Life Sciences 220 (2019) 106–116

Contents lists available at ScienceDirect

Life Sciences

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

The role of the BMP4/Smad1 signaling pathway in mesangial cell proliferation: A possible mechanism of diabetic nephropathy

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ARTICLE INFO

Keywords:
Bone morphogenetic protein
Diabetic nephropathy
High glucose
Mesangial expansion

ABSTRACT

Aims: This study explored the role of the BMP4/Smad1 signaling pathway in mesangial matrix expansion during the process of diabetic nephropathy.

Main methods: Diabetic rats were induced by high-fat feeding followed by an intraperitoneal injection of streptozotocin. Glomerular lesions were examined. Immunohistochemical analysis was performed in order to identify BMP4/Smad1 signaling proteins (BMP4, ALK3, and Smad1) and mesangial ECM proteins (Col1 and Col4) in kidney tissue. Cell proliferation and the expression of BMP4, Smad1 and Col4 were determined in cultured mesangial cells exposed to high glucose. The specific regulatory role of BMP4 was evaluated by detecting BMP4/Smad1 signaling pathway proteins and mesangial ECM proteins after blocking BMP4 both at the gene and protein levels.

Key findings: Rats with DN exhibited mesangial expansion and a thickened glomerular basement membrane. Immunohistochemical analysis of glomeruli showed increased expression of BMP4, Smad1, ALK3, Col1, and Col4 but less expression of MMP9 than observed in controls. High glucose induced slight proliferation of cultured rat mesangial cells after 48 h of incubation but there was no significant different from the control ($p > 0.05$). High glucose activated the BMP4/Smad1 signaling pathway and stimulated Col4 expression in mesangial cells. Both silencing of the *bmp4* gene (with siRNA) and blocking BMP4 protein signaling (with the BMP4 protein antagonist *Noggin*) reduced the expression of ALK3, Smad1, Col4, and Col1 in high glucose-stimulated mesangial cells.

Significance: The BMP4/Smad1 signaling pathway is crucial to the progression of mesangial expansion, and suppressing this signaling pathway may present a novel therapeutic strategy for DN.

1. Introduction

Diabetic nephropathy (DN) is on the rise worldwide. As a major complication in diabetes, this disease can lead to a decrease in the glomerular filtration rate (GFR), which ultimately progresses to end-stage renal disease (ESRD). In addition, DN is one of the highest risk factors for cardiovascular disease [1]. Currently, there are few therapeutic strategies to prevent the development and progression of DN. Thus, an in-depth study of the etiology and pathogenesis of DN is necessary in order to create effective precautionary measures and permanent treatment.

DN's pathological changes usually involve mesangial expansion and a thickened glomerular basement membrane (GBM) [2]. The mesangial matrix is largely composed of laminin, fibronectin, and types I, IV, V, and VI collagen. Among which, type IV collagen (Col4) is a major component of the thickened GBM and the expanded extracellular matrix (ECM) [3,4]. Accumulation of the mesangial matrix in DN is also associated with matrix metalloproteinases (MMPs) [5,6]. MMPs are a family of zinc-dependent proteolytic enzymes secreted in an inactive form, which later degrade ECM proteins [7]. MMP9, also known as gelatinase B (92 kDa), can proteolytically digest elastin, fibrillin, laminin, and types IV, V, XI and XVI collagens [8]. The role of MMP9 in the pathogenesis of DN has not yet been determined.

The pathogenesis of DN has multiple factors and signaling pathways. Recent studies have shown that the BMP4/Smad1 signaling pathway may be involved in the pathological process of mesangial expansion [9,10]. Bone morphogenetic proteins (BMPs) belong to the TGF-β superfamily and can regulate cellular differentiation, proliferation, migration, and apoptosis and also play a key role in embryonic development and cellular homeostasis throughout adulthood [11,12].

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https://doi.org/10.1016/j.lfs.2019.01.049
Received 11 December 2018; Received in revised form 27 January 2019; Accepted 28 January 2019
Available online 29 January 2019
0024-3205/

BMPs bind to their type I and type II receptors, which are two different types of serine/threonine kinase proteins. There are four type I receptors identified, including activin receptor-like kinase (ALK-1, -2, -3, and -6), and three type II receptors identified as the BMP type II receptor (BMPRII), activin type II receptor (ActR-II), and ActR-IIB [13]. BMPs regulate the transcription of the genes of interest through Smad and non-Smad signaling pathways [14]. For the former, BMPs bind to the type I and type II receptors, which allows the phosphorylation of Smad1, Smad5, and Smad8. Phosphorylated Smads bind to Smad4 and are subsequently transported into the nucleus, where they regulate the transcription of target genes [14,15]. Recent evidence demonstrates the crucial role Smad1 plays in in mesangial matrix expansion, contributing to the progression of DN [16]. A number of studies have explored the possibility of urinary Smad1 as a new biomarker for diagnosis and evaluation of the severity of DN [16,17]. Also, Smad1 was found to directly regulate transcription of Col4. In particular, Smad1 binds the Col4 promoter after the stimulation of advanced glycation end (AGE) product, inducing an overproduction of ECM in mesangial cells [4]. However, there are a few studies demonstrating the link between the BMP4/Smad1 signaling pathway and key mesangial ECM proteins.

This study explored the involvement of the BMP4/Smad1 signaling pathway in mesangial matrix expansion and the underlying mechanisms through in vivo and in vitro experiments. Also, we evaluated the changes in the expression of mesangial ECM proteins following inhibition of the pathway, in order to create a therapeutic strategy for treating DN.

2. Materials and methods

2.1. Animals

A total of 40 specific pathogen-free male Sprague Dawley (SD) rats (170–220 g and 6–7 weeks old) were obtained from the animal center of the Southwest Medical University. The study protocol was approved by the ethics committee of the Animal Care and Use Committee at the Southwest Medical University. 22 SD rats were fed with a high-fat diet for 4 weeks. The high-fat diet was composed of 67% of a standard diet, supplemented with 20% sugar, 2.5% cholesterol, 10% cooked lard, and 0.5% sodium cholate. Then the rats were injected intravenously with 1% streptozotocin (catalog #S0130; 35 mg/kg STZ from Sigma-Aldrich, St. Louis, MO, USA) in a vehicle (0.1% citrate buffer). After 3 days, the rats' blood glucose levels were measured where a blood glucose level of at least 16.7 mmol/L for 3 consecutive days represented development of diabetes mellitus. 2 weeks later, the rats were diagnosed with DN if the presence of the 24-hour urinary protein excretion was > 30 mg and the rats' urine volume increased by at least 50% more than the baseline volume. One rat was excluded due to a failure to reach this criteria. Another eighteen rats, serving as controls, were fed with standard chow, followed by intravenous administration of an equal volume of the vehicle. The animals were sacrificed at 8, 12, and 16 weeks, and, at each of these 3 time points, 7 rats in the DN group and 6 in the control group were sacrificed. The general condition of the animals, including food and water intake, mental state, activities, and glycemic levels were monitored throughout the experimental period. If the blood glucose levels were > 26 mmol/L, the rats were given subcutaneous injections of insulin. The body weight of each rat was weighed at 8, 12, and 16 weeks.

2.2. Determination of 24-hour urinary protein excretion

Before being sacrificed, the rats fasted for 24 h and then were placed in metabolic cages [18]. The clear cup mixed with a small amount of the preservative benzoic acid was put beneath the metabolic cage to collect urine. The next morning, the total urine volume was measured. The 24-hour urinary protein was calculated by multiplying the concentration of protein in the urine by the 24-hour urine volume.

