MicroRNA-15a modulates lens epithelial cells apoptosis and proliferation through targeting B-cell lymphoma-2 and E2F transcription factor 3 in age-related cataracts

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Abstract

Age-related cataract remains a serious problem in the aged all over the world. MicroRNAs are abnormally expressed in various diseases including age-related cataract. MicroRNA-15a (MicroRNA-15a) has been involved in various diseases and plays crucial roles in many cellular processes. However, the mechanism of microRNA-15a in the genesis of cataract remains barely known. We therefore aimed to investigate the role of microRNA-15a in the cataract. Herein, human lens epithelial cells, HLE-B3 cells were treated with 200μmol/L H₂O₂ for 24h. H₂O₂ was utilized in our study to induce HLE-B3 cells injury. We observed that cell apoptosis was induced by the treatment of H₂O₂ and meanwhile, cell proliferation was repressed by 200μmol/L H₂O₂. Then, it was found that microRNA-15a was significantly increased with the H₂O₂ exposure in vitro. Importantly, B-cell lymphoma-2 (BCL2) and E2F transcription factor 3 (E2F3) exert crucial roles in cell apoptosis and cell proliferation. We found that BCL2 and E2F3 were greatly reduced by 200μmol/L H₂O₂ in human lens epithelial cells. In addition, microRNA-15a overexpression induced cell apoptosis and repressed cell proliferation through suppressing BCL2 and E2F3. Subsequently, BCL2 and E2F3 were predicted as a direct target of microRNA-15a. The direct correlation between microRNA-15a and BCL2/E2F3 was confirmed by dual luciferase reporter assay. In conclusion, we demonstrated that microRNA-15a triggered apoptosis and repressed the proliferation of HLE-B3 cells by modulating BCL2 and E2F3.
**Key words:** age-related cataract; microRNA-15a; BCL2; E2F3

**Introduction**

Cataract has a high incidence all over the world and it can contribute to the blinding eye disease [1-3]. Numerous factors can lead to the development of cataract. But age and ocular tissue degeneration are the most common inducements. Age-related cataract can affect 46% of the 180 million visually disabled people [4-5]. Hence, identifying the effective biomarkers of cataract can reduce cataract incidence and blinding rates.

As well established, lens epithelial cell apoptosis is an early event in cataract progression [6]. Damages, such as oxidative stress to the epithelial cells can contribute a lot to age-related cataract [7]. Lens epithelial cell apoptosis induced by oxidative stress is a common cellular basis in cataract [8].

MicroRNAs are a small non-protein coding RNAs with 20-25 nucleotides, which can regulate gene expression post-transcriptionally [9]. MicroRNAs regulate mRNA degradation or translation through combining with their targeting mRNAs [10]. So far, microRNAs are involved in a variety of cell processes including proliferation, migration and apoptosis [11-13]. It has been reported that in many studies abnormal expression of miRNAs is closely associated with the pathogenesis of many age-related diseases, including cataract progression [14-16]. For example, miR-34a can promote mitochondrial dysfunction-triggered apoptosis in human lens epithelial cells via targeting Notch2 [17]. miR-26a and -26b inhibit lens fibrosis and cataract
through regulating Jagged-1/Notch signaling pathway [18]. In addition, let-7b induces lens epithelial cell apoptosis by targeting Lgr4 [19].

In our current study, we focused on the biological role of microRNA-15a in age-related cataract. We aimed to investigate the biological role of microRNA-15a in age related cataract and the mechanisms of action. Therefore, we demonstrated that microRNA-15a modulated age-related cataract progression through targeting BCL2 and E2F3 in vitro.

Materials and methods

Cell culture

Human lens epithelial B3 (HLE-B3) cells were purchased from American Type Culture Collection (ATCC; Rockville, MD, USA). Cells were cultured in minimum essential medium (MEM; GIBCO, Carlsbad, CA, USA) added with 10% fetal bovine serum (FBS; GIBCO, Carlsbad, CA, USA) in a humidified chamber with 5% CO2.

Cell transfection

MicroRNA-15a mimics or their parental negative controls (RiboBio, Nanjing, China) were transfected into the cells using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA).

Cell Counting Kit-8 (CCK-8) assay Cells were seeded in a 96-well plate for a whole night. Then, 10μL CCK8 reagents (Dojindo Molecular Technologies, Tokyo, Japan) were added to the cells and the cells were incubated for 4 hours. A microplate reader (Bio-Tek, Winooski, VT, USA) was utilized to test the absorbance at 450 nm.
5-ethynyl-2’-deoxyuridine (EdU) Assay

To detect the function of microRNA-15a on cell proliferation, EdU proliferation assay (Ribobio, Nanjing, China) was conducted. After transfection for 48 hours, cells were incubated with 50μM EdU. An Apollo and DAPI staining were employed to detect the EdU positive cells.

