**Abstract.** – **OBJECTIVE:** To evaluate the effects of miR-29a on the high glucose (HG)-induced apoptosis and the correlation between miR-29a and heme oxygenase-1 (HO-1) and the underlying molecular mechanism.

**MATERIALS AND METHODS:** The cell apoptosis was analyzed by the flow cytometry, and the cells autophagy was evaluated using the transmission electron microscopy. Luciferase reporter assay was carried out to detect the correlation between miR-29a and HO-1. Besides, reverse transcription-PCR and Western blot were applied to detect the mRNA and protein levels.

**RESULTS:** The expression of miR-29a was significantly decreased in the HG-treated podocytes. Besides, miR-29a overexpression could promote cellular autophagy and significantly reduce HG-induced podocytes apoptosis. Moreover, HO-1 was a direct target of miR-29a and the pre-autophagy and the anti-apoptotic effects of miR-29a on HG-treated podocytes could be significantly reversed by the HO-1 siRNA administration.

**CONCLUSIONS:** MiR-29a functionally promoted podocytes autophagy and inhibited apoptosis through the HO-1-dependent pathway in the HG condition.

**Key Words:** miR-29a, Heme oxygenase-1, Podocytes, Autophagy, Apoptosis.

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**Introduction**

Diabetic nephropathy (DN) is a common and severe complication of diabetes mellitus, which has become the main contributor of renal disease at end stage\(^1\,^2\). Diabetic nephropathy not only affects the patients’ life qualities, but endangers their lives\(^3\). It will provide some novel insights for the development of effective therapeutics for DN if we fully understand the underlying mechanisms of DN pathogenesis. Podocytes are cells attached to the outer surface of glomerular basement membrane, which have been considered playing a critical role in the DN development in recent years\(^4\). High glucose (HG) has been identified as an important contributor to podocytes injury, that is characterized by the increases of albumin filtration and reductions of nephrin, podocin, and the slit diaphragm-associated protein\(^5\,^7\). It has been widely reported that HG can induce podocyte apoptosis \(\text{in vivo}\) and \(\text{in vitro}\) studies\(^7\). Podocytes loss has been known as the early mechanism of DN pathogenesis\(^8\). Therefore, targeting podocyte may provide some new clues for producing more renal protective drugs for DN treatment. MicroRNAs (miRNAs) are non-coding RNAs that regulate gene expressions through targeting the 3’-untranslated region of the target gene so as to inhibit proteins translation\(^9\). In recent years, miRNAs have been widely found to participate in varieties of cellular processes, such as cell differentiation, apoptosis, and survival\(^11\,^12\). Compelling researches have showed that miRNAs play an important role in the regulation of podocytes\(^10\,^13\). Previously, studies have demonstrated that hyperglycemia could impair miR-29a expression in podocytes, which resulted in the acceleration of podocyte in-
jury and the decreased expression of nephrin and acetylated nephrin14. Overexpression of miR-29a in diabetic mice could efficiently increase nephrin levels, podocyte viability and renal function14. Since most miRNAs are highly pleiotropic and act di erentially in different cell types, the detailed function and regulation of miR-29a in the pathogenesis of podocytes injury need to be further elucidated. Heme oxygenase-1 (HO-1), as a kind of prominent stress proteins and multifunctional microsomal enzymes, can regulate many biological responses such as cell differentiation, apoptosis and cycle progression in their target cells15. Besides, studies have verified that HO-1 could inhibit cellular apoptosis through several distinct mechanisms16. Furthermore, evidence has showed that HO-1 induction could protect against podocytes apoptosis in diabetes condition7. In this study we investigated the overexpression of miR-29a potential effects on the high glucose-induced podocytes apoptosis, and explored the autophagy and apoptosis of the podocytes after the high glucose and pre-miR-29a treatment. Besides, the correlation between miR-29a and heme oxygenase-1 was also elucidated on the regulation of autophagy markers LC3BII and Beclin-land apoptosis of the mouse podocytes.

Materials and Methods

Cell Culture

Conditionally immortalized mouse podocytes were purchased from Cell Resource Center of Peking Medical College (Beijing, China). Podocytes were cultured in Roswell Park Memorial Institute 1640 (RPMI-1640) containing 10% fetal bovine serum (FBS), 50 μg/mL amphotericin B, and 100 U/mL penicillin, maintained at 37°C in 5% CO2 incubator. The control cells were untreated in medium containing vehicle dimethyl sulfoxide (DMSO). After that, podocytes were cultivated in the serum free medium containing D-glucose (high glucose, 30 mM, HG) for 24, 48 or 72 h.

