

ORIGINAL ARTICLE

Upregulation of miR-424 inhibit retinal endothelial cells proliferation under high glucose condition via cyclin D1

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Abstract

Introduction: Diabetic retinopathy is characterised by retinal vascular impairment. A number of aberrant microRNAs (miRNAs) have a role in the pathophysiology of vascular dysfunction. However, the relevance of miR-424 in retinal vascular endothelial cell dysfunction during hyperglycemia stress remains unknown. The purpose of this study is to investigate this issue. **Materials and Methods:** Rhesus macaque choroid retinal endothelial cell line (RF/6A) cells were cultivated in normal glucose (NG) and high glucose (HG) conditions. The mRNA expression of miR-424 and Cyclin D1 (CCND1) was quantified using qPCR, and the protein quantity of CCND1 was detected using Western Blot. miR-424 mimics, miR-424 inhibitors, miR-424 inhibitor+ siRNA-CCND1 or vehicle molecules were transfected into RF/6A cells. MTT test was used to assess cell proliferation, and flow cytometric analysis was used to assess cell cycle. The interaction between miR-424 and CCND1 was predicted using bioinformatics and validated using dual luciferase reporter analysis. **Results:** miR-424 was up-regulated, and cell viability was reduced in HG compared to NG. By reversing the expression of miR-424 in certain situations, the phenotypes can be changed. CCND1 has been identified as a miR-424 target gene, and it may be regulated at the transcriptional and translational levels. Manipulation of silencing CCND1 can counteract the effect of transfecting miR-424 inhibitor into RF/6A cells under HG such as proliferation stimulation. **Conclusions:** Our findings indicate that miR-424 plays an important role in hyperglycemia induced ARPE-19 cells damage, and it could be a new therapeutic target for DR by preventing retinal vascular cells from HG-induced injury.

Keywords: Diabetic Retinopathy, Diabetes Mellitus, miR-424, CCND1, Cell Cycle

INTRODUCTION

Diabetic retinopathy (DR) is a serious vision-threatening complication of diabetes mellitus (DM).^{1,2} Blood-retinal barrier breakdown (BRB) is one of the DR features that arises early in disease progression,³ and if not correctly treated, it can lead to increased vascular permeability and perhaps macular edema,^{4,5} which is the major cause of visual impairment.^{6,7} Hyperglycemia-induced vascular endothelial cell dysfunction has been connected to the pathogenic process of BRB.^{6,8} Although current BRB treatment, such as intravitreal anti-vascular endothelial growth factor (VEGF), is effective for many patients, the effect is suboptimal and does not always work.⁹⁻¹¹ Therefore, figuring out the cause of vascular endothelial dysfunction is essential to developing therapeutic strategies for BRB degradation and vascular leakage during the early stages of DR.

MicroRNAs (miRNAs) are endogenous non-coding RNAs that inhibit protein-coding gene expression by inducing mRNA degradation or translation inhibition.^{12,13} It is assumed to have an important role in the pathological angiogenesis processes of DR.¹⁴⁻¹⁶ Previous research has shown that miR-424 has a close and important relationship with vascular diseases. For example, some studies found that miR-424 promoted neovascularization in inflammation-related angiogenesis,¹⁷ as well as multiple tumors such as hemangioma,¹⁸⁻²⁰ osteosarcoma,²¹ and glioma.²² However, the role and regulatory mechanism of miR-424 in DR pathological microvascular dysfunction is unknown.

In this study, we identified miR-424 was up-regulated in the RF/6A cells at the high glucose condition and the inhibition of this molecule can protect RF/6A cells from high glucose

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induced injury. In addition, the mechanism investigation revealed that miR-424 directly targets to CCND1 and leads cell cycle to arrest at G0/G1. These results suggest the modulation of miR-424 expression in retina may be a potential therapeutic method for DR treatment.

MATERIALS AND METHODS

Cell Culture and Treatments

Rhesus macaque choroid-retinal endothelial cell line (RF/6A) was purchased from American Tissue Culture Collection (ATCC, USA). The cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium, which is supplemented with 100U/mL penicillin, 100 µg/mL streptomycin and 10% fetal bovine serum. 10-15 passages of RF/6A cell line were applied for further research. The RF/6A cells were treated with normal glucose (NG group; 5.5mmol/L D-Glucose) or high glucose (HG group; 30mmol/L D-Glucose) for 72h. The cells were cultured in 5% carbon dioxide at 37°C. The medium was replaced every two days.

