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Original Article

miR-128 protects retinal pigment epithelium in high glucose through HOXB3/PI3K/ERK-mTOR pathway

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Abstract: Diabetic retinopathy (DR) is a common ocular disease characterized by the serious microvascular complications of diabetes. Nowdays he underlying pathogenesis of diabetic retinopathy remained poorly understood. The process of abnormal cell viability or apoptosis, which was still complex and undiscovered in the DR, remains a possible target for the treatment of DR. HOX gene is involved in the migration, proliferation and differentiation of vascular endothelial cells. However, the exact role of HOXB3 in DR development and the pathological mechanism was still unclear by now. microRNAs (miRNAs) could posttranscriptionally regulate gene expression. Therefore in the present study, we assessed the effect of miR-128 on proliferation, apoptosis and the expression of HOXB3 of RPE cells in high glucose condition. In addition, we conducted several experiments to detect the detailed signaling pathways for the protective effect of miR-128. In our study, the data showed that miR-128 level was down-regulated in the high glucose group. Besides, through the analyses of the cell functions, we found that miR-128 would protect the RPE cell in both cell viability and apoptosis condition. Through western blot and luciferase analyses, we found that miR-128 would down-regulate HOXB3 expression through targeting the 3'UTR of HOXB3 gene. Moreover, through the analyses of the PI3K/AKT-mTOR pathway, it was found that miR-128 treatment decreased the phosphorylated protein level of PI3K and AKT in RPE in vivo. As mTOR was one of the downstream genes of PI3K/AKT pathway, we also found that miR-128 could down-regulate the expression of m-TOR. Collectively our data support the notion that miR-128 could protect the cell viability and apoptosis of RPE cells in high glucose. Advanced study showed that miR-128 produced the protective effect through down-regulating of HOXB3 and inhibiting PI3K/AKT-mTOR pathway. Our results demonstrate a potential for miR-128 in the development of miRNA-based therapeutics for DR treatment.

Keywords: miR-128, diabetic retinopathy, HOXB3

Introduction

Diabetes mellitus (DM) is a microangiopathic atherosclerotic disease that affects the capillary bed in many body organs, mainly the kidneys, retina, and peripheral nervous system [1]. Diabetic retinopathy (DR) is a common ocular disease characterized by the serious microvascular complications of DM [2]. DR is now one of the leading causes of blindness in working-age individuals. The causes of DR were still unclear by now and the established risk factors for DR were complex, including duration of diabetes, hyperglycemia, and hypertension [3]. Furthermore, the underlying pathogenesis of diabetic retinopathy remained poorly understood.

Despite the fact that increased glucose level is the main pathogenic factors of DR, abnormal cell viability and apoptosis in the high glucose condition is final pathway of retinal cell death in the early stage of DR condition [4]. The process of abnormal cell viability or apoptosis, which was still complex and undiscovered in the DR, remains a possible target for the treatment of DR [5].

HOX gene is involved in the migration, proliferation and differentiation of vascular endothelial cells [6, 7]. Considering the comprehensive effect of Hox gene, it could be conjectured that there was potential relationship between HOX gene group and DR incidence. More than 30 kinds of HOX gene have been reported so far [8]. Most of the Hox genes contain a period of about 180 nucleotides of the homeobox fragment, transcription with approximately 60 amino acid sequence, known as homologous

protein zones (homeodomain), and the HOX gene group is highly conserved. They are clustered in four different chromosomes (7, 17, 12 and 2), which were named as A, B, C and D four cluster [9]. Each cluster consists of a number of different sizes of gene fragments, and then they are named after the number. The Hoxb3 gene is expressed in the posterior neural tube and extended to r5, with an anterior boundary at r4/5 and this expression domain is complementary to the expression territory of Hoxb1 [10]. Based on the evolutionary conserved posterior prevalence model, Hoxb3 could be a candidate negative regulator of Hoxb1 and suppresses the expression of Hoxb1 in the neural tube caudal to r4. HOXB3 was also reported to be associated with several cancers, including acute myeloid leukemia [11], oral cancer [12] and breast cancer. For instance, a study by Chen et al showed that show that HoxB3 mRNA and protein are overexpressed in primary prostate cancer tissues compared to the adjacent normal prostate tissues [13]. Moreover, HoxB3 overexpression is associated with higher Gleason grade, clinical stage ($P < 0.001$) and PSA levels. However, the exact role of HOXB3 in DR development and the pathological mechanism was still unclear by now.

