International Journal of Biochemistry and Cell Biology 116 (2019) 105613

Contents lists available at ScienceDirect

International Journal of Biochemistry and Cell Biology

journal homepage: www.elsevier.com/locate/biocel

BMP-7/Smads-induced inhibitor of differentiation 2 (Id2) upregulation and Id2/Twist interaction was involved in attenuating diabetic renal tubulointerstitial fibrosis

Ying Xiao[a,b,*], Xiaohan Jiang[c,*], Can Peng[a,b], Yingying Zhang[a,b], Yawen Xiao[a,b,*], Dan Liang[a,b], Mingjun Shi[a,b], Yuanyuan Wang[a,b], Fan Zhang[a,b], Bing Guo[a,b]

[a] Department of Pathophysiology, Guizhou Medical University, Guiyang, Guizhou 550025, China
[b] Guizhou Provincial Key Laboratory of Pathogenesis & Drug Research on Common Chronic Diseases & State Key Laboratory of Functions and Applications of Medicinal Plants, Guizhou Medical University, China
[c] Jiangsu Taizhou People's Hospital, Taizhou, Jiangsu 225300, China

ARTICLE INFO

Keywords:
Bone morphogenetic protein-7
Diabetes mellitus
Epithelial–mesenchymal transition
Inhibitor of differentiation 2
Renal tubulointerstitial fibrosis
Twist

ABSTRACT

Renal tubular epithelial–mesenchymal transition (EMT) is the main pathological change in diabetic renal tubulointerstitial fibrosis. Mounting evidence indicates that the inhibitor of differentiation 2 (Id2) protein acts as a negative regulatory factor in organ fibrosis and can inhibit or reverse the process of fibrosis. However, its specific regulatory mechanism is not clear. Diabetes mellitus (DM) rat models were established by injecting rats with streptozotocin and sacrificing them after 16 weeks. Rat renal tubular epithelial cells (NRK-52E) were cultured with normal and high glucose. Immunohistochemical analysis, double immunofluorescence staining, co-immunoprecipitation, Western blot analysis, and real-time polymerase chain reaction were used to determine the expression of Id2, Twist, Smad1/5/8, E-cadherin, α-smooth muscle actin (α-SMA), and collagen IV. The results showed that bone morphogenetic protein-7 (BMP-7) upregulated the expression of Id2 against high-glucose-induced EMT and extracellular matrix secretion.

Moreover, only the simultaneous knockdown of Smad1, Smad5, and Smad8 downregulated the expression of Id2, which was not altered by the individual knockdown of Smad1, Smad5, and Smad8. Basic helix-loop-helix (bHLH) transcription factors were essential for Id2 to regulate the role of downstream target genes, and Twist was a bHLH transcription factor. Therefore, the expression of Twist was examined in this study. Twist was found to be highly expressed in the kidney of DM rats and renal tubular epithelial cells cultured with high glucose. The overexpression of Id2 did not alter the expression of Twist, but the interaction between Id2 and Twist was enhanced. In conclusion, the data showed the specific mechanism underlying Id2 negative regulation in diabetic renal tubulointerstitial fibrosis.

1. Introduction

Renal fibrosis is the main pathological change and a common pathway for various chronic kidney diseases to develop into end-stage renal diseases (Huynh and Chai, 2019; Leung et al., 2013). The pathogenesis of renal tubulointerstitial fibrosis in diabetic nephropathy (DN) is very complicated; hyperglycemia, hypertension, immunity, inflammation, deposition of advanced glycation products, dyslipidemia, and epithelial–mesenchymal transition (EMT) may be involved (Jain and Rains, 2011; Vallon and Thomson, 2012; Tziomalos and Athyros, 2015). Of these, EMT is one of the main reasons for the loss of intrinsic renal cells and also one of the important sources of fibroblasts. EMT plays an important role in renal tubulointerstitial fibrosis (Humphreys, 2018; Kuma et al., 2016). Recent studies have indicated that the occurrence of EMT is a central event of diabetic renal tubulointerstitial fibrosis (Loeffler and Wolf, 2014; Hills and Squires, 2011; Zhao et al., 2017a). Therefore, inhibiting or reversing the occurrence of EMT during diabetic renal tubulointerstitial fibrosis may play an essential

* Corresponding authors at: Department of Pathophysiology, Guizhou Provincial Key Laboratory of Pathogenesis and Drug Research on Common Chronic Diseases, State Key Laboratory of Functions and Applications of Medicinal Plants, Guizhou Medical University, Guizhou Medical University, Guiyang, Guizhou 550025, China.
E-mail addresses: xiaoying@gmc.edu.cn (Y. Xiao), jxiaohan99@163.com (X. Jiang), 1784690156@qq.com (C. Peng), zhangyingyinggms@gmc.edu.cn (Y. Zhang), 460522908@qq.com (Y. Xiao), 718298034@qq.com (D. Liang), smjtyf@gmc.edu.cn (M. Shi), yuanyuanwang18180@gmc.edu.cn (Y. Wang), zfan1985@yeah.net (F. Zhang), guobingbs@126.com (B. Guo).

https://doi.org/10.1016/j.biocel.2019.105613
Received 3 April 2019; Received in revised form 13 July 2019; Accepted 16 September 2019
Available online 17 September 2019
1357-2725/

role in controlling the progress of fibrosis.

Studies have confirmed that the inhibitor of differentiation 2 (Id2) is capable of resisting the fibrosis of organs, which reduces the production of extracellular matrix (ECM) by reversing or suppressing the occurrence of EMT, thereby significantly improving hepatic fibrosis (Kinoshita et al., 2007; Tajima et al., 2007; Yu et al., 2015), pulmonary fibrosis (Izumi et al., 2006a; Yang et al., 2015a), and renal fibrosis (Veerasamy et al., 2013; Gervasi et al., 2012; Vigolo et al., 2019). Moreover, its expression is significantly reduced in fibrotic organ tissues. Recent studies have revealed that bone morphogenetic protein-7 (BMP-7) exerts an anti-fibrotic effect by increasing the expression of Id2 protein, and the phosphorylation of Smad1, Smad5, and Smad8 is detected at the same time, indicating the activation of BMP-7/Smads signaling pathway (Yang et al., 2015a; Vigolo et al., 2019). However, the role of Id2 in EMT and DN is not fully defined yet.

A previous study reported that the Id family lacks the basic region directly binding to DNA, but it can bind to a basic helix-loop-helix (bHLH) transcription factor to form a heterodimer. This affects the binding of such a transcription factor to DNA and results in the loss of activity and negative regulation of the bHLH transcription factor, ultimately impacting the transcription of target genes (Wang and Baker, 2015).Therefore, it is also called a DNA-binding inhibitor. Twist, as a member of the highly conserved bHLH transcription factor family, is an important regulator in the EMT process. It can bind to the E-box on the E-cadherin promoter and inhibit the expression of E-cadherin, thereby promoting the development and progression of EMT (Rahme and Israel, 2015a; Meng et al., 2018; Bulzico et al., 2019). It was therefore hypothesized that Id2 might improve the effect of renal tubulointerstitial fibrosis in DN through inhibiting the transcription of downstream target genes via Twist.

Therefore, the present study investigated the inhibitory effect of BMP-7 on Twist-induced renal fibrosis in DM by upregulating the expression of Id2 and explored its related mechanism. Further studies on the pathogenesis of renal tubulointerstitial fibrosis were conducted in depth to provide a new therapeutic target for preventing and treating renal tubulointerstitial fibrosis in DN.

