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Original Article

MicroRNA-125b protects hyperglycemia-induced, human retinal pigment epithelial cells (RPE) from death by targeting hexokinase 2

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Abstract: Diabetic retinal disease (DR) is the main cause of visual disability and blindness in adults with diabetes mellitus. Currently, efficient prevention and treatment are still under investigation. MicroRNAs (miRNAs) are groups of short, non-coding RNAs that post-transcriptionally control their target genes' expression through complementary binding to the 3'UTR region. MiRNAs have been reported to play important roles in a variety of physiological and pathophysiological processes. However, the roles of miR-125b in DR are still unclear. In this study, we exposed human retinal pigment epithelial (RPE) cells to high glucose levels to mimic DR progression. Hyperglycemia induced RPE cell death in 1, 3 and 5 days. Meanwhile, we observed that miR-125b expressions were significantly downregulated by the high glucose treatments. We demonstrated elevated cellular glycolysis rates of RPE cells under hyperglycemia. The glycolysis key enzymes, GLUT1, Hexokinase 2 (HK2) and LDHA were upregulated by high glucose. Moreover, treatments of RPE cells with low-toxic dosages of the glycolysis inhibitor, 2-DG or Oxamate, rescued the high glucose-induced cell from death. We identified hexokinase 2 as a direct target of miR-125b in RPE cells by showing the binding of the miR-125b seed region to HK2 mRNA 3'UTR. Notably, we demonstrated that the overexpression of miR-125b significantly attenuated hyperglycemia-induced RPE cell death. This study reveals a new mechanism for miRNA-mediated cellular protection against RPE cell death, representing an effective DR-treatment approach.

Keywords: Diabetic retinal disease, microRNA-125b, retinal pigment epithelial cells, hexokinase 2

Introduction

Diabetic retinopathy (DR) is the main cause of visual disability and blindness resulting from retinal neurodegeneration and the microvascular abnormalities of diabetes [1]. Currently, DR is the leading cause of legal blindness among adults [2]. One risk factor for diabetic retinopathy is hyperglycemia, which leads to increased inflammatory responses and vascular dysfunction of the eye [3]. Currently, the molecular mechanisms underlying hyperglycemia-mediated DR are not fully understood. Available treatments for patients with proliferative diabetic retinopathy are laser photocoagulation or anti-vascular endothelial growth factor (VEGF) [4]. However, many patients show little response to these therapies [5]. Therefore, it is imperative to develop new approaches to treat diabetic retinopathy.

An elevated blood glucose level is a common characteristic of insulin-dependent diabetes [6]. In addition, studies have reported that high retinal glucose causes a "hypoxia-like" redox imbalance with increased lactate production and retinal lactate-pyruvate ratios [7], suggesting increased blood glucose results in an elevated retinal glycolysis rate.

MicroRNAs (miRNAs) are a class of short (20-25 nt) endogenous RNAs that post-transcriptionally regulate the stability of their target mRNAs by directly binding to the 3'UTR region [8]. Most studies have revealed dysregulated miRNAs is a risk factor for the development of diabetes and its associated DR [9]. It has been reported that miR-125b is closely associated with the presence of microvascular complications in type 2 diabetes mellitus [10]. Moreover, miR-125b has been described as playing an impor-

tant role during the development of early diabetic retinopathy because miR-125b expression decreases with DR progression *in vitro* and *in vivo [9]*, indicating that targeting the miR-125b-mediated pathway might contribute to the development of a new therapeutic agent to treat diabetic retinopathy. The aim of this study was to investigate the roles of miR-12-5b in DR using a high glucose-treated retina pigment epithelial cell line, ARPE-19 as an *in vitro* model. The direct target of miR-125b in human retina pigment epithelial cells will be identified. Results from this study will reveal mechanisms of the miRNA-mediated protection of RPE cells against hyperglycemia-induced cell death.

