

Suppression of Gremlin by RNA interference attenuates glucose-induced fibrogenesis in rat mesangial cells

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Abstract

Background: diabetic nephropathy (DN) is the most common cause of end-stage of renal disease. It is beneficial for us to find effective way to treat the disease. Gremlin is deemed as a key factor in the development of diabetic nephropathy at present. We hypothesized that the pathological changes might be prevented by eliminating Gremlin function in high glucose-induced renal fibrosis.

Methods: lentiviral vector targeting Gremlin was applied to inhibit Gremlin expression at a high glucose concentration which simulated diabetic nephropathy in rat mesangial cells.

Results: the shRNA vector, designated shGremlin, significantly inhibited Gremlin expression in rat mesangial cells cultured under high glucose conditions. Increase in BMP-7 as well as its downstream genes or proteins including phospholated Smad 1/5/8, type IV collagen and fibronectin was observed.

Conclusions: our work provides a valuable method to prevent glomerular/renal fibrosis with RNA interference, and also enable development of new therapies that target Gremlin.

1. Introduction

Diabetic nephropathy (DN) is a serious complication of diabetes and it is also the leading cause of end-stage renal disease [1]. At present, the DN treatments are mainly focus on the control of blood level and blood pressure. It is beneficial for us to find extra strategies to inhibit the development of DN.

At present, gremlin which is one of the bone morphogenetic protein (BMP) antagonists, has deemed as an outstanding regulator of DN [2, 3]. Recently, it has been implicated to play great roles in processes such as glomerulosclerosis, tubulointerstitial fibrosis. Several recent reports have proved that gremlin can also modulate BMP activity to affect the kidney morphology during embryonic development, and it will disappear after kidney maturity [4], however, gremlin expression can be induced again by high glucose stimulation. BMP-7 which is considered as a protective factor in DN can increase collagen IV and fibronectin expression. A number of studies show that BMP-7 might play protective roles in cultured renal cells and BMP-7 binding to its receptor can affect Smad1/5/8 phosphorylation and play roles in disease [5]. Gremlin is reported to be one of the three BMP-7 antagonists and its increased expression is testified in kidneys of diabetic rats [6]. The high expression of Gremlin indicates that it plays great roles in the development of DN [7]. As an important target, gremlin plays an important role in the development of diabetic nephropathy by modulating BMP and Smad1/5/8.

Therefore, we hypothesized that the DN pathological changes could be prevented by eliminating Gremlin function in high glucose-induced renal fibrosis. We aim to construct a lentiviral vector targeting Gremlin and examined the effect of Gremlin inhibition in rat mesangial cells (RMCs).

2. Materials And Methods

2.1 Construction of lentiviral vectors targeting Gremlin

We obtained the RNA silencing sequences targeting Gremlin from the paper listed in reference 8 [8], which was proved to be effective. The sequences of shRNAs against rat Gremlin mRNA (Genbank, accession Gremlin site NM_019282.2) are as follows:

Sense strand: 5'-CAC CGC ACT ATC ATC AAT CGC TTC TCG AAA GAA GCG ATT GAT GAT AGT GC-3';

Antisense strand: 5'-AAA AGC ACT ATC ATC AAT CGC TTC TTT CGA GAA GCG ATT GAT GAT AGT GC-3'.

Sequences of negative control:

Sense strand: 5'-CAC CGA CTA CCA TTA CCA TTG CTT CCG AAG AAG CAA TGG TAA TGG TAG TC-3';

Antisense strand: 5'-AAA AGA CTA CCA TTA CCA TTG CTT CTT CGG AAG CAA TGG TAA TGG TAG TC-3'.

All the sequences were synthesized and amplified by PCR. The PCR fragments were digested with EcoRI and XbaI, and subcloned into pUC-lentivirus vector, which was performed by Genechem Company of Shanghai, China.

2.2 Cell culture and infection

RMCs were purchased from the China Center for Type Culture Collection (CCTCC) and were cultured at 37 °C in an atmosphere of 5% CO₂ in Dulbecco's modified Eagle's medium [DMEM(Gibco-BRL, USA)] supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 mg/ml) for 3 days. The culture medium was refreshed in 24 h. Cells were washed twice with DMEM and transfected with lentivirus vectors carrying Gremlin shRNA or negative control shRNA. Enhancing agent polybrene (Genechem, China) was also used.

2.3 Grouping

After incubation at 37 °C for 24 hr, the cells were examined using fluorescence microscope to ensure ~ 90% transfection efficiency. The RMCs were grouped into 4 groups and cultured for 48hr: 1) 5.5 mmol/L glucose, 2) 30 mmol/L glucose, 3) 30 mmol/L glucose plus shGremlin, and 4) 30 mmol/L glucose plus negative control lentivirus (shNC). To examine the time-effect relationship between Gremlin and glucose concentration, RMCs were cultured separately in DMEM containing 5.5 mmol/L, 10 mmol/L, 20 mmol/L, 30 mmol/L glucose for 48 h. To examine the dose-response relationship, RMCs were cultured separately in DMEM containing 30 mmol/L glucose for 0 hr, 24 hr, 48 hr and 72 hr.

