Role of microRNA-25 in high glucose cultured Müller glia

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Abstract

- **AIM:** To investigate the role of microRNA-25 (miR-25) in proliferation and apoptosis of retinal Müller glia (MG) under high glucose condition.

- **METHODS:** The purity of the cultured cells was verified by immunocytochemistry and flow cytometry using antibodies that specifically recognized MG. The expression level of miR-25 under normal and high glucose conditions were validated by quantitative reverse transcription polymerase chain reaction (RT-qPCR). miR-25 mimics and negative control were transfected into MG and multiple functional experiments including cell counting kit-8 assay, EDU assay, and flow cytometry were conducted to explore the effects of miR-25 on the proliferation and apoptosis of high glucose cultured MG (HGMG).

- **RESULTS:** Immunocytochemistry and flow cytometry confirmed the high purity of primary cultured MG. RT-qPCR results showed that the expression level of miR-25 was significantly repressed in HGMG, while overexpression of miR-25 by miR-25 mimic markedly inhibited the high glucose induced cell apoptosis and promoted the proliferation of MG.

- **CONCLUSION:** The expression level of miR-25 is significantly downregulated in HGMG and its overexpression could attenuate the high glucose damages on MG by promoting proliferation and reducing apoptosis.

- **KEYWORDS:** microRNA-25; Müller glia; proliferation; apoptosis

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INTRODUCTION

Diabetic retinopathy (DR) is a major microvascular complication of diabetes and one of the main causes of blindness in working-age adults[1]. Multiple studies have suggested that the pathogenesis of DR is closely involved in the dysfunction of Müller glia (MG), which occurs even before vascular injury, especially in the preclinical stage of DR[2-3]. MGs are the major retinal supporting cells that participate in retinal metabolism, function, maintenance and protection by specifically expressing a variety of enzymes and ion channels. MGs also play an important role in the occurrence and development of retinal injury, making further investigation of the pathogenic role of MGs in DR of great significance for early diagnosis and treatment of DR[4-5].

MicroRNAs (miRNAs) are small non-coding RNAs which participate in posttranscriptional regulation of their target mRNAs and participates in physiological and pathological processes[6]. Accumulating evidence has shown that aberrant expressions of miRNAs are highly related to the onset and progression of various diseases, including cancer, congenital heart disease, and degeneration diseases[7]. Regarding ocular diseases, microRNA-25 (miR-25) has been reported to induce mature MGs to re-differentiate into retinal progenitor cells[8-9], but its role in DR has yet been reported. Here, we detected the expression difference of miR-25 between low and high glucose cultured Müller glia (LGMG and HGMG, respectively) and analyzed the effects of miR-25 on the HGMG. Moreover, we discussed its potential mechanism in the progress of DR in hope of identifying new targets for the treatment of DR.

MATERIALS AND METHODS

Culture of Mice Primary Müller Glia  Primary cultures of mice retinal microglia were conducted following the established protocol with minor modifications[10]. Briefly, postnatal day 6 to 7 mice were sacrificed under anesthesia and retina from 4-5 mice were harvested and digested in 70 U collagenase for 15min at 37°C. Cells were collected by centrifugation at 300×g for 5min and resuspended in Dulbecco’s modified Eagle media/nutrient mixture F-12 (DMEM/F12) medium containing 10% fetal bovine serum (FBS), 1% penicillin and streptomycin.

Evaluation of the Purity of Primary Cultured Müller Glia  Immunocytochemistry and flow cytometry were conducted to validate the purity of MG. Cells were seeded on 6-well cell
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culture plates and were fixed in 4% paraformaldehyde for 10 min at room temperature. Cells were washed three times with PBS. Afterwards, 5% bovine serum albumin (BSA) solution was added for 60 min, and samples were stained with primary antibodies (Abcam, ab16700 at 1/1000 dilution; ab64613 at 1/1000 dilution) and the second antibodies (1:200 dilution). Each sample was observed under a confocal microscope (MIC00223, LSM5 Live).

**RT-PCR** MG in different groups were harvested, and total RNA was extracted using Trizol reagent (Vazyme Biotech Co, Nanjing, China). The miRNA cDNA was synthesized using the kit (Ribo, Guangzhou, China) according to the manufacturer’s instructions. Reverse transcription quantitative polymerase chain reaction (RT-qPCR) was performed using miRNA RT-qPCR Starter Kit (Ribo, Guangzhou, China). MiR-39-3p was served as the external reference, added before total RNA extraction. All amplifications were performed in triplicate.

