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MicroRNA-770-5p is involved in the development of diabetic nephropathy through regulating podocyte apoptosis by targeting TP53 regulated inhibitor of apoptosis 1

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Abstract. – OBJECTIVE: Diabetic nephropathy (DN) is a major diabetic micro-vascular complication, and podocyte apoptosis induced by high glucose (HG) is a typical early feature of DN. Studies have shown that microRNAs (miRNAs) play a crucial role in the pathogenesis of DN. The purpose of the current study was to explore the role and molecular mechanism of miR-770-5p in podocyte apoptosis in DN.

PATIENTS AND MATERIALS AND METHODS: *In vitro* podocyte model of DN was conducted by treatment conditionally immortalized mouse podocytes with HG (30 mM D-glucose). The level of miR-770-5p in podocytes was detected using quantitative real-time PCR (qRT-PCR), and protein levels were measured using Western blot assay in our current study. The relationship between miR-770-5p and TP53 regulated inhibitor of apoptosis 1 (TRIAP1) was revealed by TargetScan and dual luciferase reporter assay. Cell proliferation ability and cell apoptosis were determined by using cell counting kit-8 (CCK-8) assay and flow cytometer (FCM), respectively.

RESULTS: We found that miR-770-5p was significantly upregulated in podocytes under HG condition. TRIAP1 was a target gene of miR-770-5p and it was down-regulated in podocytes by HG treatment. Further analysis indicated that HG induced cell proliferation ability reduction, cell apoptosis enhancement and apoptotic peptidase activating factor 1(APAF1)/Caspase9 pathway exaltation in podocytes were prevented by miR-770-5p down-regulation. More importantly, the results showed that all the effects of miR-770-5p inhibitor on HG induced podocytes were eliminated by TRIAP1 silencing.

CONCLUSIONS: We showed that miR-770-5p was upregulated in the *in vitro* model of DN, and it might promote the development of DN through regulating podocyte apoptosis by targeting TRIAP1.

Key Words:
Diabetic nephropathy, Podocyte apoptosis, miR-770-5p, TRIAP1.

Introduction

The incidence of diabetes mellitus (DM) has increased year by year worldwide, and the recent clinical epidemiology shows that the incidence of diabetes in China is 10.9%[1]. Diabetic nephropathy (DN) is a kind of diabetic complication with high incidence, and is an important cause of end-stage renal failure[2]. The incidence of DN is increasing every year, and it has become a global public health problem that endangers human health. There is a close relationship between the incidence of DN and the course of this disease. Without effective treatment, DN can easily progress to uremia, which poses a great threat to the life and health of patients and brings a large economic burden to patients. Currently, there is no specific treatment method for DN[3]. Therefore, exploring the pathogenesis of DN, finding safe and effective treatment strategies have always been a hot topic in the field of diabetes. Proteinuria is a common clinical symptom of DN and an independent risk factor for the development of

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DN[4]. Although pathological changes in DN include accumulation of extracellular matrix and thickening of the basement membrane, these changes do not fully explain the cause of proteinuria formation[5,6]. More and more studies[7,8] have shown that the injury of renal vesicle epithelial cells (podocytes) is involved in the occurrence of DN proteinuria, which is the central target of the pathogenesis and progression of DN. The mechanism of diabetic podocyte injury is complicated, and factors such as inflammation, oxidative stress and activation of renin-angiotensin-aldosterone system are involved in the occurrence of podocyte injury. In addition, a large number of researches have shown that hyperglycemia is likely to cause podocyte damage, and over-expression of reactive oxygen species may lead to podocyte apoptosis. However, the relevant mechanism of action is still unclear, and there is no suitable method to prevent podocyte apoptosis in such patients. MicroRNAs (miRNAs) are a class of small non-coding RNAs, which can post-transcriptionally regulate gene expression through the degradation or translation inhibition of their target mRNAs via completely or partially binding to the 3' untranslated regions (UTR) of their multiple target mRNAs[9,10]. Studies[10-13] have indicated that miRNAs play critical roles in a variety of biological processes, including cell differentiation proliferation, and apoptosis. In recent years, there has been increasing evidences indicted that miRNAs are involved in the pathogenesis of DN by regulating biological signaling pathways[14]. MiR-22 is involved in DN progression through promoting renal tubulointerstitial fibrosis by targeting phosphatase and tensin homolog (PTEN)[15]. MiR-29c participates in DN via regulating the production of inflammatory cytokines[16]. MiR-130b may be involved in the pathological mechanism of DN, such as lipid metabolic disorders, oxidative stress, extracellular matrix deposition and renal fibrosis[17]. MiRNAs induced DN progression involves a variety of renal pathological changes, especially the abnormal proliferation and apoptosis of podocytes[18-22]. Park et al[23] has revealed that miR-770 was significantly up-regulated in both blood and urine from patients with DN, indicating the involvement of miR-770 in the occurrence and development of DN. However, the specific role and molecular mechanism of miR-770-5p in DN remain unclear. The present work aimed to investigate whether miR-770-5p had an effect on podocyte apoptosis, and further to explore the underlying mechanism.