2.3. Biochemical analysis

At the end of 8, 12, and 16 weeks, the rats were anesthetized by an intraperitoneal injection of 2% pentobarbital sodium (catalog #P376; Sigma Chemical Co., St. Louis, MO, USA) at a dose of 30–60 mg/kg. The blood samples collected from the abdominal aorta were analyzed by an automatic biochemistry analyzer (Advia2400, Siemens, Germany) for blood glucose, serum creatinine (SCr), blood urea nitrogen (BUN), and cystatin C.

2.4. Histopathological examination

After collecting blood samples, the kidneys were harvested and fixed in 10% formaldehyde. The deparaffinized slides were stained with either hematoxylin and eosin (H&E) or periodic acid-silver methenamine (PASM). The positive glomerular surface area was calculated for 10 PASM-stained glomeruli from each rat using the software Image Pro Plus 6.0 (Media Cybernetics Inc., Rockville, MD, USA). Pathological changes in the glomeruli were examined under a light microscope.

2.5. Antibodies

Multiple antibodies were used. Rabbit anti-rat polyclonal antibodies against BMP4 (catalog #ab39973), Col4 (catalog #ab6586), and MMP9 (catalog #ab76003) were obtained from Abcam (Cambridge, MA, USA). Also, rabbit anti-rat polyclonal antibodies against ALK3 were provided by Absin Bioscience Inc. (catalog #abs136154; Shanghai, China). Next, rabbit anti-rat monoclonal antibodies against Smad1 were provided by Bioworld Tech Inc. (catalog #BS6225; Shanghai, China). Rabbit anti-rat polyclonal antibodies against Col1 were obtained from Boster Bio Tech (catalog #BA0325; Wuhan, China).

2.6. Immunohistochemistry

The paraffin-embedded slides of kidney tissue were deparaffinized and dehydrated. The slides were incubated with 3% hydrogen peroxide for 10 min. Antigen retrieval was performed by heating the samples in citrate buffer (pH = 6.0). The slides were then blocked with goat serum at room temperature for 15 min, followed by incubation with the primary antibody (Dilution, Bmp4 1:1000; Smad1 1:100; Col1 1:100; Col4 1:500; MMP9 1:1000) overnight at 4 °C, after being washed in PBS 3 times,

The slides were incubated with biotinylated goat anti-rabbit IgG with a dilution factor of 1:500 (catalog #ZB-2010; Zhongshan Golden Bridge Biotech, China) for 15 min. The slides were then washed again and covered with horseradish peroxidase (HRP)-conjugated streptavidin (catalog #BA1088; Boster Bio Tech, Wuhan, China) for 30 min at room temperature. The slides were stained with 3,3′-diaminobenzidine (catalog #AR1000; DAB, Boster Bio Tech, Wuhan, China) for 3 to 5 min. After counterstaining with hematoxylin for 2 min, the slides were mounted and analyzed. 5 high power fields were randomly selected for each slide. The slides were digitized and analyzed by Image Pro Plus 6.0. As a negative control, the staining was also performed in parallel without the primary antibody.

2.7. Cell culture

Mesangial cells (ATCC® CRL-2573™) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in DMEM media (catalog # SH30021.01; HyClone, Logan, UT, USA) with 10% fetal bovine serum (FBS) in a sterile incubator maintained at 37 °C with 5% CO_2. After 24 h of incubation in serum-free medium, the cells were then exposed to various experimental conditions: (1) a 5.6 mM glucose control media hereafter known as normal glucose (NG group); (2) an osmotic medium containing normal glucose with 24.4 mM D-mannitol (MG group); and (3) a high

glucose media containing 30 mM glucose (HG group).

The BMP4 protein antagonist Noggin (5 μg; catalog # 250-38; Peprotech, Rocky Hill, NJ, USA) was reconstituted in a 50 μL sterile vehicle control (acetic acid plus 0.1% bovine serum albumin [BSA]). For detection of the BMP4/Smad1 signaling pathway, the cells were treated with NG or HG media with various concentrations of the Noggin solution.

2.8. Cell viability assay

Cell viability was assayed using the Cell Counting Kit-8 (catalog #CK04; CCK-8, Dojindo, Japan) [19]. The cells were plated in 96-well plates at a density of 1000 cells per well. After 24 h of incubation in DMEM, supplemented with 0.5% FBS, the cells were subjected to NG, HG, and MG medium for an additional 48 h. After the 48-hour incubation, the media was replaced with 100 μL of fresh culture media containing 10 μL of CCK-8, and the cells were incubated at 37 °C for 3 h. Cell viability was determined by reading the absorbance at 450 nm using a spectrometer (Bio-Rad, San Francisco, CA, USA). All experiments were performed in triplicate.

2.9. Immunofluorescence

The cell climbing film was fixed in 4% paraformaldehyde for 10 min, and permeabilized with 0.5% Triton X-100 for 15 min. After blocking with 1% BSA, the films were incubated overnight at 4 °C with primary antibodies (Dilution, BMP4 1:400; Smad1 1:100; Col4 1:1000). Then, the cells were incubated with goat anti-rabbit IgG (catalog # ab150077; Alexa Fluor® 488; dilution 1:400; Abcam) for 1 h at room temperature. The nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, Solarbio Bio Tech, Beijing, China). A negative control was prepared using the stain without any primary antibody. The immunolabeled cells were analyzed by fluorescence microscopy (Leica, Wetzlar, Germany).

2.10. BMP4-siRNA sequence and transfection

The BMP4-siRNA sequence (target sequence GCTCCAGGAAGAAG AATAA). A scrambled siRNA was used as a negative control. The cells were seeded into 6-well plates and cultured in DMEM medium with 0.5% FBS for 24 h.

siRNA transfection was performed using riboFECT™ CP (RiboBio, China). 20 μM siRNA stock solution (7.5 μL) was added to 1× riboFECT™ CP Buffer (120 μL) and mixed with 12 μL riboFECT™ CP Reagent for 0–15 min. The plates were incubated with the siRNA complex for 6 h in a complete culture medium and then continuously cultured for another 48 h in a medium containing normal (5.6 mM) or high (30 mM) glucose.

2.11. Western blotting analysis

The cells were lysed in ice-cold Lysis RIPA buffer (catalog #P0013B; Beyotime, Shanghai, China) supplemented with proteinase inhibitor (catalog #5892791001; Roche, Germany) for 30 min. The lysates were centrifuged at 13,000 rpm for 30 min at 4 °C. The protein concentrations were quantified using a bicinchoninic acid (BCA) protein assay kit (catalog #P0010S; Beyotime, Shanghai, China). Subsequently, the protein lysates were denatured by boiling in sodium dodecyl sulfate (SDS) loading buffer at 100 °C for 10 min. The protein samples were separated by 10% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF; catalog #IPVH00010; Millipore, Bedford, MA, USA) membranes. The membranes were incubated with primary antibodies (BMP4 1:1000; ALK3 1:500; Smad1 1:500; Col4 1:1000; COL1 1:100; MMP9 1:1000) overnight at 4 °C and then incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. The band density was quantified using an image system (Bio-Rad GelDoc XR, Hercules, CA, USA) and corrected by the value for GAPDH (with a dilution factor of 1:1000; Boster Bio Tech).

2.12. Statistical analysis

All statistical analysis was performed using SPSS 17.0. The quantitative data is presented as a mean with a standard deviation (SD). The independent *t*-test and multiple analyses of variance (ANOVA) tests were used for comparing distributions between two groups and among multiple samples. A p-value < 0.05 was considered statistically significant.

