

Original Article

Hsa-miR-155 regulates the cell cycle and barrier function of corneal endothelial cells through E2F2

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Abstract: This study was aimed to determine the role of has-miR-155 and E2F2 on corneal endothelial cells. Real-time quantitative PCR and Western blot assays were carried out to determine the levels of has-miR-155 and E2F2, and Flow cytometry assay was conducted to detect cell cycle. In addition, Targetscan7.2 was adopted to analyze the internal connection between hsa-miR-155 and E2F2, and a dual luciferase reporter gene assay to determine predicted site between has-miR-155 and E2F2. Increased hsa-miR-155 resulted in decreased E2F2, while decreased hsa-miR-155 increased the level of E2F2. In addition, both increased hsa-miR-155 and decreased E2F2 led to an increase in S-phase cells and a decrease in G1-phase cells. Also, they induced an increase in the activity of barrier-related proteins MLCK and ZO-1, an up-regulation of Cyclin D1 and Cyclin E1, and a down-regulation of apoptosis proteins (Caspase 3/Bax/Bim/Bid) whereas decreased hsa-miR-155 led to an opposite change in cells, and decreased E2F2 could offset cell changes caused by increased has-miR-155. In conclusion, Has-miR-155 regulates the cell cycle of corneal endothelial cells and improves their barrier function by down regulating E2F2.

Keywords: Corneal endothelial cell, hsa-miR-155, E2F2, cell cycle, barrier function

Introduction

After the optical power of the cornea or lens loses balance with the axial length of eyes, images of distant objects are not able to focus on the plane of the retinal photoreceptor, which is called myopia [1]. Its development and progression are related to heredity, individual behaviors, social environment, and other factors [2]. In recent years, the disease has become one of the diseases be of concern because its increasing global incidence [3]. Low myopia can be corrected by glasses and other means, but the correction and improvement of high myopia pose great challenges. Patients with high myopia may be accompanied by pathological damages such as retinal detachment, distorted eyeballs, and refraction [1], whose therapeutic strategies are still in the fog. Therefore, myopia, especially high myopia, has become a major international public health problem [4, 5]. Cornea is an important tissue for forming visual images and maintaining ocular transparency [6]. Corneal endothelial cells (CECs) and tissues located on one side of ante-

rior chamber can prevent water from flowing from anterior chamber to corneal stroma [7], so the abnormal barrier function or other abnormal biological characteristics of the cells damages the corneal tissue and visual acuity. In terms of the barrier function of CECs, phosphorylated STAT3 can be expressed in CECs, and it regulates the cell barrier function by positively regulating the barrier function marker ZO-1 [7]. In addition, MLCK induces the phosphorylation of MLC, which causes an increase in the contractility of cytoskeleton of CECs, and finally destroys the integrity of barrier function [8]. Therefore, studying changes in CECs in cases with myopia may promote the development of treatment strategy for high myopia.

Genes are a risk factor for myopia [9], and gene regulation methods (including DNA methylation and microRNA regulation) are closely related to the development and progression of the disease [10-12]. CECs are regulated by microRNAs [13], so exploring microRNAs related to CEC abnormalities contributes to discovering the possible molecular mechanism of myopia and

treating it. Hsa-miR-155 is a microRNA, approximately 65 bp long and located on human chromosome 21. It is associated with the development and progression of atherosclerosis, rheumatic heart disease, and pneumonia Chlamydia infection [14-18]. It improves CEC permeability and then promotes corneal epithelial recovery in corneal epithelial injury [6]. As a regulatory factor involved in a variety of diseases and related to the cycle regulation of various cells [19-24], transcription factor E2F2 promotes non-proliferative CECs to proliferate by regulating cell cycle [25, 26].

In this paper, we found that serum hsa-miR-155 increased but E2F2 decreased in cases with myopia when comparing the serum samples between myopic patients and healthy individuals. The phenomenon was similar when comparing the corneal tissue between normal and myopic rats. Therefore, we speculated that miR-155 and E2F2 may be related to myopia. However, the correlations of the two with the disease, especially with CECs, are not yet clear, so this study was carried out to discover a possible mechanism of the development of myopia.

Methods

Myopic patients

One hundred and sixty-two volunteers were recruited by Tianjin Eye Hospital for this study. According to diopter, visual acuity, axial length, slit lamp, dilated fundus, and other examinations, the myopia severity of the participants was determined. Those suffering from eye diseases or other hereditary diseases were excluded. Finally, 97 myopic patients and 65 healthy individuals undergoing physical examinations were enrolled in this study. Serum samples were collected from the participants and stored at -80°C for testing. All participants signed the informed consent form. This study was approved by the Hospital Ethics Committee and was in line with the *Declaration of Helsinki*.