microRNAs (miRNAs), an abundant class of 17-25 nucleotides small noncoding RNAs, post-transcriptionally regulate gene expression through directly binding to the 30 untranslational region (3' TR) of target mRNAs. Till now, over 1000 miRNA genes have been identified in mammals, but revealing their roles in physiology and pathology is still an ongoing process. Recently, miRNAs have been suggested to participate in the regulation of diverse biological processes, and their deregulation or dysfunction plays important roles in kinds of physiopathological progresses [14, 15]. Even several studies reported the role of miRNA in the development of DR [16, 17], the detailed mechanism remain largely illusive. Recently, emerging evidence has suggested that deregulated miR-128 was involved in the pathogenesis of kinds of diseases [14]; however the role of miR-218 in the DR was still unknown.

Therefore in the present study, we determined the expression levels of miR-128 in RPE cells under high glucose condition. Then we assessed the effect of miR-128 on proliferation, apoptosis and the expression of HOXB3 of RPE cells in high glucose condition. In addition, we conducted several experiments to detect the detailed signaling pathways for the protective effect of miR-128.

Materials and methods

Cell culture

RPE cell line (ARPE-19) was purchased from Shanghai Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China) and cells at 6 to 9 passages were used in this study. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Grand Island, NY) and supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO, USA) and a 1% antibioticantimycotic solution containing 1×10^4 units penicillin, 10 mg streptomycin at 37°C in a humidified atmosphere of 5% CO_2 95% air.

Cell model and miR-128 treatment

After being incubated in the presence of normal glucose (1 g/L) or high glucose (4.5 g/L) culture condition, the normal conduction and high glucose condition were used for advanced studies. ARPE-19 in normal and high glucose condition were treated with miR-128 mimic (miR-126-mimic) or miR-128 inhibitor (miR-126-inhibitor). ARPE-19 cells were transfected with 100 nM miR-128 mimic and 200 nM miR-126 inhibitor using lipofectamine 2000 reagent (Invitrogen Corporation, Carlsbad, CA, USA) following the manufacturer instructions. ARPE-19 cells were pretreated with miR-128 mimics, miR-128 inhibitors, or negative controls for 6 h in high glucose. It RNA extraction was conducted from the treated cells in each group. Then the treated cells and clear supernatant liquid were obtained for the advanced studies, including cell viability, western blot, flow cytometry and et al.

RNA extraction and qRT-PCR

Total RNA was extracted from cultured cells with TRIzol reagent. The extracted RNA was quantified using the NanoDrop 2000 Spectrophotometer (Thermo Scientific, Walthan, MA). The first-strand cDNA was synthesized using SuperScript III reverse transcriptase according to manufacturer's instructions (In-

vitrogen, San Diego, CA). The qRT-PCR was performed using SYBR Green-based assays with the ABI Prism 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA). The analysis of miR-29b expression was performed using stem loop real-time RT-PCR. All reactions were measured in triplicates in a final volume of 10 μL. Cycling conditions were chosen according to the manufacturer's protocols. For miRNA detection, the relative level of miRNA was calculated against U6 RNA (internal control) using the $2^{-\Delta\Delta Ct}$ method.

Cell viability assay

Cultured ARPE-19 cells in each group were collected for the cell viability assays. ARPE-19 cells in each gropp was plated at a density of about 10^5 cells per wells in 12-well plate containing medium with 10% FBS and cultured overnight. The medium was exchanged to one without FBS, and the cells were cultured for 24 h. All the cells were digested and collected for advanced. ARPE-19 cells were re-seeded in 96-well, flat-bottom plates at a density of 10^3 cells/100 μL. Cell viability was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium (Sigma, St. Louis, MO, USA) assay according to the manufacturer's protocol. For the MTT assay, cultured ARPE-19 cells were treated with 0.5 mg/mL of MTT for 6 h of the culture period in the dark and were tested at 37°C. After the cells were washed, the absorbance was recorded on a microplate reader at a wavelength of 540 nm All the experimental repeated triple times and the mean values were used for the study.

