## *Review Article*
# Expression and effects of miR-26a-5p in regulating the PTEN/AKT pathway in renal tissues and cells of diabetic mice

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**Abstract:** This study aims to investigate the expression and effects of miR-26a-5p in regulating the PTEN/AKT pathway in renal tissues and cells of diabetic mice. A total of 40 mice were stochastically divided into a normal control group (n=10) and a diabetes model group (DM group, n=30). Normal diet was adopted in the normal group, while high-fat diet was adopted in the DM group. The expression changes of miR-26a-5p and PTEN/AKT in the murine renal tissues were dynamically measured by real-time fluorescence quantitative PCR. The histopathological changes of the renal tissues were observed with H&E under colored light microscope. Through detecting miR-26a-5p and PTEN/AKT expression in mouse tissues and renal cells and transfecting the them into cells by up-regulating or down-regulating their expression; the levels of apoptosis-related proteins Caspase 3, Caspase 9 and Bax/Bcl-2 were analyzed, and cell proliferation, invasion and apoptosis mechanisms were studied. miR-26a-5p was less expressed in the DM group but had more highly expressed PTEN/AKT. miR-26a-5p down-regulation or PTEN/AKT up-regulation could notably promote malignant proliferation and invasion in diabetic mice, inhibit cell apoptosis levels, and down-regulate the expression and protein levels of Caspase 3, Caspase 9, Bax/Bcl-2. Dual luciferase reporter confirmed that miR-26a-5p had targeted relationship with PTEN. When up-regulating miR-26a-5p and down-regulating PTEN/AKT at the same time, the malignant progression of cells was not different from miR-NC transfected cells with unrelated sequences. miR-26a-5p promotes cell survival in diabetic mice by down-regulating PTEN/AKT, which may become a new direction for diagnosis and gene therapy of diabetes.

**Keywords:** miR-26a-5p, PTEN/AKT, diabetic mice, cell expression

## Introduction

Diabetes is a metabolic disease mainly characterized by hyperglycemia [1]. As the body of diabetic patients is in a high glucose state for a long time, it will lead to lowered immune function; thus causing a series of pathological changes in organs, blood vessels and tissues [2, 3]. As people's living standards improve, the incidence of diabetes is also rising year by year. Some studies have shown that the pathogenesis of diabetes is affecting younger and younger people, but there is still a risk of disease in all age groups [4, 5]. At present, there is no complete cure for diabetes in the clinic, and blood sugar is maintained only with a long cycle of medication [6]. Studies have shown that there

is a lack of standardized measures for the unified diagnosis and disease-related complications of diabetes [7]. Therefore, we continue to clarify the relevant mechanisms of diabetes and to seek new potential therapeutic targets to improve the prognosis of diabetic patients [8].

miRNA is a short non-coding single-stranded RNA that regulates gene expression through base pairing and 3' UTR binding [9]. There are significant differences between miRNA in different tissues and at different growth stages, indicating that miRNA has different spatio-temporal expression patterns [10]. Moreover, some studies have shown that miRNA can be used as a negative regulator of gene expression to regu-

late a series of biological functions, including cell proliferation, apoptosis, and tumor growth and metastasis [11, 12]. Studies have shown that miRNA produces a marked effect on the occurrence and progression of diabetes and its related complications [13]. For example, in the study of Yu C Y et al [14], up-regulation of miR-152b-5p inhibits JNK signaling pathway through negatively mediating DACT1, thus improving insulin sensitivity of diabetic patients and enhancing pancreatic β cell function. Furthermore, in the research of Duan X et al [15], hyperglycemia can down-regulate miR-223 and miR-146a expression, thus causing platelet activation in diabetic patients. It is also mentioned in their study that low expression of miR-223 and miR-146a may be risk factors for diabetic patients. miR-26a-5p participates in the pathogenesis of diabetes and shows an abnormal down-regulation in diabetes, suggesting that the disease can be regulated by miR-26a-5p through regulation of β cell function and insulin signaling pathways in a diabetic mouse model; and as such miR-26a-5p may be a new strategy in treating diabetes [16]. PTEN is a nucleoprotein and cytoplasmic protein [17] and an effective tumor inhibitor whose loss of function is often observed in hereditary and sporadic cancers. It is phosphatase dependent (and active) in cells and controls multifarious biological processes, such as cell survival, metabolism and proliferation [18]. Moreover, studies have shown that PTEN is a candidate gene for susceptibility to type 2 diabetes [19]. The PTEN/AKT signaling pathway is involved in mediating high glucose treatment to induce X-box binding protein 1, which has an antagonistic effect on increased cell apoptosis and generation of reactive oxygen species [20].

We found that miR-26a-5p and PTEN have targeted sites on the target gene prediction website, and there may be a potential targeted relationship between the two. In this case, we hypothesized that both of them could mediate the PTEN/AKT pathway and have potential protective effects on renal tissues and cells of diabetic rats, which was verified as follows.

