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BMP-7 inhibits renal fibrosis in diabetic nephropathy via miR-21 downregulation

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ABSTRACT

Epithelial-mesenchymal transition (EMT) and extracellular matrix (ECM) deposition in renal tubular epithelial cells are critical to diabetic nephropathy (DN) pathogenesis, but the underlying mechanisms remain undefined. Bone morphogenetic protein 7 (BMP-7) inhibits EMT and ECM accumulation in renal tubular epithelial cells cultured in presence of high glucose. Meanwhile, miRNA-21 (miR-21) downregulates Smad7, promoting EMT and ECM deposition. However, the association of BMP-7 with miR-21/Smad7 in DN is unknown. Here, NRK-52E cells incubated in presence of high glucose and STZ-induced C57BL diabetic mice were considered *in vitro* and *in vivo* models of DN, respectively. In both models, BMP-7 (mRNA/protein) amounts were decreased as well as Smad7 protein expression, while miR-21 expression and TGF-β1/Smad3 pathway activation were enhanced, accompanied by enhanced EMT and ECM deposition. Further, addition of BMP-7 human recombinant cytokine (rhBMP-7) and injection of the BMP-7 overexpression plasmid in diabetic mice markedly downregulated miR-21 and upregulated Smad7, reduced Smad3 activation without affecting TGF-β1 amounts, and prevented EMT and ECM accumulation. MiR-21 overexpression in the *in vitro* model downregulated Smad7, promoted EMT and ECM accumulation without affecting BMP-7 amounts, and miR-21 downregulation reversed it. By interfering with BMP-7 and miR-21 expression in high glucose conditions, miR-21 amounts and Smad3 phosphorylation were further decreased. Smad7 was then upregulated, and EMT and ECM deposition were inhibited; these effects were reversed after miR-21 overexpression. These findings suggest that BMP-7 decreases renal fibrosis in DN by regulating miR-21/Smad7 signaling, providing a theoretical basis for the development of novel and effective therapeutic drugs for DN.

1. Introduction

Diabetes mellitus (DM) is a critical chronic non-infectious disease, and 25%–30% of patients develop diabetic nephropathy (DN) [1]. DN represents a common microvascular complication of DM and a cause of end-stage kidney failure. Its pathological changes include glomerular sclerosis and tubular-interstitial fibrosis, which eventually develop into renal fibrosis [2,3]. However, due to many factors and complex processes, the current pathogenesis of DN has not been fully elucidated, and no effective prevention and control measures are currently available. Therefore, exploring the mechanisms of DN occurrence and development is very significant.

Other scholars and our team previously showed that transforming growth factor-β1 (TGF-β1) constitutes an essential fibrogenic cytokine in the development of DN [4–7]. By activating the Smad2/3 signaling pathway, renal tubular epithelial cells are induced to undergo

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epithelial-mesenchymal transition (EMT), which promotes extracellular matrix (ECM) synthesis and deposition, eventually developing extensive renal tissue fibrosis. This process has many influencing factors and complex effects; therefore, its molecular mechanism remains largely unclear. It was found that Smad7 can competitively bind Smad2/Smad3 to the activate TGF-β type I receptor (tβRI) in the cytoplasm, directly or indirectly reducing the activation levels of Smad2 and Smad3; meanwhile, blocking TGF-β1 activates the downstream signaling pathway and reduces the development of renal fibrosis [8,9]. In the process of renal fibrosis and inflammation, Smad7 is one of the targets of miR-21, which contributes to fibrosis by targeting Smad7 regulation [10]. In addition [11–13] miR-21 has a function in DN by enhancing ECM deposition and α-SMA expression, and shows a close association with the TGF-β1/Smads signal transduction pathway. Further studies by Kantharidis P and Wang JY et al. [14,15] showed that overexpression of miR-21 upregulates α-SMA and downregulates E-cadherin, which may occur by up-regulating *p*-Smad3 and down-regulating Smad7.

In recent years, reports [16,17] have indicated that during renal fibrosis, bone morphogenetic protein 7 (BMP-7) exerts anti-tubular-interstitial fibrotic effects by inhibiting ECM deposition. Belonging to the TGF-β superfamily, BMP-7 expression in the kidney is mainly concentrated in podocytes and renal tubular epithelial cells. Under high glucose conditions, the transcription and protein levels of BMP-7 in renal tubular epithelial cells are markedly decreased, while BMP-7 increase could inhibit EMT and ECM accumulation in renal tubular epithelial cells [18]. In addition, other reports [19,20] demonstrated that increased BMP-7 expression also attenuates TGF-β1-associated renal tubular epithelial EMT and ECM deposition; however, the specific mechanism has not been clarified so far. Currently, studies have shown that BMP-7 and Smad7 may be effective for the treatment of kidney disease, but little is known about the relationship and possible mechanism of BMP-7 and Smad7 in DN associated renal fibrosis. The latest studies on peripheral nervous system referred to BMP-7 and its downstream regulatory miRNAs, including miR-21, but did not explore the specific mechanisms [21]. In addition, the functions and regulatory mechanisms of BMP-7 and miR-21/Smad7 in DN associated renal fibrosis remain largely unknown.

Here, the associations of BMP-7 with miR-21 and Smad7 were demonstrated, as well as its regulatory mechanisms *in vitro* (renal tubular epithelial cells grown under high glucose conditions) and *in vivo* (STZ-induced diabetic mice), indicating that the three molecules are critical to the development of DN renal fibrosis.

