Biochemical and Biophysical Research Communications 460 (2015) 164–169

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

MicroRNA-26a overexpression protects RGC-5 cells against H_2O_2-induced apoptosis

Ye Kang [a, 1], Ping Jia [b, 1], Huaqi Zhao [c], Chunyan Hu [c], Xiangze Yang [d, *]

[a] Department of Ophthalmology, Yantaishan Hospital, 91 Jiefang Road, Yantai, 264000, Shandong, China
[b] Department of Ophthalmology, Yantai Development Area Hospital, Yantai, 264006, Shandong, China
[c] Department of Ophthalmology, People's Hospital of Cao County, Heze, 274400, Shandong, China
[d] Department of Ophthalmology, Weihai Municipal Hospital, 70 Heping Road, Weihai, 264200, Shandong, China

ARTICLE INFO

Article history:
Received 4 February 2015
Available online 7 March 2015

Keywords:
RGC-5
miR-26a
PTEN
Apoptosis
Neuroprotection

ABSTRACT

Background: We intended to examine the functional role of microRNA 26 (miR-26a) in regulating H_2O_2-induced cytotoxicity and apoptosis in RGC-5 cells *in vitro*.
Method: Various concentrations of H_2O_2 (0–1000 μM) were added in RGC-5 culture. Cell cytotoxicity was monitored by viability assay and gene expression level of miR-26a examined by qRT-PCR. MicroRNA-26a mimic was then applied in the RGC-5 culture to examine its effect on upregulating endogenous miR-26a and rescuing H_2O_2-induced cytotoxicity. TUNEL immunostaining assay was used to further assess the protective effect of upregulating miR-26a on H_2O_2-induced apoptosis in RGC-5 cells. Direct targeting of miR-26a on Phosphatase and tensin homolog (PTEN) signaling pathway was assessed by luciferase assay and western blotting. PTEN was then ectopically over-expressed in RGC-5. And its effects on miR-26a mediated apoptosis protection in RGC-5 were investigated by western blot and TUNEL assay.
Results: H_2O_2 induced cytotoxicity and down-regulated miR-26a in dose-dependent manner in RGC-5 cells. MiR-26a-mimic upregulated endogenous miR-26a gene levels, and then reduced H_2O_2-induced cytotoxicity, as well as H_2O_2-induced apoptosis in RGC-5 cells. PTEN was directly targeted by miR-26a. PTEN protein was upregulated, and phosphorylated AKT protein down-regulated while miR-26a was upregulated to reduce H_2O_2-induced apoptosis. Finally, overexpressing PTEN reversed the protective effect of miR-26a upregulation on RGC-5 apoptosis.
Conclusion: Upregulating miR-26a protects RGC-5 cell against cytotoxicity and apoptosis, probably through down-regulation of PTEN.

1. Introduction

Glaucoma is the most common cause of blindness worldwide. In glaucoma patients, loss of vision often starts with a gradual but irreversible degeneration of retinal ganglion cells (RGCs), a specialized group of sensory neurons convey visual signals from the eyes to the brain. Many factors may contribute to glaucoma progression, such as increased intraocular pressure [1,2], aging [3,4], genetic defects [5,6] or vascular dysfunction [7,8]. As the result, RGCs start to degenerate with pathological responses such as neurotoxicity or apoptosis [9,10]. For the past decades, both experimental and clinical studies had revealed many of the signaling pathways involved in the degeneration/regeneration of peripheral sensory neurons including RGCs, such as PTEN/Akt/ERK pathway, BDNF/TrkB pathway, or RGMa/neogenin pathway [11–15]. However, the complete molecular or cellular profile of RGC degeneration, as well as the clinically feasible treatment to prevent RGC degeneration, remains elusive.

MicroRNAs (miRNAs) are groups of small (18–22 nucleotides) noncoding RNAs that suppress gene expression through the binding of 3′ untranslated regions (3′ UTR) of target genes [16]. In mammalian retina, miRNA clusters of miR-183/96/182 and miR132/212 were shown to be regulating retinal synaptogenesis [17,18], and miR-132 was shown to regulate RGC neurite development through the association of neurotrophin factor BDNF [19]. Among many of the neuronal miRNA regulators, microRNA-26a (miR-26a) is shown to regulate cortical neurite growth in Alzheimer's disease [20]. In non-mammalian retina, miR-26a was shown to regulate the development of voltage-gated calcium channels in photoreceptors

* Corresponding author.
E-mail address: xiangzey@aol.com (X. Yang).
[1] These authors contribute equally.

http://dx.doi.org/10.1016/j.bbrc.2015.02.164
0006-291X/

[21]. In mammalian retina, though miR-26a was shown to be abundantly expressed [22], its function was largely unknown.

Phosphatase and tensin homolog (PTEN) is a known tumor suppressor. In mammalian neural development, PTEN is often to be a negative regulator in modulating neurogenesis, synaptogenesis or neural regeneration [23,24]. In RGCs, knock-down of PTEN/mTOR pathway was shown to promote axon regeneration [25]. Yet, there has been no study demonstrating functional association of PTEN and miR-26a in regulating RGC apoptosis or cytotoxicity.