## Data and methods

### Experimental animals, materials and reagents

Forty 16-week-old C57BL/6 male mice (Junke Biological Co., Ltd., Nanjing, China, J006) with an average body weight of (27±3) g were pur-

chased. Their environmental adaptation conditions were as follows: The mice were housed for 12 h in light and 12 h in darkness, with an indoor temperature range of (22±2)°C and a humidity range of 50-65%. Water and food were provided according to animal care standard procedures, with one week of adaptation time. Materials and reagents used in this experiment were as follows: Human proximal renal tubular cells (ZEPING Bioscience & Technologies Co., Ltd., Beijing, China, CC-2553), microplate reader (Yanhui Biotechnology Co., Ltd., Shanghai, China, HBS-1096A), flow cytometer (Ranger Apparatus Co., Ltd., Shanghai, China, NovoCyte), ultraviolet spectrophotometer (PKU healthcare, China, UV-1100), PCR instrument (Image Trading Co., Ltd., Beijing, China, 100-073), dual luciferase reporting system (Top Biotech Co., Ltd., Shenzhen, China). Annexin V-FITC/PI double staining apoptosis kit, trypsin, PVDF membrane (Chreagen Biotechnology Co., Ltd., Beijing, China, 120248, 14260, ISEQ00-011), phosphate buffer (PBS), BCA kit, ECL luminescent reagent (Junrui Biotechnology Co., Ltd., Shanghai, China, JR01521, LCB004, UFML05294), DMEM comprising 10% fetal bovine serum (FBS) (Xinyu Biotech Co., Ltd., Shanghai, China, 19-0040-100). Lysate (Zhenyu Biotechnology Co., Ltd., Shanghai, China, PS0033), primary antibody diluent (Chreagen Biotechnology Co., Ltd., Beijing), horseradish peroxidase labeled goat anti-rabbit secondary antibody (Bersee Science and Technology Co., Ltd., Beijing, China, BHR201). Trizol kit (Mingjing Biology Co., Ltd., Shanghai, China, 50030-50), reverse transcription kit (Qiming Biotechnology Co., Ltd., Shanghai, China, OX02700), SYBR Premix Ex Taq TM kit (Yihui Biological Technology Co., Ltd., Shanghai, China, HRR-420A), Lipofectamine™ 2000 (Mito Biotechnology Co., Ltd., Shanghai, China, 11668019), TUNEL apoptosis detection kit (Chreagen Biotechnology Co., Ltd., Beijing, China, 10462). Gas analyzer, modular incubator (Yuyan Instruments Co., Ltd., Shanghai, 53222, customized).

This study has been approved by the animal experimental ethics committee of Laboratory Animal Center, Southern Medical University.

### Modeling

Forty mice were stochastically and averagely divided into the healthy control group, diabetes group (DM), DM+miR-26a-5p-mimics group,

and DM+si-PTEN group. Model establishment [21]: mice in the ND group were fed with common feed and grew completely naturally, and mice in the other groups were fed with self-prepared high-sugar and high-fat food. After a single injection of STZ (35 mg/kg), blood was collected from the caudal apex vein of all mice after fasting for 12 h to measure the fast blood glucose (FBG) of the mice. If the FBG value was ≥11.1 mmol/L, the modeling was successful.

*Cell culture and transfection*

The purchased human proximal renal tubular cells were placed in DMEM comprising 10% FBS, and cultured in a 5% $CO_2$ and 37°C incubator. Transfection was carried out after cell passaging. Mimics (over-expression sequence of miR-26a-5p), inhibitor (inhibition sequence of miR-26a-5p), miR negative control (miR-NC), targeted PTEN RNA (si-PTEN), targeted over-expression PTEN RNA (sh-PTEN), and negative control RNA (si-NC) were respectively transfected into the cells. DM models were established in all groups except the control group. After the cells of each group adhered to the wall and grew to a certain density, the culture solution was discarded, and cultured in DMEM with 25 mmol/L glucose.

*Observation of renal histomorphology*

Renal tissue was fixed with 4% neutral formaldehyde, and paraffin sections were cut after embedding and fixation. The histomorphological changes of the renal tissues were observed with H&E under colored light microscope.

*RT-PCR detecting the expression of miR-26a-5p and PTEN*

The total RNA was extracted using the Trizol kit. RNA purity, concentration and integrity were determined by UV-1500 spectrophotometer and agarose gel electrophoresis. RNA was reverse-transcribed into cDNA with the reverse transcription kit. SYBR Premix Ex Taq TM kit was used in this study. With GAPDH or U6 as internal reference, the PCR reaction was carried out. PCR amplification conditions were as follows: first, pre-denaturation at 95°C for 10 min, then denaturation at 95°C for 15 s, and annealing/extension at 60°C for 60 seconds, a total of 40 cycles were performed. The data were obtained after three replicate experiments, and the relative expression was calculated using 2-ΔΔCT. See **Table 1**.

