

# Role of microRNA-25 in high glucose cultured Müller glia

Yu Liu, Han Shen, Song-Tao Yuan, Qing-Huai Liu

Department of Ophthalmology, the First Affiliated Hospital of Nanjing Medical University, Nanjing 210029, Jiangsu Province, China

**Correspondence to:** Qing-Huai Liu and Song-Tao Yuan. Department of Ophthalmology, the First Affiliated Hospital of Nanjing Medical University, Nanjing 210029, Jiangsu Province, China. liuqh@njmu.edu.cn; yuansongtao@vip.163.com  
Received: 2020-12-17 Accepted: 2021-02-18

## 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-PCR 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

**DOI:10.18240/ijo.2021.05.02**

**Citation:** Liu Y, Shen H, Yuan ST, Liu QH. Role of microRNA-25 in high glucose cultured Müller glia. *Int J Ophthalmol* 2021;14(5): 643-648

## INTRODUCTION

Diabetic retinopathy (DR) is a major microvascular complication of diabetes and one of the main causes of blindness in working-age adults<sup>[1]</sup>. 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<sup>[2-3]</sup>. 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<sup>[4-5]</sup>.

MicroRNAs (miRNAs) are small non-coding RNAs which participate in posttranscriptional regulation of their target mRNAs and participates in physiological and pathological processes<sup>[6]</sup>. 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<sup>[7]</sup>. Regarding ocular diseases, microRNA-25 (miR-25) has been reported to induce mature MGs to re-differentiate into retinal progenitor cells<sup>[8-9]</sup>, 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<sup>[10]</sup>. 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

culture plates and were fixed in 4% paraformaldehyde for 10min at room temperature. Cells were washed three times with PBS. Afterwards, 5% bovine serum albumin (BSA) solution was added for 60min, 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 5min 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 mimic or mimic NC (100 nmol/L) using Lipofectamine 3000 (Invitrogen Technologies). The 24h after transfection, luciferase activities of podocytes were ensured using a Dual-Luciferase Report Assay Kit (Promega, Madison, WI).

**Western Blot Analysis** MGs (1×10<sup>5</sup> cells) were lysed in RIPA buffer, followed by 12 000×g centrifugation for 15min and concentration quantification using BCA protein assay kit (Cell Signaling Technology, USA). After blocking with 5% BSA, 0.45 μm polyvinylidene fluoride (PVDF) membranes were incubated with antibodies against Fbsw7 (Abcam, ab192328 at 1/500 dilution) and GAPDH (Abcam, ab8245 at 1/1000 dilution). Protein bands were detected with a Tanon 4100 Multi Fluorescence Imager.

**Statistical Analysis** All data were expressed as mean±standard deviation. The *t*-test, two-way repeated-measure analysis of

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

Name	Disease	PMID
miR-25	Diabetic nephropathy	33107695
	Cyanotic congenital heart disease	3328191
	Angiogenesis in aging	33201836
	Esophageal cancer	33103502
	Multiple sclerosis	32914363
	Neurogenesis	32585676
	Fetal growth restriction	31383796
	Lung cancer	33253979

