miR-424 up-regulation inhibiting RF/6A cells function under high glucose condition via CCND1

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Research Article

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Abstract

**Backgrounds:** Retinal vascular dysfunction is an important factor to the progression of diabetic retinopathy (DR). Multiple abnormal microRNAs (miRNAs) contributes to the pathogenesis of vascular dysfunction. However, the role and underlying mechanism of miR-424 in retinal vascular endothelial cells dysfunction under hyperglycemia stress remain obscure.

**Methods:** Rhesus macaque choroid retinal endothelial cell line (RF/6A) cells were cultured under normal glucose (NG) and high glucose (HG) condition. qPCR was used to quantify the mRNA expression of miR-424 and Cyclin D1 (CCND1) and western blot was applied to detect the protein amount of CCND1. RF/6A cells were transfected with miR-424 mimics, miR-424 inhibitor, miR-424 inhibitor+ siRNA-CCND1 or with vehicle molecules. The cell proliferation, wound healing, tube formation ability and cell cycle were evaluated by MTT assay, scratch wound healing assay, tube formation assay and flow cytometric analysis, respectively. Interaction between miR-424 and CCND1 was predicted with bioinformatics and confirmed by Dual Luciferase Reporter Analysis.

**Results:** Compared with NG, miR-424 was up-regulated and cell phenotype such as proliferation, wound healing and tube formation were inhibited in HG. The phenotypes can be reversed by inverting miR-424 expression under different conditions. CCND1 was confirmed as one of target genes of miR-424 and it can be modulated at transcriptional or translation level. Manipulation of silencing CCND1 can reverse the influences, such as promotion in proliferation, scratch wound tube formation and cell cycle, induced by transfecting miR-424 inhibitor into RF/6A cells under HG.

**Conclusions:** Overexpression of miR-424 in RF/6A cells under HG stress significantly inhibited the cell function such as cell proliferation, wound healing, tube formation through suppressing CCND1 and blocking cell cycle.

Background

Diabetic retinopathy is a major sight-threatening complication of Diabetes Mellitus [1, 2] (DM). The development of diabetic retinopathy (DR) is divided into multiple phases. As one of the DR characters, blood-retinal barrier breakdown (BRB) may occur at DR's early stage [3], if not proper treated it may lead to increased vascular permeability and even macular edema [4, 5], which is one of the biggest cause of vision impairment in DR [6, 7]. The impairment of vascular endothelial cells due to hyperglycemia was associated with the pathological process of BRB [6, 8]. Although current therapies for BRB, such as intravitreal anti-vascular endothelium growth factor (VEGF) or retinal photocoagulation are effective to many victims, but the effect is somewhat unsatisfactory and it is not always work [9–11]. Therefore, the search for the mechanism of vascular endothelial dysfunction is of primary clinical importance to provide interventional modality to the BRB breakdown and vascular leakage during early DR.

MicroRNAs (miRNAs) are highly conserved and short endogenous non-coding RNAs, which suppress protein-coding gene expression by inducing mRNA degradation or translation inhibition [12, 13]. It is
supposed to play a significant role in pathological angiogenesis processes of DR [14–16]. As previously researches shown that miR−424 poses a close and crucial relationship with vascular function. Some studies reported that miR−424 promoted neovascularization in angiogenesis related diseases, for example, inflammation related angiogenesis [17], multiple tumors as hemangioma [18–20], osteosarcoma [21] and glioma [22]. However, the role and regulative mechanism of miR−424 on pathological microvascular injury of DR remains obscure.

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 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 strategy for DR treatment.

**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; 11mmol/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 SYRB qPCR mix kit (TAKATA, Japan). The comparative Ct(△△Ct) method was applied to calculate the relative gene expression.
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’-CACAGCTACTTGGTTGTTGTTCT-3’ |
|         | Reverse 5’-GCCTCGAAGTCTGCTTACA-3’ |
| GAPDH   | Forward 5’-GCCCCCGGGTTTCTATAAATTG-3’ |
|         | Reverse 5’-TGCGGCTAACTCTCGAACAG-3’ |

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 manufacture’s protocol. Equal amount of protein samples was separated by sodium dodecyl sulfate-polyacrylamidegel electrophoresis and transferred onto polyvinylidenedifluoride transfer membranes. Then, the membrane was incubated with primary antibody: CCND1, β-actin (all 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.
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−424 inhibitor + scrambled-siRNA | RF/6A cells co-transfected with miR−424 inhibitor and the vehicles of siRNA-CCND1 as control |

**MTT Assay**

The RF/6A cells, at the density of 1×10⁴ cells/well, 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).

**Wound Healing Assay**

RF/6A cells migration was assessed by scratch wound healing assay. Briefly, each group was added with 1mL mitomycin after 72h incubation. Then, the confluent monolayer was scratched with a 200μL yellow micropipette tip. Followed by further 72h incubation, the pictures were captured at 0h, 72h. The quantitative data of wound healing rate (%) was analyzed by Image J.