Apoptosis Detection

Cell apoptosis was assessed using an Annexin V-FITC/PI apoptosis detection kit. Briefly, 1 × 10^4 cells were grown in six-well plates per well. Afterward, cells were digested using trypsin without EDTA, harvested, and washed three times using PBS diluted in 500μL Annexin binding buffer. For each sample, 5μL Annexin-V-FITC and 5μL propidium iodide were added to cell suspension and then the cells were incubated for 15 minutes in the dark. Cell apoptosis was evaluated by quantifying the Annexin V-FITC-positive cells. Subsequently, flow cytometry data were plotted and analyzed using the fluorescence activated cell-sorting (FACS-Vantage) system and Cell Ouest software (Becton-Dickinson, San Jose, CA, USA).

Caspase-3 Activity Assay

Caspase-3 activity was detected using a caspase-3 assay kit (Abcam, Cambridge, UK). Cells were lysed in 50μL of chilled cell lysis buffer. 50μL of cell lysis buffer containing 100μg protein was added. 50μL 2× reaction buffer, 0.5μL 10 mmol/L DL-dithiothreitol (DTT) and 5μL caspase-3 catalytic substrate DEVD-pNA substrate were used. After incubation, the absorbance in each well was tested at 405 nm using a microplate ELISA reader.
Western Blot Analysis

Cell extracts were prepared using RIPA buffer containing protein inhibitor cocktail (Roche, Penzberg, Germany). Samples were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto PVDF membranes. Then, the membranes were blocked using 5% skim milk in TBS-T buffer at room temperature for 1 hour. The membrane was incubated with rabbit monoclonal antibody against human BCL2 and E2F3 (Abcam, Cambridge, UK) followed by horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG (Southern Biotech, AL, USA). The protein bands were exposed with the ECL chemiluminescence kit (Pierce, Rockford, IL, USA).

Quantitative real time PCR (qRT-PCR)

The RNAiso Plus (TaKaRaBio Technology, Dalian, China) was used to extract RNA. RNA reverse transcription was carried out using Prime Script TM RT Master Mix and qPCR was performed using SYBR Premix Ex Taq II (TaKaRa Bio Technology, Dalian, China). Primers for qRT-PCR were displayed in Table 1. Applied Biosystems 7900 Real Time PCR System (Applied Biosystems, Foster City, CA, USA) was used.

Luciferase activity assay

The wild-type (WT) or mutant (MUT) BCL2/E2F3 binding microRNA-15a was sub-cloned into pGL3 Basic vector (Promega, Madison, WI, USA). microRNA-15a mimics (RiboBio, Guangzhou, China) were co-transfected with 10μg pLUC-WT-BCL2/E2F3 or pLUC-MUT-BCL2/E2F3 into the cells.

Statistical analysis
Statistical data analysis was carried out using GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA) statistical packages. Each experiment was performed in triplicate and data were presented as mean ± SD. Statistical analysis was performed using Student’s t-test or one-way analysis of variance. Statistical significance was considered when p-value was less than 0.05.

Results

H₂O₂ induced apoptosis and repressed proliferation in human lens epithelial cells.

Firstly, as shown in Figure. 1A and 1B, HLE-B3 cell apoptosis was increased by 200μmol/L H₂O₂ for 24 hours. In addition, we observed that HLE-B3 cell proliferation was significantly inhibited by 200μmol/L H₂O₂ in vitro (Figure. 1C and 1D). These findings indicated that H₂O₂ induced apoptosis and depressed proliferation in HLE-B3.

H₂O₂ up-regulated microRNA-15a and down-regulated BCL2, E2F3 in HLE-B3 cells.

Then, a significant increase of microRNA-15a was observed in H₂O₂-treated HLE-B3 cells compared to the control group (Figure. 2A). Additionally, BCL2 mRNA and protein expression was obviously decreased in HLE-B3 cells incubated with 200μmol/L H₂O₂ (Figure. 2B and 2C). In addition, E2F3 mRNA and protein expression was also greatly decreased by H₂O₂ in vitro (Figure. 2D and 2E). These implied that microRNA-15a/BCL2/E2F3 was involved in cataract development.
MicroRNA-15a regulated human lens epithelial cell apoptosis and cell proliferation. Then, further investigation was conducted to explore the effect of microRNA-15a on apoptosis and proliferation. HLE-B3 cells were transfected with microRNA-15a mimics or mimic controls for 48 hours. As shown in Figure 3A, microRNA-15a was significantly increased by microRNA-15a mimics in HLE-B3 cells. Subsequently, flow cytometry assay indicated that overexpression of microRNA-15a induced cell apoptosis (Figure 3B). In addition, caspase-3 activity assay showed that microRNA-15a mimic group significantly elevated caspase-3 activity (Figure 3C). Next, CCK8 assay was carried out and it was found that cell viability was repressed by microRNA-15a mimics in Figure 3D. These further revealed that microRNA-15a regulated the proliferation and apoptosis of human lens epithelial cells.