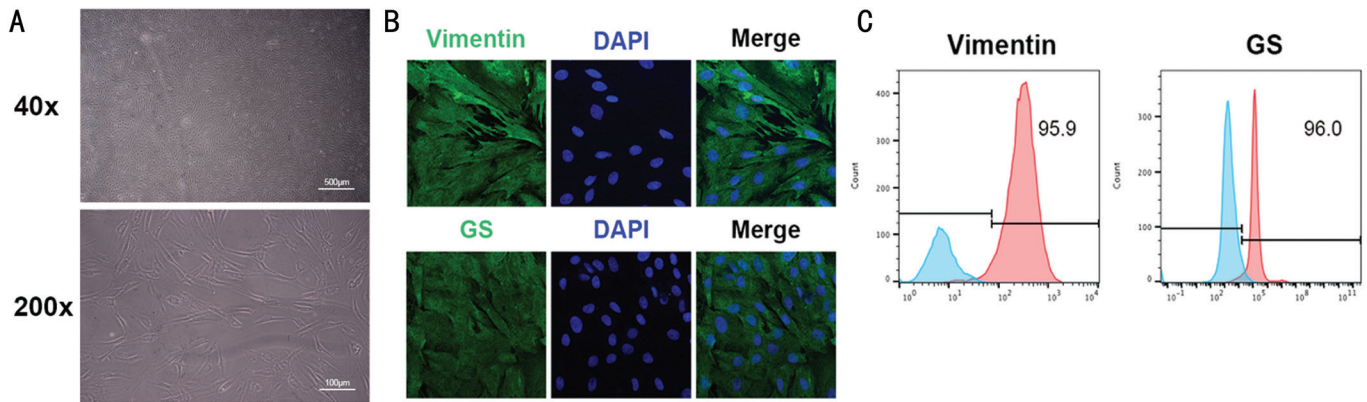
variance (ANOVA), or curve fit. All statistical analyses were performed using SPSS 21.0 (SPSS, Chicago, USA). *P*-values <0.05 were considered statistically significant. All experiments were conducted in triplicate.

**RESULTS**

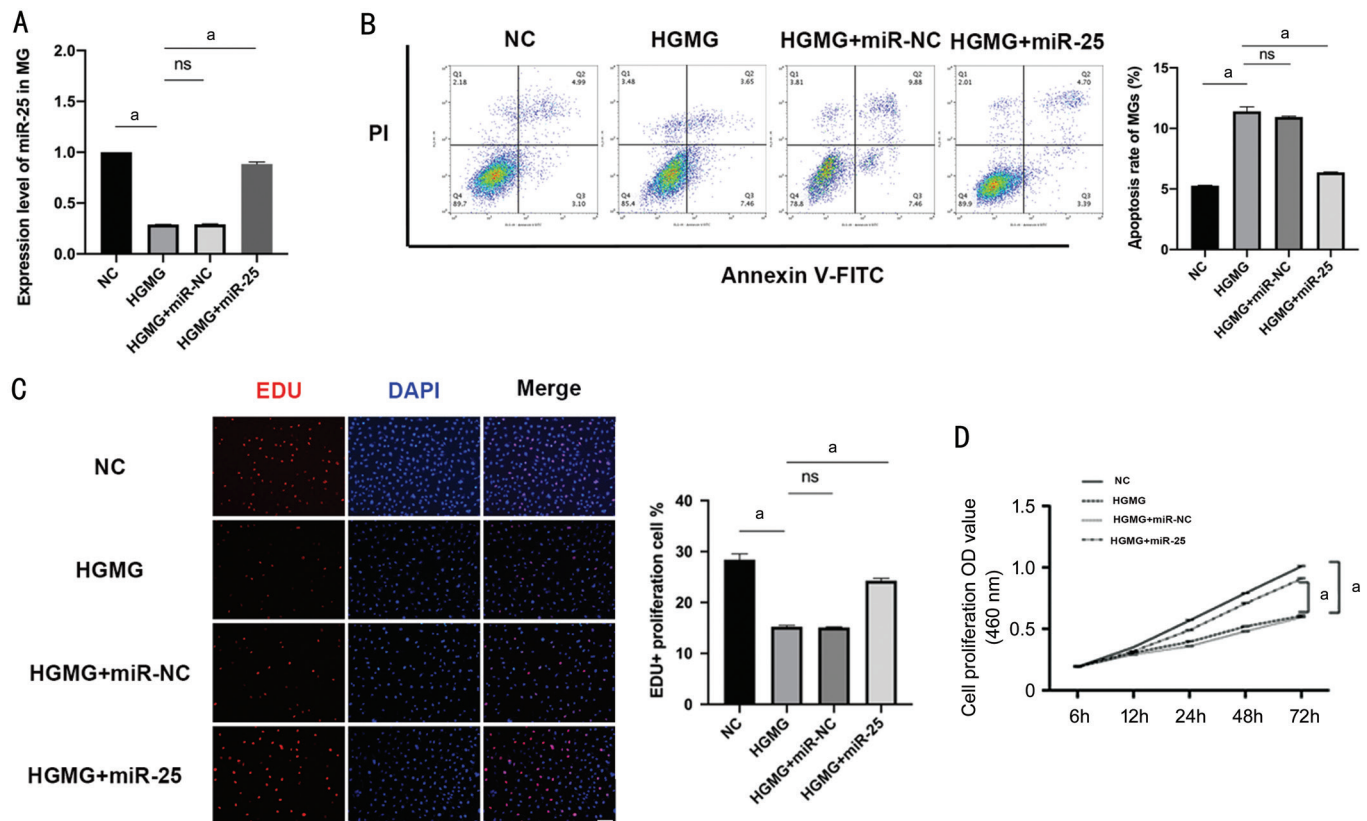
**Characterization of Cultured Retinal Müller Glia** As shown in Figure 1A, primary cultured MG exhibited typical morphological features of glia cells. Flow cytometry and immunocytochemistry both confirmed that glutamine synthetase (GS) and vimentin were positively expressed in primary cultured MG (Figure 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*<sup>[11]</sup> and Huang *et al*<sup>[12]</sup> reported that M2 macrophage-derived exosomal miR-25-3p 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 48h. As shown in Figure 2B, flow cytometry analysis results showed that up-regulation of miR-25 significantly alleviate 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