**Tube Formation Assay**

The 96-well plate was coated with 50μL Matrigel Matrix (BD Bioscience, USA) and placed at 37 °C for 30 min. The RF/6A cells were seeded into the Matrigel Matrix at the density of 2×10⁵ cells/mL. Followed by further 6h incubation, the pictures were captured and the tube formation was analyzed by Image J.
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 (http://mirdb.org/, http://www.targetscan.org/, http://mirtarbase.mbc.nctu.edu.tw/php/index.php).

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 instruction.

| 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 |

Statistical Analysis

All the experiments are replicated three times. The data was presented as the mean ± 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, suggesting HG induced miR−424 overexpression in RF/6A cells (Fig. 1A).
To explore the biological roles of miR−424, we transfected miRNA mimics and inhibitor into RF/6A cells (Fig. 1B). Afterwards, cell function tests, including Wound Healing Assay, MTT Assay and Tube Formation Assay, were applied to each group. For Wound Healing Assay, the migration ability of RF/6A cells was significantly impaired in HG in contrast to NG. However, the ability can be reversed by the treatment of miR−424 mimic to RF/6A cells in NG or miR−424 inhibitor in HG, (Fig. 1C and D). After 72h incubation, decreased RF/6A cell viability was observed in HG compared with NG, and it can be reversed by transfecting RF/6A cells with miR−424 mimic in NG or miR−424 inhibitor in HG (Fig. 1E and F). Moreover, Tube Formation Assay showed tube formation of RF/6A cells was inhibited in HG in contrast with NG. The transfection of miR−424 inhibitor to RF/6A in HG can regain the ability of tube formation while transfection of miR−424 mimic in NG can lead to decreasing ability (Fig. 1G and H). These results suggest that HG induced RF/6A cells dysfunction through miR−424.

**miR−424 inhibit Cell Cycle by target CCND1**

To further investigate the mechanism, we predicted the potential target genes of miR−424 by Bioinformatics. A total of 189 genes, including CCND1, co-exist in the three Database (Fig. 2A). Because of its significant role in angiogenesis, CCND1, as a putative target gene, was supposed to be involved in RF/6A cells dysfunction. Luciferase assay was performed to identify the hypothesis. CCND1−3’UTR luciferase activity was significantly repressed in the group transfected with miR−424 mimic. This repression, however, was not seen with mutated CCND1−3’UTR (Fig. 2B and C). These results confirmed binding of miR−424 with 3’UTR of CCND1 mRNA. Moreover, qPCR and Western blot were performed to test the expression of CCND1 in RF/6A cells incubated in different glucose conditions. The results revealed a reduced expression of CCND1 in HG compared with NG at the transcription (Fig. 2D) and translation level (Fig. 2E and F). Subsequently, cell cycle progression of RF/6A cells was evaluated by flow cytometry analysis. In contrast to cells in NG, there was a significantly increased percentage at G0/G1 phase and decreased at S phase in HG, as the results shown (Fig. 2G, H and I).

To validate the mechanism that miR−424 suppresses cell cycle through CCND1, we transferred RF/6A with miR−424 inhibitor or siRNA-CCND1 in HG (Fig. 3A and B). Significant decrease percent in G0/G1 phase and increase in S phase was observed in cells transferred with miR−424 inhibitor, however, the effect can be reversed after blocking CCND1 (Fig. 3C-H).

**miR−424 induced RF/6A cells dysfunction via CCND1**

We further identify the mechanism that miR−424 inhibit RF/6A cells through CCND1. Cells in HG were transfected with miR−424 inhibitor or siRNA-CCND1, then the cell functions were observed. For wound healing assay, RF/6A cells migration was significantly up-regulated after miR−424 inhibitor transfected, but it can be reversed by the suppression of CCND1 (Fig. 4A and B). In addition, the transfection of siRNA-CCND1 can reverse the positive effect of cell viability (Fig. 4C and D) caused by miR−424 inhibitor. Moreover, the function of tube formation was reversed by siRNA-CCND1 towards the effect of miR−424 inhibitor (Fig. 4E and F).
Discussion

miR−424 was crucial to pathological vascular related disease. In the study of infantile skin hemangioma, Yang [20] found that miR−424 could suppress the bFGF/FGFR1 pathway, thereby inhibit ERK1/2 phosphorylation, and thus inhibit cell proliferation, migration and tube formation capabilities and the development of infantile skin hemangioma. Furthermore, Lee [17] reported that miR−424 is essential for regulation of inflammation mediated angiogenesis by reducing LPS induced endothelial cells(EC) sprouting, migration and tube formation. As DR is one of the most common DM complications, it is manifested with the microvascular impairment, which can lead to BRB and retinal ischemia, thus retinal angiogenesis. However, the relationship between miR−424 and DR remains poorly understood, and further investigation is required.

