miR-132 mediates cell permeability and migration by targeting occludin in high-glucose-induced ARPE-19 cells

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**Abstract.** This study investigated the effects and mechanisms of miR-132 related to the permeability and mobility of human retinal pigment epithelium ARPE-19 cells in high-glucose (HG) condition. ARPE-19 cells were cultured in normal and HG condition and identified by immunofluorescence staining. Cell viability was assessed by the MTT assay, cell permeability was assessed by the FITC-dextran assay and cell mobility was assessed by the wound healing assay. Different miRNA and mRNA expression levels were determined by quantitative real-time polymerase chain reaction (RT-qPCR). The expression of tight junction-related proteins was determined by Western blot assay and immunofluorescence. The interaction between occludin and miR-132 was confirmed by a dual-luciferase reporter assay. We revealed that HG-treated ARPE-19 cells exhibited significantly increased miR-132 expression, decreased expression of the tight-junction markers including occludin and E-cadherin, and increased cell mobility and permeability. Occludin is a direct target of miR-132, which could regulate cell viability, mobility and permeability under HG condition through the JAK/STAT3 signaling pathway. These are the first data to suggest that miR-132 may contribute to the progression of diabetic retinopathy (DR) and that targeting the effect of miR-132 on occludin and the JAK/STAT3 pathway could represent a novel effective DR-treatment strategy.

**Key words:** Diabetic retinopathy, JAK/STAT3 pathway, miR-132, Occludin

However, there is still little known about the molecular mechanisms involved in increased retinal vascular permeability.

At present, miRNAs have been reported to play pivotal roles in the regulation of apoptosis, proliferation and migration of retinal cells and participate in regulating DR-related neovascularization [12]. A number of studies have investigated the roles of miRNAs in DR using HRECs and RPE cells, and *in vivo* models of streptozotocin (STZ)-induced diabetic retinas. In addition, they have identified a series of miRNAs whose expression is altered in the retinas and retinal endothelial cells (RECs) of STZ-induced diabetic rats 3 months after the onset of diabetes [13]. In microvascular endothelial cells (ECs), miR-200b, which directly targeted VEGF, was decreased after treatment with HG [14]. miR-146 was first implicated in DR and can modulate the NF-κB inflammatory response that played a crucial role in the early phases of DR development [15]. miR-126, miR-29b and miR-195 were also reported to play roles in the DR process. Moreover, aberrant miRNAs could be biomarkers in the serum of patients with DR [16], and six miRNAs were discovered to be significantly overexpressed and related to fibrosis and angiogenesis in DR [17].

AS ONE of the most serious and common chronic eye complications of diabetes, diabetic retinopathy (DR) has become the leading cause of blindness in adults [1, 2]. Progressive loss of vascular cells and slow dissolution of inter-vascular junctions are the main characteristics of DR [3]. DR is associated with diabetic macular edema (DME), and it presents microvascular and macrovascular changes [4]. Human retinal pigment epithelial cells, components of the blood–retinal barrier (BRB), could be impaired by the adverse effects of high glucose (HG) in the early stage of this disease, which induces dysfunction of the BRB, thus contributing to progression of DR [5, 6]. Recently, to elucidate the mechanism of DR, significant efforts [7, 8] have been focused on vascular endothelial growth factor (VEGF) [9], defects of which contribute prominently to the clinical features of DR, and have led to the inference of the general microvascular nature of this diabetic complication[10, 11].
Occludin is a protein of the claudin group and the key component of tight junction [18], playing a pivotal role in maintaining their barrier properties. Therefore, absence or mutation of occludin could increase epithelial leakiness, which is a critical barrier to cancer prevention [19], and it can also cause reduced adhesion and tight junction function and increased invasion in breast cancer tissues [20]. In addition, occludin participates in the regulation of apoptosis, disruption of which is an important aspect of a number of diseases [21]. It has been reported that tight junction abnormalities resulting from its disruption are closely associated with the progression of retinal pathology in DR. Reduced expression of occludin has been implicated in the breakdown of the BRB [22]. Compromised tight junction in endothelial cells was also found to be present on the retinas of diabetic rats [23, 24] and those of diabetic patients [25, 26]. Thus, we infer that preventing or reversing the downregulation of occludin may be a potential therapeutic target for DR. Moreover, recent studies have shown that occludin is also the key factor through which the JAK/STAT3 pathway may regulate the process of epithelial-mesenchymal transition (EMT) [27]. Whether this pathway is involved in occludin in the process of DR needs further investigation.

