Diabetic retinopathy (DR) is the leading cause of visual impairment in developed nations. Though plasma microRNA-93 (miR-93) is associated with the risk of DR, the function and regulatory mechanism of miR-93 during DR remains unclear. Blood samples were collected from 12 DR patients and 12 healthy controls. Primary human retinal pigment epithelium (RPE) cells and ARPE-19 cells were cultured in 5 mmol/L or 33 mmol/L D-glucose medium. Long noncoding (lnc) RNA MEG3 and miR-93 expression was detected by real-time quantitative PCR. The effect of MEG3 and miR-93 on high glucose (HG)-induced apoptosis was detected by MTT and flow cytometry. IL-6 and tumor necrosis factor-α levels were detected by enzyme-linked immunosorbent assay. The relationships among MEG3, miR-93, and Nrf2 (also known as NFE2L2) were explored via dual-luciferase reporter assay. lncRNA MEG3 and Nrf2 were decreased and miR-93 was increased in blood samples of DR patients and HG-treated human RPE and ARPE-19 cells. Overexpression of miR-93 inhibited cell proliferation and promoted apoptosis, whereas overexpression of Nrf2 or MEG3 promoted proliferation and suppressed apoptosis and inflammation. In addition, MEG3 targeted miR-93 and down-regulated miR-93. Moreover, miR-93 directly targeted Nrf2 and negatively regulated Nrf2. This study suggests that lncRNA MEG3 depresses HG-induced apoptosis and inflammation of RPE via miR-93/Nrf2 axis, providing a novel perspective on the genesis and development of DR.

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normal physiological conditions, and is becoming more and more important in degenerative retinal diseases in which chronic subclinical inflammation is considered to be the main pathogenic factor.16,17 Early detection and treatment of the disease can decrease the risk of developing complications. Therefore, studying the mechanism of apoptosis and regulation of RPE cells will provide theoretical basis for potential therapeutic targets of DR.

As a group of RNA transcripts, long noncoding RNA (lncRNAs) are not translated into protein products.18 Studies over the past few years have also pointed out that lncRNA is also involved in the development of DR.19 Furthermore, a significantly lower level of maternally expressed gene 3 (MEG3) was observed in diabetic mice and patients than healthy controls.20 However, there are still many unknowns regarding the function and molecular mechanism of MEG3 during the progression of DR.

It is well known that the miRNAs are involved in the pathogenesis of diabetes and relevant micro- and macrovascular complications, and play an important role in many pathophysiological processes, for example, glucose angiogenesis, homeostasis, and the regulation of the inflammatory response.21 However, little has been reported about direct involvement of miRNA during DR.21 Lin et al14 have confirmed that miR-29 plays a vital role in the HG-induced apoptosis of RPE cells through the inverse correlation of PTEN. Twenty-four hours after the onset of diabetes, a down-regulated level of miR-126 was noticed in diabetic retinas of streptozotocin-induced diabetic rats.22 A previous study illustrated that miR-195 is up-regulated and SIRT1 is down-regulated in human retinal microvascular endothelial cells exposed to HG.23 A significantly higher level of miR-93 (MIR93) was noticed in eyes with proliferative DR in another study, suggesting that miR-93 may have a role in angiogenesis and fibrosis.24

In view of miR-93 as an important regulator of DR, this study aimed to further examine the latent function of miR-93 during the pathological process of DR.

Materials and Methods

Patients

Twelve DR patients with a mean course of disease for 8.23 ± 2.14 years were included in this study. All of the patients were diagnosed according to the diagnostic criteria established by the Chinese Medical Association in 2014. Inclusion criteria: after routine fundus examination and fundus fluorescence angiography examination, type 2 diabetes mellitus patients with any one of the following conditions: hemangioma, a few small bleeding points, neovascularization, vitreous hemorrhage, or secondary retinal detachment in the retina. Exclusion criteria: patients with acute complications such as diabetic ketois, hyperglycemic coma, severe stress such as recent cardiovascular events, trauma operation, acute or chronic infection, hepatic disease, and other endocrine metabolic disease. Meanwhile, 12 healthy controls were selected as a control group. No significant differences were found between the two groups in terms of age and sex. This study was approved by the Ethics Committee of Provincial People’s Hospital Affiliated to Nanchang University. All patients signed informed consent. Fasting blood (20 mL) was extracted from each participant in the morning. Blood samples were kept at room temperature for 1 hour, followed by centrifugation at 2500 × g for 20 minutes to collect plasma samples.