3. Results

3.1. General condition of the animals

The appearance of rats in the control group presented as normal. At 8 weeks after establishment of the model, rats in the DN group showed typical diabetic symptoms, such as dull fur and reduced activity. The diabetic rats became thinner and developed an abdominal bulge and tail ulcers at 12 weeks, and these symptoms became worse at 16 weeks.

The body weight of normal rats gradually increased over time. In contrast, rats in the DN group exhibited a substantial weight loss with the progression of the disease, leading to a significantly lower body weight for DN rats than for their control counterparts ($p < 0.05$ at 8, 12, and 16 weeks; Fig. 1A).

As expected, the DN rats' blood glucose levels were significantly higher than the control rats' ($p < 0.05$ at all three time points; Fig. 1B). Similarly, the DN rats exhibited significantly higher levels of BUN, SCr, and cystatin C ($p < 0.05$ at 12 and 16 weeks; Fig. 1C–E) than those of the controls. Also, the DN rats had a remarkably higher urinary protein excretion, almost five times greater than that in the control group at 16 weeks (Fig. 1F). These results indicated a successful DN rat model.

3.2. Histological findings

For the control group, gross observations of the kidneys showed no apparent abnormalities in kidney size and morphology. At the end of 8 weeks, the kidneys of rats in the DN group were larger than those of the control group. At 12 weeks, there was no obvious size difference between the kidneys of the control and DN rats. However, the kidneys of the DN rats appeared slightly pale with an uneven outline. When observed at 16 weeks, the kidneys of DN rats were smaller than those of the control group.

Histological analysis by H&E staining was performed at 8, 12, and 16 weeks. The glomeruli of the control rats appeared normal in size and morphology, without any thickened GBM or mesangial matrix hyperplasia (Fig. 2A). At the end of 8 weeks, the glomeruli of the DN rats mildly increased in volume, associated with a slight thickening of the GBM and widening of the mesangial region. With the progression of the disease, the GBM extensively thickened along with diffuse hyperplasia of the mesangial matrix, and segmental sclerosis was visible at 16 weeks.

PASM staining was used to examine the mesangial matrix. No apparent abnormalities in the glomeruli were observed in the control group. A small amount of black collagen deposition was observed in the mesangial area; however, there was no thickening of the GBM (Fig. 2B). After 8 weeks, the DN group developed a greater amount of collagen deposition in the mesangium compared with the control group ($p < 0.05$; Fig. 2C). As the disease progressed, the DN rats' mesangial matrix clearly expanded ($p < 0.05$ vs. control at 12 and 16 weeks; Fig. 2C), presenting as a diffuse or nodular distribution with a distinct thickening of the GBM.

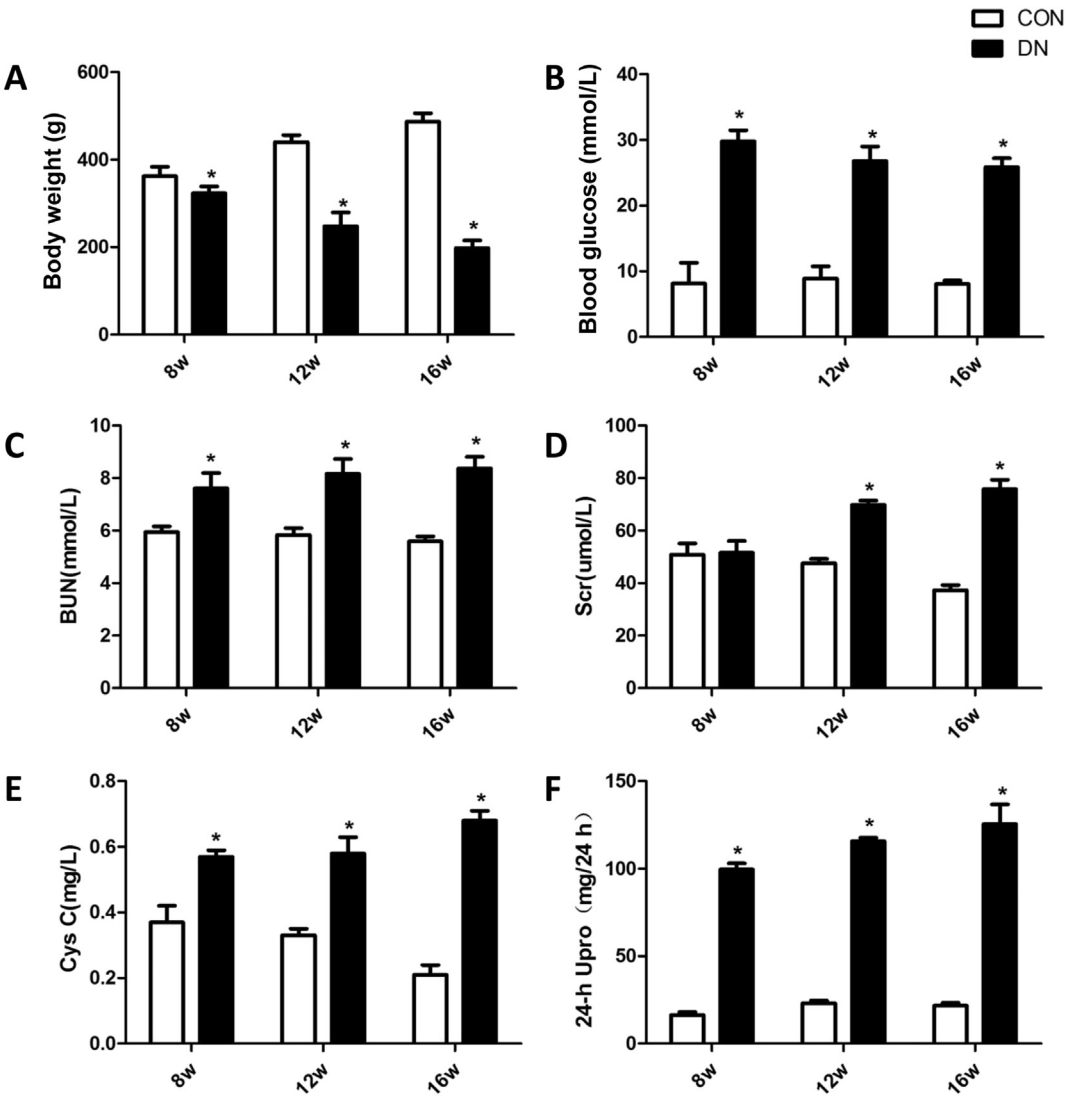

Fig. 1. Comparison of body weight and biochemical indicators between the control (CON) and DN groups. (A) Body weight; (B) blood glucose; (C) BUN; (D) SCr; (E) cystatin C; and (F) 24-hour urine protein excretion. The rats were evaluated at the end of 8, 12, and 16 weeks ($n = 7$ in the DN group and $n = 6$ in the CON group at each time point). *$p < 0.05$ vs. CON group, which was compared by independent *t*-test.

3.3. Immunohistochemical analysis of BMP4/Smad1 signaling proteins and mesangial ECM proteins in kidney tissue

The immunohistochemical analysis of the glomeruli showed that the BMP4 and ALK3 proteins were mainly expressed in the cytoplasm, while Smad1 was distributed in the cytoplasm and nuclei. The immunoreactivity of BMP4, ALK3, and Smad1 were significantly higher in DN rats at 8 weeks than in the control rats ($p < 0.05$; Fig. 3). And the areas of positive staining consistently increased with the progression of the disease, which directly correlated with the severity of the glomerular lesions. Quantitative analysis of the immunoreactivity of BMP4, ALK3, and Smad1 was performed by calculating the percent area of positive glomerular staining. This analysis showed stronger immunoreactivity of BMP4, ALK3, and Smad1 in DN rats than in the control rats at all time points (8, 12, and 16 weeks).