In present study, we cultured RGC-5 cells *in vitro*, and evaluated the gene expression levels of miR-26a in response to H_2O_2-induced RGC-5 cytotoxicity and apoptosis. Then, we used synthetic miRNA mimics to upregulate miR-26a in RGC-5 cells, and assessed the functional effects of miR-26a upregulation on H_2O_2-induced RGC-5 cytotoxicity and apoptosis. Moreover, the molecular target of miR-26a, PTEN was over-expressed in RGC-5 cells to assess its effects on miR-26a mediated apoptosis protection. Our data would help elucidate the interaction between microRNAs and their molecular pathways in hypoxia-induced neurotoxicity in retina.

2. Materials and methods

2.1. Culture of RGC-5 cells and H_2O_2 treatment

The retinal ganglion cell (RGC)-like RGC-5 is a rat retinal ganglion progenitor cell line, kindly provided by Dr. Neeraj Agarwal at the University of North Texas Health Science Center in the United States. The cells were maintained in Dulbecco's Medium Eagle's medium (DMEM) (Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin under a humidified chamber of 5% CO_2 at 37 °C. RGC-5 cells were passaged using 0.25% Trypsin every 2–3 days.

The treatment of H_2O_2 is known to induce hypoxia-like apoptosis in RGC-5 cells *in vitro* [26]. In the viability experiment of our study, RGC-5 cells were treated with series concentrations of H_2O_2 (1 μM–1 mM) for 24 h to induce cell death. In the apoptosis experiment of our study, 200 μM H_2O_2 was added into RGC-5 culture for 4 h to induce apoptosis.

2.2. Cell viability assay

RGC-5 viability was evaluated by an MTT assay. Briefly, RGC-5 cells were seeded in a 96-well plate (1×10^5/well). Various concentrations of H_2O_2 were then added to the culture for 24 h. After discarding the supernatant, fresh DMEM containing 10% MTT (Sigma Aldrich, USA) was added to the culture for 4 h. The MTT-formazan crystals were then dissolved in DMSO, and the absorption at 570 nm was measured using a Synergy HT multi-detection microplate reader (Bio-Rad, USA).

2.3. RNA isolation, reverse transcription, and quantitative real-time PCR

Total RNA was extracted from suspended RGC-5 cells from culture using Trizol reagent (Invitrogen, USA). The quantity of RNA was verified by a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA) at 260/280 and an Agilent 2100 Bioanalyzer (Agilent Technologies, USA). To quantitatively measure miR-26a expression, total RNA was reversely transcribed using NCode miRNA First-Strand cDNA Synthesis kit (Invitrogen, USA). Quantitative real-time PCR (qRT-PCR) was then carried on with an SYBR Green PCR master mix on a 7500 Real-time PCR System (Applied Biosystems, USA). U6 snRNA was used as endogenous control. The relative expression levels were calculated as folder changes ($2-\Delta\Delta Ct$) to endogenous controls [27].

2.4. MicroRNA-26a overexpression assay

The miR-26a mimics (miR-26a-mimic) and a non-specific miR control (NC-miR) were synthesized and purified by RiboBio (Guangzhou, China). MiRNA mimics (50 nM) were then transfected into RGC-5 culture by Lipofectamine 2000 reagent (Invitrogen, USA) according to manufacturer's instruction. Twenty-four hours after transfection, culture media were changed to fresh DMEM + 10% FBS.

2.5. Apoptosis assay

The apoptosis of RGC-5 cells was assessed by TUNEL assay. Briefly, RGC-5 cells were fixed and permeabilized with 1% Triton X-100 (Sigma Aldrich, USA) and 4% paraformaldehyde (PFA) (Millipore, USA) in phosphate-buffered saline (PBS) (Invitrogen, USA) for 1 h. An Apo-BrdU In Situ DNA Fragmentation Assay Kit (BioVision, USA) was applied to assess apoptosis. Transferred bromolated deoxyuridine triphosphate nucleotides (Br-dUTP) to the free 3′-OH of cleaved DNA by terminal deoxynucleotide transferase (TdT) were immuno-stained by the anti-BrdU-FITC antibody. A nuclear marker (DAPI) was also used to immune-stain RGC-5 nuclei. The fluorescent images were taken on an SP5 confocal microscopy (Zeiss, Germany). The degree of apoptosis was presented as the percentage of non-apoptotic RGC-5 cells (TUNEL-negative) against all cells (DAPI positive) within 1 mm^2 region.

2.6. Luciferase reporter assay

The wild type PTEN 3′-UTR luciferase reporter vector (Luc-PTEN-WT) was constructed by amplifying the rat PTEN 3′-UTR from genomic DNA through regular PCR. The fragment was then inserted into the psiCHECK-2 vector downstream from the renilla luciferase cassette. The Predicted miR-26a-binding sites on PTEN 3′-UTR were mutated using the Site-Directed Mutagenesis Kit (SBS Genetech, China), and the mutated luciferase reporter vector (Luc-PTEN-MU) was generated accordingly. An empty luciferase reporter vector (Luc-E) was used as internal control. HEK293T cells were then transfected with miR-26a-mimic and one of the three luciferase reporter vectors for 24 h. Cell lyses we analyzed using the Dual-Luciferase Reporter Assay System (Promega, USA). Luciferase assays were read in a Veritas Microplate Luminometer (Turner Biosystems, USA), and normalizing to firefly luciferase signal of Luc-E.