Quantitative Real-Time Polymerase Chain Reaction

Total RNAs were extracted from the incubated cells by TRIzol reagent (Invitrogen, USA). After testing the purity and concentration, RNAs were reversely transcribed into cDNA using RT reagent Kit (TAKATA, Japan). Primers of miR-424 and CCND1 were shown as Table 1. Quantitative Real-time Polymerase Chain Reaction (qPCR) was performed to detect the expression level by following the instruction of SYBR qPCR mix kit (TAKATA, Japan). The comparative Ct ($\Delta\Delta C_t$) method was applied to calculate the relative gene expression.

Western Blot Analysis

Total protein was extracted after RF/6A cells were treated with RIPA Lysis Buffer (Beyotime, Haimen, China). The BCA protein assay kit

(KeyGEN Bitech, NanJing, China) was applied to quantify the amount of the extract according to the manufacturer's protocol. Equal amount of protein samples was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride transfer membranes. Then, the membrane was incubated with primary antibody: CCND1, β -actin (both from Abcam, Cambridge, UK), at 4°C overnight. After washed with TBST for 3 times, second antibody was applied and incubated for 1 hour at room temperature. The membrane was treated with ECL luminescence reagent (Thermo Fisher Scientific, Pittsburgh, PA, USA) and Protein signals were quantified using Quantity One software (Bio-Rad, Hercules, CA, USA).

Cell Group and Transfection

RF/6A cells were transfected with miR-424 mimic, miR-424 inhibitor or negative control of the two (all from KeyGEN Bitech, NanJing, China) in Cell Phenotype study. The cells were transfected with siRNA-CCND1, miR-424 inhibitor or negative control of the two (all from KeyGEN Bitech, NanJing, China) in Rescue experiment, shown as Table 2. The transfection processes were performed using Lipofectamine RNAiMAX reagent (Thermo Fisher Scientific, Pittsburgh, PA, USA) followed the manufacturer's protocol.

MTT Assay

The RF/6A cells were planted in 48-well plates with serum free RPMI1640 for 2h. Afterwards, the cells were incubated individually in NG or HG condition for 72h. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Beyotime, Haimen, China) reagent was added to each well before the end of incubation. Finally, the purple formazan crystals were dissolved in dimethyl sulfoxide (DMSO). The absorbance was recorded at 570 nm and calculated as optical density (OD).

Table 1: Primers used in real time quantitative PCR

Primers		Sequence (5'→3')
miR-424	Forward	5'- TGACAAAACGTGAGGCGC -3'
	Reverse	5'-GCAGGGTCCGAGGTATTC -3'
CCND1	Forward	5'-CACAGCTACTTGGTTTGTGTTCT -3'
	Reverse	5'-GCCTCGAAGTCCTGCTTACA -3'
GAPDH	Forward	5'-GCCCCCGGGTTTCTATAAATTG -3'
	Reverse	5'- TGCGGCTAACTCTCGAACAG -3'

Table 2: The group and treatment of RF/6A cells

Group	Treatment
NG	RF/6A cells cultured in normal glucose condition
HG	RF/6A cells cultured in high glucose condition
Cell phenotype experiment	
miR-424 mimic	RF/6A cells transfected with miR-424 mimic
miR-424 MC	RF/6A cells transfected with vehicles of miR-424 mimic as control
miR-424 inhibitor	RF/6A cells transfected with miR-424 inhibitor
miR-424 IC	RF/6A cells transfected with vehicles of miR-424 inhibitor as control
Rescue experiment	
miR-424 inhibitor	RF/6A cells transfected with miR-424 inhibitor
inhibitor -NC	RF/6A cells transfected with vehicles of miR-424 inhibitor as control
miR-424 inhibitor+ siRNA-CCND1	RF/6A cells co-transfected with miR-424 inhibitor and siRNA-CCND1
miR-424inhibitor+scrambled-siRNA	RF/6A cells co-transfected with miR-424 inhibitor and the vehicles of siRNA-CCND1 as control

Flow Cytometry Analysis

The RF/6A cells were fixed in 70% ethanol at 4 °C overnight. Then, the cells were treated with RNase-A (Thermo Fisher Scientific, Pittsburgh, PA, USA) and were stained with propidium iodide (PI; Sigma-Aldrich, St. Louis, MO, USA). Cell cycle analysis was performed by the FACSCanto II flow cytometer (BD Biosciences, USA).