Cell Culture

Primary human RPE (HRPE) cells were purchased from Cell Systems (Kirkland, WA) and cultured in an endothelial growth medium-2 kit (Lonza, Basel, Switzerland). The human RPE cell line ARPE-19 was obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The complete culture medium for ARPE-19 cells consisted of Dulbecco’s modified Eagle’s medium/F-12 (volumetric ratio of 1:1; Hyclone, Logan, UT) and 10% fetal bovine serum (Hyclone). ARPE-19 and HRPE cells were cultured at 37°C in humidified air with 5% CO₂. The culture medium was renewed every other day. For HG treatment, ARPE-19 and HRPE cells were grown in complete culture medium until approximately 75% confluent. Subsequently, the culture medium was replaced by fetal bovine serum—free Dulbecco’s modified Eagle’s medium/F-12 for 24 hours before switching to the HG condition (33 mmol/L D-glucose). The cell culture medium was not renewed during the 48 hours of HG treatment. When the cells were routinely cultured, the medium was renewed every other day.

Quantitative Real-Time PCR Analysis

Total RNA was extracted according to the manufacturer’s instructions. Then, 1 μg of RNA was reverse transcribed to cDNA with the Prime Script RT Kit (Fermentas, Burlington, ON, Canada). Quantitative real-time PCR (qPCR) was performed by using the SYBR Premix Ex Taq (Takara Bio Inc., Kusatsu, Japan) with a Roche 480 Light Cycler (Roche, Mannheim, Germany). The threshold cycle for each reaction was recorded as quantity of gene expression. The levels of target mRNA expression were analyzed by the comparative 2–ΔΔCt method using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control, whereas the miR-93 expression was calculated using U6 as a control.

Western Blot Analysis

After the indicated treatment, cells were lysed with radiolabeling precipitation assay buffer, and then centrifuged. The protein concentrations were measured by using a BCA kit (Pierce, Rockford, Illinois). The protein sample was separated by 8% SDS-PAGE gels, and then they were
transferred onto 0.45-µm polyvinylidene difluoride membranes. After the polyvinylidene difluoride membranes had been incubated with primary antibody at 4°C overnight, they were then incubated with secondary antibodies. Protein bands were obtained using a FluorChem E System (Bio-Rad, Singapore). An antibody against GAPDH was used for equivalent loading of total proteins. The following antibodies were used: anti-cleaved caspase-3 antibody (ab2302; Abcam, Cambridge, UK), anti-Nrf2 (NFE2L2) antibody (ab137550; Abcam), anti-Bax (BCL2 associated X, apoptosis regulator) antibody (ab32503; Abcam), anti-Bcl-2 (BCL2 apoptosis regulator) antibody (ab32124; Abcam); anti-GAPDH antibody (ab8245; Abcam); and sheep anti-rabbit IgG (horse radish peroxidase) (ab6795; Abcam).

**Cell Viability Assay**

ARPE-19 cells were seeded in 96-well culture plates (Corning, Corning, NY); 20 µL of MTT (Beyotime Biotech, Shanghai, China) was added to each well, and the plates were then incubated at 37°C for another 4 hours. After removing the supernatant, 100 µL of dimethyl sulfoxide was added to each well. The optical density at 560 nm was obtained on a microplate reader (Promega, Madison, WI).