Materials and Methods

Podocytes Culture and Treatment

Conditionally immortalized mouse podocytes (Cat No. C0906) were obtained from Shanghai Guandao Biological Engineering Co., Ltd. (Shanghai, China). Podocytes were grown in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA), 1% penicillin-streptomycin and 10 U/ml γ-interferon, and incubated at 33°C with 5% CO^2. After cells were 80-90% confluent, the podocytes were cultured in 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C with 5% CO2 in the absence of γ-interferon for 10-14 days to induce cell differentiation. After 12 hours of serum starvation, the podocytes were cultured for 24, 48 or 72 hours under conditions of high glucose (HG, 30 mM D-glucose) or normal glucose (NG, 5 mM D-glucose).

Quantitative Real-time PCR (qRT-PCR)

Total RNA from cells was isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. QRT-PCR was performed for the measurement of gene expression using the Mir-X miRNA First-Strand Synthesis and SYBR qRTPCR Kits (TaKaRa, Otsu, Shiga, Japan) according to the manufacturer's instructions. U6 and GAPDH were used as internal reference for the relative expression of miRNA and mRNA. Relative gene expression was calculated by using the 2-ΔΔCt method (Table I).

Western Blot Analysis

Proteins from podocytes from different groups were isolated using RIPA buffer (Beyotime Institute of Biotechnology, Shanghai, China) as per the manufacturer's instructions. We used the bicinchoninic acid assay kit (BCA; Pierce Biotechnology, Rockford, IL, USA) to detect the concentration of the protein samples. Protein samples (30 μg per lane) were separated on 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Merck Millipore, Billerica, MA, USA). After blocking

Table I. Primer sequences for PCR.

Sequence (5′-3′)
U6 forward, 5′-GCTTCGGCAGCACATATACTAAAAT-3′; Reverse, 5′-CGCTTCACGAATTTGCGTGTCAT-3′. GAPDH forward, 5′-CTTTGGTATCGTGGAAGGACTC-3′; Reverse, 5′-GTAGAGGCAGGGATGATGTTCT-3′. miR-770-5p forward, 5'-CCAGTACCACGTGTCAG-3'; Reverse, 5'-GAACATGTCTGCGTATCTC-3'. TRIAP1 forward, 5′-GCACCGACCTCTTCAAGC-3′; Reverse, 5′-CCATGAACTCCAGTCCTTCA3′. APAF1 forward, 5′-AACCAGGATGGGTCACCATA-3′; Reverse, 5′-ACTGAAACCCAATGCACTCC-3′. Caspase3 forward, 5′-TGTCGATGCAGCAAACCTCA-3′; Reverse, 5′-GACTTCTACAACGATCCCCTC-3′. Caspase7 forward, 5′-GATCAGCCTTGTGGGATGGCAGA-3′; Reverse, 5′-GTACTGATATGTAGGCACTCG-3′. Caspase9 forward, 5′-TGTTTCCGAGCGAGGGATTT-3′; Reverse, 5′-CGCAGGAAGGTTTTGGGGTA-3′.

with 5% non-fat milk at room temperature for 1 h, the membranes were incubated with primary antibodies (TRIAP1, APAF1, Caspase^3, Caspase^7, Caspase^9 and β-actin) (Cell Signaling Technology Inc., Danvers, MA, USA) at 4°C overnight. Then, the membranes were incubated with the horseradish peroxidase(HRP)-conjugated secondary antibody, anti-rabbit IgG, HRP-linked antibody (Cell Signaling Technology Inc., Danvers, MA, USA) at room temperature for 2 h. Finally, the enhanced chemiluminescence (ECL) Western blotting detection kits (Merck Millipore, Billerica, MA, USA) was used to visualize protein bands according to the manufacture's protocol.

Cell Transfection

Podocytes were transfected with miR-770-5p inhibitor, inhibitor control, control-siRNA, TRIAP1-siRNA, or miR-770-5p inhibitor+TRIAP1-siRNA using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. 48 h after cell transfection, transfection efficiency was detected using qRT-PCR.