2.4 Quantitative real-time (qRT)-PCR

Total RNA was extracted from cultured RMCs with Trizol (TAKARA, Dalian, China) according to the manufacturer's instructions. Briefly, cells were dissolved in Trizol, mixed with chloroform and centrifuged at 12,000 rpm for 20 min. After that, in order to isolate total RNA, iso-propylalcohol was added into the supernatant. First strand cDNA was synthesized with oligo-d(T) primer and M-MLV reverse transcriptase

(TAKARA, Dalian, China), followed by qRT-PCR on a LightCycler480 sequence detection system. The primer sequences are: Gremlin forward primer, 5'-TCC TTT CAG TCT TGC TCC TTC T-3', backward primer, 5'-GCG TGT GAC TCT TTT CTT CTT G-3'; rat GAPDH forward primer, 5'- ACC ACA GTC CAT GCC ATC AC-3', backward primer, 5'- TCC ACC ACC CTG TTG CTG TA -3'. Polymerase chain reaction was carried out under the following conditions: 1 cycle at 95 °C for 10 min and 30 cycles at 94 °C (10 s), 65 °C (30 s) and 72 °C (10 s). The gremlin expression was normalized to GAPDH mRNA levels.

2.5 Western blot analysis

Cells were lysed in buffer (50 mM Tris [pH8.0], 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 100 mM PMSF, 1 M DTT, 10 mg/ml leupeptin, 10 ml/ml aprotinin; all from Sigma, St. Louis, USA) and centrifuged at 12,000 x g for 30 min after cooling on ice for 30 min. Protein concentration was measured by the Bradford protein assay (BioRad, Hercules, USA). SDS-PAGE was performed on 15% polyacrylamide gels at 100 V for 2 h. The resolved proteins were transferred onto a PVDF membrane and probed with anti-Gremlin (1:200) (Abcam, UK), anti-BMP-7 (1:1000) (Abcam, UK), anti-phospho-Smad 1/5/8(1:500) (Cell Signaling, USA), anti-total Smad 1/5/8(1:500) (Santa Cruz, USA) and anti-Actin (1:10000) (Abcam, UK). And then, they were incubated with secondary antibodies conjugated with horseradish peroxidase (1:2000) (Amersham Bioscience, UK) for 1 h at room temperature. Finally, they were detected with enhanced chemiluminescence reagents (Amersham Bioscience, UK).

2.6 ELISA

FN and Col IV expression levels in culture medium were measured by commercial ELISA kits (Uscn Life Science Inc, Wuhan, China) according to the manufacturer's instructions. The absorbance was measured at 492 nm using a microplate reader (Model 680, Bio-Rad). The results were expressed in nanograms per milliliter according to the calibration curve obtained with serial dilutions of a known quantity of sample, and these were then normalized to the β -actin content of the corresponding cells. The procedure was performed in triplicate with each sample.

2.7 Statistical analysis

Data are presented as mean \pm standard deviation (SD). Statistical analysis was performed by one-way ANOVA with F-test. P value of < 0.05 was considered significant. The data were analyzed with SPSS II for Windows (release 11.0.1J, SPSS Japan Inc.). All experiments were performed at least three times.

3. Results

3.1 Overexpression of Gremlin mRNA

Induction of Gremlin expression was achieved by exposing mesangial cells to 5.5 mM glucose, 10 mM glucose, 20 mM glucose and 30 mM glucose in DMEM media for 24 hr. Gremlin mRNA expression was determined by qRT-PCR. The expression level of Gremlin increased by about 5.40-fold in 30 mM glucose (Fig. 1).

3.2 Overexpression of Gremlin mRNA under exposure to 30mM glucose

Mesangial cells were cultured in DMEM media containing 30mM glucose for 0 hr, 24 hr, 48 hr and 72 hr. Gremlin mRNA expression was determined by qRT-PCR. The expression level of Gremlin increased by about 4.94-fold after 48 hr (Fig. 2).

3.3 Efficacy of Infection with lentiviral vector carrying green fluorescein protein-labeled shRNAs

To determine the efficacy of infection with Gremlin and negative control shRNAs (shNC), green fluorescein protein (GFP)-labeled shRNAs were transfected into RMCs. The cells were then evaluated by fluorescence microscopy. Most of the RMCs exhibited strong fluorescence (Fig. 3).

3.4 Gremlin shRNA Inhibits Gremlin Expression in RMCs

As seen in Figure 4, Gremlin mRNA expression in the high glucose-treated group was about 3.92-fold greater compared with the normal glucose control group (N). Treatment with Gremlin shRNA significantly inhibited Gremlin expression induced by HG conditions, but the negative control shRNA group was no different than N group.

3.5 Effect of Gremlin shRNA on the level of BMP-7, pSmad 1/5/8, total Smad 1/5/8 protein in high glucose (HG)-induced RMCs

We investigated the mechanism of inhibition of Gremlin expression in fibrosis, by analyzing the expression of BMP-7-Smad signal pathway in RMCs cultured under HG conditions. Incubation of cultured cells under HG conditions over 48 hours revealed a gradual increase in Gremlin expression with associated decrease in BMP-7. Similarly, the level of phosphorylated Smad1/5/8, markers of BMP-7 activity, was significantly and gradually decreased while total Smad1/5/8 protein levels remained constant. Significant changes in BMP-7 expression were seen after infection of cells with Gremlin shRNA. Gremlin shRNA prevented the decrease in phosphorylation of Smad1/5/8 (Fig.5). These results suggest that the protective effects of shRNA-induced inhibition of Gremlin expression on RMCs were, at least partially, mediated by BMP-7.