The expression of mesangial ECM proteins, including Col1, Col4, and MMP9, was measured. Col1 and Col4 expression levels were significantly higher in DN rats than in those of the controls and steadily increased (Fig. 4). MMP9 expression level was lower in DN rats and continuously decreased throughout the experimental period. These results suggest that the expression of mesangial ECM proteins changes during the progression of DN.

3.4. In vitro detection of mesangial cell proliferation

The proliferation of mesangial cells in the NG, MG, and HG groups was detected by a CCK-8 assay after 48 h in culture. The results showed that the HG group had higher cell proliferation compared with the NG and MG groups, shown by a higher optical density (OD) value (Table S1), but there was no significant statistical significance ($p > 0.05$).

3.5. Activation of the BMP4/Smad1 signaling pathway in rat mesangial cells with a high glucose concentration

Using immunofluorescence analysis, it was observed that mesangial cells in the NG and MG groups expressed weak staining of BMP4, Smad1, and Col4, and there was no significant change over time. In contrast, the HG group exhibited strong staining of BMP4, Smad1, and Col4 after exposure to high glucose (Figs. 5 and S1), which became most pronounced at 48 h. This indicates that a high glucose concentration could activate the BMP4/Smad1 signaling pathway and stimulate Col4 expression in rat mesangial cells (Fig. 5). Accordingly, we selected 48 h as the optimal time length for subsequent experiments.

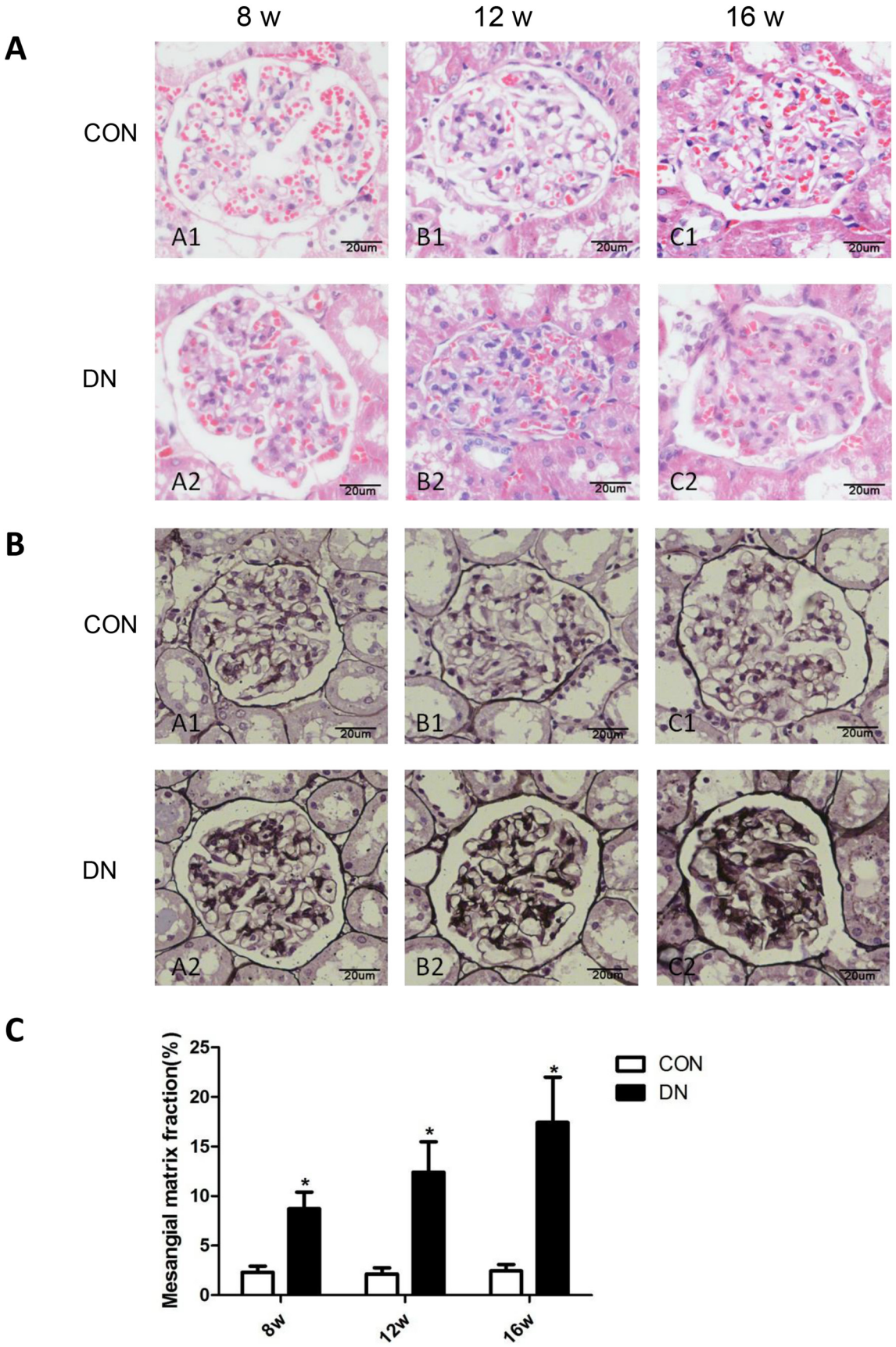

Fig. 2. Histological findings. (A) Histological analysis by H&E staining and (B) PASM staining were performed at 8, 12, and 16 weeks. Rats in the DN group developed a thickened glomerular basement membrane and mesangial matrix expansion, which became worse as DN progressed. (C) The mesangial matrix fraction was significantly higher in the DN group than in the control (CON) group ($p < 0.05$ at all-time points). *$p < 0.05$ vs. CON group, which was compared by an independent *t*-test.