**Measurement of Müller Glia Proliferation** Cell proliferation was measured using the cell counting kit-8 (CCK8; MCE, USA) and the EdU cell proliferation kit (Ribo, Guangzhou, China). Forty-eight hours after transfection with miR-25 mimics or negative control, cell proliferation was detected using the CCK8 kit and EdU kit following the manufacturer’s instructions.

**Detection of Müller Glia Apoptosis** Cell apoptosis assay was performed using the Annexin V-FITC/PI apoptosis detection kit (Vazyme Biotech Co, Nanjing, China) according to the manufacturer’s protocol. MGs in different experimental groups were collected by centrifugation at 300×g for 5 min followed by being washed with PBS for three times. The 300 μL of 1×binding buffer was added and samples were mixed for analyze with a fluorescence activating cell sorter (FACS; BD Biosciences).

**Luciferase Activity Assay** The luciferase activity assay was conducted in 96-well plates. Wild-type Fbsw7 (Fbsw7-WT) or its mutation (Fbsw7-Mut) were co-transfected into MGs with miR-25 mimics or negative control, cell proliferation was detected using the EdU staining as well as CCK8 assay confirmed that MG proliferation was significantly downregulated in high glucose compared with the high glucose treated alone group. Also, results showed that up-regulation of miR-25 significantly alleviated apoptosis rate of MG treated with high glucose compared with the high glucose treated alone group. Also, the EdU staining as well as CCK8 assay confirmed that MG proliferation was significantly downregulated in high glucose medium, while miR-25 mimics treated group showed no variance (ANOVA), or curve fit. All statistical analyses were performed using SPSS 21.0 (SPSS, Chicago, USA). P-values <0.05 were considered statistically significant. All experiments were conducted in triplicate.

**RESULTS**

**Characterization of Cultured Retinal Müller Glia** As shown in Figure 1A, primary cultured MG exhibited typical morphological features of glia cells. Flow cytometry and immunocytochemistry both confirmed that glutamine synthetase (GS) and vimentin were positively expressed in primary cultured MG (Figure 1B and 1C). Nuclei were stained with DAPI. These results altogether confirmed our cultured cells were primarily MG.

**miR-25 Downregulated in Müller Glia Under High Glucose** The roles of miR-25 have been reported in multiple diseases including cancer, degeneration, and genesis of neural progenitor cells (Table 1). To evaluate the potential role of miR-25 in MGs, we investigated the expression level of miR-25 in MGs under high glucose. Results showed that the expression of miR-25 was significantly downregulated in high glucose (25 mmol/L) compared to its in low glucose medium (5 mmol/L; P<0.05, Figure 2A). These results indicate that the dysregulation of miR-25 expression in HGMG may have a regulatory role in the dysfunction of DR.

**miR-25 Alleviated HG-induced Injury on Müller Glia** Increasing evidence confirmed that miRNAs regulate apoptosis of retina cells exposed to high glucose. Shi et al. reported that M2 macrophage-derived exosomal miR-25 mimics attenuated high glucose-induced podocytes injury in diabetic nephropathy. Herein, we investigated the effects of miR-25 on MGs proliferation and apoptosis in high glucose medium. The 100 nmol/L miR-25 mimic, and negative control miRNA-NC were transfected into cells using Lipofectamine 3000 for 48 h. As shown in Figure 2B, flow cytometry analysis results showed that up-regulation of miR-25 significantly alleviated apoptosis rate of MG treated with high glucose compared with the high glucose treated alone group. Also, the EdU staining as well as CCK8 assay confirmed that MG proliferation was significantly downregulated in high glucose medium, while miR-25 mimics treated group showed no variance (ANOVA), or curve fit. All statistical analyses were performed using SPSS 21.0 (SPSS, Chicago, USA). P-values <0.05 were considered statistically significant. All experiments were conducted in triplicate.