2. Results

2.1. EMT and ECM occurred in DN rats and NRK-52E cells

The successful establishment of the DM model and the development of fibrosis following the exposure of NRK-52E cells to high glucose were confirmed by biochemical, histopathological, and Western blot analyses were performed. The levels of 24-h urine protein and blood glucose were significantly higher in DM rats than in normal rats (24 h urine protein: 913.08 ± 311.42 vs. 33.48 ± 11.71 mg; blood glucose: 19.00 ± 0.82 vs. 8.30 ± 0.30 mmol/L) ($P < 0.05$). Hematoxylin and eosin (HE) and Masson staining showed an increase in the tubulointerstitium and tubular basement membrane thickening in DM rats. Moreover, the renal tubule cavity was obviously expanded, and most renal tubular epithelial cells disintegrated with vacuolar degeneration (Fig. 1A and B). The expression of E-cadherin, alpha-smooth muscle actin(α-SMA), and Col-IV, which is the marker of EMT and ECM, increased in the DM group compared with the NC group (Fig. 1C and D). In addition, the results revealed that the expression of E-cadherin protein decreased significantly in the HG group compared with the NC group. However, the expression of α-SMA and Col-IVproteins significantly increased in the HG group compared with the NC group, but with no time dependence (Fig. 1E and F). These findings indicated that the DM model was successfully established, and the exposure of NRK-52E cells to high glucose caused increased extracellular matrix secretion. This resulted due to the loss of the epithelial phenotype of NRK-52E cells and the expression of myofibroblastic markers.

2.2. Significant downregulation of the expression of Id2 and effect of renal fibrosis on DN rats and NRK-52E cells

Next, the expression of Id2 was detected in DN rats and NRK-52E cells. The results indicated that the expression of Id2 protein and mRNA significantly decreased in kidney tissues of DN rats (Fig. 2A–C). Immunohistochemical analysis showed that compared with NC group, the expression of Id2 in the kidney of rats in the DM group was reduced and distributed mainly in renal tubules, while insignificant expression was found in renal glomeruli (Fig. 2D and E). Moreover, the expression of Id2 protein decreased in HG-induced NRK-52E cells (Fig. 2F). Further, NG- or HG-induced NRK-52E cells were transfected with Id2-HA or corresponding control plasmids. Western blot analysis results showed high expression of HA protein in the overexpressed Id2 group, indicating that the expression of Id2 increased in cells clearly (Fig. 2G). The expression of α-SMA and Col-IVsignificantly increased while that of E-cadherin significantly decreased in the HG group compared with the NG group. The expression of E-cadherin protein significantly increased while the expression of α-SMA and Col-IV protein reduced in NG- and HG-cultured NRK-52E cells with Id2 overexpression compared with the corresponding NC group (Fig. 2H and I). These data suggested that the expression of Id2 significantly reduced in renal tubular epithelial cells in high glucose condition. Also, Id2 could inhibit or reverse the EMT process and reduce the deposition of ECM, indicating its negative effects on the regulation of renal tubular interstitial fibrosis in high glucose condition.

2.3. ExogenousrhBMP-7 could promote the expression of Id2 in NRK-52E cells

The optimum concentration of anti-renal fibrosis was screened by adding 50, 100, 150, and 200 ng/mL exogenous rhBMP-7 cytokines to the HG-cultured NRK-52E cells for 48 h. The expression of E-cadherin protein significantly reduced in the HG group compared with the NC group [Fig. 3A(1)]. When exogenous rhBMP-7 cytokines were added with HG exposure, the expression of E-cadherin significantly increased in a dose-dependent manner [Fig. 3A(1)]. In contrast, the expression of α-SMA and Col-IV proteins significantly increased compared with the NC group [Fig. 3A(2) and (3)]. On adding different concentrations of exogenous rhBMP-7 cytokines to HG-cultured NRK-52E cells, the expression of α-SMA and Col-IV decreased in a concentration-dependent manner [Fig. 3A(2) and (3)]. Therefore, 200 ng/mL rhBMP-7 was used for further analysis.

Next, NG- and HG-cultured NRK-52E cells were incubated with rhBMP-7. Western blot analysis results showed that the expression of Id2 in HG-induced NRK-52E cells significantly decreased when exogenous rhBMP-7 was added (Fig. 3B and C). The results of quantitative polymerase chain reaction (PCR) were similar to those of Western blot analysis (Fig. 3D). The data suggested that BMP-7 was involved in the expression of Id2 in NRK-52E cells.

2.4. Smad1/5/8 were involved in the BMP-7-regulated expression of Id2 in HG-cultured NRK-52E cells

The mechanism through which BMP-7 regulates the expression of Id2 was identified by transfecting NRK-52E cells with Smad1/Smad5/Smad8 small hairpin RNA (shRNA) and control shRNA. The expression of Id2 protein decreased but not significantly in the knockdown Smad1 group compared with the normal group (Fig. 4A). These conditions also applied to Smad5 and Smad8 plasmids, and the results were similar (Fig. 4B and C). Thus, NRK-52E cells were successfully co-transfected with Smad1, Smad5, and Smad8 plasmids. The expression of Id2 significantly decreased after Smad1/5/8 knockdown. It did not increase with the addition of exogenous rhBMP-7 cytokines (Fig. 4D and E). All data indicated that the expression of Id2 was upregulated by BMP-7 in HG-cultured NRK-52E cells through the BMP-7/Smads signaling