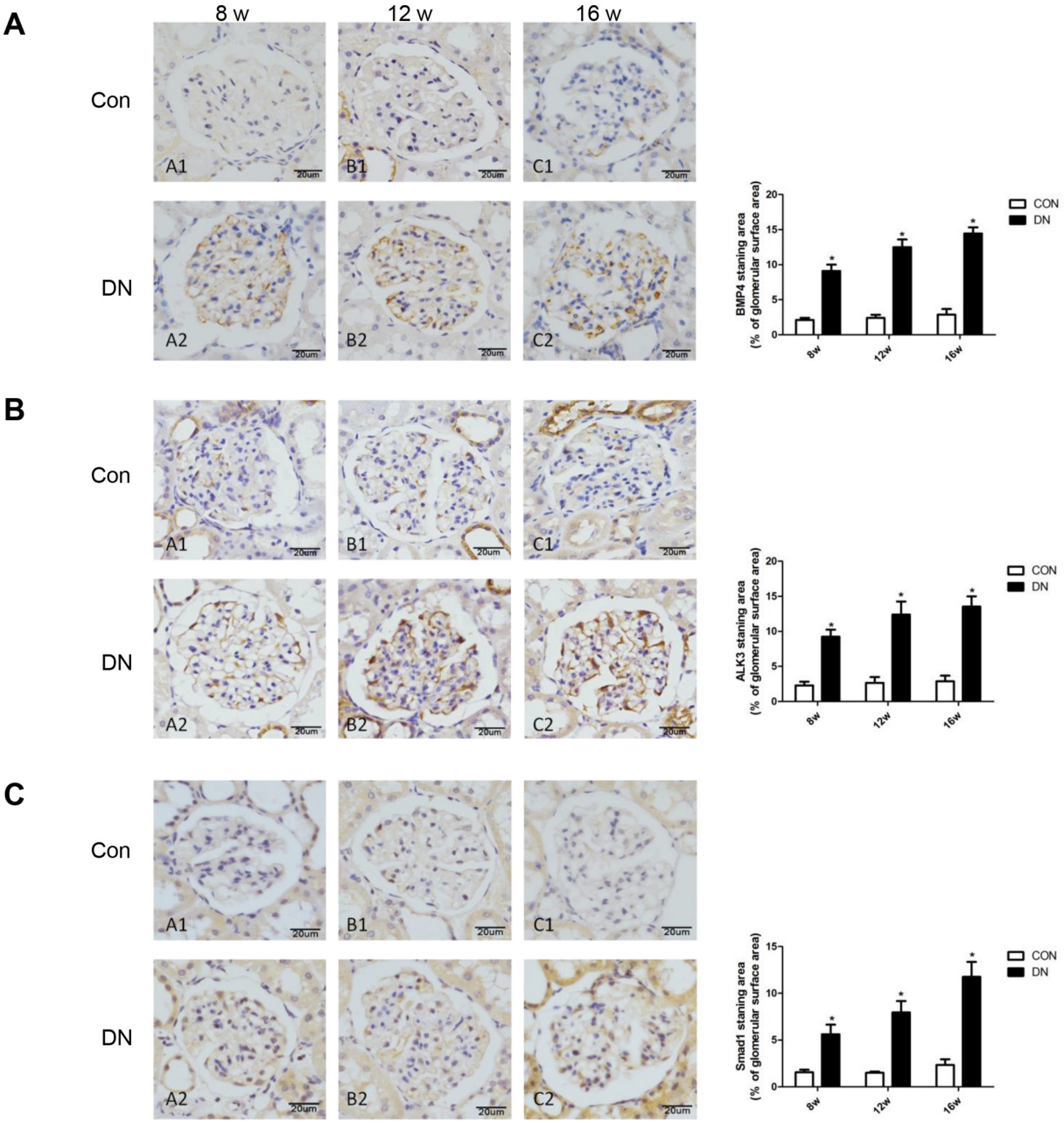

Fig. 3. Immunohistochemical analysis of the BMP4/Smad1 signaling proteins in kidney tissue. (A) BMP4; (B) ALK3; and (C) Smad1. The right panel shows the quantitative analysis of immunoreactivity for BMP4, ALK3, and Smad1 by calculating the percent area of positive glomerular staining ($n = 7$ rats in the DN group and $n = 6$ rats in the control [CON] group at each time point). The expression levels of BMP4, ALK3, and Smad1 steadily increased throughout the experimental period. The data indicates stronger immunoreactivity of BMP4, ALK3, and Smad1 in DN rats than in the CONs at 8, 12, and 16 weeks. *$p < 0.05$ vs. CON group, which was compared by independent *t*-test.

3.6. Silencing of bmp4 gene restored the expression of BMP4/Smad1 and mesangial ECM proteins exposed to a high glucose concentration

Cells exposed to a high glucose concentration were pretreated with siRNA-BMP4 (BMP4-si group), scramble siRNA (scramble-si group), or no transfection (HG group). Cells exposed to normal glucose (NG group) served as the control group. After 48 h of incubation, a Western blotting analysis showed that the expression of BMP4, ALK3, Smad1, Col4, and Col1 was significantly higher in the HG group ($p < 0.05$) and the expression of MMP9 was lower ($p < 0.05$) than in the NG group (Fig. 6). However, BMP4, ALK3, Smad1, Col4, and Col1 expression levels were significantly reduced in the BMP4-si group ($p < 0.05$), while MMP9 expression was higher than in the HG group ($p < 0.05$). Even when the cells were exposed to abnormally high glucose concentrations, these results suggest that silencing the *bmp4* gene may return the expression levels of the BMP4/Smad1 signaling pathway and mesangial ECM proteins to levels closer to those associated with a normal glucose concentration.

3.7. Blocking BMP4 protein signaling restored the expression of BMP4/Smad1 and mesangial ECM proteins exposed to high glucose

Different cell samples were exposed to normal glucose or high glucose for 48 h. Cells exposed to high glucose were pretreated with either the BMP4 protein antagonist *Noggin* or BSA. Pretreatment with *Noggin* led to a reduction in the expression of BMP4 downstream

