Abstract. Previous reports have indicated that microRNAs (miRNAs) function as regulators of gene expression. miRNAs inhibit protein translation by interacting with the 3'-untranslated region (UTR) of the mRNA of target genes (8,9). Therefore, miRNAs are involved in the regulation of various cellular processes, including cell proliferation, survival, apoptosis and differentiation (10). Furthermore, an increasing number of studies have indicated that miRNAs participate in the pathogenesis of various diseases and represents novel biomarkers for diagnosis, prognosis and treatment (11-13). Certain miRNAs have been reported to be associated with the pathogenesis of DN, which highlights their potential as promising therapeutic targets for DN (14,15). In addition, recent studies have demonstrated that several miRNAs, including miRNA-34c (miR-34c), miR-30a and miR-218, are aberrantly expressed in podocytes induced by HG and have important roles in regulating podocyte apoptosis (16,17). However, the exact role of miRNAs in podocyte apoptosis remains largely unknown.

The sirtuin family of proteins consists of seven members (SIRT1-7), which are nicotinamide adenine dinucleotide oxidized form-dependent deacetylases that have important roles in the regulation of various biological processes, including metabolism, DNA repair, inflammation, stress response, cell cycle and apoptosis (18,19). Sirtuin 7 (SIRT7) is the most recently characterized sirtuin, however, important roles for SIRT7 in several pathological processes have been recently reported (20). SIRT7 regulates lipid metabolism, chromatin remodeling and protein synthesis (20). SIRT7 has been reported to have a key role in cellular survival in response to a variety of stress conditions, including hypoxia, and endoplasmic reticulum and genomic stress (21-23). However,
whether SIRT7 regulates podocyte apoptosis remains to be established.

It has been hypothesized that miR-20b may be an miRNA that is associated with apoptosis (24). Recently, miR-20b was reported to be a response gene under hyperglycemic conditions in retinal endothelial cells (25). However, whether miR-20b participates in podocyte apoptosis under hyperglycemic conditions is yet to be determined. The present study aimed to investigate the biological role and potential underlying mechanism of miR-20b in podocyte apoptosis induced by HG. The results demonstrated that miR-20b was significantly upregulated in HG-treated podocytes. Suppression of miR-20b significantly inhibited podocyte apoptosis induced by HG. In addition, SIR7 was identified as a functional target of miR-20b that may contribute to the protective effect of miR-20b suppression on HG-induced apoptosis. These results indicate that miR-20b may contribute to HG-induced podocyte apoptosis by targeting SIRT7, therefore representing a potential therapeutic target for DN.

Materials and methods

Cell cultures and treatments. Conditionally immortalized mouse podocytes were purchased from the Cell Resource Center of Peking Union Medical College (Beijing, China). Podocytes were routinely cultured as previously described (26). Briefly, podocytes were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 10 U/ml interferon-γ (Sangon Biotech Co., Ltd., Shanghai, China), 100 U/ml Penicillin and 0.1 mg/ml Streptomycin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at 33°C in a 5% CO2 atmosphere with relative humidity of 95%. Once cells reached a confluence of ~80%, the cells were cultured in the medium described above without interferon-γ for 14 days at 37°C in a 5% CO2 atmosphere. Prior to experiments, the podocytes were cultured in serum-free medium for 24 h at 37°C in a 5% CO2 atmosphere and relative humidity of 95% to synchronize cell growth. Subsequently, podocytes were cultured in serum-free medium containing 5, 10, 15, 20, 25 or 30 mM D-glucose for 6, 12 or 24 h at 37°C. HEK293T cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco’s modified Eagle’s medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS and 100 U/ml Penicillin and 0.1 mg/ml Streptomycin in a humidified atmosphere with 5% CO2 at 37°C.