Animals

Sprague Dawley (SD) rats (0.25~0.35 kg) were purchased from Hunan Slack Jingda Experimental Animal Co., Ltd. and confirmed to be without abnormal behaviors and manifestations. They were divided into a myopia group

and a control group based on standards from the Association for Research in Vision and Ophthalmology (ARVO). Rats were fed in a sterile culture room with constant temperature & humidity for 3 h, during which they were allowed to drink and eat freely.

Rats in the myopia group were induced for myopia modeling by referring to one study by Guo and et al. [10]. The experiments were finished according to the protocols and the National Institutes of Health guidelines. This study was approved by the Hospital Ethics Committee of Tianjin Eye Hospital, and carried out in accordance with the ethical plan approved by the Committee. Rat corneal tissues were extracted after myopia induction. In short, during this process, the rats were intraperitoneally injected with 150 mg/kg pentobarbital sodium to make them respiratory and cardiac arrest. Then, corneal tissues were extracted from them after euthanasia. All experiments with animals were carried out at SPF animal laboratory of Hangzhou Hebei Technology Co., Ltd. (ethical number: YYLL-2019-63).

Cell culture and transfection

CECs purchased from ATCC were cultured in DMEM (Gibco, USA) in an animal incubator (37°C, 5% CO₂) until they grew well. Cell transfection: The cells were transfected using a Lipofectamine 3000 kit (Invitrogen, USA). The plasmids (E2F2 siRNA/miR-155-inhibitor/miR-155-mimics/NC siRNA/NC-mimics/NC-inhibitor) were purchased from Tiangen Co., Ltd. (Beijing, China).

Flow cytometry

The cells were fixed in 70% ethanol (0°C, 30 min). After the ethanol solution removed, the cell granulations were incubated in Annexin V-FITC/PE solution. The FACScan flow cytometer (Becton Dickinson, USA) was used to analyze the apoptosis. Operating steps of cell cycle were similar to those of the apoptosis. However, propidium iodide/RNase/0.1% Triton X-100 solution was used for staining after cell fixation at 25°C for 30 min.

qPCR

miRNeasy Serum/Plasma Kit (QIAGEN, Germany) was adopted for extracting RNA from the

serum, while Trizol reagent was adopted for the extraction from rat corneal tissues and CECs. The FastKing One-Step RT-qPCR kit (Tiangen, Beijing, China) and ABI PRISM 7000 instrument (Applied Biosystems, USA) were used for the reverse transcription and amplification of total RNA samples. Primers for hsa-miR-155 and E2F2 mRNA were designed and synthesized by Tiangen Co., Ltd (Beijing, China). U6 and GAPDH were used as the control of gene expression. The relative expression was normalized by $2^{-\Delta\Delta Ct}$. The upstream primer of hsa-miR-155 (5'-3'): GCGGTTAATGCTAATCGTGAT. The downstream primer of hsa-miR-155 (5'-3'): GTGCAG-GGTCCGAGGT. The upstream primer of E2F2 mRNA (5'-3'): GAACGAGTCCCTCTTCACTTTC. The downstream primer of hsa-miR-155 (5'-3'): GTTCCGATCTGCTGCATATAACT.

Western blot

RIPA lysis buffer was used to lyse the cells. After the lysis buffer was centrifuged (4°C, $1.6 \times 10^4 \times g$) for 20 min, the BCA protein assay kit (Thermo Fisher) was used to determine the concentration of protein in supernatant. After being separated with SDS-PAGE electrophoresis with a loading quantity of 20 mg sample, the protein was transferred to PVDF membrane (EMD Millipore) to blot, and 5% skimmed milk-PBS buffer was used to block the membrane at 4°C for overnight). Subsequently, the antibody of protein to be detected and β -actin were added onto PVDF membrane for standing overnight at 4°C. And then goat anti-rabbit antibody was added onto the membrane for standing at room temperature for 1 hour. Finally, ECL luminescent solution was used for visualizing the blots. The antibodies of proteins to be tested included anti-Caspase 3 antibody (1:5000), anti-cleaved Caspase 3 antibody (1:500), anti-Cyclin D1 antibody (1:200), anti-Cyclin E1 antibody (1:2000), anti-MLC antibody (1:10000), anti-MLC phosphorylation antibody (1:500), anti-MLCK antibody (1:1000), STAT3 (1:2000), anti-STAT3 phosphorylation antibody (1:10000), anti-ZO-1 antibody (1:1000) and anti-E2F2 antibody (1:10000). All antibodies above were acquired from Abcam (USA).

Dual luciferase reporter gene assay (DLRGA)

Targetscan7.2 was used to analyze the sites between hsa-miR-155 and E2F2. The cells were seeded into a 12-well plate. According to

the predicted sites, E2F2 mutation site was designed, and E2F2-wt (without mutation site) and E2F2-mut (with mutation site) were constructed, with a negative control group being set up. The above three were co-transfected with hsa-miR-155-inhibitor and hsa-miR-155-mimics into the cells, respectively. The dual luciferase reporter gene assay system (Promega, USA) was used to determine the luciferase intensity of cells.