Cell Counting kit-8 (CCK-8) Assay

The proliferation ability was determined using CCK-8 assay in the current study. Briefly, after specific treatments, podocytes were seeded in 96-well plates at a density of 5×103 cells per well and cultured at 37°C with 5% CO2. 24 h later, each well was added with 10 μl CCK-8 solution (Keygen, Nanjing, Jiangsu, China) and incubated at 37°C for additional 30 minutes. At last, absorbance at 450 nm was detected by FLUOstar® Omega Microplate Reader (BMG LABTECH, Ortenberg, Germany) to evaluate cell proliferation ability. This test was repeated three times.

Cell Apoptosis Assay

The apoptosis of podocytes was analyzed by flow cytometer (FCM) using the Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit (Cat. No. 70-AP101-100; MultiSciences, Hangzhou, Zhejiang, China) according to the manufacturer's instructions. Podocytes were per-transfected with miR-770-5p inhibitor, inhibitor control, or miR-770-5p inhibitor+TRIAP1-siRNA for 6 h and then cultured under condition of high glucose (HG, 30 mM D-glucose) for 48 h. Cells from the control group were grown in normal glucose (NG, 5 mM D-glucose). Podocytes were harvested with 0.25% Trypsin and centrifuged at 1000 rpm for 10 min. After washing with PBS three times and the cells were then stained with 5 μl Annexin V- FITC and 5 μl PI for 30 min at room temperature without light. At the end of the experiment, cell apoptosis was analyzed by using flow cytometer (Becton Dickinson, Franklin Lake, NJ, USA) according to the manufacturer's instructions.

Dual Luciferase Reporter Assay

We used TargetScan bioinformatics software (www.targetscan.org/vert_71) to predict the target genes of miR-770-5p in the current study, and binding sites between TRIAP1 and miR-770-5p were observed. Then, to validate whether miR-770-5p directly targets the 3'-untranslated region (3'-UTR) of TRIAP1, we performed dual luciferase reporter assay. The wild type (WT-TRIAP1) and mutant (MUT-TRIAP1) 3'-UTR of TRIAP1 were cloned into a pmiR-RB-ReportTM dual luciferase reporter gene plasmid vector (Guangzhou RiboBio Co., Ltd., Guangzhou, Guangdong, China) according to the manufacturer's instructions. Podocytes were co-transfected with WT-TRIAP1 or MUT-TRIAP1 and miR-770-5p mimic or mimic control using Lipofectamine® 2000 (Invitrogen, Carlsbad, CA, USA) as per the manufacturer's protocols. 48 h later, the dual-luciferase assay system (Promega Corporation, Madison, WI, USA) was applied to determine the luciferase activity. Luciferase activity was normalized to the Renilla luciferase activity.

Statistical Analysis

All the experiments in the current study were performed three times. Data were presented as the mean ± SD and analyzed by SPSS software version 17.0 (SPSS Inc., Chicago, IL, USA). Comparison

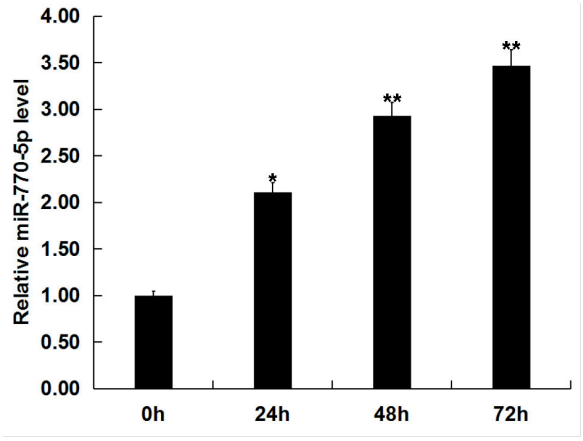

Figure 1. MiR-770-5p was up-regulated in HG-treated podocytes. The level of miR-770-5p in podocytes, which were cultured under high glucose (HG, 30 mM D-glucose) condition for 0, 24, 48 or 72 hours respectively, was detected using qRT-PCR. 0 h indicated podocytes were cultured in normal glucose (NG, 5 mM D-glucose) condition. Data were displayed as mean ±SD. *, **p<0.05, 0.01 *vs.* 0 h.