2. Experimental procedures

2.1. Cell culture and transfections

Rat renal tubular epithelial (NRK-52E) cells (Kunming, China) were maintained at 37 °C in an environment with 5% CO_2 in Dulbecco's modified eagle medium (Hyclone, USA) containing 10% fetal bovine serum (FBS; Gibco, USA). They were then cultured under normal (NG, 5.5 mmol/L) and high (HG, 25 mmol/L) glucose conditions, respectively, in media containing 10% FBS. NRK-52E cells were transiently transfected with Lipofectamine™ 3000 (Invitrogen, USA) as directed by the manufacturer. MiR-21 and NC mimics, and miR-21 and NC inhibitors were purchased from Ribobio (China).The *rh*-BMP-7 (*h*BMP-7) was purchased from Sigma (USA). After 48 h of culture, cells were randomly assigned to the normal glucose control (NG), high-glucose (HG), normal glucose + *h*BMP-7 (NB, 100 ng/ml), high glucose + *h*BMP-7 (HB, 100 ng/ml), high glucose + Con-miR-21 mimics (HCmimics), high glucose + Con-miR-21 inhibitor (HCinhibitor), high glucose + miR-21 mimics (HM), high glucose + miR-21 inhibitor (HI), high glucose + *h*BMP-7 + miR-21 mimics (HBM) and high glucose + *h*BMP-7 + miR-21 inhibitor (HBI) groups.

2.2. Immunofluorescent staining

NRK-52E cells underwent fixation in 4% formalin (4 °C), blocking with bovine serum antigen (30 min in ambient conditions) and incubation overnight with anti-E-cadherin and anti-SMA at 4 °C. E-cadherin detection was performed with DyLight 488-linked goat anti-rabbit IgG (H + L) (Abbkine, USA), and SMA was detected with 594-linked goat anti-rabbit IgG (H + L) (Abbkine). DAPI was used for nuclear staining, and samples were analyzed under a Leica DM4000B microscope (Germany).

2.3. Experimental animals

SPF male C57BL mice purchased from Beijing Sibefu Biotechnology Co., Ltd. were randomized into normal (NC, n = 36) and diabetes (DM, n = 36) groups. The mice of the DM group were intraperitoneally injected with 55 mg/kg streptozotocin (STZ, Sigma); mice in the NC group were injected with the same amount of pH 4.5 sterile citric acid-sodium citrate buffer (lysozyme) for 5 consecutive days. Fasting blood glucose levels in mice were assessed at 72 h after treatment, and values ≥ 16.7 mmol/L indicated that DM mice were successfully modeled. After 4 weeks of feeding, the normal and diabetic mice were randomized into the normal control (NC + Control), normal empty (NC + Vector), normal BMP-7 (NC + BMP-7), diabetic control (DM + Control), diabetic empty (DM + Vector) and diabetic BMP-7 (DM + BMP-7) groups (n = 10 per group). Subsequently, the BMP-7 treatment and empty groups were injected with 15 μg BMP-7 overexpression and empty plasmids, respectively, via the tail vein, once weekly for a ttal of 6 weeks. The pHAGE-BMP-7 plasmid was purchased from Addgene (USA). The animals were euthanized at 12 weeks following STZ administration. Urine samples were obtained and measured for volume in the 24-hr period preceding euthanasia. All mice were fasted for 6 h prior to sacrifice. Blood specimens were collected from the femoral artery and centrifuged for preparing serum, kept at −20 °C for biochemical assessment. Both kidneys were removed, with one stored at −80 °C (RNA and protein preparations) and the other submitted to fixation with 4% formalin for histological and immunohistochemical evaluations. All animal studies complied with the regulations and guidelines of Guizhou Medical University institutional animal care, and followed the AAALAC and IACUC guidelines.

2.4. Biochemical analyses

Serum glucose and urinary microalbumin/creatinine amounts were assessed on an automated biochemical analyzer (Beckman Instruments 1650, USA).

2.5. Histopathology and immunohistochemistry

Kidney tissue specimens were fixed in 4% paraformaldehyde, paraffin embedded, and sectioned at 3 μm. This was followed by hematoxylin-eosin (H&E) staining for histological evaluation. Renal fibrosis was assessed by Masson's trichrome staining. The Two Step Immunoassay assay kit (ZSBIO, China) was employed for immunochemical staining, as directed by the manufacturer. Sample analysis was carried out by two independent and blinded investigators.

2.6. Immunoblot

Protein concentrations were assessed using the BCA protein assay kit (Beyotime, China). Equal amounts of total protein underwent separation by sodium dodecyl (SDS) sulfate-polyacrylamide gel electrophoresis (PAGE) and transfer onto polyvinylidene difluoride membranes (Millipore, Germany). Then, the membranes were incubated with antibodies raised against BMP-7 (1:1000, Proteintech, China), Smad7 (1:1000, Abcam, USA), TGF-β1 (1:300, Santa Cruz, USA),

Smad3 and Phospho-Smad3 ($^{Ser423/425}$) (1:1000,Cell Signaling, USA), E-cadherin (1:1000, Cell Signaling), α-SMA (1:200, Santa Cruz), Collagen III (1:1000, Sigma), and β-actin (1:1000, Pumei, China) at 4 °C overnight. This was followed by incubation with appropriate secondary antibodies for 1 h and detection with the clarity™ Western ECL substrate; quantification was carried out on a Bio-Rad gel imaging system (Bio-Rad, USA).

2.7. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was purified from kidneys and cells with TRIzol reagent (Invitrogen, USA) as directed by the manufacturer. The Bulge-Loop™ miRNA qRT-PCR primer kit (Ribobio) was used to quantitate miR-21. Meanwhile, qPCR was carried out with SuperReal PreMix (SYBR Green) (Tiangen, China) and iQ SYBR Green SuperMix (Bio-Rad). Gene expression levels were normalized to β-actin or U6 amounts. The $2^{-\Delta\Delta Ct}$ method was employed for quantification.

2.8. Statistical analysis

SPSS 19.0 (SPSS, USA) was employed for statistical analyses. Data were expressed as mean ± standard deviation (SD), and assessed by Student's Newman-Keuls test and One-way ANOVA. $P < 0.05$ indicated statistical significance.