Statistical analysis

The experiment was repeated 3 times, and their results were showed as the Mean \pm SD. SPSS 21.0 (IBM, USA) was used for the analysis of data difference. The independent samples t test and one-way analysis of variance were applied for comparison of difference between groups, and dunnett's multiple comparisons test was used for post hoc pairwise comparison. The difference was significant in statistics at 95% confidential interval when $P < 0.05$.

Results

The expression of hsa-miR-155 and E2F2 in myopic groups

In this paper, serum samples were collected from 97 patients with high myopia and 65 healthy individuals, in which the expression of hsa-miR-155 and E2F2 mRNA was detected by qPCR. **Figure 1A** and **1B** showed that hsa-miR-155 rose but E2F2 mRNA declined in the patients. We also compared the corneal tissues between the normal and highly myopic rats. **Figure 1C** and **1D** showed that hsa-miR-155 rose but E2F2 mRNA declined in highly myopic rats. These findings suggest that both increasing hsa-miR-155 and reducing E2F2 may be related to myopia.

E2F2 as the downstream target of hsa-miR-155

Figure 2A showed a sequence site capable of binding to hsa-miR-155 at 1540-1547 of E2F2 3'UTR. Based on this predicted site, E2F2-wt and E2F2-mut were constructed. **Figure 2B** and **2C** showed that after E2F2-wt was co-transfected with hsa-miR-155-inhibitor and hsa-miR-155-mimics, respectively, its luciferase intensity tended to rise and reduce correspondingly. This suggests that hsa-miR-155 can bind to

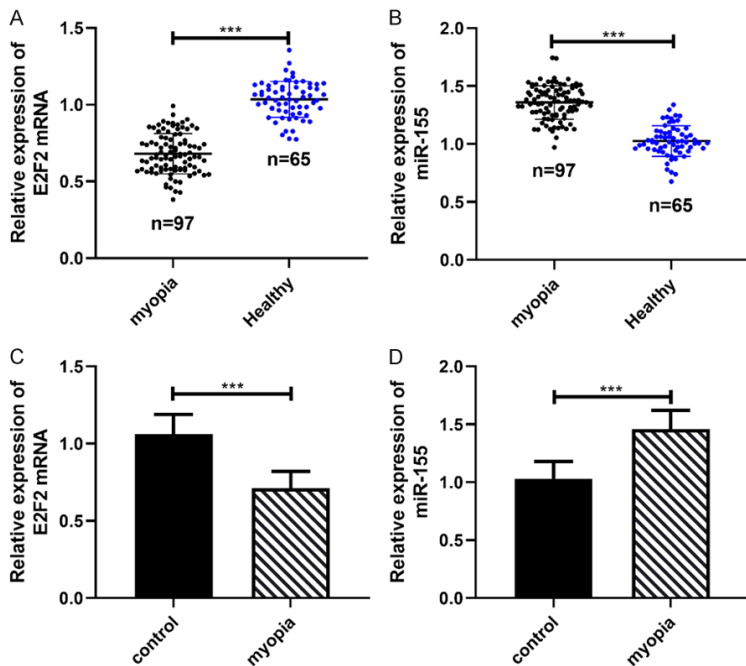


Figure 1. miR-155 and E2F2 expression in myopic groups. *** indicates $P < 0.001$. A and B: E2F2 mRNA and miR-155 expression in the serum samples collected from 97 patients with high myopia and 65 healthy individuals. C and D: E2F2 mRNA and miR-155 expression in the corneal tissues collected from normal and highly myopic rats, $n = 10$ in each group.

E2F2 through the predicted site and negatively regulate its expression. **Figure 2D** and **2E** showed that the upregulation of hsa-miR-155 inhibited E2F2 mRNA, while its downregulation increased this mRNA. These findings suggest that hsa-miR-155 can specifically inhibit E2F2.

Effects of hsa-miR-155 on barrier function and cell cycle

Figure 3A showed that the upregulation of hsa-miR-155 increased the apoptotic rate, while its downregulation reduced the rate. **Figure 3B-E** and **3H** showed that the downregulation of hsa-miR-155 reduced Caspase-3, cleaved Caspase-3 (c-Caspase-3), Bax, MLCK, and p-MLC, but increased Bcl-2, Cyclin D1, Cyclin E1, ZO-1, and p-STAT3; its upregulation had an opposite effect. **Figure 3F** showed that the upregulation of hsa-miR-155 increased G1 phase cells and reduced S1 phase cells, while its downregulation had an opposite effect. These findings suggest that hsa-miR-155 can damage the barrier function of CECs, inhibit their transition from G1 phase to S phase, and promote their apoptosis. **Figure 3G** showed the morphology of corneal endothelia cells via optical microscope.

