2/3 signaling pathways were activated by 10 ng/ml TGF-β1 in a time-dependent manner, and this effect was abrogated in the HK-2 cells overexpressing BMP-7. (B) TGF-β1 activated the Wnt3/β-catenin and TGF-β1/Smad2/3 signaling pathways in a dose-dependent manner in HK-2 cells, whereas this effect was reversed in cells overexpressing BMP-7. BMP-7, bone morphogenetic protein-7; HK-2, human renal proximal tubular epithelial cells; GSK-3β, glycogen synthase kinase-3β; TGF-β1, transforming growth factor β1; LV-Control, pGCL-green fluorescent protein-lentivirus carrying a non-targeting sequence; LV-BMP-7, pGCL-green fluorescent protein-lentivirus carrying full-length human BMP-7 cDNA sequence.

overexpression prevented this transformation from an epithelial to mesenchymal phenotype when treated with TGF-β1. Therefore, the results of the present study revealed that BMP-7 overexpression may notably impede EMT, suggesting that BMP-7 exhibits a protective potentially tumor-suppressive effect in RIF.

The Wnt3/β-catenin signaling pathway is a highly conserved, extremely complex pathway which is thought to be associated with the pathogenesis of RIF (38,39). Previously, studies have revealed that Wnt3/β-catenin signaling is associated with proteinuria, renal dysfunction and renal fibrosis in a variety of chronic kidney diseases (40,41). An increased mesenchymal-like phenotype, consisting of reduced E-cadherin expression, and increased β-catenin and N-cadherin expression, have been demonstrated to serve a key function in EMT progression in renal fibrosis (14,34). It was hypothesized that the BMP-7 mediated attenuation of TGF-β1-induced EMT may be through inhibition of the Wnt3/β-catenin signaling pathway. BMP-7 overexpression substantially attenuated the activation of the Wnt3/β-catenin signaling pathway, which was induced by TGF-β1. These results are consistent with previous studies demonstrating that blocking the Wnt3/β-catenin signaling attenuates EMT in RIF (39,42,43).

Numerous studies have demonstrated that the TGF-β1/Smad2/3 signaling pathway is an important molecular pathway which regulates EMT in RIF (6,44,45). The results of the present study demonstrated that HK-2 cells overexpressing BMP-7 overexpression displayed the notably reduced phosphorylation of Smad2/3 when treated with TGF-β1. This result suggested that BMP-7 overexpression inhibited TGF-β1-induced EMT through inhibiting the Smad2/3 signaling pathway. However, the precise mechanisms underlying the inhibition of Wnt3/β-catenin and TGF-β1/Smad2/3 signaling by BMP-7 requires further study.

In summary, the present study identified a novel mechanism by which BMP-7 overexpression significantly attenuated TGF-β1-induced EMT, by suppressing Wnt3/β-catenin and TGF-β1/Smad2/3 signaling. The results highlight a potentially novel mechanism for preventing EMT in patients with RIF.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YS, GL and CP designed the research. YS, SLv, FW, XL, JC, SLi and XW conducted the experiments. WC and GG analyzed the data. YS and CP wrote the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Berchtold L, Ponte B, Moll S, Hadaya K, Seyde O, Bachtler M, Vallee JP, Martin PY, Pasch A and de Seigneux S: Phosphocalcic markers and calcification propensity for assessment of interstitial fibrosis and vascular lesions in kidney allograft recipients. PLoS One 11: e0167929, 2016.
2. Zheng GH, Wang YJ, Wen X, Han XR, Shen M, Wang S, Zhuang J, Zhang ZF, Wang L, Hu B, *et al*: Silencing of SOCS-1 and SOCS-3 suppresses renal interstitial fibrosis by alleviating renal tubular damage in a rat model of hydronephrosis. J Cell Biochem 119: 2200-2211, 2018.
3. Mengel M: Deconstructing interstitial fibrosis and tubular atrophy: A step toward precision medicine in renal transplantation. Kidney Int 92: 553-555, 2017.
