MicroRNA-182-5p protects human lens epithelial cells against oxidative stress-induced apoptosis by inhibiting NOX4 and p38 MAPK signaling

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Abstract
Background: MicroRNAs (miRNAs) are abnormally expressed in varying ocular diseases, including age-related cataract. However, the roles of miR-182-5p in age-related cataract progression remains unclear.

Methods: The expression of miR-182-5p in HLE-B3 cells were detected by qRT-PCR. HLE-B3 cells were transfected with miR-182-5p mimics. CCK-8, EdU, flow cytometry, 2’,7’-dichlorodihydrofluorescein diacetate, JC-1 kit, and Western bolt were used to assess the cell viability, proliferation, apoptosis, reactive oxygen species (ROS) level, mitochondrial membrane potential (MMP), and protein expression, respectively, in vitro. The target relationship of miR-182-5p and NOX4 was confirmed using dual-luciferase reporter gene analysis.

Results: We found that miR-182-5p was significantly decreased by the H2O2 exposure. Overexpression of miR-182-5p promoted cell proliferation, inhibited ROS production and apoptosis in H2O2-induced HLE-B3 cells. Moreover, p-p-38, p-ERK, and p-JNK were up-regulated in H2O2-treated HLE-B3 cells, and overexpression of miR-182-5p reversed the effect of H2O2 on HLE-B3 cells. In addition, dual-luciferase reporter assay substantiated that NOX4 was a direct target and downregulated by miR-182-5p.

Conclusions: We concluded that miR-182-5p inhibited lens epithelial cells apoptosis through regulating NOX4 and p38 MAPK signaling, providing a novel biomarker for treatment of age-related cataract.

Background
Cataract is characterized by progressive opacity of the ocular lens, which can lead to blindness[1]. Approximately 50% of blindness in middle-income and low-income countries are caused by cataracts[2]. Until now, multiple risk factors like aging, diabetes, genetic, oxidative stress and UV exposure are relevant to pathogenesis of age-related cataract[3]. Although cataract removal and intraocular lens implants surgery are effective procedure, letting patients see the light again[4]. However, there are disadvantages in replacing artificial tissues and organs with artificial materials. Surgery may result in severe postoperative complications, including wound leakage, corneal abrasion,
and ocular hypertension, especially in the elderly[5]. Owing to the prevalence of the disease among ageing populations, cataract surgeries amount to a significant portion of healthcare cost, especially remote and poor areas in developing countries[2]. Therefore, in-depth study of the pathogenesis of age-related cataracts by preventing the occurrence of cataracts or delaying their development has become a very useful subject.

As previously reported, the apoptosis of human lens epithelial cells (LECs) has been accepted as an early event of cataract[6], while oxidative damage to the LECs can be one of the major factors leading to apoptosis[7].

MicroRNAs (miRNAs) are single-stranded, short, non-coding molecules that have vital roles in the negatively regulation of target genes, leading to the repression of the translation process[8]. MiRNAs are involved in numerous fundamental cellular processes, including cell differentiation, proliferation and apoptosis. miR-182 (miR-182-5p) is reported to play an important role in ophthalmic disorder, including pterygium [9], high-tension glaucoma[10], congenital cataract[11], retinoblastoma[12], macular degeneration[13] and so on. However, the exact roles of miR-182-5p and its mechanism in age-related cataract progression remain poorly understood.

In the present study, we measured the expression of miR-182-5p in LECs upon exposure to H2O2 and explored whether miR-182-5p might affect LECs apoptosis by regulating NOX4 and p38 MAPK signaling.

Methods
Cell culture
Human lens epithelial B3 (HLE-B3) cells were obtained from American Type hCulture Collection (ATCC, Rockville, MD, USA). Cells were cultured in eagle's minimum essential medium (EMEM, Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco) and were incubated at 37 °C in a humidified chamber with 5% CO2.

Cell transfection
MiR-182-5p mimics or negative controls (RiboBio, Guangzhou, China) were transfected into the cells according to the instructions on the Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA).
HLEC-B3 cells were treated with pcDNA3.1-NOX4 (oe-NOX4) or pcDNA3.1 negative control (oe-NC) (RiboBio, Guangzhou, China), followed by treatment with the miR-182-5p mimics or negative controls. At 48h after transfection, HLE-B3 cells were treated with H$_2$O$_2$ (250 μmol/L) for 12h.

**Luciferase assays**

The putative binding sites of miR-182-5p and NOX4 were predicted by targetscan human 7.2. The 3’untranslated regions (3’UTR) sequences containing wild-type or mutant binding sites of NOX4 were subcloned into pmirGlO luciferase reporter vector (Promega, Madison, WI, USA) to generate the wild-type plasmids (NOX4-WT) or mutant-type plasmids (NOX4-MUT), respectively. The miR-NC, miR-182-5p mimics was cotransfected with reporter plasmids into HLE-B3 cells using Lipofectamine 3000. Luciferase activities were analyzed 24h after transfection using the Dual-luciferase Reporter Assay Kit (Promega, Madison, USA).

