

Regulation role of miR-204 on SIRT1/VEGF in metabolic memory induced by high glucose in human retinal pigment epithelial cells

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Abstract

• **AIM:** To examine the regulatory role of microRNA-204 (miR-204) on silent information regulator 1 (SIRT1) and vascular endothelial growth factor (VEGF) under high-glucose-induced metabolic memory in human retinal pigment epithelial (hRPE) cells.

• **METHODS:** Cells were cultured with either normal (5 mmol/L) or high D-glucose (25 mmol/L) concentrations for 8d to establish control and high-glucose groups, respectively. To induce metabolic memory, cells were cultured with 25 mmol/L D-glucose for 4d followed by culture with 5 mmol/L D-glucose for 4d. In addition, exposed in 25 mmol/L D-glucose for 4d and then transfected with 100 nmol/L miR-204 control, miR-204 inhibitor or miR-204 mimic in 5 mmol/L D-glucose for 4d. Quantitative reverse transcription-polymerase chain reaction (RT-qPCR) was used to detect miR-204 mRNA levels. SIRT1 and VEGF protein levels were assessed by immunohistochemical and Western blot. Flow cytometry was used to investigate apoptosis rate.

• **RESULTS:** It was found that high glucose promoted miR-204 and VEGF expression, and inhibited SIRT1 activity, even after the return to normal glucose culture conditions. Upregulation of miR-204 promoted apoptosis inhibiting SIRT1 and increasing VEGF expression. However, downregulation of miR-204 produced the opposite effects.

• **CONCLUSION:** The study identifies that miR-204 is the upstream target of SIRT1 and VEGF, and that miR-204 can

protect hRPE cells from the damage caused by metabolic memory through increasing SIRT1 and inhibiting VEGF expression.

• **KEYWORDS:** human retinal pigment epithelial; metabolic memory; microRNA-204; silent information regulator 1; vascular endothelial growth factor; high-glucose
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INTRODUCTION

Diabetes, a significant public health concern globally, has a profound impact on patient well-being due to its retinopathy-related complications^[1]. The serious damage caused by persistent hyperglycemia fluctuation to the function and morphology of microvessels results in retinal ischemia and hypoxia, ultimately leading to neovascularization^[2-3]. Repeated vitreous hemorrhage^[4] and macular edema further exacerbate the damage to retinal pigment epithelial cells (RPE) and photoreceptors and the patient's vision is further damaged^[5-7]. Research has found that this series of injuries is closely linked to oxidative stress and the increase of vascular endothelial growth factor (VEGF)^[8-9]. Moreover, it has been observed that even after blood glucose levels return to normal, there may still be persistent cell damage in the presence of persistent hyperglycemia, this phenomenon is known as metabolic memory^[10-11].

Mounting evidence suggests that microRNAs play a role in the metabolic memory process associated with diabetic complications^[11-14]. MicroRNAs have been shown to directly or indirectly regulate the expression of VEGF^[15-16] or silent information regulator 1 (SIRT1)^[17] genes in diabetic retinopathy. microRNA-204 (miR-204), in particular, regulates SIRT1 and participates in metabolic memory formation in diabetic retinopathy^[18-19]. SIRT1 is a class III histone deacetylase that depends on nicotinamide adenine dinucleotide (NAD⁺) and is

involved in oxidative stress and neovascularization in diabetic retinopathy^[20-21], and the key mediator of neovascularization is VEGF^[9]. Therefore, in this study, we focused on exploring the effects of high glucose and metabolic memory formation on miR-204/SIRT1/VEGF regulation. We analyzed the impact of miR-204 expression on the expression levels of SIRT1 and VEGF in RPE.

MATERIALS AND METHODS

Cell Culture and Infection The human retinal pigment epithelial (hRPE) cell line (ARPE-19) was obtained from Saibaikang of Shanghai and was cultured in standard Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 100 units/mL penicillin and 100 µg/mL streptomycin (Gibco, Rockville, MD, USA) in a humidified 5% CO₂ incubator at 37°C. The cells were maintained in media containing 5 mmol/L D-glucose for 8d (normal glucose group, C), or 25 mmol/L D-glucose for 8d (high glucose group, H), or 25 mmol/L D-glucose for 4d followed by 5 mmol/L D-glucose for 4d (metabolic memory group, M)^[10], or 25 mmol/L D-glucose for 4d followed by transfected 100 nmol/L miR-204 control, a miR-204 mimic, or a miR-204 inhibitor plasmid (GENE, Shanghai, China) with 5 mmol/L D-glucose for 1d, the transfected cells were screened by the addition of 1.0 µg/mL puromycin and incubated with 5 mmol/L D-glucose for 3d.