4. Farris AB and Colvin RB: Renal interstitial fibrosis: Mechanisms and evaluation. Curr Opin Nephrol Hypertens 21: 289-300, 2012.
5. Liu Y: New insights into epithelial-mesenchymal transition in kidney fibrosis. J Am Soc Nephrol 21: 212-222, 2010.
6. Song Y, Peng C, Lv S, Cheng J, Liu S, Wen Q, Guan G and Liu G: Adipose-derived stem cells ameliorate renal interstitial fibrosis through inhibition of EMT and inflammatory response via TGF-β1 signaling pathway. Int Immunopharmacol 44: 115-122, 2017.
7. Huang S and Susztak K: Epithelial plasticity versus EMT in kidney fibrosis. Trends Mol Med 22: 4-6, 2016.
8. Xu XY, Chai JJ, Chen YP, Rui HL, Wang YY, Dong HR, Man YL and Cheng H: Hirsutella sinensis attenuates aristolochic acid-induced renal tubular epithelial-mesenchymal transition by inhibiting TGF-β1 and snail expression. PLoS One 11: e0149242, 2016.
9. Li A, Zhang X, Shu M, Wu M, Wang J, Zhang J, Wang R, Li P and Wang Y: Arctigenin suppresses renal interstitial fibrosis in a rat model of obstructive nephropathy. Phytomedicine 30: 28-41, 2017.
10. Sutariya B, Jhonsa D and Saraf MN: TGF-β: The connecting link between nephropathy and fibrosis. Immunopharmacol Immunotoxicol 38: 39-49, 2016.
11. Ninichuk V, Gross O, Segerer S, Hoffmann R, Radomska E, Buchstaller A, Huss R, Akis N, Schlöndorff D and Anders HJ: Multipotent mesenchymal stem cells reduce interstitial fibrosis but do not delay progression of chronic kidney disease in collagen4A3-deficient mice. Kidney Int 70: 121-129, 2006.
12. Cao J, Wang W, Li Y, Xia J, Peng Y, Zhang Y and Xia A: Artesunate attenuates unilateral ureteral obstruction-induced renal fibrosis by regulating the expressions of bone morphogenetic protein-7 and uterine sensitization-associated gene-1 in rats. Int Urol Nephrol 48: 619-629, 2016.
13. Wang Z, Zhao J, Zhang J, Wei J, Zhang J and Huang Y: Protective effect of BMP-7 against aristolochic acid-induced renal tubular epithelial cell injury. Toxicol Lett 198: 348-357, 2010.
14. Meng XM, Chung AC and Lan HY: Role of the TGF-β/BMP-7/Smad pathways in renal diseases. Clin Sci (Lond) 124: 243-254, 2013.
15. Ohigashi M, Imai N, Toba H, Kobara M and Nakata T: Pitavastatin exhibits protective effects on podocytes accompanied by BMP-7 up-regulation and Rho suppression. Pharmacology 97: 265-276, 2016.
16. Kabuto Y, Morihara T, Sukenari T, Kida Y, Oda R, Arai Y, Sawada K, Matsuda K, Kawata M, Tabata Y, *et al*: Stimulation of rotator cuff repair by sustained release of bone morphogenetic protein-7 using a gelatin hydrogel sheet. Tissue Eng Part A 21: 2025-2033, 2015.
17. Sugiyama O, An DS, Kung SP, Feeley BT, Gamradt S, Liu NQ, Chen IS and Lieberman JR: Lentivirus-mediated gene transfer induces long-term transgene expression of BMP-2 in vitro and new bone formation in vivo. Mol Ther 11: 390-398, 2005.
18. Duangkumpha K, Techasen A, Loilome W, Namwat N, Thanan R, Khuntikeo N and Yongvanit P: BMP-7 blocks the effects of TGF-β-induced EMT in cholangiocarcinoma. Tumour Biol 35: 9667-9676, 2014.