MicroRNA-15a regulated BCL2 and E2F3 expression in human lens epithelial cells. After HLE-B3 cells were transfected with microRNA-15a mimics or mimic controls for 48 hours, BCL2 and E2F3 mRNA expression and protein expression were detected. As compared to the mimic control group, BCL2 expression were significantly decreased in microRNA-15a mimics group (Figure 4A and 4B). In addition, E2F3 mRNA (Figure 4C) and protein (Figure 4D) levels were also significantly reduced compared to the control group. These data suggested that microRNA-15a regulated BCL2 and E2F3 expression in HLE-B3 cells. BCL2 and E2F3 were the Target of microRNA-15a.
To investigate the direct target gene of microRNA-15a, bioinformatic analysis (TargetScan, Starbase, miRanda and miRDB database) was performed. BCL2 and E2F3 were predicted as the direct targets of microRNA-15a. Binding regions between microRNA-15a and BCL2 was shown in Figure. 5A and the Luciferase reporter plasmids of wild type BCL2 and mutant BCL2 binding sites were displayed. Co-transfection of the luciferase reporter plasmid containing the wild type with microRNA-15a mimics decreased the reporter activity in HLE-B3 cells (Figure. 5B). In addition, the Luciferase reporter plasmids of wild type E2F3 and mutant E2F3 binding sites were exhibited in Figure. 5C. Consistently, co-transfection of the luciferase reporter plasmid containing the wild type with microRNA-15a mimics also suppressed the reporter activity (Figure. 5D). The results showed that BCL2 and E2F3 directly targeted microRNA-15a.

Discussion

MicroRNAs are involved in various pathological processes including cell growth and apoptosis and they can also represent a potential target for the diagnosis, prevention and treatment of age-related cataracts [20-22]. In our study, we observed microRNA-15a was greatly up-regulated in HLE-B3 cells indicated with 200μmol/L H2O2. Meanwhile, BCL2 and E2F3 was obviously down-regulated by 200μmol/L H2O2 in HLE-B3 cells. Overexpression of microRNA-15a was able to inhibit cell proliferation and induce cell apoptosis by targeting BCL2 and E2F3. Moreover, the negative interaction between BCL2, E2F3 and microRNA-15a was proved in our
study. A novel mechanism of microRNA-15a/BCL2/E2F3 axis in age-related cataracts was revealed in our present study.

Oxidants can trigger apoptosis and contribute to cataract development [23]. In our present research, we found that 200μmol/L H₂O₂ induced apoptosis and inhibited cell proliferation of HLE-B3 significantly. miR-15 family are clustered on three separate chromosomes [24] and the miR-15 family can play a significant role in various cancers [25, 26]. For instance, knock down microRNA-15a promotes the development and induces the EMT process of NSCLC cells [27]. MicroRNA-15a inhibits endometrial cancer cell growth through Wnt/β-catenin signaling by repressing WNT3A [28]. Recently, microRNA-15a in age-related cataract patients is reported to be increased [29].

The BCL2 gene family and its related protein bcl-2 were the first apoptosis-related genes to be studied [30]. BCL2 family genes can play a regulatory role in apoptosis. For example, miR-34a can promote apoptosis of human lens epithelial cells through down-regulating BCL2 [31]. Using informatics analysis, we found that the famous anti-apoptotic gene BCL2 might act as a direct target of microRNA-15a.

The E2F transcription factor family contains 8 members, which plays an important role in cellular proliferation, differentiation and apoptosis [32]. E2F1-3 transcription factors exert essential roles in cellular proliferation [33]. For example, miR-203a can suppress cell proliferation through targeting E2F 3 in human gastric cancer [34]. MiR-217 inhibits pancreatic cancer cell proliferation via targeting E2F3 [35]. In addition, miR-34a can suppress proliferation and induce apoptosis of human lens...
epithelial cells through targeting E2F3 [36].

In summary, the results of our present study demonstrated that microRNA-15a was able to modulate lens epithelial cells apoptosis and proliferation through targeting BCL2 and E2F3 in age-related cataracts. Our observations can provide novel insights into the potential therapeutic applications for age-related cataracts treatment.

Data Availability Statement
All data are available upon request.

Author Contribution Statement
QHL conceived and designed the study, QL performed the experiments and collected the data, HTP did the analysis, QL wrote the manuscript, QHL revised the manuscript, all of the authors approved the final proof.