In this study, we found that miR-132 is one of the miRNAs targeting occludin through bioinformatics analysis, and we provide the first evidence of the regulatory role of miR-132 in retinal barrier function in DR through targeting occludin and activating the JAK/STAT3 pathway, suggesting a promising alternative therapeutic strategy for treating vascular permeability in DR.

Materials and Methods

Cell culture

The human retinal pigmented epithelium cell line ARPE-19 was obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) plus 10% fetal bovine serum (FBS, Gibco, CA) at 37°C with 5% CO₂. For HG treatment, the ARPE-19 cells were exposed to D-glucose (Sigma) at a concentration of 30 mmol/L in cultures as described in previous publications [28]. For normal glucose treatment, ARPE-19 cells were exposed to D-glucose (5.6 mmol/L) and D-mannitol (Sigma, 24.4 mmol/L) in cultures.

Immunofluorescence

Permeable Transwell filters were used to seed ARPE-19 cells. Cells were fixed and permeabilized in 1% bovine serum albumin (blocking buffer) was used to block cells for 1 h at room temperature (RT). Primary antibodies were cultured overnight in a 1:250 dilution (mouse anti-keratin 0.25 mg/mL; mouse anti-E-cadherin 0.25 mg/mL; mouse anti-Vimentin 0.25 mg/mL; mouse anti-occludin 0.25 mg/mL). All the antibodies were purchased from Zymed, San Francisco, USA. Coverslips were then washed three times with PBS the next day. Corresponding secondary fluorophore-labelled antibodies in PBS/1% BSA were then added and incubated for 60 min at room temperature. Cells were washed twice with PBS and mounted in phenylethylamine and were analyzed with a Zeiss LSM510 laser scanning confocal microscope (Zeiss, Germany).

Cell transfection

The miR-132 mimics and inhibitors and the scrambled negative control (si-NC, NC mimics and NC inhibitor) were purchased from Shanghai GenePharma, China. The pcDNA3.1-occludin and pcDNA3.1 empty vector (Vector) were purchased from Generay (Shanghai, China). Transfections were performed with Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer’s instructions. We optimized the concentration of transfection reagents to permit the highest transfection efficiency with the least cell death (<10%).

Quantitative real-time polymerase chain reaction (RT-qPCR)

TRIzol (Invitrogen, USA) was used to isolate total RNA. Then, to generate first-strand cDNA, the Improm-II Reverse Transcription System (Promega, USA) and random primers or Q-miRT primers were used [29]. We then carried out a RT-qPCR test of mRNA with SYBR Premix Ex Taq (TaKaRa, Japan) and gene-specific primers. GAPDH was used for normalization. RT-qPCR for microRNA analysis was performed as described before [29] and U6 snRNA was employed as an endogenous control. The comparative Ct method was carried out to calculate the relative expression levels of RNAs. Primers used for real-time PCR were as follows: E-cadherin-F: 5'-GCCCCCATCAGGCCTCCGTTT-3', E-cadherin-R: 5'-ACCTCGTCCTCTTGTCTTGTTGA-3'. Vimentin-F: 5'-CCTGAACCTGAGGGAACTAA-3', Vimentin-R: 5'-GCCAAAGGGACCTGGAACG-3'. occludin-F: 5'-CCCAAGGTTCCATCCGAAGCA-3', occludin-R: 5'-GCGTGTTAAAAGGAACCCGC-3'. hsa-miR-132: 5'-GGAGAAGGAGGACCTGGAAC-3'. GAPDH-F: 5'-GGAGAAGGAGGACCTGGAAC-3'. GAPDH-R: 5'-GCGTGTTAAAAGGAACCCGC-3'. hsa-miR-132: 5'-GGAGAAGGAGGACCTGGAAC-3'. GAPDH-F: 5'-GGAGAAGGAGGACCTGGAAC-3'. GAPDH-R: 5'-GCGTGTTAAAAGGAACCCGC-3'. hsa-miR-21-3p: 5'-GCGTGTTAAAAGGAACCCGC-3'. GAPDH-F: 5'-GGAGAAGGAGGACCTGGAAC-3'. GAPDH-R: 5'-GCGTGTTAAAAGGAACCCGC-3'. hsa-miR-21: 5'-GCGTGTTAAAAGGAACCCGC-3'. GAPDH-F: 5'-GGAGAAGGAGGACCTGGAAC-3'. GAPDH-R: 5'-GCGTGTTAAAAGGAACCCGC-3'. hsa-miR-21-3p: 5'-GCGTGTTAAAAGGAACCCGC-3'. GAPDH-F: 5'-GGAGAAGGAGGACCTGGAAC-3'. GAPDH-R: 5'-GCGTGTTAAAAGGAACCCGC-3'.
CTGTAGCCAAATTCGTTG-3', U6-F: 5'-CGCTTCGGCAGCACATATACTA-3', U6-R: 5'-CGCTTCACGAATT TGCGTGTCA-3'.