**Figure 1 MG characterization** A: Two days after seeding, light images were taken at 40× and 200× magnification; B: Immunocytochemistry confirmed that expressions of vimentin and glutamine synthetase (GS) in MG; C: Flow cytometric analysis of characteristic cell markers of MGs. Blue curves represent isotype controls for fluorescein isothiocyanate (FITC) and red curves represent measured markers (vimentin and GS, respectively). This experiment was repeated triplicate independently.

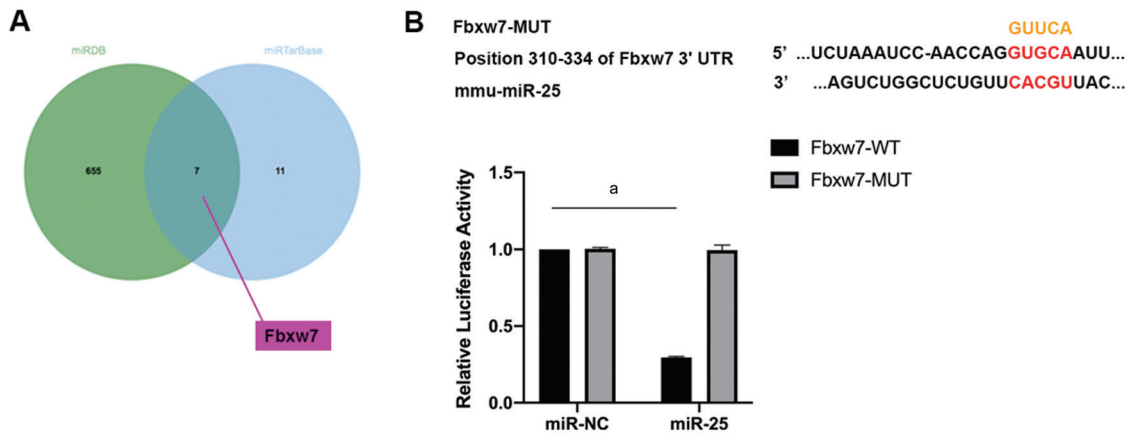


**Figure 2 miR-25 attenuated HG-induced injury on MG** A: RT-qPCR results showed that high glucose medium could significantly downregulated the expression level of miR-25 in MG, while 100 nmol/L miR-25 mimics was efficient to overexpress it; B: FACS results showed that miR-25 overexpression attenuated HG-induced MGs apoptosis; C and D: EdU staining and CCK8 assay both verified that miR-25 rescued MGs proliferation to a basal level. Scale bar=100  $\mu$ m. <sup>a</sup> $P$ <0.001,  $n$ =3.