3.6 Transfection with Gremlin shRNA reduces over-expression of FN and Col IV accumulation induced by high glucose

To evaluate the impact of Gremlin inhibition on fibrosis and the underlying molecular mechanisms, cultured RMCs were again infected with Gremlin shRNA or negative control and subjected to stimulation with high glucose. FN and Col IV levels in the culture medium were determined by ELISA (Table 1). Significant accumulation of these fibrotic factors in the culture medium was seen in the HG and HG + shNC groups, while shGremlin infection significantly reduced these changes.

4. Discussion

The molecular pathogenesis of diabetic nephropathy has not been fully characterized. Novel molecular targets and mechanisms are still under investigation. Bone morphogenetic proteins active in development include homodimeric members of the TGF- β superfamily of cysteine-knot cytokines [9]. The TGF- β superfamily comprises over twenty BMPs, of which BMP-7 is the most prominent member involved in renal development and disease. In the adult life, BMP-7 is primarily expressed in kidney tubules, as well as glomeruli. Loss of endogenous BMP-7 expression occurs in diabetic rats and is associated with fibrosis [10]. In the streptozotocin-induced diabetes model, BMP-7 was reduced by 50% at 15 weeks and continued to decline further to 10% by 30 weeks [11]. In cultured tubular cells, TGF- β decreased BMP-7 expression, which suggests that a rise in tubular TGF- β levels during the evolution of diabetic nephropathy contributes to the loss of BMP7 and BMP7 type I and II receptors [12]. Morrissey and associates [13] showed that exogenously administered recombinant human (rh) BMP-7 may even resolve, at least partially, glomerular and interstitial fibrosis in experimental diabetic nephropathy. BMP-7 activity in the kidney is also determined by a balance of agonists, such as Kielin/chordin-like protein (KCP) or BMP receptors [14], and antagonists, such as Gremlin, noggin, or uterine sensitization-associated gene-1 (USAG-1) [15] that prevent binding of BMPs with their cognate receptors. Of the three BMP antagonists, only Gremlin increased in kidneys of diabetic rats. We proposed that inhibition of Gremlin may induce therapeutic effects by promoting efficient binding of endogenous BMP-7 with receptors.

Our data demonstrate that expression of Gremlin was closely related with matrix glucose level and with exposure time. Gremlin shRNA significantly suppressed FN, collagen type IV, PAI-1 and MMP-2 accumulation, indicating beneficial effects of Gremlin inhibition in DN. Based on our data, the expression level of BMP-7 was dramatically reduced by suppression of Gremlin, as well as its downstream signal proteins, phosphorylated Smad 1/5/8. However, a physical interaction between BMP-7 and Gremlin was demonstrated by immunoprecipitation, and phosphorylated Smad-5, a marker of BMP-7 activity, was up-regulated by Gremlin siRNA transfection [16]. BMPs binding with their receptors prevent Smad signaling, which is revealed by the phosphorylation of Smads. Smad 1, 5 and 8 are receptor-regulated Smads (R-Smads) activated by BMPs [17]. Smad5 was the preferred BMP-7-induced receptor-activated Smad signal in kidney. Loss of BMP-7 signaling activity, as illustrated by decreased Smad 5 protein phosphorylation, was observed in experimental DN [18]. Our results with mesangial cells cultured under high glucose conditions demonstrate a gradual increase in Gremlin protein levels from 0 h to 72 h after HG stimulation and depended on exposure time. Transfection of cells with Gremlin shRNA resulted in significantly increased levels of phosphorylated Smad 1/5/8.

Our data support that Gremlin inhibition in DN leads to the recovery of BMP-7 activity. BMP-7 is involved in ameliorating renal damage due to mesangial proliferation by suppression of mesangial cell mitosis via Smad1/5/8 signaling. BMP-7 is also able to prevent metanephric mesenchymal cells and renal epithelial cells from undergoing apoptosis and then protecting renal function [19]. Evidence suggests that early renal hypertrophy acts as a pacemaker for subsequent irreversible structural changes, such as glomerulosclerosis and tubulointerstitial fibrosis [20]. Maintenance of BMP-7 activity by inhibition of Gremlin expression may result in blockade of extracellular matrix (ECM) accumulation. BMP-7 reduced TGF- β -induced ECM protein accumulation in cultured mesangial cells by maintaining the levels and