*WB detection*

Fifty mg of renal tissue was taken, and 500 µL of lysate was added for lysis. After homogenization in an ice bath, centrifugation was carried out at 12,000× g, at 4°C for 20 min. The supernatant was taken and the BCA kit was used to determine the protein concentration. Twelve % SDS-PAGE electrophoresis was applied for separation. Afterwards it was transferred to a PVDF membrane, placed in 5% skim milk for sealing, and the immune reaction was conducted. Incubation of the membrane was conducted with primary antibody with a dilution of 1:1000 at 4°C overnight. Then the membrane was washed to remove the primary antibody, and horseradish peroxidase-labeled goat anti-rabbit secondary antibody with a dilution of 1:1000 was added to it, incubated at 37°C for 1 h, and rinsed with PBS for 3 times, 5 min each time. When the ECL luminescent reagent was developed and fixed, the image was taken by the Quantity One infrared imaging system. The relative expression level of the protein to be detected = gray value of the band to be detected/gray value of the internal reference protein band.

*Cell proliferation detection (CCK-8)*

Cells were collected 24 hours after transfection, adjusted to $4*10^6$ cells, and inoculated into 96-well plates. Then they were cultured for 24 h, 48 h, 72 h and 96 h, respectively. Ten µL CCK solution and 90 µL basic medium (DMEM) were added to each well, culturing for 2 h at 37°C. The OD value of each group of cells was measured under 570 nm absorbance using an enzyme reader.

*Cell apoptosis detection (flow cytometry)*

Digestion of transfected cells was performed by the aid of 0.25% trypsin, and then they were rinsed twice with PBS. Binding buffer (100 µL) was added to it to configure a suspension of $1*10^6$/mL. Annexinv-FITC and PI were successively added, incubating at room temperature in the dark for 5 min. Determination was conducted with the help of flow cytometry, and the experiment was repeated 3 times, to take the average value.

**Table 1.** Primer sequences

| | Forward primer | Reverse primer |
|---|---|---|
| miR-26a-5p | 5'-ACACTCCAGCTGGGTTCAAGTAATCCAGGA-3' | 5'-TGGTGTCGTGGAGTCG-3' |
| U6 | 5'-CTCGCTTCGGCAGCACA-3' | 5'-ACGCTTCACGAATTTGCGT-3' |
| PTEN | 5'-AAAGGGACGAACTGGTGTATAG-3' | 5'-TGGTCCTTACTTCCCCATAGAA-3' |
| GAPDH | 5'-CGGAGTCAACGGATTTGGTCGTATTGG-3' | 5'-GCTCCTGGAAGATGGTGATGGGATTTCC-3' |

*Detection of dual luciferase activity*

miR-26a-5p downstream target genes were predicted via Targetscan7.2. PTEN 3' UTR-Wt, PTEN 3' UTR-Mut, miR-26a-5p-mimics and miR-NC were transferred into human proximal renal tubular cells using the Lipofectamine™ 2000 kit, and dual luciferase reporter kit was used for detection of luciferase activity.

*Statistical analysis*

In this study, GraphPad 6 was used for data analysis and image rendering. Independent sample t test was applied for comparison between two groups, and one-way ANOVA was adopted for comparison among groups, represented by F. LSD-t test was utilized for pairwise comparison afterwards. Repeated measurement ANOVA was utilized for multiple time points, represented by F. Bonferroni was used for back testing. When $P < 0.05$, statistical difference was indicated.

**Results**

*Expression of miR-26a-5p, PTEN and AKT in renal tissues of diabetic mice*

miR-26a-5p expression in renal tissues of the two groups of mice was detected. The miR-26a-5p expression in the DM group was remarkably reduced when compared with the control group ($P < 0.05$). However, the phosphorylation results of the PTEN/AKT pathway proteins in the renal tissues of the two groups revealed that the levels of p-PTEN and p-AKT were considerably increased in the DM group when compared with the control group ($P < 0.05$). After H&E staining, the renal histomorphology of the control group and the DM group was observed. In the control group, the mouse renal tubules were clear in structure, the basement membrane was intact, the epithelial cells of the tubules were arranged orderly, and there was no infiltration of inflammatory cells in the stroma. In the DM group, some renal tubular epithelial cells were vacuolated, inflammatory cells were infiltrated in the stroma, and lumen of some renal tubules were dilated. As shown in **Figure 1**.

*Effect of over-expression of miR-26a-5p on renal cell apoptosis and related proteins*

Cell proliferation and invasion ability in the DM group decreased notably, while the apoptosis rate increased when compared with the control group. The expression of Caspase-3, Caspase-9, Bax and Bcl-2 was remarkably elevated, while the expression of Bcl-2 was notably reduced. However, miR-26a-5p up-regulation in the DM+mimics group reversed the proliferation, invasion, apoptosis and protein phosphorylation in the PTEN/AKT pathway of the above cells, with statistically significant differences ($P < 0.05$). As shown in **Figure 2**.