3. Results

3.1. BMP-7 inhibits EMT and deposition of ECM by regulating the expressions of miR-21 and Smad7, without affecting the expression of TGF-β1 in vitro

To assess BMP-7, miR-21 and Smad7 amounts in the high glucose environment, we used rat renal tubular epithelial cells (NRK-52E cells) maintained for 48 h under normal and high glucose conditions, respectively, as study objects. First, to observe cell morphology, cell immunofluorescence was employed to identify the cells used, and E-cadherin and α-SMA amounts were determined. The results showed that E-cadherin in the NG group was mainly expressed in the cell membrane, and α-SMA expression levels were very low. In the high glucose (HG) group, α-SMA amounts were markedly elevated and E-cadherin levels were reduced (Fig. 1A), which was further validated in subsequent immunoblot data. Next, we found that TGF-β1 expression and Smad3 phosphorylation (*p*-Smad3 $^{(Ser423/425)}$) were remarkably enhanced in the HG group in comparison with the NG group (Fig. 1 B), indicating that TGF-β1/Smad3 signaling was activated in the HG environment. In addition, E-cadherin protein amounts in the HG group were starkly reduced while α-SMA and Collagen III protein levels were significantly elevated (Fig. 1E), suggesting enhanced EMT and ECM deposition in NRK-52E cells. Then, BMP-7, miR-21 and Smad7 were assessed by Western blot, cellular immunochemistry and/or qRT-PCR. The results showed that under NG conditions, BMP-7 was mainly expressed in the cytoplasm, but significantly decreased under HG conditions (Fig. .1B–D). Compared with the NG group, under HG conditions, miR-21 was remarkably upregulated, while Smad7 protein expression was significantly reduced (Fig. 1B, D).

The above results suggested that under HG conditions, the TGF-β1/Smad3 signaling pathway is activated, accompanied by the occurrence of EMT and ECM deposition. In addition, BMP-7 mRNA and protein levels were reduced, as well as Smad7 protein expression, while miR-21 was upregulated. In order to further clarify the associations among these molecules, experiments were carried out by adding BMP-7 cytokines (rhBMP-7, hBMP-7) to NRK-52E cells in NG and HG environments. Immunoblot and qRT-PCR showed that BMP-7 protein was upregulated, while comparable mRNA amounts were found in NG and HG groups, suggesting effective hBMP-7 intervention (Fig. 1B). Immunoblot revealed that under HG conditions, BMP-7 increase could

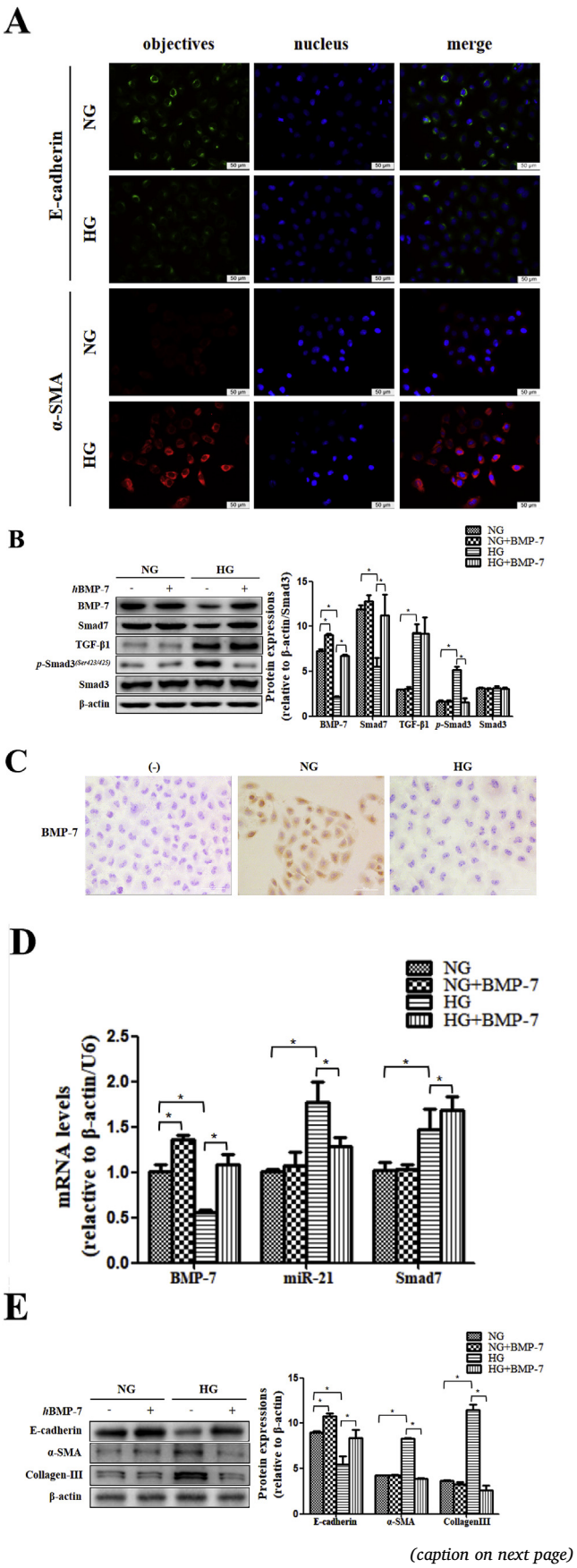

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Fig. 1. BMP-7 inhibits EMT and deposition of ECM by regulating the expressions of miR-21 and Smad7, without affecting the expression of TGF-β1 *in vitro*. **1A** Immunofluorescent detection of E-cadherin and α-SMA in NRK-52E cells cultured under NG or HG conditions (magnification, ×400).**1B** Immunoblot assessment of BMP-7, Smad7, TGF-β1, *p*-Smad3$^{(Ser423/425)}$ and Smad3 in NRK-52E cells cultured under NG or HG conditions. Data were presented as mean ± SD (n = 3). *$P < 0.05$ vs NG or HG group. **1C** Immunohistochemical staining of BMP-7 in NRK-52E cells (magnification, ×200). **1D** Expression levels of BMP-7, miR-21 and Smad7 in NRK-52E cells as analyzed by qPCR. Mean ± SD, n = 3, *$P < 0.05$ vs NG or HG group. **1E** Immunoblot assessment of E-cadherin, α-SMA and collagen III in NRK-52E cells. Mean ± SD, n = 3, *$P < 0.05$ vs NG or HG group.

significantly downregulate the *p*-Smad3 $^{(Ser423/425)}$ protein without affecting the protein expression levels of TGF-β1 (Fig. 1B). In addition, BMP-7 increase significantly upregulated the E-cadherin protein and downregulated the α-SMA and Collagen III proteins (Fig. 1E), suggesting that BMP-7 could inhibit the activation of TGF-β1/Smad3 signaling as well as EMT occurrence and ECM deposition. Interestingly, we also found that BMP-7 significantly downregulated miR-21 (Fig. 1D) and upregulated Smad7 at the gene and protein levels (Fig. 1 B, D).

