Original Article Suppression of microRNA-128 attenuates high glucose-induced podocyte apoptosis through activation of Nrf2-ARE signaling pathway

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Abstract: MicroRNAs (miRNAs) have been emerging as critical regulators of podocyte apoptosis, which plays an important role in the pathogenesis of diabetic nephropathy. miR-128 has been suggested as an apoptosis-related miRNA involved in various pathological processes. However, the role of miR-128 in podocyte apoptosis induced by high glucose (HG) remains largely unknown. In this study, we aimed to investigate the potential role of mliR-128 in vitro. The suppression of miR-128 inhibited cell apoptosis and oxidative stress induced by HG. Nuclear factor erythroid 2-related factor 2 (Nrf2) was identified as the functional target of miR-128 by a dual-luciferase activity reporter assay. The suppression of miR-218 can upregulate Nrf2 expression and activate the Nrf2-ARE signaling pathway. However, Nrf2 knockdown markedly abrogated the protective effects of miR-128 suppression against HG in podocytes. In conclusion, our study suggests that the suppression of miR-128 alleviates HG-induced oxidative stress and apoptosis in podocytes by activating the Nrf2-antioxidant response elements signaling pathway, providing a potential molecular target for the treatment of diabetic nephropathy.

Keywords: Apoptosis, diabetic nephropathy, miR-128, Nrf2, podocyte

Introduction

Hyperglycemia is a major characteristic of diabetes mellitus, causing various severe complications such as diabetic nephropathy (DN) [1, 2]. In recent years, DN has become the leading cause of end-stage renal disease [1]. Hyperglycemia induced oxidative stress and apoptosis of podocytes contributes to the development and progression of DN [3-6]. Podocytes attached to the outer surface of the glomerular basement membrane are terminally differentiated cells that preserve glomerular capillary integrity [7]. Gain a better understanding of the podocyte loss induced hyperglycemia may provide novel insight into understanding the ND pathogenesis and help development of potential therapeutic strategy for DN.

MicroRNAs (miRNAs), a class of small, non-coding RNAs consisting of ~22 nucleotides, are emerging as novel regulators of gene expression [8, 9]. miRNAs can directly recognize and interact with the 3'-untranslated region (UTR) of the target mRNAs which leads to the translation inhibition [8, 9]. Through inhibition of the targets, miRNAs regulates various cellular processes including proliferation, apoptosis, differentiation and cell cycle [10]. Amounting evidences have indicated that miRNAs play an important role in the development and progression of DN [11, 12]. Various miRNAs, such as miR-218 [13] and miR-34c [14], have been reported to be involved in regulating podocyte apoptosis. However, the role of miRNAs in regulating podocyte apoptosis involved in DN remains poorly understood.

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a critical transcription factor regulating various antioxidant genes to protect against oxidative stress [15, 16]. Under basal conditions, Nrf2 is expressed in the cytoplasm and underwent degradation by ubiquitination [17]. Upon activation, Nrf2 translocates to the nucleus and binds to the antioxidant response elements

(ARE) to activate the expression of antioxidant genes, such as heme oxygenase-1 (HO-1) and NADPH-quinone oxidoreductase 1 (NQO1) [17]. The Nrf2-ARE signaling pathway has been dysregulated in cardiac cells [18], retinal endothelial cells [19], and mesangial cells [20] induced by hyperglycemia. Recent study has suggested that the Nrf2-ARE signaling pathway plays an important role in the development of DN [21, 22]. The activation of Nrf2-ARE signaling inhibits podocyte apoptosis and shows a protective effect against DN [23-25]. Therefore, targeting Nfr2-ARE signaling pathway may provide novel strategy for treatment of DN.

miR-128 has been reported as an apoptosisrelated miRNAs involved in various pathological processes [26, 27]. However, whether miR-128 is involved in podocyte apoptosis under hyperglycemic conditions remains unclear. In this study, we aimed to investigate the potential role of miR-128 in regulating podocyte apoptosis induced by high glucose (HG) in vitro. We demonstrated that miR-128 was markedly induced by HG in podocytes. Suppression of miR-128 remarkably prevented apoptosis and oxidative stress induced by HG in podocytes. Interestingly, Nrf2 was identified as the target gene of miR-218. Suppression of miR-128 can activate the Nrf2-ARE signaling pathway to alleviate oxidative stress and apoptosis induced by HG in podocytes. Taken together, our data indicate that miR-128 regulate HG-induced oxidative stress and apoptosis through regulating Nrf2-ARE signaling pathway.