Quantitative Reverse Transcription-Polymerase Chain Reaction Analysis miRNA was extracted using the SanPrep Column miRNA Extraction Kit (B518811, Sangon, Shanghai, China), and then converted into cDNA using the Avian Myeloblastosis Virus Reverse Transcriptase First Strand cDNA Synthesis Kit (SK2445, Sangon, Shanghai, China). Real-time polymerase chain reaction (PCR) was performed using 2X SG Fast qPCR Master Mix (B639273, Sangon, Shanghai, China) with the ABI 7500 system (Applied Biosystems, Foster City, CA, USA). The Sequence Detection Software (7500 system, Applied Biosystems) was used for data analysis. The results are means±standard deviation (SD) of 2^{-ΔΔCt}. Expression levels of miR-204 were normalized against the levels of an endogenous control U6. All reactions were performed in triplicate. The following primer sequences (Sangon, Shanghai, China) were used: miR-204 forward 5'-CGCGTTCCTTTGTCATCCT-3' and reverse 5'-AGTGCAGGGTCCGAGGTATT-3'; U6 forward 5'-CTCGCTTCGGCAGCACCA-3' and reverse 5'-AACGCTTCACGAATTGCGT-3'.

Western Blot Analysis Anti-VEGF (PB0084) and anti-SIRT1 (BM2175) antibodies were obtained from Wuhan Boster Biological Technology (China). Anti-β-actin (AB1001) and peroxidase-conjugated goat anti-rabbit IgG (AB10058) were purchased from Shanghai Sangon Biotech (China).

Cells were lysed in ice-cold RIPA lysis buffer (WB0061, Dingguo, Beijing, China) and protein from the samples was electrophoresed on SDS-PAGE (WB0201, Dingguo, Beijing, China) in a Bio-Rad mini slab gel apparatus (BioRad, Hercules, CA, USA) before electrophoretic transfer to PVDF membranes (Millipore, Billerica, MA, USA). The PVDF membranes were blocked with non-fat milk and then incubated with the primary antibodies, including anti-SIRT1 (1:1000), anti-VEGF (1:1000), and anti-β-actin (1:1000), followed by the secondary antibody (1:3000). Immunoreactive bands were visualised by chemiluminescence imaging (Bio-Rad, Mississauga, ON, Canada). Protein bands were quantified by densitometry using Image J software. The protein level of β-actin acted as an internal control.

Cellular Immunohistochemistry Staining ARPE-19 cells were plated on coverslips in 6-well plates and treated for 8d before fixing in 4% paraformaldehyde for 10min. The cells were permeabilized with 1% Triton X-100, blocked with normal goat serum, and incubated with 50 µL anti-SIRT1 (1:500) or anti-VEGF antibodies (1:500) for 2h at room temperature (RT). After washing with phosphate buffered solution (PBS), the cells were then incubated with 50 µL goat anti-rabbit secondary antibody (1:100, KIT-9706, Boster, Wuhan, China) for 30min at RT. Finally, cells were incubated with 50 µL streptavidin biotin-peroxidase complex (SABC;1:100, AR2002, Boster, Wuhan, China) for 20min at RT before adding 50 µL 3,3'-diaminobenzidine (AR1022, Boster, Wuhan, China) for 5min. Images were viewed and photographed using a fluorescence microscope (BX53, Olympus, Tokyo, Japan). Data were analysed using Image-Pro Plus Software.

Flow Cytometry Analysis ARPE-19 cells from each group were collected and washed three times with PBS and resuspended in 0.5 mL cold binding buffer containing Annexin V-FITC and PI (KGA107, KeyGEN, Jiangsu, China). After incubation for 15min in the dark at RT, the cells were examined by flow cytometry (BD AccuriC6, San Jose, CA, USA). CytExpert 2.0 was used for data analysis.

Statistical Analysis All results were obtained from at least three independent experiments and presented as mean±SD. Comparisons among all groups were tested using one-way ANOVA followed by the least significant difference (LSD) and Dunnett's post-hoc test. All results were analysed using SPSS 24.0 Software (IBM, Armonk, NY, USA) and GraphPad Prism 6.0 Software (GraphPad Software, La Jolla, CA, USA). *P*<0.05 was considered statistically significant.