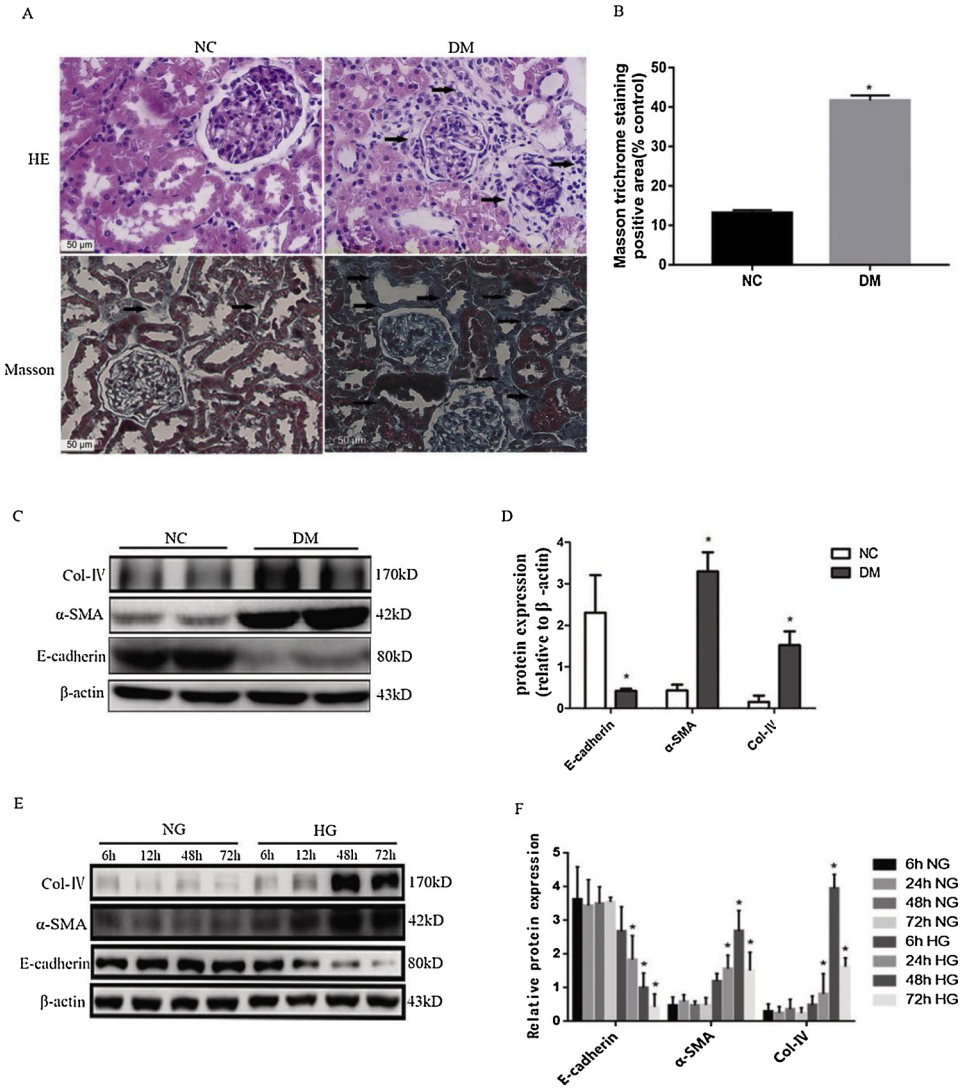

Fig. 1. EMT and ECM occurred in DN rats and NRK-52E cells. (A) Histomorphological changes in kidneys in the control group (NC group) and diabetic group (DM group) (hematoxylin and eosin and Masson staining, 50 μm). (B) The changes in Masson-stained cells were analyzed using the number of brown yellow cells($n = 6$). $^*P < 0.05$ versus the NC group. (C) Expression of E-cadherin, α-SMA, and Col-IVwas detected using Western blot analysis in kidney tissues (D) and analyzed using ImageJ ($n = 6$). $^*P < 0.05$ versus the NC group. (E) Expression of E-cadherin, α-SMA, and Col-IVwas detected using Western blot analysis in NRK-52E cells (F) and analyzed using ImageJ ($n = 3$). $^*P < 0.05$ versus the NG group (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

pathway.

2.5. Expression of Twist increased obviously in DN rats and HG-induced NRK-52E cells

The expression of Twist in DN rat kidney tissues was detected using Western blot analysis and quantitative PCR. The results indicated that the expression of Twist significantly increased in DN rat kidney tissues (Fig. 5A–C). The immunohistochemical analysis showed that the expression of Twist in the kidney of DM rats increased and was distributed mainly in renal tubules compared with the NC group (Fig. 5D and E). Further, the expression of Twist protein increased in HG-induced NRK-52E cells, but with no time dependence (Fig. 5F). NRK-52E cells were transfected with Twist shRNA for 48 h. Western blot analysis results showed that Twist T1 shRNA was knocked down obviously (Fig. 5G). The expression of α-SMA and Col-IVsignificantly increased, while the expression of E-cadherin significantly decreased, in the HG group compared with the NG group. The expression of E-cadherin protein significantly increased, while the expression of α-SMA and Col-IV protein decreased, in NG- and HG-cultured NRK-52E cells with Twist knockdown compared with the corresponding NC group (Fig. 5H and I). These data indicated that Twist could promote the EMT process and reduce the deposition of ECM, and Twist knockdown inhibited renal tubular interstitial fibrosis in high glucose condition.

2.6. Id2 interacted with Twist

For the dysregulation of Id2 and Twist in DN, their relationship in NRK-52E cells was further explored. Western blot analysis was used to examine the expression of Twist protein. The overexpression of Id2 did not affect the expression of Twist in NRK-52E cells (Fig. 6A and B). Confocal microscopy was used to analyze the samples. Immunofluorescence results indicated that Id2 was expressed mainly in the nucleus (red fluorescence). The expression of Twist was significantly higher in the HG group than in the NG group. It was expressed mainly in the nucleus, with some expression in the cytoplasm (green fluorescence). The results indicated that Id2 and Twist had similar subcellular localization, indicating that these two proteins could interact and had a similar distribution in the nucleus (Fig. 6C). Subsequently, using the co-immunoprecipitation method, Twist protein was found in the complex precipitated by Id2 antibody. Also, Id2 protein was found in the complex precipitated by Twist antibody, indicating a mutual binding between Id2 and Twist proteins. The precipitates were examined using Western blot analysis (Fig. 6D). Id2 was not regulated by the expression of Twist; it interacted with Twist by forming an allodimer, thereby affecting the regulatory effect of Twist on the downstream target genes, inhibiting the EMT process and ECM deposition, and improving DN tubular interstitial fibrosis.

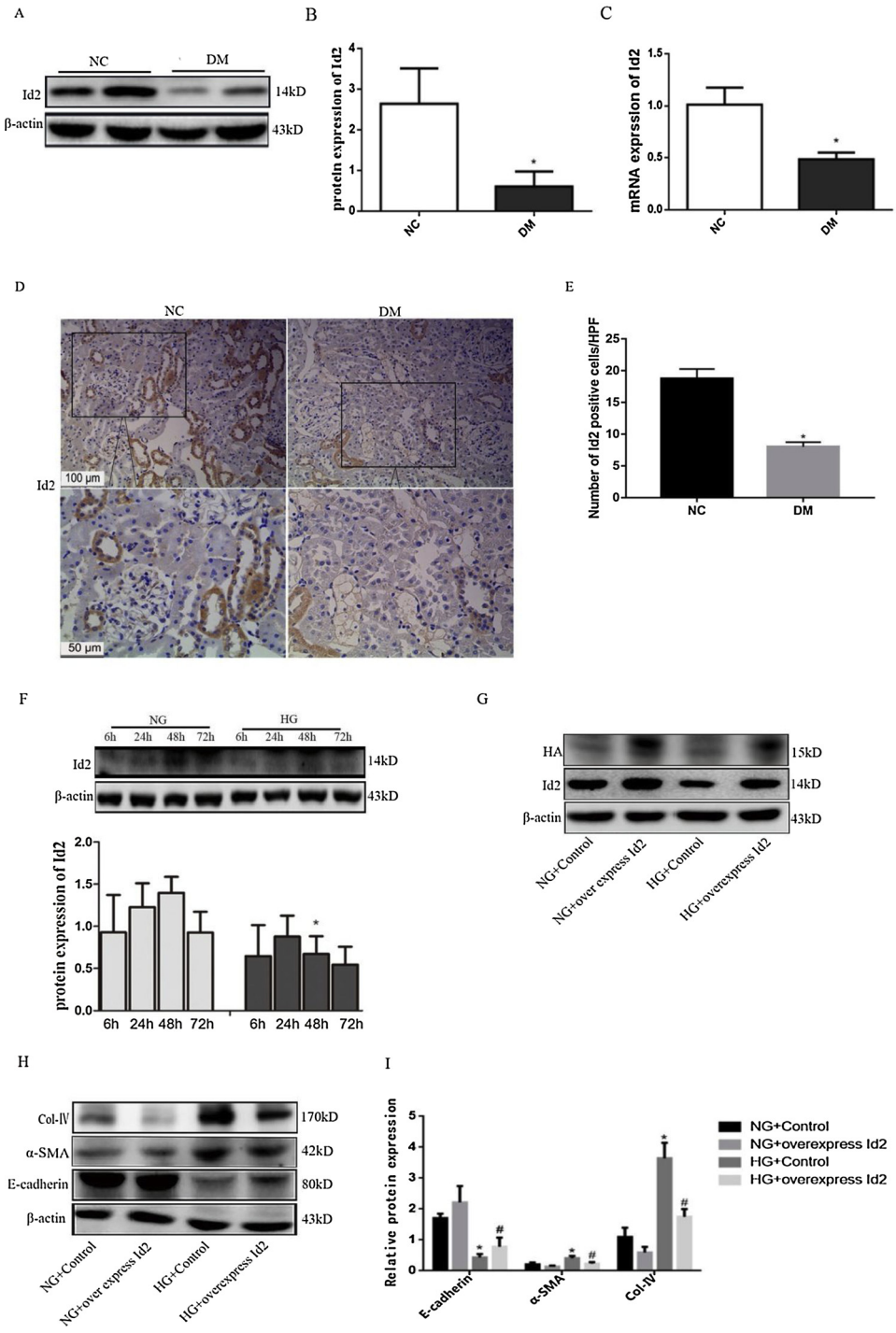

Fig. 2. Significant downregulation of Id2 and the effect of renal fibrosis on DN rats and NRK-52E cells. (A and B) Expression of Id2 was determined using Western blot analysis in kidney tissues ($n = 6$). $^{*}P < 0.05$ versus the NC group. (C) Expression of Id2 was determined using quantitative polymerase chain reaction (PCR) analysis in kidney tissues ($n = 6$). $^{*}P < 0.05$ versus the NC group. (D) The expression of Id2 in the renal tissues of rats in the NC and DM groups was detected by immunohistochemical staining (100 and 50 μm) (E)and analyzed using the position number of cells ($n = 6$). $^{*}P < 0.05$ versus the NC group. (F) NRK-52E cells were cultured with NG and HG for 6, 24, 48, and 72 h. The expression of Id2 protein was detected using Western blot analysis. (G) NRK-52E cells were transfected with the Id2-over-expressing plasmids with HA tag. High expression of HA protein was found in the Id2-over-expressing group using Western blot analysis. (H and I) Expression of E-cadherin increased and that of α-SMA and Col-IVdecreased after transfecting cells with Id2-overexpressing plasmids ($n = 3$). $^{*}P < 0.05$ versus the NG group; $^{\#}P < 0.05$ versus the HG group.