2.7. Western blotting assay

The protein was extracted and purified from RGC-5 lysates in a cell disruption buffer (Ambion, USA). Twenty micrograms of protein was denatured for 10 min at 95 °C in a volume of 95% Laemmli sample buffer (Bio-Rad, USA) and 5% β-mercaptoethanol solution. Proteins were then separated on 10% SDS-PAGE gel and electrophoretically transferred to the nitrocellulose membranes (Schleicher & Schuell, Germany). Filters were blocked for 1 h at RT in a blocking solution containing PBS, 0.1% Tween 20, and 2% ECL Advance blocking agent (Amersham Biosciences, USA). For primary antibodies, the membranes were incubated with mouse anti-PTEN monoclonal antibody (Sigma Aldrich, USA), and a rabbit anti phosphor-AKT ([Ser473]-Akt) polyclonal antibody (Sigma Aldrich, USA) for 1 h at RT (1:500 dilution). Then, the membranes were washed 3 X10 min with 1% Tween 20 in PBS, and incubated with horseradish peroxidase-conjugated secondary antibodies (Sigma Aldrich, USA) for another 1 h at room temperature (1:2000

dilution). The blots were visualized with an enhanced iluminesence system (Amersham Biosciences, USA).

2.8. PTEN overexpression assay

The 3′-UTR sequence of rat PTEN (NCBI: NM_031606) was amplified and cloned into the pcDNA3.1-hygro(+) vector (Promega, Madison, WI, USA) to generate PTEN overexpression vector (pcDNA3.1-PTEN). An empty vector (pcDNA3.1-E) was used as control. RGC-5 cells were transfected with either pcDNA3.1-PTEN or pcDNA3.1-E by Lipofectamine 2000 for 24 h, followed by culturing with fresh DMEM + 10%FBS for another 24 h. The efficiency of endogenously upregulating PTEN by overexpression assay was examined by western blot.

2.9. Statistical analysis

All experiments were repeated in triplicates. Statistical analysis was performed using SPSS 11.0. Data are presented as the mean ± standard deviation. Statistical analyses were performed by analysis of unpaired two-tail Student's t test. P value <0.05 was termed as statistically significant.

3. Results

3.1. Cell viability was decreased and miR-26a was downregulated by H_2O_2 in RGC-5 cells

The effect of H_2O_2-iduced hypoxia on RGC-5 viability was examined by an MTT assay. The RGC-5 cells were treated with different concentrations of H_2O_2 for 24 h. The results showed that H_2O_2 had no toxic effect on RGC-5 cells at the concentrations between 0 and 10 μM, whereas significant toxicity was observed between concentrations of 50–1000 μM (Fig. 1A). We also examined the endogenous expression level of miR-26a gene in RGC-5 cells while they were treated with H_2O_2. We found that, H_2O_2 significantly decreased the expression levels of miR-26a in RGC-5 cells at concentrations between 10 and 1000 μM (Fig. 1B).

3.2. MiR-26a upregulation protects H_2O_2-induced cytotoxicity in RGC-5 cells

As we discovered that H_2O_2 induced cytotoxicity, as well as down-regulated miR-26a in RGC-5 cells, we suspected that miR-26a might have a functional role in regulating H_2O_2-induced cytotoxicity in RGC-5 cells. We thus used miR-26a mimic (miR-26a-mimic) to endogenously overexpress miR-26a genes in RGC-5 cells. The efficiency of miR-26a upregulation was examined by qRT-PCR. We found that, miR-26a gene expression level was markedly upregulated by miR-26a-mimic (50 nM) in RGC-5 cells, as compared to the expression level in cells transfected with a non-specific mimic miRNA (NC-miR, 50 nM) (Fig. 1C). We then pre-treated RGC-5 cells with miR-26a-mimic at concentration range of 0–200 nM for 24 h, followed by another 24 h treatment of 200 μM H_2O_2. We found that miR-26a-mimic reduced H_2O_2-induced RGC-5 cytotoxicity at concentrations between 10 and 200 nM (Fig. 1D). Thus, our results clearly demonstrated that miR-26a upregulation restored cell viability, which was decreased by H_2O_2-induced toxicity in RGC-5 cells.

3.3. MiR-26a upregulation rescued H_2O_2-induced apoptosis in RGC-5 cells

Next, we examined whether overexpressing miR-26a would also protect H_2O_2-induced apoptosis in RGC-5 cells. RGC-5 culture was