Dual Luciferase Reporter Analysis

Potential target genes of miR-424 were predicted based on Bioinformatics analysis tools, such as miRDB (<http://mirdb.org/>), TargenScan (<http://www.targetscan.org/>), miRTarBase (<https://mirtarbase.cuhk.edu.cn/>).

As one of the hypothetical genes, CCND1 was confirmed with Luciferase reporter analysis. Briefly, RF/6A cells were co-transfected with the 3'-UTR constructs and either miR-424 mimic (all from KeyGEN Bitech, NanJing, China) or the negative control using Lipofectamine 3000 (Thermo Fisher Scientific, Pittsburgh, PA, USA), shown as Table 3. After 36h of incubation, the luciferase activities were measured using the Dual-Luciferase Assay kit (KeyGEN Bitech, NanJing, China) according to the manufacturer's instructions.

Statistical Analysis

All the experiments are replicated three times. The data was presented as the mean \pm standard

deviation (SD). The differences between variables were analyzed using *t*-test between two groups and one-way ANOVA among multiple groups. A value of $P < 0.05$ was considered to indicate statistically significant differences.

RESULTS

miR-424 overexpression induced RF/6A cells Dysfunction in HG

qPCR was conducted firstly to evaluate the expression of miR-424 in NG and HG. As the results shown, increased miR-424 was observed in HG compared with NG (Fig 1 A).

We transfected RF/6A cells in NG with miRNA mimics or miRNA inhibitors and subsequently cultured the cells in HG to examine the biological roles of miR-424 (Fig 1B). Cell viability was then assessed in each group. The viability of RF/6A cells in HG was much lower than in NG. It can, however, be reversed by treating RF/6A cells in NG with a miR-424 mimic or HG with a miR-424 inhibitor. After 72 hours of incubation, HG showed poorer RF/6A cell viability than NG, which could be reversed by transfecting RF/6A cells with miR-424 mimic in NG or miR-424 inhibitor in HG (Fig 1 C and D). These results suggest that HG induced RF/6A cell dysfunction via miR-424.

miR-424 inhibit Cell Cycle by target CCND1

In order to further investigate the mechanism, we used bioinformatic methods to identify the

Table 3: The groups and treatment of luciferase reporter analysis

Group	Treatment
miR-424 mimic + WT -CCND1	RF/6A cells co-transfected with miR-424 mimic and CCND1 3'UTR wild type
miR-NC + WT-CCND1	RF/6A cells co-transfected with CCND1 3'UTR wild type and vehicles of miR-424 mimic
miR-424 mimic + MUT-CCND1	RF/6A cells co-transfected with miR-424 mimic and CCND1 3'UTR Mutant
miR-NC + MUT-CCND1	RF/6A cells co-transfected with CCND1 3'UTR Mutant and vehicles of miR-424 mimic

potential miR-424 target genes. A total of 189 genes, including CCND1, co-exist in the three Database (Fig 2 A). Because of its significant role in cell proliferation, CCND1 was hypothesized to be a viable target gene for RF/6A cell dysfunction. The luciferase assay was used to confirm the hypothesis. The activity of CCND1-3'UTR luciferase was significantly reduced in the group transfected with miR-424 mimics, but not in the mutant CCND1-3'UTR (Fig 2 B and

C). RF/6A cells were also tested for CCND1 expression using qPCR and Western blot after being exposed to different glucose levels. The results showed that CCND1 expression was lower in HG at both transcription (Fig. 2 D) and translation levels as compared to NG (Fig 2 E and F). The cell cycle process of RF/6A cells was then studied using flow cytometry. The results revealed that HG had a much higher percentage of G0/G1-phase cells and a significantly lower

