PC-1 NF suppresses high glucose-stimulated inflammation and extracellular matrix accumulation in glomerular mesangial cells via the Wnt/β-catenin signaling

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Abstract. Diabetic nephropathy (DN) is the leading cause of end-stage renal disease worldwide with high morbidity and mortality. Glomerular mesangial cell (MC) proliferation, inflammatory cell infiltration and extracellular matrix (ECM) accumulation are the main pathological characteristics of DN. A previous study revealed that polycystin-1 N-terminal fragment (PC-1 NF) fusion protein could inhibit ECM accumulation in a mesangial proliferative glomerulonephritis model. However, the role of PC-1 NF fusion protein in DN remains unknown. The results of the present study indicated that PC-1 NF fusion protein significantly abolished high glucose (HG)-induced glomerular MC viability over three time points measured (24, 48 and 72 h). In addition, PC-1 NF suppressed the levels of monocyte chemotactic peptide-1 and tumor necrosis factor α, as well as the expression of fibronectin and collagen IV, in HG-stimulated MCs. Furthermore, PC-1 NF fusion protein efficiently inhibited the activation of Wnt/β-catenin signaling pathway in HG-stimulated MCs. Taken together, these data indicated that PC-1 NF fusion protein inhibited HG-induced MC proliferation, inflammation, and ECM expression via the modulation of the Wnt signaling pathway. The present study indicated that PC-1 NF fusion protein may be a potential agent in treating DN.

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Introduction

Diabetic nephropathy (DN) is one of the most common complications of diabetes with high morbidity and mortality (1). It is the leading cause of end-stage renal disease worldwide (1). Over the past three decades, numerous studies were performed to elucidate the pathology, progression, mediators, and treatment of DN (2-4). Studies demonstrated an important role of high glucose (HG) in the pathogenesis of DN; HG leads to glomerular mesangial cell (MC) proliferation, inflammatory cytokine infiltration and extracellular matrix (ECM) accumulation (5,6). Thus, suppression of MC proliferation, inflammation and ECM accumulation may be a promising approach for treating DN.

Polycystin-1 (PC-1) is a membrane-bound protein encoded by the polycystic kidney disease gene 1. PC-1 acts as a G protein-coupled receptor; downstream it also interacts with transcriptional activator TCF/LEF and components of the canonical Wnt/β-catenin signaling pathway (7). PC-1 N-terminal fragment (PC-1 NF) fusion protein encodes the cell wall integrity and stress response component domains and part of the leucine-rich repeat of the PC-1 extracellular region (8). A previous study demonstrated that PC-1 NF fusion protein promoted ECM degradation in a mesangial proliferative glomerulonephritis rat model (7). However, little is known about the role of PC-1 NF fusion protein in treating DN.

The Wnt/β-catenin pathway plays an essential role in nephron formation and renal development (9). In the adult kidney, Wnt/β-catenin signaling pathway becomes functionally silent after differentiation. However, available evidence indicates that Wnt/β-catenin is re-activated after kidney injury, including obstructive nephropathy, focal segmental glomerulosclerosis and DN (10-12). Wnt proteins interact with their receptors and co-receptors and induce downstream signaling events, leading to the dephosphorylation and stabilization of β-catenin. Stabilized β-catenin in turn leads to matrix production and renal fibrosis. In this context, the inhibition of Wnt/β-catenin signaling can be a rational strategy for the therapeutic intervention of DN (12,13). Previous studies revealed that the blockade of the Wnt/β-catenin pathway by

a variety of approaches ameliorates proteinuria, kidney injury and renal fibrosis (14).

The present study investigated the effects of PC-1 NF fusion protein on HG-induced proliferation, inflammatory cytokine infiltration and ECM accumulation in rat glomerular MCs. It provided evidence that PC-1 NF fusion protein could be a novel therapy for DN.

Materials and methods

Cell culture and treatment. The normal rat glomerular MC line was purchased from the Shanghai Academy of Life Sciences. The cells were cultured in DMEM supplemented with 10% fetal bovine serum/F12 medium (Gibco; Thermo Fisher Scientific, Inc.) in a humidified atmosphere of 5% CO_2 at 37˚C. Rat MCs in three to eight generations were selected for the following experiments.