Materials and methods

Cell culture and hyperglycemia treatment

Human RPE cell line ARPE-19 was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco's modified Eagle medium/F-12 (Hyclone, Beijing, China) containing 10% FBS (Gibco; Thermo Fisher Scientific, Waltham, MA, USA). Cell cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. For hyperglycemia treatment, ARPE-19 cells were plated at 5×10^5 cell/well in 6-well plates (Corning, Acton, MA, USA) and treated with normal glucose (NG) at 5.5 mmol/L as a control, or with high glucose (HG) at 25 mmol/L, or the osmotic control mannitol at 19.5 mmol/L for 1, 3 and 5 days to mimic the early stage of DR. The media were refreshed daily.

Antibodies and reagents

Rabbit monoclonal antibody against Hexokinase 2 (#2867), GLUT1 (#12939) and LDHA (#3582) were purchased from Cell Signaling (Beverly, MA, USA). Mouse monoclonal antibody against β-actin (sc-47778) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). 2-DG and Oxamate were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell death assay

The cell death assay was performed using a LDH Cytotoxicity Detection Kit (Clontech, Mountain View, CA, USA) according to the manufacture's instruction. Briefly, after treatments, 50 μl of cell culture supernatants was collected. Cells were lysed with 200 μl of 0.1% Triton X-100, and 50 μl of lysate was subjected to analysis. LDH activities in culture supernatants and cell lysates were determined by adding 50 μl of 'substrate solution' from the kit, followed by incubation at 37°C for 15 min. The reaction was stopped by the addition of 100 μl of 'stopping solution'. Absorbance at 550 nm was measured. Cell damage was calculated as a percentage of LDH leakage from damaged cells by the following formula: LDH leakage (%) = (Sup)/(Sup + Cell) ×100. Each experiment was performed in triplicate.

Measurement of cellular glycolysis rate

The cellular glycolysis rate was examined using the Glucose Uptake Assay Kit (Colorimetric) (ab136955) and the L-Lactate Assay Kit (Colorimetric) (ab65331, Abcam, Cambridge, United Kingdom) according to the manufacturer's instructions. Results were normalized accoring to the protein concentrations of each sample.

Total RNA isolation and qRT-PCR

To analyze the miRNA-125b and glycolysis enzymes mRNA expressions, total RNA was isolated using a RNeasy Mini Kit (#74104, Qiagen, Hilden, Germany) according to the manufacturer's instructions. For detection of miR-125b expressions, 1.5 μg of total RNA was polyadenylated and reverse transcribed using an TaqMan MicroRNA Reverse Transcription Kit (#4366596, ThermoFisher, Waltham, MA, USA). The obtained cDNA was used for a further, quantitative, real-time polymerase chain reaction (qPCR). miRNA levels were measured using an mirVana™ qRT-PCR miRNA Detection Kit (#AM1558, ThermoFisher, Waltham, MA, USA). Measurements were performed in triplicate and RNA U6 was used as a normalization control for miRNA expression. For mRNA analysis, 500 ng of total RNA was reverse transcribed using a reverse transcribed into cDNA with TransScript First-Strand cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China). The primer sequences for GLUT1: forward: 5'-AACTCTTCAGCCAGGGTCCAC-3'; reverse: 5'-CACAGTGAAGATGATGAAGAC-3'; HK2: forward: 5'-CAAAGTGACAGTGGGTGTGG-3'; reverse: 5'-GCCAGGTCCTTCACTGTCTC-3'; LDHA: forward: 5'-AGCCCGATTCCGTTACCT-3'; reverse: 5'-CACCAGCAACATTCATTCCA-3'. β-actin: forward: 5'-A-