*Inhibition of PTEN on renal cell apoptosis and related proteins*

Cell proliferation and invasion ability in the DM group decreased notably, while the apoptosis rate increased when compared with the control group. The expression of Caspase-3, Caspase-9, Bax and Bcl-2 were elevated remarkably, while Bcl-2 expression reduced notably. The levels of p-PTEN and p-AKT increased considerably, while DM+si-PTEN after downward adjustment of PTEN could reverse the results of proliferation, invasion, apoptosis and protein phosphorylation in the PTEN/AKT pathway, with a statistically significant difference ($P < 0.05$). As shown in **Figure 3**.

*Identification of miR-26a-5p target genes*

Targeted binding sites were found between PTEN and miR-26a-5p by Targetscan7.2. The results of the dual luciferase reporter showed that PTEN 3' UTR-Wt luciferase activity decreased remarkably after miR-26a-5p up-regulation ($P < 0.05$), but it did not affect PTEN 3' UTR-
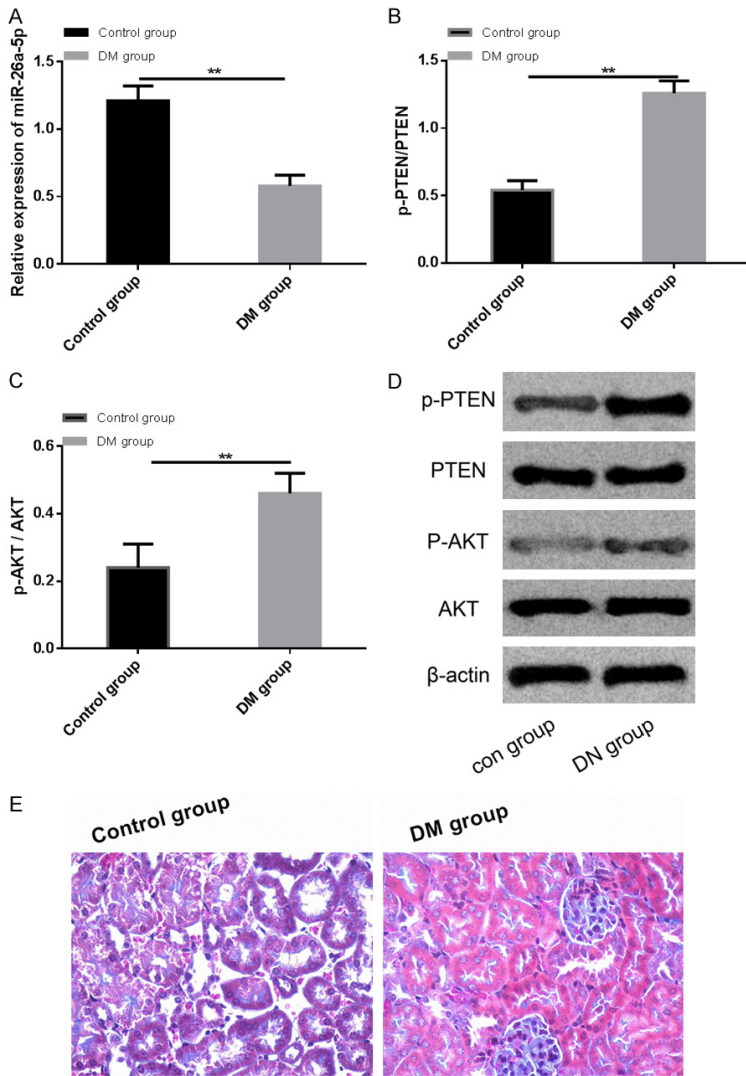
**Figure 1.** Expression of miR-26a-5p, PTEN and AKT in renal tissues of diabetic mice. A. Expression level of miR-26a-5p in tissues of mice in each group. B, C. Expression level of PTEN/AKT pathway protein phosphorylation in tissues of mice in each group. D. Protein map of PTEN/Akt pathway related proteins. E. The morphological observation of renal tissues in control group and DM group. Notes: Comparison with the control group or between the two groups, **P<0.01.