3.2. Under high glucose conditions, miR-21 regulates p-Smad3 $^{(Ser423/425)}$, EMT and ECM deposition via Smad7 modulation

To further explore the associations among BMP-7, miR-21 and Smad7 under HG conditions and explore the possible mechanisms, we next assessed the function and mechanisms of miR-21 in DN renal fibrosis. To this end, miR-21 mimics and inhibitor, and their corresponding negative controls (con-miR-21 mimics and con-miR-21 inhibitor) were assessed under HG conditions. The results showed that miR-21 was markedly upregulated in the HG group in comparison with the NG group, and miR-21 amounts were further increased by treatment with miR-21 mimics in the HG group. On the contrary, miR-21 inhibitor markedly reduced miR-21 amounts, while con-miR-21 mimics and con-miR-21 inhibitor did not affect the experimental results (Fig. 2B), suggesting that miR-21 intervention was effective. Other scholars and our team demonstrated miR-21 is a fibrotic factor in DN renal fibrosis and closely related to TGF-β1/Smad3 signaling. In this study, miR-21 overexpression remarkably upregulated *p*-Smad3$^{(Ser423/425)}$, α-SMA and Collagen III (Fig. 2A), while significantly downregulating E-cadherin at the protein level. Conversely, inhibiting miR-21 expression could reverse the above effects (Fig. 2C), suggesting that under HG conditions, miR-21 increased the activation level of the *p*-Smad3$^{(Ser423/425)}$ protein and participated in DN occurrence by promoting EMT and ECM deposition, corroborating previous findings. Meanwhile, Smad7 is known as a downstream target of miR-21. Therefore, we further analyzed the regulatory association of miR-21 with Smad7. In this study, miR-21 overexpression significantly downregulated Smad7 mRNA and protein under HG conditions, while opposite effects were obtained after miR-21 inhibition, without affecting the expression of BMP-7(Fig. 2A and B).

3.3. Under high glucose conditions, miR-21 mediates the regulatory effects of BMP-7 on Smad7, p-Smad3 $^{(Ser423/425)}$, EMT and ECM

The above results suggested under HG conditions, BMP-7 could affect the levels of the downstream molecules miR-21 and Smad7, and miR-21 could regulate its downstream target gene Smad7 without affecting the expression of BMP-7 itself. To explore the mechanisms by which the three molecules contribute to the development of DN renal fibrosis, hBMP-7, hBMP-7 with miR-21 mimics, and hBMP-7 with miR-21 inhibitor were added, respectively, to NRK-52E cells cultured for 48 h in high glucose media. As shown in Fig. 3A, in comparison with the NG group, Smad3 activation was enhanced in the HG group; meanwhile, miR-21, and α-SMA and Collagen III protein amounts were increased, and Smad7 and E-cadherin protein levels were decreased (Fig. 3A–C). In comparison with the HG group, BMP-7 upregulation significantly inhibited Smad3 protein activation and reduced the expression levels of miR-21, and remarkably increased Smad7 mRNA and protein amounts, inhibiting EMT and ECM deposition (Fig. .2A–C). It is worth noting that the experimental results also showed that Smad3 protein phosphorylation and miR-21 amounts were further decreased in NRK-52E cells administered the miR-21 inhibitor compared with the HG group incubated with hBMP-7 alone, while Smad7 mRNA and protein amounts were further increased, further inhibiting EMT and ECM deposition. In contrast, after incubation with miR-21 mimics, Smad3 activation and miR-21 levels were restored, and Smad7 mRNA and protein amounts were decreased compared with the hBMP-7 alone group, enhancing EMT and ECM deposition. These results suggested that BMP-7 inhibited renal fibrosis via regulation of miR-21 and Smad7.

3.4. BMP-7 inhibits EMT and deposition of ECM by regulating the expressions of miR-21 and Smad7, without affecting the expression of TGF-β1 in vivo

The above *in vitro* results suggested the antifibrotic effects of BMP-7 were achieved by regulating miR-21-Smad7/Smad3. In order to further confirm this mechanism of action *in vivo*, STZ-induced C57BL diabetic mice were injected the *BMP-7* plasmid via the tail vein, and the corresponding control group was set up. In comparison with NC group mice, the DM group showed weight loss, polydipsia, polyphagia and polyuria, with significantly increased blood glucose and urine microalbumin/urinary creatinine (ACR). Meanwhile, the *BMP-7* group showed significantly recovered ACR, but the effect on blood glucose was not statistically significant (Table 1). In addition, H&E and MASSON's staining results revealed kidney tubules in the control group had a clear structure; in addition, renal tubular epithelial cells were full and neatly arranged, and the glomeruli were clearly visible. In the DM group, the renal tissues showed partial dilatation of the renal tubule, vacuolar degeneration of renal tubular epithelial cells, enhanced infiltration of inflammatory cells in the renal interstitial space, cell atrophy, shedding, and fibrotic glomeruli and renal tubules. Administration of the *BMP-7* plasmid could alleviate these effects (Fig. 4A). Immunohistochemistry showed that compared with the NC group, the DM group had reduced E-cadherin amounts and elevated α-SMA levels, and treatment with *BMP-7* plasmid could restore E-cadherin expression and reduce α-SMA amounts (Fig. 4B). The above results were further confirmed by Western blot (Fig. 4E).