Materials and methods

Podocyte culture and treatment

Conditionally immortalized mouse podocytes were purchased from the Institute of Biochemistry and Cell Biology (Shanghai, China) and routinely cultured as previously described [28]. To induce podocyte growth, podocytes were cultured in RPMI 1640 medium (Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS; Gibco), 1% penicillin/ streptomycin mix (Sigma, St. Louis, MO, USA) and 10 U/ml y-interferon (Sangon Biotech, Shanghai, China) at 33°C in a humidified chamber containing 5% CO, atmosphere. After cells reach a confluence of 70~80%, the cells were cultured in RPMI 1640 complete medium without y-interferon for 2 weeks at 37°C in a humidified chamber containing 5% CO₂ atmosphere

to induce podocyte differentiation. Then, podocytes treated with 5, 10, 20 and 30 mM of D-glucose for indicated times.

Real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Reverse transcription of mRNA was performed by Moloneymurine leukemia virus (M-MLV) reverse transcriptase (BioTeke, Beijing, China). Reverse transcription of miRNA was performed by miScript Reverse Transcription Kit (QIAGEN, Dusseldorf, Germany). The RT-qPCR assays were conducted using an Applied Biosystems AB7500 Real Time PCR system and SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The fold-change of gene expression was calculated by 2-DACt method, using GAPDH or U6 as endogenous control. The primers used for examination were listed as follows: Nrf2 forward, 5'-TTTTCCATTCCCGAATT-ACAGT-3'and reverse, 5'-AGGAGATCGATGAGTA-AAAATCCT-3'; HO-1 forward, 5'-CGTGCTCGA-ATGAACACTCT-3' and reverse, 5'-GGAAGCTG-AGAGTGAGGACC-3': NOO1 forward, 5'-CAAG-TTTGGCCTCTCTGTGG-3' and reverse, 5'-AAGC-TGCGTCTAACTATATGT-3'; GAPDH, 5'-TGTGTCCG-TCGTGGATCTGA-3' and reverse, 5'-TTGCTGTT-GAAGTCGCAGGAG-3'; miR-128 forward, 5'-CG-CGCTCACAGTGAACCG-3' and reverse, 5'-GTGC-AGGGTCCGAGGT-3'; U6 forward, 5'-TGCGGG-TGCTCGCTTCGGCAGC-3' and reverse, 5'-CCAG-TGCAGGGTCCGAGGT-3'.

Cell transfection

miR-128 inhibitor, miR-128 mimics and negative control (NC) were synthesized by Gene-Pharma (Shanghai, China) and transfected into cells at a final concentration of 50 nM using Lipofectamine2000 (Invitrogen). Nrf2 siRNA and NC siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and transfected into cells according to the recommended protocols by the manufacturer.

MTT assays

Cells were seeded into 96-well plates $(5 \times 10^3 \text{ cells/well})$ and cultured for 48 h after transfection. Then, each well was added with 20 μ l of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 5 mg/ml; Sigma). After



Figure 1. miR-128 is induced by HG. A. miR-128 expression in podocytes exposed to 5, 10, 20, and 30 mM of glucose for 24 h was detected by RT-qPCR. *P<0.05 vs. 5 mM. B. miR-128 expression in podocytes exposed to 30 mM of glucose for 6, 12, and 24 h was detected by RT-qPCR. *P<0.05 vs. 0 h.

incubation for 4 h, the medium was removed and dimethyl sulfoxide (200 μ l/well) was added to dissolve the formazan product. After agitation for 15 min, the absorbance at a wavelength of 490 nm was measured by an ELISA reader (Bio-Rad, Hercules, CA, USA).