**Cell Transfection**

Human miR-93 mimics (5’-CAAGAGUCGUUCUGUGUCAGGUAG-3’), normal control (NC) mimics (5’-UGAGACUGGAUGACCAGUGUG-3’), miR-93 inhibitor (5’-CUACCCUGCACGAACAGCAGCUUUG-3’), and NC inhibitor (5’-UUCUCGAACGUGUCACGUTT-3’) were obtained from RiboBio (Guangzhou, China). pcDNA3.1 vector for MEG3, siRNA for MEG3 (si-MEG3), pcDNA3.1 vector for Nrf2 (p-Nrf2), pcDNA3.1 vector for MEG3/Nrf2 normal control (p-NC), and siRNA for MEG3/Nrf2 normal control (si-NC) were obtained from RiboBio. miRNA mimics or a miRNA inhibitor was transfected into the ARPE-19 cells in serum-free Opti-MEM by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA).
To explore the relationship between miR-93-5p and MEG3, miR-93-5p and Nrf2, the reporter vector was constructed by amplifying the 3'-UTR sequence of the MEG3 or Nrf2 gene including miR-93-5p binding sites and inserting into the pMIR-REPORT luciferase vector. To remove the complementarity, the two miR-93-5p complementary sequences UCGUGAAA or GUGAAA in the 3'-UTR were mutated singly or simultaneously. The mutants were called pMIR-MEG3/mut and pMIR-Nrf2/mut, respectively. ARPE-19 cells were transfected with the appropriate reporter plasmid and miRNA or expression plasmids in serum-free Opti-MEM by Lipofectamine 2000 (Invitrogen). The luciferase activity was detected on a Dual-Luciferase Reporter Assay System (Promega).

To explore the interactions among miR-93, IncRNA MEG3, and Nrf2, the apoptosis rate in each group was measured by flow cytometry. The cells were incubated with PI-conjugated anti-Annexin V antibodies (Hanbio, Shanghai, China) for 20 minutes under darkness. The cell apoptosis rate was then measured by flow cytometry (Becton Dickinson, Franklin Lakes, NJ).

Levels of IL-6 and tumor necrosis factor-α (TNF-α) were detected by the corresponding enzyme-linked immunosorbent assay (ELISA) kit (Sigma-Aldrich, Darmstadt, Germany) according to the instructions.
Statistical Analysis

Data are expressed as means ± SD and analyzed using SPSS version 14.0 software (SPSS 14.0 for Windows; SPSS Inc., Chicago, IL). Unpaired t-test was used for the comparisons between two groups, and the differences among more than two groups were tested by one-way analysis of variance. P < 0.05 indicates statistical significance.

Results

HG Damages RPE Cells and Up-Regulates miR-93 Expression

A previous study has shown that the miR-93 is up-regulated during DR. Therefore, qPCR was used to detect the expression of miR-93 in DR patients and normal subjects. Up-regulated expression of miR-93 was detected in DR patients (P < 0.001) (Figure 1A). HRPE and ARPE-19 cells were cultured in 5, 15, 25, 35, and 45 mmol/L D-glucose for 48 hours. MTT assay indicated that 25, 35, and 45 mmol/L D-glucose significantly inhibited the activities of HRPE and ARPE-19 cells (P < 0.05) (Figure 1B). HRPE and ARPE-19 were cultured in 0.3 mmol/L H2O2 for 4 hours, and apoptosis was detected by flow cytometry. The results showed that H2O2 induced apoptosis significantly (P < 0.01) (Figure 1F). In terms of the expression of miR-93, the results from qPCR suggest that there is no significant difference between the control group and the H2O2 group (not significant) (Figure 1G). Together, the data illustrate that HG stimulated expression of miR-93 in DR.