Effects of E2F2 on barrier function and cell cycle

Figure 4A showed that the downregulation of E2F2 increased the apoptotic rate. **Figure 4B-E** and **4H** showed that the downregulation of E2F2 increased Caspase-3, c-Caspase-3, Bax, MLCK, and p-MLC, but reduced Bcl-2, Cyclin D1, Cyclin E1, ZO-1, and p-STAT3. **Figure 4F** showed that the downregulation of E2F2 increased G1 phase cells and reduced S1 phase cells. These findings suggest that E2F2 can contribute to the barrier function of CECs, and promote their transition from G1 phase to S phase. **Figure 4G** showed the morphology of corneal endothelia cells via optical microscope.

Rescue experiment

CECs were divided into a NC group, a hsa-miR-155-inhibitor group (transfected with hsa-miR-155-inhibitor), and a hsa-miR-155-inhibitor+E2F2 si group (transfected with hsa-miR-155-inhibitor+E2F2 si). **Figure 5** showed that the downregulation of E2F2 could offset the changes caused by the downregulation of hsa-miR-155.

Discussion

As a serious public health problem, myopia shows an annually increasing incidence [12], and it may occur in both children and adults [11]. High myopia is the second common cause of blindness, possibly giving rise to many complications [27]. The proliferation of CECs is limited, and the cell density decreases with the increase of human age [25, 26]. In addition, the barrier function of the cells is important for maintaining visual acuity, but any abnormality of the barrier-related proteins may damage the function [6, 7]. MicroRNAs can bind to mRNAs of downstream target genes and then regulate the gene expression at post-transcriptional level. They probably manage cell proliferation and barrier function through this regulatory mechanism in CECs, thus participating in the

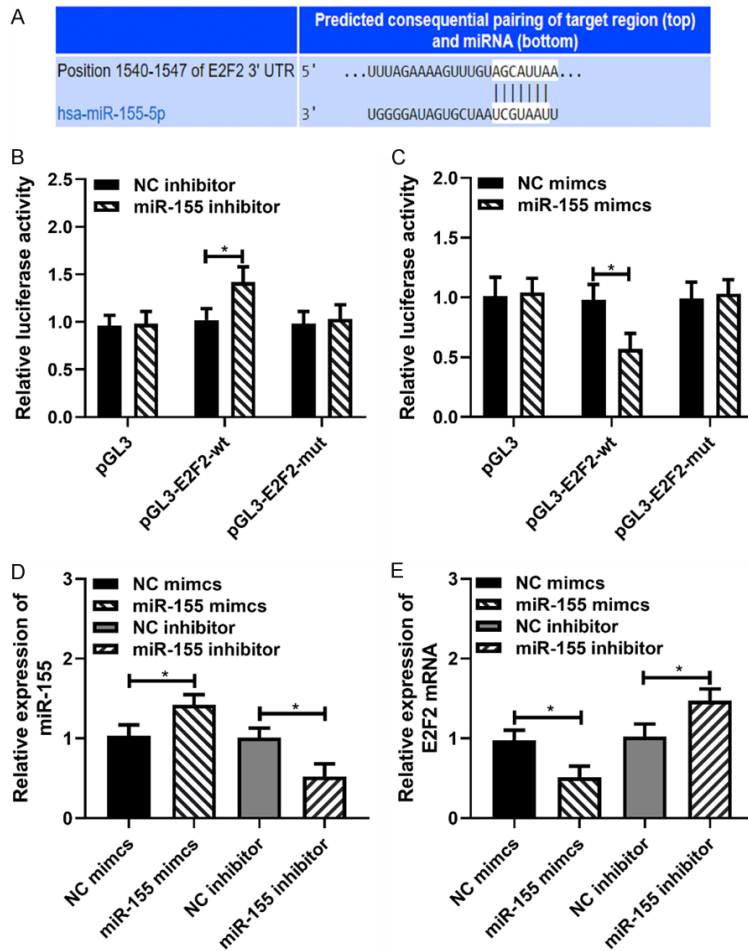


Figure 2. E2F2 as the downstream target of miR-155. * indicates $P < 0.05$. A: TargetsCan7.2 predicted the binding site between miR-155 and E2F2. B and C: Results of DLRGA. D: MiR-155-mimics upregulated miR-155 but miR-155-inhibitor downregulated it. E: MiR-155 upregulation inhibited E2F2 mRNA, while its downregulation increased this mRNA.

development and progression of myopia. Therefore, understanding correlations of microRNAs with the cells is helpful to discover the molecular mechanism of the disease.