activity of MMP-2, partially through prevention of TGF- β -dependent upregulation of PAI-1 [20, 21]. Our data showed that treatment with Gremlin shRNA resulted in a significant reduction of FN and Col IV level in mesangial cells cultured under HG conditions. Whether Gremlin manifests BMP-7-independent effects in the pathogenesis of diabetic nephropathy needs to be determined. The proliferative activity of mesangial cells is associated with the expression level of Gremlin. It was reported that Gremlin increased DNA synthesis and cell counts and accelerated cell cycle progression of vascular smooth muscle cells (VSMC) via p27(kip1) down-regulation [22]. Gremlin was also overexpressed in various human tumors and widely expressed by cancer-associated stromal cells, and promotes tumor cell proliferation [23], suggesting potential stimulation of proliferation. We supposed that Gremlin might regulate cell growth via a BMP-7-independent pathway. Overexpression of Gremlin in diabetic kidneys suggests its role for the developmental programs in DN. Other developmental genes including FMN1, a gene with a Gremlin transcriptional enhancer should be investigated as well [24]. Knockdown of Gremlin by siRNA plasmid might not affect the expression and function of FMN1. Given the role of FMN1 in gremlin transcriptional regulation, it would be interesting and meaningful to investigate the role of FMN1 in DN.

In summary, in addition to expanding our knowledge of the of diabetic nephropathy pathophysiology, our data should enable development of new therapies that target Gremlin.

List Of Abbreviations

diabetic nephropathy (DN); bone morphogenetic protein (BMP); rat mesangial cells (RMCs); Kielin/chordin-like protein (KCP); uterine sensitization-associated gene-1 (USAG-1).

Declarations

Acknowledgements

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Authors'contributions

Xiujuan zhang is mainly responsible for the writing and submission work. Li Zhang, Xinzhe Wang, Yuwen Song and Kai Lou are for the exact experiment procedure. Yong He and Wenxia Han are for the main experimental design. All of the authors have made substantive contributions to the experimental design and preparation.

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Availability of data and materials

All data generated or analysed during this study are included in this published article or are available from the corresponding author on reasonable request.

Ethics approval

The animal experimental protocol was approved by the Animal Ethics Committee of Shandong Provincial Hospital affiliated with Shandong University (Jinan, China).

Consent for publication

All authors support the submission to this journal.

Competing interests

The authors declare that they have no competing interests.

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Table

Table 1

Effect of Gremlin shRNA on the level of FN, and Col IV protein in high glucose-induced RMCs $\bar{x} \pm S$

Group	FN (ng/ml)	Col IV (ng/ml)
5.5mmol/L glucose	70.33±6.59*	50.32±1.18#
30mmol/L glucose	110.64±5.60	76.33±1.25
HG+shGremlin	75.97±6.28**	48.02±2.11##
HG+shNC	114.21±4.95	79.95±1.60

*P < 0.05 vs 30mmol/L glucose group **P < 0.05 vs 30mmol/L glucose group

#P < 0.05 vs 30mmol/L glucose group ##P < 0.05 vs 30mmol/L glucose group.