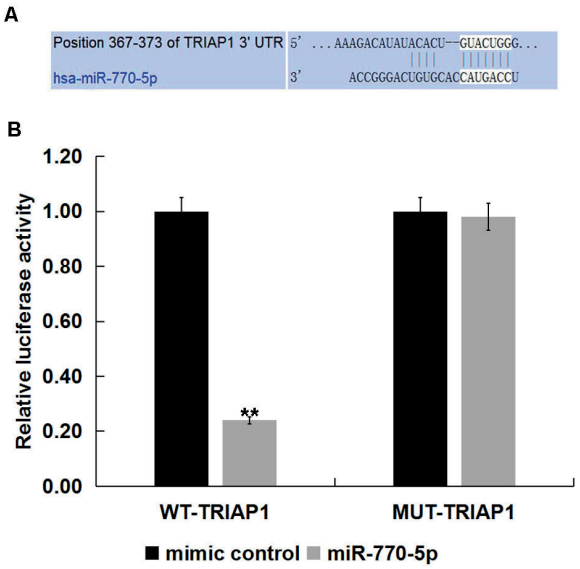

Figure 2. TRIAP1 is a direct target of miR-770-5p. ***A,*** Interaction between miR-770-5p and 3'UTR of TRIAP1 was predicted using TargetScan; ***B,*** Luciferase activity of a reporter containing a wild-type TRIAP1 3'UTR or a mutant TRIAP1 3' UTR are presented. "MUT-TRIAP1" indicates the TRIAP1 3' UTR with a mutation in the miR-770-5p binding site. UTR, untranslated region. All data are presented as the mean±SD of three independent experiments. ** p<0.01 vs. mimic control.

between groups was made by one-way analysis of variance with Tukey's post-hoc test or Student's *t*-test. p<0.05 indicated statistically significant.

Results

MiR-770-5p was Upregulated in Podocytes Under High Glucose Condition

To investigate the role of miR-770-5p in diabetic nephropathy, we explored the potential function of miR-770-5p in HG-treated podocytes. Firstly, we detected the level of miR-770-5p in podocytes, which were cultured under high glucose (HG, 30 mM D-glucose) condition for 0, 24,

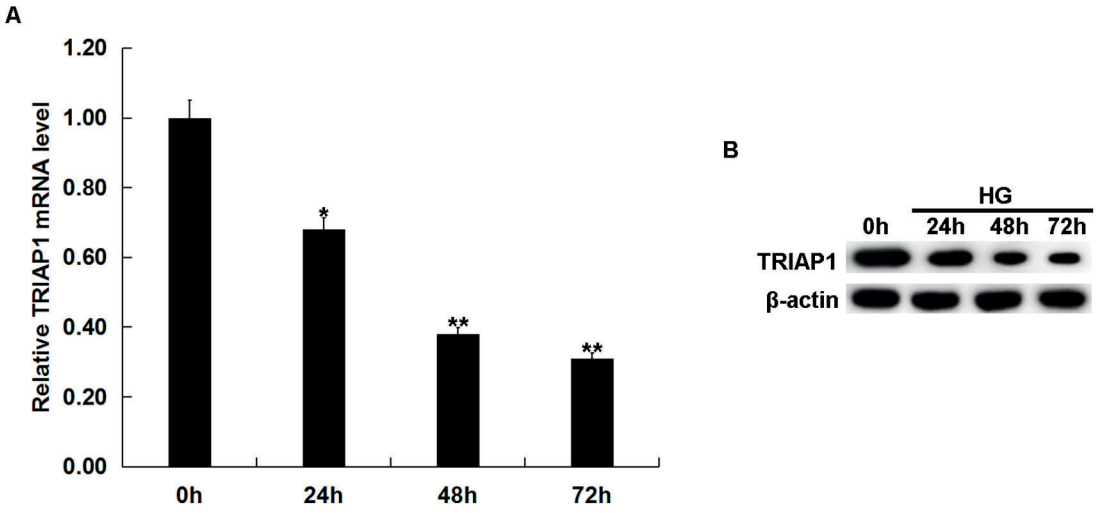

Figure 3. TRIAP1 was downregulated in HG-treated podocytes. The mRNA ***(A)*** and protein ***(B)*** level of TRIAP1 in podocytes, which were cultured under high glucose (HG, 30 mM D-glucose) condition for 0, 24, 48 or 72 hours respectively, was detected using qRT-PCR and Western blotting respectively. 0 h indicated podocytes were cultured in normal glucose (NG, 5 mM D-glucose) condition. Data were displayed as mean ±SD. *, **p<0.05, 0.01 *vs.* 0 h.