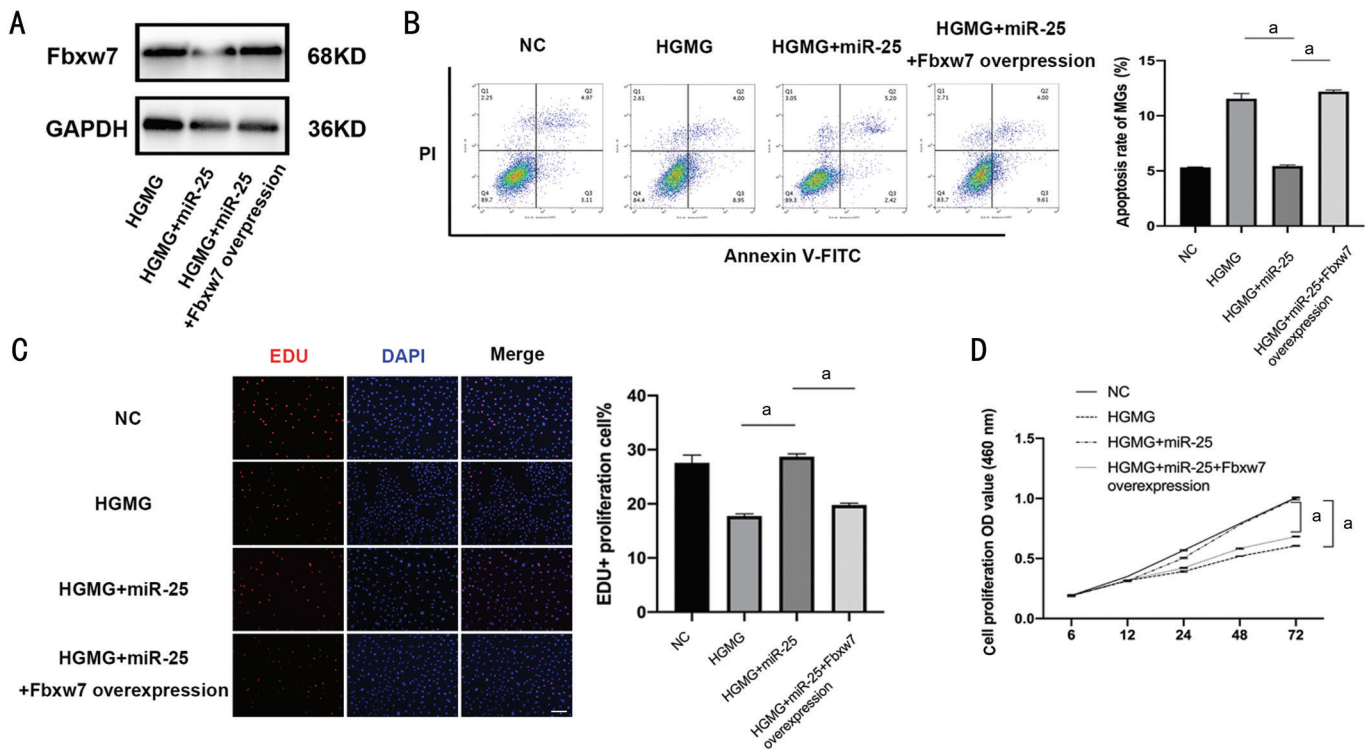
significant difference compared with NC group ( $P$ <0.001, Figure 2C and 2D). Our data reveal that MG were damaged in high glucose, while 500 nmol/L miR-25 could efficiently attenuate high glucose-induced MG injury.

**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



**Figure 3** Fbxw7 was characterized as the target of miR-25. A: Predicted duplex formation between mouse Fbxw7 3'UTR and miR-25. Seven genes were recognized as miR-25 targets verified by two different datasets (miRDB and miRTarBase). B: Dual luciferase reporter assay on Fbxw7-WT or Fbxw7-MUT transfected with NC or miR-25 mimics to explore the possible regulation mechanisms. The luciferase activities were measured 48h later. <sup>a</sup> $P < 0.001$ ,  $n = 3$ .