In our study, hsa-miR-155 was higher but E2F2 was lower in the serum of myopic patients and in the corneal tissue of myopic rats, which suggests that increasing hsa-miR-155 and reducing E2F2 may be both related to myopia. For studying whether hsa-miR-155 and E2F2 were related, TargetsCan7.2 was adopted to analyze their base sequence information. **Figure 2A** showed a hsa-miR-155 binding site at E2F2 3'UTR, so this miR probably regulates E2F2 through this site. **Figure 2B** and **2C** showed that E2F2-wt luciferase intensity could be changed by both upregulation and downregulation of

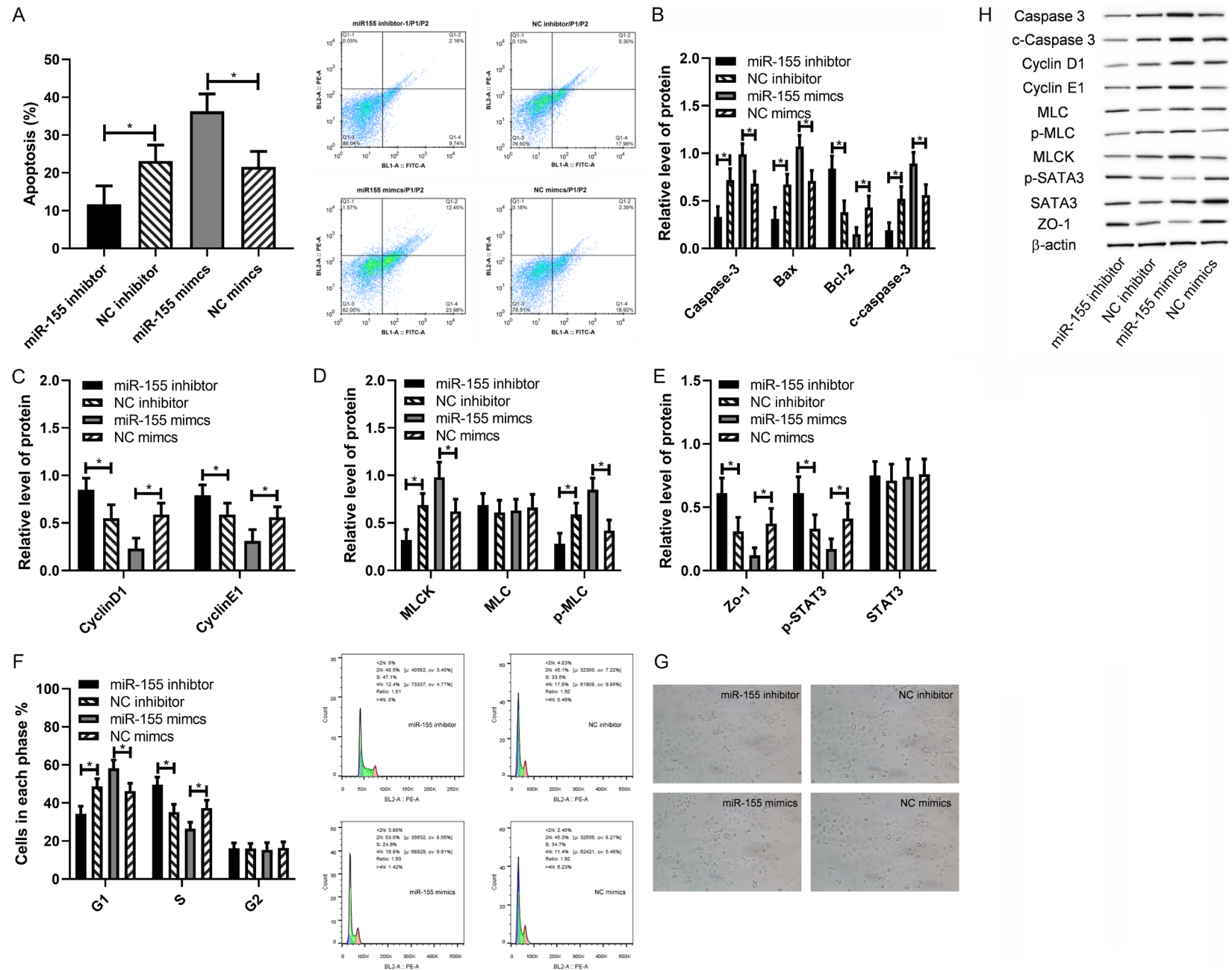
hsa-miR-155, indicating that this miR can regulate E2F2 by binding to E2F2 3'UTR. **Figure 2D** and **2E** showed that hsa-miR-155 negatively regulated E2F2. Accordingly, it is speculated that upregulated hsa-miR-155 may be involved in the regulation of CECs by downregulating E2F2 in myopia.