miR-93 Is Up-Regulated under HG Condition

To explore the effects of miR-93 on HG-induced apoptosis in RPE cells, miR-93 mimics or miR-93 inhibitors were transfected into ARPE-19 cells. Results from qPCR revealed that miR-93 mimics and miR-93 inhibitor worked well (P < 0.05) (Figure 2A). The cell viability of the above cells was further measured by MTT. It was found that overexpression of miR-93 markedly suppressed the viability of HG-treated cells, whereas knockdown of miR-93 enhanced it (P < 0.01) (Figure 2B). Apoptosis was detected by flow cytometry.
cytometry. Overexpression of miR-93 significantly promoted apoptosis induced by HG (P < 0.001), whereas inhibiting the expression of miR-93 alleviated HG-induced apoptosis (P < 0.05) (Figure 2C). Concurrently, proapoptotic protein caspase-3 and Bax were significantly increased in the miR-93 mimics group (P < 0.01; P < 0.05), and down-regulated in the miR-93 inhibitors group (P < 0.01); antiapoptotic protein Bcl-2 was remarkably down-regulated in the miR-93 mimics group (P < 0.05) and up-regulated in the miR-93 inhibitors group (P < 0.01) (Figure 2D). The expression of IL-6 and tumor necrosis factor (TNF)-α was detected by enzyme-linked immunosorbent assay (ELISA). The results show that Nrf2 can inhibit the secretion of inflammatory cytokines induced by high glucose, and overexpression of Nrf2 reverses the proinflammatory effect of overexpressing miR-93. The experiments were performed under high glucose (HG) conditions. Data are expressed as means ± SD. n = 3 (A–E). *P < 0.05, **P < 0.01, and ***P < 0.001. FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Nrf2 Is a Target Gene of miR-93 in ARPE-19 Cells

To elucidate the latent mechanism of miR-93 overexpression aggravating HG-induced RPE apoptosis, miRNA target prediction was performed using StarBase v2.0.27 Consistent with the literature, this study found the binding site of miR-93 and Nrf2 (Figure 3A). Further, a high level of miR-93 reduced the fluorescence intensity of wild-type Nrf2 in ARPE-19 cells, and silencing of miR-93 led to opposite results (P < 0.01), but little effect was seen on the fluorescence intensity of Mut-Nrf2 (not significant) (Figure 3B). Moreover, qPCR and Western blot results showed that up-regulation of miR-93 inhibited the expression of Nrf2, whereas miR-93 inhibitors significantly increased the expression of Nrf2 (P < 0.05) (Figure 3, C and D). These results indicated that miR-93 directly targets and negatively regulated Nrf2.

Nrf2 Overexpression Alleviates the HG-Induced Apoptotic Effect of ARPE-19 Cells Mediated by miR-93

The overexpression vector of Nrf2 (p-Nrf2) and its empty vector (p-NC) was transfected into the cells. Increased expression of Nrf2 was observed in p-Nrf2 group (P < 0.001) (Figure 4A). MTT assay showed that up-regulation of Nrf2 increased cell viability and reversed the inhibitory effect of miR-93 on cell viability (P < 0.05) (Figure 4B). Further, overexpression of Nrf2 significantly inhibited HG-induced apoptosis (P < 0.05) and reversed the proapoptotic effect of miR-93 (P < 0.001) (Figure 4C). The expression of Nrf2 was detected by Western blot. The
results from this analysis showed that overexpression of miR-93 inhibited the expression of Nrf2 and reversed the effect of Nrf2 overexpression on the protein level of Nrf2 (P < 0.05) (Figure 4D). Overexpression of Nrf2 inhibited the expression of cleaved caspase 3 and Bax, and promoted the expression of Bcl-2; and overexpression of Nrf2 reversed the effect of miR-93 on the promotion of cleaved caspase 3 and Bax, and the inhibitory effect on Bcl-2 (P < 0.05) (Figure 4D). The expression of inflammatory factors IL-6 and TNF-α was detected by ELISA. The results showed that Nrf2 can inhibit the secretion of inflammatory factors induced by HG, and overexpression of Nrf2 reversed the proinflammatory effect of miR-93 overexpression (P < 0.05) (Figure 4E). The above results indicate that Nrf2 overexpression could attenuate miR-93-mediated apoptosis induced by HG in retinal pigment epithelial cells.