2.7. Discussion

Some cytokines, such as transforming growth factor-β (TGF-β), platelet-derived growth factor, mitogen-activated protein kinases, connective tissue growth factor, Twist, E2A, and Snail, can promote the occurrence and development of tubulointerstitial fibrosis (Sato et al., 2003; Ostendorf et al., 2014; Rodriguez-Pena et al., 2008; Gupta et al., 2000; Sun et al., 2012; Slattery et al., 2006; Zhao et al., 2017b). However, others, such as BMP-7, hepatocyte growth factor, Smad7, Ski-related novel protein N, and Id, can inhibit the occurrence and development of tubulointerstitial fibrosis (Patel and Dressler, 2005; Mizuno et al., 1998; Fukasawa et al., 2004, 2006; Roschger and Cabrele, 2017). It is probably because these fibrosis-promoting and anti-fibrosis cytokines in the microenvironment lead to changes in the expression of certain genes (Qi et al., 2016). Recent studies reported that Id2 protein reduced the production of ECM by reversing or inhibiting the occurrence of EMT (Wen et al., 2018; Zeng et al., 2017; Zhou et al., 2015). In this study, Id2 affected the occurrence and development of EMT and the accumulation of ECM in renal tubular epithelial cells and negatively regulated renal tubulointerstitial fibrosis in DN.

BMP-7, as a member of the TGF-β superfamily, is currently recognized as an anti-fibrotic cytokine. It can reverse EMT and reduce ECM accumulation, thus alleviating the fibrosis of organs (Zeisberg et al., 2003; Yu et al., 2009). However, the mechanism underlying its specific mechanism against fibrosis remains unclear. In TGF-β1-induced mouse pulmonary fibrosis, BMP-7 ameliorated pulmonary fibrosis in

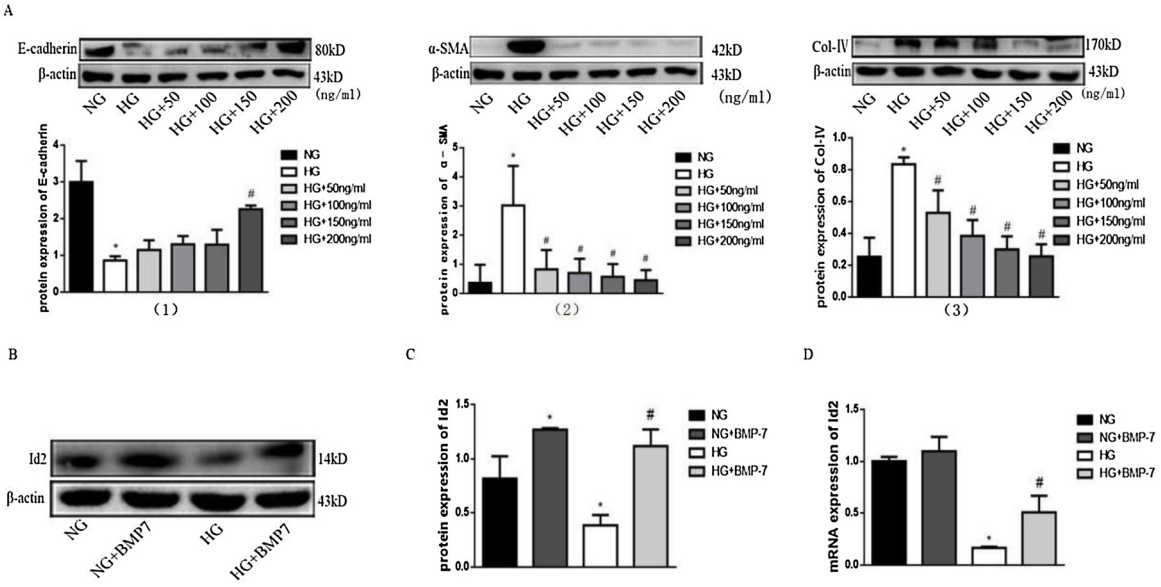

Fig. 3. Exogenous rhBMP-7 promoted the expression of Id2 in NRK-52E cells. (A) 50, 100, 150, and 200 ng/mL exogenous rhBMP-7 cytokines were added to NRK-52E cells cultured in high glucose. Western blot analysis results showed that the expression of E-cadherin increased and the expression of α-SMA and Col-IV decreased in a dose-dependent manner in NRK-52E cells ($n = 3$). $^{*}P < 0.05$ versus the NC group; $^{\#}P < 0.05$ versus the HG group. (B and C) The increase in the expression of Id2 by exogenous rhBMP-7 cytokines was determined using Western blot analysis in NRK-52E cells ($n = 3$). $^{*}P < 0.05$ versus the NC group; $^{\#}P < 0.05$ versus the HG group. (D) The expression of Id2 mRNA was detected using quantitative PCR ($n = 3$). $^{*}P < 0.05$ versus the NG group; $^{\#}P < 0.05$ versus the HG group.

mice by inducing the expression of Id2 and Id3 (Izumi et al., 2006b). In addition, the increased expression of Id2 and Id3 after overexpression of BMP-7 in injured lens epithelial cells in mice led to the reversal of EMT (Saika et al., 2006). However, the specific mechanism underlying the upregulation of the expression of Id2 by BMP-7 has not been fully understood. In this study, NRK-52E cells cultured under an and HG environment were exogenously treated with an rhBMP-7 cytokine. The results showed that BMP-7 could significantly promote the recovery of the expression of Id2, implying that BMP-7 could promote the expression of Id2 in renal tubular epithelial cells under an HG environment. Moreover, the promoting effect of BMP-7 on the expression of Id2 was only suppressed after the joint knockdown of Smad1, Smad5, and