Cell Transfection

Podocytes (1*10^5/well) were transfected with the pre-miR-control or the pre-miR-29a (GenePharma, Shanghai, China) following the manufacturer’s protocol at a final concentration of 30 nM using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Transfections of the HO-1 siRNA and the negative control siRNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were performed according to the same protocol. After podocytes were transfected for 48 h, they were carefully collected and conserved for further analysis.

Cell Apoptosis Analysis

After they were transfected with pre-miR-29a, pre-miR-control, HO-1 siRNA and siNC and incubated for 48 h, podocytes for the apoptosis analysis were firstly resuspended in the Annexin-V binding buffer, then stained with the FITC-conjugated Annexin-V and PI simultaneously at 37°C for 15 min in dark before adding the binding buffer. Next, the apoptotic podocytes were carefully identified using the flow cytometry analysis on a FACS Calibur system (Becton-Dickinson FacsScan, San Jose, CA, USA).

Transmission Electron Microscopy (TEM)

For the TEM analysis of cell morphological features, cells were first fixed in 3% glutaraldehyde followed by 1% osmium tetroxide; then, they were dehydrated in the graded alcohols and were embedded in the Epon (Agar Scientific, Stansted, UK). The sections were stained with lead citrate and uranyl acetate and were finally observed with the Morgagni 268D electron microscope (FEI, Hillsboro, OR, USA).

Luciferase Reporter Assay

Fragments from the HO-13’-UTR containing the binding sequences for miR-29a were amplified and sub-cloned into the pGL3 luciferase promoter vector (Promega, Madison, WI, USA). The site-directed mutagenesis of miR-29a binding sites in the 3’-UTR of HO-1 was also performed following the Site-Directed Mutagenesis Kit (Stratagene, Hangzhou, Zhejiang, China). The 0.1 μg pGL3 vector containing mutated or wild type 3’-UTR fragments was co-transfected with pre-miR-29a (50 nM) in the human embryonic kidney 293 (HEK293) cells. After the podocytes were collected and lysed, luciferase activity was measured with the luciferase reporter assay kit (Promega, Madison, WS, USA).

Western blot

After treatment, the cells were collected and the protein was extracted with RIPA lysis buffer. The protein concentrations were quantified by the bicinchoninic acid (BCA) method. 30 ug protein samples were run on 10% gels, and then transferred to the polyvinylidene fluoride (PVDF) membrane. After 1 h of blocking with the 5% non-fat milk, the membranes were incubated with
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the primary rabbit anti-LC3BII, the rabbit anti-Beclin-1 antibody, the rabbit anti-HO-1 antibody, and the rabbit anti-GAPDH antibody (1:1000, Abcam, Cambridge, MA, USA) at 4°C overnight. After the membranes were washed in tris-buffered saline-tween (TBST) for three times, they were incubated with a horse radish peroxidase (HRP) labeled secondary antibody (1:5000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h. The bands were washed again, enhanced with chemiluminescence reagents and visualized with the ChemiDocTM MP Imaging System (Bio-Rad, Hercules, CA, USA).

Reverse-Transcription PCR

After treatment, the podocytes were collected to determine the mRNA levels by RT-PCR. Cell total RNA was carefully extracted with the Trizol RNA extraction reagent (Invitrogen, Carlsbad, CA, USA) following the protocols, and quantified by spectrophotometer method. Purified RNA with equal volume was reverse transcribed (RevertAid First Strand cDNA Synthesis Kit, Thermo Fisher Scientific, K1622, Waltham, MA, USA). RT-PCR analysis was carried out by a PCR thermal cycler Dice instrument (TaKaRa, Otsu, Shiga, Japan).

Statistical Analysis

All data were expressed as the mean ± standard error of the mean (SEM), and analyzed using a one-way analysis of variance followed by Bonferroni-Dunn correction. Statistical analysis was performed using the SPSS software, version 20.0 (SPSS Inc., Chicago, IL, USA).