In this paper, hsa-miR-155-mimics, hsa-miR-155-inhibitor, and E2F2 siRNA were constructed to explore the correlations of hsa-miR-155 and E2F2 with CECs, respectively. **Figures 3** and **4** showed that both hsa-miR-155 upregulation and E2F2 downregulation increased CEC apoptosis, blocked most cells in G1 phase, and reduced S phase cells and ZO-1, as well as upregulated MLCK and MLC. However, hsa-miR-155 downregulation reduced the apoptosis and G1 phase cells, increased S phase cells, ZO-1, and p-STAT3, and down-regulated MLCK and p-MLC. **Figure 5** showed that E2F2 downregulation could offset hsa-miR-155 downregulation-induced apoptosis reduction, cell cycle transition from G1 phase to S phase, and barrier function recovery. Inhibiting

the growth and proliferation of CECs may lead to visual impairment. Apoptosis mediated by Caspase 3 cleavage and cell cycle mediated by Cyclin D1/E1 are important processes to regulate the growth and proliferation of CECs. Moreover, the cell barrier function regulated by STAT3/ZO-1 pathway and MLCK/MLC pathway may destroy the microenvironment in which CECs are located, resulting in damage to corneal tissues, and eventually causing visual decline [6-8]. Therefore, these results reveal that both hsa-miR-155 upregulation and E2F2 downregulation contribute to the cycle arrest and barrier dysfunction of CECs.

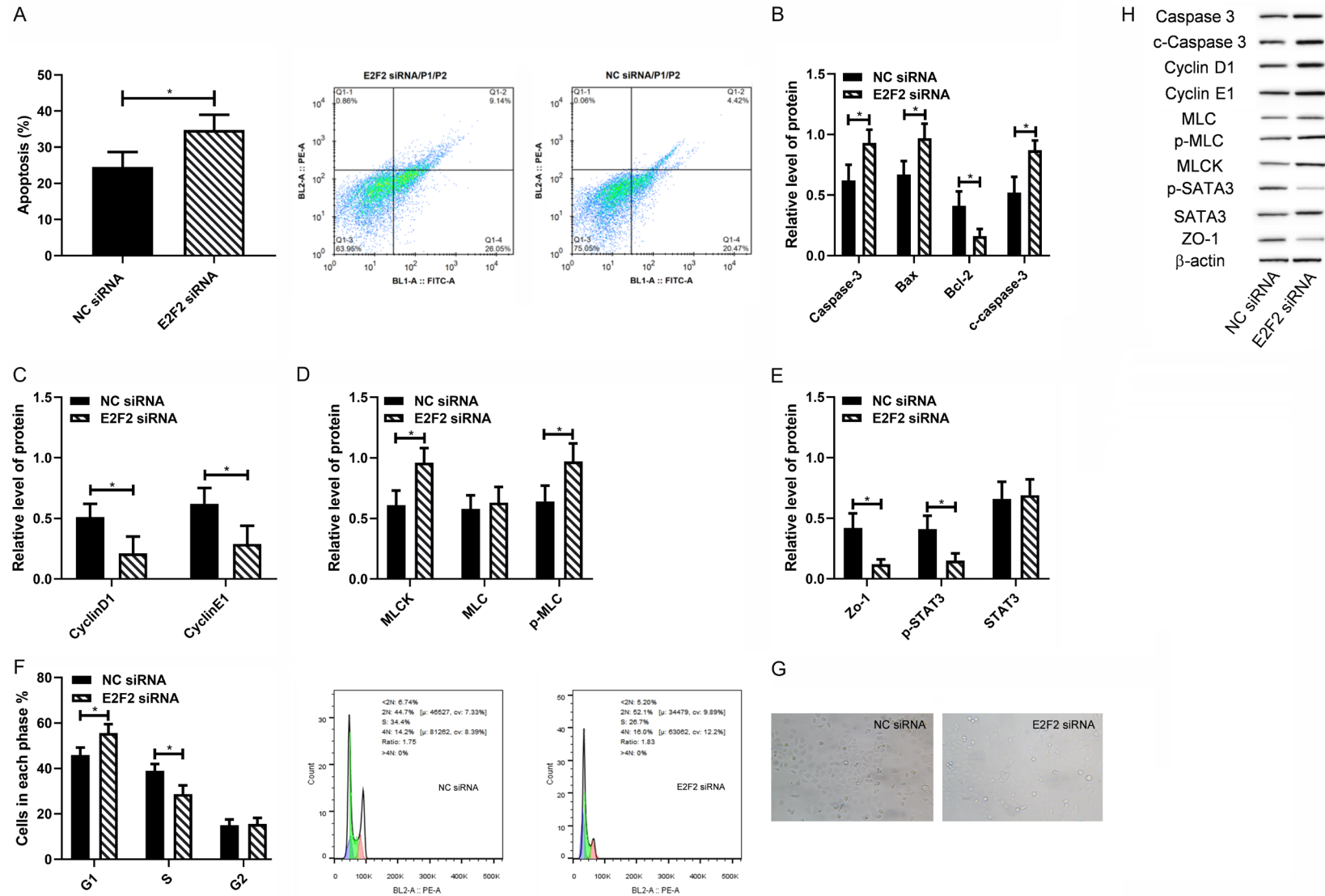
This paper discussed the correlation of hsa-miR-155/E2F2 axis with CECs. hsa-miR-155 downregulation can promote ZO-1 to upregu-