Mut luciferase activity (P>0.05). WB assay showed that PTEN protein expression was notably reduced after transfection of miR-26a-5p-mimics (P<0.05). As shown in **Figure 4**.
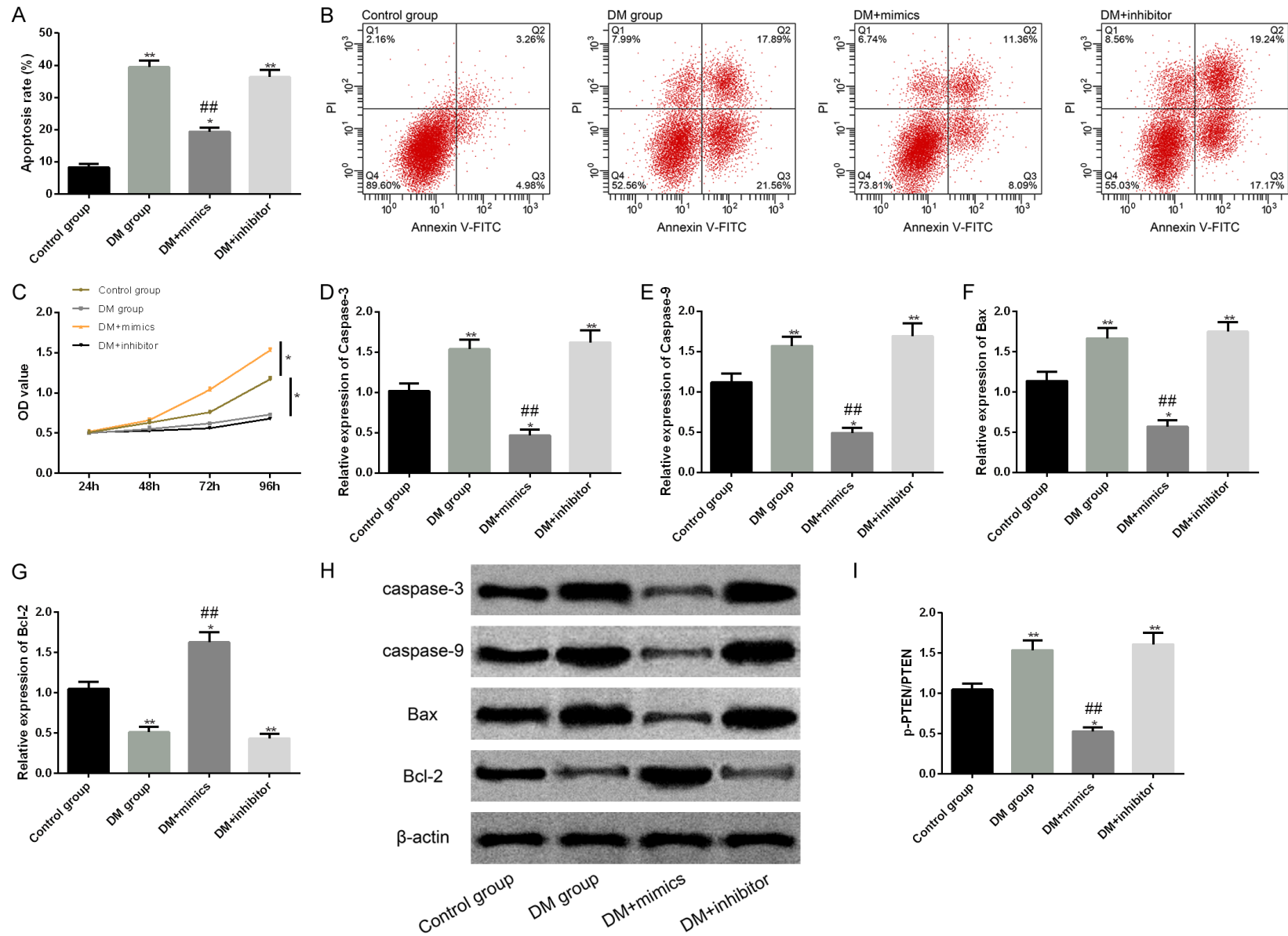
## Discussion

Diabetes, as a chronic disease with one of the highest incidences in the world, poses a great threat to human health [22]. Studies have reported that diabetes affects about one in eleven adults worldwide [23]. At present, it has