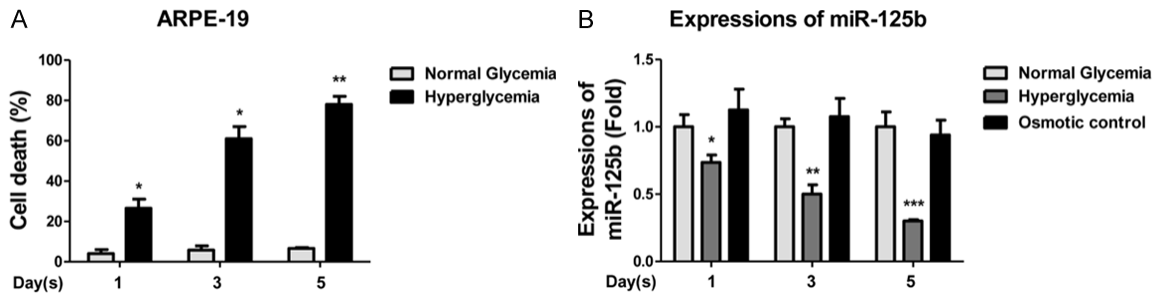

Figure 1. Effects of high glucose (Hyperglycemia) on the cell death and miR-125b expression in ARPE-19 cells. A. Hyperglycemia-induced cell death of RPE cells on days 1, 3 and 5. B. Hyperglycemia-induced miR-125b downregulation in RPE cells on days 1, 3 and 5. *P < 0.05; **P < 0.01; ***P < 0.001.

GCCATGTACGTAGCCATCC-3'; reverse: 5'-ACCCTCATAGATGGGCACAG-3'. β-actin was used as internal control. Reactions were mixed in a 20 μL reaction volume with 1 μL cDNA, 10 μmol/L qPCR primers (forward and reverse), 10 μL of 2× Fast SYBR Green master mix (Roche Diagnostics) and 8.5 μL water. Each sample was performed in triplicate. The relative expression levels of miRNAs and mRNAs were calculated using the $2^{-\Delta\Delta Ct}$ method.

Luciferase assay

The wild type or mutant 3'UTR of human HK2 was cloned into pMIR-REPORT vector (Ambion). The miR-125b mimic or control mimic and pMIR-WT-HK2 or pMIR-mutant-HK2 plasmids were cotransfected into RPE cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Forty-eight hours after transfection, luciferase activity was measured using the Dual-Light Chemiluminescent Reporter Gene Assay System (Applied Biosystems, Carlsbad, CA) following the manufacturer's instructions. Luciferase activity was read using Chemiluminescent SpectraMax M5 (Molecular Devices, Sunnyvale, CA). All experiments were performed in triplicate.

Western blot

ARPE-19 cells were lysed using a RIPA cell lysis buffer from ThermoFisher Scientific, (Waltham, MA, USA). Lysate was placed on ice for 30 min, and total protein was collected by centrifuging the lysates at 12,000 rpm for 10 min at 4°C. Protein concentrations were determined by the Bradford method. An equal amount protein was denatured with the SDS sample buffer then loaded in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. After running, proteins from the gel were transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked in 5% BSA for 1 h at room temperature, incubated with primary antibodies at 4°C for overnight. The membrane was then incubated with secondary antibodies for 1 hour at room temperature. An enhanced chemiluminescence plus kit (Millipore, Billerica, MA, USA) was used to visualize the bands. All experiments were repeated three times.

Statistical analysis

Experimental data are presented as means ± standard deviation. The statistical analysis was performed using Prism 6.0 software (GraphPad Software, San Diego, CA, USA). A Student T-test was used to compare the significance between the control and experimental groups. The results were considered statistically significant at $P < 0.05$.

Results

Hyperglycemia induces RPE cell death and downregulates miR-125b expression

To assess the effects of hyperglycemia on the *in vitro* viability of RPE cells, we conducted experiments on ARPE-19 cells by exposing them to normal glucose conditions or hyperglycemia at 25 mmol/L glucose. Expectedly, RPE cells exhibited significant cell death under exposure to hyperglycemia on days 1, 3 and 5 (**Figure 1A**). We next investigated the putative mechanisms underlining the hyperglycemia-induced cell death. Recent studies reported groups of differentially expressed microR-