The role of Mir-155 in corneal endothelial cells



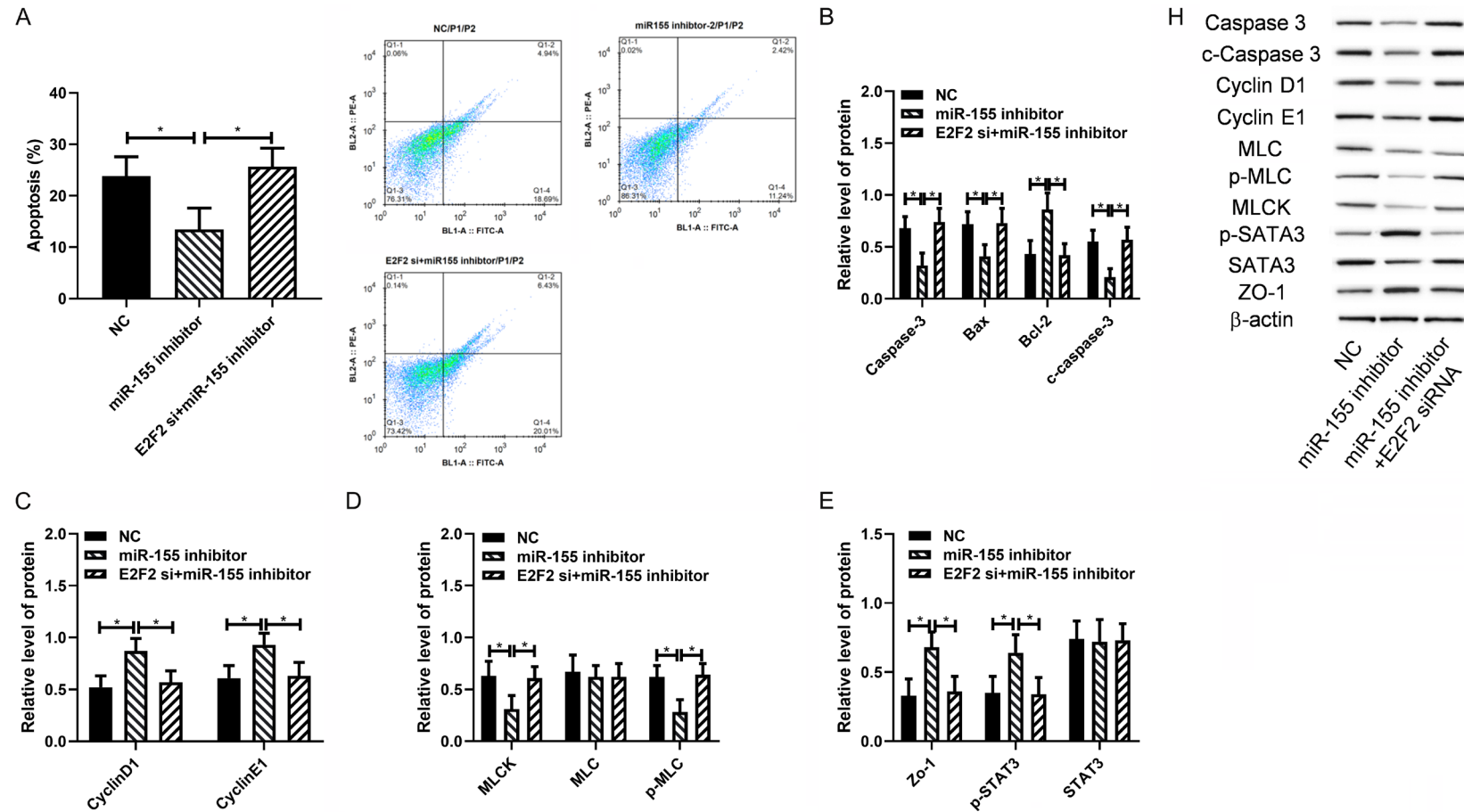
The role of Mir-155 in corneal endothelial cells

Figure 3. Function of miR-155 on barrier function and cell cycle. * indicates $P < 0.05$. A: Effects of miR-155 on the apoptotic rate. B: Effects of miR-155 on Caspase-3, Bax, Bcl-2, and c-Caspase-3. C: Effects of miR-155 on Cyclin D1 and Cyclin E1. D: Effects of miR-155 on MLCK, MLC, and MLC phosphorylation (p-MLC). E: Effects of miR-155 on ZO-1, STAT3, and STAT3 phosphorylation (p-STAT3). F: Effects of miR-155 upregulation on G1 phase cells and S1 phase cells. G: The morphology of corneal endothelia cells via optical microscope. H: Western blot.