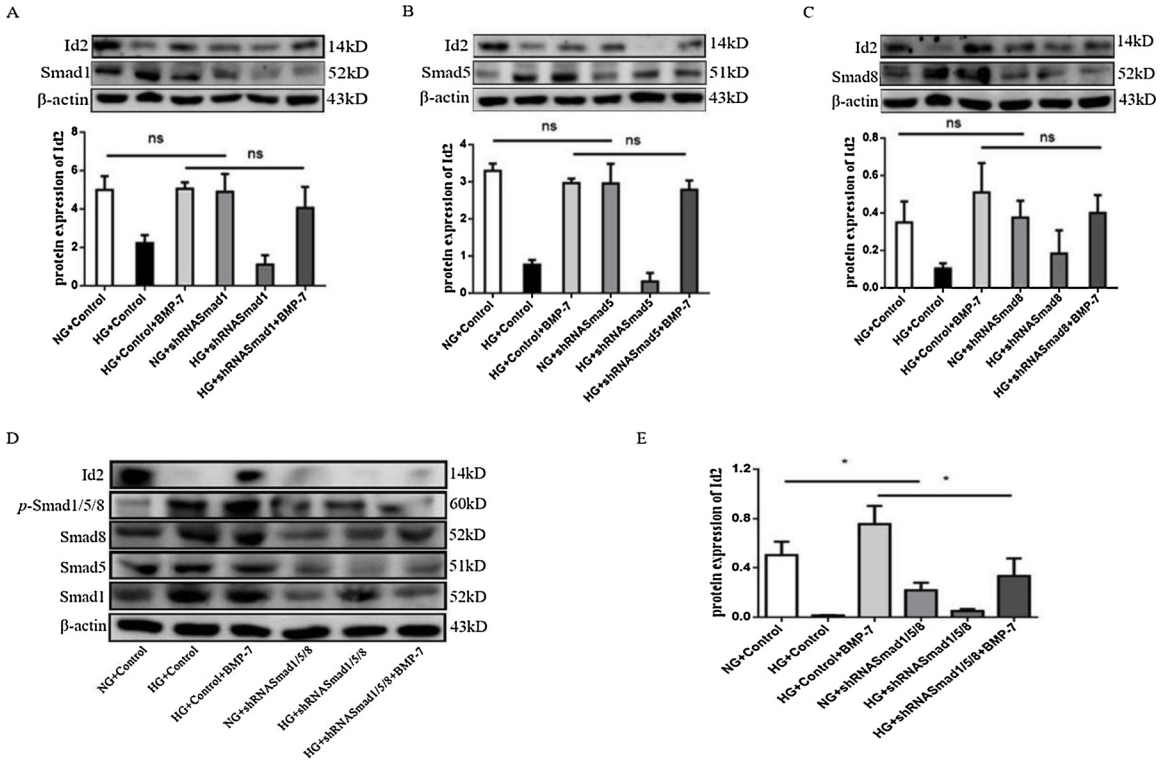

Fig. 4. Smad1/5/8 were involved in the BMP-7-regulated expression of Id2 in HG-cultured NRK-52E cells. (A–C) Cells transfected with Smad1, Smad5, and Smad8 plasmids after treatment with normal glucose, high glucose, and 200 ng/mL exogenous rhBMP-7 cytokines for 48 h. The expression of Id2 was determined using Western blot analysis in NRK-52E cells. (D and E) Cells were cotransfected with Smad1, Smad5, and Smad8 plasmids after treatment with NG, HG, and 200 ng/mL exogenous rhBMP-7 cytokines. Western blot analysis was used to detect the expression of Id2.

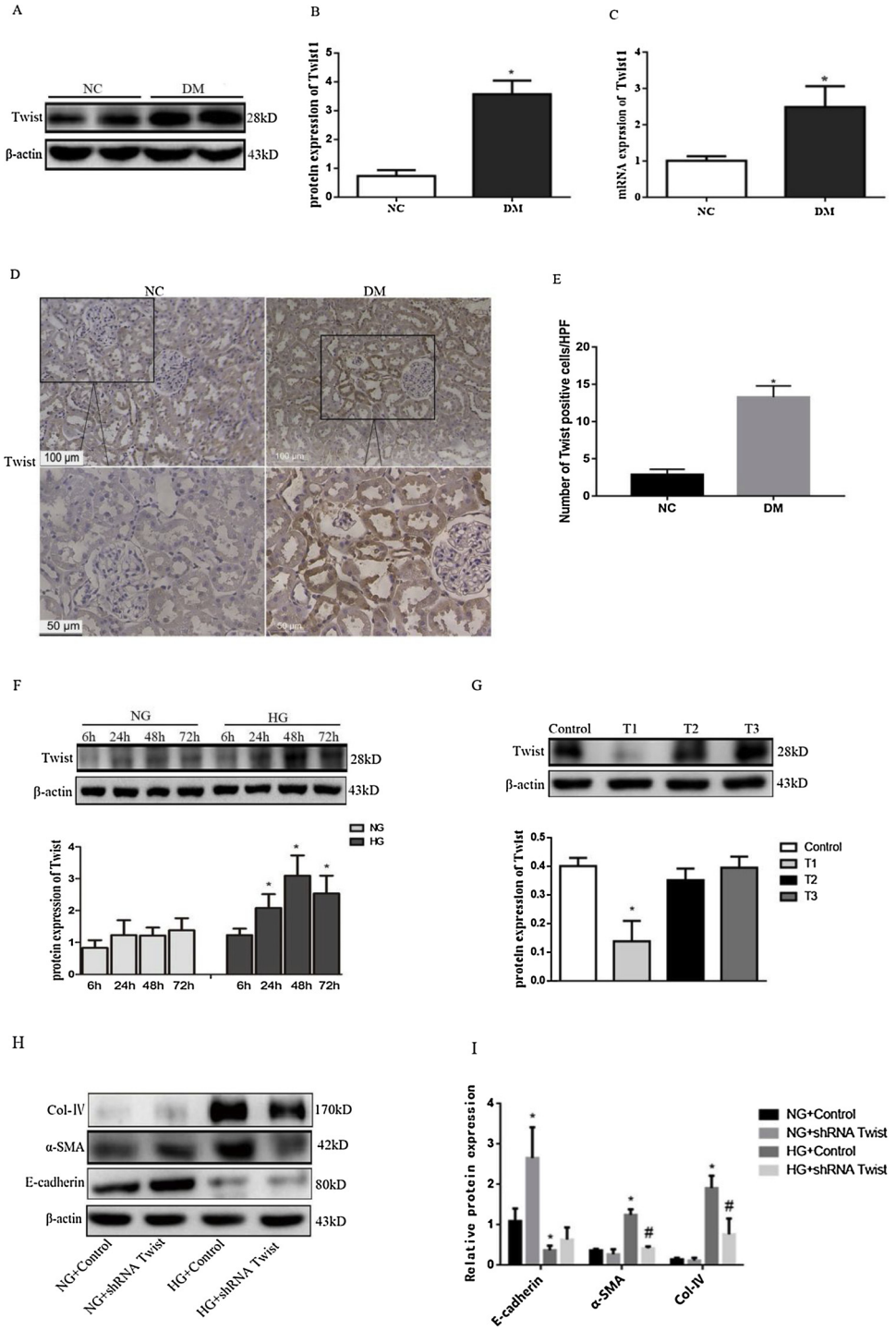

Fig. 5. Expression of Twist decreased obviously in DN rats and HG-induced NRK-52E cells. (A and B) Expression of Twist was determined by Western blot analysis in kidney tissues ($n = 6$). $^{*}P < 0.05$ versus the NC group. (C) Expression of Twist was determined using quantitative PCR analysis in kidney tissues ($n = 6$). $^{*}P < 0.05$ versus the NC group. (D) The expression of Twist in the renal tissues of the NC and DM rats detected by immunohistochemical staining (100 and 50 μm) (E) and analyzed using the position number of cells ($n = 6$). $^{*}P < 0.05$ versus the NC group. (F) NRK-52E cells were cultured with NG and HG for 6, 24, 48, and 72 h. The expression of Twist protein was detected using Western blot analysis. (G) NRK-52E cells transfected with plasmidswith Twist knockdown. The most effective knockdown was detected using Western blot analysis. (H and I) Expression of E-cadherin increased and the expression of α-SMA and Col-IV decreased after transfecting the cells with plasmids with Twist knockdown ($n = 3$). $^{*}P < 0.05$ versus the NG group; $^{\#}P < 0.05$ versus the HG group.

Smad8. Therefore, BMP-7 upregulated the expression of Id2 through the BMP-7/Smads signaling pathway in NRK-52E cells cultured with HG medium.