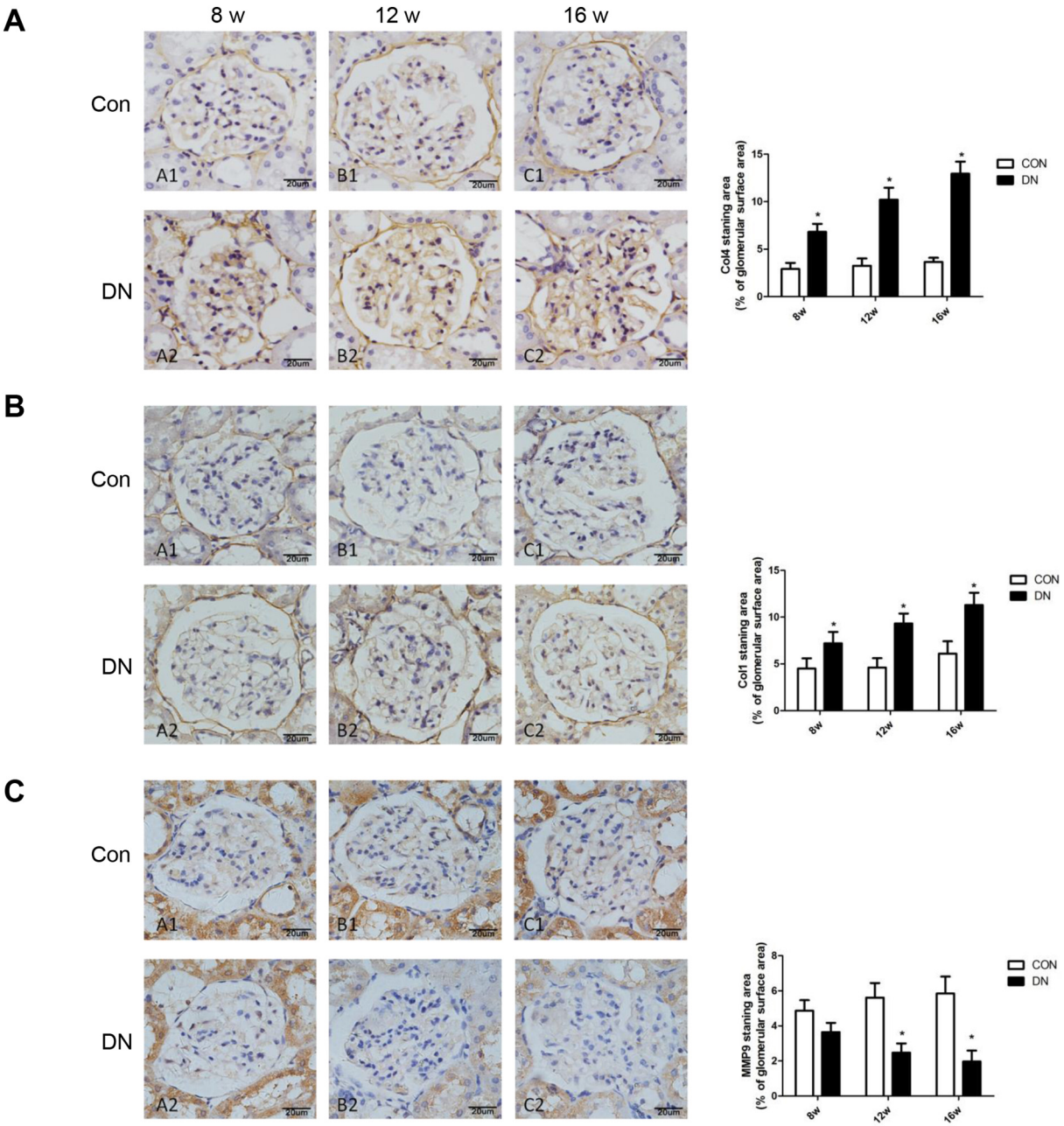

Fig. 4. Immunohistochemical analysis for mesangial ECM proteins in kidney tissue. (A) Col1; (B) Col4; and (C) MMP9. The right panel shows the quantitative analysis of immunoreactivity for Col1, Col4, and MMP9 by calculating the percent area of positive glomerular staining ($n = 7$ rats in the DN group and $n = 6$ rats in the control [CON] group at each time point). The expression levels of Col1 and Col4 steadily increased, while the MMP9 expression level continuously decreased throughout the experimental period. The data indicate stronger immunoreactivity of Col1 and Col4 but weakened immunoreactivity of MMP9 in DN rats than in the CONs at 12 and 16 weeks. *$p < 0.05$ vs. CON group, which was compared by an independent *t*-test.

proteins (ALK3 and Smad1) as well as mesangial ECM proteins (Col4 and Col1) along with an increase in MMP9 expression in high glucose-stimulated mesangial cells (Fig. 7). These results further suggest that inhibition of BMP4 signaling could reduce the expression of key molecules that stimulate mesangial cell proliferation.

4. Discussion

In this study, we observed increased expression of BMP4, ALK3, Smad1, Col4, and Col1 as well as decreased expression of MMP9 in the glomeruli of STZ-induced diabetic rats, accompanied by glomerular mesangial matrix expansion and thickening of the GBM. In vitro experiments showed that a high glucose concentration activates the BMP4/Smad1 signaling pathway and induces expression of Col4. Importantly, blocking BMP4, both at the gene and protein levels, inhibits the expression of Col1 and Col4, two key molecules that stimulate mesangial cell proliferation. These findings suggest new therapeutic possibilities for DN by specifically inhibiting the BMP4/Smad1 signaling pathway.

Although the underlying molecular mechanisms remain unclear, the activation of the BMP4/Smad1 signaling pathway may be a pathogenic factor in DN [20,21]. Among BMPs, BMP4 may act as an upstream regulator involved in ECM accumulation associated with DN. Tominaga et al. found that BMP4 phosphorylated downstream Smad1 in cultured mesangial cells and induced the expression of Col4 [9]. In particular, several traits are associated with inducible transgenic *bmp4*

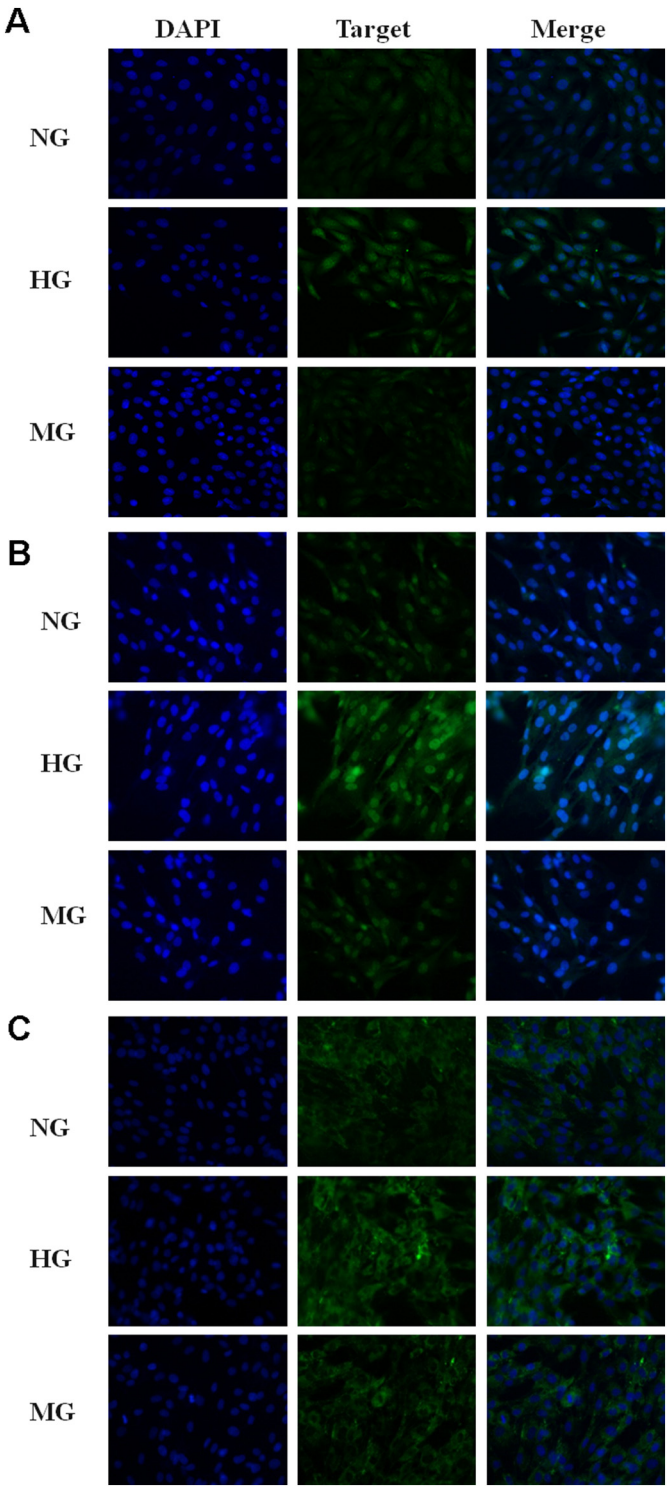

Fig. 5. Immunofluorescence analysis of BMP4/Smad1 and Col4 proteins in rat mesangial cells under high glucose at 48 h. After a 24-hour incubation in serum-free media, the mesangial cells were exposed to normal glucose (5.6 mM; NG group), an osmotic control with D-mannitol (MG group), or a high glucose concentration (HG group) for 48 h. Mesangial cells in the HG group exhibited significantly stronger staining of (A) BMP4, (B) Smad1, and (C) Col4, compared with the NG and MG groups.

overexpression and these are glomerulosclerosis, Smad1 activation, and an elevated expression of Col1 and Col4. Furthermore, mice with inducible transgenic overexpression of *bmp4* manifested typical DN features, such as proteinuria and podocyte effacement. This suggests that BMP4 is involved in the development of DN [9]. In another study, DN symptoms, such as podocyte injury and mesangial matrix expansion, were observed in tamoxifen-induced *bmp4*-overexpressing mice. In contrast, *bmp4* knockout mice exhibited improvement in injury of podocytes and proteinuria, suggesting that BMP4 also regulates podocyte loss in an early stage of DN [21]. In this study, DN rats were induced by high-fat feeding followed by intraperitoneal injection of STZ. The levels of blood glucose, BUN, SCr, and 24-hour urinary protein were significantly higher than those of the control group and steadily increased as the disease progressed. Consistent with previous studies, we found that, compared with the normal rats, the expression levels of BMP4 and its receptor ALK3 as well as downstream Smad1, Col1, and Col4 were significantly higher in the glomeruli of DN rats than in normal rats, which correlates with the severity of glomerular lesions. High expression of these proteins also positively correlates with mesangial expansion and GBM thickening. In addition, upon the stimulation of high glucose, cultured mesangial cells exhibited significant upregulation of BMP4, Smad1, and Col4. These results suggest that the BMP4/Smad1 signaling pathway is activated during the process of mesangial expansion in DN. The mechanism for how BMP4 regulates its downstream molecules during the process of DN progression needs additional investigation.