**Cell migration assay**
Cells were scratched to create a wound with a 1 mm initial width and cultured under normal condition. We measured the migrated distances at 48 h after scratching.

**MTT assay**
After culturing for 2 days, cell viability of ARPE-19 cells was determined with a colorimetric MTT assay kit (Sigma, USA) following the instructions of the kit. Absorbance at 490 nm was determined using a microplate reader.

**Cell permeability measurement**
FITC-dextran was used to measure ARPE-19 cell permeability. 10% chloral hydrate was used to anaesthetized the cultured cells and FITC-dextran (1 mL, 50 mg/mL, dissolved in sterile 0.9% NaCl solution) was then added for 10 min. Cells were soaked in 4% paraformaldehyde solution for 24 h at 4°C. After that, Cells were then embedded with optimal cutting temperature (OCT) compound. Finally, a microplate reader (Gemini® EM, Molecular Devices, USA) was used to detect cell permeability.

**Western-blot analysis**
IP lysis buffer (Pierce, USA) containing protease inhibitors (Sigma, USA) was used to lyse cells. Equal amounts of protein were then separated by 10% SDS-PAGE after BCA (Pierce, USA) quantification, electrophoretically transferred to nitrocellulose membrane (Millipore, USA), and incubated with specific primary antibodies (1:1,000) followed by corresponding secondary antibodies (1:5,000) (Pierce, USA). Chemiluminescent substrate (Millipore, USA) was then used for signal visualization and film exposure. Occludin, E-cadherin, Vimentin, JAK, STAT3 (Santa Cruz Biotechnology, USA); p-JAK, p-STAT3 (CST, USA); and GAPDH (Proteintech, USA) were used for this assay.

**Dual-luciferase reporter assay**
The fragments from the 3'-untranslated region (3'-UTR) of occludin containing predicted miR-132 binding sites and their mutant sequences were sub-cloned to the pmirGLO plasmids (Promega, USA) to form occludin wild-type (WT-occludin), and occludin mutated-type (MUT-occludin). miR-132 mimics (50 pmol/well) and luciferase reporter plasmids (800 ng/well) were co-transfected into HEK-293T cells by Lipofectamine 3000 (Invitrogen, USA). Then, the relative luciferase activities were measured with a dual-luciferase assay system (Promega, USA) 48 h after transfection.

**Statistical analysis**
Data are presented as the mean ± SD from at least three independent experiments. One-way analysis of variance (one-way ANOVA) was used for multiple comparisons to assess the significant differences between groups. Student’s t-test was performed for comparisons between two groups. Statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Prism, USA). *p < 0.05* was considered statistically significant.

**Results**

**High-glucose treatment alters ARPE-19 cell viability, mobility and permeability**
After 3 days of cell culture, the cells attached to the vessel wall to form abundant proliferating colonies. The proliferating cells were deformed into polygons, and the un-proliferated cells were bark-shaped. After one week of cell culture, the monolayer cells tended to fuse, and the morphology was uniform, with a polygonal paving stone appearance and a central nucleus. After immunocytochemical staining of ARPE-19 cells (light microscopy) with a cytokeratin antibody, the cytoplasm of cultured ARPE-19 cells had a red coloration, and keratin expression was positive (Fig. 1A, top). ARPE-19 cells were then treated with HG to create an *in vitro* model of DR.