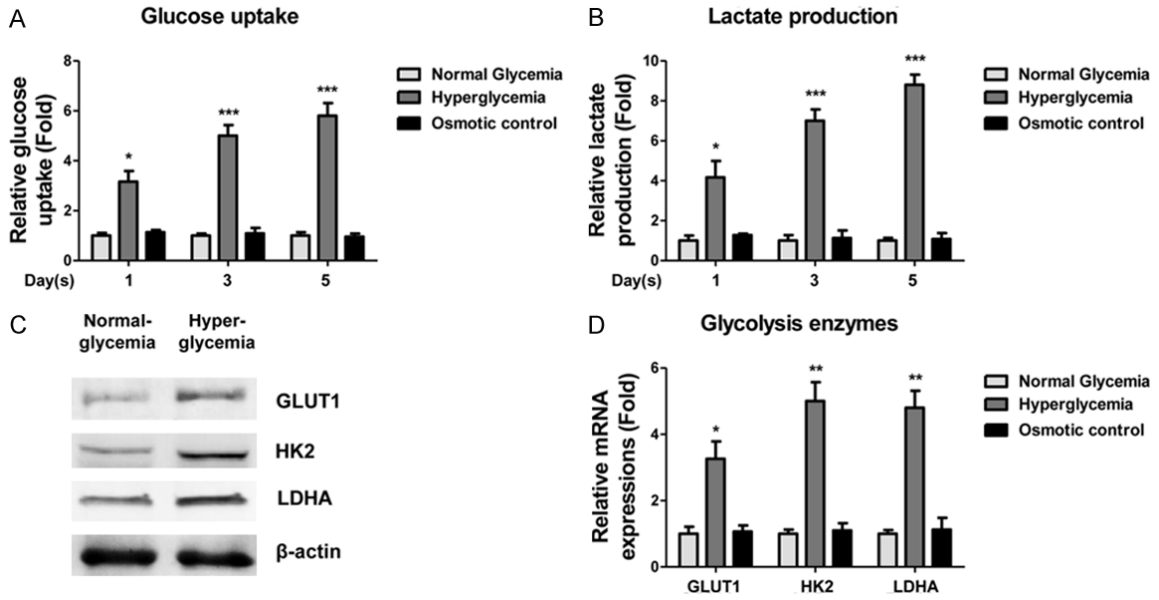

Figure 2. Effects of hyperglycemia on the cellular glycolysis of ARPE-19 cells. ARPE-19 cells were treated with normal glycemia, hyperglycemia and osmotic control conditions for 1, 3 and 5 days. The (A) glycose uptake and (B) lactate production were detected. (C) ARPE-19 cells were treated with normal glycemia or hyperglycemia for 3 days, the protein and (D) expressions of GLUT1, HK2 and LDHA were measured by Western blotting and qRT-PCR, respectively. β-actin is a loading control. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

NAs in early diabetic retinopathy [9], suggesting miRNAs might play important roles during hyperglycemia. Consistent with previous reports, we observed that miR-125b expressions were significantly suppressed under hyperglycemia conditions at days 1, 3 and 5 (**Figure 1B**). These results indicate miR-125 could be a selected target against RPE cell death under high glucose.

Glycolysis rate of RPE cells were promoted by hyperglycemia

It is known that dysregulated cellular metabolism leads to the induction of intrinsic cell death pathways [11]. To explore the roles of miR-125b during hyperglycemia, we first measured the glycolysis rate of RPE cells under normal and hyperglycemia conditions. During high glucose treatments at days 1, 3 and 5, the glucose uptake and lactate production were significantly increased (**Figure 2A**, **2B**). Meanwhile, key enzymes (GLUT1, HK2 and LDHA) which catalyze the speed-limiting reactions of glycolysis pathways, were significantly upregulated at both the protein (**Figure 2C**) and mRNA (**Figure 2D**) levels. Taken together, the above results indicate that targeting dysregulated glycolysis induced by hyperglycemia might prevent RPE cell death.

Inhibition of glycolysis attenuates the hyperglycemia-induced RPE cell death

To assess the effects of glycolysis suppression on high glucose-induced RPE cell death, we treated ARPE-19 cells with two glycolysis inhibitors, 2-DG which inhibits glycolysis via its actions on hexokinase [12], and Oxamate, an analog of pyruvate, which inhibits lactate dehydrogenase (LDH) [12]. Under 50 or 100 μM 2-DG treatment, RPE cells did not demonstrate cell death (data not shown). Intriguingly, inhibition of glycolysis by 2-DG significantly attenuated hyperglycemia-induced cell death at days 1, 3 and 5 (**Figure 3A**). Similar results were observed in RPE cells with the treatment of Oxamate at 0.5 or 1 mM (little toxicity to cells) under hyperglycemia conditions (**Figure 3B**), suggesting that the inhibition of dysregulated glycolysis of RPE cells could prevent high glucose-induced cell death.