Consistent with the above *in vitro* results, TGF-β1 and phosphorylated Smad3 (*p*-Smad3 $^{(Ser423/425)}$) amounts were markedly elevated in the DM group in comparison with the NC group (Fig. 4C), indicating that TGF-β1/Smad3 signaling is activated in DM; E-cadherin protein levels were significantly reduced in the DM group and α-SMA and Collagen III protein amounts were markedly elevated (Fig. 4E), suggesting the occurrence of EMT and increased ECM deposition in C57BL mice. In addition, in comparison with the NC group, the DM group showed starkly decreased BMP-7 mRNA and protein levels, elevated miR-21 amounts and reduced Smad7 protein levels, as assessed by immunoblot and qRT-PCR (Fig. 4C and D). Western blot and qRT-PCR data revealed increased BMP-7 mRNA and protein amounts in both NC and DM groups, with no significant differences in the empty plasmid group, suggesting the effects of BMP-7 were significant and the empty plasmid did not affect *in vivo* results (Fig. 4C and D). Western blot showed that under DM conditions, overexpression of BMP-7 significantly downregulated the *p*-Smad3$^{(Ser423/425)}$ protein without affecting the protein expression of TGF-β1 (Fig. 4C), markedly upregulated the E-cadherin protein, and starkly downregulated α-SMA and Collagen III at the protein level (Fig. 4E), suggesting that BMP-7 could inhibit the activation of TGF-β1/Smad3 signaling, EMT occurrence and ECM deposition. Moreover, consistent with *in vitro* results, overexpression of BMP-7 *in vivo* downregulated miR-21 (Fig. 4D) and

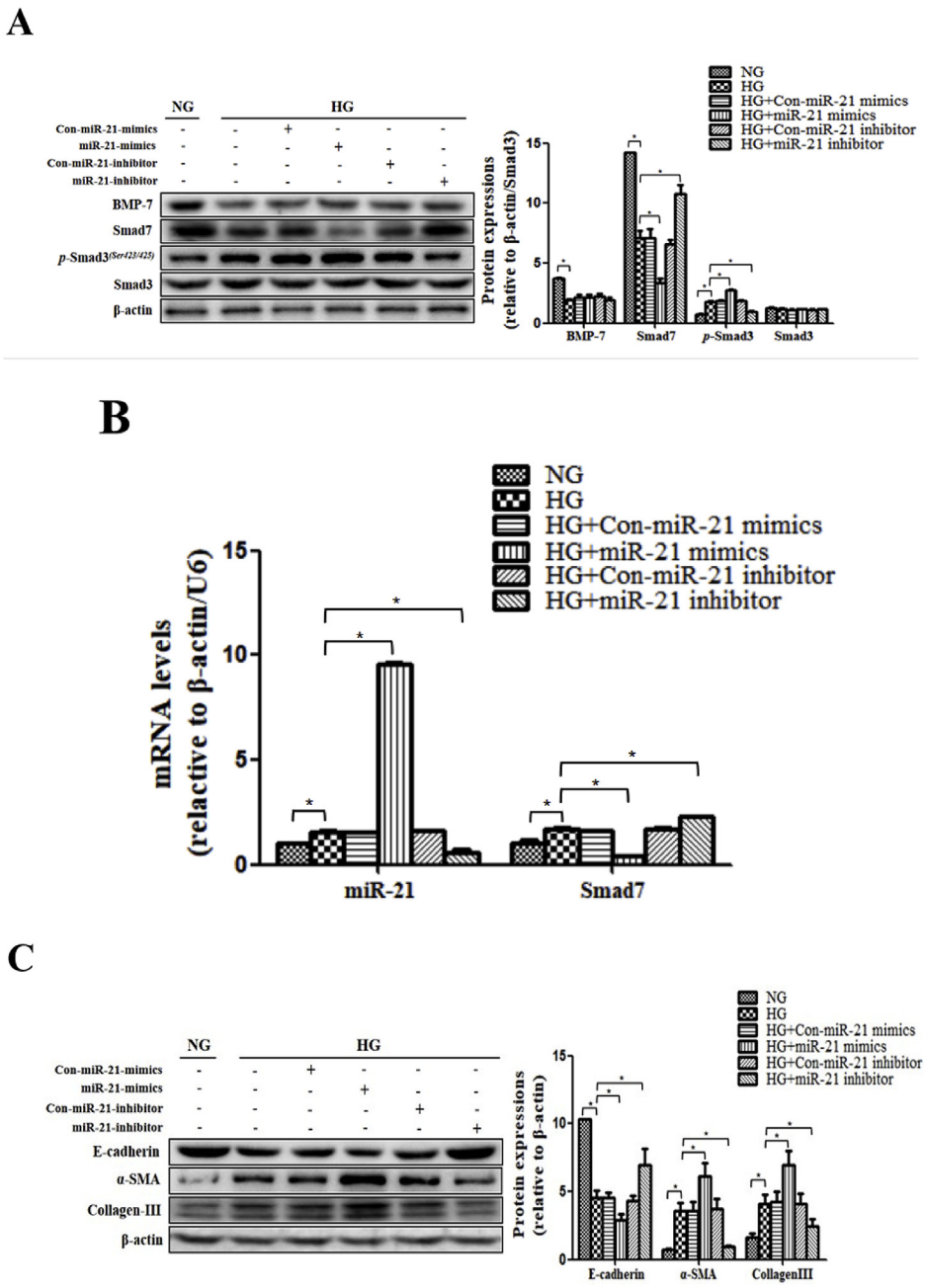

Fig. 2. Under high glucose conditions, miR-21 regulates *p*-Smad3 $^{(Ser423/425)}$, EMT and ECM deposition via Smad7 modulation. 2A Western analysis of BMP-7, Smad7, *p*-Smad3$^{(Ser423/425)}$ and Smad3 in NRK-52E cells cultured under NG or HG conditions. Data were presented as mean ± SD (n = 3).*$P < 0.05$ vs NG or HG group. **2B** Expression amounts of miR-21 and Smad7 in NRK-52E cells as analyzed by qPCR. Mean ± SD (n = 3). *$P < 0.05$ vs NG or HG group. **2C** Immunoblot assessment of E-cadherin, α-SMA and collagen III in NRK-52E cells. Mean ± SD (n = 3). *$P < 0.05$ vs NG or HG group.

upregulated Smad7 at the mRNA and protein levels (Fig. 4C and D).