MEG3 Directly Targets miR-93 in ARPE-19 Cells

Complementary binding sites between MEG3 and miR-93 were found according to bioinformatics analysis (Figure 5A). To verify their correlation, wild-type MEG3 and Mut-MEG3 luciferase reporter plasmids were designed. Fluorescence intensity was significantly lower in the ARPE-19 cells co-transfected with wild-type MEG3 and miR-93 mimics than cells co-transfected with Mut-MEG3 and miR-93 mimics (P < 0.001) (Figure 5B). qPCR results illustrated that si-MEG3 significantly inhibited the expression of MEG3 (P < 0.01), whereas p-MEG3 promoted the expression of MEG3 (P < 0.001) (Figure 5C). Further, after transfection of si-MEG3, the expression of miR-93 significantly increased (P < 0.01), whereas overexpression of MEG3 significantly inhibited the expression of miR-93 (P < 0.01) (Figure 5D). The above results indicate that in ARPE-19, MEG3 targets and negatively regulates miR-93.

MEG3 Is Down-Regulated under HG Condition, Overexpression of MEG3 Alleviates HG-Induced Apoptosis through the miR-93/Nrf2 Axis

The expression of MEG3 was measured in DR patients and healthy controls, and a lower level of MEG3 was discovered in DR patients (P < 0.01) (Figure 6A). HRPE and ARPE-19 cells were cultured with NG and HG, respectively. qPCR results showed that MEG3 was significantly down-regulated in the HG group (P < 0.01) (Figure 6B). The HG treatment groups were: control, si-NC, si-MEG3, p-NC, p-MEG3, and p-MEG3+miR-93 mimics. The results revealed that the expression of Nrf2 mRNA was down-regulated when MEG3 was knocked down (P < 0.01) and up-regulated when MEG3 was overexpressed (P < 0.001), but the regulation of Nrf2 by MEG3 was reversed by overexpression of miR-93 (P < 0.001) (Figure 6C). MTT assay showed that knockdown of MEG3 increased the inhibitory effect of HG on cell viability (P < 0.01), whereas overexpression of MEG3 reduced the induction of HG on cell viability (P < 0.01); overexpression of miR-93 reversed the promoting effect of MEG3 on cell viability (P < 0.05) (Figure 6D). The expression of Nrf2 in ARPE-19 cells after 48 hours of si-MEG3, p-MEG3, and p-MEG3+miR-93 mimics transfection was detected by Western blot. The results revealed that the expression of Nrf2 was down-regulated when MEG3 was knocked down (P < 0.01) and up-regulated when MEG3 was overexpressed (P < 0.01), but the regulation of Nrf2 by MEG3 was reversed by overexpression of miR-93 (P < 0.05) (Figure 6E). Flow cytometry showed that knockdown of MEG3 aggravated HG-induced apoptosis (P < 0.01); overexpression of MEG3
attenuated HG-induced apoptosis ($P < 0.001$), and overexpression of miR-93 reversed the antiapoptotic effect of MEG3 ($P < 0.05$) (Figure 6F). Western blot results revealed that knockdown of MEG3 increased the expression of cleaved caspase 3 and Bax, and decreased the expression of Bcl-2. Overexpression of MEG3 depressed the expression of cleaved caspase 3 and Bax, and up-regulated the expression of Bcl-2, whereas overexpression of miR-93 reversed this trend ($P < 0.05$) (Figure 6G). The results of ELISA demonstrated that knockdown of MEG3 stimulated the expression of IL-6 and TNF-α, whereas overexpression of MEG3 showed the opposite results. However, overexpression of miR-93 reversed the anti-inflammatory effect of MEG3 ($P < 0.05$) (Figure 6H). The above results indicate that MEG3 was down-regulated under the HG condition; overexpression of MEG3 alleviated HG-induced apoptosis and inflammation through the miR-93/Nrf2 axis (Figure 6I).

**Discussion**

The main problem with DR is the occurrence of metabolic, vascular, and neurological complications. The RPE cell apoptosis is an early event in DR. In the current study,
up-regulated expression of miR-93 was noticed in DR patients, as well as in HG-exposed HRPE and ARPE-19 cells, indicating that miR-93 plays a potential role in occurrence and development of DR. Therefore, the function and regulation mechanism of miR-93 were further studied.