Figures

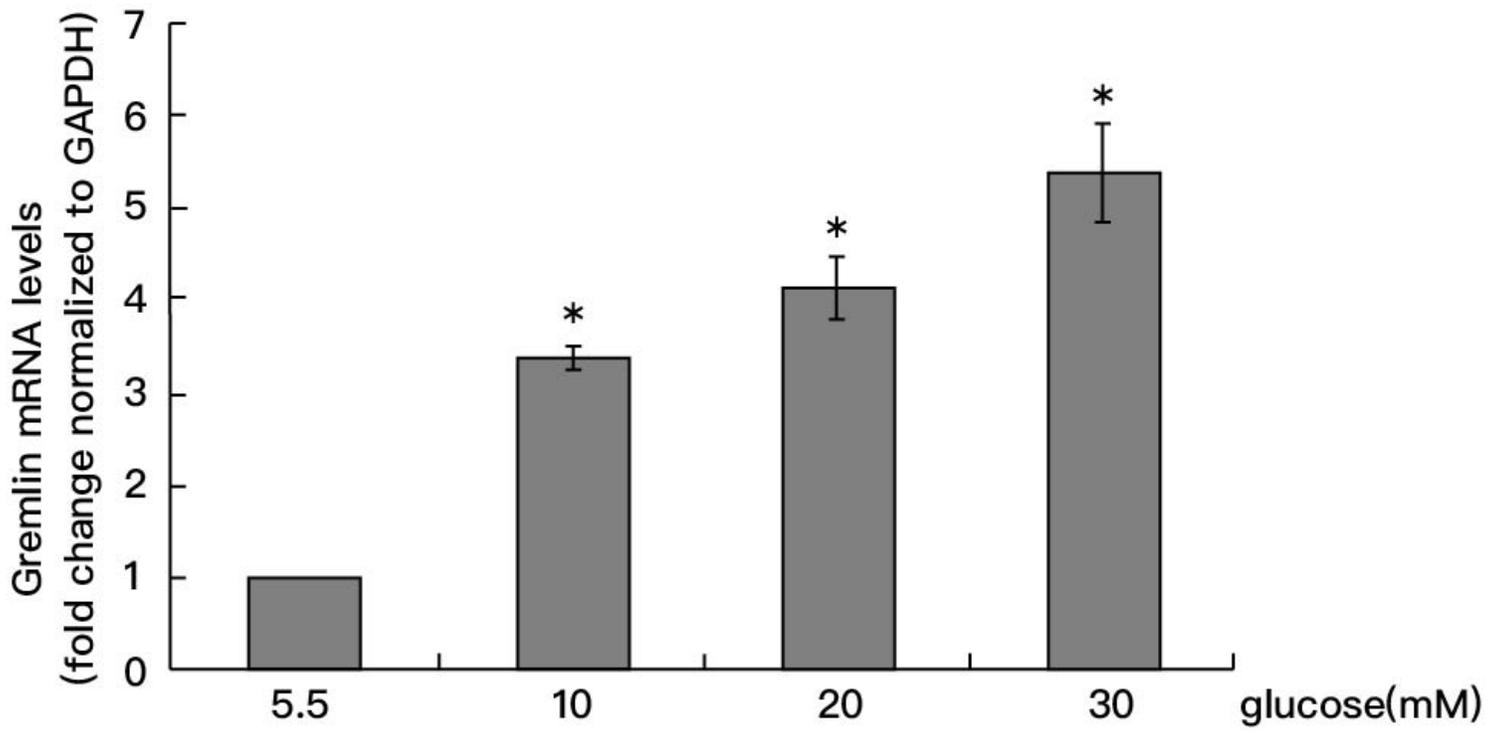


Figure 1

Gremlin stimulated by 5.5, 10, 20 and 30 mM glucose in RMCs. After incubation for 24 hr, total RNA was isolated, reverse transcribed and amplified by quantitative RT-PCR. Gremlin levels under each of the conditions were normalized to GAPDH. * P < 0.05 versus 5.5 mM glucose group.

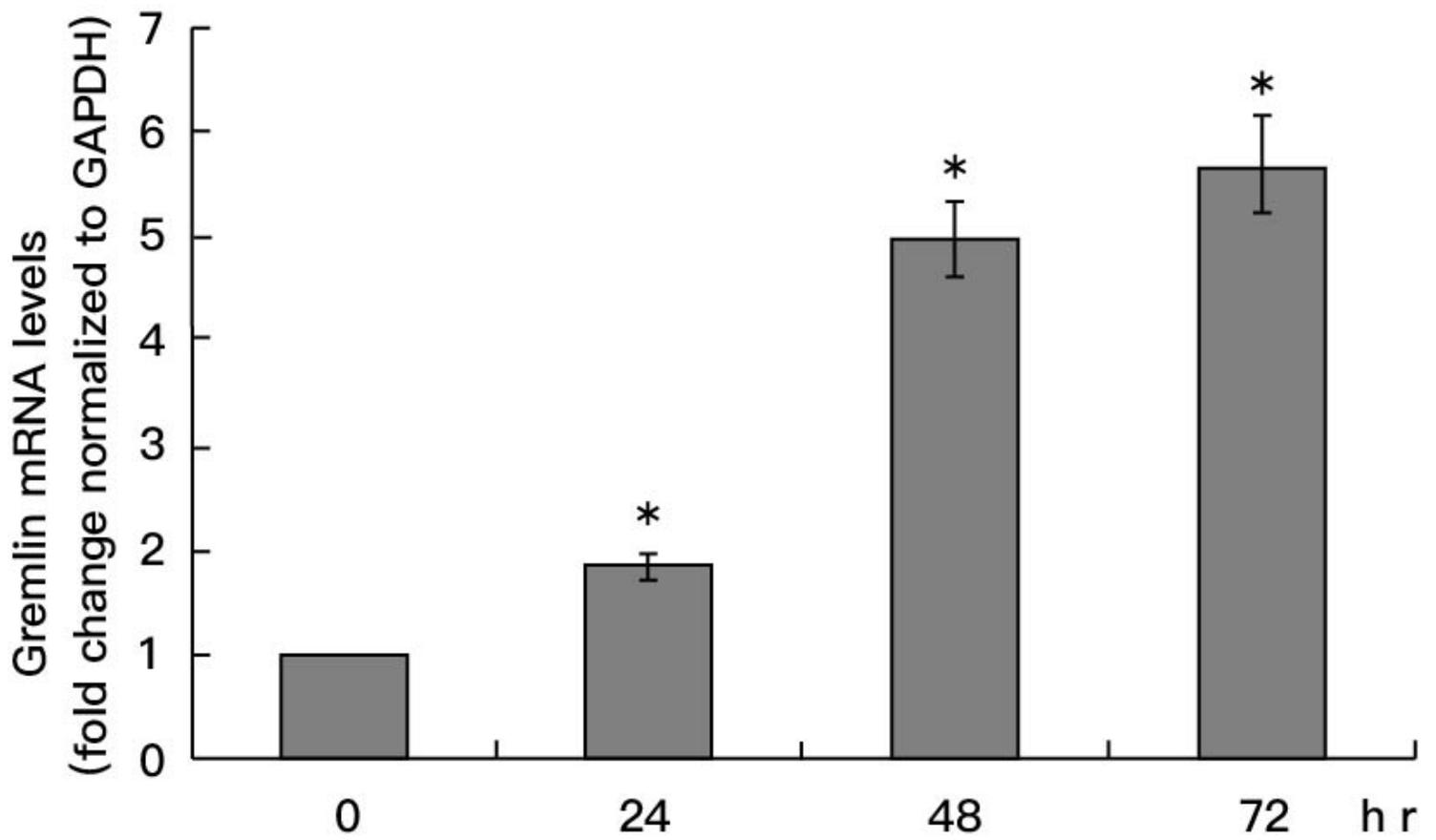


Figure 2

Gremlin stimulation with 30 mM glucose exposure After incubation of RMCs with 30 mM glucose for 0, 24, 48 and 72 hr, the total RNA was isolated, reverse transcribed and amplified by quantitative RT-PCR. Gremlin levels for each of the conditions were normalized to GAPDH. * P < 0.05 versus 0 hr group.