4. Discussion

Renal fibrosis is a common feature of chronic renal disease (CRD) progression to end-stage kidney failure, characterized by the deposition of renal interstitial ECM and tubular-interstitial fibrosis. Studies [22,23] have shown that during the progressive development of DN, epithelial cell transdifferentiation into mesenchymal cells can lead to an abnormal accumulation of ECM, which is a marker of interstitial fibrosis and ultimately constitutes renal fibrosis. Here, renal tubular epithelial cells grown *in vitro* with high glucose and STZ-induced diabetic mice were assessed. Morphological examinations revealed EMT occurrence, ECM deposition and renal fibrotic lesions both *in vivo* and *in vitro*, as well as decreased E-cadherin protein expression and increased α-SMA and Collagen III protein amounts. In addition, TGF-β1 and *p*-Smad3$^{(Ser423/425)}$ protein levels were increased in both models, indicating TGF-β1/Smad3 signaling is activated. Moreover, this study also demonstrated that under high glucose and diabetes conditions, BMP-7 mRNA and protein levels were decreased, miR-21 amounts were elevated, and Smad7 mRNA were increased but protein expression was decreased, corroborating previous reports [24–26]. The ubiquitin-proteasome system affects the expression of Smad7, which induces the expression of Smad7 mRNA under HG conditions; meanwhile, upregulation of E3 ubiquitin ligase Smurf1, Smurf2 and Arkadia occurs, promoting the degradation of the Smad7 protein. This is precisely because protein degradation is stronger than transcriptional activation, resulting in a decrease in Smad7 protein levels.

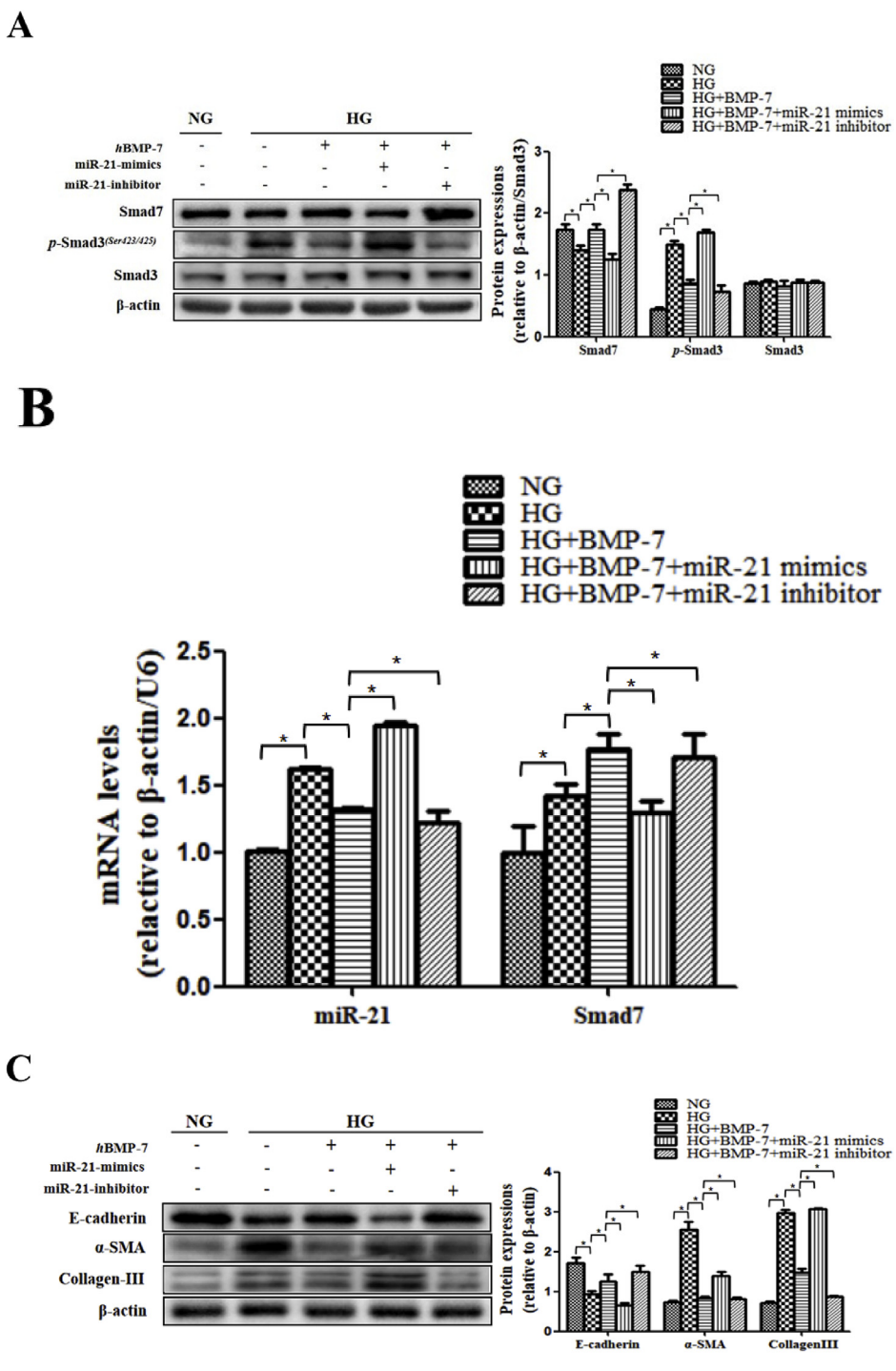

Fig. 3. Under high glucose conditions, miR-21 mediates the regulatory effects of BMP-7 on Smad7, *p*-Smad3 $^{(Ser423/425)}$, EMT and ECM. 3A Immunoblot assessment of Smad7, *p*-Smad3$^{(Ser423/425)}$ and Smad3 in NRK-52E cells cultured under NG or HG conditions. Data were presented as mean ± SD (n = 3). $^{*}P < 0.05$ vs NG, HG or HG + BMP-7 group. **3B** Expression levels of miR-21 and Smad7 in NRK-52E cells as analyzed by qPCR. Mean ± SD (n = 3). $^{*}P < 0.05$ vs NG, HG or HG + BMP-7 group. **3C** Immunoblot analysis of E-cadherin, α-SMA and collagen III in NRK-52E cells. Mean ± SD (n = 3). $^{*}P < 0.05$ vs NG, HG or HG + BMP-7 group.