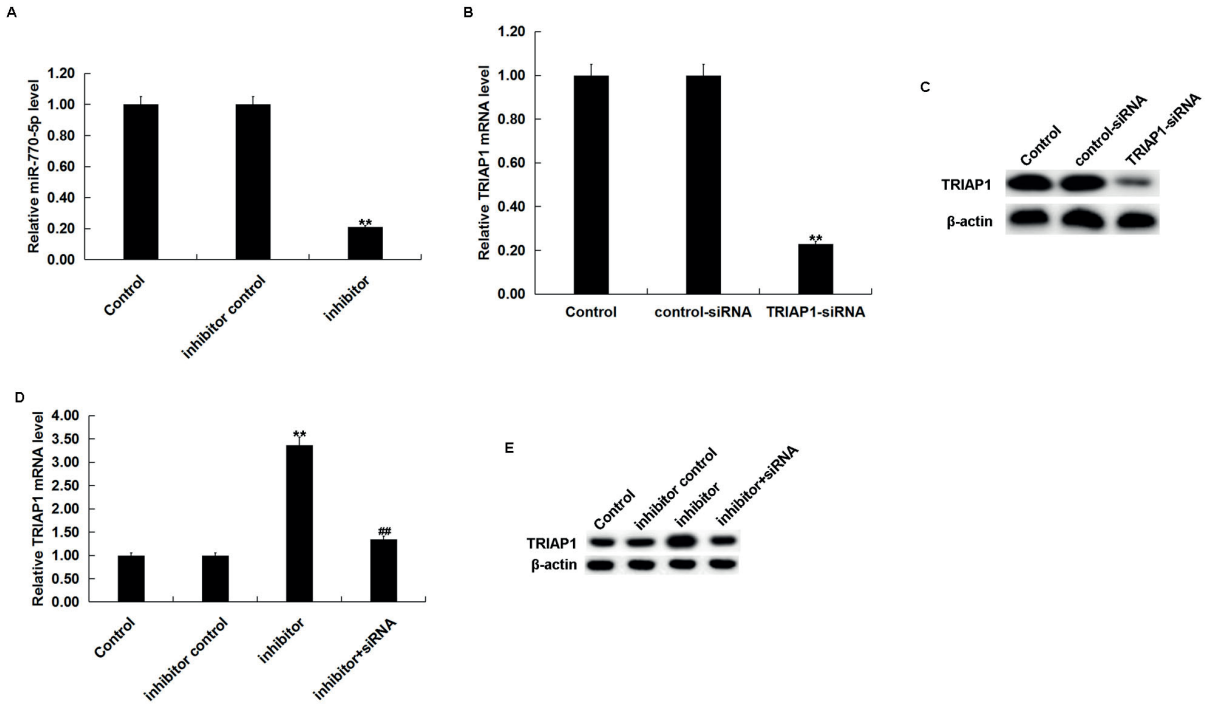

Figure 4. miR-770-5p inhibitor enhanced TRIAP1 expression in podocytes. ***A*,** Relative miR-770-5p expression in podocytes in different groups; ***B*** and ***D***: Relative TRIAP1 mRNA expression in podocytes in different groups; ***C*** and ***E:*** Protein level of TRIAP1 in podocytes in different groups. Data were displayed as mean ±SD. ***p*<0.01 ***vs***. control group; ##*p*<0.01 *vs.* inhibitor group.

48 or 72 hours respectively, using qRT-PCR. 0 h indicated that podocytes were cultured in normal glucose (NG, 5 mM D-glucose) condition. The results showed that the level of miR-770-5p in podocytes was up-regulated after HG treatment in a time-dependent manner (Figure 1), indicating that miR-770-5p might play an important role in HG-treated podocytes.

MiR-770-5p Direct Targets TRIAP1

The data of TargetScan (http://www.targetscan.org) showed the binding sites between TRIAP1 and miR-770-5p (Figure 2A). To reveal the potential binding sites of TRIAP1 and miR-770-5p, luciferase reporter assay was performed. Compared with cells co-transfection with MUT-TRIAP1 and miR-770-5p mimic, the luciferase activity was significantly decreased in cells co-transfected with WT-TRIAP1 and miR-770-5p mimic (Figure 2B). The data indicated that miR-770-5p directly targets TRIAP1.

TRIAP1 was Down-Regulated in Podocytes Under High Glucose Condition

Then, we used qRT-PCR and Western blot assay to measure the mRNA and protein expression of TRIAP1 in podocytes at different time-points (0, 24, 48, and 72 h) following HG stimulation. Results indicated that both mRNA and protein levels of TRIAP1 in podocytes were down-regulated after HG treatment in a time-dependent manner (Figure 3).

MiR-770-5p Inhibition Promoted the Proliferation of Podocytes by Targeting TRIAP1

Then, to study the effect of miR-770-5p on the proliferation ability of podocytes, podocytes were pre-transfected with miR-770-5p inhibitor, inhibitor control, control-siRNA, TRIAP1-siRNA, or miR-770-5p inhibitor+TRIAP1-siRNA for 6 h and then cultured under condition of high glucose (HG, 30 mM D-glucose) for 48 h. The transfection efficiency was measured by qRT-PCR. As shown in Figure 4A-C, miR-770-5p inhibitor significantly down-regulated the miR-770-5p level in podocytes, and both the protein and mRNA levels of TRIAP1 in podocytes were significantly decreased by TRIAP1-siRNA. Moreover, miR-770-5p inhibitor significantly increased both the mRNA and protein levels of TRIAP1, and these increases were eliminated by TRIAP1-siRNA (Figure 4D