The cells were divided into the following groups: i) Normal glucose (NG; 5 mmol/l glucose); ii) osmotic pressure (OP; 5 mmol/l glucose + 25 mmol/l mannitol); iii) HG, 30 mmol/l glucose; iv) NG + PC-1 NF (endotoxin level <1.0 endotoxin units (EU)/μg; Zishanzhu; Iyunbio, Inc.) at 4 μg/ml, which was based on a previous study (7); v) NG + dickkopf-related protein 1 (DKK1; 400 ng/ml; endotoxin level <1.0 EU/μg; Sigma-Aldrich; Merck KGaA); vi) HG + PC-1 NF at 4 μg/ml; and vii) HG + DKK1 at 400 ng/ml. All treatments were carried out at 37˚C. The cells were incubated with PC-1 NF or DKK1 for 30 min either in NG or before being exposed to HG. Each test was independently repeated more than three times. The cells were subsequently subjected to various assays.

Cell proliferation assay. Cell proliferation was measured using the MTT assay. In brief, MCs were seeded into 96-well plates at a density of 1,000 cells per well and incubated in serum-free DMEM for 24 h. Cells were incubated with HG in the presence or absence of PC-1 NF fusion protein for 24, 48 and/or 72 h before 20 μl of MTT (5 mg/m; Sigma-Aldrich; Merck KGaA) was added to each well and incubation was continued at 37˚C for 4 h. The cells were re-suspended in 150 μl of dimethylsulfoxide (Sigma-Aldrich; Merck KGaA) to dissolve the formazan crystals. The absorbance was measured at a wavelength of 490 nm using a microplate reader (Bio-Rad Laboratories, Inc.).

Western blot analysis. Protein expression in MCs was analyzed using western blot analysis as described previously (14). The following primary antibodies were used: Rabbit polyclonal anti-fibronectin (FN; 1:10,000; cat. no. F3648; Sigma-Aldrich; Merck KGaA), rabbit polyclonal anti-collagen I (1:10,000; cat. no. 234167, EMD Millipore), rabbit monoclonal anti-β-catenin (1:1,000; cat. no. ab32572; Abcam), rabbit monoclonal anti-β-actin (1:1,000; cat. no. 4970; Cell Signaling Technology, Inc.) and mouse monoclonal anti-α-tubulin (1:1,000; cat. no. T9026; Sigma-Aldrich; Merck KGaA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA isolation from rat glomerular MCs was performed using the TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The first strand of complementary DNA was synthesized using 1 μg of RNA in 20 μl of reaction buffer containing Moloney Murine Leukemia Virus Reverse Transcriptase (Promega Corporation), dNTPs and random primers at 37˚C for 50 min. RT-qPCR analyses of Wnt mRNA expression were performed as described previously (7). Specifically, RT-qPCR was performed under the following thermocycling conditions: Initial denaturation at 95˚C for 30 sec; 5 cycles of denaturation at 95˚C for 10 sec, annealing at 60˚C for 30 sec and extension at 70˚C for 20 sec; 35 additional cycles of denaturation at 95˚C for 5 sec and annealing at 60˚C for 30 sec to reach the fluorescent signal detection point; and another 40 cycles of denaturation at 95˚C for 1 min and annealing at 55˚C for 1 min (with the temperature increasing by 0.5˚C/cycle). The target gene expression was calculated from the respective standard curves. The sequences of the primer pairs were as follows: Collagen IV forward, 5'-CCTGGTAGT CGTGGGAGACATTG-3' and reverse, 5'-CCTTTCCTGCTT CACCCTTTG-3'; FN forward, 5'-CGAGGTGACAGAGAC CACAA-3' and reverse, 5'-CTGGAGTCAAGCCAGACA CA-3'; α-smooth muscle actin (SMA) forward, 5'-TGAACC CTAAGGCCAACCG-3' and reverse, 5'-TCCAGAGTCCAG CACAATACCA-3'; monocyte chemotactic peptide-1 (MCP-1) forward, 5'-GATGATCCCAATGAGTCGGC-3' and reverse, 5'-TGATCTCACTTGGTTCTGGTCC-3'; tumor necrosis factor (TNF)-α forward, 5'-ACTCCCAGAAAAGCAAGCAA-3' and reverse, 5'-CGAGCAGGAATGAGAAGAGG-3'; transforming growth factor (TGF)-β1 forward, 5'-ACCGCAACAACGCAA TCTATG-3' and reverse, 5'-GCAGCTCTGCACGGGACA-3'; Wnt 1 forward, 5'-CTGGAGCCCGAAGACCCT-3' and reverse, 5'-CGTCCACTGTACGTGCAGAAGT-3'; Wnt 3 forward, 5'-TGGTGTAGCCTTCGCAGTCA-3' and reverse, 5'-CCG TGCATCCGCAAACTC-3'; Wnt 4 forward, 5'-CCCTTCCGT CAGGTTGGC-3' and reverse, 5'-CCTCATCCGTATGTGGCT TG-3'; Wnt 5a forward, 5'-ACGCACGAGAAAGGGAACG-3' and reverse, 5'-GAGGCTACAGGAGCCAGACACT-3'; CTGF forward, 5'-TAGCAAGAGCTGGGTGTGTG-3' and reverse, 5'-TTCACTTGCCACAAGCTGTC-3'; β-actin forward, 5'-CAG CTGAGAGGGAAATCGTG-3' and reverse, 5'-GAGGCTACA GGAGCCAGACACT-3'. The relative mRNA level expression of each gene was determined using the comparative CT method ($2^{-\Delta\Delta Cq}$) with the mRNA levels of each gene calculated after normalization to those of β-actin (15) using an iCycler Thermal Cycler (Bio-Rad Laboratories, Inc.).