Recent studies have manifested that Twist can promote the transdifferentiation of epithelial cells to mesenchymal cells during the development of EMT, thus leading to the migration of tumor cells (Ansieau et al., 2008; Cieply et al., 2013; Castanon and Baylies, 2002). Id2 can block the TGF-β_1-mediated expression of typeIcollagen by inhibiting Twist, thus providing protection from lung fibrosis (Yang et al., 2015b). Moreover, a previous study also suggested that Id4 decreased the expression of matrix metalloproteinases via direct inhibitory interaction with Twist1 in patients with glioblastoma multiforme (Rahme and Israel, 2015b). The data showed that Twist was highly expressed in the kidney of DM rats and renal tubular epithelial cells cultured with HG. It promoted the occurrence and development of EMT and increased the deposition of ECM. Furthermore, Id2 did not affect the expression of Twist but bound to Twist. Twist protein formed a complex precipitate with Id2 antibody, and Id2 protein formed a complex precipitate with Twist antibody, suggesting that Id2 and Twist interacted with each other.

In conclusion, this study demonstrated that in NRK-52E cells cultured with HG medium and DN rat renal tissues, the expression of Id2 was upregulated via the BMP-7/Smad1/5/8 signaling pathway, and Id2 affected the regulatory effect of Twist on downstream target genes by

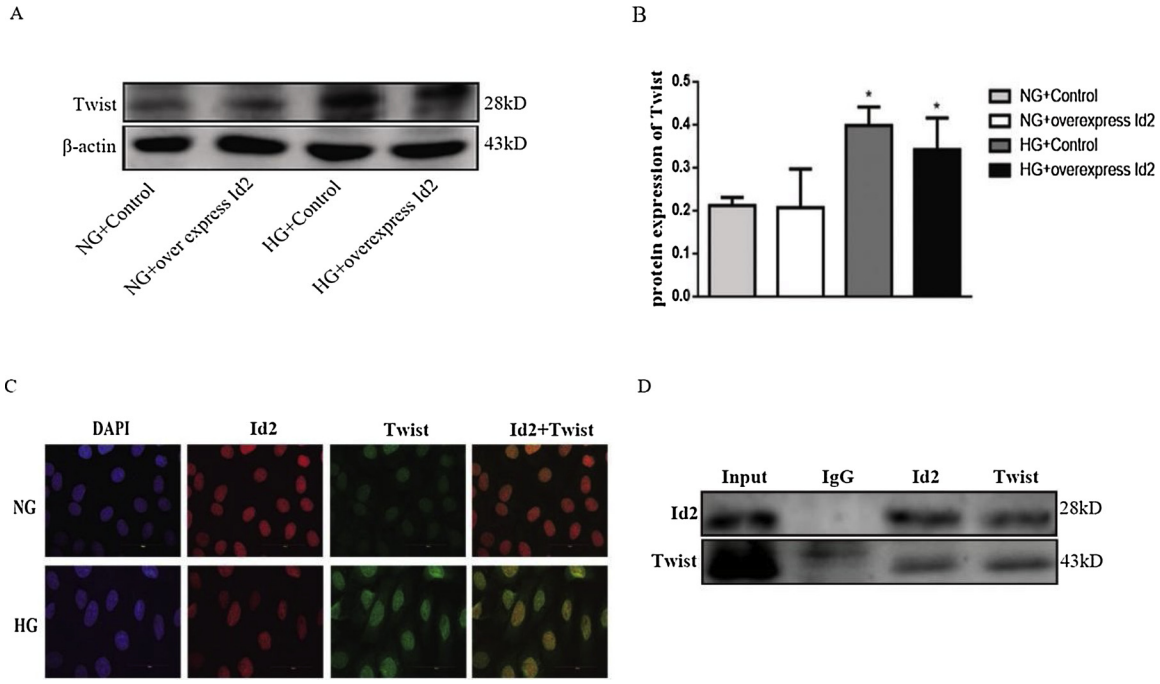

Fig. 6. Id2 interacted with Twist. (A–B) Expression of Twist was determined using Western blot analysis in NRK-52E cells ($n = 3$). *$P < 0.05$ versus the NC group. (C) Immunofluorescence double staining of Id2 and Twist displayed colocalization (merged image, **orange)** of Id2 and Twist in NRK-52e cells. (D) Co-immunoprecipitation showed the interaction of Id2 with Twist.

forming a heterodimer with Twist rather than regulating the expression of Twist (Fig. 7). Targeting Id2 may be a novel strategy in reversing or inhibiting EMT and DN.

3. Materials and methods

3.1. Experimental animals

Twenty healthy and specific pathogen-free male Sprague–Dawley rats weighing 180 ± 20 g were purchased from Beijing HFK Bioscience Co. Ltd. (Beijing, China) and housed in the animal center of Guizhou Medical University (Guizhou, China). The research complied with the guidelines of the National Health and Medical Research Council of China's Code for the care and use of animals for scientific purpose. All rats were randomly divided into a diabetic group (DM group, $n = 10$) and a control group (NC group, $n = 10$). DM rat models were established by injecting streptozotocin (STZ, prepared with sterile citric acid–sodium citrate buffer, pH 4.5; Sigma, MO, USA) into the tail vein at a dose of 55 mg/kg, while NC rats were injected with an equal volume of solvent. After 48 h, the high blood glucose level (fasting blood glucose ≥16.7 mM) indicated that the DM rat models were established successfully. Sixteen weeks after STZ or solvent injection, 24-h urine and femoral arterial blood from each rat were collected before the rats were sacrificed and urine protein and various biochemical indices were measured. The kidneys of each rat were harvested; one was fixed in 4% paraformaldehyde for paraffin sections, and the other one was snap-frozen in liquid nitrogen and stored at −80 °C for RNA and protein extractions.

3.2. Biochemical assays

Blood glucose was measured by the oxidase method, and 24-h urinary protein was measured by the Coomassie Brilliant Blue method. All tests were analyzed using a 1650 automatic biochemical analyzer (Beckman Instruments, CA, USA).

3.3. Cell culture

NRK-52E cells were cultured in Dulbecco's modified Eagle's medium (Gibco, CA, USA) supplemented with 10% fetal bovine serum (Gibco) containing normal glucose (NG, 5.5 mM glucose) or high glucose (HG, 30 mM glucose) with 5% CO_2 at 37 °C for 6, 24, 48, and 72 h for further study.

3.4. Transfection

The gene expression of Smad1, Smad5, Smad8, and Twist were knocked down in NRK-52E cells by shRNA plasmid and pCMVPuro04-Id2 plasmid designed by YILE Biotech (Shanghai, China). NRK-52E cells were seeded into six-well plates and transfected with the aforementioned plasmids according to the manufacturer's protocols. The cells were harvested 48 h after transfection.

3.5. Immunohistochemical analysis

Kidneys were fixed with paraformaldehyde and embedded in paraffin. The sections (4 μm) were observed by HE and Masson trichrome staining under a light microscope. Paraffin was removed and the sections were put in an oven for 2 h at 60 °C and dehydrated with gradient ethanol. Then, the sections were incubated overnight at 4 °C with the primary antibodies anti-Twist (1:80, Sigma, USA) and anti-Id2 (1:100, Santa Cruz, USA) diluted with phosphate-buffered saline (PBS), and then with secondary antibody goat anti-rabbit immunoglobulin G (IgG) for 1 h at room temperature. The sections incubated with PBS served as the negative control.