RESULTS

Hyperglycemia-Induced Increases of miR-204 Expression High-glucose treatment for 8d resulted in increased relative expression levels of miR-204 mRNA compared with the normal glucose group (Figure 1). Similar high miR-204

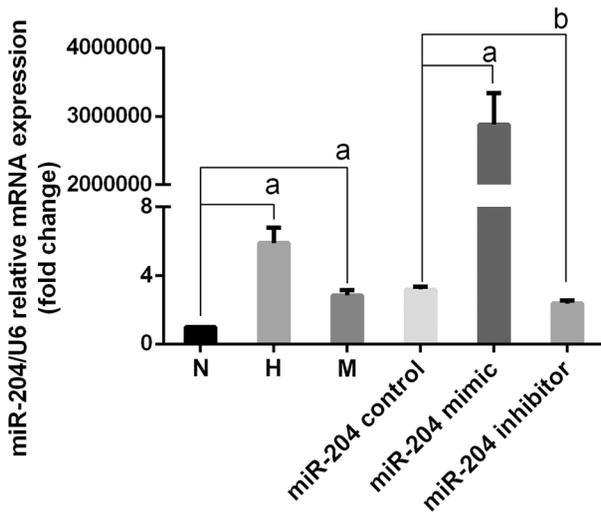


Figure 1 Analysis of miR-204 mRNA expression ^a $P < 0.05$; ^b $P < 0.01$. N: Normal glucose group; H: High glucose group; M: Metabolic memory group; miR-204: microRNA-204.

expression levels were found in cells treated with high glucose for 4d followed by normal glucose for 4d. In addition, we performed quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis of miR-204 expression after transfection with different plasmids. The results showed that the relative expression levels of miR-204 mRNA were further up-regulated by transfection with the miR-204 mimic compared with the miR-204 control in the metabolic memory group. However, there was a significant reduction in miR-204 expression after transfection with the miR-204 inhibitor compared with the miR-204 control.

SIRT1 Expression in Transfected Cells in Relation to Metabolic Memory Induction ARPE-19 cells that were incubated with continuous high glucose showed decreased levels SIRT1 protein (Figure 2A, 2B). SIRT1 protein were lower in cells treated with high glucose for 4d followed by normal glucose for 4d than in cells exposed to continuous normal glucose concentrations (Figure 2A, 2B). Immunohistochemical staining showed that SIRT1 was expressed mainly in the nucleus with only small amounts in the cytoplasm. It can be seen that there is a reduction in the number of SIRT1-positive cells containing brown particles in both the metabolic memory and high glucose groups compared with the normal glucose group (Figure 2D).

We then verified the association between miR-204 and SIRT1 by transfection of the miR-204 mimic or the miR-204 inhibitor. Transfection of the miR-204 inhibitor effectively up-regulated both SIRT1 protein but no such effect was observed in cells transfected with the miR-204 control (Figure 2A, 2B). In contrast, transfection of the miR-204 mimic down-regulated SIRT1 protein compared with the miR-204 control group, and it was noted that the numbers of SIRT1-positive cells were further diminished (Figure 2D).

VEGF Expression in Transfected Cells in Relation to Metabolic Memory Induction

We performed Western blot and cellular immunohistochemical staining analysis to examine the VEGF expression levels in cells transfected with the miR-204 control, the miR-204 mimic, or the miR-204 inhibitor and exposed to normal glucose, high glucose, and metabolic memory conditions. As shown in Figure 3, continuous treatment with high glucose treatment for 8d significantly increased the VEGF expression levels (Figure 3A, 3B). Expression levels of VEGF were also increased in the M cells treated with transiently high glucose compared with the C group.

In contrast, transfection of the miR-204 inhibitor significantly suppressed both VEGF protein levels. This was not observed after transfection of the miR-204 control (Figure 3A, 3B). In addition, immunohistochemical staining showed that VEGF was expressed mainly in the cytoplasm and also that the numbers of VEGF-positive cells containing brown particles were decreased compared with the miR-204 control group (Figure 3D). In contrast, transfection of the miR-204 mimic further up-regulated the VEGF protein compared with the miR-204 control group.