Statistical analysis. All data are presented as the mean ± standard deviation. Statistical analyses were performed using SPSS software (version 19.0 for Windows; IBM, Corp.). Comparisons between groups were made using one-way analysis of variance, followed by the Student-Newman-Keuls test or Dunnett's T3 test when the assumption of equal variances did not hold. P<0.05 was considered to indicate a significantly significant difference.

Results

HG treatment induces MC proliferation. Cell proliferation was detected using the MTT assay. As indicated in Fig. 1A, exposure of MCs to HG increased cell viability after 72 h compared with that in the NG group (P<0.05). RT-qPCR results demonstrated that HG treatment upregulated the expression level of ECM components fibronectin and collagen IV (Fig. 1B and C) and fibrotic factors α-SMA, TGF-β1 and CTGF (Fig. 1D-F) in MCs compared with that in the NG group (all P<0.05). No
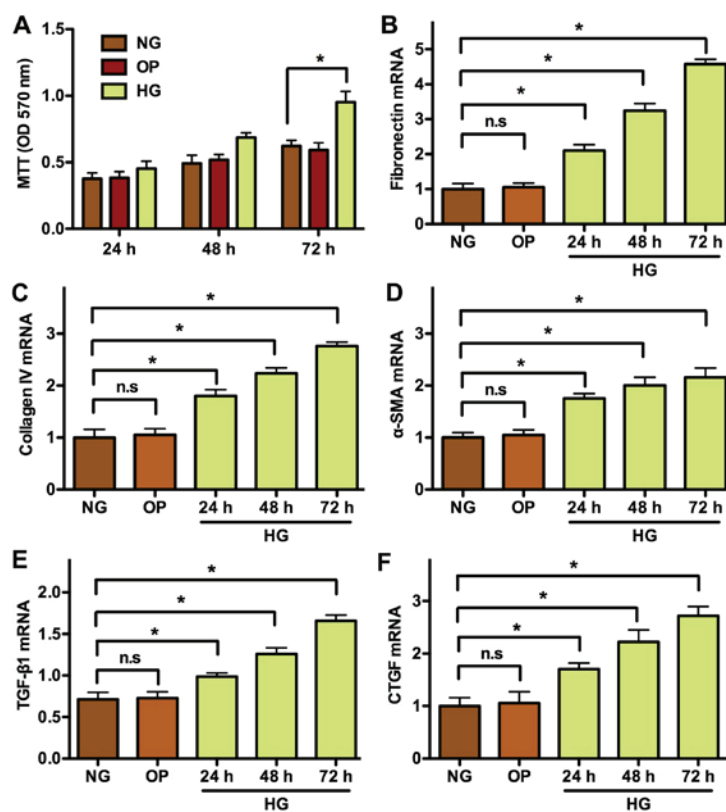
Figure 1. HG treatment induces MC proliferation in a time-dependent manner. (A) Glomerular MCs were treated with NG, OP and HG for 24-72 h and then subjected to MTT analyses. HG treatment induced mRNA expression of (B) fibronectin, (C) collagen IV, (D) α-SMA, (E) TGF-β1 and (F) CTGF in MCs. *P<0.05 (n=3). NG, normal glucose; OP, osmotic pressure; HG, high glucose; MC, mesangial cell; TGF-β1, transforming growth factor β1; CTGF, connective tissue growth factor; n.s., not significant; α-SMA, α-smooth muscle actin; OD, optical density.

difference in cell proliferation and ECM induction was found between the NG and OP groups.