19. Liang D, Wang Y, Zhu Z, Yang G, An G, Li X, Niu P, Chen L and Tian L: BMP-7 attenuated silica-induced pulmonary fibrosis through modulation of the balance between TGF-β/Smad and BMP-7/Smad signaling pathway. Chem Biol Interact 243: 72-81, 2016.
20. Liu JH, He L, Zou ZM, Ding ZC, Zhang X, Wang H, Zhou P, Xie L, Xing S and Yi CZ: A novel inhibitor of homodimerization targeting MyD88 ameliorates renal interstitial fibrosis by counteracting TGF-β1-induced EMT in vivo and in vitro. Kidney Blood Press Res 43: 1677-1687, 2018.
21. Peng C, Zhao H, Chen W, Song Y, Wang X, Li J, Qiao Y, Wu D, Ma S, Wang X and Gao C: Identification of SHCBP1 as a novel downstream target gene of SS18-SSX1 and its functional analysis in progression of synovial sarcoma. Oncotarget 7: 66822-66834, 2016.
22. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
23. Yan D, Deng S, Gan W, Li S and Li Y: Curcumol attenuates epithelial-mesenchymal transition of nasopharyngeal carcinoma cells via TGF-β1. Mol Med Rep 17: 7513-7520, 2018.
24. Peng C, Song Y, Chen W, Wang X, Liu X, Wang F, Wu D, Ma S, Wang X and Gao C: FLVCR1 promotes the proliferation and tumorigenicity of synovial sarcoma through inhibiting apoptosis and autophagy. Int J Oncol 2018.
25. Loboda A, Sobczak M, Jozkowicz A and Dulak J: TGF-β1/Smads and miR-21 in renal fibrosis and inflammation. Mediators Inflamm 2016: 8319283, 2016.
26. Moore LD, Isayeva T, Siegal GP and Ponnazhagan S: Silencing of transforming growth factor-β1 in situ by RNA interference for breast cancer: Implications for proliferation and migration in vitro and metastasis in vivo. Clin Cancer Res 14: 4961-4970, 2008.
27. Wei SY, Wang YX, Zhang QF, Zhao SL, Diao TT, Li JS, Qi WR, He YX, Guo XY, Zhang MZ, *et al*: Multiple mechanisms are involved in salt-sensitive hypertension-induced renal injury and interstitial fibrosis. Sci Rep 7: 45952, 2017.
28. Wang YY, Jiang H, Pan J, Huang XR, Wang YC, Huang HF, To KF, Nikolic-Paterson DJ, Lan HY and Chen JH: Macrophage-to-myofibroblast transition contributes to interstitial fibrosis in chronic renal allograft injury. J Am Soc Nephrol 28: 2053-2067, 2017.
29. Cao J, Li Y, Peng Y, Zhang Y, Li H, Li R and Xia A: Febuxostat prevents renal interstitial fibrosis by the activation of BMP-7 signaling and inhibition of USAG-1 expression in rats. Am J Nephrol 42: 369-378, 2015.
30. Liang D, An G, Zhu Z, Wang Y, Yang G, Li X, Niu P, Chen L and Tian L: The protective effects of bone morphogenetic protein-7 against epithelial injury and matrix metalloproteases upregulation induced by silica in vitro. Hum Exp Toxicol 36: 892-900, 2017.
31. Li X, An G, Wang Y, Liang D, Zhu Z, Lian X, Niu P, Guo C and Tian L: Anti-fibrotic effects of bone morphogenetic protein-7-modified bone marrow mesenchymal stem cells on silica-induced pulmonary fibrosis. Exp Mol Pathol 102: 70-77, 2017.
32. Wang LP, Dong JZ, Xiong LJ, Shi KQ, Zou ZL, Zhang SN, Cao ST, Lin Z and Chen YP: BMP-7 attenuates liver fibrosis via regulation of epidermal growth factor receptor. Int J Clin Exp Pathol 7: 3537-3547, 2014.