Caspase-3 activity assays

Caspase-3 activity was measured using a commercial kit (Beyotime, Haimen, China) in accordance with the recommended protocols. Briefly, cells were lysed and the supernatant was harvested. A total of 100 μ g protein and 5 μ l DEVDpNA substrate (4 mM) were incubated with 50 μ l reaction buffer at 37°C for 2 h. The absorbance value at a wavelength of 405 nm was determined using an ELISA reader (Bio-Rad).

ROS measurement

ROS (reactive oxygen species) production in podocytes was measure with the fluorescent probe 2',7'-dichlorofluoresceindiacetate (DCFH-DA; Beyotime). Briefly, cells were harvested and incubated with 50 μ M DCFH-DA at 37°C for 30 min in dark place. After washes with phosphate buffer solution, the mean fluorescence intensity was measured using fluorescence spectrophotometer (Bio-tek Instruments, Winooski, VT, USA) with an emission wavelength of 530 nm and excitation wavelength of 485 nm.

Detection of MDA and SOD levels

Oxidative stress markers, including malondialdehyde (MDA) and superoxide dismutase (SOD) were measured using commercially available kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to manufacturer instructions.

Luciferase reporter assays

To determine the correlation of miR-128 and Nrf2-3'-UTR, a cDNA fragment of the Nrf2-3'-UTR mRNA containing the seed sequence of the mature miR-128-binding site or a mutated binding site of the 3'-UTR sequence were cloned into the pmirGLO dual-luciferase vector (Promega, Madison, WI, USA). The dual-luciferase vector constructed was cotransfected with miR-128 mimics or NC mimics into 293T cells (ATCC, Manassas, VA, USA) using Lipofectamine2000 (Invitrogen). After 48 h of transfection, the luciferase activity was measured using the Dual-Luciferase Assay System (Promega). The activity of Nrf2 antioxidant pathway was detected by the ARE Reporter kit as the per the manufacturer's instructions. Briefly, podocytes were cotransfected with ARE Reporter and miR-128 inhibitor or NC inhibitor. After incubation for 24 h, the luciferase activity was measured by the Dual-Luciferase Assay System (Promega).

Western blot analysis

Total proteins were extracted from cells by the addition of lysis buffer (Beyotime). For western blot analysis, a total of 50 μ g proteins were separated by 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis. The separated proteins on the polyacrylamide gel were then transferred to a polyvinylidene fluoride membrane (Millipore, Boston, MA, USA). The



Figure 2. Suppression of miR-128 inhibits HG-induced podocytes. Podocytes were transfected miR-128 inhibitor or NC inhibitor and then incubated with 5 mM of glucose (normal glucose, NG) or 30 mM of glucose (high glucose, HG) for 24 h. A. The expression of miR-128 was detected by RT-qPCR. B. Cell viability was determined by MTT assays. C. Cell apoptosis was detected by caspase-3 activity assays. *P<0.05 vs. NG; #P<0.05 vs. HG and HG+NC inhibitor.

membrane was immersed in 5% nonfat milk for 1 h at 37°C before incubation with primary antibodies (anti-Nrf2 and anti-GAPDH, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight at appropriate dilutions according to the recommended instructions. After rinsing in TBST buffer (Tris buffer saline containing 0.1% Tween-20), the membrane was incubated with horseradish peroxidase-conjugated secondary antibodies (1:2000; Santa Cruz Biotechnology) for 1 h at 37°C. After the final wash, the protein bands were developed by an ECL Western blotting kit (Millipore). Densitometric analysis was conducted by Image-Pro Plus 6.0 software.

Data analysis

All data were reported as means \pm standard deviation and the data were analyzed by SPSS version 11.5 (SPSS Inc., Chicago, IL, USA). Differences were analyzed by Student's *t* test and one-way analysis of variance according to the data characteristics. Differences were considered as statistically significant when the *P* value was lower than 0.05.

Results

HG induces miR-128 expression in cultured podocytes

To explore the relevance of miR-128b in podocytes, we initially examined the expression level of miR-128 in podocytes exposed to HG *in vitro* by RT-qPCR. The results showed that miR-128 was significantly induced in podocytes exposed to HG (20 and 30 mM) for 24 h treatment (**Figure 1A**). Moreover, miR-128b was markedly upregulated post-time challenge in HG-induced podocytes (30 mM) (**Figure 1B**). Therefore, these data imply that miR-128 is involved in podocytes in the response to HG.