Evaluation of apoptosis

The apoptotic rates were analyzed with flow cytometry (Flow-Count, Beckman Coulter, CA, USA) using the Annexin V-Fluorescein isothiocyanate (FITC)/Propidium iodide (PI) kit (Roche, Penzberg, Germany). All the operations were performed according to the manufacturers. Hep-2 cells without any treatment were used as an internal control, and the experiments were repeated at least three times. A dot plot of the X-axis (FL4), being the log of Annexin V fluorescence, and the Y-axis (FL2), which reflects the PI fluorescence, was constructed.

Luciferase activity assay

Luciferase activity assay was performed as previously described [18]. Briefly, ARPE-19 cells were cultured in 12-well plate (1×10^5 cells/well) and co-transfected with wild type or mutated 3'-UTRs of HOXB3 (WT and Mut, respectively) luciferase reporter constructs and miR-128 or control mimic with Lipofectamine 3000. After the treatment for 24 h, cells were harvested and luciferase activity was examined by Dual-Luciferase Reporter Assay Kit (Promega, Wisconsin, WI, USA).

Western blot analysis

ARPE-19 cells were harvested and resuspended with PBS. After centrifugation at 2000 rpm for 5 min, the cells were lysed in ice cold Lysis Buffer (Thermo Scientific) for 30 min. The supernatant was collected after 10 min of centrifugation at 12×10^3 rpm, equaled by spectrophotometry, denatured with sample loading buffer for 10 min at 95°C and stored at -80°C for future use. Proteins were separated by 10% SDS-PAGE gels and transferred to PVDF membrane, and blocked with 5% skimmed milk/TBST for 1 h at room temperature, then incubated with primary antibodies of interest at 4°C overnight. The appropriate HRP-conjugated secondary antibody at a dilution of 1:10000 in blocking buffer and incubated for 1 h at room temperature, then added ECL (HRP substrate) and exposed at dark room. The HOXB3, total PI3K, phospho-PI3K, total AKT, and p-AKT used at a 1:1000 dilution were purchased from Cell Signaling Technology (Boston Globe, MA, USA). The expression levels of proteins were analyzed using Image J software and normalized to that of GAPDH protein.

Statistical analysis

The data are expressed as the mean ± SD. Differences between the groups were assessed with the student *t*-test or one-way ANOVA test. The SPSS 13.0 package was used to analyze the continuous variable (IBM, Chicago, IL). $P < 0.05$ was considered statistically significant.

Results

miR-128 expression level was decreased in high glucose condition

In order to determine the expression of miR-128 in the high glucose condition, the quantitative RT-PCR detection was conducted between the RNA extracted from culture ARPE-19 in high

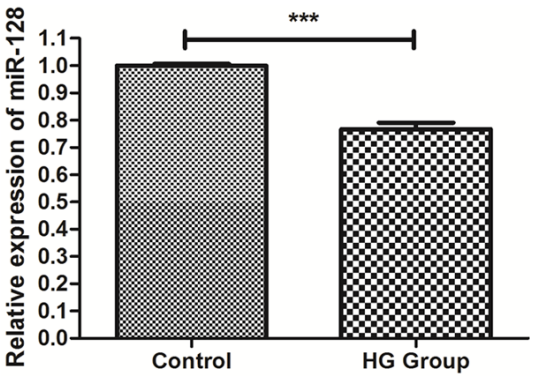

Figure 1. Different expression level of miR-128 in control and high glucose condition.

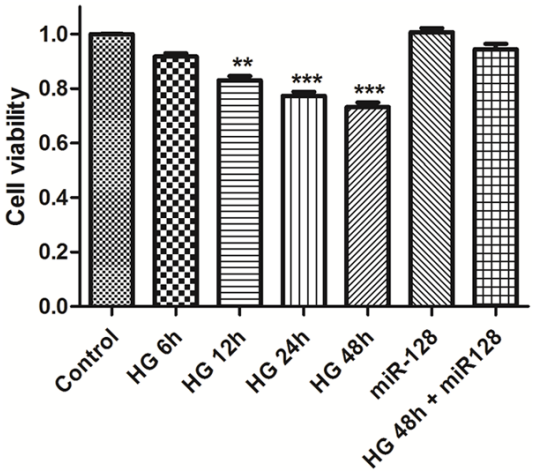

Figure 2. Effect of high glucose, miR-128 and miR-128 inhibitor on the ARPE-19 cell viability in high glucose. The cell viability detected by MTT method. Mean ± SD, n = 6.

glucose compared with the normal condition. Our data showed that miR-128 was significantly decreased in ARPE-19 cells in high glucose compared with the control group (**Figure 1**).