**Figure 4** miR-25 attenuated HG-induced MG injury via miR-25 downregulated Fbxw7 expression. A: Western blot verified that the protein level of Fbxw7 was decreased in the miR-25 overexpression group (HGMG+miR-25) compared with HGMG group; B: Cell apoptosis were detected by FACS; C, D: Cell proliferation of MGs after PBS, HGMG, HGMG+miR-25 and HGMG+miR-25+Fbxw7 administration were measured by EDU staining and CCK8 assay. Scale bar=100  $\mu$ m. <sup>a</sup> $P < 0.001$ ,  $n = 3$ .

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<sup>[13-14]</sup>. Numerous studies indicated the significant role of miRNAs in regulating the onset and progression of diabetes and its complications<sup>[15]</sup>. 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*<sup>[16]</sup> 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 Fbxw7 genes *via* related pathways<sup>[17]</sup>. Multiple studies have discussed the role of miR-25 in ocular diseases. Zhang *et al*<sup>[18]</sup> 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*<sup>[8]</sup> 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 Fbxw7, 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<sup>[19-20]</sup>. As the major glial cell type in the retina, MGs are closely related to DR<sup>[21-23]</sup>. To date, numerous studies indicate that dysfunction of the retinal MGs precedes the microvascular dysregulation, especially in early stage of DR<sup>[24]</sup>. 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<sup>[25]</sup>.

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.

#### ACKNOWLEDGEMENTS

We thank colleagues in Jiangsu Province Hospital Core Facility Center for critical input and suggestions.

**Foundation:** Supported by National Key Research and Development Program of China (No.2017YFA0104101).

**Conflicts of Interest:** Liu Y, None; Shen H, None; Yuan ST, None; Liu QH, None.

#### REFERENCES

- 1 Jampol LM, Glassman AR, Sun J. Evaluation and care of patients with diabetic retinopathy. *N Engl J Med* 2020;382(17):1629-1637.
- 2 Coughlin BA, Feenstra DJ, Mohr S. Müller cells and diabetic retinopathy. *Vision Res* 2017;139:93-100.
- 3 Abcouwer SF. Müller cell-microglia cross talk drives neuroinflammation in diabetic retinopathy. *Diabetes* 2017;66(2):261-263.
- 4 Malone JI, Morrison AD, Pavan PR, Cuthbertson DD; Diabetic Control and Complications Trial. Prevalence and significance of retinopathy in subjects with type 1 diabetes of less than 5 years' duration screened for the diabetes control and complications trial. *Diabetes Care* 2001;24(3):522-526.
- 5 Roy S, Kern TS, Song B, Stuebe C. Mechanistic insights into pathological changes in the diabetic retina: implications for targeting diabetic retinopathy. *Am J Pathol* 2017;187(1):9-19.
- 6 Andreeva K, Cooper NG. MicroRNAs in the neural retina. *Int J Genomics* 2014;2014:165897.
- 7 Zhang L, Tong Z, Sun Z, Zhu G, Shen E, Huang Y. MiR-25-3p targets PTEN to regulate the migration, invasion, and apoptosis of esophageal cancer cells via the PI3K/AKT pathway. *Biosci Rep* 2020;40(10):BSR20201901.
- 8 Wohl SG, Hooper MJ, Reh TA. MicroRNAs miR-25, let-7 and miR-124 regulate the neurogenic potential of Müller glia in mice. *Development* 2019;146(17):dev179556.
- 9 Cheng LC, Pastrana E, Tavazoie M, Doetsch F. miR-124 regulates adult neurogenesis in the subventricular zone stem cell niche. *Nat Neurosci* 2009;12(4):399-408.
- 10 Hicks D, Courtois Y. The growth and behaviour of rat retinal Müller cells *in vitro*. 1. An improved method for isolation and culture. *Exp Eye Res* 1990;51(2):119-129.
- 11 Shi Q, Chen X, Sun G, Wang L, Cui L. Ginsenoside Rg1 protects human retinal pigment epithelial ARPE-19 cells from toxicity of high glucose by up-regulation of miR-26a. *Life Sci* 2019;221:152-158.
- 12 Huang HH, Liu HY, Tang JZ, Xu WQ, Gan HX, Fan QW, Zhang W. M2 macrophage-derived exosomal miR-25-3p improves high glucose-induced podocytes injury through activation autophagy via inhibiting DUSP1 expression. *IUBMB Life* 2020;72(12):2651-2662.
- 13 Raffort J, Lareyre F, Clement M, Mallat Z. Micro-RNAs in abdominal aortic aneurysms: insights from animal models and relevance to human disease. *Cardiovasc Res* 2016;110(2):165-177.
- 14 Wu C, Bardes EE, Jegga AG, Aronow BJ. ToppMiR: ranking microRNAs and their mRNA targets based on biological functions and context. *Nucleic Acids Res* 2014;42(Web Server issue):W107-W113.
- 15 Montoya V, Fan H, Bryar PJ, Weinstein JL, Mets MB, Feng G, Martin J, Martin A, Jiang H, Laurie NA. Novel miRNA-31 and miRNA-200a-mediated regulation of retinoblastoma proliferation. *PLoS One* 2015;10(9):e0138366.
- 16 Ning L, Zhang M, Zhu Q, Hao F, Shen W, Chen D. miR-25-3p inhibition impairs tumorigenesis and invasion in gastric cancer cells *in vitro* and *in vivo*. *Bioengineered* 2020;11(1):81-90.