MiR-93 often functions as a promoter of tumor progression in several human carcinomas such as glioblastoma and breast cancer. Moreover, Hirota et al. pointed out that miR-93 was highly expressed in patients with proliferative DR. A previous study demonstrated that plasma miR-93 participated in the type 2 DR progression. Consistent with previous studies, up-regulated miR-93 was observed in DR patients, and HG-exposed HRPE and ARPE-19 cells, indicating that miR-93 may be a potential key factor involved in DR progression.

Although apoptosis has been proved to be one of the main characteristics of DR progression in RPE cells, there is no direct evidence that the up-regulation of miR-93 has any effect on RPE. In the previous literature, the regulatory role of miR-93 in apoptosis is controversial. miR-93 was up-regulated in oxygen and glucose deprivation/reoxygenation model, and miR-93 inhibition ameliorated oxygen and glucose deprivation/reoxygenation-induced hypoxia/reoxygenation injury apoptosis in cells by targeting Nrf2. However, in cardiomyocytes, miR-93-3p functioned as a protective factor against inflammation and apoptosis. Therefore, the expression of miR-93 is inconsistent under different disease states, sometimes manifested as pro-apoptosis and sometimes as antiapoptosis. The current study confirmed that miR-93 inhibited the viability and increased apoptosis and inflammation in HG-exposed ARPE-19 cells.

This study further investigated how miR-93 regulates apoptosis in RPE cells. It is well documented that Nrf2 plays a critical protective role against oxidative stress and inflammation. In addition, Niture and Jaiswal have found that Nrf2 protein could up-regulate antiapoptotic protein Bcl-2 and prevent cellular apoptosis. Another study has indicated that Nrf2 is an important protective factor regulating the progression of DR, and it suggested that up-grading of the Nrf2 pathway may act as a potential therapeutic method for DR. Evidence concerning a potential protective role for Nrf2 in the retina is increasing. Consistent with the previous literature, the present study revealed that overexpression of Nrf2 can reverse the proapoptotic effect of miR-93.

As a tumor suppressor gene, functionality of MEG3 has been widely studied in different types of human cancer. A previous study pointed out that the expression of MEG3 was down-regulated in HG-treatment ARPE-19 cells, MEG3 overexpression reduced the increased expression levels of VEGF and TGF-β1 induced by HG treatment. Therefore, it was concluded that lncRNA MEG3 overexpression may inhibit the development of DR by inhibiting TGF-β1 and VEGF expression. Consistent with previous reports, down-regulated MEG3 expression was detected in DR patients, and HG-exposed HRPE and ARPE-19 cells. Therefore, the authors speculated that MEG3 may play a potential regulatory part during DR. A study on diabetic mice showed significantly down-regulated expression of MEG3 and aggravated retinal vascular dysfunction. However, it is unclear whether MEG3 can regulate apoptosis in RPE cells. Wang et al. discovered that lncRNA MEG3 could inhibit apoptosis in ethanol-induced AML-12 cells and hepatic steatosis. Another study suggested that inhibition of lncRNA MEG3 was conducive to protect rat brain microvascular endothelial cells against oxygen and glucose deprivation/reoxygenation-induced apoptosis. Consistent with the above studies, this current study reveals that in HG-cultured ARPE-19 cells, MEG3 enhanced cell viability and inhibited apoptosis and inflammation, and it is further demonstrated that MEG3 functions through the miR-93/Nrf2 axis.

To sum up, the present study demonstrates that lncRNA MEG3 and Nrf2 were decreased and miR-93 was increased under DR and HG conditions. Nrf2 is a target gene of miR-293, Nrf2 overexpression alleviates the HG-induced apoptotic effect of ARPE-19 cells mediated by miR-93. MEG3 directly targets miR-93, overexpression of MEG3 alleviates HG-induced apoptosis and inflammation through the miR-93/Nrf2 axis. A mouse model of DR will be established to investigate the role of these genes/proteins in the development of DR in future studies by the authors.

References

11. Vinores SA, Van Niel E, Swardloff JL, Campochiaro PA: Electron microscopic immunocytochemical demonstration of blood-retinal barrier breakdown in human diabetics and its association with...