Effect of miR-128 on the ARPE-19 cell viability in high glucose

First, the cell viabilities in different time points were presented in **Figure 2**. Compared with the control group, cell group with high glucose treated for 6 h showed no significant difference. However, with the increase of high glucose treatment duration, the cell viability reduced significantly. In the 12 h group, the cell viability was significantly decreased after high glucose treated ($P < 0.01$). In the longer treatment group, the cell viability was depressed more significantly ($P < 0.001$). Considering that the 48 h demonstrated a significantly and stably decreased cell viability level, this time point was used in the following experiment. Besides, we conducted advanced study to detect the effect of miR-128 on the ARPE-19 cells. The cell viability in the miR-128 treated in the normal condition ARPE-19 cells was not significant different from control group. While it was detected that miR-128 application in the high glucose group improved the ARPE-19 cells viability significantly.

Protection of miR-128 on ARPE-19 from apoptosis

Apoptosis plays key roles in kinds of abnormal pathologic processes, including the decreased cell viability induced by high glucose. To investigate whether miR-128 protect the ARPE-19 cells from apoptosis induced by high glucose, flow cytometry was used for the detection of apoptotic rate of cultured cells. The data showed that ARPE-19 cells incubated with high glucose for 48 h (**Figure 3B**) showed significant higher apoptosis rate compared with the control group (**Figure 3A**, $P < 0.001$). However, ARPE-19 cells in high glucose treated with miR-128 for 6 h showed significantly decreased apoptosis rate ($P < 0.001$, **Figure 3C**). Besides, the application of miR-128 inhibitor would increase the apoptosis rate compared with the RPE cells in the high glucose condition ($P < 0.001$, **Figure 3D**). All the detailed data was presented in the summary histogram (**Figure 3E**).

miR-128 directly targets HOXB3 in ARPE-19 cells

We used TargetScan 6.2 software to search the potential target gene of miR-128. HOXB3 was predicted to be a target of miR-128 (**Figure 4A**). In the western blot analysis, it was found that HOXB3 was up-regulated in the high glucose group. In the miR-128 treatment group, miR-128 could down-regulate the expression HOXB3 in the ARPE-19 cells. Besides the miR-128 inhibitor would lead to the up-regulation of HOXB3 (**Figure 4B**). To confirm this correlation between miR-128 and HOXB3, the miR-128 binding sequences present at the 3'-UTR of HOXB3 mRNA (WT-3'-UTR), its mutant site (HOXB3-3'UTR-mut) were subcloned downstream of the luciferase reporter gene and then