Suppression of miR-128 inhibits HG-induced podocytes

To investigate the biological role of miR-128 in HG-treated podocyte, we detected the effect of miR-128 suppression on HG-induced podocyte apoptosis. miR-128 induced by HG was significantly suppressed by transiently transfecting miR-128 inhibitor (**Figure 2A**). MTT assays showed that suppression of miR-128 significantly rescued the decreased cell viability induced by HG (**Figure 2B**). Furthermore, we detected cell apoptosis by caspase-3 activity assay. The results showed that HG-induced podocyte apoptosis was markedly prevented by miR-128 suppression (**Figure 2C**). These results indicate that HG-induced miR-128 contributes to podocyte apoptosis.

Suppression of miR-128 inhibits oxidative stress by HG

To further investigate the biological role of miR-128 in HG-treated podocyte, we detected the



Figure 3. Suppression of miR-128 inhibits oxidative stress by HG. (A) ROS levels was detected by DCFH-DA assays. The contents of SOD (B) and MDA (C) were measured by commercially available kits. *P<0.05 vs. NG; #P<0.05 vs. HG and HG+NC inhibitor.



Figure 4. miR-128 targets the 3'-UTR of Nrf2. A. Schematic diagram of the seed sequences between miR-128 and Nrf2-3'-UTR. B. The relative luciferase activity generated by the luciferase reporter vectors containing Nrf2-3'-UTR WT or Nrf2-3'-UTR MT in 293T cells with miR-128 mimics was determined using the dual-luciferase assays. *P<0.05 vs. NC mimics.

effect of miR-128 suppression on oxidative stress. The results showed that the excessive ROS level induced by HG was markedly decreased by miR-128 suppression (Figure 3A). Furthermore, we detected the oxidative stress markers, SOD and MDA. The results showed that SOD was inhibited by HG (Figure 3B) while MDA was promoted by HG (Figure 3C) in podocytes. However, these effects were reversed by miR-128 suppression (Figure 3B and 3C). These results indicate that miR-128 contributes to oxidative stress in HG-induced podocytes.

Nrf2 is characterized as a target of miR-128

To identify the functional target gene of miR-128 in regulating podocyte apoptosis, we performed bioinformatics analysis to predict the potential targets of miR-128. Among these genes, Nrf2, an important antioxidant gene, induced our interest. The predicted binding sites of miR-128 in the 3'-UTR of Nrf2 (Nrf2-3'-UTR WT) were depicted in Figure 4A. The complementary seed sequences were mutated to generate the 3'-UTR mutant (Nrf2-3'-UTR MT), which should not bind miR-128. To confirm this prediction, we performed dual-luciferase reporter assays. The results showed that the luciferase activity generated by the reporter vector with Nrf2-3'-UTR WT was markedly suppressed after co-transfection with miR-128 mimics (Figure 4B). However, the activity generated by the reporter vector with Nrf2-1-3'-UTR MT was not obviously affected by miR-128 overexpression (Figure 4B). These results suggested that miR-128 might inhibit the expression of Nrf2 by directly binding to the 3'-UTR. The following RT-gPCR and western blot analyzes demonstrated that the mRNA and protein of Nrf2 were markedly upregulated by miR-128 suppression in podocytes treated with HG (Figure 5A and 5B). Therefore, these results



Figure 5. Suppression of miR-128 upregulates Nrf2 expression. The mRNA (A) and protein (B) expression of Nrf2 were detected by RT-qPCR and western blot analysis, respectively. Podocytes were transfected with miR-128 inhibitor or NC inhibitor and then incubated with NG or HG for 24 h. After treatment, cells were harvested for analysis. *P<0.05 vs. NG; #P<0.05 vs. HG and HG+NC inhibitor.

suggest that miR-128 might repress Nrf2 by targeting the 3'-UTR of Nrf2.

