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. 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.

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 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-β 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. ab76055), anti-α-SMA (cat. no. ab5694), anti-FSP-1 (1:1,000), anti-collagen I (1:1,000), anti-vimentin (1:1,000), anti-zeta-Catenin (Ser33/37/Thr41) (cat. no. 9561), anti-β-Catenin (Ser33/37/Thr41) (cat. no. sc-8432) antibody was purchased from Santa Cruz Technology, Inc. (Danvers, MA, USA). Anti-β-Catenin (cat. no. 23227). Equal quantities of protein (20 µg) were loaded on a 10-12% SDS-gel and resolved using SDS-PAGE. 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.; cat. no. 23227). The cells were 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).

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^(-ΔΔCt) 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), anti-phospho-Smad3 (1:1,000), anti-Smad2 (1:1,000), anti-Smad3 (1:1,000), anti-α-SMA (1:1,000), anti-vimentin (1:1,000) and anti-β-Catenin (1:1,000) antibodies were purchased 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% CO2 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).
anti-GSK-3β concentrations of TGF-β1 (0, 2, 5 and 10 ng/ml) at 37˚C for a 24 h incubation period, cells were treated with different (1:1,000) and anti-BMP-7, bone morphogenetic protein-7.

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

| Gene     | Forward | Reverse |
|----------|---------|---------|
| α-SMA    | 5'-CCGAGATCTCACGACTACC-3' | 5'-TCCAGAGCGACATAGCACAG-3' |
| FSP-1    | 5'-ACCCTCTTGTACAGACTTC-3' | 5'-GAACCTTGTCACCCTTGTTGC-3' |
| Collagen I | 5'-ACATGCCGAGACCTTGAGCTA-3' | 5'-GCATCCATAGTACATCTTGTTAGG-3' |
| E-cadherin | 5'-CACACTGATGTGAGGTACAAGG-3' | 5'-GGGCTTCAGGAACACATACATGG-3' |
| Vimentin | 5'-GGCTTCTCAGGAACACATACATGG-3' | 5'-AGCGAGAGTGGCAGAGGA-3' |
| BMP-7    | 5'-TGCAGCATCCAATGACAAGACTCC-3' | 5'-TTCCCTTTCACAGACACACCATGTC-3' |
| GAPDH    | 5'-ACAAGATGGTGAGGTCGGTG-3' | 5'-AGAAGGCGACGCTTGGTAACC-3' |

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

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

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).

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, 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 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 genes (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 determined by measuring the absorbance at 450 nm on a spectrophotometer.

Cell Counting Kit-8 and cell morphology assays. A total of 3x10^4 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).
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.

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.
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
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
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.

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