MMP9 plays an important role in maintaining the balance between synthesis and degradation of ECM proteins. But whether MMP9 promotes or inhibits the development of DN is still controversial. There is evidence that high glucose decreases expression of MMP9 in rat mesangial cells as well as increases expression of the tissue inhibitor of metalloproteinase-1 (TIMP-1), an endogenous, specific inhibitor of MMP9 [22]. Down-regulation of MMP9 contributes to mesangial matrix accumulation in the kidneys of diabetic rats [23]. In other studies, DN was associated with enhanced MMP9 protein production and activity in the glomeruli, and, using genetic or pharmacological interventions, reducing renal MMP9 expression attenuates DN [24–26]. In this study, immunohistochemical staining of the kidneys showed that the MMP9 protein expression level in DN rats was not significantly different from the controls at 8 weeks, but significantly lower than the controls at 12 weeks. Similarly, in vitro experiments showed a significantly decreased MMP9 expression after exposure to a high glucose concentration for 48 h. These changes in MMP9 expression may be associated with regulation through the MMPs/TIMPs system [27,28]. In an experimental study using immunohistochemical staining, the ratio of MMP9/TIMP-1 was reduced in the kidneys of DN rats, while upregulation of the MMP9/TIMP-1 ratio facilitated mesangial ECM degradation [28]. Another possible reason for the decrease of MMP9 expression in DN is that STZ treatment might increase cortical TGF-β expression and reduce renal MMP9 protein expression [29]. Contrary to our findings, some studies found increased levels of serum, urinary, or renal MMP9 expression at early stages of DN [30,31]. This is probably due to an excessive response to inflammatory cytokines. We speculate, that in the early stages of DN, mesangial hyperglycemia induces an increase in MMP9 expression which promotes the degradation of ECM protein. MMP9 then gradually decreases with the progression of diabetic damage in the kidneys, leading to mesangial matrix accumulation and thickening of the GBM.

It is still unknown whether the BMP4/Smad1 signaling pathway is a specific regulatory molecular mechanism directly involved in DN. In a study, the heterozygous *bmp4* knock-out mice exhibited decreased renal Col4 expression and an attenuation of mesangial matrix expansion compared with wild type diabetic mice, indicating that BMP4 may act as a critical upstream determinant for the process of ECM accumulation and the development of DN [9]. In another study, BMP4 and its receptors (BMPRII and Alk3 but not Alk6) were increased in the glomeruli of diabetic mice. Blocking BMP4 with neutralizing antibody decreased the phosphorylation of Smad1 and ameliorated the glomerular injuries, especially mesangial matrix expansion, in both Smad1 transgenic diabetic mice and wild type diabetic mice. In addition, Smad1-

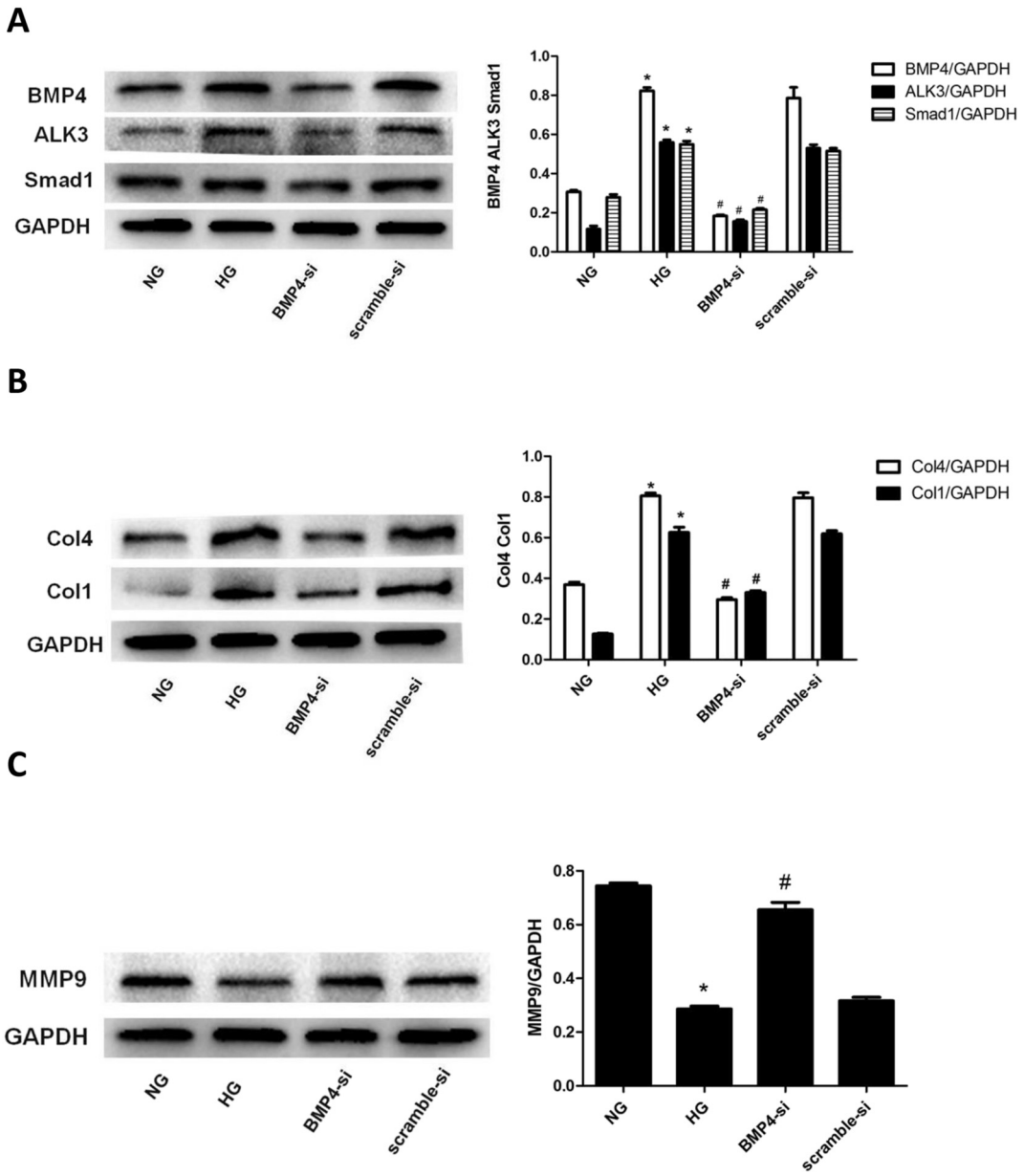

Fig. 6. The expression of BMP4/Smad1 and mesangial ECM proteins after the silencing of the *bmp4* gene. The cells were incubated for 48 h with normal glucose (NG group), high glucose (HG group), pretreatment with siRNA-BMP4 transfection + high glucose (BMP4-si group), and pretreatment with scramble siRNA transfection + high glucose (scramble-si group). A Western blotting analysis was used for determining the expression levels of (A) BMP4, ALK3, and Smad1; (B) Col4 and Col1; and (C) MMP9, and their corresponding quantitative results are showed in the right column. $^{*}p < 0.05$ vs. NG group; $^{\#}p < 0.05$ vs. HG group; the difference was compared by multiple comparison in ANOVA.