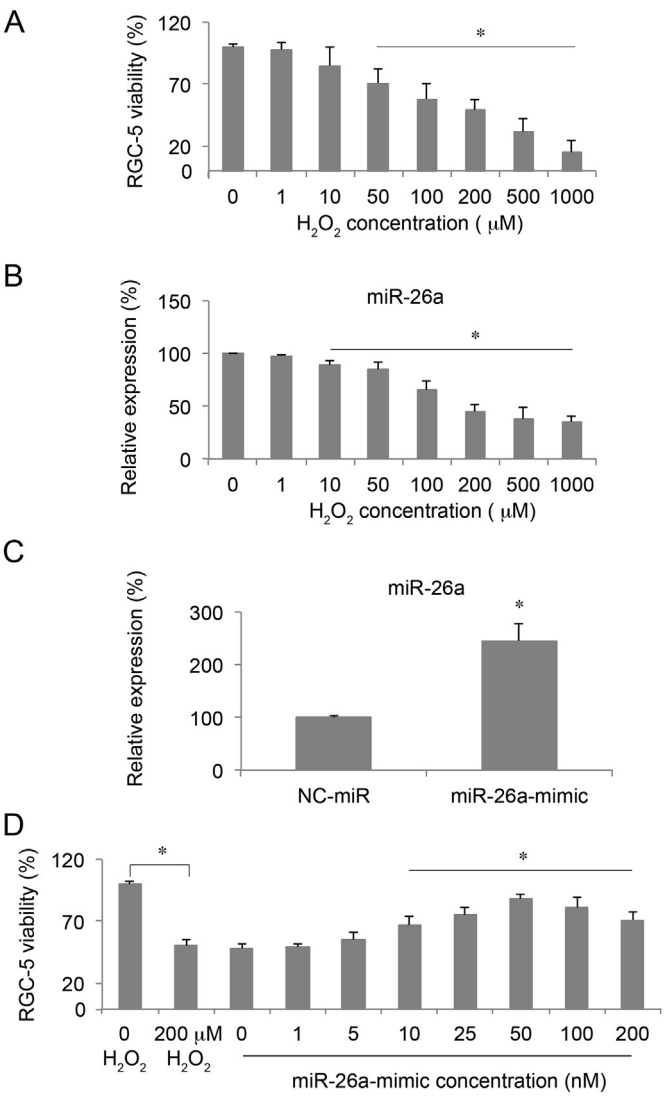

Fig. 1. Protective effect of miR-26a upregulation against H_2O_2-induced toxicity in RGC-5. RGC-5 cells were treated with H_2O_2 at concentrations between 0 and 1000 μM for 24 h. **(A)** An MTT assay was used to examine cell viability. $^*P < 0.05$ vs. 0 μM. **(B)** The relative change of miR-26a gene expression level, induced by H_2O_2, was measured by qRT-PCR. $^*P < 0.05$ vs. 0 μM. **(C)** RGC-5 cells were transfected with a microRNA-26a mimic oligonucleotides (miR-26a-mimic, 50 nM) for 24 h. The endogenous gene expression level of miR-26a was measured by qRT-PCR, and compared to the miR-26a expression level in RGC-5 cells transfected with a non-specific miRNA oligonucleotides (NC-miR, 50 nM). $^*P < 0.05$. **(D)** In RGC-5 culture, cells were treated with 0 μM H_2O_2, 200 μM H_2O_2, or 200 μM H_2O_2 with 24-h pre-treatment of different concentrations of miR-26a-mimic. Twenty-four hours later, MTT assay was used to examine RGC-5 cell viability. $^*P < 0.05$ vs. 0 nM miR-26a-mimic.

divided into three groups. In one group (Control), RGC-5 received neither miRNA transfection nor H_2O_2 treatment. In second group, RGC-5 cells were transfected with 50 nM NC-miR for 24 h, followed by 4-h treatment of 200 μM H_2O_2 treatment. In 3rd group, RGC-5 cells were transfected with 50 nM miR-26a-mimic for 24 h, followed by 4-h treatment of 200 μM H_2O_2. At the end of H_2O_2 treatment, cells were washed with fresh culture media of DMEM + 10% FBS and continuously cultured for additional 24 h, followed by TUNEL assay to evaluate apoptosis. The results showed that while there was no TUNEL positive (indicative of apoptosis) RGC-5 in control group (Fig. 2A, Control), 200 μM H_2O_2 induced significant apoptosis in RGC-5 cells (Fig. 2A, 200 μM H_2O_2/NC-miR). We also discovered that H_2O_2-induced apoptosis was reduced