MiR-125b directly targets glycolysis enzyme hexokinase 2

Our above results revealed a correlation between the hyperglycemia-induced glycolysis and -downregulated miR-125b expressions in RPE cells. To investigate whether miR-125b directly regulated glycolysis during hyperglyce-

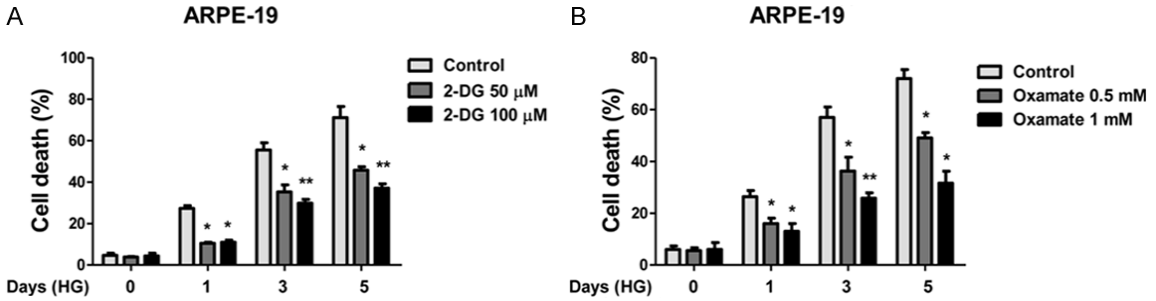

Figure 3. Inhibition of glycolysis protects RPE cells against hyperglycemia induced-cell death. A. ARPE-19 cells were treated with control, 2-DG at 50 or 100 μM under hyperglycemia conditions for 0, 1, 3 and 5 days. Cell death rates were measured. B. ARPE-19 cells were treated with the control, Oxamate, at 0.5 or 1 mM under hyperglycemia conditions for 0, 1, 3 and 5 days. Cell death rates were measured. **P* < 0.05; ***P* < 0.01.

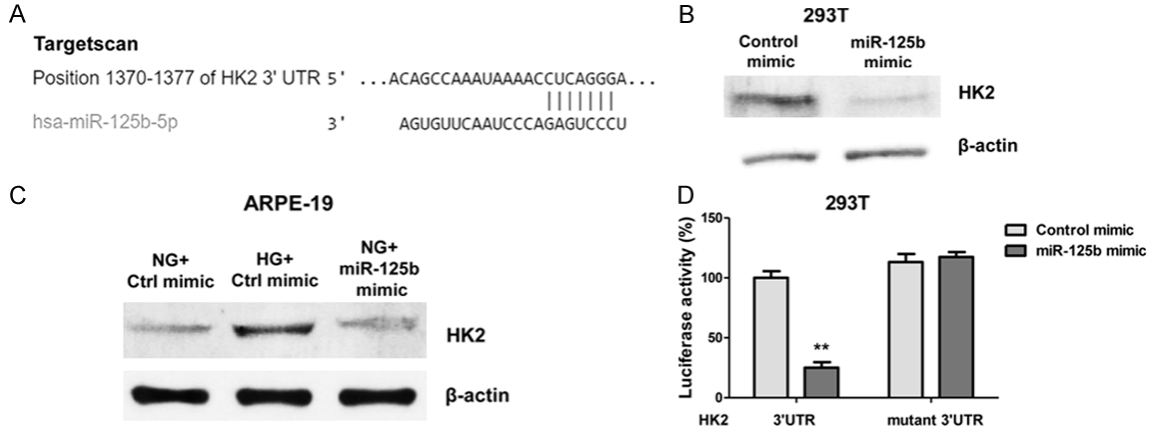

Figure 4. MiR-125b directly targets Hexokinase 2 in RPE cells under hyperglycemia. A. Targetscan predicted seed regions of miR-125b in the 3'UTR of HK2 mRNA. B. Expression of the HK2 protein was significantly suppressed by transfection with a miR-125b mimic in 293T cells compared with the control mimic transfection. β-actin was used as a loading control. C. Expression of the HK2 protein was significantly suppressed by miR-125b overexpression in RPE cells under hyperglycemic conditions compared with control mimic transfection under normal and hyperglycemia. D. A luciferase assay showed binding of HK2 3'-UTR with miR-125b, whereas mutant HK2 3'-UTR abrogated the inhibitory effects of miR-125b. ***P* < 0.01.