HG treatment induces the expression of various Wnts in MCs. Since the Wnt/β-catenin pathway plays an important role in the development of DN (12), the present study investigated the Wnt/β-catenin pathway in MCs exposed to HG. As indicated in Fig. 2A-D, HG treatment significantly induced the expression of a variety of Wnts in MCs, including Wnt 1, Wnt 3, Wnt 4, and Wnt 5a, compared with the NG group (P<0.05). Furthermore, as indicated in Fig. 2E and F, HG treatment significantly increased the expresion of proinflammatory factors, including MCP-1 and TNF-α, compared with the NG group (P<0.05). However, there was no difference in mRNA expression levels of the different Wnts between the NG and OP groups.

PC-1 NF fusion protein inhibits HG-induced proliferation and ECM accumulation in glomerular MCs. Based on the preliminary RT-qPCR and MTT data, no difference was observed in cell viability and the expression of fibrotic factors after 24, 48 and 72 h between the NG and OP groups (data not shown). Therefore, for subsequent experiments data from the OP group were not included. As indicated in Fig. 3A, pretreatment with PC-1 NF fusion protein significantly suppressed HG-induced MC proliferation compared with the HG group (P<0.05). It has been previously demonstrated that dysregulated expression of ECM components is associated with DN progression (6). Therefore, the present study investigated the effect of PC-1 NF

on HG-stimulated ECM component expression in MCs. As shown in Fig. 3B-F, PC-1 NF treatment significantly reduced the mRNA and protein expression levels of ECM components, including FN, collagen IV and α-SMA, compared with the HG group (all P<0.05).

PC-1 NF fusion protein inhibits the HG-induced expression of TGF-β1 and CTGF in glomerular MCs. Both TGF-β1 and CTGF are important fibrotic factors in DN (16). The present study investigated the effect of PC-1 NF on the expression of TGF-β$_1$ and CTGF. As shown in Fig. 4A and B, the mRNA expression levels of TGF-β1 and CTGF were significantly inhibited in the HG + PC-1 NF group compared with the HG group (P<0.05). Western blot analysis also demonstrated that PC-1 NF inhibited the protein expression levels of TGF-β1 and CTGF proteins in HG-induced MCs (Fig. 4C and D; P<0.05). The study subsequently assessed the renal filtration of inflammatory cytokines in glomerular MCs. As shown in Fig 4E and F, RT-qPCR revealed that treatment with PC-1 NF significantly reduced both MCP-1 and TNF-α mRNA expression in HG-induced MCs (P<0.05).