Suppression of miR-128 activates the Nrf2-ARE signaling pathway

To further confirm the regulatory effect of miR-128 on Nrf2, we detected the effect of miR-128 suppression on Nrf2-ARE signaling pathway by ARE reporter assays. The results showed that miR-128 suppression significantly promoted the activation of Nrf2-ARE signaling pathway (**Figure 6A**). Furthermore, we detected the expression of Nrf2-ARE signaling downstream targets, HO-1 and NQO1 by RT-qPCR. The results showed that suppression of miR-128 significantly upregulated the expression of HO-1 (**Figure 6B**) and NQO1 (**Figure 6C**) in HG-treated podocytes. Thus, these results suggest that suppression of miR-128 activates Nrf2-ARE signaling pathway.

Silencing Nrf2 abrogates the protective effect of miR-128 suppression

To further verify whether Nrf2 is involved in miR-128 suppression-mediated protective effect against HG, we cotransfected podocytes with miR-128 inhibitor and Nrf2 siRNA. The results showed that the promoted effect of miR-128 suppression on Nrf2 expression was significantly blocked by Nrf2 knockdown (Figure 7A). The protective effects of miR-128 suppression against HG-induced apoptosis (Figure 7B) and ROS generation (Figure 7C) were markedly reversed by Nrf2 knockdown. Moreover, the promoted effect of miR-128 suppression on activation of Nrf2-ARE signaling pathway was also abolished by Nrf2 knockdown (Figure 7D). Taken together, these results suggest that Nrf2 contributes to the miR-128 suppression-mediated protective effects against HG in podocytes.

Discussion

MiRNAs play a critical role in regulating podocyte apoptosis and the progression of DN and can be used as diagnosis biomarkers and therapeutic targets [11, 12]. In this study, we identified that miR-128 was a novel miRNA that was involved in regulating podocyte apoptosis induced by HG *in vitro*. We found that miR-128 was induced by HG and suppression of miR-128 alleviated HG induced apoptosis and oxidative stress. Nrf2 was found as the target gene of miR-128. The protective effect of mIR-128 suppression was mediated through activation of the Nrf2-ARE signaling pathway. Taken together, our data suggest that miR-128 is a novel regulator for podocyte apoptosis.

Various miRNAs were deregulated in DN and regulate podocyte apoptosis *in vitro* and *in vivo* [29, 30]. miR-195 is increased in DN and HG-treated podocytes and contributes to podocyte apoptosis by inhibiting anti-apoptotic protein Bcl-2 [31]. Overexpression of miR-29a ameliorates HG-induced podocyte apoptosis by inhibiting histone deacetylase 4 signaling pathway [30]. Liu et al. reported that miR-34c inhibits HG-induced podocyte apoptosis by targeting Notch signaling pathways [14]. A recent study reveals that miR-218 promotes HG-induced



Figure 6. Suppression of miR-128 activates Nrf2-ARE signaling pathway. (A) The activity of Nrf2-ARE signaling pathway was examined by ARE reporter assay. Podocytes were cotransfected with ARE reporter and miR-128 inhibitor or NC inhibitor and then incubated with NG or HG for 24 h. The mRNA expression of HO-1 (B) and NQO-1 (C) was detected by RT-qPCR analysis. *P<0.05 vs. NG; #P<0.05 vs. HG and HG+NC inhibitor.



Figure 7. Nrf2 knockdown blocks the protective effect of miR-128 suppression. A. Western blot analysis of Nrf2 protein expression in podocytes cotransfected with miR-128 inhibitor and Nrf2 siRNA. NC inhibitor, cells were transfected with NC inhibitor; miR-128 inhibitor + NC siRNA, cells were cotransfected with miR-128 inhibitor and Nrf2 siRNA; miR-128 inhibitor+Nrf2 siRNA, cells were cotransfected with miR-128 inhibitor and Nrf2 siRNA. After the transfection, cells were incubated with HG for 24 h. B. Podocyte apoptosis was detected by caspase-3 activity assays. *P<0.05 vs. C. ROS generation was determined by DCFH-DA assays. D. The activity of Nrf2-ARE signaling pathway was examined by ARE reporter assay. *P<0.05 vs. NC inhibitor; "P<0.05 vs. miR-128 inhibitor + NC siRNA.