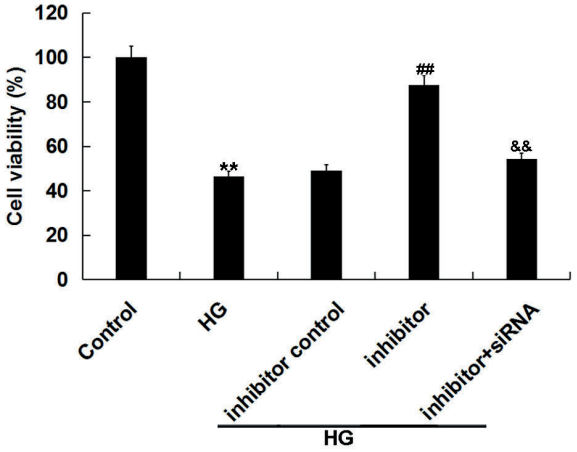

Figure 5. miR-770-5p inhibitor enhanced the cell viability of podocytes. Podocytes were pre-transfected with miR-770-5p inhibitor, inhibitor control, or miR-770-5p inhibitor+TRIAP1-siRNA for 6 h and then treated with high glucose (HG, 30 mM D-glucose) for 48 h. Then, cell viability was detected using MTT assay. Data were displayed as mean ±SD. **p<0.01 vs. control group; ## p<0.01 vs. HG treatment group; && p<0.01 vs. inhibitor group.

and E). Podocytes were pre-transfected with miR-770-5p inhibitor, inhibitor control, or miR-770-5p inhibitor+TRIAP1-siRNA for 6 h and then treated with high glucose (HG, 30 mM D-glucose) for 48 h. Next, to determine the proliferation ability of podocytes, CCK-8 assay was performed. Findings suggested that HG induced cell proliferation ability decrease was significantly inhibited by miR-770-5p inhibitor, and this effect was eliminated by TRIAP1-siRNA (Figure 5).

MiR-770-5p Inhibition Inhibited the Apoptosis of Podocytes by Targeting TRIAP1

Podocytes were pre-transfected with miR-770-5p inhibitor, inhibitor control, or miR-770-5p inhibitor+TRIAP1-siRNA for 6 h and then treated with high glucose (HG, 30 mM D-glucose) for 48 h. Then, to analyze cell apoptosis, FCM was performed. We found that compared with the control group, the apoptosis of podocytes significantly increased in HG treated podocytes. While miR-770-5p inhibitor significantly inhibited the apoptosis of podocytes, this inhibition was eliminated by TRIAP1 silencing (Figure 6).

MiR-770-5p Inhibition Inhibited APAF1/Caspase9 Pathway in Podocytes by Targeting TRIAP1

Finally, to explore the molecular mechanism of the effect of miR-770-5p on apoptosis in