PC-1 NF fusion protein inhibits the activation of the Wnt signaling pathway in HG-stimulated MCs. The present study examined the effect of PC-1 NF on the Wnt/β-catenin signaling pathway in HG-treated MCs to expound the mechanism underlying the PC-1 NF-mediated inhibition of DN development. As presented in Fig. 5, PC-1 NF inhibited the mRNA
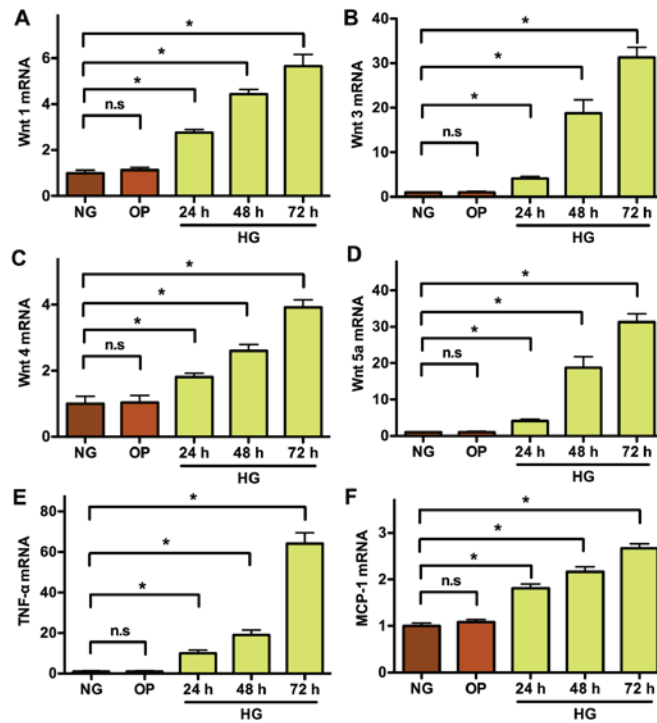
Figure 2. HG treatment induces various Wnts in MCs. RT-qPCR analyses revealed that HG induced the expression of various Wnts in MCs, including (A) Wnt 1, (B) Wnt 3, (C) Wnt 4, and (D) Wnt 5a. HG treatment induced mRNA expression of (E) TNF-α and (F) MCP-1 in MCs. *P<0.05 (n=3). NG, normal glucose; OP, osmotic pressure; HG, high glucose; MC, mesangial cells; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; TNF-α, tumor necrosis factor-α; MCP-1, monocyte chemotactic peptide-1.
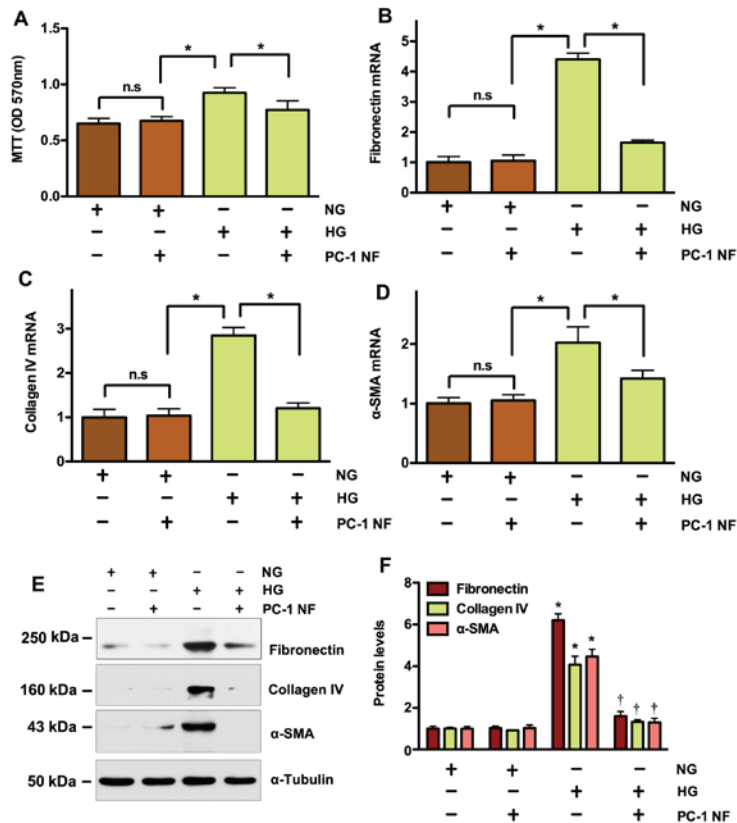