mia, we searched potential miR-125b targets that may have roles in regulating cellular glycolysis. From the miRNA target prediction tool TargetScan, we found Hexokinase 2 was among the commonly predicted targets with putative miR-125b interaction sites presenting in the 3'-UTR of HK2 mRNA (**Figure 4A**). Since HK2 was already shown as a target of miR-125a in multiple cancer cells [13, 14], we were interested in investigating whether miR-125b also targeted HK2 in human RPE cells. Thus, an miR-125 mimic or control mimic was transfected into 293T cells. As we expected, Western blotting results demonstrated the HK2 protein level was approximately 80% decreased in miR-125 mimic transfected cells compared to the control mimic transfected cells (**Figure 4B**). Notably, with the introduction of miR-125b into RPE cells, HK2 protein expressions were also significantly inhibited under hyperglycemia condition compared with normal glucose and control miRNAs transfected under hyperglycemia (**Figure 4C**). To validate miR-125b targeting of 3'UTR of HK2 mRNA, a luciferase reporter assay was performed by co-transfection miR-125b mimic or control mimic with plasmid containing a constitutively active promoter and miR-125b binding sites of HK2 3'-UTR or binding sites mutated 3'-UTR. Expectedly, ectopic overexpression of miR-125b led to a significant reduction of luciferase reporter activity of plasmid with HK2 3'UTR compared to the control miRNAs transfection (**Figure 4D**). Moreover, the overexpression of miR-125b into

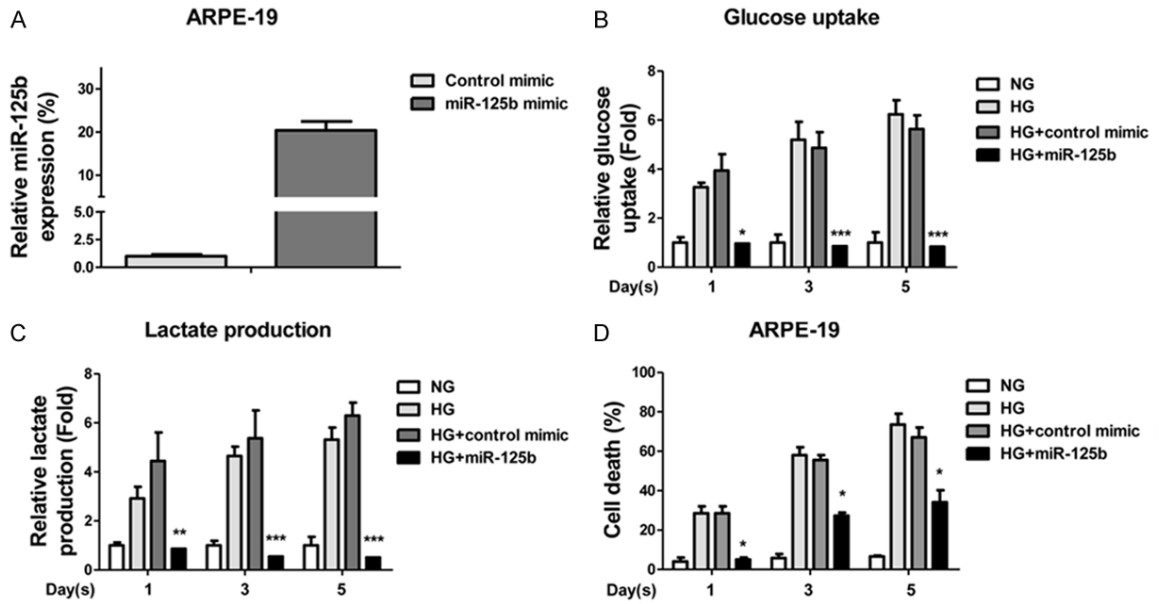

Figure 5. Overexpression of miR-125b prevents hyperglycemia-induced RPE cell death. (A) ARPE-19 cells were transfected with a control mimic or with the miR-125b mimic for 48 hours, and the expressions of miR-125b were detected by qRT-PCR. (B) ARPE-19 cells were transfected with a control mimic or the miR-125b mimic for 48 hours, followed by measurements of glucose uptake, (C) lactate production and (D) cell death under normal or high glucose conditions. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

293T cells with mutant HK2 3'UTR elicited no effects (**Figure 4D**). These results verified HK2 was a direct target of miR-125b in human RPE cells.

