Overexpression of KLF5 inhibits puromycin-induced apoptosis of podocytes

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Received January 18, 2018; Accepted June 28, 2018

DOI: 10.3892/mmr.2018.9366

Abstract. Diabetic nephropathy (DN) is one of the most common microvascular complications associated with diabetes mellitus (DM); the incidence has been predicted to reach 7.7% by 2030 on a global scale. Krüppel-like factor 5 (KLF5) is involved in numerous important biological processes; however, the potential effects of KLF5 on podocytes in patients with diabetic nephrotic (DN) have not yet been investigated. In the present study, synaptopodin expression in podocytes was investigated using an immunofluorescence assay. Following this, the proliferation of podocytes was investigated using an MTT assay. In addition, KLF5 was overexpressed in podocytes, and cell cycle arrest and apoptosis was subsequently investigated using flow cytometry. Western blotting and reverse transcription-quantitative polymerase chain reaction assays were performed to detect the expression levels of genes involved in the cell cycle and apoptosis, and the extracellular signal-regulated protein kinase (ERK)/p38 mitogen-activated protein (MAP) kinase pathway. The results demonstrated that treatment with puromycin aminonucleoside (PAN) suppressed the proliferation of podocytes in a dose- and time-dependent manner, and overexpression of KLF5 induced cell cycle arrest of podocytes regulated by PAN. Furthermore, overexpression of KLF5 was revealed to have inhibited PAN-induced apoptosis of podocytes, and that overexpression of KLF5 suppressed the ERK/p38 MAP kinase pathway in podocytes induced by PAN. Therefore, the results of the present study suggested that KLF5 may represent a potential therapeutic target for treatment of patients with DN.

Introduction

Diabetic mellitus (DM) is a progressive disease and is usually associated with numerous complications (1-4). The incidence of DM is predicted to reach 7.7% by 2030 on a global scale (5,6). Diabetic nephropathy (DN) is one of the most common microvascular complications associated with DM and has become the second leading cause of end-stage renal disease in China (7). Numerous studies have demonstrated that well-managed blood glucose, blood pressure and blood lipid levels, and medicinal application of angiotensin-converting enzyme inhibitors or angiotensin receptor blockers, may postpone disease progression (8,9). The exact mechanisms underlying the pathogenesis of DN remain unclear. A previous study revealed that the onset of DN may be pertinent to oxidative stress in living cells (10). Podocytes are considered to be the most critical component of glomerular permselectivity (11). Changes in podocyte morphology and dysfunction are closely associated with renal diseases (12,13). The apoptosis of podocytes has been regarded as an important factor in the progression of DN (14). Therefore, investigating the molecular mechanism associated with the inhibition of podocyte injury is important for the development of novel therapeutic strategies for the treatment of patients with DN.

Kruppel-like factors (KLFs) are a type of transcriptional regulatory factor. KLFs are associated with cell proliferation, migration, apoptosis and tissue remodeling (15,16). Furthermore, KLFs are associated with the development of numerous diseases, including cardiovascular disease and cancer (17-19). KLF5, a member of the KLF family, can be regulated via phosphorylation, acetylation and ubiquitination following translation (20,21). KLF5 is associated with hypertensive nephropathy and diabetic retinopathy (22). However, the effects of KLF5 on DN are not fully understood.

Puromycin aminonucleoside (PAN) may disrupt the morphology of podocytes, trigger the overproduction of reactive oxygen species and induce nephrosis. Therefore, PAN is frequently used to establish a nephropathy model (23,24). The aim of the present study was to investigate the effect of KLF5 on PAN-induced injury of podocytes and to determine the underlying molecular mechanism. Therefore, the results of the present study may further the understanding of molecular mechanisms associated with DN and provide a potential therapeutic target for DN.
Materials and methods

Cell culture and treatment. Podocytes (MPC-5 cells) were acquired from the Bena Culture Collection (Beijing, China). Podocytes were cultured in Ham Nutrient Mix F12-Dulbecco modified Eagle medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 1% penicillin-streptomycin G (Biochrom, Ltd, Cambridge, UK) and 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.). Podocytes were seeded in 6-well plates at a concentration of 3x10^4 cells/ml and subsequently treated with PBS (control), or with PAN (0, 5, 10, 20, 40, 60, 80 and 100 µg/ml) for 0, 6, 12, 24 and 48 h time intervals at 37˚C.