3.6. Immunofluorescence double staining

The NRK-52E cells were fixed with 4% paraformaldehyde at 4 °C and incubated with 5% bovine serum albumin for 30 min. The cells were incubated with anti-Id2 (1:100, Santa Cruz) and anti-Twist (1:80, Sigma) overnight at 4 °C. They were incubated with CY3 goat anti-mouse (green) and IgG FITC goat anti-rabbit IgG (red) (1:200, Santa

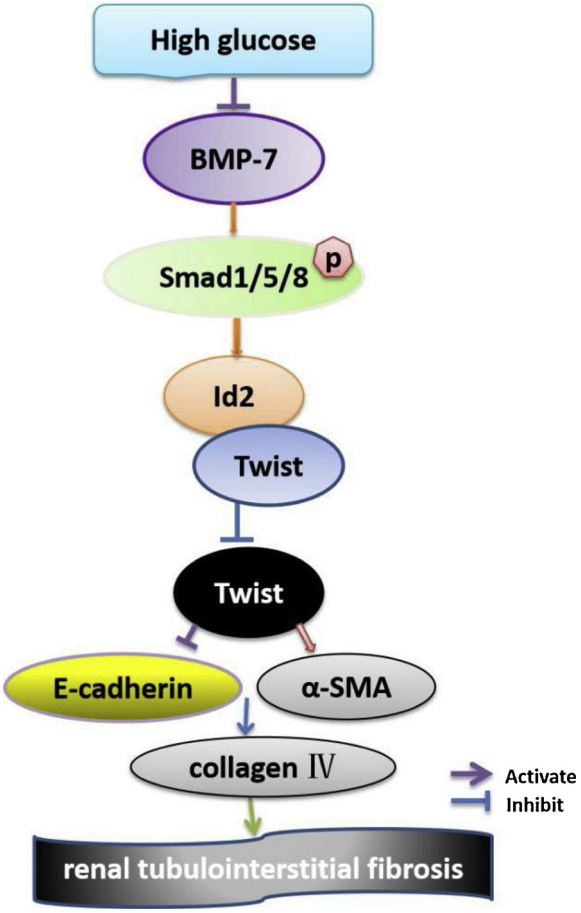

Fig. 7. Principal signaling pathways involved in the effect of HG on renal tubulointerstitial fibrosis. HG promoted the expression of α-SMA and Col-IV via suppressing the BMP-7/Smads/Id2 signaling pathway, thereby reversing or inhibiting EMT, reducing the accumulation of ECM, and alleviating renal tubulointerstitial fibrosis in DN. The Id2 protein formed a heterodimer with Twist to affect the regulatory effect of Twist on downstream target genes. Once the effect of Twist was suppressed by binding to Id2, renal tubulointerstitial fibrosis in DN was also ameliorated.

Cruz) at 37 °C for 1 h in the dark. The fluorescence was observed under a laser scanning confocal microscope (Olympus FV1000, Olympus, Japan).

3.7. Western blot analysis

Kidney tissue homogenates and cell culture samples were dissolved in RIPA lysis buffer (Beyotime, Jiangsu, China). The primary antibodies were incubated with anti-β-actin (1:400, Santa Cruz), anti-collagen-IV (1:800, Sigma), anti-Smad3 (1:1000, Cell Signaling Technology, USA), anti-Twist(1:800, Sigma)anti-Id2 (1:400, Santa Cruz), anti-Smad5 (1:800, Sigma), anti-Smad1 (1:400, Santa Cruz), anti-Smad8 (1:400, Santa Cruz), anti-Id2 (1:400, Santa Cruz), anti-α-SMA (1:400, Santa Cruz), anti-E-cadherin (1:400, Boss, Beijing, China), anti-p-Smad1 (Ser463/465)/Smad5 (Ser463/465)/Smad8 (Ser465/467) (1:1000, Cell Signaling Technology), anti-HA (1:200, PMK, Wuhan, China) overnight at 4 °C. Then, they were incubated with HRP-conjugated secondary antibody for 1 h at room temperature. The signals were enhanced using a chemiluminescence system (Millipore). The target of protein was quantified using Image Lab 5.1 software.

3.8. Real-time PCR

Total RNA was extracted from renal tissues and NRK-52E cells using the TRIzol reagent (Ambion, USA). Then, cDNA was synthesized using the RevertAId First Strand cDNA Synthesis Kit (Thermo, USA). Quantitative PCR was performed using 2 × SuperReal PreMix Plus SYBR Green (Tiangen, Beijing, China) with iQ SYBR Green SuperMix (Bio-Rad). The gene expression was referenced to the β-actin mRNA levels. The data were processed using the $2^{-\Delta\Delta Ct}$ method.

Name	Sequences of the primer (5′– 3′)	Annealing temperature, °C
Twist	*F:* ACCCTCACACCTCTGCATTC *R:* CAGTTTGATCCCAGCGTTTT	56
Id2	*F:* CTCCAAGCTCAAGGAACTGG *R:*ATGCTGATGTCCGTGTTCAG	56
β-actin	*F:* GCCAACACAGTGCTGTCT *R:* AGGAGCAATGATCTTGATCTT	56

3.9. Co-immunoprecipitation

Co-immunoprecipitation experiments were performed using the Pierce Co-Immunoprecipitation Kit (26149, Thermo Scientific, IL, USA) according to the manufacturer's protocols. Briefly, to minimize non-specific binding, 200 μg protein lysate was precleared using a control agarose resin. After mixing 100 μL (20 μg) of rabbit anti-mouse Id2 polyclonal antibody with 100 μL of 1 × cross-linked buffer and adding resin-containing column (Id2 antibody precipitation group), 20 μL (20 μg) of rabbit anti-mouse Twist polyclonal antibody and 180 μL of 1 × cross-linked buffer were mixed and added to the resin-containing column (Twist antibody precipitation group). At the same time, 20 μL (20 μg) of normal rabbit IgG + 180 μL of 1 × cross-linked buffer were added to the resin-containing column as control (IgG control group). The lysates were applied to the columns containing 1 μg immobilized antibodies (P2 × 2 or Pirt) covalently linked to an amine-active resin and incubated overnight at 4 °C. Equal volumes of the lysates were also applied to the columns containing control resin and processed in the same manner as the antibody coupling resin for negative controls. The **co-**immunoprecipitate was then eluted and analyzed using Western blot analysis along with the input controls.

3.10. Statistical analysis

Data were statistically analyzed using SPSS19.0. Mean ± standard deviation was used to represent the data. One-way analysis of variance was used for comparison between groups, and the Student two-tailed *t* test was used for comparison between the two groups. A *P* value less than 0.05 indicated a statistically significant difference.

Authors' contributions

Ying Xiao, Xiaohan Jiang, and Can Peng contributed equally to this study.

Declaration of Competing Interest

None.

Acknowledgment

This study was supported by the National Natural Science Foundation of China (grant no. 81760131 and 81860656) and the Guizhou Province Science and Technology Foundation [grant no. (2016) 7355].

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biocel.2019.105613.

References

Huynh, P., Chai, Z., 2019. Transforming growth factor β (TGFβ) and related molecules in chronic kidney disease (CKD). Clin. Sci. 133 (2), 287–313.

Leung, K.C., Toneli, M., James, M.T., 2013. Chronic kidney disease following acute kidney injury-risk and outcomes. Nat. Rev. Nephrol. 9 (2), 77–85.

Jain, S.K., Rains, J.L., 2011. Toll-like receptor-4 and vascular inflammation in diabetes: editoria. Cytokine. 55 (3), 446–447.

Vallon, V., Thomson, S.C., 2012. Renal function in diabetic disease models: the tubular system in the pathophysiology of the diabetic kidney. Annu. Rev. Physiol. 74 (1), 351.

Tziomalos, K., Athyros, V.G., 2015. Diabetic nephropathy: new risk factors and improvements in diagnosis. Rev. Diabet. Stud. 12 (1–2), 110–118.

Humphreys, B.D., 2018. Mechanisms of renal fibrosis. Annu. Rev. Physiol. 80, 309–326.

Kuma, A., Tamura, M., Otsuji, Y., 2016. Mechanism of and Therapy for Kidney Fibrosis. J. UOEH 38 (1), 25–34.

Loeffler, I., Wolf, G., 2014. Transforming growth factor-β and the progression of renal disease. Nephrol. Dial. Transplant. 29 (suppl. 1), i37–i45.

Hills, C.E., Squires, P.E., 2011. The role of TGF-β and epithelial-to mesenchymal transition in diabetic nephropathy. Cytokine Growth Factor Rev. 22 (3), 131–139.

Zhao, Y., et al., 2017a. MiR-30c protects diabetic nephropathy by suppressing epithelial-to-mesenchymal transition in db/db mice. Aging Cell 16 (2), 387–400.