Cell transfection. miR-20b inhibitor (5'-cuaccucacuauacgcacag-3'), miR-20b mimic (5'-caauacuugauccagcaggg-3') and negative control (NC; 5'-agcuacuacuacagaacgagc-3') were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China) and transfected into cells at a final concentration of 30 nM using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) for 24 h at 37°C. SIRT7 small interfering RNA (siRNA) and NC siRNA were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA) and transfected into cells at a final concentration of 50 nM using Lipofectamine 2000 (Invitrogen). For SIRT7 overexpression, the open reading frame of SIRT7 cDNA was inserted into pcDNA3.1 plasmids (BioVector NTCC, Inc., Beijing, China) to generate pcDNA3.1/SIRT7 overexpressing vectors. The pcDNA3.1/SIRT7 vectors were transfected into cells at a final concentration of 1 µg/ml using Lipofectamine 2000. Empty pcDNA3.1 vectors were used for the control group. Following transfection for 24 h, cells were subjected to the subsequent experiments.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted by using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer’s protocol. RT of mRNA and miRNA were performed by using M-MLV reverse transcriptase (Takara Biotechnology Co., Ltd., Dalian, China) or a One Step PrimeScript® miRNA cDNA Synthesis kit (Takara Biotechnology Co., Ltd.), respectively. For the RT of mRNA, a mixture of RNA, Oligo (dT)12-18 primer, dNTP mixture, M-MLV buffer (all from Takara Biotechnology Co., Ltd.), and M-MLV reverse transcriptase was incubated at 42°C for 1 h and then at 70°C for 15 min. For the RT of miRNA, a mixture of RNA, miRNA Reaction Buffer Mix, Universal Adaptor Primer, and miRNA PrimeScript® RT Enzyme Mix (all from Takara Biotechnology Co., Ltd.) was incubated at 37°C for 60 min and 85°C for 5 sec. Gene expression was detected by using the Power SYBR Green PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) on an Applied Biosystems AB7500 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) under the following conditions: 95°C for 5 min; 30 cycles of 94°C for 20 sec, 55°C for 30 sec and 72°C for 35 sec; and 72°C for 5 min. The primers used were as follows: SIRT7, 5'-agaaagaccagctcagctag-3' (forward) and 5'-tgagaagggagtagcgtgtggtcag-3' (reverse); β-actin, 5'-tctctttgggtatggaattact-3' (forward) and 5'-ggagctgccctttggtcagaag-3' (reverse); and U6, 5'-tgggcttcgaagcttctttg-3' (forward) and 5'-cgaaatggtatcctgcac-3' (reverse). miR-20b, 5'-gtcgtggagtcg-3' (reverse); and U6, 5'-tgggcttcgaagcttctttg-3' (forward) and 5'-cgaaatggtatcctgcac-3' (reverse). β-actin or U6 were used as the internal controls for normalization. Data were obtained from three independent experiments. Gene expression was calculated by using 2-ΔΔCq (27), normalized against β-actin or U6 and compared with the control group.

Caspase-3 activity assay. Caspase-3 activity was measured using a Caspase-3 Activity assay kit (Beyotime Institute of Biotechnology, Haimen, China), according to the manufacturer’s protocol. Briefly, 2x10⁶ cells were lysed using radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology) and the supernatant was collected. The protein concentration was measured using Enhanced BCA Protein Assay kit (Beyotime Institute of Biotechnology). A total of 100 µg protein was incubated with 5 µl DEVD-pNA substrate (4 mM) in 50 µl reaction buffer at 37°C for 2 h. The absorbance value at a wavelength of 405 nm was detected using an ELISA reader (BioTek Instruments, Inc., Winooski, VT, USA).

TUNEL assay. Cell apoptosis was detected by using Roche In Situ Cell Death Detection kit (Roche Diagnostics, Indianapolis, IN, USA), according to the manufacturer’s protocol. Briefly, slides of 1x10⁶ cells were fixed with 4% paraformaldehyde for
30 min at room temperature and permeabilized with 0.2% Triton-X-100 for 5 min at room temperature. The cells were then incubated with 450 µl TUNEL label solution and 50 µl TUNEL enzyme solution at 37˚C for 1 h in the dark. Subsequently, the slides were observed under a fluorescence microscope (Olympus Corporation, Tokyo, Japan). The number of apoptotic cells in five random fields per slide were counted and averaged.

Western blot analysis. Cells were harvested and lysed in radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology) and protein concentration was determined by a BCA kit (Beyotime Institute of Biotechnology). A total of 40 µg protein was separated by 12% SDS-PAGE and electrotransferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). Following incubation with 3% nonfat milk for 1 h at 37˚C, the membranes were incubated with the following rabbit primary antibodies: Anti-SIRT7 (cat. no. ab78977; 1:250; Abcam, Cambridge, MA, USA) and anti-β-actin (cat. no. ab8227; 1:1,000; Abcam) at 4˚C overnight. Subsequently, the membrane was washed thrice with Tris-buffered saline with 0.05% Tween-20 and incubated with horseradish peroxidase-conjugated secondary antibodies (cat. no. bs-0295G-HRP; 1:1,000; BIOSS, Beijing, China) for 1 h at 37˚C. The immunoblots were visualized by a Pierce ECL Western Blotting Substrate (Pierce; Thermo Fisher Scientific, Inc.). Densitometric analysis of the protein bands was performed using Image-Pro Plus software version 6.0 (Media Cybernetics, Inc., Rockville, MD, USA).