Plasmid construction. Mouse KLF5 complementary (c)DNA clone was purchased from GeneCoppoeia Inc. (Rockville, MD, USA). KLF5 was amplified with PrimSTAR DNA polymerase (Takara Bio, Inc., Otsu, Japan) according to the manufacturer's instructions. After 12 h following transfection, the cells were bilized with 0.2% Triton X-100 for 3 min. Following this, 20 µl of MTT solution was added into each well and incubated for 30 min. A total of 150 µl dimethyl sulfoxide was subsequently was added into each well. Finally, a microplate reader was used to determine the absorbance at 490 nm. Treatment with 60 µg ml PAN for 6 h at 37˚C. KLF5 overexpression (1 ug) was subsequently determined using the SYBR-Green PCR Master Mix kit (Takara Bio, Inc.) and the ABI 7500 system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions were set as: 5 min pretreatment at 95˚C, followed by 28 cycles of 95˚C for 15 sec and 60˚C for 30 sec, a final extension at 72˚C for 10 min. The specific primers used were as follows: KLF5 forward, 5'-TTTCTGTCCCTACCCACGAG-3' and reverse, 5'-AGTAGTGCGGCTGTGTGGAG-3'; Bax forward, 5'-AGATGTGAGGATGATTGC-3' and reverse, 5'-CCAATGGCCACGGCAGTG-3'; Bcl-2 forward, 5'-GCCCTTTAGTGCTGGTG-3' and reverse, 5'-GAATCCAAACAGGCGGCA-3'; Caspase-3 forward, 5'-TTGCCACCTGGCGGTGTTGA-3' and reverse, 5'-AGGAGTGATGCTTGCTC-3'; caspase-8 forward, 5'-TTTGTGGCCCTACCCACGAG-3' and reverse, 5'-AGTAGTGAGGATGATTGC-3'; GAPDH forward, 5'-GGCTTGGTTGGA-3'; Caspase-9 forward, 5'-GCCCTCTTCTGCACTTG-3' and reverse, 5'-CATTTCGCGCCCTGTAGT-3'. Data were quantified using the 2^-ΔΔCT method (25).

Western blot analysis. Total proteins were prepared using a radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Shanghai, China) containing a protease inhibitor cocktail (P8340; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Protein concentrations were determined using a Bradford Protein Assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Proteins (30 µg) were separated via 10% SDS-PAGE gels and then transferred onto polyvinyl difluoride (PVDF) membranes (PerkinElmer, Inc., Waltham, MA, USA). Following this, the membranes were blocked with 5% skimmed milk (BD Biosciences, Franklin Lakes, NJ, USA) at room temperature for 2 h. The membranes were subsequently incubated with the following primary antibodies overnight at 4˚C: Anti-GAPDH (1:2,000; cat. no. ab8245; Abcam, Cambridge, UK), anti-KLF5 (1:1,000; cat. no. ab24331; Abcam), anti-B cell lymphoma 2 (Bcl-2) associated X (Bax) (1:1,000; cat. no. ab32503; Abcam), Bcl-2 (1:1,000; cat. no. ab32124; Abcam), anti-caspase-3 (1:1,500; cat. no. ab32586; Abcam), anti-caspase-8 (1:1,500; cat. no. ab25901; Abcam), anti-caspase-9 (1:1,500; cat. no. ab25901; Abcam), anti-cyclin D1 (1:1,000; cat. no. ab13475; Abcam), anti-c-myc (1:1,000; cat. no. ab39688; Abcam), anti-phosphorylated (p)-extracellular signal-regulated protein kinase (ERK)1/2 (1:200; cat. no. 4370; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-ERK1/2 (1:200; cat. no. ab84699; Abcam), anti-p-p38 (1:1,000; cat. no. ab47363; Abcam), anti-p-p38 (1:1,000; cat. no. ab170099; Abcam). PVDF membranes were subsequently incubated with a horseradish peroxidase-conjugated secondary antibody (1:5,000; cat. no. sc-2000, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at room temperature for 1 h. Finally, the proteins were visualized using ECL Western Blotting Substrate (Pierce; Thermo Fisher Scientific, Inc.) in an enhanced chemiluminescence detection system (GE Healthcare, Chicago, IL, USA). The gray value was determined by Quantity One 4.6.2 software (Bio-Rad Laboratories, Inc.).