**Cell Counting Kit-8 (CCK-8) assay**

Cells were seeded in a 96-well plate (1×10$^4$) and incubated for 24h. Then 10μL CCK8 reagents (Beyotime Institute of Biotechnology, Jiangsu, China) was added to the cells. 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 miR-182-5p on cell proliferation, EdU proliferation assay (RiboBio, Guangzhou, China) was conducted. Cells were incubated with 50μM EdU. The EdU positive cells were then visualized under a fluorescence microscope (leica, Germany).

**Apoptosis Detection**

Cells were collected and incubated with Annexin V-FITC/PI apoptosis detection kit (KeyGEN Biotech, Nanjing, China) in the dark for 15min. Cell apoptosis were analyzed by using a flow cytometer (A60-Micro, Apogee, UK).

**Detection of MMP (mitochondrial membrane potential)**

Cells were added to 6-well plates(1×10$^6$) and divided into groups as described above. Then, the changes of cell MMP in different groups of cells were measured using 5 μg/mL JC-1 (Beyotime
Biotechnology, Shanghai, China). The cells were washed with PBS buffer and detected by flow cytometer (Apogee).

**Detection of Oxidative Stress Products**

The concentrations of reactive oxygen species (ROS) in the cells were measured by adding 200μL DCFH-DA (5μmol/L final concentration, Sigma-Aldrich, St.Louis, MO, USA). After washed, cells were detected by the flow cytometer (Apogee). The malondialdehyde (MDA) content, superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px) activities were detected by using measurement kits (Nanjing Jiancheng Bioengineering Institute) separately.

**Real time quantitative RT-PCR (RT-qPCR)**

Total RNA was isolated from LECs using TRIzol reagent and converted into cDNA (TaKaRa, Dalian, China). For quantitative real-time PCR (qRT-PCR), the SYBR (Roche) was used according to the manufacturer’s protocol with the Analytik-jena qTOWER PCR System (Jena, Germany). Primers were listed as follows, miR-182-5p (ACACTCCAGCTGGGTTTGGCAATGGTAGAACT and TGGTGTCGTGGAGTCG), U6 (CTCGCTTCGCGAGCACA and AACGCTTCACGAATTTCGT), NOX4 (CGATTCCGGGATTTGCTACTG and CCTCAAATGGGCTTCCAATG), β-actin (TGAGCGCGGCTACAGCTT and TCTTAATGTACGCACGATT).

**Western blot**

Cells were lysed in lysis buffer to extract protein samples. 50μg total protein was separated by SDS-PAGE and transferred onto PVDF membranes. The membranes were probed with appropriate primary antibodies, including Cleaved caspase 3(#9664, CST,1:1000), Cleaved caspase9(#9509,CST,1:1,000), p-p38(#4511,CST,1:1000), p38(#8690,CST,1:1000), p-ERK(#4370, CST,1:1000), ERK (#4695,CST,1:1000), p-JNK(#9255,CST,1:1000), JNK(#9252,CST,1:1000), NOX4 (ab133303,abcam 1:1000), β-actin (#3700, CST, 1:5000.). Then, membranes were incubated with secondary antibodies for 2h. Finally, the protein bands were detected by chemiluminescence reagents.

**Statistical analysis**

GraphPad Prism 7 (GraphPad, San Diego, CA, USA) was applied for statistical analysis. All experiments were repeated three times. Data are shown as the mean ± SD. Differences between multiple groups
were assessed by one-way ANOVA and Tukey’s multiple comparisons test. Differences between groups were considered significant when P < 0.05.

Result

**Overexpression of miR-182-5p alleviates the inhibition of proliferation induced by H$_2$O$_2$.**

As detected by qRT-PCR (Fig.1A), miR-182-5p was decreased by H$_2$O$_2$ in the HLE-B3 cells. Cells transfected with miR-182-5p mimics exhibited higher miR-182-5p expression, indicating a high transfection efficiency. CCK-8 and EdU analysis showed that the H$_2$O$_2$ treatment triggered the proliferative inhibition of HLE-B3 cells, and the miR-182-5p mimics transfection partially attenuated the proliferative inhibition (Fig.1B and C).