Results

MiR-29a Level is Decreased in the HG-treated Podocytes

To explore the role of miR-29a in the HG-treated podocytes, we firstly detected the miR-29a levels in podocytes after the HG challenge through RT-qPCR analysis. Results suggested that the miR-29a expression was dramatically decreased in the podocytes after HG stimulation for 24 h, and then continuously reduced at 48 and 72 h time points (Figure 1). The results showed that miR-29a played an important role in the HG-treated podocytes.

MiR-29a Overexpression Promotes Autophagy in Podocytes

Electron microscopy demonstrated that 48 h HG treatment reduced autophagic vacuole formation, while pre-miR-29a alleviated the HG-induced reduction (Figure 2A). Levels of two autophagy markers were further measured by both qPCR and Western blot analysis. Data showed that both LC3BII and Beclin-1 were significantly decreased on the mRNA and protein levels in the HG treated podocytes, while they were increased in the HG+pre-miR-29a cells compared the control and HG group (Figure 2B).

MiR-29a Overexpression Inhibits the HG-induced Podocytes Apoptosis

Flow cytometry analysis was applied to explore the miR-29a contribution to the HG-induced podocyte apoptosis. Data suggested that the HG group exhibited a significant increase in the apoptotic cells as compared with the control group, while the pre-miR-29a administration could remarkably decrease HG induced podocytes apoptosis (Figure 3). The results implied that miR-29a was associated with the HG-induced podocytes apoptosis.

HO-1 is a Target of miR-29a

Results of luciferase reporter suggested that the transient transfection of pre-miR-29a with the luciferase expression plasmids in HEK293 cell markedly increased 3’-UTR luciferase activity of

Figure 1. The miR-29a levels in HG-treated podocytes. Podocytes were incubated with HG (30 nM) and the miR-29a expression was analyzed by RT-qPCR at different time points. Statistical analysis was calculated by one-way ANOVA. N = 3, *p<0.05, **p<0.01 vs. control.
HO-1 compared with the pre-miR-control, whereas 3'-UTR containing mutations of HO-1 at the miR-29a binding sites lost the response (Figure 4A). To further investigate the miR-29a effect on HO-1, podocytes were transfected with pre-miR-29a and treated with HG for 48 h. RT-qPCR and Western blot analysis showed that HG treatment could significantly decrease the HO-1 level in the podocytes. However, the HG effect on HO-1 expression was reduced if cells were transfected with pre-miR-29a at the same time (Figure 4B). Therefore, the results showed that miR-29a could
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mediate HO-1 expression, and HO-1 is a direct target of miR-29a.

**HO-1 Knockdown Inhibits the Autophagy Effect of miR-29a on Podocytes**

To explore if miR-29a functions via HO-1, we detected the effect of HO-1 siRNA on the miR-29a-mediated podocytes autophagy. In this experiment, both the pre-miR-29a and the HO-1 siRNA were co-transfected into the high glucose (HG)-treated podocytes. Our results suggested that the pre-autophagy effect of pre-miR-29a on the HG-treated podocytes was greatly reversed by the HO-1 siRNA administration (Figure 5A-B). The results showed that miR-29a could functionally promote podocytes autophagy through the HO-1 dependent pathway.

**HO-1 Knockdown Inhibits the Anti-Apoptotic Effect of Pre-miR-29a on Podocytes**

To further verify if miR-29a functions via HO-1, we then examined the HO-1 siRNA effect on the miR-29a-mediated podocytes apoptosis. In the study, both the pre-miR-29a and the HO-1 siRNA were co-transfected into the HG-treated podocytes. As shown in Figure 6, the anti-apoptotic effect of pre-miR-29a on the HG-treated podocytes was significantly reversed by the HO-1 siRNA administration, further implying that miR-29a functionally inhibited podocytes apoptosis through the HO-1 dependent pathway.