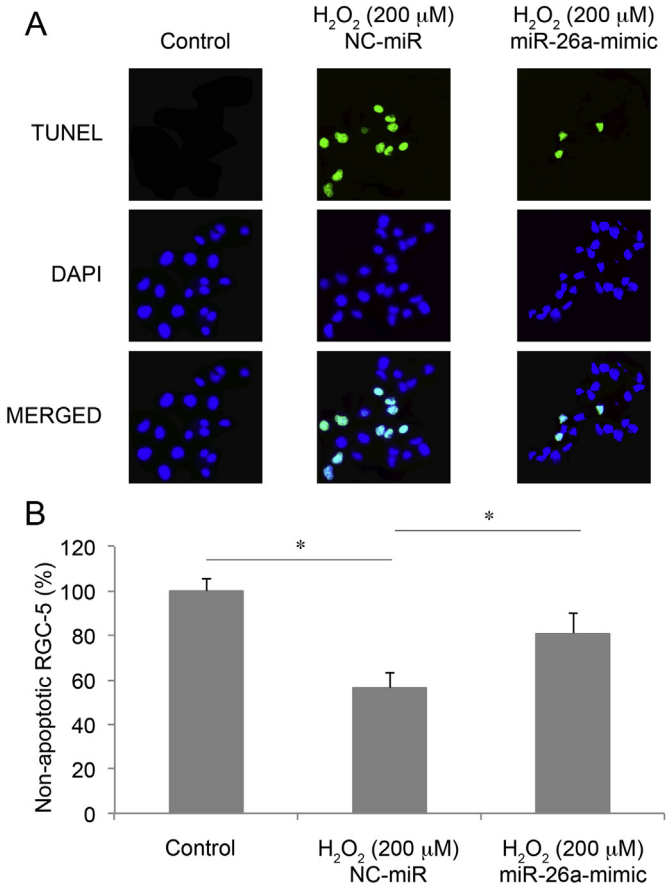

Fig. 2. Protective effect of miR-26a upregulation against H_2O_2-induced apoptosis in RGC-5. **(A)** RGC-5 cells were treated without H_2O_2, or with H_2O_2 (200 μM) for 4 h preceded by 24-h transfection of either NC-miR (50 nM) or miR-26-mimic (50 nM). Twenty-four hours after H_2O_2 treatment, TUNEL assay was performed to assess apoptosis in RGC-5 cells. DAPI staining was used to identify RGC-5 nuclei. **(B)** The statistic result of counting the percentages of non-apoptotic (TUNEL-negative) RGC-5 showed that H_2O_2 induced severe apoptosis, whereas upregulating miR-26a rescued H_2O_2-induced apoptosis in RGC-5 cells. $^*P < 0.05$.

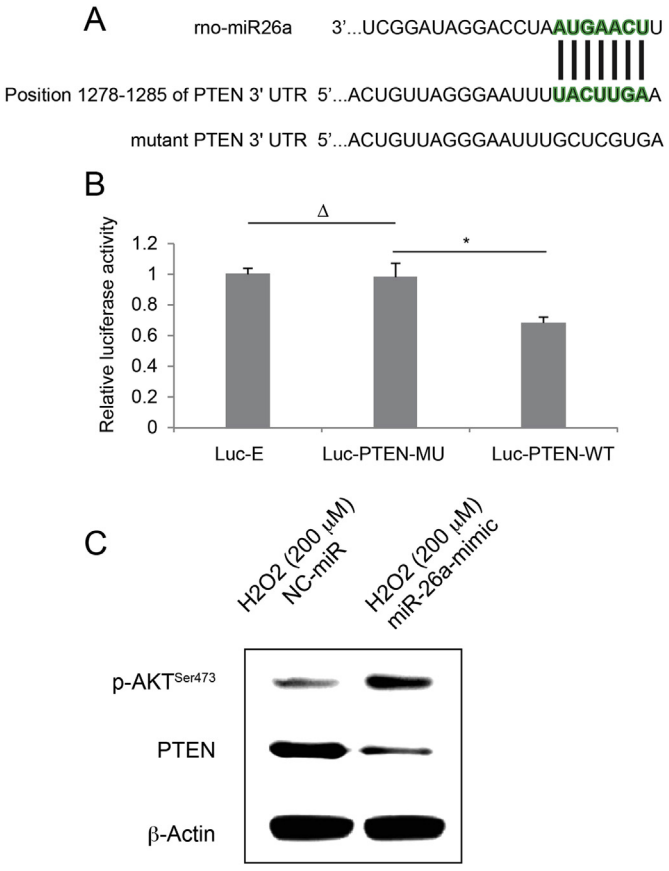

Fig. 3. PTEN pathway was targeted by miR-26a upregulation during H_2O_2-induced apoptosis in RGC-5. **(A)** The Schematic binding site on PTEN 3′-UTR was shown for rat miR-26a. A mutant PTEN 3′-UTR with modified binding sequence was also shown. **(B)** Using a luciferase report assay, HEK293T cells were transfected with an empty psiCHECK-2 vector (Luc-E), psiCHECK-2 vector containing PTEN 3′-UTR (Luc-PTEN-WT), or psiCHECK-2 vector containing mutated PTEN 3′-UTR (Luc-PTEN-MU), along with miR-26a-mimic. Twenty-four hours after transfection, the relative luciferase activities were assessed. $\Delta P > 0.05$. $P < 0.05$. **(C)** In RGC-5 culture, cells were pre-transfected with 50 nM NC-miR or miR-26a-mimic for 24 h, then treated with 200 μM H_2O_2 for 4 h, followed by western blotting analysis on PTEN and phosphor-AKT (ser473).

while miR-26a was upregulated in RGC-5 cells (Fig. 2A, 200 μM H_2O_2/miR-26a-mimic). The quantitative assessment verified our findings with immunostaining of TUNEL assay, as 200 μM H_2O_2 markedly reduced the percentage of non-apoptotic (or healthy) RGC-5 cells, whereas the additional upregulation of miR-26a rescued the apoptotic effect by H_2O_2 (Fig. 2B). Thus, our results demonstrated that MiR-26a upregulation could rescue H_2O_2-induced hypoxia-like apoptosis in RGC-5 cells.

3.4. MiR-26a upregulation modulates PTEN signaling pathway during H_2O_2-induced apoptosis in RGC-5 cells

Next, we assessed the possible molecular pathways involved in miR-26a regulation on RGC-5 apoptosis. Using online miRNA binding predication software (Target Scan, http://www.targetscan.org/), we found that PTEN signaling pathway was very likely to be the target of miR-26a, as miR-26a directly binds the 3′-UTR of PTEN gene (Fig. 3A). Based on this information, we then performed a luciferase reporter assay by transfecting HEK293T cells with miR-26a-mimic and one of the three-luciferase reporter (psiCHECK-2) vectors, an empty non-specific vector (Luc-E), a vector containing the binding sequences of wild type PTEN 3′-UTR (Luc-PTEN-WT), or a vector containing the mutated binding sequences of PTEN 3′-UTR (Luc-PTEN-MU). The results showed that relative luciferase activity of Luc-PTEN-WT was significantly reduced, whereas the luciferase activity of Luc-PTEN-MU was un-affected, by miR-26a upregulation, suggesting miR-26a directly binds PTEN (Fig. 3B).