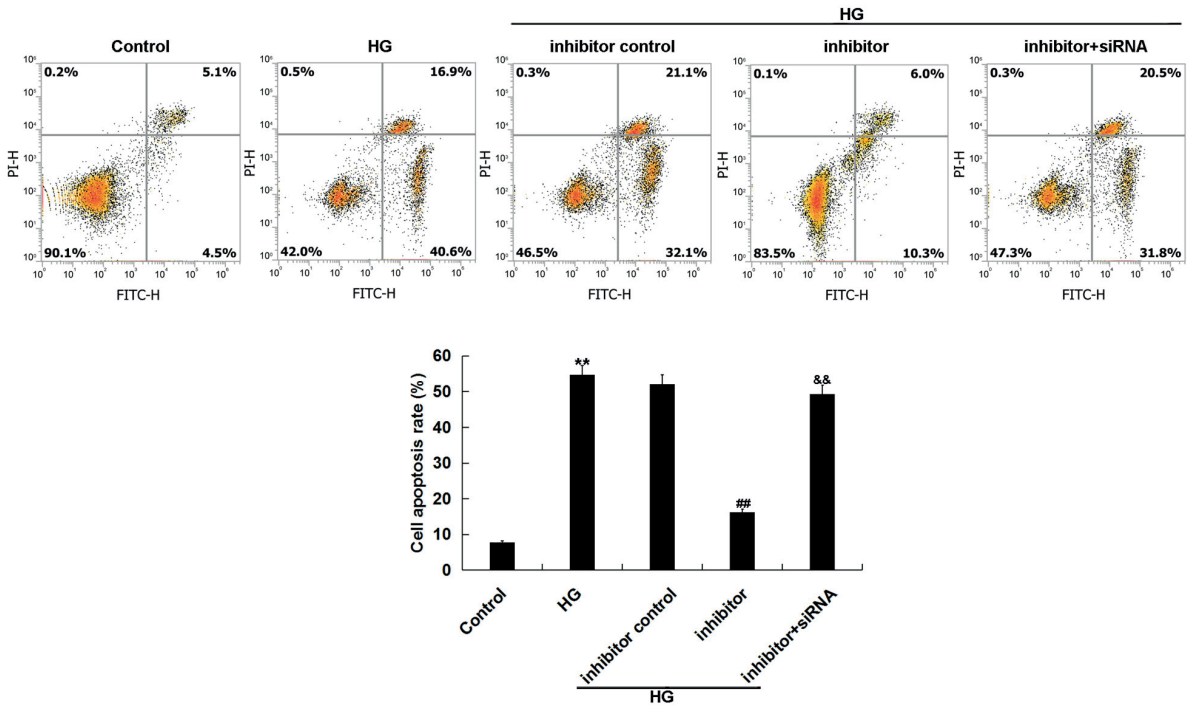

Figure 6. miR-770-5p inhibitor inhibited the cell apoptosis of podocytes. Podocytes were pre-transfected with miR-770-5p inhibitor, inhibitor control, or miR-770-5p inhibitor+TRIAP1-siRNA for 6 h and then treated with high glucose (HG, 30 mM D-glucose) for 48 h. Then cell apoptosis was analyzed using FCM and cell apoptosis rate was calculated. Data were displayed as mean ±SD. ** p<0.01 vs. control group; ## p<0.01 *vs.* HG treatment group; && p<0.01 *vs.* inhibitor group.

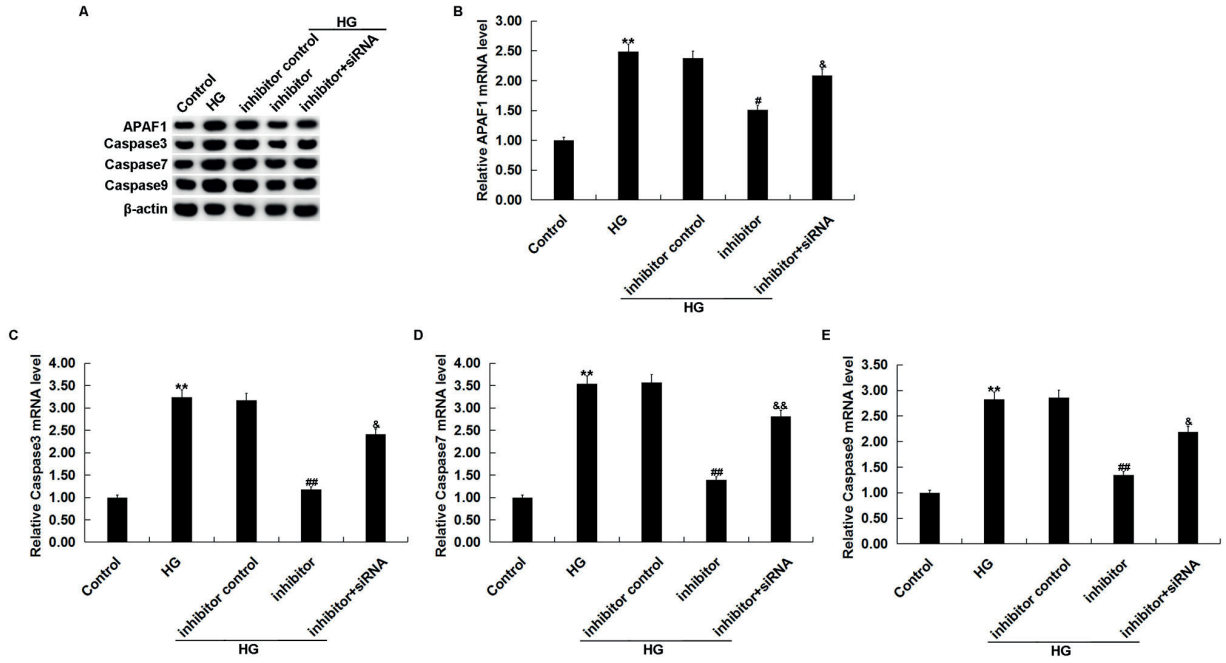

Figure 7. miR-770-5p inhibitor inhibited APAF1/Caspase9 pathway in podocytes. Podocytes were pre-transfected with miR-770-5p inhibitor, inhibitor control, or miR-770-5p inhibitor+TRIAP1-siRNA for 6 h and then treated with high glucose (HG, 30 mM D-glucose) for 48 h. Then, the protein and mRNA levels of APAF1, Caspase3, Caspase7 and Caspase9 in podocytes were detected using Western blotting and qRT-PCR respectively. Data were displayed as mean ±SD. ** p<0.01 vs. control group; #, ## p<0.05, 0.01 *vs.* HG treatment group; &, && p<0.05, 0.01 *vs.* inhibitor group.

podocytes, the APAF1/Caspase9 pathway was analyzed in our study. As shown in Figure 7A, HG treatment induced up-regulations of APAF1, Caspase3, Caspase7, and Caspase9 protein levels in podocytes were markedly inhibited by miR-770-5p inhibitor, and this inhibition was reversed by TRIAP1 silencing. Similar results were obtained from qRT-PCR (Figure 7B-E).