Table 1
Levels of blood glucose and urine microalbumin/urinary creatinine (ACR) in each groups (Mean ± SD, n = 10). Note.BG:blood glucose; ACR:urine microalbumin/urinary creatinine.

Group	BG(mmol/L)	ACR (mg/umol)
NC + Control	7.64 ± 1.75	0.13 ± 0.04
DM + Control	31.56 ± 3.26*	41.25 ± 8.89*
DM + Vector	29.66 ± 2.48*	40.10 ± 7.75*
DM + BMP-7	30.39 ± 2.89*	20.30 ± 6.01*$^{\#}$

$^{*}P < 0.05$ versus NC + Control group; $^{\#}P < 0.05$ versus DM + Control group.

It has been reported [6,27] that under high glucose conditions, increased expression of TGF-β1 activates the downstream Smad3 via its transmembrane types II (TβRII) and I (TβRI) serine/threonine kinase receptors. The active Smad3 forms a complex with Smad4 and moves to the nucleus to induce EMT and promote excessive deposition of the ECM. Smad7 not only suppresses TGF-β activation by inhibiting Smad3 protein phosphorylation [6,27,28], but also inhibits TGF-β signaling by recruiting the TGF-β receptor E3 ubiquitin ligase Smurf1, Smurf2 and Arkadia [26,29]. Furthermore, overexpression of Smad7 attenuates ECM deposition due to high glucose by inhibiting the level of Smad3 activation [30]. Taken together, these findings clarified the significant antifibrotic effect of Smad7 in the development of DN. However, whether there is a certain correlation between BMP-7 and Smad7,

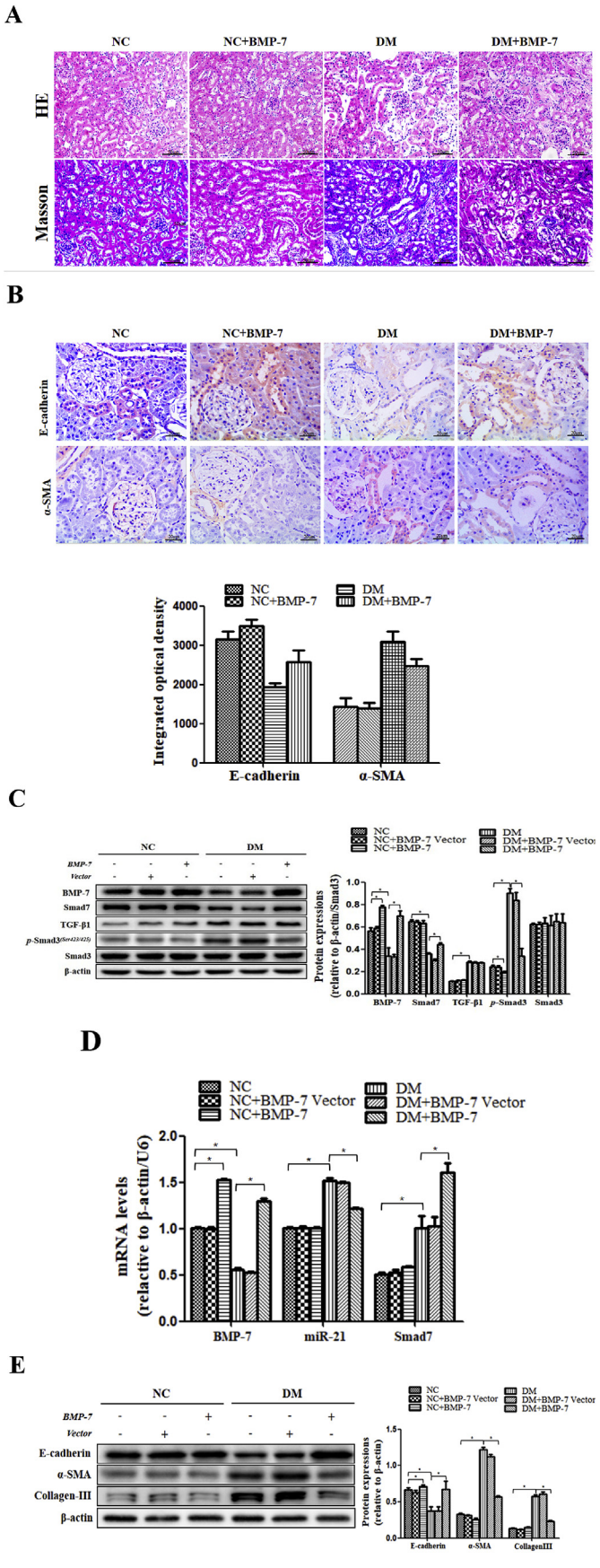

Fig. 4. BMP-7 inhibits EMT and deposition of ECM by regulating the expressions of miR-21 and Smad7, without affecting the expression of TGF-β1 *in vivo*. **4A** Histological changes in kidneys from mice at 12 weeks as assessed by H&E and Masson's staining (magnification, ×200). **4B** Immunohistochemistry for E-cadherin and α-SMA detection in renal tissue samples from mice at 12 weeks (magnification, ×400). **4C** Immunoblot assessment of BMP-7, Smad7, TGF-β1, *p*-Smad3$^{(Ser423/425)}$ and Smad3 in renal tissues of NC and DN mice. Band intensities were quantified with Image-Pro Plus 6.0 and quantity one. Data were presented as Mean ± SD (n = 10). $^*P < 0.05$ vs NC or DM group. **4D** Expression levels of BMP-7, miR-21 and Smad7 in kidney tissue specimens from NC and DN mice as evaluated by qPCR. Mean ± SD (n = 10). $^*P < 0.05$ vs NC or DM group. **4E** Immunoblot analysis of E-cadherin, α-SMA and collagen III in renal tissues of NC and DN mice. Data are Mean ± SD (n = 10). $^*P < 0.05$ vs NC or DM group.

which are both reduced under high glucose conditions and diabetes, remains unclear.