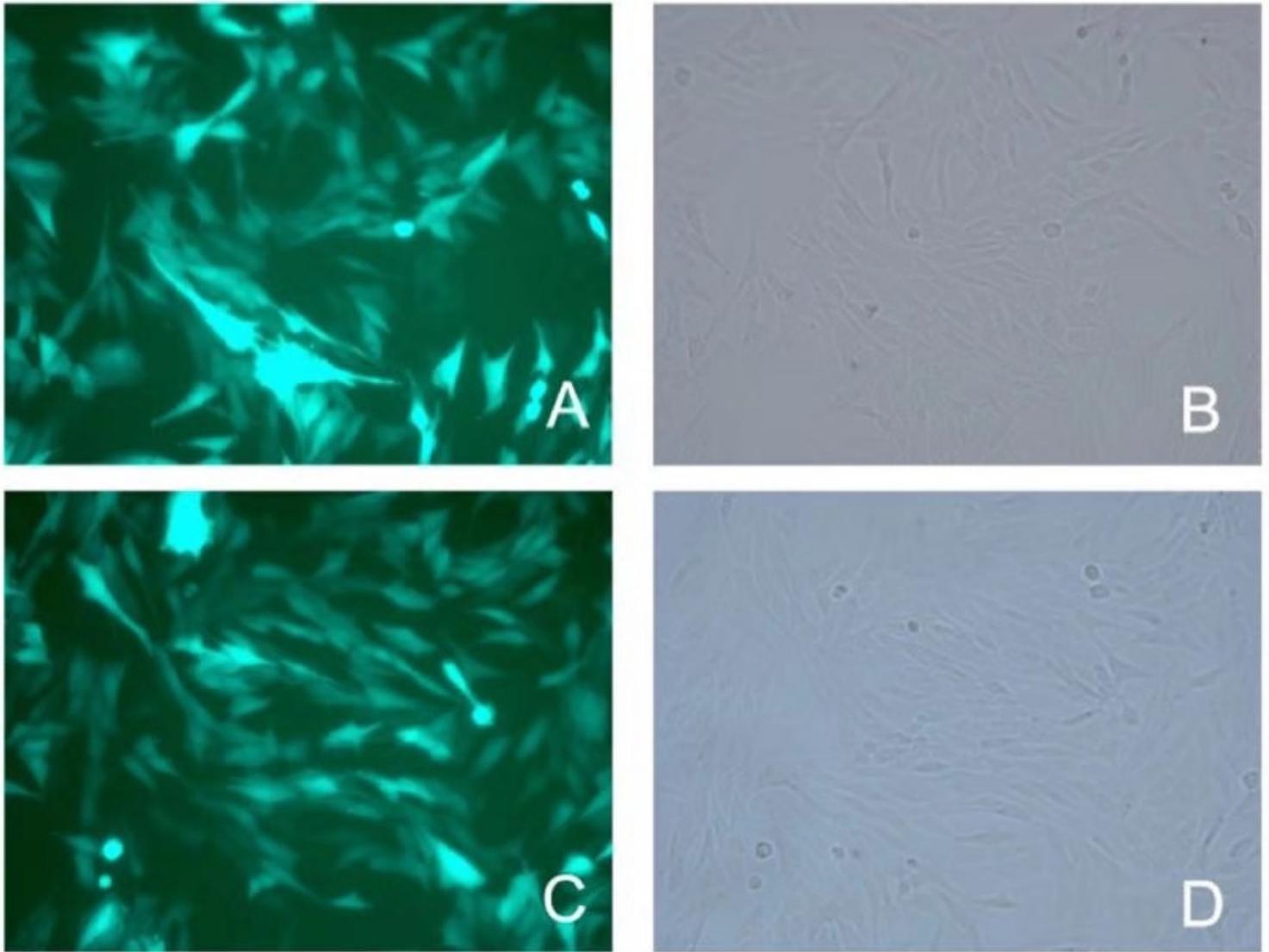


Figure 3

Fluorescence microscopy photograph of RMCs transfected with Gremlin shRNAs or negative control shRNAs labeled with green fluorescent protein (GFP) ($\times 200$). A. Expression of GFP after transfection of shGremlin for 48 hr in RMCs; B. bright field; C. expression of GFP after transfection of shNC for 48 hr in RMCs ; D. bright field.