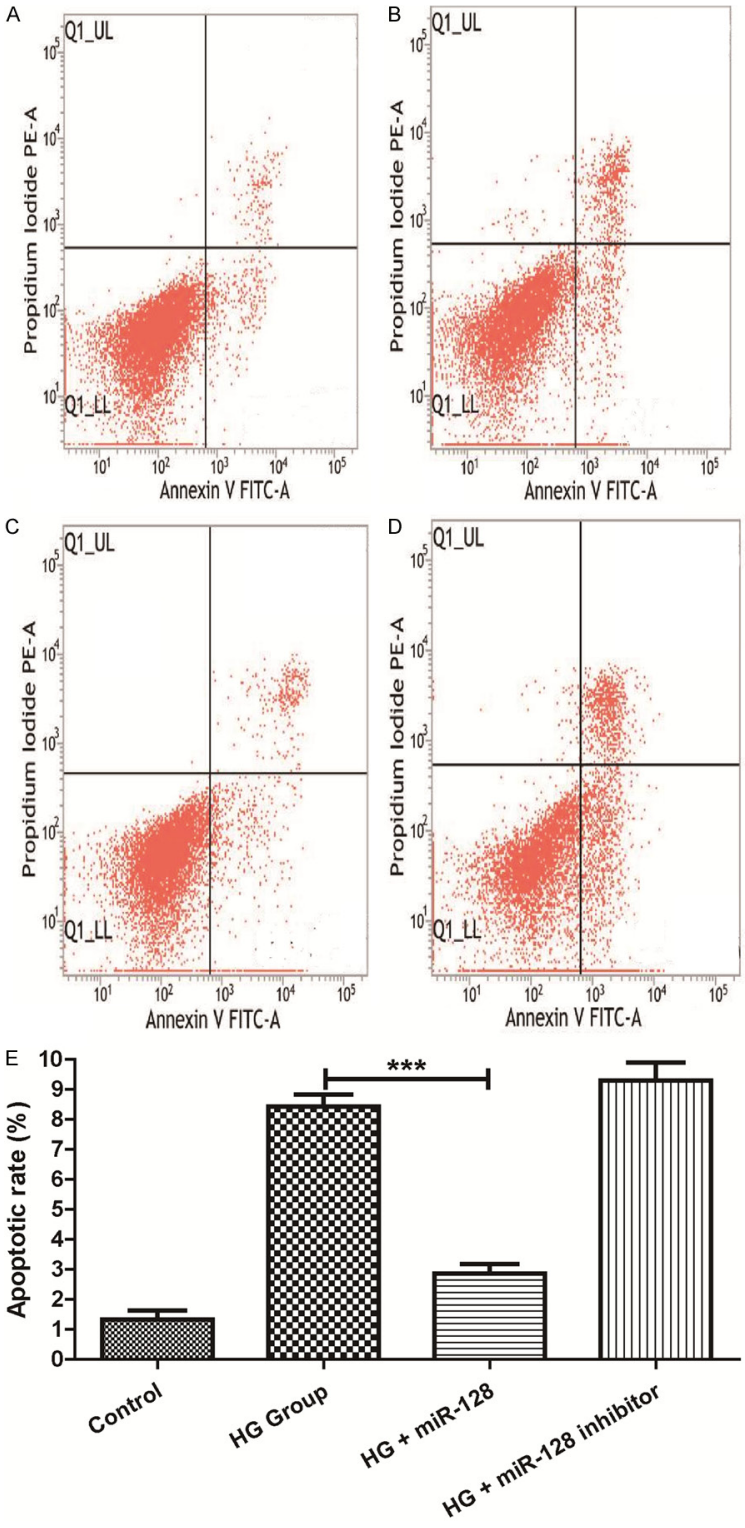

Figure 3. Modulation in high glucose induced apoptosis by miR-128. (A) Control group. (B) High glucose group; (C) miR-128 treated group; (D) miR-128 inhibitor treated group and (E) data of the apoptotic rate in each group.

co-transfected into ARPE-19 cells. The relative luciferase activity of the reporter that contained wild-type 3'-UTR was decreased to 54.6% when miR-128 was co-transfected. However then luciferase activity assay showed that miR-128 significantly suppressed the WT 3'-UTR but not that of Mut 3'-UTR of PTEN luciferase activity in ARPE-19 cells (**Figure 4C**).

Effect of miR-128 treatment on the PI3K/AKT-mTOR pathway in ARPE-19 cell line

To further investigate the mechanism of miR-128 protecting RPE cells in high glucose condition, we evaluated the protein levels of PI3K/AKT-mTOR pathway. The PI3K/AKT-mTOR pathway was associated with HOXB3 gene function and played key roles in different pathologic processes. The total protein of PI3K and AKT was not significantly different in each group. However, the phosphorylation of PI3K and AKT were significantly increased in the high glucose group. Besides miR-128 treatment prevented the increase of p-PI3K and p-AKT. Besides the miR-128 inhibitor treatment would increase the expression of p-PI3K and p-AKT. For the m-TOR expression level, it would be up-regulated in the high glucose group; however, miR-128 would significantly reduce the m-TOR expression (**Figure 5**).