**Overexpression of miR-182-5p suppresses H$_2$O$_2$-induced oxidative stress**

The intracellular ROS levels were presented as the mean fluorescent intensity (MFI), as performed by the DCFH-DA method (Fig.2A). The MFI of intracellular ROS was increased in the HLE-B3 cells from the H$_2$O$_2$ group, but was significant decreased after transfection with miR-182-5p mimics. Moreover, a higher level of MDA was observed in the H$_2$O$_2$ group compared with the control group, but was significant decreased after transfection with miR-182-5p mimics (Fig.2B). Besides, H$_2$O$_2$ also inhibited the activities of SOD and GSH-Px in HLE-B3 cells, which could be obviously reversed by miR-182-5p mimics (Fig. 2C and D).

**Overexpression of miR-182-5p protects HLE-B3 cells against oxidative stress-induced apoptosis.**

MiR-182-5p mimics reduced H$_2$O$_2$-induced HLE-B3 cells apoptosis (Fig.3A). The western blotting showed that cleaved caspase-3 and cleaved caspase-9 were upregulated by H$_2$O$_2$ treatment, while was reversed with the transfection of miR-182-5p mimics(Fig.3B). Consequently, after 12h treatment with 250μmol/L of H$_2$O$_2$, the MMP of HLE-B3 cells was significantly lower than that in the control group, while the transfection of miR-182-5p mimics could abolish the H$_2$O$_2$-induced decrease in MMP in HLE-B3 cells (Fig.3C).

**Overexpression of miR-182-5p suppresses MAPK signals in H$_2$O$_2$-treated HLE-B3 cells.**
Our data showed that treatment with H$_2$O$_2$ led to extensive increase of p-p38, p-ERK, and p-JNK. As expected, pretreatment with miR-182-5p mimics can apparently reverse the effect (Fig.4).

**miR-182-5p binds NOX4 directly**

Through bioinformatics analysis using targetscan human 7.2, we found that miR-182-5p could bind NOX4 3'UTR (Fig.5A). Luciferase report assay showed that co-transfection of the wild type plasmid with miR-182-5p mimics suppressed the luciferase reporter activity (Fig.5B). We then detected NOX4 protein expression after transfecting miR-182-5p mimics or inhibitor. NOX4 protein expression was significantly up-regulated by miR-182-5p mimics and down-regulated by miR-182-5p inhibitor (Fig.5C).

**NOX4 overexpression reverses protective the effects of miR-182-5p mimics in H$_2$O$_2$-treated HLE-B3 cells**

Compared with the control group, H$_2$O$_2$ treatment increased the NOX4 expression and miR-182-5p mimics could decrease the NOX4 expression in the HLE-B3 cells (Fig.6A). The results showed that NOX4 overexpression could reverse the promotion effect of miR-182-5p mimics on the cell proliferation of the HLE-B3 cells treated by H$_2$O$_2$(Fig.6B and C). The results also showed that NOX4 overexpression could reverse the inhibition effect of miR-182-5p mimics on the cell apoptosis of the HLE-B3 cells treated by H$_2$O$_2$(Fig.6D).

**Discussion**

It is believed that oxidative stress takes part in the pathogenesis of age-related cataract[14]. This study studied the effects of miR-182-5p protects HLE-B3 cells against oxidative stress through inhibiting NOX4 expression and the activation of the p38 MAPK pathway.

Growing evidence indicates that aberrant expression of miRNAs is detected after oxidative stress induction. One study reported that microRNA-15a was significantly increased with the H$_2$O$_2$ exposure in HLE-B3 cells[15]. Another study demonstrated that the expression of miR-34a was up-regulated in HLE-B3 cells treated by H$_2$O$_2$[16]. In this study, the expression of miR-182-5p were significantly downregulated by the treatment of H$_2$O$_2$ in HLE-B3 cells, which was consistent with previous
work[17]. Emerging evidence suggests that miR-182-5p contribute to anti-apoptotic and anti-oxidative processes. MiR-182-5p inhibited oxidative stress-induced apoptosis via targeting TLR4[18]. In this article, miR-182-5p weakened H₂O₂-treated HLE-B3 cells apoptosis by inhibiting the decline of MMP. The balance of MMP was important for maintaining the normal function of mitochondria. Decreased MMP triggered mitochondrial swelling and rupturing of outer membrane, and ultimately led to the apoptosis of cells[19, 20].

Oxidative stress can specifically activate ERK, JNK or p38 MAPK under different conditions [21–23]. Inhibition of phosphorylation of p38 reduced H₂O₂-induced cellular apoptosis and inhibited the generation of ROS[23]. We found that miR-182-5p could suppress both the activation of p38 MAPK and ROS production in HLE-B3 cell treated by H₂O₂. Peng J al also identified that p-Coumaric Acid suppresses H₂O₂-induced LECs apoptosis through by suppressing phosphorylation of p-38, ERK, and JNK[24].