Immunofluorescence (IF) staining. Podocytes were fixed with 4% paraformaldehyde at 4˚C for 20 min, and then permeabilized with 0.2% Triton X-100 for 3 min. Following this, cells were washed with PBS and then blocked using 10% goat

Cell viability assay. Treated podocytes (2x10^4 cells/well) were seeded into a 96-well plate. The cells were treated with PAN at different concentrations (0, 5, 10, 20, 40, 60, 80 and 100 µg/ml) and maintained at 37˚C for 0, 6, 12, 24 and 48 h time intervals. Following this, 20 µl of MTT solution was added into each well and incubated for 30 min. A total of 150 µl dimethyl-sulfoxide was subsequently was added into each well. Finally, a microplate reader was used to determine the absorbance at 490 nm. Treatment with 60 µg/ml PAN for 6 h was selected for subsequent analysis as this resulted in decreased cell viability (described below).

Cell transfection. Podocytes were plated in 6-well plates at a density of 1x10^6 cells per well. Cells were treated with PBS or 60 µg/ml PAN for 6 h at 37˚C. KLF5 overexpression (1 ug) or negative control plasmids (pcDNA 3.1; 1 µg) were transduced into podocytes using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer’s instructions. After 12 h following transfection, the cells were used for subsequent experimentation.

RT-qPCR assay. Total RNA was obtained using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.). RNA was reversely transcribed to cDNA using a miScript II RT Kit (Qiagen GmbH, Hilden, Germany). The temperature protocol or RT was 25˚C for 5 min, 37˚C for 60 min, 85˚C for 5 min and then held at 4˚C. The mRNA expression levels were subsequently determined using the SYBR-Green PCR Master Mix kit (Takara Bio, Inc.) and the ABI 7500 system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions were set as: 5 min pretreatment at 95˚C, followed by 28 cycles of 95˚C for 15 sec and 60˚C for 30 sec, a final
serum (Thermo Fisher Scientific, Inc.) for 30 min at room temperature. Cells were subsequently incubated with 4 µg/ml anti-synaptopodin antibodies (cat. no. ab220345; Abcam) over-night at 4˚C. Following this, cells were washed using PBS and then incubated with 500 µl propidium iodide (PI)/RNase buffer (BD Biosciences, Franklin Lakes, NJ, USA) at room temperature for 30 min. Finally, a FACS-Calibur flow cytometer (BD Biosciences) was used to investigate cell cycle distribution, and ModFit LT 2.0 software (Verity Software House, Inc., Topsham, ME, USA) was used to analyze the results. To investigate apoptosis, cells were washed with PBS and then re-suspended with 0.5 ml binding buffer containing 5 µl Annexin V-fluorescein isothiocyanate and PI double stain (BD Biosciences) for 20 min at room temperature in the dark. Following this, the apoptotic rate was determined using a FACS-Calibur flow cytometer (BD Biosciences) and ModFit LT 2.0 software.