been proven to be one of the inducements of multiple diseases, such as nephropathy, chronic fatty liver, cerebrovascular disease and retinopathy [24]. The incidence of diabetes is not only high, but also seriously affects the physical health, mental health and quality of life of patients [25]. Currently, multiple studies have shown that miRNA is closely related to the biological function of diabetic renal cells. For example, when the glucose concentration in HK-2 cells increases, miR-155-5p expression will also increase, indicating that miR-155-5p up-regulation may suppress Sirtl, activate P53 and form a positive feedback loop [26]. miR-26a-5p has been found to be down-regulated in diabetic glomerular cells, and its down-regulation participates in the development of diabetic nephropathy in humans and in mice by enhancing TGF-β/CTGF signaling [27]. The mechanism of miR-26a-5p mediated PTEN/AKT pathway regulation in diabetic renal tissues and cells has not been clarified, and that is also a reason why this study paid attention to miR-26a-5p for relevant exploration.
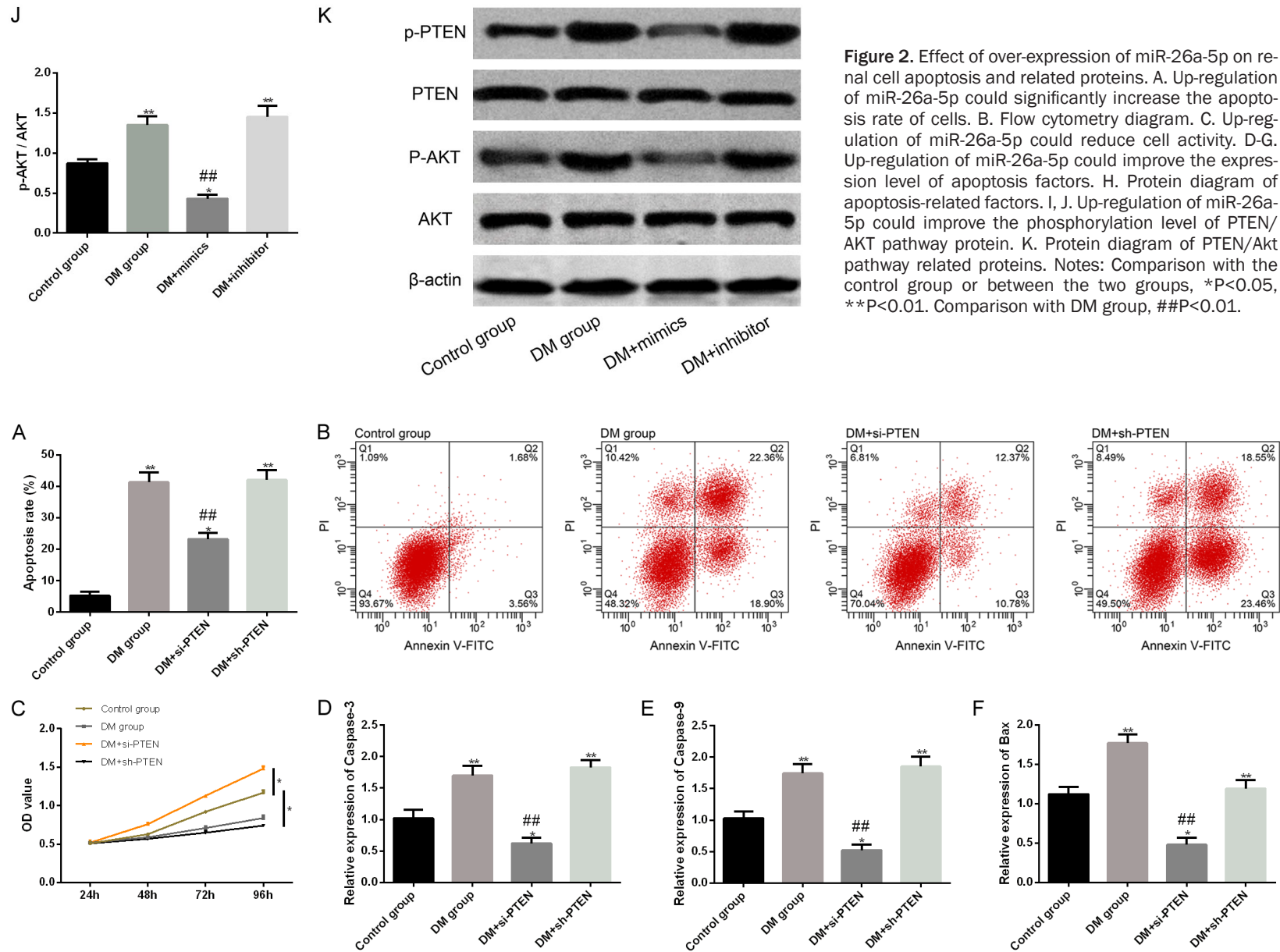
More and more research has paid attention to the regulatory mechanisms of miR-26a-5p in diabetes, and have carried out related studies. Such as the report of Ma H et al [28], in which miR-26a-5p is revealed to inhibit autoreactive T cells and expand Treg in diabetic rats *in vivo* and *in vitro*, and can partially inhibit autoimmune diabetes in non-obese diabetic mice by promoting the expression of regulatory T cells. miR-26a-5p is reported to have the ability to improve myocardial apoptosis-related proteins in diabetic rats by regulating PTEN and prevent myocardial injury in diabetic rats [29]. In this study, miR-26a-5p expression notably decreased PTEN/AKT path-