Apoptosis Rate in Transfected Cells in Relation to Metabolic Memory

We used flow cytometry to measure apoptosis rate in ARPE-19 cells (Figure 4A, 4B). Continuous exposure to high glucose for 8d significantly increased apoptosis rate compared with the normal glucose group (Figure 4A). Apoptosis rate was also increased in the metabolic memory cells induced by high glucose. Furthermore, transfection of the miR-204 inhibitor decreased the metabolic memory-mediated apoptosis compared with the miR-204 control group while this effect was reversed by transfection of the miR-204 mimic.

DISCUSSION

The probability of developing proliferative diabetic retinopathy is significantly heightened in patients with persistently poor glycemic control^[22-23]. The recurrence of late-stage vitreous hemorrhage^[4,24], neovascularization^[25], and macular edema^[26] seriously threaten vision. These pathological reactions are associated with vascular endothelial cell damage, disruption of the blood-retinal barrier, and the consequent massive release of VEGF, exacerbating neovascularization^[27-29]. Even if brought under strict control glucose, these pathological processes cannot be reversed^[10,30].

In our study, we found that sustained high-glucose exposure induces miR-204 expression in hRPE cells, leading to VEGF release and SIRT1 suppression, which exacerbates hRPE cells apoptosis. Following removal of high-glucose exposure, the levels of miR-204, VEGF, and SIRT1 did not fully recover, and hRPE cells apoptosis only partially improved. These findings suggest that miR-204, VEGF, and SIRT1 are associated with

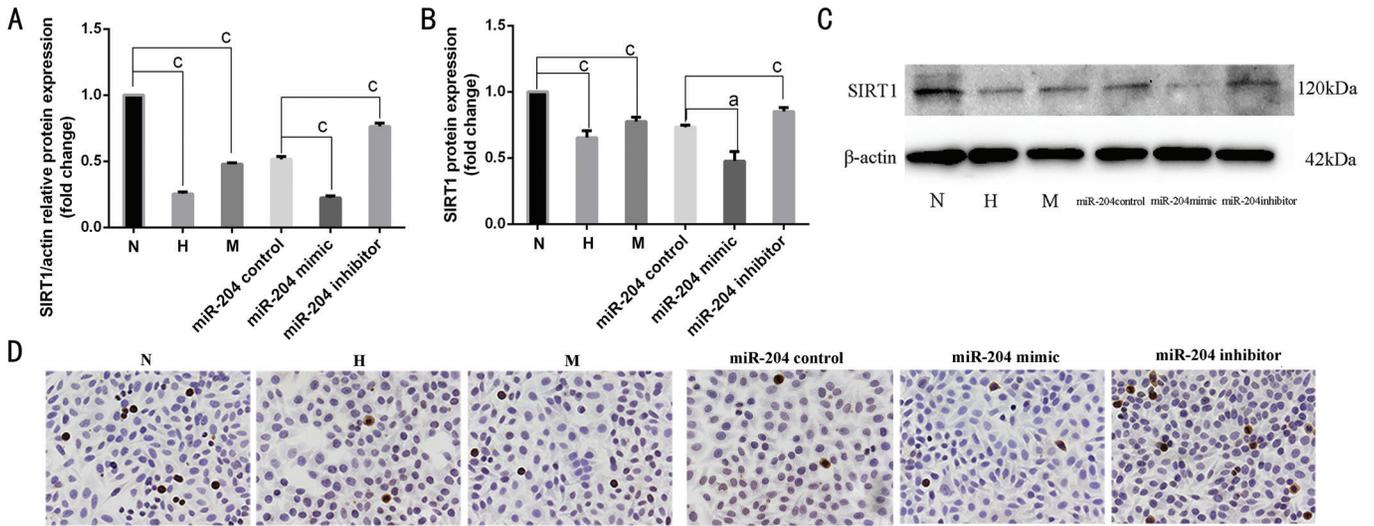


Figure 2 Expression of SIRT1 protein A: Semi-quantitative SIRT1 protein levels of Western blotting; B: Semi-quantitative SIRT1 protein levels of immunohistochemistry staining; C: Western blotting protein bands; D: Cellular immunohistochemistry staining picture (200 \times). ^a*P*<0.05, ^c*P*<0.001. N: Normal glucose group; H: High glucose group; M: Metabolic memory group; miR-204: microRNA-204; SIRT1: Silent information regulator 1.