Figure 3. PC-1 NF fusion protein inhibits HG-induced proliferation and ECM component accumulation in glomerular MCs. (A) MTT analyses revealed that incubation with PC-1 NF fusion protein at 4 μg/ml for 72 h, inhibited HG-induced MC proliferation. *P<0.05 (n=3). Reverse transcription-quantitative polymerase chain reaction demonstrated that PC-1 NF blocked HG-induced expression of (B) fibronectin, (C) collagen IV and (D) α-SMA in MCs. *P<0.05 (n=3). (E) Western blot analysis demonstrated that PC-1 NF inhibited the expression of fibronectin, collagen IV and α-SMA proteins induced by HG in MCs. (F) Western blotting results were quantified. *P<0.05 vs. NG + PC-1 NF (n=3). †P<0.05 vs. HG (n=3). NG, normal glucose; HG, high glucose; PC-1 NF, polycystin-1 N-terminal fragment; MC, mesangial cells; α-SMA, α-smooth muscle actin; n.s. not significant; OD, optical density; kDa, kilodalton; ECM, extracellular matrix.
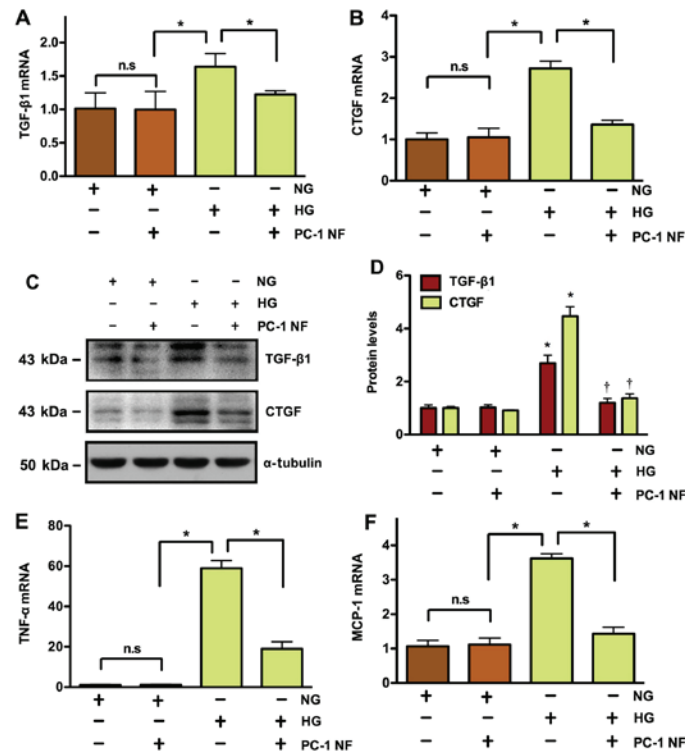
Figure 4. PC-1 NF fusion protein inhibits HG-induced TGF-β1 and CTGF expression in glomerular MCs. RT-qPCR demonstrated that PC-1 NF blocked HG-induced expression of (A) TGF-β1 and (B) CTGF in MCs. *P<0.05 (n=3). (C) Western blotting and (D) quantitative analysis demonstrated that PC-1 NF inhibited TGF-β1 and CTGF induction by HG in MCs. *P<0.05 vs. NG + PC-1 NF (n=3). †P<0.05 vs. HG (n=3). RT-qPCR demonstrated that PC-1 NF blocked HG-induced expression of (E) TNF-α and (F) MCP-1 in MCs. *P<0.05 (n=3). NG, normal glucose; HG, high glucose; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; PC-1 NF, polycystin-1 N-terminal fragment; MC, mesangial cells; TGF-β1, transforming growth factor β1; CTGF, connective tissue growth factor; TNF-α, tumor necrosis factor-α; MCP-1, monocyte chemotactic peptide-1; n.s. not significant; kDa, kilodalton.
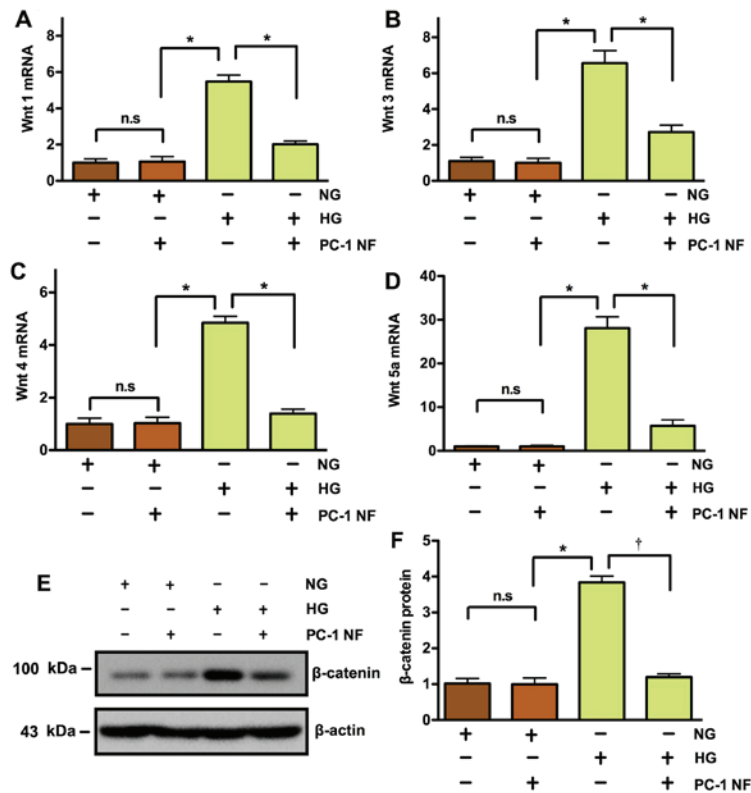