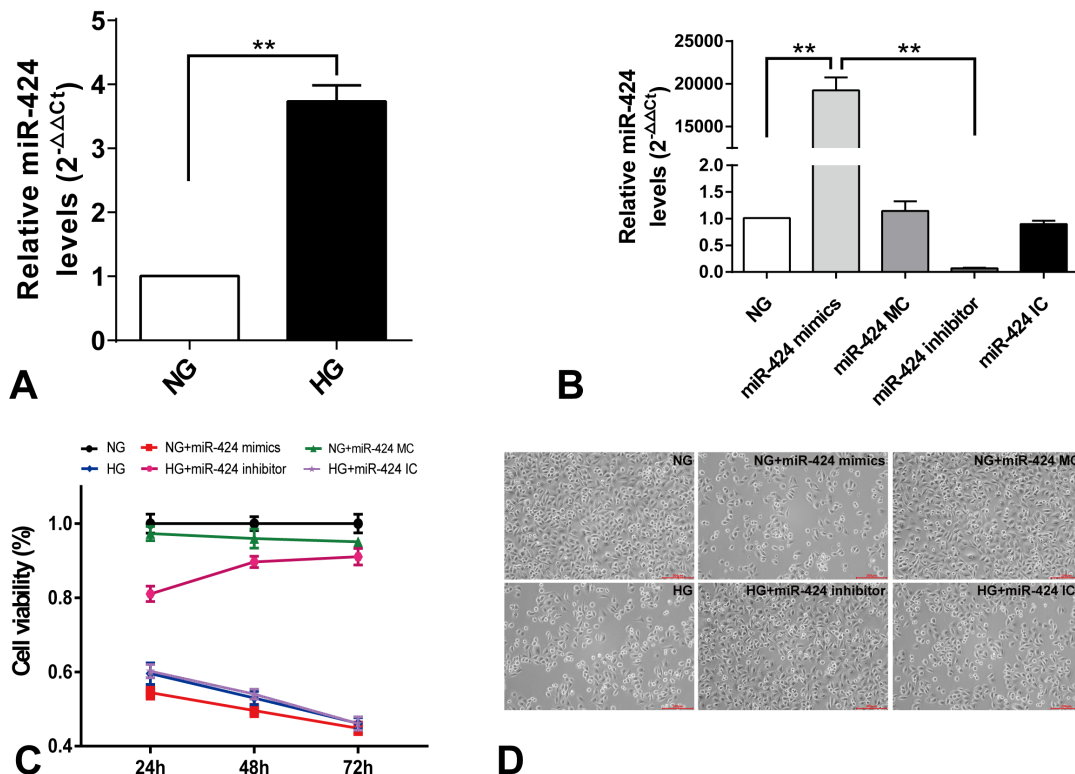


Fig. 1: miR-424 expression and its effect under different culture conditions. (A) The expression level of miR-424 in NG and HG; (B) miR-424 expression in response to transfection; (C) MTT assay in response to transfection; (D) Photograph of cells at 72h. Results were presented as mean ±SD (n = 3). *P < 0.05, **P < 0.01.

percentage of S-phase cells than NG (Fig 2 G, H and I).

To validate the mechanism by which miR-424 induced cell cycle arrest via CCND1, we transplanted RF/6A in HG with miR-424 inhibitor or siRNA-CCND1 (Fig 3 A and B). A

considerable increase in S phase and a decrease in G0/G1 phase percentage was seen in cells transplanted with miR-424 inhibitor. However, by blocking CCND1, the effect can be reversed (Fig 3 C-H).