**Statistical analysis.** All data were analyzed by SPSS 13.0 (SPSS, Inc., Chicago, IL, USA) using one-way analysis of variance with Tukey’s test. Data are presented as the mean ± standard deviation. All experiments were performed in triplicate. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**PAN inhibits the proliferation of podocytes.** Initially, synaptopodin expression in podocytes was investigated using immunofluorescence. The results revealed that the expression of synaptopodin was positive in podocytes (Fig. 1A). To further investigate the effect of PAN on the proliferation ability of podocytes, podocytes were treated with PBS (control) or PAN (5, 10, 20, 40, 60, 80, and 100 µg/ml) for 0, 6, 12, 24 and 48 h time intervals. The results revealed that the viability of podocytes were markedly inhibited following treatment with PAN in a dose- and time-dependent manner. (A) Synaptopodin expression in podocytes was investigated via immunofluorescence assay using a fluorescence microscope (magnification, x200). (B) Cell viability in podocytes was analyzed using an MTT assay following treatment with PBS (control), or PAN (5, 10, 20, 40, 60, 80, and 100 µg/ml) for 0, 6, 12, 24 and 48 h time intervals. PAN, puromycin aminonucleoside. *P<0.05 vs. 0 h.

**Overexpression of KLF5 inhibits cell cycle arrest of PAN-treated podocytes.** The results revealed that the proportion of cells in the G1 phase was increased in the PAN group compared with the control group (Fig. 3A). The number of cells in the G1 phase was decreased in the KLF5+PAN group compared with the NC+PAN group (Fig. 3A, P<0.05). In addition, the results demonstrated that treatment with PAN promoted cell cycle arrest in podocytes (P<0.05); however, this effect was markedly attenuated following overexpression of KLF5 (Fig. 3A, P<0.05). In addition, the expression levels of cyclin D1 and c-myc were investigated via RT-qPCR and western blot assays, and the results revealed that cells treated with PAN exhibited significantly decreased expression levels of cyclin D1 and c-myc compared with the NC group (P<0.01), whereas cells overexpressing KLF5 exhibited significantly increased expression levels of cyclin D1 and c-myc compared with the NC + PAN group (Fig. 3B-D, P<0.05).

**Overexpression of KLF5 suppresses the apoptosis of PAN-treated podocytes.** Flow cytometry was performed to determine whether KLF5 has an important role in the apoptosis of podocytes. The results demonstrated that the apoptosis of podocytes was significantly increased in the PAN group compared with the NC group (P<0.001; Fig. 4A); however, this effect was significantly attenuated following overexpression of KLF5 (P<0.05; Fig. 4A). Furthermore, the
expression levels of genes associated with apoptosis (Bax, Bcl-2, caspase-3, caspase-8 and caspase-9) were investigated using RT-qPCR and western blot assays. The results revealed that treatment with PAN significantly enhanced the expression levels of Bax, caspase-3, caspase-8 and caspase-9 exhibited by podocytes (P<0.01 and P<0.001; Fig. 4B and C); however, overexpression of KLF5 significantly attenuated this effect (P<0.05 and P<0.01; Fig. 4B and C). In addition, podocytes treated with PAN exhibited significantly decreased Bcl-2 expression, and overexpression of KLF5 significantly attenuated this effect (P<0.05, P<0.01 and P<0.001; Fig. 4B and C).

Overexpression of KLF5 suppresses the activation of ERK/p38 mitogen-activated protein (MAP) kinase pathway in PAN-treated podocytes. MAP kinases have important roles in numerous cellular functions and are regulated via independent upstream activation cascades (26). In the present study, the effects of KLF5 and PAN on the ERK/p38 MAP kinase pathway in podocytes were investigated. The protein levels of p-ERK1/2,
ERK1/2, p-p38 and p38 in podocytes were investigated using a western blot assay. The results revealed that treatment with PAN significantly enhanced ERK1/2 and p38 phosphorylation compared with the control group; however, overexpression of KLF5 significantly attenuated these effects (P<0.001; Fig. 5).

Discussion

DN is one of the most common complications associated with diabetes and has become the most frequent causative factor resulting in the development of end-stage renal disease (27).
The pathogenesis of DN arises from glucose and lipid metabolic disorders, abnormal hemodynamics and oxidative stress (28,29). The results of the present study demonstrated that podocyte injury is an important factor resulting in the development of proteinuria and glomerular sclerosis, and has significant effects on the pathogenesis of DN (30). Considering that synaptopodin is a podocyte marker (31), the expression levels of synaptopodin in podocytes was investigated in the present study, and the results revealed that synaptopodin was highly expressed in podocytes.