Figure 5. PC-1 NF fusion protein inhibits activation of the Wnt signaling pathway in HG-stimulated MCs. Reverse transcription-quantitative polymerase chain reaction demonstrated that PC-1 NF blocked HG-induced expression of (A) Wnt 1, (B) Wnt 3, (C) Wnt 4, and (D) Wnt 5a in MCs. *P<0.05 (n=3). (E) Western blotting and (F) quantitative analysis demonstrated that PC-1 NF inhibited β-catenin induction by HG in MCs. *P<0.05 (n=3). †P<0.05 vs. HG (n=3). NG, normal glucose; HG, high glucose; PC-1 NF, polycystin-1 N-terminal fragment; MC, mesangial cells; kDa, kilodalton; n.s. not significant.
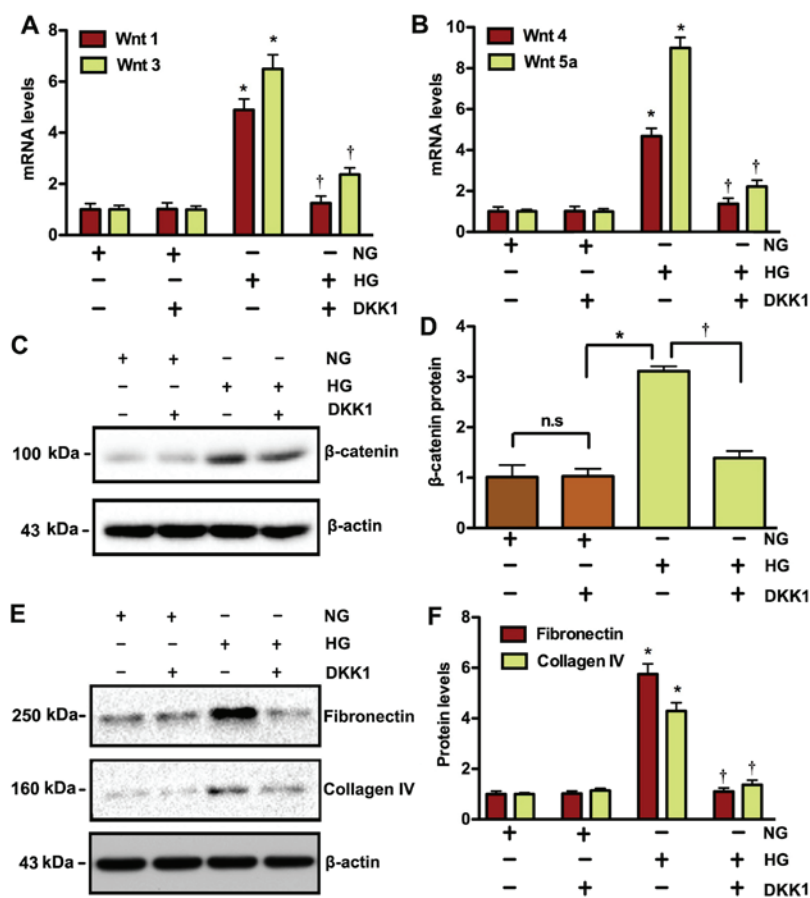
Figure 6. DKK1 inhibits the activation of the Wnt signaling pathway and ECM induction in HG-stimulated MCs. Reverse transcription-quantitative polymerase chain reaction demonstrated that DKK1 inhibited the HG-induced expression of (A) Wnt 1 and Wnt 3, and (B) Wnt 4 and Wnt 5a in MCs. *P<0.05 vs. NG + DKK1 (n=3). †P<0.05 vs. HG (n=3). (C) Western blotting and (D) quantitative analysis demonstrated that DKK1 inhibited β-catenin induction by HG in MCs. *P<0.05 (n=3). †P<0.05 vs. HG (n=3). (E) Western blotting and (F) quantitative analysis demonstrated that DKK1 inhibited fibronectin and collagen IV induction by HG in MCs. *P<0.05 vs. NG + DKK1 (n=3). †P<0.05 vs. HG (n=3). NG, normal glucose; HG, high glucose; DKK1, Dickkopf-related protein 1; ECM, extracellular matrix; MC, mesangial cells; kDa, kilodalton.

expression levels of Wnt 1, 3, 4 and 5a, and β-catenin protein expression in MCs compared with the HG group (all P<0.05). Furthermore, the mRNA expression levels of the aforementioned Wnts and protein expression levels of β-catenin were significantly reduced after treatment with the Wnt inhibitor, DKK1 in MCs (all P<0.05; Fig. 6A-D). In addition, DKK1 significantly reduced HG-induced expression of ECM components, including fibronectin and collagen IV (Fig. 6E and F; P<0.05).

Discussion

The present study demonstrated that HG treatment significantly promoted cell proliferation, inflammatory cytokine infiltration and ECM production in MCs. However, pretreatment with PC-1 NF efficiently suppressed the proliferation and ECM induction in HG-stimulated MCs. Furthermore, PC-1 NF significantly reduced HG-induced Wnt/β-catenin activation in MCs. The present study provided novel insights into the mechanism by which PC-1 NF protected the MCs from developing DN after HG stimulation.

Hyperglycemia is an important pathogenic factor in diabetes (17). High glucose-induced glomerular MC proliferation is a major pathological feature of DN. MC proliferation

can lead to excessive ECM accumulation and subsequent progression of DN (18,19). The present study revealed that HG stimulation significantly induced MC proliferation; however, pretreatment with PC-1 NF significantly reduced HG-induced MC proliferation. These results suggested that PC-1 NF exerted a protective effect against HG-induced MC proliferation.