The role of Mir-155 in corneal endothelial cells

Figure 4. Function of E2F2 on barrier function and cell cycle. * indicates $P < 0.05$. A: Effects of E2F2 on the apoptotic rate. B: Effects of E2F2 on Caspase-3, Bax, Bcl-2, and c-Caspase-3. C: Effects of E2F2 on Cyclin D1 and Cyclin E1. D: Effects of E2F2 on MLCK, MLC, and p-MLC. E: Effects of E2F2 on ZO-1, STAT3, and p-STAT3. F: Effects of E2F2 downregulation on G1 phase cells and S1 phase cells. G: The morphology of corneal endothelia cells via optical microscope. H: Western blot.



The role of Mir-155 in corneal endothelial cells

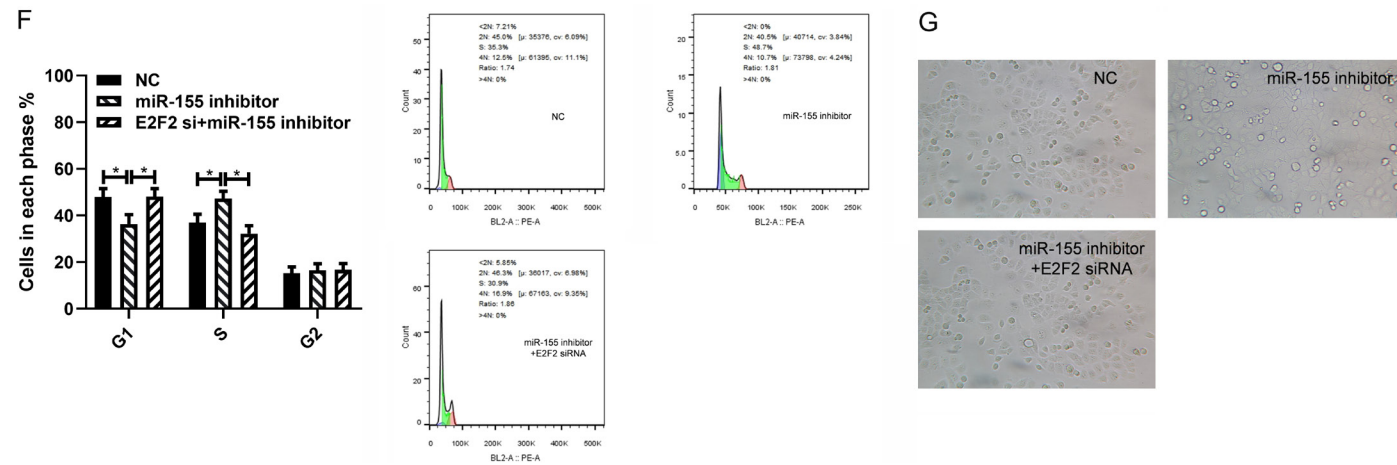


Figure 5. Rescue experiment. * indicates $P < 0.05$. A: Apoptotic rate. B: Changes in Caspase-3, Bax, Bcl-2, and c-Caspase-3. C: Changes in Cyclin D1 and Cyclin E1. D: Changes in MLCK, MLC, and p-MLC. E: Changes in ZO-1, STAT3, and p-STAT3. F: Changes in G1, S and G2. G: The morphology of corneal endothelia cells via optical microscope. H: Western blot.

late, induce cell proliferation, and inhibit apoptosis by up regulating E2F2 in the cells. However, the influence of the axis on the cells was discussed with respect to only barrier function and cell cycle. In fact, the inflammatory responses and immune specialization of CECs are also involved in the cell injury [7], so does the axis also affect inflammation and immunity? This issue deserves further discussion in future studies. Additionally, upstream regulatory factors of hsa-miR-155 will also be explored in subsequent studies. The expression of hsa-miR-155 or E2F2 may be also used to diagnose myopia severity in children, because genetic information is helpful to predict myopia progression in the children [9]. Therefore, the correlation of hsa-miR-155 or E2F2 expression with myopia severity in children will be further investigated in future studies.

Conclusion

In summary, we have studied biological functions of CECs, and found that hsa-miR-155 regulates the barrier function and cycle of the cells through E2F2. This finding demonstrates that the hsa-miR-155/E2F2 axis may be a therapeutic target for corneal endothelial injury in high myopia.

Disclosure of conflict of interest

None.

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