The prediction of target genes is a key step toward understanding the function of specific miRNAs. We found that miR-182-5p could bind the 3’UTR of NOX4 mRNA. Moreover, miR-182-5p mimics could increase and inhibitor could decrease the expression of NOX4. These results indicated that miR-182-5p may act via NOX4 to regulate cataract formation. The NADPH oxidase 4 (NOX4) is a member of NOX family that is the primary source of ROS[25]. NOX4-derived ROS play an important role in p38 MAPK signaling[26], regulation of mitochondrial function[27]. A previous results indicate that dapagliflozin decreases NOX4 levels in the LECs from fructose-fed rats, thereby reducing ROS generation during fructose-induced diabetic cataracts[28]. We confirmed that miR-182-5p inhibited H₂O₂-stimulated HLE-B3 cells apoptosis, while apoptosis was reversed by overexpressing of NOX4. It was in accordance with previous findings that NOX4 reverses the protective effect of miR-423-5p in diabetic kidney diseases[29].

Conclusion
In summary, we found that miR-182-5p alleviate H₂O₂-induced LECs injury. The protective effects of miR-182-5p on LECs injury were mediated through directly targeting NOX4. MiR-182-5p decreased
ROS production and p38 MAPK activity. This study may provide novel insights for age-related cataract therapy.

**Abbreviations**

reactive oxygen species (ROS); mitochondrial membrane potential (MMP); human lens epithelial cells (LECs); Human lens epithelial B3 (HLE-B3); pcDNA3.1-NOX4 (oe-NOX4); pcDNA3.1 negative control (oe-NC); wild-type plasmids (NOX4-WT); mutant-type plasmids (NOX4-MUT); malondialdehyde (MDA) content; superoxide dismutase (SOD); glutathione peroxidase (GSH-Px).

**Declarations**

**Ethics approval and consent to participate**

Not Applicable

**Consent for publication**

Not Applicable

**Availability of data and material**

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

**Competing Interests**

The authors declare that they have no conflicts of interest.

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Not applicable.

**Author contributions**

ZF Y designed the study; ZN L and MX G performed the research, analyzed data, and wrote the paper.

All authors have read and approved the manuscript.

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Figures

**Figure 1**

Overexpression of miR-182-5p alleviates the inhibition of proliferation induced by H2O2. A. The expression of miR-182-5p was detected by qRT-PCR. B. Cell viability was detected by Cell Counting Kit-8 (CCK-8) assay. C. EdU assay illustrated the proliferation of HLE-B3 cells.

** P<0.01 vs. control group, # P<0.05 vs. H2O2 group, ## P<0.01 vs. H2O2 group.
Overexpression of miR-182-5p suppresses H2O2-induced oxidative stress. A. The MFI of ROS in HLE-B3 cells determined by DCFH-DA assay. B. Effect of miR-182-5p on MDA content, SOD (C) and GSH-Px (D) activities in HLE-B3 cells. ** P<0.01 vs. control group, # P<0.05 vs. H2O2 group, ## P<0.01 vs. H2O2 group.
Figure 3

Overexpression of miR-182-5p protects HLE-B3 cells against oxidative stress-induced apoptosis. A. Cell apoptosis was examined by flow cytometric analysis. B. Cleaved caspase 3 and cleaved caspase 9 expression in HLE-B3 cells was detected by western blot. C. The ratio of green/monomeric forms of JC-1 was calculated with flow cytometry. ** P<0.01 vs. control group, ## P<0.01 vs. H2O2 group.
Overexpression of miR-182-5p suppresses p38 MAPK signals in H2O2-treated HLE-B3 cells. p-p-38, p-ERK, and p-JNK expressions were measured using Western blot. ** P<0.01 vs. control group, ## P<0.01 vs. H2O2 group.
MiR-182-5p could bind NOX4 directly. A. Putative miR-182-5p binding site in the 3’UTR region of NOX4. B. The luciferase activity among NOX4-3’UTR-wt, NOX4-3’UTR-mut, mimics control and miR-182-5p mimics. ** P<0.01 vs. NOX4-WT+ mimics NC group. C. The expression of NOX4 protein regulated by upregulated or downregulated miR-182-5p. ** P<0.01 vs. mimics NC group, ## P<0.01 vs. inhibitor NC group.
NOX4 overexpression reverses protective the effects of miR-182-5p mimics in H2O2-treated HLE-B3 cells. A. The expression of NOX4 was detected by qRT-PCR. B. Cell viability was detected by Cell Counting Kit-8 (CCK-8) assay. C. EdU assay illustrated the proliferation of HLE-B3 cells. D. Cell apoptosis was examined by flow cytometric analysis. ** P<0.01 vs. control group, # P<0.05 vs. H2O2 group, ## P<0.01 vs. H2O2 group.