**Discussion**

In our present research, the miR-29a potential effects on the high glucose-induced podocytes apoptosis were evaluated. Our results suggested that in the HG-treated mouse podocytes, the expression of miR-29a was significantly decreased, suggesting an important role for miR-29a in the HG-treated podocytes. Besides, the miR-29a overexpression could promote autophagy in podocytes, supported by the electron microscopy and the increases of autophagy markers. Moreover, flow cytometry experiments confirmed that the pre-miR-29a treatment could significantly re-
duce HG-induced cell apoptosis in podocytes. In addition, we found that miR-29a could mediate the expression of HO-1, implying that HO-1 may be a direct target of miR-29a. Most importantly, we further explored that the pre-autophagy effect and the anti-apoptotic effect of pre-miR-29a on the HG-treated podocytes were largely attenuated by the HO-1 siRNA administration. Therefore, the above results showed that miR-29a could functionally promote podocyte autophagy and inhibited apoptosis through the HO-1 dependent pathway in the HG condition. Diabetic nephropathy has turned into one of the most common contributors to chronic kidney diseases (CKD)\(^{18}\). Meanwhile, podocyte injury has been widely recognized as an early predictor of DN on account of its slit diaphragm structure. Recently, podocytes have been identified to play a critical role in the maintaining of the integrity of the glomerular filtration barrier\(^{19,20}\). Autophagy is a common cell process including the recycling and removal of misfolded proteins, bulk cytoplasmic constituents, and damaged the intracellular organelle to maintain the cell homeostasis\(^{21,22}\). The functions of autophagy in the podocytes have recently obtained more and more attention. Studies indicated that autophagy plays a critical role in the homeostasis maintenance and cellular integrity in podocyte. Hartleben et al\(^{23}\) once found that autophagy had an impact on the susceptibility of glomerular diseases and maintained podocytes homeostasis in the aging mice. Although several studies\(^{24,25}\) have proved that autophagy participated in the DN pathogenesis, the exact role of autophagy and

**Figure 4.** HO-1 is a target of miR-29a. Relative luciferase activity of reporters containing wild type or mutated type with miR-29a target sites in HEK293 cells. Levels of HO-1 mRNA and protein (B) in podocytes transfected with pre-miR-29a as indicated and treated for 48 h with HG. *p<0.05, **p<0.01 vs. the HG; *p<0.05, **p<0.01 vs. the control.
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The underlying mechanisms in podocytes apoptosis in diabetic condition remained to be further investigated. MicroRNAs play a key role in both maintaining of cellular homeostasis and regulation of deterioration of renal tissues. Hsu et al. reported that the miR-29a knockdown could promote nephrin deacetylation and podocytes apoptosis, which was consistent with our results. HO-1 is one of the oxidative stress inducible defense enzymes, which convert free heme into iron, biliv-

Figure 5. HO-1 knockdown blocks the autophagy effect of premiR-29a on podocytes. The different groups of cells were subjected to TEM examination and the relative levels of Beclin-1, LC3BII expression in individual groups of cells were determined by RT-PCR and Western blot. Data are representative images or expressed as the mean ± S.D. of each group from three separate experiments. (A) TEM analysis. (Magnification 10,000x). (B) Western blot analysis. *p<0.05, **p<0.01 vs. the pre-miR-29a group.
erdin, and carbon monoxide. HO-1 possesses some powerful anti-apoptotic characteristics by itself or through the enzymatic product. The potential relationship between autophagy and HO-1 in some vital organs was reported; however, its precise role remained controversial. The new results in our present work were that the miR-29a overexpression could remarkably promote autophagy and significantly reduce the HG-treated podocytes apoptosis. Also, we found that miR-29a could regulate HO-1 level, implying that HO-1 may be a direct target of miR-29a. Moreover, we investigated that the pre-autophagy effect and the anti-apoptotic effect of pre-miR-29a on the HG-treated podocytes could be significantly attenuated by the HO-1 siRNA administration. Consequently, we suggested that miR-29a could functionally promote podocyte autophagy and inhibit apoptosis through a HO-1 dependent pathway. The close correlation between the podocytes function and levels of miR-29a and HO-1 may provide some experimental evidence for the possible effect of the miR-29a and HO-1 overexpressions against the high glucose-induced podocytes apoptosis. The clear mechanisms underlying the miR-29a action and its utility for the treatment of diabetic nephropathy in human beings still need to be investigated further.

**Conclusions**

MiR-29a overexpression could remarkably promote autophagy and significantly reduce the HG-treated podocytes apoptosis in mouse. The effects of pre-miR-29a on the HG-treated podocytes could be significantly reversed by the HO-1 siRNA administration. Our findings suggested that miR-29a could serve as an innovative and prospective therapeutic target for DN.

**Conflict of Interest**
The Authors declare that they have no conflict of interests.

**References**

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