Discussion

In the current study, we found that miR-770-5p was significantly up-regulated in conditionally immortalized mouse podocytes under high glucose condition. TRIAP1 was a direct target of miR-770-5p, and it was down-regulated in podocytes under high glucose condition. MiR-770-5p down-regulation eliminated HG induced cell proliferation ability inhibition and cell apoptosis of podocytes by targeting TRIAP1, indicating the inhibitory effect of miR-770-5p inhibition on podocyte apoptosis. In addition, our results indicated that miR-770-5p down-regulation inhibited the up-regulations of APAF1, Caspase3, Caspase7, and Caspase9 in podocytes induced by high glucose treatment. The data of our current study indicated that miR-770-5p inhibited podocyte apoptosis by targeting TRIAP1, and miR-770-5p/TRIAP1 axis may be a promising target for the treatment of DN. DN is a serious complication of DM and one of the leading causes of death and disability in diabetic patients. At present, the therapeutic effect of DN is still unsatisfactory. In recent years, the emerging roles of miRNAs in the pathogenesis of DN have been highlighted[14-22,24,25]. However, the mechanisms by which miRNAs modulate this disease are complex and further investigations are required to clarify the role of these miRNAs in DN. So far, there have been few studies on miR-770-5p. Li et al[26] reported that miR-770-5p down-regulation could inhibit cell migration and proliferation in Hirschsprung's disease. Lee et al[27] suggested that miR-770-5p could inhibit tumor growth and could make tumors more sensitive to radiation by negatively regulating PI3K. Besides, miR-770-5p has been found[28] to inhibit drug chemoresistance in human ovarian cancer by targeting ERCC2. Moreover, miR-770 was found to be up-regulated in both blood and urine from DN patients[23]. However, to the best of our knowledge, the role of miR-770-5p

in DN remains unclear. Therefore, we performed the current study, and we aimed to explore the role of miR-770-5p in podocytes in DN *in vitro*. Firstly, we detected the level of miR-770-5p in an in vitro podocyte model of DN that was conducted by treatment conditionally immortalized mouse podocytes with HG (30 mM D-glucose). The findings suggested that miR-770-5p was upregulated in podocytes under HG condition, indicating the potential role of miR-770-5p in the development of DN. Then, to investigate the precise role of miR-770-5p in DN, we predicted the target genes of miR-770-5p, and further analysis confirmed that TRIAP1 was a direct target of miR-770-5p and it was downregulated in podocytes under HG condition. TRIAP1 is a small, conserved protein containing 76 amino acids that is induced by TP53 under low levels of genotoxic stress, helping to reduce cell death[29]. Podocyte apoptosis induced by high glucose (HG) is a typical early feature of DN[7,8]. Therefore, next, we studied the effect of miR-770-5p down-regulation on the proliferation and apoptosis of podocytes. Findings of our study indicated that the cell proliferation ability inhibition and cell apoptosis induction of podocytes caused by HG treatment were prevented by miR-770-5p down-regulation. TRIAP1 has been report to regulate the apoptotic pathway through interaction with heat shock protein 70 (HSP70), preventing the formation of APAF1, cytochrome c and Caspase9 apoptotic complexes[30]. We then investigated whether APAF1/Caspase9 pathway was involved the effect of miR-770-5p on podocyte apoptosis, and we found that HG treatment induced the up-regulations of APAF1, Caspase3, Caspase7, and Caspase9 in podocytes were markedly inhibited by miR-770-5p inhibition. It was worth mentioning that all the effects of miR-770-5p inhibitor on podocytes were eliminated by TRIAP1 silencing. In summary, results from our current study suggested that miR-770-5p was up-regulated in podocytes under HG condition in vitro, and its down-regulation significantly prevented HG induced podocyte apoptosis by targeting TRIAP1.

Conclusions

We found that miR-770-5p down-regulation plays a protective effect on diabetic nephropathy *in vitro*; therefore, it may be a promising therapeutic target for the treatment of diabetic nephropathy.

Conflict of Interest

The Authors declare that they have no conflict of interest.

Acknowledgment

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