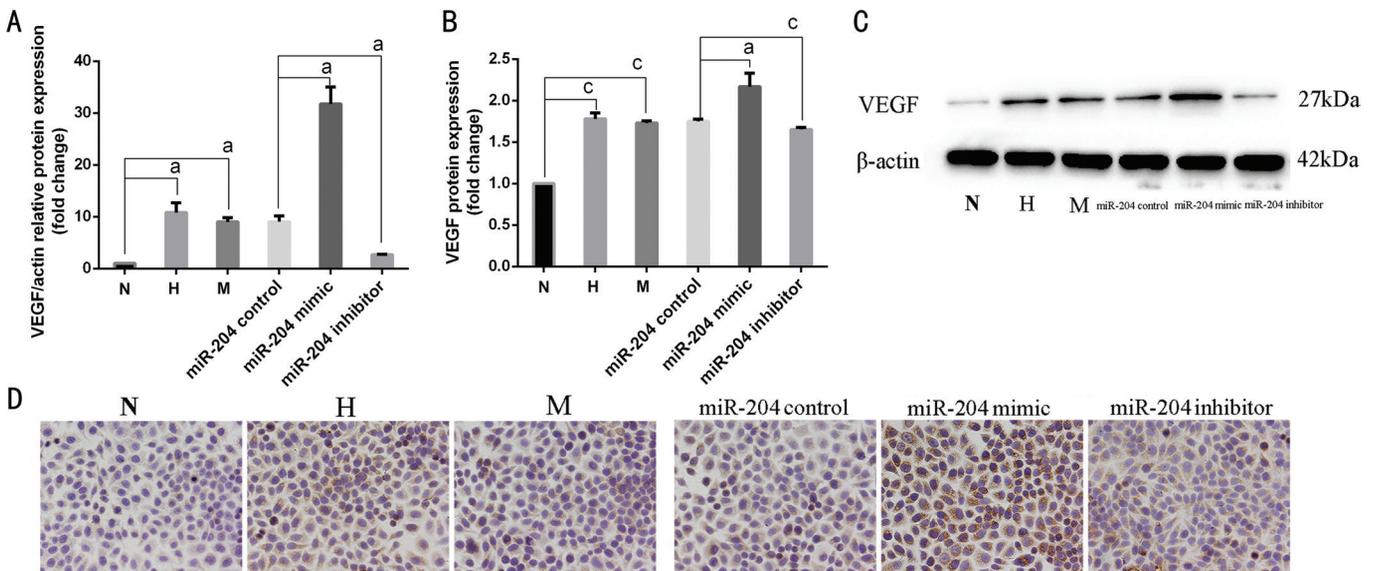


Figure 3 Expression of VEGF protein A: Semi-quantitative VEGF protein levels of Western blotting; B: Semi-quantitative SIRT1 protein levels of immunohistochemistry staining; C: Western blotting protein bands; D: Cellular immunohistochemistry staining picture (200 \times). ^a*P*<0.05; ^c*P*<0.001. N: Normal glucose group; H: High glucose group; M: Metabolic memory group; miR-204: microRNA-204; VEGF: Vascular endothelial growth factor.

high-glucose damage and its associated metabolic memory. Therefore, miR-204, VEGF, and SIRT1 may participate in the formation of high-glucose-induced cellular metabolic memory. To further understand the correlation between miR-204 and VEGF, SIRT1, we conducted an overexpression experiment of miR-204. The results indicate that during the formation of metabolic memory induced by high glucose, the expression of SIRT1 decreases while VEGF expression increases in hRPE cells, leading to increased cell apoptosis. This suggests that miR-204 may serve as an upstream regulator of SIRT1 and VEGF, capable of regulating their expression. It is inferred that the mechanism of cell damage caused by high blood glucose may be associated with miR-204 overexpression.

In the context of VEGF, a key component in the formation of pathological angiogenesis, its release is significantly influenced by tissue ischemia and hypoxia^[31]. SIRT1, on the other hand, its ability to improve tissue oxygenation and reduce oxidative stress-induced damage, has been identified to be associated with VEGF regulator^[32-33]. Our experiments show that persistent hyperglycemia increase miR-204 expression, which subsequently downregulates SIRT1 expression. This results in exacerbated ischemia and hypoxia, augmented oxidative stress, and accelerated angiogenesis, ultimately leading to increased cell apoptosis. Moreover, the upregulation of miR-204 would also promote the release of VEGF, further driving the formation of