Previous studies showed a key role of inflammation in the development and progression of DN (20,21). Furthermore, strategies targeting inflammatory molecules have been shown to be beneficial in treating DN (18-20). Therefore, the present study investigated the levels of proinflammatory cytokines TNF-α and MCP-1 in HG-induced MCs. RT-qPCR results from the present study demonstrated that HG significantly reduced the mRNA expression levels of TNF-α and MCP-1 in MCs. However, this amplification of inflammatory cytokines induced by HG in MCs was significantly abrogated by PC-1 NF treatment, suggesting that PC-1 NF may protect against DN by inhibiting inflammation.

ECM deposition is a key event in the progression of DN (19). FN has been recognized as a major component of ECM. Its overexpression contributes to glomerular basement membrane thickening and ECM accumulation in DN (22). Furthermore, HG is the most potent stimulus leading to the synthesis of ECM, which results in the deposition of collagen

and FN in glomerular cells (23). The present study found that exposure of MCs to HG significantly induced the production of collagen IV and FN, consistent with the results of previous studies (22,23). However, this induction was ameliorated by PC-1 NF treatment. These results suggested that PC-1 NF could protect against DN through inhibiting ECM production in MCs.

TGF-β1 and CTGF are key regulators in promoting glomerular sclerosis in DN (16). HG can be used as a profibrotic factor in MCs to promote the synthesis and secretion of TGF-β1 and CTGF (24). The present study found that HG could induce the mRNA and protein expression of TGF-β1 and CTGF, suggesting that HG promoted glomerular sclerosis and renal fibrosis in DN. PC-1 NF decreased the expression of TGF-β1 and CTGF induced by HG in MCs. These results suggested that PC-1 NF potentially offered a therapeutic option for treating DN through inhibiting renal fibrosis.

The Wnt/β-catenin signaling pathway plays an important role in the pathogenesis of DN (9). Furthermore, numerous studies demonstrated that specific blockade of the Wnt/β-catenin pathway prevented DN progression, including abolishing cell proliferation and ECM expression in glomerular MCs (12,25,26). The Wnt family comprises of 19 different Wnt ligands (13). A previous study demonstrated that several canonical Wnts, including Wnt 1, 3, 4 and 5a, promoted the development of kidney disease, such as diabetic kidney disease (13). Thus, blocking the activation of the Wnt/β-catenin signaling pathway is an important strategy for preventing DN. In the present study results revealed that HG significantly induced the activation of the Wnt/β-catenin pathway in MCs. However, PC-1 NF inhibited Wnt/β-catenin activation in HG-stimulated MCs. Furthermore, DKK1 significantly suppressed HG-induced Wnt/β-catenin activation and ECM component expression in MCs. On the basis of these data, it was hypothesized that PC-1 NF inhibited HG-induced glomerular MC proliferation and ECM expression via suppressing the Wnt/β-catenin signaling pathway.

The present study had certain limitations. Only *in vivo* analysis was performed and no animal model was used for confirmation. Future studies are warranted to investigate the effect of PC-1 NF on diabetic nephropathy in mice. Another limitation of the present study was the use of RT-qPCR instead of ELISA as an indicator of post-translational modifications. As ELISA is more accurate than RT-qPCR for assessing the secretion of inflammatory factors in chronic kidney disease, more studies are required to fully address this issue. Although DKK1 is an inhibitor of Wnt and its effect on Wnt has been previously confirmed (27), the DKK1 + PC-1 NF experimental group was not included, which is a further limitation. In future studies, these experiments should be performed.

In conclusion, the present study revealed that HG could induce cell proliferation, inflammatory cytokine infiltration and ECM component expression in glomerular MCs. However, PC-1 NF could abolish these effects by blocking Wnt/β-catenin pathway activation. A previous study revealed that PC-1 could combine with β-catenin and regulate Wnt/β-catenin signal activation (28). Another study demonstrated that PC-1 NF inhibited glomerular MC proliferation and induced G_0/G_1 phase arrest and apoptosis *in vitro* (7). The present study provided a novel mechanism by which PC-1 NF may inhibit ECM component expression by regulating the Wnt/β-catenin activation in HG-induced glomerular MCs. In this context, the administration of PC-1 NF could be a rational strategy for the treatment of DN.

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

LX, YY, YC, BX, and QG performed the experiments, collected data and drafted the manuscript. PC, TZ and TG performed the statistical analysis and designed the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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