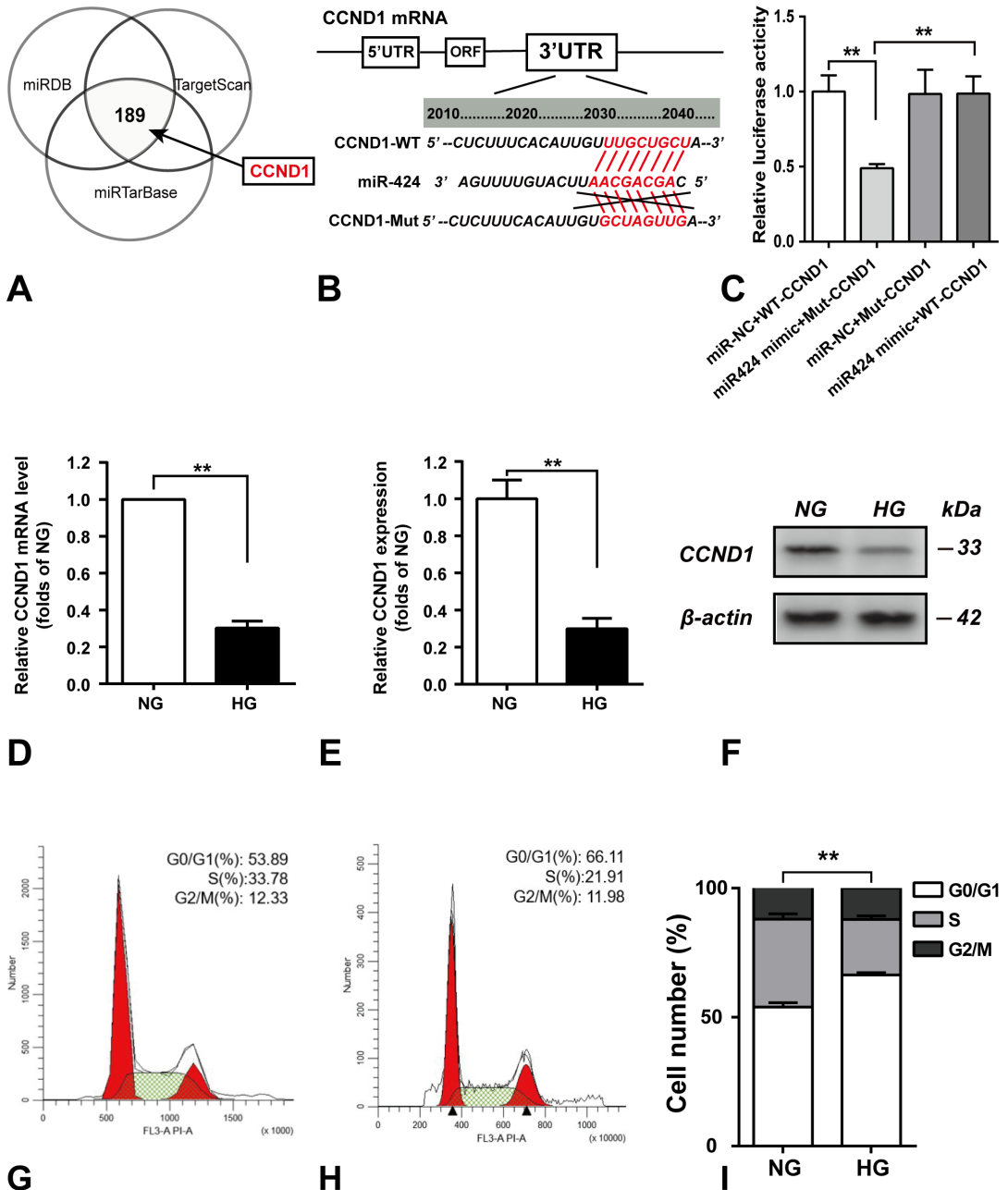


Fig. 2: Identification and confirmation of miR-424's target gene. (A) Bioinformatics assay predicts CCND1 as the potential target gene of miR-424; (B,C) Dual Luciferase Reporter Analysis confirmed the interaction between miR-424 and CCND1; (D-F) CCND1 was suppressed in both transcription and translation level under HG stress; (G) Cell cycle distribution of RF/6A cells in NG; (H) Cell cycle distribution of RF/6A cells in HG; (I) RF/6A cells in G1 stage increased under HG condition. Results were presented as mean \pm SD (n = 3). * $P < 0.05$, ** $P < 0.01$.

miR-424 inhibits RF/6A cells proliferation via CCND1

After miR-424 inhibitor or siRNA-CCND1 were transfected into HG cells, the proliferation of the transfected cells was evaluated. As demonstrated, miR-424 inhibitor transfection dramatically increased the vitality of RF/6A cells, however this effect can be reversed by CCND1 suppression (Fig 4 A and B).