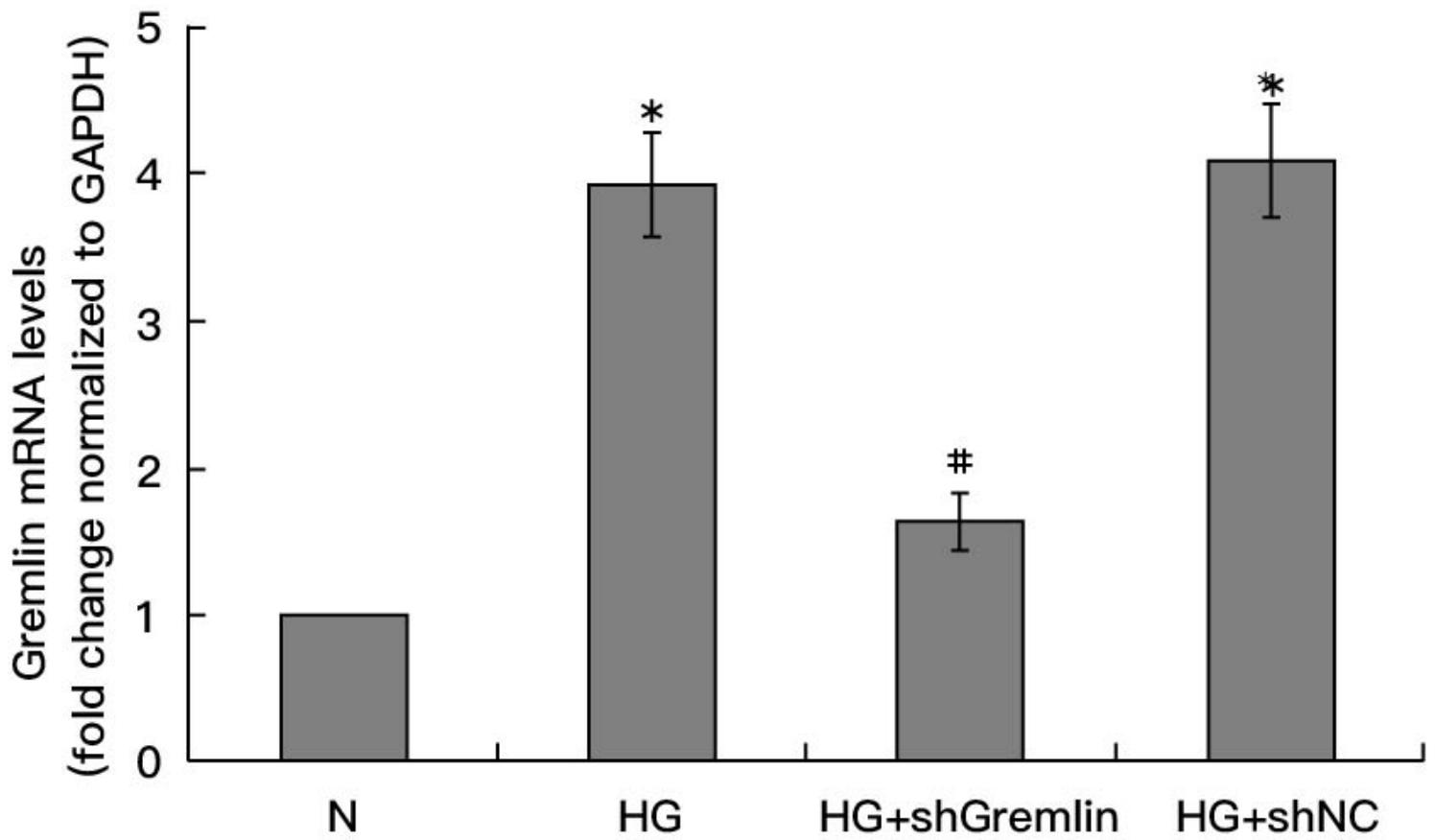


Figure 4

Effect of shGremlin on high glucose-induced Gremlin mRNA upregulation in RMCs (qRT-PCR). N: control (5.5 mM); HG: stimulated with high glucose (30 mM); HG+shGremlin: 30 mM glucose plus shGremlin; HG+shNC: 30 mM glucose plus negative control shRNA. Gremlin levels under the different conditions were normalized to GAPDH. * $P < 0.05$ versus N group, # $P < 0.05$ versus HG group.