Expression and effect of miR-26a-5p in renal tissues and cells of diabetic mice

**Figure 2.** Effect of over-expression of miR-26a-5p on renal cell apoptosis and related proteins. A. Up-regulation of miR-26a-5p could significantly increase the apoptosis rate of cells. B. Flow cytometry diagram. C. Up-regulation of miR-26a-5p could reduce cell activity. D-G. Up-regulation of miR-26a-5p could improve the expression level of apoptosis factors. H. Protein diagram of apoptosis-related factors. I, J. Up-regulation of miR-26a-5p could improve the phosphorylation level of PTEN/AKT pathway protein. K. Protein diagram of PTEN/Akt pathway related proteins. Notes: Comparison with the control group or between the two groups, *P<0.05, **P<0.01. Comparison with DM group, ##P<0.01.
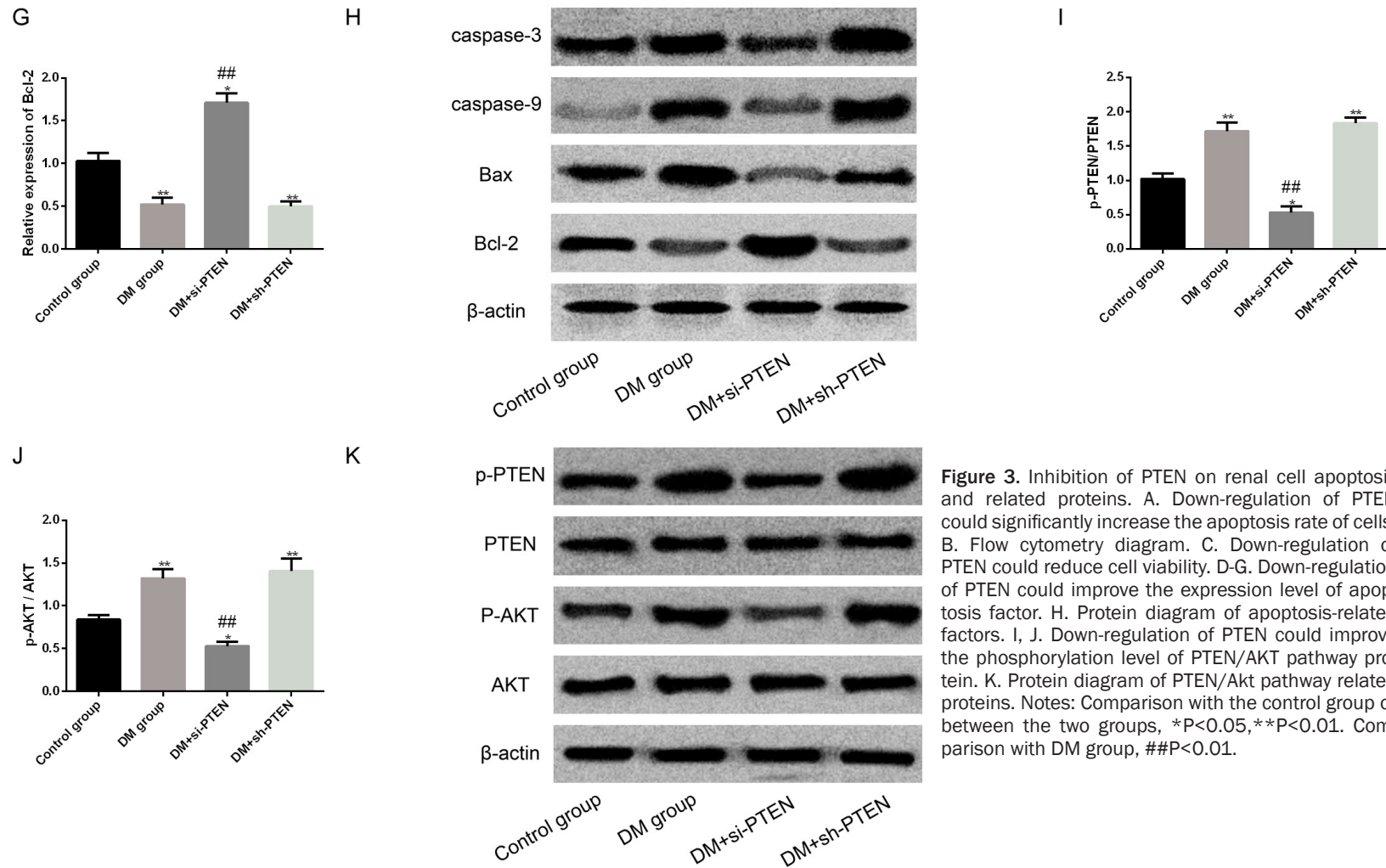
Figure 3. Inhibition of PTEN on renal cell apoptosis and related proteins. A. Down-regulation of PTEN could significantly increase the apoptosis rate of cells. B. Flow cytometry diagram. C. Down-regulation of PTEN could reduce cell viability. D-G. Down-regulation of PTEN could improve the expression level of apoptosis factor. H. Protein diagram of apoptosis-related factors. I, J. Down-regulation of PTEN could improve the phosphorylation level of PTEN/AKT pathway protein. K. Protein diagram of PTEN/Akt pathway related proteins. Notes: Comparison with the control group or between the two groups, *P<0.05,**P<0.01. Comparison with DM group, ##P<0.01.
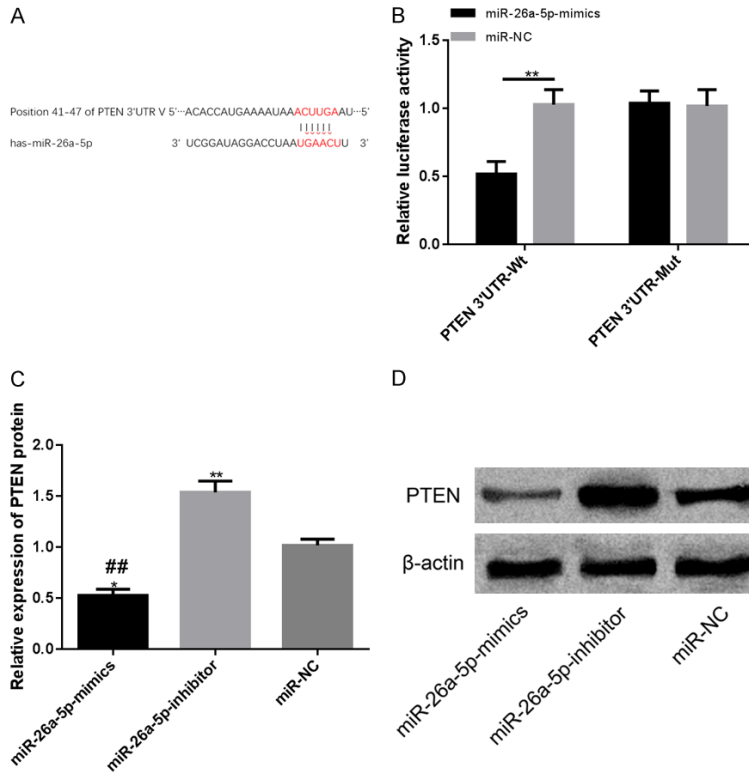
**Figure 4.** Detection of dual luciferase reporter. A. Targeted binding sites existed between miR-26a-5p and PTEN. B. Relative luciferase activity-dual luciferase reporter. C. Expression of PTEN protein in proximal renal tubular cells after transfection. D. Protein diagram. Note: **P<0.01.