Overexpression of miR-125b prevents the hyperglycemia-induced RPE cell death through suppression of glycolysis

To examine the functions of miR-125b during the hyperglycemia-induced RPE cell death, we transfected miR-125b mimic or control mimic in ARPE-19 cells. The transfection efficiency was confirmed by analyzing the expressions of miR-125b (**Figure 5A**). We then examined the glycolysis rates by overexpression of miR-125b under normal or high glucose conditions. The glucose uptake (**Figure 5B**) and lactate production (**Figure 5C**) were significantly suppressed by overexpression in miR-125b under hyperglycemic conditions. Therefore, we hypothesized that the overexpression of miR-125b could protect against hyperglycemia-induced RPE cell death. To test this, ARPE-19 cells were transfected with a control mimic or miR-125b, and then the cells were exposed to normal- or hyperglycemia conditions for 1, 3 and 5 days. We obtained consistent results that under hyperglycemia at 1, 3 and 5 days, the overexpression of miR-125b significantly decreased RPE cell death, compared to the control miRNA transfection (**Figure 5D**). In summary, the above results demonstrated that the overexpression of miR-125 could prevent high glucose induced RPE cell death, making miR-125b a potential therapeutic agent for diabetic retinopathy.

Discussions

Diabetic retinopathy is a serious eye complication associated with diabetes mellitus [1]. Notably, patients with DR also exhibit an increased risk of microvascular complications [1, 2]. Since abnormally high levels of blood glucose is the leading cause of type 2 diabetes mellitus, hyperglycemia has been recognized as a representative *in vitro* model to study DR [9]. During hyperglycemia, which mimics the pathologic changes of the early stage of DR, RPE cells are affected and impaired by decreased cell viability and increased cell death, resulting in retina dysfunction and contributing to DR progression [15]. In this study, we treated ARPE-19 cells with normal or high glucose and found that, under hyperglycemic conditions, ARPE-19 cells underwent cell death. Meanwhile, hyperglycemia suppressed the miR-125b expressions, suggesting a negative correlation

between miR-125b expression and RPE cell death under hyperglycemia.

A recent study reported that miR-125b was found downregulated during diabetic retinopathy in a rat model [9]. However, the molecular mechanisms underlying DR are not fully understood. We also obtained consistent results in human ARPE-19 cells. Although miR-125b directly targets HK2 in multiple cancer types [13, 14], we first verified that HK2 could be targeted by miR-125b in human RPE cells under hyperglycemic conditions, indicating that targeting the dysregulated glucose metabolism of RPE cells with DR might lead to therapeutic improvement.

High blood glucose promotes RPE cells to uptake glucose and further increases pyruvate and lactate products. In cancer cells, increased cellular glucose metabolism gives them higher survival advantages, particularly under extra stress [11]. Moreover, increased glucose metabolism leads to lactate production and secretion. However, hyperglycemia-induced glycolysis results in lactate accumulation, which is toxic to cells. We therefore hypothesized that the suppression of high glucose promoted the imbalance of the cellular glucose metabolism could promote the survival of RPE cells. As we expected, either glycolysis inhibitor treatment or overexpression of miR-125b rescued RPE cells under hyperglycemia. Although we report consistent functions and mechanisms of miR-125b-meidated RPE cell protection under hyperglycemia, this *in vitro* system still has limitation that could not fully reproduce *in vitro* results into *in vivo* system. We will continue to investigate the mechanisms and test the above *in vitro* results using streptozotocin-injected rats as an *in vivo* diabetes model. In general, our study revealed the protective function of miR-125b during high glucose induced RPE cell death, presenting a new mechanism for development of therapeutic approaches against diabetic retinopathy.

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Disclosure of conflict of interest

None.

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