- 17 Peng G, Yang C, Liu Y, Shen C. miR-25-3p promotes glioma cell proliferation and migration by targeting FBXW7 and DKK3. *Exp Ther Med* 2019;18(1):769-778.
- 18 Zhang J, Wang J, Zheng L, *et al.* miR-25 mediates retinal degeneration via inhibiting ITGAV and PEDF in rat. *Curr Mol Med* 2017;17(5):359-374.
- 19 Kim D, Lewis CS, Sarthy VP, Roy S. High-glucose-induced Rab20 upregulation disrupts gap junction intercellular communication and promotes apoptosis in retinal endothelial and Müller cells: implications for diabetic retinopathy. *J Clin Med* 2020;9(11):3710.
- 20 Chen Z, Yang Z, Li X, Wang H, Wang Y, Ding C, Yang J, Ni N, Mei Y, Zhang S. MicroRNA-320a prevent Müller cells from hypoxia injury by targeting aquaporin-4. *J Cell Biochem* 2020. Epub ahead of print.
- 21 Zeng K, Wang Y, Yang N, *et al.* Resveratrol Inhibits diabetic-induced müller cells apoptosis through microRNA-29b/specificity protein 1 pathway. *Mol Neurobiol* 2017;54(6):4000-4014.
- 22 Rajaram K, Harding RL, Bailey T, Patton JG, Hyde DR. Dynamic miRNA expression patterns during retinal regeneration in zebrafish: reduced dicer or miRNA expression suppresses proliferation of Müller glia-derived neuronal progenitor cells. *Dev Dyn* 2014;243(12):1591-1605.
- 23 Wang J, He C, Zhou T, Huang ZJ, Zhou LL, Liu XL. NGF increases VEGF expression and promotes cell proliferation via ERK1/2 and AKT signaling in Müller cells. *Mol Vis* 2016;22:254-263.
- 24 Ghaseminejad F, Kaplan L, Pfaller AM, Hauck SM, Grosche A. The role of Müller cell glucocorticoid signaling in diabetic retinopathy. *Graefes Arch Clin Exp Ophthalmol* 2020;258(2):221-230.
- 25 Zeng K, Yang N, Wang D, *et al.* Resveratrol prevents retinal dysfunction by regulating glutamate transporters, glutamine synthetase expression and activity in diabetic retina. *Neurochem Res* 2016;41(5):1050-1064.