podocyte apoptosis by inhibiting HO-1 [13]. These reports highlight an important role of miRNA in regulating podocyte apoptosis. In this study, we found that miR-128 was a novel miRNA involved in regulating HG-induced podocyte apoptosis. Suppression of miR-128 alleviated HG-induced podocyte apoptosis suggesting a novel molecular target for preventing podocyte apoptosis. However, the underlying molecular mechanism needs to be elucidated.

miR-128 has been reported as a pro-apoptotic miRNA in various pathological processes.

Several studies reported that miR-128 promotes cancer cell apoptosis by targeting insulin receptor substrate 1 [32], silent information regulator 1 [27], or Bmi-1 [33]. Overexpression of miR-128 induces human embryonic kidney cell apoptosis [34]. Interestingly, miR-128 was upregulated by ischemia/reperfusion injury in cardiomyocytes and suppression of miR-128 showed a protective effect against ischemia/ reperfusion injury through activation of peroxisome proliferator-activated receptor gamma [26]. In this study, we found that miR-128 was induced by HG and suppression of miR-128 prevented HG-induced apoptosis through activation of Nrf2 signaling. Our results showed that miR-128 directly targeted the 3'-UTR of Nrf2, as detected by dual-luciferase reporter assay. Suppression of miR-128 promoted Nrf2 expression as well as activation of Nrf2-ARE signaling pathway. Silencing of Nrf2 could markedly block the protective effect of miR-128 suppression. Our data suggest that Nrf2 is a novel target of miR-128 by which miR-128 regulates cell apoptosis.

Nrf2-ARE signaling is an important component of cellular defensive machinery against oxidative stress [15]. Increasing evidence has suggested that Nrf2 is extensively associated with hyperglycemia-induced pathological processes [35]. Activation of Nrf2 signaling effectively inhibits HG-induced oxidative stress and apoptosis in renal tubular epithelial cells [36, 37], coronary artery endothelial cells [38], and cardiomyocytes [18, 39] etc. Activation of Nrf2 signaling attenuates the progression of diabetic nephropathy [24, 40, 41]. In cultured podocytes, activation of Nrf2-ARE signaling reduces HG-induced oxidative stress and apoptosis [25]. Therefore, targeting Nrf2-ARE signaling pathway is a promising therapeutic strategy for preventing DN.

In addition to chemical molecules and natural drug molecules [25, 37], the miRNAs represent novel tools for activation of Nrf2-signaling pathway [42]. The overexpression of miR-153 significantly inhibited Nrf2 and its downstream genes in breast cancer cells [43]. miR-155 suppression results in activation of Nrf2-dependent pathway that reduces fluorooctane sulfonateinduced oxidative hepatic damage [44]. Inhibition of miR-27a promotes Nrf2 expression and protects myocardium against sepsis injury [45]. Suppression of miR-144 attenuates ROS formation and apoptosis induced by HG in cultured cardiomyocytes [46]. Otherwise, targeting and inhibiting the kelch-like ECH-associated protein 1 (Keap1), an inhibitor gene of Nrf2, also activates Nrf2 signaling pathway. It is reported that miR-200a [47, 48], miR-141 [49], miR-28 [50] and miR-7 [51] can activate Nrf2 signaling pathway by targeting and inhibiting Keap1. Here, we revealed that miR-128 was a novel miRNA for modulating Nrf2 signaling pathway.

Here we present for the first time findings suggesting that miR-128 plays an important role in

regulating HG-induced podocyte apoptosis. We found that Nrf2 was a direct target of miR-128; miR-128 suppression activated the NRf2-ARE antioxidant signaling pathway to inhibit the oxidative stress and apoptosis induced by HG. Our findings may shed new light on miR-128 in the regulation of DN pathogenesis. Therefore, miR-128 might serve as a promising therapeutic target for the treatment of DN. However, further *in vivo* and *in vitro* studies will be necessary to fully understand the precise role of miR-128 in regulating DN development and progression.

Disclosure of conflict of interest

None.

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