overexpressing diabetic mice displayed increased glomerular expression of Col1, Col4, and α-SMA, while heterozygous *smad1* knockout diabetic mice displayed attenuated mesangial expansion and sclerosis [10]. These results suggest a specific regulatory role of BMP4/Smad1 in the pathogenesis of DN. In this study, a high glucose concentration induced the expression of BMP4/Smad1 signaling molecules and mesangial ECM proteins (Col1 and Col4) and reduced the expression of MMP9. While a high glucose concentration induced abnormally high expression of BMP4/Smad1 signaling molecules and Col1 and Col4, inhibiting *bmp4* in mesangial cells significantly reduced the expression of these proteins. In accordance with these findings, blocking the BMP4 protein signal with its specific antagonist *Noggin* also prevented abnormally high levels of Col1 and Col4. These results demonstrate the role of BMP4 in mesangial cell proliferation, based on the observation that blocking BMP4, both at the gene and protein levels, could inhibit the expression of key molecules involving mesangial cell proliferation.

There are a few shortcomings in this study. We did not determine the changes in the expression of phosphorylated Smad1 as well as TIMP-1 level in the glomeruli of DN rats, compared with the glomeruli of control rats. Since both BMP4 and Smad1 expression behaved in a similar way, the present study could not determine whether diabetes-induced and high glucose-induced changes in the expression level of ECM proteins were mediated via Smad1-dependent or Smad1-independent mechanisms. Also, we did not examine to what extent glomerular lesions would be rescued by blocking the BMP4/Smad1 signaling pathway. These unsolved questions warrant further investigation.

In conclusion, this study provides evidence that the BMP4/Smad1 signaling pathway is crucial to the progression of mesangial expansion, and inhibiting this signaling pathway may represent a novel therapeutic

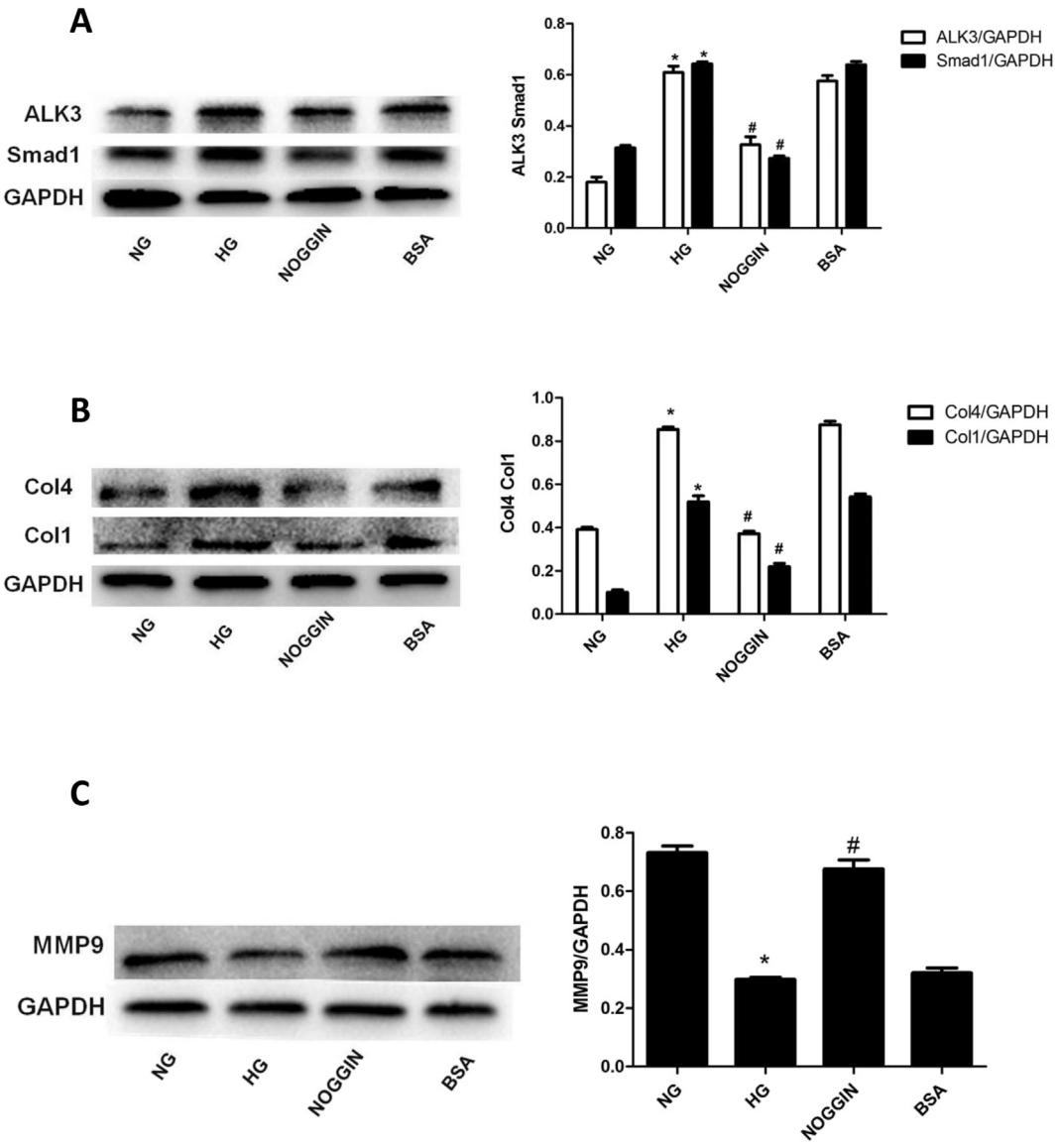

Fig. 7. The expression of BMP4/Smad1 and mesangial ECM proteins after blocking BMP4 protein signaling. The cells were cultured for 48 h with normal glucose (NG group), high glucose (HG group), pretreatment with *Noggin* (a BMP4 protein antagonist) + high glucose (*Noggin* group), and pretreatment with bovine serum albumin (BSA) + high glucose (BSA group). A Western blotting analysis was used for determining the expression levels of (A) ALK3 and Smad1; (B) Col4 and Col1; and (C) MMP9, and their corresponding quantitative results are showed as right column. $^{*}p < 0.05$ vs. NG group; $^{\#}p < 0.05$ vs. HG group; the difference was compared by multiple comparison in ANOVA.

strategy for treating DN.

Acknowledgments

The study is supported by the grant from the Education Department of Sichuan Province (No.18ZB0631).

Author contributions

Methodology, CC; Software, JRL; Validation, LL and TTZ; Formal Analysis, LCG; Investigation, WHW; Resources, CC; Data Curation, QL; Writing – Original Draft Preparation, CC; Writing – Review & Editing, STO; Visualization, CC and JRL; Supervision, STO; Project Administration, STO; Funding Acquisition, STO.

Conflict of interest

The authors declare that they have no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.01.049.

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