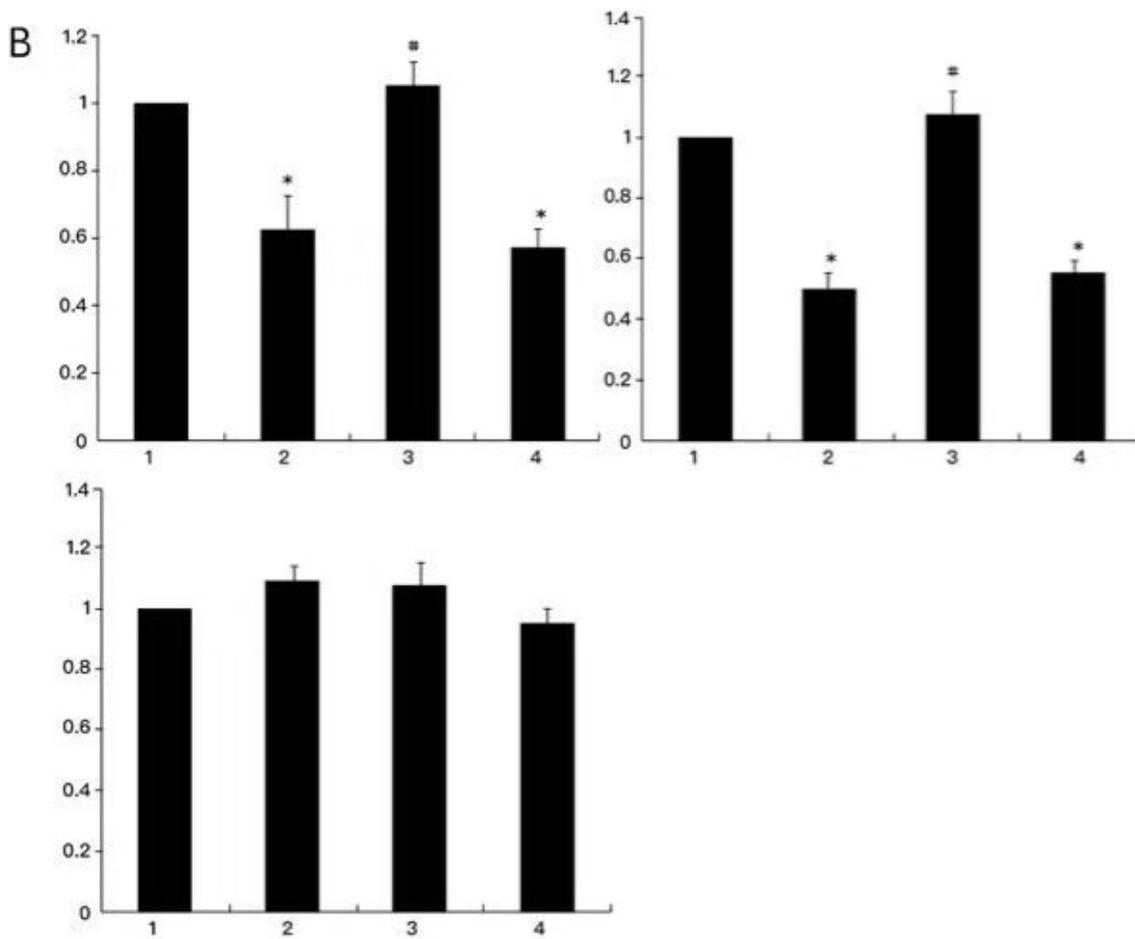
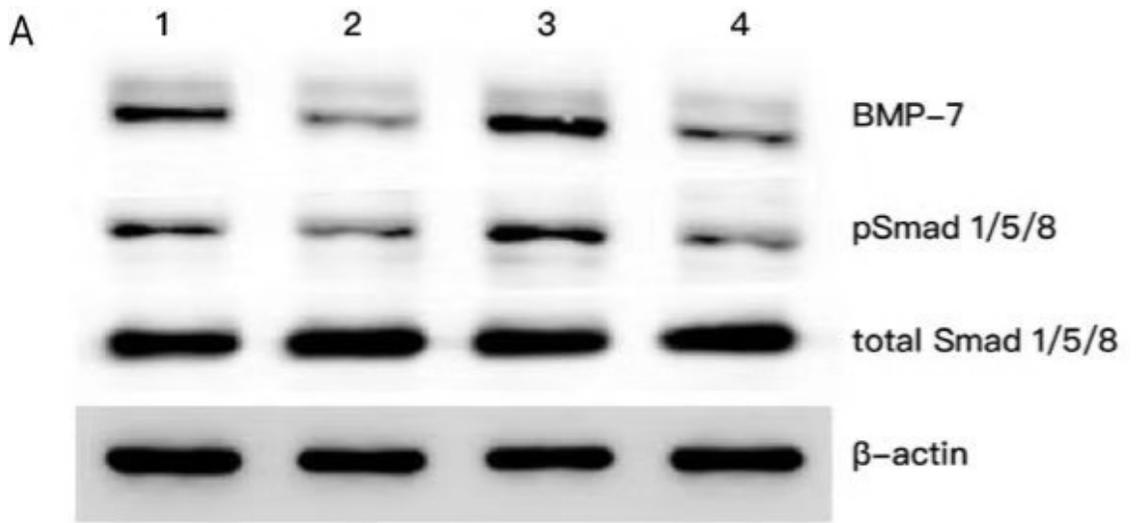


Figure 5

Effect of shGremlin on high glucose-induced BMP-7, pSmad 1/5/8 and total Smad 1/5/8 protein expression in RMCs (Western blot). (A) Lane 1: control (5.5 mM). Lane 2: stimulated with high glucose (30 mM). Lane 3: shGremlin infection plus high glucose (30 mM) stimulation for 48 hr. Lane 4: shNC infection plus high glucose (30 mM) stimulation for 48 hr. (B) Values are presented as means \pm SEM of triplicate experiments. * $P < 0.05$ compared to normal glucose; # $P < 0.05$ compared to high glucose.