The group with normal glucose treatment was used as a control. Optical microscopy was used to observe cell morphology within different groups. Compared with the normal glucose treatment group, cells in the hypertonic group had larger size with a normal morphology, while cells in the HG group had a slender form and scattered arrangement (Fig. 1A, bottom). The MTT assay showed that cells in the HG group had lower viability compared with that of the other two groups (Fig. 1B). E-cadherin, Vimentin and occludin are protein markers that are associated with tight junction. We analyzed their mRNA and protein expression by RT-qPCR, Western-blot and immunofluorescence. These analyses showed that E-cadherin and occludin were downregulated under HG treatment while Vimentin showed an opposite effect (Fig. 1C–E), which indicated that the tight junction of cells was impaired by HG. Moreover, the wound healing assay showed that cell migration was significantly induced by HG (Fig. 1F), and the FITC-dextran assay revealed that cell permeability was greatly increased in the HG group compared with that of the normal glucose and hypertonic groups (Fig. 1G).
MiR-132 is upregulated in ARPE-19 cells under high glucose condition and targets occludin

To identify miRNAs that could target occludin, we searched published studies of changes in miRNAs expression in DR [13]. 5 miRNAs, including miR-132, miR-383, miR-425, miR-7 and miR-212, were selected. RT-qPCR analysis was then applied to identify which miRNAs could be upregulated in ARPE-19 cells by HG treatment. As shown in Fig. 2A, miR-132, miR-212 and miR-7 were upregulated in the HG condition, while miR-383 and miR-425 did not show a significant change. In addition, no changes in the expression levels of the 5 miRNAs were observed in the hypertonic group. Of the three upregulated miRNAs, miR-132 expression was increased the most, and therefore we chose it to investigate its function in the regulation of DR. In addition, we detected the expression of miR-132 at different time points (day 1, 3, 5 and 7). Fig. 2B shows that miR-132 expression was increased with the prolongation of HG treatment time ($p < 0.05$), and its expression was significantly increased at day 7, to almost 8 times that of the normal control group ($p < 0.001$). However, no changes in the expression level of miR-132 were observed in the hypertonic group. In addition, TargetScan (http://www.targetscan.org) was used to predict the binding site between miR-132 and occludin, and the dual-luciferase
reporter assay was used to confirm the relationship. The results showed that there is a binding site for miR-132 in the 3'-untranslated region (UTR) of occludin. Moreover, miR-132 mimics can significantly reduce the luciferase activity of transfection with wild-type 3'UTR occludin, but there is no significant effect of transfection with mutant type 3'UTR occludin (Fig. 2C). Taken together, there is a direct targeting relationship between miR-132 and occludin.

miR-132 alters cell viability, mobility, and permeability under high glucose condition

To investigate the role of miR-132 in DR, miR-132 mimics and NC mimics were transfected into ARPE-19 cells. Compared with the NC mimics group, miR-132 was upregulated in the miR-132 mimics group (Fig. 3A) and the miR-132 mimics group showed lower viability compared with the NC mimics group under HG condition (Fig. 3B), the mRNA and protein levels of occludin and E-cadherin were downregulated by miR-132 mimics, while the mRNA and protein levels of Vimentin were upregulated (Fig. 3C–D), which indicated that tight junction of cells was decreased by miR-132 mimics. Moreover, the wound healing assay showed that cell migration was significantly increased by miR-132 mimics (Fig. 3E). Meanwhile, the FITC-dextran assay also revealed that cell permeability was greatly increased (Fig. 3F). Therefore, by integrating the results above, it can be inferred that miR-132 could regulate cell viability, mobility and permeability under HG condition.