We then examined whether the direct binding of miR-26a on PTEN gene would result in the change of PTEN protein, as well as modulate PTEN-associated signaling pathways during H_2O_2-induced apoptosis in RGC-5 cells. We pre-transfected RGC-5 cells with either NC-miR (50 nM) or miR-26a-mimic (50 nM) for 24 h, then 200 μM H_2O_2 for 4 h. The western blot showed that miR-26a upregulation significantly reduced PTEN protein, as well as increased phosphorylated-AKT protein during H_2O_2-induced apoptosis in RGC-5 cells (Fig. 3C). Thus, our results strongly suggest that miR-26a upregulation modulated PTEN signaling pathway after H_2O_2-induced apoptosis in RGC-5 cells.

3.5. Overexpressing PTEN reversed the protective effect of miR-26a upregulation on H_2O_2-induced apoptosis in RGC-5 cells

Finally, we asked whether PTEN was also involved in the regulation of miR-26a on RGC-5 apoptosis. We transfected RGC-5 cells with a PTEN overexpression vector (pcDNA3.1-PTEN). One day later, western blot verified that pcDNA3.1-PTEN significantly upregulated

endogenous PTEN protein, as well as down-regulated p-AKT(ser473) protein in RGC-5 cells (Fig. 4A).

We then used pcDNA3.1-PTEN to upregulate PTEN while miR-26a was upregulated during H_2O_2-induced apoptosis in RGC-5 cells. To do so, we transfected RGC-5 cells with 50 nM miR-26-mimic for 24 h, followed by 4-h 200 μM H_2O_2 treatment to induce apoptosis. Twelve hours before the beginning of H_2O_2 treatment, pcDNA3.1-PTEN or pcDNA3.1-E were added into RGC-5 culture. Then, 24 h after H_2O_2 treatment, TUNEL assay was performed to assess apoptosis in RGC-5 cells. The results showed that more apoptotic RGC-5 cells were seen while PTEN was upregulated (Fig. 4B-C). Thus, our results demonstrated that, not only PTEN was regulated by miR-26a, but also overexpressing PTEN could reverse the protective effect of miR-26a upregulation on H_2O_2-induced apoptosis in RGC-5 cells.

4. Discussions

In the present study, we cultured RGC-5 cells *in vitro*, and induced cytotoxicity and apoptosis by applying H_2O_2 to mimic hypoxia injury in RGC-5 cells. We showed that H_2O_2 induced significant cytotoxicity and apoptosis in concentration-dependent manner. Theses results are in line with previous studies showing oxidative stress induced neurotoxicity in RGCs both *in vitro* and *in vivo* [9,28].

Beyond H_2O_2–induced cytotoxicity, we demonstrated in the present study that miR-26a was down-regulated during this process. These result are consistent with a previous report showing miR-26a was highly expressed in normal mammalian eye [22]. However, our data is the first to reveal that miR-26a gene expression level could be modified, most likely reduced, under pathological conditions such as H_2O_2–induced hypoxia. Most importantly in the study, we presented new evidence of miR-26a with a functional role in regulating H_2O_2 –induced cytotoxicity and apoptosis in RGC-5 cells. The family of miR-26 genes, including both miR-26a and miR-26b, are active cancer regulators, serving as either oncogenes to promote cancer development and migration, or tumor suppressor to inhibit tumor proliferation and metastasis [29,30]. In central nervous system, miR-26a overexpression was shown to promote neurite outgrowth in Alzheimer's disease [20]. In peripheral nervous system, miR-26a was shown to be abundantly expressed in spinal cord, yet its function was unknown [31]. Thus, the data of our study showing that upregulating miR-26a reduced H_2O_2–induced apoptosis in RGC-5 cells, not only reinforce the notion that miR-26a is a positive factor in neuroprotection or neuro-development, but also highlight the novel mechanism of miR-26a regulation in retina ganglion cells.

Also in the study, we demonstrated that PTEN signaling pathway was directly associated with the protection of miR-26a upregulation on H_2O_2–induced RGC-5 apoptosis. First, luciferase reporter assay demonstrated that PTEN gene was directly associated with miR-26a gene. Then, ectopic PTEN overexpression upregulated PTEN protein, down-regulated phosphor-AKT, and reduced the protective effect of overexpressing miR-26a on H_2O_2–induced apoptosis. PTEN has been shown to be an important regulator in retinal ganglion development and regeneration [13,25]. Thus, our