33. Liu J, Zhong Y, Liu G, Zhang X, Xiao B, Huang S, Liu H and He L: Role of Stat3 signaling in control of EMT of tubular epithelial cells during renal fibrosis. Cell Physiol Biochem 42: 2552-2558, 2017.
34. Chen Q, Yang W, Wang X, Li X, Qi S, Zhang Y and Gao MQ: TGF-β1 induces EMT in bovine mammary epithelial cells through the TGFβ1/Smad signaling pathway. Cell Physiol Biochem 43: 82-93, 2017.
35. Bai J, Xiao X, Zhang X, Cui H, Hao J, Han J and Cao N: Erythropoietin inhibits hypoxia-induced epithelial-to-mesenchymal transition via upregulation of miR-200b in HK-2 cells. Cell Physiol Biochem 42: 269-280, 2017.
36. Huang S, Liu F, Niu Q, Li Y, Liu C, Zhang L, Ni D and Pu X: GLIPR-2 overexpression in HK-2 cells promotes cell EMT and migration through ERK1/2 activation. PLoS One 8: e58574, 2013.
37. Zhou J, Cheng H, Wang Z, Chen H, Suo C, Zhang H, Zhang J, Yang Y, Geng L, Gu M and Tan R: Bortezomib attenuates renal interstitial fibrosis in kidney transplantation via regulating the EMT induced by TNF-α-Smurf1-Akt-mTOR-P70S6K pathway. J Cell Mol Med 23: 5390-5402, 2019.
38. Nlandu-Khodo S, Neelisetty S, Phillips M, Manolopoulou M, Bhave G, May L, Clark PE, Yang H, Fogo AB, Harris RC, *et al*: Blocking TGF-β and β-Catenin epithelial crosstalk exacerbates CKD. J Am Soc Nephrol 28: 3490-3503, 2017.
39. Lin X, Zha Y, Zeng XZ, Dong R, Wang QH and Wang DT: Role of the Wnt/β-catenin signaling pathway in inducing apoptosis and renal fibrosis in 5/6-nephrectomized rats. Mol Med Rep 15: 3575-3582, 2017.

40. Xue H, Xiao Z, Zhang J, Wen J, Wang Y, Chang Z, Zhao J, Gao X, Du J and Chen YG: Disruption of the Dapper3 gene aggravates ureteral obstruction-mediated renal fibrosis by amplifying Wnt/β-catenin signaling. J Biol Chem 288: 15006-15014, 2013.
41. Jiang MQ, Wang L, Cao AL, Zhao J, Chen X, Wang YM, Wang H and Peng W: HuangQi decoction improves renal tubulointerstitial fibrosis in mice by inhibiting the up-regulation of Wnt/β-catenin signaling pathway. Cell Physiol Biochem 36: 655-669, 2015.
42. Hsu YC, Chang PJ, Ho C, Huang YT, Shih YH, Wang CJ and Lin CL: Protective effects of miR-29a on diabetic glomerular dysfunction by modulation of DKK1/Wnt/β-catenin signaling. Sci Rep 6: 30575, 2016.
43. Tsujimura T, Idei M, Yoshikawa M, Takase O and Hishikawa K: Roles and regulation of bone morphogenetic protein-7 in kidney development and diseases. World J Stem Cells 8: 288-296, 2016.
44. Huang Y, Tong J, He F, Yu X, Fan L, Hu J, Tan J and Chen Z: miR-141 regulates TGF-β1-induced epithelial-mesenchymal transition through repression of HIPK2 expression in renal tubular epithelial cells. Int J Mol Med 35: 311-318, 2015.
45. Peng C, Zhao H, Song Y, Chen W, Wang X, Liu X, Zhang C, Zhao J, Li J, Cheng G, *et al*: SHCBP1 promotes synovial sarcoma cell metastasis via targeting TGF-β1/Smad signaling pathway and is associated with poor prognosis. J Exp Clin Cancer Res 36: 141, 2017.