Dual-Luciferase reporter assay. Bioinformatics analysis for miR-20b was performed using a web server of TargetScan: Prediction of microRNA targets (http://www.targetscan.org/). The 3’-untranslated region (UTR) of SIRT7 containing miR-20b binding sites was synthesized and cloned into the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega Corporation, Madison, WI, USA). The 3’-UTR of SIRT7 containing mutations in the miR-20b recognition sites was synthesized by QuikChange Multi Site-Directed Mutagenesis kit (Agilent Technologies, Inc., Santa Clara, CA, USA) and cloned into the pmirGLO Dual-Luciferase miRNA Target Expression Vector. The constructed pmirGLO vectors were cotransfected into HEK293T cells with miR-20b mimics (30 nM) or NC mimics (30 nM) by using Lipofectamine 2000. Following transfection for 48 h, the cells were immediately harvested and subjected to detection of luciferase activity. The relative luciferase activity was analyzed by using a Dual-Glo Luciferase assay system (Promega Corporation), according to the manufacturer’s protocol. Relative luciferase activity was calculated according to the formula: Firefly luciferase/Renilla luciferase.

Statistical analysis. Quantitative data are presented as the mean ± standard deviation. Statistical analyses were performed by one-way analysis of variance followed by the Bonferroni post-hoc test using SPSS software version 11.5 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

miR-20b expression is upregulated by HG in podocytes. To investigate the relevance of miR-20b in podocytes, the present study determined the expression pattern of miR-20b in podocytes exposed to HG in vitro by RT-qPCR. As demonstrated in Fig. 1A, the expression level of miR-20b was significantly and dose-dependently upregulated in podocytes exposed to concentrations of glucose >20 mM after 24 h treatment, compared with treatment with 5 mM glucose. Furthermore, miR-20b was significantly increased after 6, 12 and 24 h treatment with 30 mM glucose (Fig. 1B), compared with the 0 h treatment group. These results indicate that miR-20b may be involved in the response to HG in podocytes.

Suppression of miR-20b attenuates apoptosis of podocytes. To investigate the precise biological role of miR-20b in HG-induced podocyte apoptosis, the current study suppressed the expression of miR-20b by transiently transfecting podocytes with miR-20b inhibitor. The results demonstrated that miR-20b expression was significantly suppressed by miR-20b inhibitor transfection in cultured podocytes treated with HG, compared with podocytes treated with HG only (Fig. 2A). The effect of miR-20b suppression on apoptosis was subsequently detected by TUNEL (Fig. 2B) and caspase-3 activity (Fig. 2C) assays. The results demonstrated
that HG exposure significantly induced podocyte apoptosis compared with cells treated with normal glucose (5 mM), whereas suppression of miR-20b significantly prevented podocyte apoptosis induced by HG (Fig. 2B and C). These results indicate that miR-20b may be involved in podocyte apoptosis induced by HG.

**SIRT7 is a target of miR-20b.** To elucidate the underlying molecular mechanism by which miR-20b regulates podocyte apoptosis, the present study aimed to identify the potential target gene for miR-20b using bioinformatics analysis. Notably, SIRT7, an important stress adaptor molecule for cell survival, was identified as a putative target of miR-20b (Fig. 3A). To verify whether SIRT7 is a direct target gene of miR-20b, a Dual-Luciferase reporter assay was performed. Cotransfection of wild-type SIRT7 3’-UTR construct with miR-20b mimics into HEK293T cells resulted into a significant decrease in luciferase activity compared with cells cotransfected with NC mimics (Fig. 3B). However, miR-20b mimics exhibited no significant effect on the luciferase activity when cotransfected with mutant SIRT7 3’-UTR construct (Fig. 3B). The results indicate that miR-20b directly targets the 3’-UTR of SIRT7. To further confirm this prediction, the direct effect of miR-20b on SIRT7 expression was investigated. The results demonstrated that the mRNA and protein expression of SIRT7 was significantly upregulated by miR-20b suppression in podocytes treated with HG (Fig. 4). Taken together, these results indicate that SIRT7 is a direct target gene of miR-20b.

**SIRT7 is involved in HG-induced podocyte apoptosis.** To investigate whether SIRT7 is involved in HG-induced podocyte apoptosis, the present study investigated the effect of SIRT7 knockdown or SIRT7 overexpression on podocyte apoptosis. For knockdown of SIRT7, podocytes were transfected with SIRT7 siRNA. The results demonstrated that transfection with SIRT7 siRNA significantly decreased the protein expression of SIRT7 in HG-treated cells (Fig. 5A). Furthermore, knockdown of SIRT7 significantly increased podocyte apoptosis induced by HG (Fig. 5B). Conversely, SIRT7 overexpression led to significantly increased protein levels of SIRT7 in HG-treated cells (Fig. 5C) and markedly inhibited podocyte apoptosis.
apoptosis induced by HG (Fig. 5D), which mimicked the effect of miR-20b suppression. These results indicate that SIRT7 may be involved in the protection of podocytes from HG-induced apoptosis.