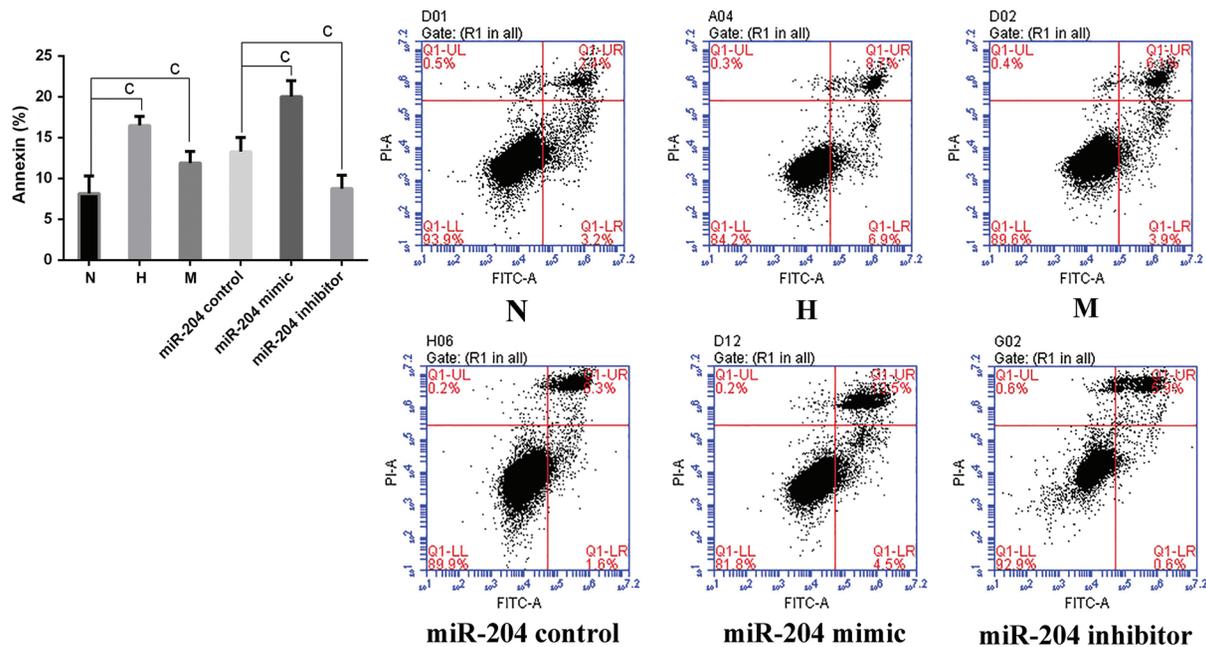


Figure 4 Analysis of apoptosis rate in ARPE-19 cells $P < 0.0001$. N: Normal glucose group; H: High glucose group; M: Metabolic memory group; miR-204: microRNA-204; hrPE: Human retinal pigment epithelial.

pathological neovascularization. Notably, even after removal of hyperglycemic conditions, cells are unable to entirely regain the regulation of miR-204, maintaining suppressed SIRT1 levels and promoting VEGF release, the ischemic and hypoxic state of the cells has not been reversed. It's indicated that the damage of cell metabolic memory induced by high glucose may be related to the inhibition of SIRT1 expression and VEGF release caused by the upregulation of miR-204. Our findings also suggest that downregulating miR-204 expression can help inhibit VEGF release, promote SIRT1 expression, improve the ischemic and hypoxic state of cells, inhibit the formation of pathological angiogenesis, and reduce cell apoptosis and metabolic memory damage.

This study focused on exploring the expression of miR-204/SIRT1/VEGF in hRPE cells during the process of metabolic memory induced by high-glucose conditions. The findings indicate that a high-glucose environment exacerbates ischemia, hypoxia, and oxidative stress in hRPE cells, leading to the formation of metabolic memory. As the upstream target, miR-204, plays a crucial role in SIRT1 up-regulation and VEGF down-regulation. By suppressing miR-204 expression, the ischemia, hypoxia, and oxidative stress in cells can be alleviated, thus inhibiting the formation of pathological angiogenesis. In summary, the regulation of miR-204 holds significant promise in preventing metabolic memory in hRPE cells, and the data suggest that miR-204 may serve as a novel therapeutic target for preventing hRPE cell metabolic memory damage.

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Conflicts of Interest: Lai QL, None; Xie T, None; Zheng WD, None; Huang Y, None.

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