Kinoshita, K., et al., 2007. Adenovirus-mediated expression of BMP-7 suppresses the development of liver fibrosis in rats. Gut. 56 (5), 706–714.

Tajima, K., et al., 2007. Importance of inhibitor of DNA binding/differentiation 2 in hepatic stellate cell differentiation and proliferation. Hepatol. Res. 37 (8), 647–655.

Yu, F., et al., 2015. MicroRNA-200a suppresses epithelial-to-mesenchymal transition in rat hepatic stellate cells via GLI family zinc finger 2. Mol. Med. Rep. 12 (6), 8121–8128.

Izumi, N., et al., 2006a. BMP-7 opposes TGF-beta1-mediated collagen induction in mouse pulmonary myofibroblasts through Id2. Am. J. Physiol. Lung Cell Mol. Physiol. 290 (1), 120–126.

Yang, J., et al., 2015a. Overexpression of inhibitor of DNA-binding 2 attenuates pulmonary fibrosis through regulation of c-Abl and Twist. Am. J. Pathol. 185 (4), 1001–1011.

Veerasamy, M., Phanish, M., Dockrell, M.E., 2013. Smad mediated regulation of inhibitor of DNA binding 2 and its role in phenotypic maintenance of human renal proximal tubule epithelial cells. PLoS One 8 (1), e51842.

Gervasi, M., et al., 2012. JunB contributes to Id2 repression and the epithelial-mesenchymal transition in response to transforming growth factor-β. J. Cell Biol. 196 (5), 589–603.

Vigolo, E., et al., 2019. Canonical BMP signaling in tubular cells mediates recovery after acute kidney injury. Kidney Int. 95 (1), 1–15.

Wang, L.H., Baker, N.E., 2015. E Proteins and ID Proteins: Helix-Loop-Helix Partners in Development and Disease. Dev. Cell 35 (3), 269–280.

Rahme, G.J., Israel, M.A., 2015a. Id4 suppresses MMP2-mediated invasion of glioblastoma-derived cells by direct inactivation of Twist1 function. Oncogene. 34 (1), 53–62.

Meng, J., et al., 2018. Twist1 regulates Vimentin through Cul2 circular RNA to promote EMT in hepatocellular carcinoma. Cancer Res. 78 (15), 4150–4162.

Bulzico, D., Pires, B.R.B., DE Faria, P.A.S., Neto, L.V., Abdelhay, E., 2019. Correlates with epithelial-mesenchymal transition markers and in adrenocortical tumors. Anticancer Res. 39 (1), 173–175.

Sato, M., Muragaki, Y., Saika, S., Roberts, A.B., Ooshima, A., 2003. Targeted disruption of TGF-beta1/Smad3 signaling protects against renal tubulointerstitial fibrosis induced by unilateral ureteral obstruction. J. Clin. Invest. 112 (10), 1486–1494.

Ostendorf, T., Boor, P., Van Roeyen, C.R., Floege, J., 2014. Platelet-derived growth factors (PDGFs) in glomerular and tubulointerstitial fibrosis. Kidney Int. Suppl. (2011) 4 (1), 65–69.

Rodriguez-Pena, A.B., et al., 2008. Activation of Erk1/2 and Akt following unilateral ureteral obstruction. Kidney Int. 74 (2), 196–209.

Gupta, S., Clarkson, M.R., Duggan, J., Brady, H.R., 2000. Connective tissue growth factor: potential role in glomerulosclerosis and tubulointerstitial fibrosis. Kidney Int. 58 (4), 1389–1399.

Sun, S., et al., 2012. Twist is a new prognostic marker for renal survival in patients with chronic kidney disease. Am. J. Nephrol. 35 (2), 141–151.

Slattery, C., McMorrow, T., Ryan, M.P., 2006. Overexpression of E2A proteins induces epithelial-mesenchymal transition in human renal proximal tubular epithelial cells suggesting a potential role in renal fibrosis. FEBS Lett. 580 (17), 4021–4030.

Zhao, Y., et al., 2017b. MiR-30c protects diabetic nephropathy by suppressing epithelial-to-mesenchymal transition in db/db mice. Aging Cell 16 (2), 387–400.

Patel, S.R., Dressler, G.R., 2005. BMP7 signaling in renal development and disease. Trends Mol. Med. 11 (11), 512–518.

Mizuno, S., et al., 1998. Hepatocyte growth factor prevents renal fibrosis and dysfunction in a mouse model of chronic renal disease. J. Clin. Invest. 101 (9), 1827–1834.

Fukasawa, H., et al., 2004. Down-regulation of Smad7 expression by ubiquitin-dependent degradation contributes to renal fibrosis in obstructive nephropathy in mice. Proc. Natl. Acad. Sci. U. S. A. 101 (23), 8687–8692.

Fukasawa, H., et al., 2006. Ubiquitin-dependent degradation of SnoN and Ski is increased in renal fibrosis induced by obstructive injury. Kidney Int. 69 (10), 1733–1740.

Roschger, C., Cabrele, C., 2017. The Id-protein family in developmental and cancer-associated pathways. Cell Communication & Signaling Ccs. 15 (1), 7–33.

Qi, X., Zhang, L., Lu, X., 2016. New insights into the epithelial-to-Mesenchymal transition in Cancer. Trends Pharmacol. Sci. 37 (4), 246–248.

Wen, X.F., et al., 2018. Inhibitor of DNA binding 2 inhibits epithelial-mesenchymal transition via up-regulation of Notch3 in breast Cancer. Transl. Oncol. 11 (5), 1259–1270.

Zeng, S., et al., 2017. BMP4 promotes metastasis of hepatocellular carcinoma by an induction of epithelial-mesenchymal transition via upregulating ID2. Cancer Lett. 390, 67–76.

Zhou, J.P., et al., 2015. Snail interacts with Id2 in the regulation of TNF-α-induced cancer cell invasion and migration in OSCC. Am. J. Cancer Res. 5 (5), 1680–1691.

Zeisberg, M., et al., 2003. BMP-7 counteracts TGF-beta1-induced epithelial-to-mesenchymal transition and reverses chronic renal injury. Nat. Med. 9 (7), 964–968.

Yu, M.A., et al., 2009. HGF and BMP-7 ameliorate high glucose-induced epithelial-to-mesenchymal transition of peritoneal mesothelium. J. Am. Soc. Nephrol. 20 (3), 567–581.

Izumi, N., et al., 2006b. BMP-7 opposes TGF-beta1-mediated collagen induction in mouse pulmonary myofibroblasts through Id2. Am. J. Physiol. Lung Cell Mol. Physiol. 290 (1), 120–126.

Saika, S., et al., 2006. Adenoviral gene transfer of BMP-7, Id2, or Id3 suppresses injury-induced epithelial-to-mesenchymal transition of lens epithelium in mice. Am. J. Physiol., Cell Physiol. 290 (1), C282–C289.

Ansieau, S., et al., 2008. Induction of EMT by twist proteins as a collateral effect of tumor-promoting inactivation of premature senescence. Cancer Cell 14 (1), 79–89.

Cieply, B., Farris, J., Denvir, J., Ford, H.L., Frisch, S.M., 2013. Epithelial-mesenchymal transition and tumor suppression are controlled by a reciprocal feedback loop between ZEB1 and Grainyhead-like-2. Cancer Res. 73 (20), 6299–6309.

Castanon, I., Baylies, M.K., 2002. A Twist in fate: evolutionary comparison of Twist structure and function. Gene. 287 (1–2), 11–22.

Yang, J., et al., 2015b. Overexpression of inhibitor of DNA-binding 2 attenuates pulmonary fibrosis through regulation of c-Abl and Twist. Am. J. Pathol. 185 (4), 1001–1011.

Rahme, G.J., Israel, M.A., 2015b. Id4 suppresses MMP2-mediated invasion of glioblastoma-derived cells by direct inactivation of Twist1 function. Oncogene. 34 (1), 53–62.