DISCUSSION

miR-424 played a key role in various pathological vascular diseases. Yang²⁰ discovered that miR-424 inhibits ERK1/2 phosphorylation in bFGF/FGFR1 pathway, which in turn decreases cell proliferation, migration, and tube forming abilities. Additionally, Lee¹⁷ demonstrated that miR-424 is a mediator of LPS-induced

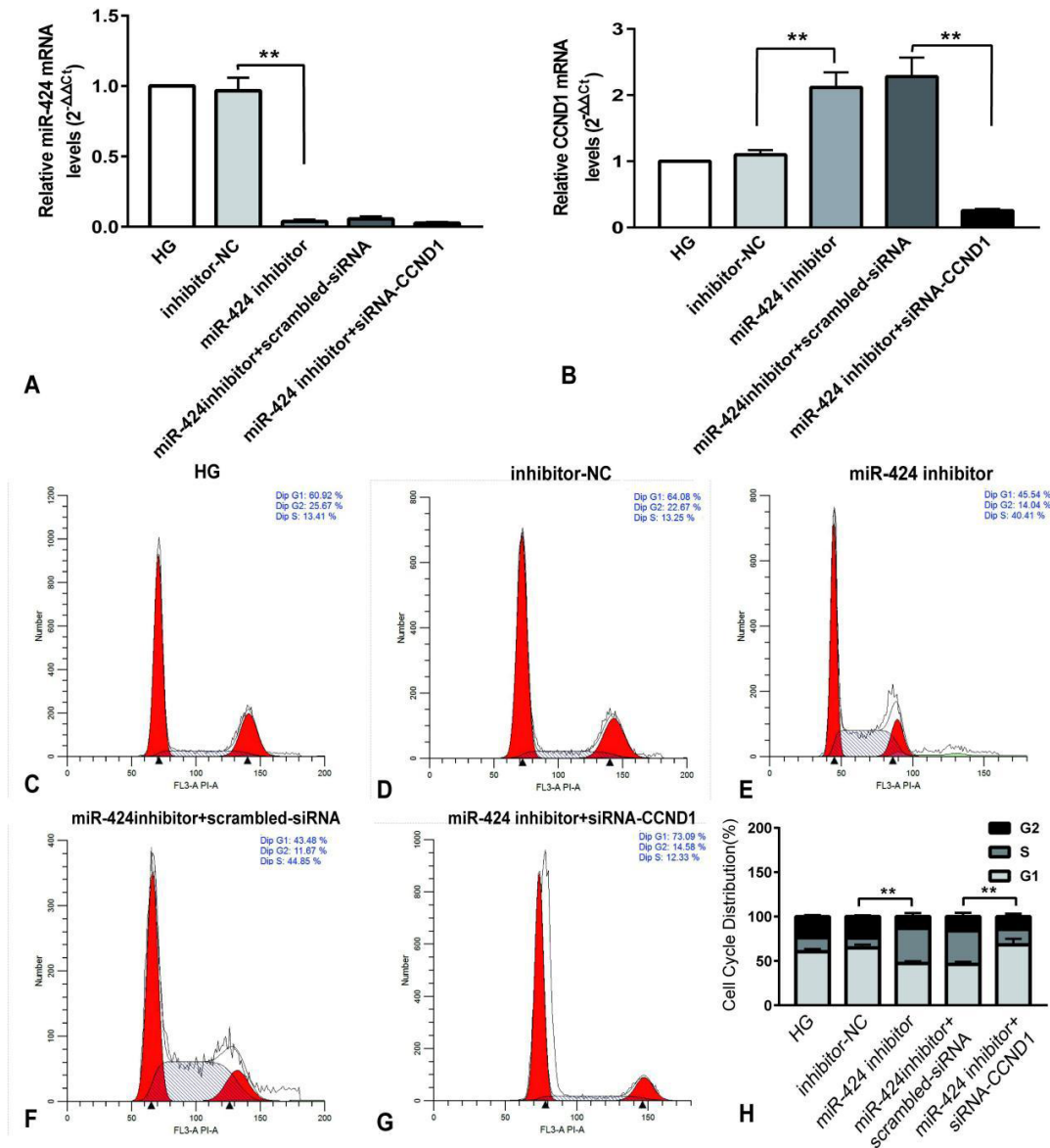


Fig. 3: The cell cycle of RF/6A cells after manipulating miR-424 and CCND1 expression. (A) miR-424 expression after cells transfection; (B) CCND1 expression after cells transfection; (C) Cell cycle of RF/6A cells in HG; (D) Cell cycle of RF/6A cells transfected with inhibitor-NC in HG; (E) Cell cycle of RF/6A cells transfected with miR-424 inhibitor; (F) Cell cycle of RF/6A cells transfected with miR-424 inhibitor + scrambled -siRNA in HG; (G) Cell cycle distribution of RF/6A cells transfected with miR-424 inhibitor + siRNA-CCND1 in HG; (H) Cell cycle of RF/6A cells with or without transfection. Results were presented as mean ±SD (n = 3). *P < 0.05, **P < 0.01.