Knockdown of SIRT7 blocks the protective effect of miR-20b suppression. To verify whether SIRT7 is involved in the observed miR-20b-mediated protective effect, podocytes were cotransfected with miR-20b inhibitor and SIRT7
siRNA. The results demonstrated that the increase in SIRT7 expression following miR-20b suppression was significantly reduced by SIRT7 knockdown (Fig. 6A). In addition, the protective effect of miR-20b suppression against HG-induced apoptosis was significantly blocked by SIRT7 knockdown (Fig. 6B).

**Discussion**

Previous evidence has indicated that miRNAs have an important role in DN and that miRNAs may serve as diagnosis biomarkers and therapeutic candidates (14,15). Therefore, the identification of DN-associated miRNAs is essential for the development of novel therapeutic strategies. The present study, to the best of our knowledge, is the first to demonstrate that miR-20b may be a hyperglycemia-responsive miRNA as it was induced by HG in podocytes and suppression of miR-20b provided a protective effect against HG-induced apoptosis. The present study also identified that SIRT7 is a functional target of miR-20b that contributed to the miR-20b suppression-mediated protective effect against apoptosis. In conclusion, the results of the present study revealed a novel miRNA-based mechanism for regulating podocyte apoptosis.

An increasing number of studies have reported important roles for miRNAs in the regulation of podocyte apoptosis (28). Among these, certain studies have concluded that HG-induced podocyte apoptosis was attenuated by miR-29a overexpression (29) and overexpression of miR-29c strongly induced podocyte apoptosis by targeting sprouty homolog 1 (30). In addition, Chen et al (31) reported that miR-195 was increased in diabetic mice and promoted podocyte apoptosis induced by HG by inhibiting Bcl-2. Recently, miR-34c and miR-218 were reported to contribute to HG-induced podocyte apoptosis by targeting Notch signaling or heme oxygenase-1, respectively (16,32). The present study demonstrated that miR-20b was significantly induced by HG in cultured podocytes and suppression of miR-20b provided a protective effect against HG. The current study has identified a potential novel regulator of podocyte apoptosis.

Previous reports have demonstrated that miR-20b is extensively involved in several pathological processes (33,34). The inhibition of oncogenes in various cancer cell lines has revealed an antiproliferative properties of miR-20b (35-37). In addition, suppression of miR-20b provided cardioprotection against ischemia/reperfusion injury (38), and overexpression of miR-20b promoted apoptosis of P19 cells by inducing mitochondrial impairment (24). The present study demonstrated that miR-20b contributed to HG-induced podocyte apoptosis, further confirming the proapoptotic role of miR-20b. However, the underlying mechanism of miR-20b in regulating apoptosis remains to be elucidated.

The current study identified that SIRT7 is a functional target of miR-20b in the regulation of podocyte apoptosis. SIRT7 is the seventh member of the sirtuin family and has an important role in cell proliferation, stress and disease (20). Cumulative evidence has indicated that SIRT7 functions as an important regulator of cell survival and apoptosis (20,23,39). SIRT7 was also reported to prevent cardiomyocyte apoptosis by inhibiting the p53 proapoptotic signaling pathway (40), and promote myocardial tissue repair following myocardial infarction and hind-limb ischemia (41). Furthermore, knockdown of SIRT7 induced gastric cancer cell apoptosis (39), and promoted cellular survival and inhibited apoptosis induced by genomic stress via repression of stress-activated kinases (p38 and c-Jun N-terminal kinase) and the p53 signaling pathway (23,42). SIRT7 was also reported to have a vital role in sensing cellular energy levels and conserving energy during stress (43). Thus, SIRT7 aids cellular survival in response to stress conditions. The present study demonstrated that SIRT7 overexpression protected podocytes against HG-induced apoptosis, while SIRT7 silencing promoted apoptosis, indicating that SIRT7 may promote the survival of podocytes under HG conditions.
Regulation of SIRT7 by specific miRNAs has been reported by recent studies (44,45). Several studies have revealed that miR-125b inhibits cancer development by targeting SIRT7 in bladder cancer (46) and hepatocellular carcinoma (47,48). In addition, suppression of SIRT7 by miR-3666 induced cell apoptosis of non-small cell lung cancer (49), and Gu et al (50) reported that miR-152 induced human dental pulp stem cell senescence by targeting SIRT7. The results of the current study indicated that miR-20b may be a novel regulator of SIRT7, indicating that SIRT7 undergoes epigenetic regulation by various miRNAs, which may exert effects in various pathological processes.

To the best of our knowledge, the present study is the first to demonstrate that miR-20b may be an important regulator of HG-induced podocyte apoptosis. The results demonstrated that SIRT7 is a direct target of miR-20b in the regulation of podocyte apoptosis. The results of the current study may provide novel insight into the effects of miR-20b in DN. Therefore, miR-20b may serve as a promising therapeutic target for the treatment of DN. However, further in vitro and in vivo studies are required to fully elucidate the precise role and molecular mechanism of miR-20b in DN.

References