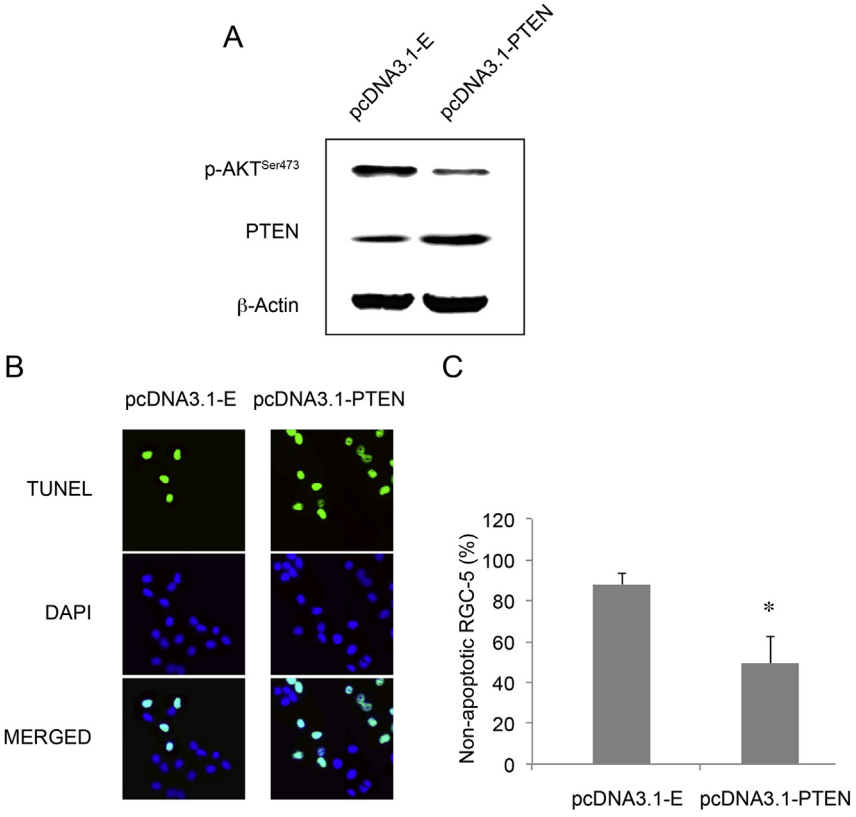

Fig. 4. PTEN pathway was directly involved in miR-26a modulation on H_2O_2-induced apoptosis in RGC-5. **(A)** RGC-5 cells were transfected with a PTEN overexpression vector (pcDNA3.1-PTEN). In controlled RGC-5 culture, cells were transfected with an empty overexpression vector (pcDNA3.1-E). Twenty-four hours after transfection, the endogenous protein levels PTEN and phosphor-AKT(ser473) were then examined by western blot. **(B)** RGC-5 cells were transfected with 50 nM miR-26-mimic for 24 h, followed by 4-h 200 μM H_2O_2 treatment. Twelve hours before the H_2O_2 treatment, pcDNA3.1-PTEN or pcDNA3.1-E were added in the culture. Twenty-four hours after H_2O_2 treatment, TUNEL assay was performed to assess apoptosis in RGC-5 cells. **(C)** The statistic result of counting the percentages of non-apoptotic (TUNEL-negative) RGC-5 showed that PTEN overexpression reversed the protective effect of miR-26a upregulation on H_2O_2–induced apoptosis in RGC-5 cells. $^*P < 0.05$.

data showing PTEN was down-regulated during apoptosis protection and vice versa, upregulating PTEN promoted apoptosis, thus support the notion that PTEN was a negative regulator in retinal ganglion growth.

In summary, our study revealed new mechanism of miR-26a in regulating H_2O_2-induced cytotoxicity and apoptosis in retinal ganglion cells. The protective mechanism of upregulating miR-26a, possibly through the inhibition of PTEN signaling pathway, could be a new method to protect hypoxia-induced neural injury and degeneration in retina.

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.02.164.