To further reveal the function of miR-132 and assess whether inhibiting the miR-132 level in ARPE-19 could rescue HG-induced perturbation of cell viability, cell mobility and permeability, miR-132 inhibitor and inhibitor NC were transfected into ARPE-19 cells. First, we evaluated the expression of miR-132 by RT-qPCR to verify the inhibitory effect, and the results demonstrated the level of miR-132 was downregulated after transfection with miR-132 inhibitor (Fig. 4A). Then, cell viability, mobility, and permeability were assessed. Compared with the inhibitor NC group, cells in the miR-132 inhibitor group had higher viability under HG condition (Fig. 4B). Both the mRNA and protein levels of occludin and E-cadherin were upregulated by miR-132 inhibitor, while the mRNA and protein levels of Vimentin were downregulated (Fig. 4C–D), indicating that tight junction of cells was enhanced by inhibiting miR-132 under HG condition. Moreover, the wound healing assay showed that cell migration was significantly decreased by miR-132 inhibitor (Fig. 4E), and the FITC-dextran assay also revealed that cell permeability was greatly suppressed in the miR-132 inhibitor group compared to the
inhibitor NC group under HG condition (Fig. 4F, \( p < 0.01 \)). We also detected the expression of miR-132 in normal glucose condition or hypertonic condition and determined the viability and tight junction related gene expression after ARPE-19 cells were transfected with miR-132 mimics. The results showed that compared with the blank control group, the expression of miR-132 under normal glucose condition or hypertonic condition did not change significantly (Fig. 4G), and after overexpression of miR-132 in the normal glucose condition or hypertonic condition, the proliferation and the expression of tight junction related genes in ARPE-19 cells did not change significantly (Fig. 4H and 4I).

**miR-132 activates the JAK/STAT3 signaling pathway through the regulation of occludin in ARPE-19 cells under high glucose condition**

To investigate the downstream molecular mechanism of miR-132, ARPE-19 cells were then transfected with an occludin-overexpressing vector, and the results
Fig. 4  MiR-132 knockdown upregulated cell viability and downregulated cell mobility and permeability under high-glucose condition

A. RT-qPCR analysis to detect the expression of miR-132 after transfected with miR-132 inhibitor for 72 h. B. Viability of cells under different treatments was analysed by the MTT assay. C-D. RT-qPCR and Western blot analysis of occludin, E-cadherin and Vimentin. RNA and protein levels were normalized to GAPDH. E. Cell migration changes were detected by the wound healing assay. F. FITC-dextran assay to detect cell permeability of different groups of cells. G. RT-qPCR analysis to detect the expression of miR-132 in ARPE-19 cells under normal glucose condition or or hypertonic condition. H. Viability of cells transfected with miR-132 mimics (mimics NC) under normal glucose condition or hypertonic condition was analysed by the MTT essay. I. RT-qPCR analysis of occludin, E-cadherin and Vimentin of ARPE-19 cells transfected with miR-132 mimics (mimics-NC) under normal condition or hypertonic condition. RNA levels were normalized to GAPDH. Data are presented as the mean ± standard error (SD) (n = 3). * p < 0.05, ** p < 0.01. normal glucose, NG.
demonstrated that overexpression of occludin elevated the decreased level of occludin and lowered the increased cell permeability caused by miR-132 mimics (Fig. 5A–B). Additionally, we performed Western blot to evaluate the related molecules regulated by occludin, and found compared with NC mimics, both p-JAK2 and p-STAT3 were upregulated by miR-132 mimics under HG condition (Fig. 5C–D), and this upregulation was further enhanced by si-occludin and reversed by occludin overexpression. The results indicated that miR-132 activated the JAK2/STAT3 signaling pathway through regulation of occludin in ARPE-19 cells under high-glucose condition.

**Discussion**

As a main cause of visual disability, DR mainly results from abnormal retinal blood vessels [3, 30]. Diabetic macular edema (DME) is the most common cause of central vision loss in diabetics [31]. It leads to vasogenic edema and pathological retinal thickening and is triggered secondarily to the deterioration of the BRB and the consequent increase in extravasation of fluids [32]. The BRB, comprising the retinal vasculature and pigment epithelium, protects and allows the retina to regulate its extracellular chemical composition. Retinal capillaries form the inner BRB and it features endothelial cells harboring tight junctions that are almost impermeable to protein transport and help maintain the BRB, whose impairment could cause increased vascular permeability [33].