**Table 1 Published miR-25 related diseases**

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<th>Name</th>
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**Statistical Analysis** All data were expressed as mean±standard deviation. The t-test, two-way repeated-measure analysis of
significant difference compared with NC group \((P<0.001, \text{Figure 2C and 2D})\). Our data reveal that MG were damaged in high glucose, while 500 nmol/L miR-25 could efficiently attenuate high glucose-induced MG injury.

**miR-25 Alleviated HG-induced Müller Glia Injury via miR-25 Targeting Fbxw7**

Fbxw7, which encodes F-box and WD repeat domain-containing 7 protein, was characterized as the target of miR-25 based on two datasets (miRbase, and miRTarBase; Figure 3A). Dual luciferase report assay was conducted to investigate whether miR-25 directly targeted Fbxw7. Co-transfecting miR-25 and 3’UTR-Fbxw7-WT vectors significantly decreased Fbxw7 luciferase activity, while 3’UTR-Fbxw7-MUT did not affect the luciferase activity (Figure 3B). To determine whether above effects on MGs depend specifically on Fbxw7 suppression, we overexpressed Fbxw7. Western blot results showed greater expression of
Fbxw7 in HG group than in miR-25 overexpression group (Figure 4A). FACS results showed that miR-25 overexpression attenuated HG-induced MGs apoptosis (Figure 4B). EdU staining as well as CCK8 assay showed that miR-25 mimics could protect MGs from HG-induced injury (Figure 4C and 4D). Our data suggest that after miR-25 overexpression, a decrease in Fbxw7 is essential for MGs to show increased proliferation and less apoptosis in high glucose medium (Figure 4B-4D).

DISCUSSION
The miRNAs play important roles in regulating the complex physiological and pathological processes by inhibiting the transcription and translation of the target mRNAs or promote its degradation by splicing the target mRNAs[13-14]. Numerous studies indicated the significant role of miRNAs in regulating the onset and progression of diabetes and its complications[15]. However, few studies have yet reported the elusive relationship between miRNA levels and the pathological development
of DR. In our study, we found that high glucose notably decreased the expression level of miR-25 in MG, indicating its potential role in modulating MG damages in DR. Furthermore, overexpression of miR-25 by miR-25 mimics (100 nmol/L) attenuated apoptosis and improved proliferation in MG under HG condition. Findings from our current study suggest that further investment in miR-25 may serve as a novel biomarker for the early detection of DR and shed light on the treatment for DR.

Ning et al.\textsuperscript{16} once reported that miR-25-3p could enhance the cell proliferation and migration of mouse glioma cells, impair tumorigenesis and regulate the invasion of gastric cancer cells. Also, miR-25-3p was verified to regulate cancer cell processes by targeting DKK3 and Fbsw7 genes via related pathways.\textsuperscript{17} Multiple studies have discussed the role of miR-25 in ocular diseases. Zhang et al.\textsuperscript{18} once reported that the increased miR-25 was confirmed to regulate the degeneration of retinal pigment epithelium in retinal degeneration disease. A previous research conducted by Wohl et al.\textsuperscript{19} demonstrated that increasing the level of miR-25 with a specific mimic causes an increase in MG-derived neuronal cells which means miR-25 may have a general role in maintaining the neural progenitor phenotype. Based on our data, miR-25 mimic was proven to protect MGs from high glucose induced damages via down-regulating Fbsw7, making maintaining the miR-25 expression a useful strategy in preventing MGs death associated with DR. MGs are responsible for a broad variety of significant functions including uptake and recycling of neurotransmitters, maintaining the water balance, ionic environment, and supply of nutrients for the retinal neurons.\textsuperscript{19-20} As the major glial cell type in the retina, MGs are closely related to DR.\textsuperscript{21-23} To date, numerous studies indicate that dysfunction of the retinal MGs precedes the microvascular dysregulation, especially in early stage of DR.\textsuperscript{24} Our results clearly showed that Müller cells are damaged in high glucose. Treatment of 25 mmol/L glucose resulted in a significant decrease in proliferation and increase in apoptosis compared with normal glucose. The apoptosis of MGs could initiate oxidative stress, producing reactive oxygen species.\textsuperscript{25}

To conclude, our current data demonstrate the function of miR-25 as a protective miRNA in MGs against HG-induced injury, while the specific mechanism of miR-25 exerting a rescue effect on MGs needs to be further investigated.

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