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Competing Interests
The authors declare that there are no competing interests associated with the manuscript.
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Figure Legends:

**Figure 1: H$_2$O$_2$ induced apoptosis and inhibited proliferation in HLE-B3 cells.**

(A-B) Flow cytometry analysis of the apoptosis induced by H$_2$O$_2$. Cells were treated with 200 μmol/L H$_2$O$_2$ for 24 hours. Flow cytometry was performed to test cell apoptosis. (C-D) Analysis of the proliferation induced by H$_2$O$_2$. EDU assay was
performed to test cell proliferation. Three independent experiments were carried out. Error bars stand for the mean ± SD of at least triplicate experiments. *P<0.05.

Figure 2: Expression of microRNA-15a, BCL2 and E2F3 in HLE-B3 cells incubated with 200μmol/L H₂O₂. (A) MicroRNA-15a expression in HLE-B3 cells. Cells were indicated with 200μmol/L H₂O₂ for 24 hours. (B) BCL2 mRNA expression in HLE-B3 cells. (C) BCL2 protein expression in HLE-B3 cells. (D) E2F3 mRNA expression in HLE-B3 cells. (E) E2F3 protein expression in HLE-B3 cells. Three independent experiments were carried out. Error bars stand for the mean ± SD of at least triplicate experiments. *P<0.05.

Figure 3: MicroRNA-15a regulated human lens epithelial cell proliferation and apoptosis. (A) MicroRNA-15a expression in HLE-B3 cells. Cells were transfected with microRNA-15a mimics for 48 hours. (B) Flow cytometry analysis of the apoptosis in HLE-B3 cells. (C) Caspase-3 activity in HLE-B3 cells. (D) Cell viability measured by the CCK-8 assay in HLE-B3 cells. Three independent experiments were carried out. Error bars stand for the mean ± SD of at least triplicate experiments. *P<0.05.

Figure 4: BCL2 and E2F3 expressions were inhibited by overexpression of microRNA-15a in HLE-B3 cells. (A) Quantitative RT-PCR data of BCL2 mRNA in HLE-B3 cells. (B) Western blot data of protein levels of BCL-2 in HLE-B3 cells. (C) E2F3 mRNA expression in HLE-B3 cells. (D) Protein levels of E2F3 in HLE-B3 cells. Three independent experiments were carried out. Error bars stand for the mean ± SD of at least triplicate experiments. *P<0.05.

Figure 5: BCL2 and E2F3 were direct
targets of microRNA-15a.

(A) The luciferase reporter constructs containing the wild type (WT-BCL2) or mutant BCL2 (MUT-BCL2) sequence. (B) WT-BCL2 or MUT-BCL2 was co-transfected into HLE-B3 cells with microRNA-15a mimics or their corresponding negative controls.

(C) The luciferase reporter constructs containing the wild type (WT-E2F3) or mutant E2F3 (MUT-E2F3) sequence. (D) WT-E2F3 or MUT-E2F3 was co-transfected into HLE-B3 cells with microRNA-15a mimics or their corresponding negative controls.

Three independent experiments were carried out. Error bars stand for the mean ± SD of at least triplicate experiments. *P<0.05.
Table 1. Primers for real-time PCR

| Genes          | Forward (5’-3’)       | Reverse (5’-3’)        |
|----------------|-----------------------|------------------------|
| GAPDH          | AAGAAGGTGGTGAGCACAGGC | GTCAAAAGTGAGGAGGTGGG   |
| U6             | CTCGCTTCGGCAGCACATA   | CAGTGCAGGGTCCGAGGTA    |
| microRNA-15a   | CGCCTAGGCAGACATATGG   | AGTGCAGGGTCCGAGGTAT    |
| BCL2           | CTGCACCTGACGCCCTTCACC | CACATGACCCACCGAACTCAAGA|
| E2F3           | CGGTCTGCTCACAAGAAGT   | CCTCTTCTGCACCTTGAAGCA  |
Figure 2

(A) Relative expression of microRNA-15a (fold change) in control and H$_2$O$_2$ treated groups.

(B) Relative mRNA expression of BCL2 (fold change) in control and H$_2$O$_2$ treated groups.

(C) Western blot analysis showing BCL2 and GAPDH expression levels in control and H$_2$O$_2$ treated groups.

(D) Relative mRNA expression of E2F3 (fold change) in control and H$_2$O$_2$ treated groups.

(E) Western blot analysis showing E2F3 and GAPDH expression levels in control and H$_2$O$_2$ treated groups.
Figure 3

A. Relative expression of microRNA-15a

B. Apoptotic ratio

C. Caspase-3 activity

D. Absorbance OD450 nm
Figure 4

(A) Relative mRNA expression of BCL2 fold change

(B) Western blot analysis of BCL2

(C) Relative mRNA expression of E2F3 fold change

(D) Western blot analysis of E2F3