miRNAs are a class of endogenous, small non-coding RNAs with 20-23 nucleotides long that are regulators of genes involving in the post-transcriptional regulation process [34]. They regulate gene expression by binding to the 3’UTR of the mRNAs, which in turn lead to translation inhibition. Aberrantly expressed miRNAs associated with cellular dysfunction in early DR as well as their targets were identified, indicating that miRNAs...
miR-132 in cell permeability

represent potential effective DR therapeutic targets [13]; among these, miR-132 was a potential DR-related miRNA, a result that needs further validation. The expression of miR-132 was increased by HG treatment in bone marrow stem cells [35]. On the contrary, the expression of miR-132 was significantly decreased in HG-treated vascular smooth muscle cells (VSMCs) [36]. In this study, we explored the function and downstream mechanism of miR-132 in an in vitro cell model of DR and showed that it promotes cell mobility and permeability by regulating occludin and activating the JAK/STAT3 pathway.

Occludin is a protein encoded by the OCLN gene [37], the knockout of which conferred morphological stability of epithelial tissues, chronic inflammation, hyperplasia in the gastric epithelium and other effects [38, 39], suggesting that its function is quite complicated. The JAK/STAT3 signaling pathway is involved in many processes including cell division, immunity, cell death and tumour formation [37]. Studies also showed that the JAK2/STAT5 axis may partially mediate retinal vascular endothelial cell tolerance to hypoxia, indicating that it played a critical role during retinal angiogenesis. It is of much significance to endothelial cell survival to maintain the inappropriate retinal neovascularization common in DR during tissue hypoxia [40]. In our study, we for the first time revealed occludin is the target of miR-132. Additionally, to our knowledge there is at present no research on the JAK/STAT3 pathway affecting cell migration and leakage in DR.

Although this study reveals a new molecular mechanism of miR-132 regulation of DR based on a HG treated ARPE-19 cell model, much more needs to be done to deeply understand the pathological mechanism of DR. First, whether this molecular mechanism could be applied to an animal model needs further verification. Next, to better realize the potential of miRNA detection in DR, in future work, we will collect serum and vitreous humour samples from DR patients and detect the expression of miR-132 in these samples to verify its potential as a serum marker. Besides, HG could induce the expression of miR-132 and reproduce DR-related phenotypes, meanwhile, miR-132 mimics alone without HG treatment cannot reproduce DR-related phenotypes, indicating that the miR-132 induction is not sufficient to reproduce DR-related phenotypes or even to suppress occludin and the co-existence of HG seems necessary for both and may have additional unknown effects, which needs further investigation. In conclusion, we for the first time revealed that the upregulation of miR-132 in ARPE-19 cells could enhance cell migration and permeability, and our findings suggested that the modulation of occludin targeting by miR-132 via the JAK/STAT3 pathway could represent novel effective DR-treatment strategies.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (NO. 81660164), the National Science Foundation Key Program of Jiangxi Province (NO. 20171ACB20036), the Natural Science Foundation of Jiangxi Province (NO. 2015BAB205026 and NO. 2015BBG70171), the Scientific Research Program of the Second Affiliated Hospital of Nanchang University (NO. 2016YNZJ12001), the Scientific Research Program of Jiangxi Health and Family Planning Commission (NO. 2017A238) and Doctor Start-up Fund of the Second Affiliated Hospital of Nanchang University (NO. B1728#).

Ethics Approval and Consent to Participate

Not Applicable. This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of Interest Statement

The authors declare that they have no conflict of interest.

Authorship

guarantor of integrity of the entire study: Shu-Hua Fu study concepts: Shu-Hua Fu study design: Shu-Hua Fu; Shan-Shan Wang definition of intellectual content: Xing Liao literature research: Fei Liu clinical studies: Shan-Shan Wang experimental studies: Shan-Shan Wang data acquisition: Qian Zhang data analysis: Shan-Shan Wang, Xing Liao statistical analysis: Jing-Jing Qiu, Qian Zhang manuscript preparation: Shan-Shan Wang, Fei Liu manuscript editing: Shu-Hua Fu manuscript review: Shu-Hua Fu

Endocrine Journal Advance Publication
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