Discussion

High glucose induced RPE cells abnormality is a key pathological alteration in the development of DR. As hyperglycemia was once of the leading dysfunctions of DM, the treatment for the RPE cells in the high glucose condition would help in the research and development of new drugs. In our study, the data showed that miR-128 level was down-regulated in the high glucose group. Besides,

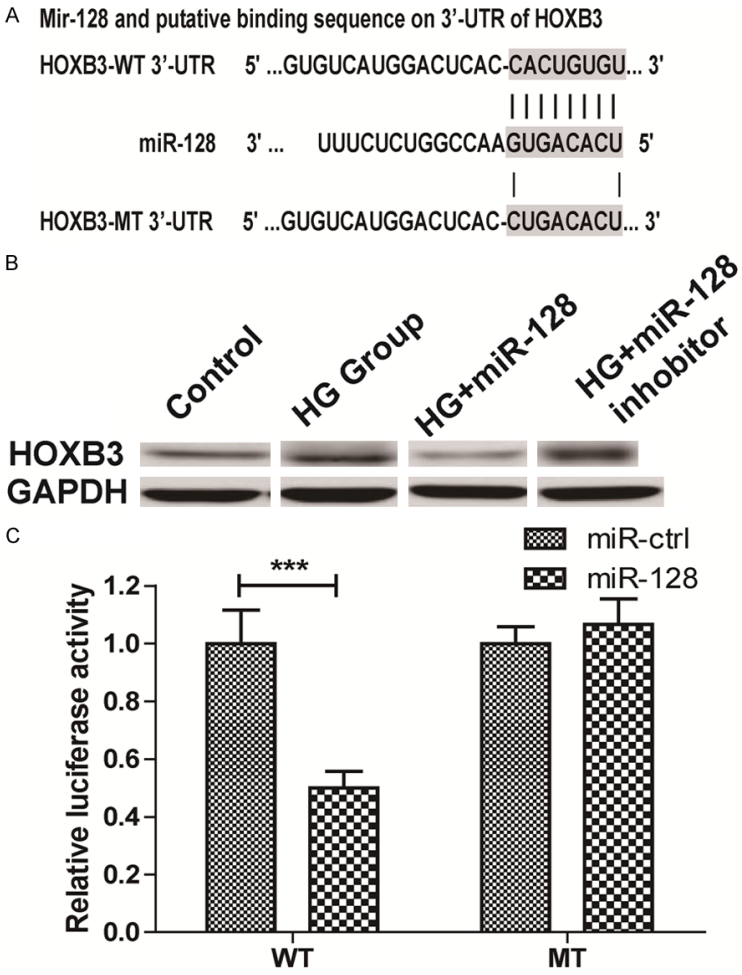

Figure 4. miR-128 directly targets HOXB3 in ARPE-19 cells. A. miR-128 and its putative binding sequence in the 3'-UTR of HOXB3 gene. The seed sequence of miR-29 family is shown in the box. B. Effect of miR-128 and miR-128 inhibitor on HOXB3 in high glucose inducedARPE-19 cells. C. The relative luciferase activity of the reporter that contained wild-type and mutation-type 3'-UTR.

through the analyses of the cell functions, we found that miR-128 would protect the RPE cell in both cell viability and apoptosis condition. Through western blot and luciferase analyses, we found that miR-128 would down-regulate HOXB3 expression through targeting the 3'UTR of HOXB3 gene. Moreover, through the analyses of the PI3K/AKT-mTOR pathway, it was found that miR-128 treatment decreased the phosphorylated protein level of PI3K and AKT in RPE in vivo. As mTOR was one of the downstream genes of PI3K/AKT pathway, we also found that miR-128 could down-regulate the expression of m-TOR. These results demonstrated that miR-128 could produce a marked effect through down-regulating of HOXB3 and inhibiting PI3K/AKT-mTOR pathway.

RPE constitute a simple layer of cuboidal cells that are localized behind the photoreceptor (PR) cells. The main sites of light energy absorption are the melanin pigment granules in RPE cells, which are most susceptible to oxidative stress in many primary and secondary retinal dysfunctions. In the development of DR, the dysfunction of RPE cells also demonstrated significant effects. HOX gene is involved in the migration, proliferation and differentiation of vascular endothelial cells. However, no previous study investigated the role of HOXB3 in DR development by now. In this study, we found that HOXB3 was up-regulated in the high glucose treated RPE cells. Considering the potential role of HOXB3 in the development of DR, an inhibitor of HOXB3 might produce significant therapeutic effect of DR.