BMP-7 exerts its biological effects through signaling pathways such as Smads, ERK, and p38MAPK axes, and its antifibrotic effect has been confirmed to be involved in renal fibrosis [31,32]. Further studies [16,33] showed that the antifibrotic effect of BMP-7 occurs not only through ECM deposition inhibition in renal tubular epithelial cells, but also by preventing the fibrotic effect induced by TGF-β1/Smads signaling. However, the definite mechanism of action is not known. Therefore, to explore the antifibrotic mechanism of BMP-7 in renal fibrosis, we first assessed the relationship between BMP-7 and Smad7. Initially, we induced the expression of the BMP-7 protein by administering human recombinant BMP-7 (rhBMP-7) cytokines to NRK-52E cells cultured under normal or high glucose conditions. As shown above, BMP-7 protein amounts were markedly elevated, suggesting significant intervention *in vitro*. A study demonstrated that in HG environment, rhBMP-7 reduces the occurrence of EMT and ECM accumulation in renal tubular epithelial cells [31], with no effect after TGF-β1 induction under high glucose conditions [34]. In this work, effective rhBMP-7 intervention under high glucose showed that BMP-7 not only significantly upregulated E-cadherin protein and downregulated α-SMA and collagen III proteins, but also remarkably downregulated the *p*-Smad3 protein, without affecting the expression of TGF-β1 itself, suggesting that BMP-7 could inhibit EMT and ECM deposition, as well as Smad3 protein activation level, without affecting the expression of TGF-β1. Most importantly, our experimental results also revealed that BMP-7 could significantly upregulate Smad7 at the gene and protein levels. The above effects were further confirmed by effective intervention of the BMP-7 overexpression plasmid in diabetic mice. *In vivo*, normal control and successfully established diabetic C57BL mice were injected the BMP-7 overexpression and corresponding empty plasmids via the tail vein. As shown above, BMP-7 gene and protein amounts were markedly elevated, and the corresponding empty plasmid had no effect on the experimental results, suggesting effective intervention *in vivo*. Although increased BMP-7 amounts upregulated Smad7, the underpinning mechanism of action is largely undefined. For further exploration, we noted that Smad7 is a downstream target of miR-21 as confirmed by many studies. Liu et al. [35] found that overexpression of miR-21 promotes the increase of collagen I and α-SMA induced by TGF-β1 activation in the process of myocardial infarction, and its inhibition could reverse these effects. Further, application of the dual luciferase reporter gene assay confirmed Smad7 as a downstream target gene of miR-21. Lan HY [4] found that TGF-β1 upregulates miR-21 and downregulates Smad7 by increasing Smad3 levels during the development of DN associated renal fibrosis. Their follow-up study [10] with Smad7 immunohistochemical staining and FITC-miR-21 *in situ* hybridization showed that Smad7 is negatively correlated with miR-21 during renal fibrosis, and miR-21 overexpression in epithelial cells could decrease Smad7 mRNA levels. Finally, these authors performed dual luciferase reporter gene assays with tumor cells to verify that miR-21 can directly bind to the 3′UTR of Smad7. The conclusion that Smad7

represents a downstream target gene of miR-21 has been continuously confirmed by other scholars in recent years [14,15,36]. The current study also found that in HG-induced renal tubular epithelial cells, miR-21 mimics could significantly reduce Smad7 gene and protein expression levels, while the miR-21 inhibitor had opposite effects. In addition, miR-21 mimics could increase α-SMA and collagen III protein amounts and decrease E-cadherin protein levels, while the α-SMA and collagen III proteins were downregulated by miR-21 inhibition, and E-cadherin protein was upregulated, verifying the significant fibrotic effects of miR-21. Notably, the miR-21 mimics and inhibitor had no effects on BMP-7 expression, and Smad7, EMT, and ECM regulation.

Moreover, both NRK-52E cells in high-glucose administered rhBMP-7 and C57BL diabetic mice injected with the BMP-7 overexpression plasmid showed significantly downregulated miR-21 expression. This may suggest that BMP-7 regulates Smad7 by regulating the downstream miR-21, thereby exerting antifibrotic effects in the kidney. However, there are no reports investigating the roles and mechanisms of BMP-7 and miR-21/Smad7 in DN associated renal fibrosis. Therefore, we further administered rhBMP-7 to NRK-52E cells cultured in high glucose media, and treated with miR-21 mimics and miR-21 inhibitor, respectively. The results showed that BMP-7 increase not only led to decreased miR-21 amounts, reduced activation level of the Smad3 protein and upregulated Smad7 at the mRNA and protein levels, but also significantly inhibited EMT and ECM deposition. Treatment with miR-21 inhibitor, not only reduced miR-21 levels, further downregulated the phosphorylation of the Smad3 protein, and upregulated Smad7 (mRNA and protein), but also further inhibited EMT and ECM deposition. These effects were reversed after administration of miR-21 mimics. The above results indicated that BMP-7 exerts antifibrotic effects by regulating miR-21/Smad7.

In conclusion, this study demonstrated that BMP-7 is involved in the antifibrotic process in DN by regulating miR-21/Smad7 and affecting TGF-β1/Smad3 signaling *in vitro* and *in vivo*. However, in-depth mechanisms by which BMP-7 regulates miR-21 require more researches. Further exploration of the mechanism involved in the occurrence and development of the fibrotic process could provide insights into the mechanism of DN and help develop effective therapeutic drugs.

Declaration of competing interest

All authors declare that there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.116957.

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