Podocytes and the slit diaphragm within foot processes are critical components of the glomerular filtration barrier (32). Associated molecules, including nephrin, podocin, CD2 associated protein and α-actinin-4 have important roles in the development of proteinuria (33). A human podocyte cell line was used in the present study to investigate podocyte injury in vitro. Furthermore, the present study established a podocyte injury model in vitro via administration of PAN. This model provided the possibility to further investigate molecular mechanisms associated with DN (34-36). The results revealed that treatment with PAN markedly inhibited the proliferation of podocytes in a dose- and time-dependent manner.

KLF-5 is an important member of the KLF protein family, and is located at chromosome 13q21 and encodes a 55 kDa protein that contains 457 amino acids (37). In normal tissue, KLF5 extensively regulates numerous cellular processes, such as proliferation, differentiation, movement, inflammation and pluripotency (38,39). However, the role of KLF5 in DN remains unclear. In the present study, the results revealed that PAN induced cell cycle arrest in podocytes; however, overexpression of KLF5 significantly attenuated this effect via upregulation of cyclin D1 and c-myc expression levels. In addition, a previous study demonstrated that KLF5 inhibited the cell cycle progression of vascular smooth muscle cells via activation of cyclin D1 (40). Furthermore, overexpression of KLF5 was revealed to significantly suppress PAN-mediated apoptosis of podocytes. At a molecular level, the results of the present study revealed that PAN enhanced the expression levels of Bax, caspase-3, caspase-8 and caspase-9; however, treatment with PAN significantly reduced Bcl-2 expression. Furthermore, overexpression of KLF5 significantly decreased Bax, caspase-3, caspase-8 and caspase-9 expression levels, whereas overexpression of KLF5 significantly increased Bcl-2 expression in PAN-treated podocytes. A previous study revealed that treatment with PAN induces apoptosis of glomerular podocytes (41). A further study demonstrated that the downregulation of KLF5 is associated with G1 phase cell cycle arrest (42). The results of the present study were therefore consistent with previous studies. Therefore, it was concluded that overexpression of KLF5 alleviates PAN-mediated podocyte injury.

MAP kinases are serine/threonine-specific protein kinases (43-45). p38 MAP kinase and ERK are factors of the MAP kinase protein family (46,47). The results of the present study suggested that treatment with PAN induced the phosphorylation of ERK1/2 and p38. Overexpression of KLF5 suppressed the phosphorylation of ERK/p38 MAP kinase in PAN-treated podocytes. These results suggested that overexpression of KLF5 protected podocytes from injury via inhibited activation of the ERK1/2 and p38 pathways. However, a previous study demonstrated that KLF5 may promote the activation of ERK1/2 in breast cancer cells (48). This discrepancy may be due to the use of different cells.

Limitations of the present study included that all experiments were performed in vitro, and that the exact mechanism underling the regulation of KLF5 remains unclear. Thus, future studies should perform further in vivo investigations to determine the role of KLF5 in DN. In conclusion, the results of the present study demonstrated that PAN inhibited the proliferation of podocytes, and that overexpression of KLF5 attenuated PAN-induced cell cycle arrest and apoptosis of podocytes. Furthermore, the results demonstrated that overexpression of KLF5 suppressed the ERK/p38 MAP kinase pathway in PAN-treated podocytes. The present study revealed that a potential therapeutic strategy for the treatment of DN may comprise the upregulation of KLF5 expression.

Acknowledgements
Not applicable.

Funding
The present study was supported by the Natural Science Fund project in Shandong Province (grant no. 2017GSF21116).

Availability of data and materials
All data generated and/or analyzed during this study are included in this published article.

Authors’ contributions
YL wrote the main manuscript. YL, XH and XS performed the experiments. YL and ZH designed the study. XS and XH performed data analysis. YL, XS and ZH contributed to manuscript revisions. All authors reviewed the 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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