In this work, we identified miR−424 was up-regulated in retinal vascular endothelial cells and subsequently led to retinal vascular dysfunction in HG. The ability of proliferation, wound healing and tube formation of RF/6A cells is suppressed under HG stress compared with NG, and the EC function can be reversed in the two groups by reversing miR−424 expression. This result indicated that HG stress resulted in vascular dysfunction through elevating miR−424 expression in RF/6A cells. To further study the involved molecular mechanism, we applied bioinformatic methods to explore the target genes of miR−424, and 189 genes including CCND1 were gained. As a potential target of miR−424, CCND1 encodes protein called Cyclin D1, which is crucial in the cell cycle promotion during G1 phase. Inhibition of CCND1 causes cells to be arrested at G0/G1 phase [23–26]. CCND1 is a key factor to cell biological process. Previous studies have shown the significant role of CCND1 in pathology of DR. Devi's research [27] demonstrated cyclin D1 mediated insulin-like growth factor (IGF)−1 induced retinal endothelial dysfunction. Tang's study [28] suggests cyclin D1 might be involved in the pathological process of DR. 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 to confirm the regulation of miR−424 upon vascular endothelium function through CCND1. The results exhibited a decrease ratio of RF/6A cells in G0/G1 phase after transfected with miR−424 inhibitor, and it can be reversed by supplemental inhibition of CCND1. In addition, the biological function of vascular endothelium cells such as proliferation, wound healing and tube formation can be reversed according to the manipulation of miR−424 and CCND1 in RF/6A cells. 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. Suppression of RF/6A cells proliferation by miR−424 can be largely account for cell cycle arrest induced by CCND1 inhibition based on previously studies proved [29, 30]. Also, our reverse assay displayed that CCND1 not only contribute to the cell proliferation but also to wound healing and tube formation. The mechanism for the phenomenon maybe that cell proliferation posed a positive interaction with cell migration and tube formation, which need to be further investigated.

This study has several limitations. Because of the interaction of miRNA and its target genes as well as their regulatory roles is very complicated, we can't exclude the possibility that other target genes of
miR−424 are involved in the pathology of DR. Also, the expression of CCND1 can also be regulated by other miRNAs. Therefore, further studies are needed to elucidate the underlying multiple interaction mechanism involved in DR.

Conclusions

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.

Abbreviations

DR: diabetic retinopathy; miRNAs: microRNAs; NG: normal glucose; HG: high glucose; RF/6A: Rhesus macaque choroid retinal endothelial cell line; CCND1: Cyclin D1; DM: Diabetes Mellitus; BRB: blood-retinal barrier breakdown; VEGF: vascular endothelium growth factor; RPMI: Roswell Park Memorial Institute; DMSO: dimethyl sulfoxide; OD: optical density; MTT: 3,4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide; SD: standard deviation; EC: endothelial cells

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All the data used to support the findings of this study are included within the article and are available from corresponding author by a reasonable request.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

All the authors listed participated in the manuscript and have read and approved the final submission. CY, WMQ and HZR designed research. CY, CHY performed research. CY, WMQ and HZR analyzed data. CY wrote the paper.
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**Figures**
Figure 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,D) Scratch wound healing assay in respond to transfection; (E,F) MTT assay in response to transfection; (G,H) Tube formation assay in response to transfection. Results were presented as mean ±SD (n = 3). *P < 0.05, **P < 0.01.
Figure 2

Identification and validation of CCND1 as the direct target of miR-424. (A) Bioinformatics assay predict 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, the grouping of blots cropped from different gel and full-length gels and blots are presented in Supplementary Figure 1; (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 phase increased under HG condition. Results were presented as mean ±SD (n = 3). *P < 0.05, **P < 0.01.

Figure 3

miR-424 inhibit cell cycle through CCND1. (A) miR-424 expression in respond to transfection; (B) CCND1 expression in respond to transfection; (C) Cell cycle distribution of RF/6A cells in HG as blank control; (D) Cell cycle distribution of RF/6A cells transfected with inhibitor-NC in HG as negative control; (E) Cell
cycle distribution of RF/6A cells transfected with miR-424 inhibitor; (F) Cell cycle distribution of RF/6A cells transfected with miR-424 inhibitor + scrambled -siRNA in HG as negative control; (G) Cell cycle distribution of RF/6A cells transfected with miR-424 inhibitor + siRNA-CCND1 in HG; (H) Cell cycle distribution of RF/6A cells with or without transfection. Results were presented as mean ±SD (n = 3). *P < 0.05, **P < 0.01.
miR-424 induced vascular endothelial dysfunction through CCND1. (A,B) Scratch wound healing assay in response to transfection; (C,D) MTT assay in response to transfection; (E,F) Tube formation assay in response to transfection. Results were presented as mean ±SD (n = 3). *P < 0.05, **P < 0.01.

**Supplementary Files**

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