MicroRNAs (miRNAs), a class of small noncoding RNA molecules, result in translational repression or degradation and contribute to the inhibition of gene expression. miR-128 is a brain-enriched miRNA [19]. It has been found that the expression of miR-128 has tissue-specific and developmental-specific expression patterns, mainly in neurons rather than in atrocities. A study by Hauser showed that miR-128 acted as a tumor suppressor inhibiting the head and neck squamous cell carcinoma growth by directly mediating the expression of putative targets [20]. In another studym it was found that miR-128 expression obviously decreased in prostate cancer tissues compared with paired normal tissues. Restored miR-128 expression sensitized prostate cancer cells to cisplatin and inhibited the invasion. Furthermore, there was an inverse expression pattern between miR-128 and zinc-finger E-box-binding homeobox 1 in prostate cancer cells

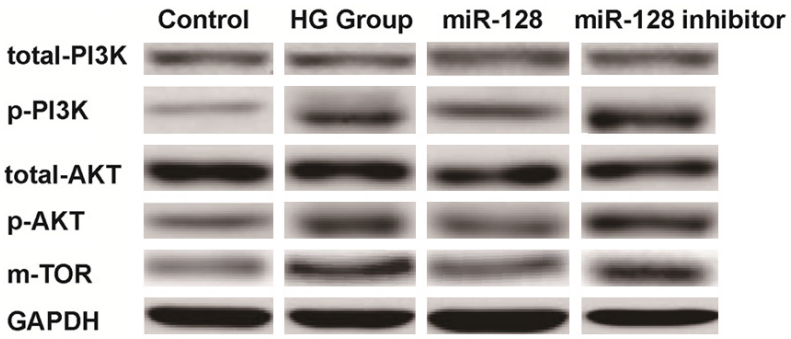

Figure 5. Effect of miR-128 on PI3K-AKT-mTOR pathway in high glucose induced ARPE-19 cells. The expressions of total PI3K, p-PI3K, total AKT, p-AKT and mTOR in each group were presented.

and tissues, and zinc-finger E-box-binding homeobox 1 was identified as a direct target of miR-128 in prostate cancer [21]. From bioinformatics analyses and luciferase assay, we found that HOXB3 was the target gene of miR-128.

The effect of HOXB3 on the RPE cells was unclear. Considering that PI3K/AKT pathway was reported to play an essential role in the different pathological processes and it was associated with HOX gene expression in the the AP body axis in the developing embryo [22]. PI3K/AKT was reported to be associated with the development of DR. An in vitro study showed that high glucose induced the expression of fibronectin, collagen IV, and laminin through PI3K/Akt signaling pathway in RPE cells, and the PI3K/Akt signaling pathway may contribute to the formation of fibrotic membrane during the development of DR [23]. The treatment of PI3K/AKT was also frequently detected. Four weeks after intravitreal injection, KH902-treated rats had better retinal electrophysiological function, less retinal vessel leakage and lower levels of VEGFR2, PI3K, AKT, p-AKT, p-ERK and p-SRC than PBS or Avastin-treated rats [24].

The PI3K/AKT/mTOR pathway is a complex signalling pathway involved in crucial cellular functions such as cell proliferation, migration and angiogenesis [25]. A previous study showed that ocular neovascularization, a common pathological feature of wet age-related macular degeneration (AMD), proliferative and diabetic retinopathy (PDR) leads to fluid and blood leakage, scar formation and ultimately blindness. The phosphatidylinositol-3-kinase (PI3K) pathway is an alternative therapeutic target in angiogenic diseases. The PI3K/Akt/mTOR pathway orchestrates an array of normal cellular processes, including growth, survival and angiogenesis [26]. In this study, it was found that miR-128 could down-regulate the expression of m-TOR and it demonstrated that miR-128 could produce a marked effect through down-regulating of HOXB3 and inhibiting PI3K/AKT-mTOR pathway. This result showed the potential therapeutic of miR-128 for the DR.

Collectively our data support the notion that miR-128 could protect the cell viability and apoptosis of RPE cells in high glucose. Advanced study showed that miR-128 produced the protective effect through down-regulating of HOXB3 and inhibiting PI3K/AKT-mTOR pathway. Our results demonstrate a potential for miR-128 in the development of miRNA-based therapeutics for DR treatment.

Disclosure of conflict of interest

None.

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