way protein phosphorylation expression which was remarkably elevated after high glucose induction in mice compared with the control group. These findings indicate that miR-26a-5p and PTEN/AKT may participate in the pathological mechanisms of diabetic mice. To this end, we conducted an in-depth exploration to understand the potential regulatory mechanisms of the two. In *in vitro* experiments, we transfected miR-26a-5p in human proximal tubular cells after high glucose intervention, and analyzed apoptosis, proliferation, and the PTEN/AKT signal transduction pathway. The results revealed that the DM group showed a remarkably higher apoptosis rate. Except for Bcl-2, other apoptosis-related factors Caspase-3, Caspase-9 and Bax also showed higher levels. The cell activity was notably inhibited, and the levels of p-PTEN and p-AKT proteins in PTEN/AKT pathway were remarkably increased. In the study of team of Jiang Z [30], Caspase-3, Caspase-9 and Bax, as apoptosis-promoting factors, showed higher levels in the

high glucose-induced group, while Bcl-2, as an anti-apoptosis factor, exhibits the opposite results. After treatment and intervention, the above results were reversed, which is similar to our research results.

At present, there is little research on the specific protective mechanism of PTEN/AKT signaling pathway for diabetic renal cell apoptosis. It is understood that PTEN is a tumor suppressor gene which mutates frequently in numerous cancers [31]. Its function in the cytoplasm mostly depends on the activity of its lipid phosphatase, and it can also antagonize the carcinogenic pathway of P13K-AKT [32, 33]. In this study, after transfection of PTEN, the DM group was found to have a considerably higher apoptosis rate. Except for Bcl-2, the other apoptosis-related factors Caspase-3, Caspase-9 and Bax showed higher levels, the cell activity was remarkably inhibited, and the levels of p-PTEN and p-AKT proteins in the PTEN/AKT pathway increased considerably. However, when miR-26a-5p and PTEN were over-expressed and knocked down, apoptosis, proliferation and PTEN/AKT pathway protein phosphorylation level induced by high glucose were significantly reversed. Dual luciferase reporter confirmed that miR-26a-5p had a targeted relationship with PTEN in this paper. If miR-26a-5p expression was increased, PTEN protein level would be notably inhibited. The above studies show that miR-26a-5p up-regulation can inhibit PTEN regulation of the PTEN/AKT pathway in a targeted manner, which has protective effects on renal cell function induced by high glucose.

To sum up, miR-26a-5p promotes cell survival in diabetic mice by down-regulating PTEN/AKT, which may become a new direction for diagnosis and gene therapy of diabetes. However, there is still room for improvement in this study. First of all, we can supplement the research on whether miR-26a-5p and PTEN have

inhibitory effects on inflammatory factors and explore their potential protective effects on diabetic kidney cells. In addition, we can also increase their regulation of oxidative stress indicators to explore whether they participate in the protective mechanism of oxidative stress injury.

## Acknowledgements

## Disclosure of conflict of interest

None.

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