References

[1] The Advanced Glaucoma Intervention Study (AGIS): 7, The relationship between control of intraocular pressure and visual field deterioration. The AGIS Investigators, Am. J. Ophthalmol. 130 (2000) 429–440.
[2] J. Caprioli, A.L. Coleman, Intraocular pressure fluctuation a risk factor for visual field progression at low intraocular pressures in the advanced glaucoma intervention study, Ophthalmology 115 (2008), 1123–1129 e1123.
[3] R. Klein, B.E. Klein, The prevalence of age-related eye diseases and visual impairment in aging: current estimates, Invest Ophthalmol. Vis. Sci. 54 (2013) ORSF5–ORSF13.
[4] E.K. Akpek, R.A. Smith, Overview of age-related ocular conditions, Am. J. Manag. Care 19 (2013) S67–S75.
[5] M. Takamoto, M. Araie, Genetics of primary open angle glaucoma, Jpn. J. Ophthalmol. 58 (2014) 1–15.
[6] P. Bettin, F. Di Matteo, Glaucoma: present challenges and future trends, Ophthalmic Res. 50 (2013) 197–208.
[7] L.P. Cohen, L.R. Pasquale, Clinical characteristics and current treatment of glaucoma, Cold Spring Harb. Perspect. Med. 4 (2014).
[8] J.R. SooHoo, L.K. Seibold, M.Y. Kahook, Recent advances in the management of neovascular glaucoma, Semin. Ophthalmol. 28 (2013) 165–172.
[9] M. Almasieh, A.M. Wilson, B. Morquette, J.L. Cueva Vargas, A. Di Polo, The molecular basis of retinal ganglion cell death in glaucoma, Prog. Retin Eye Res. 31 (2012) 152–181.
[10] J. Qu, D. Wang, C.L. Grosskreutz, Mechanisms of retinal ganglion cell injury and defense in glaucoma, Exp. Eye Res. 91 (2010) 48–53.
[11] M. Tong, A. Brugeaud, A.S. Edge, Regenerated synapses between postnatal hair cells and auditory neurons, J. Assoc. Res. Otolaryngol. 14 (2013) 321–329.
[12] A. Brugeaud, M. Tong, L. Luo, A.S. Edge, Inhibition of repulsive guidance molecule, RGMa, increases afferent synapse formation with auditory hair cells, Dev. Neurobiol. 74 (2014) 457–466.
[13] R.L. Zhu, K.S. Cho, C.Y. Guo, J. Chew, D.F. Chen, L. Yang, Intrinsic determinants of optic nerve regeneration, Chin. Med. J. Engl. 126 (2013) 2543–2547.
[14] D. Fischer, M. Leibinger, Promoting optic nerve regeneration, Prog. Retin Eye Res. 31 (2012) 688–701.
[15] H. Gao, H.L. Zhang, J. Shou, L. Chen, Y. Shen, Q. Tang, J. Huang, J. Zhu, Towards retinal ganglion cell regeneration, Regen. Med. 7 (2012) 865–875.
[16] R.S. Pillai, MicroRNA function: multiple mechanisms for a tiny RNA? RNA 11 (2005) 1753–1761.
[17] S. Lumayag, C.E. Haldin, N.J. Corbett, K.J. Wahlin, C. Cowan, S. Turturro, P.E. Larsen, B. Kovacs, P.D. Witmer, D. Valle, D.J. Zack, D.A. Nicholson, S. Xu, Inactivation of the microRNA-183/96/182 cluster results in syndromic retinal degeneration, Proc. Natl. Acad. Sci. U. S. A. 110 (2013) E507–E516.
[18] J. Remenyi, M.W. van den Bosch, O. Palygin, R.B. Mistry, C. McKenzie, A. Macdonald, G. Hutvagner, J.S. Arthur, B.G. Frenguelli, Y. Pankratov, miR-132/212 knockout mice reveal roles for these miRNAs in regulating cortical synaptic transmission and plasticity, PLoS One 8 (2013) e62509.
[19] K.J. Marler, P. Suetterlin, A. Dopplapudi, A. Rubikaite, J. Adnan, N.A. Maiorano, A.S. Lowe, I.D. Thompson, M. Pathania, A. Bordey, T. Fulga, D.L. Van Vactor, R. Hindges, U. Drescher, BDNF promotes axon branching of retinal ganglion cells via miRNA-132 and p250GAP, J. Neurosci. 34 (2014) 969–979.
[20] B. Li, H. Sun, miR-26a promotes neurite outgrowth by repressing PTEN expression, Mol. Med. Reports 8 (2013) 676–680.
[21] L. Shi, M.L. Ko, G.Y. Ko, Rhythmic expression of microRNA-26a regulates the L-type voltage-gated calcium channel alpha1C subunit in chicken cone photoreceptors, J. Biol. Chem. 284 (2009) 25791–25803.
[22] D.G. Ryan, M. Oliveira-Fernandes, R.M. Lavker, MicroRNAs of the mammalian eye display distinct and overlapping tissue specificity, Mol. Vis. 12 (2006) 1175–1184.
[23] M. Groszer, R. Erickson, D.D. Scripture-Adams, R. Lesche, A. Trumpp, J.A. Zack, H.I. Kornblum, X. Liu, H. Wu, Negative regulation of neural stem/progenitor cell proliferation by the Pten tumor suppressor gene in vivo, Science 294 (2001) 2186–2189.
[24] X. Luo, K.K. Park, Neuron-intrinsic inhibitors of axon regeneration: PTEN and SOCS3, Int. Rev. Neurobiol. 105 (2012) 141–173.
[25] K.K. Park, K. Liu, Y. Hu, P.D. Smith, C. Wang, B. Cai, B. Xu, L. Connolly, I. Kramvis, M. Sahin, Promoting axon regeneration in the adult CNS by modulation of the PTEN/mTOR pathway, Science 322 (2008) 963–966.
[26] S.-Y. Li, A.C. Lo, Lutein protects RGC-5 cells against hypoxia and oxidative stress, Int. J. Mol. Sci. 11 (2010) 2109–2117.
[27] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method, Methods 25 (2001) 402–408.
[28] Q. Liu, W.-K. Ju, J.G. Crowston, F. Xie, G. Perry, M.A. Smith, J.D. Lindsey, R.N. Weinreb, Oxidative stress is an early event in hydrostatic pressure–induced retinal ganglion cell damage, Investigative Ophthalmol. Vis. Sci. 48 (2007) 4580–4589.
[29] W. Shen, M. Song, J. Liu, G. Qiu, T. Li, Y. Hu, H. Liu, MiR-26a promotes ovarian cancer proliferation and tumorigenesis, PloS One 9 (2014) e86871.
[30] P. Liu, H. Tang, B. Chen, Z. He, M. Deng, M. Wu, X. Liu, L. Yang, F. Ye, X. Xie, miR-26a suppresses tumour proliferation and metastasis by targeting metadherin in triple negative breast cancer, Cancer Lett. 357 (2015) 384–392.
[31] H. Hohjoh, T. Fukushima, Expression profile analysis of microRNA (miRNA) in mouse central nervous system using a new miRNA detection system that examines hybridization signals at every step of washing, Gene 391 (2007) 39–44.