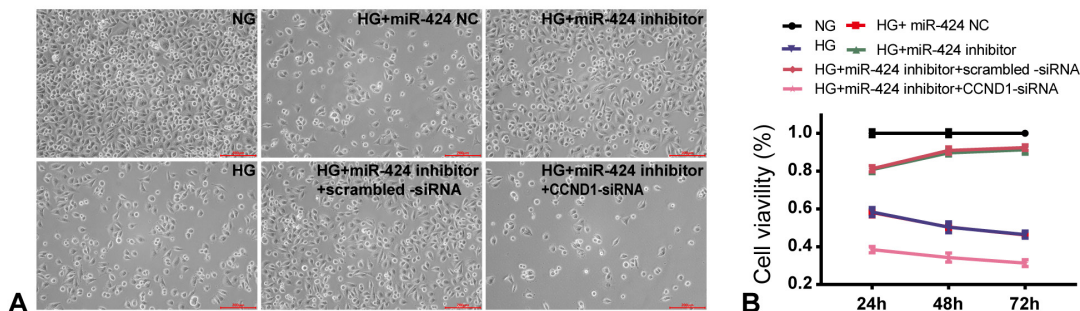


Fig. 4: The cells viability of RF/6A cells after manipulate miR-424 and CCND1 expression. (A) MTT assay in response to transfection; (B) Photograph of cells at 72h. Results were presented as mean \pm SD (n = 3). * P < 0.05, ** P < 0.01.

endothelial cells' (EC) sprouting, migration, and tube formation in inflammation-induced angiogenesis. As one of the most common DM complications, DR is characterized by microvascular dysfunction, which can lead to BRB and retinal ischemia. However, little is known about the connection between miR-424 and DR.

In this study, we discovered that miR-424 was up-regulated in retinal vascular endothelial cells, which resulted in retinal vascular dysfunction in HG. Under HG stress, RF/6A cells proliferate less than they do in NG, and by switching the expression of miR-424, the function of the EC can be changed in both groups. This finding demonstrated that HG stress increased the expression of miR-424 in RF/6A cells, which in turn caused vascular dysfunction. CCND1 is essential for cell cycle progression during the G1 phase, which functions as regulators of CDK kinases and contributes to the temporal coordination of each mitotic event. Cells can be arrested in G0/G1 when CCND1 is inhibited.²³⁻²⁶ The molecule was indicated as a predictor of retinal microvascular endothelial cells.²⁷ And it was demonstrated to be mediated with insulin-like growth factor (IGF)-1 induced retinal endothelial dysfunction.²⁸ In the present research, we demonstrated that miR-424 targets 3'UTR of CCND1 mRNA, resulting in a repress on both transcription and translation level. Furthermore, we performed rescue experiment and observed changes of cell proliferation according with variation of the ratio of RF/6A cells in G0/G1 phase. These findings indicate miR-424 negatively regulates the expression of CCND1 under high glucose stress, leading cell cycle to halt in G1 phase, subsequently leads to vascular endothelial dysfunction.

This study has several limitations. As it is a

preliminary study, the animal experiment into the role of miR-424 in DR was not performed and the expression of the molecule in clinical tissue samples with DR was not determined.

CONCLUSION

In conclusion, the aberrantly up-regulation of miR-424 in RF/6A cells under high glucose stress can significantly inhibit CCND1 expression and halts cell cycle, leading to vascular endothelial dysfunction. Based on these results, we speculated miR-424 may be a potential therapeutic target for DR. This assumption requires more researches to validate and further clinical trials to test.

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Authors' contribution: YC and ZH conceived and designed the project. KC and WZ acquired the data. KC, WZ and JC analyzed and interpreted the data. YC wrote the paper. All authors read and approved the manuscript.

Conflict of interest: The authors declare no conflict of interest.

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