European Review for Medical and Pharmacological Sciences 2019; 23: 3173-3182

Long non-coding RNA ANRIL down-regulates microRNA-7 to protect human trabecular meshwork cells in an experimental model for glaucoma

J. ZHAO[1], H. SUN[2], J.-M. ZHANG[1], M. WANG[1], X.-J. DU[3], J.-L. ZHANG[4]

[1]Department of Ophthalmology, Linyi People's Hospital, Linyi, Shandong, China
[2]Department of Ophthalmology, The First People's Hospital of Tancheng, Tancheng County, Shandong, China
[3]Department of Ophthalmology, Eye Institute of Shandong University of Traditional Chinese Medicine, Affiliated Eye Hospital of Shandong University of TCM, Jinan, Shandong, China
[4]Department of Ophthalmology, The People's Hospital of Pingyi County, Pingyi County, Shandong, China

Abstract. – OBJECTIVE: Although the potential involvements of INK4 locus reported in glaucoma, the effects of long non-coding RNA (lncRNA) antisense noncoding RNA in the INK4 locus (ANRIL) on trabecular meshwork (TM) cells remain unclear. In this study, we aimed to explore the effects of lncRNA ANRIL on the oxidative injury of human TM cells as well as the underlying mechanisms.

MATERIALS AND METHODS: Oxidative injury of human TM cells was induced by H_2O_2 stimulation. Cell viability, apoptotic cells, expression of proteins related to apoptosis, and reactive oxygen species (ROS) level were testified by CCK-8 assay, flow cytometry assay, Western blot analysis, and DCFH-DA staining, respectively. LncRNA ANRIL was overexpressed, and its effects on H2O2-injured TM cells were analyzed. Afterward, miR-7 expression in lncRNA ANRIL overexpressing-cells was determined by RT-qPCR. Moreover, it was verified whether lncRNA ANRIL affected H_2O_2-treated TM cells via miR-7, followed by the measurements of the involved signaling pathways.

RESULTS: H_2O_2-induced decrease of cell viability and the increases in apoptosis and ROS generation were significantly attenuated by lncRNA ANRIL overexpression. miR-7 expression was down-regulated by lncRNA ANRIL, and miR-7 overexpression abrogated the effects of lncRNA ANRIL on H_2O_2-treated TM cells. Phosphorylation levels of the key kinases in the mTOR and MEK/ERK pathways were enhanced by lncRNA ANRIL via down-regulating miR-7 in H2O2-treated TM cells.

CONCLUSIONS: LncRNA ANRIL attenuated oxidative injury of human TM cells and activated the mTOR and MEK/ERK pathways, possibly through down-regulating miR-7.

Key Words:
Glaucoma, Oxidative stress, LncRNA ANRIL, MicroRNA-7, MTOR/MEK/ERK.

Introduction

Being a neurodegenerative disease of the eye, the glaucoma is the second leading cause of irreversible blindness that behind cataract and affects more than 70 million individuals globally[1,2]. In patients with glaucoma, retinal ganglion cells which convey visual messages from the retina to the brain, as well as the visual field, are selectively lost, and the retinal ganglion cell axons and somas are degenerated[3-5]. Glaucoma is asymptomatic at the beginning, and it may not be diagnosed until a substantial loss of vision has occurred. Therefore, the actual number of patients with glaucoma may be even higher than the numbers estimated, making the exploration of the effective therapies for glaucoma important[6].

Several risk factors may contribute to glaucoma, including age, intraocular pressure (IOP), reduced ocular perfusion pressure, and genetic mutations[7]. Among them, IOP is the only modifiable risk factor for the development and progression of glaucoma, and lowing IOP is the only therapy which can effectively reduce glaucomatous progression[8,9]. IOP homeostasis is previously defined

Corresponding Author: Jinlian Zhang, MD; e-mail: jinlianzhang0131@sina.com

as corrective adjustments of the aqueous humor outflow resistance in response to pressure changes[10]. IOP is regulated by the resistance to aqueous humor outflow since the aqueous humor inflow is relatively constant and pressure-insensitive. The trabecular meshwork (TM) is a series of fenestrated beams and sheets of the extracellular matrix, through which aqueous humor leaves the anterior chamber of the eye[11]. Previous studies[12,13] have pointed out that the probable site of the resistance to aqueous humor outflow is located within the juxtacanalicular and Schlemm's canal. Therefore, the regulatory mechanism in TM cells isolated from the juxtacanalicular is of great importance for the clinical treatment of glaucoma.

Long non-coding RNAs (lncRNAs) are a group of RNAs which are not involved in the protein generation while they are involved in the regulatory processes[14]. Currently, an increasing number of lncRNAs are identified to participate in the development of the glaucoma, such as lncRNA-MEG3 and lncRNA-MALAT1[15,16]. The INK4 locus at chromosome 9p21, which encodes three tumor suppressor genes and the lncRNA antisense noncoding RNA in the INK4 locus (ANRIL), has been reported to be related to primary open angle glaucoma (POAG) and normal-tension glaucoma[17,18]. Therefore, we hypothesized that there might be a relationship between lncRNA ANRIL and glaucoma.

In this study, we induced *in vitro* oxidative injury model using human TM cells and studied the effects of lncRNA ANRIL on H_2O_2-treated human TM cells as well as the regulatory mechanism. We aimed to explore the potential regulatory mechanism of lncRNA ANRIL in glaucoma, assisting in the identification of novel therapies for glaucoma.

Materials and Methods

Cell Culture and Treatment

Primary human TM cells, isolated from the juxtacanalicular and corneoscleral regions of the human eye and cryopreserved at P0, were purchased from Sciencell Research Laboratories (Carlsbad, CA, USA). Cells were maintained in Trabecular Meshwork Cell Medium (TMCM) containing 2% fetal bovine serum (FBS), 1% trabecular meshwork cell growth supplement and 1% penicillin/streptomycin solution (all from Sciencell Research Laboratories, Carlsbad, CA, USA), and were plated in poly-L-lysine-coated surface (2 μg/cm^2). Cell maintaining was performed in a humidified incubator with 5% CO_2 and 95% air at 37°C. Cells in passages 2-5 were used for experiments. Culture medium was refreshed every three days until the culture reached 70% confluence, whereas it was refreshed every other day when the confluence of cells was 70%-90%. For the stimulation with H_2O_2, the cells were cultured in TMCM containing 50-400 μM H_2O_2 (Sigma-Aldrich, St. Louis, MO, USA) for 24 h after they reached 80-85% confluence.

Cell Transfection

Full-length human lncRNA ANRIL sequences were ligated into a pcDNA3.1 plasmid (Invitrogen, Carlsbad, CA, USA), and the recombined plasmid was sequenced and referred to as pc-ANRIL. Either pcDNA3.1 or pc-ANRIL was transfected into TM cells using the lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) following the instructions provided by the manufacturer. The stable transfected cell lines were harvested after selection in the culture medium containing 0.5 mg/mL G418 (Sigma-Aldrich, St. Louis, MO, USA) for approximately 4 weeks. For the transient transfection with microRNAs (miRs), the scramble miRs or miR-7 mimic, synthesized by GenePharma (Shanghai, China), were transfected into TM cells using the lipofectamine 3000 reagent.

Cell Viability Assay

TM cell viability was examined using a Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Gaithersburg, MD, USA). In brief, TM cells were seeded in poly-L-lysine-coated 96-well plates at 5×10^3 cells per well. Then, the cells were incubated at 37°C overnight for growth. After cell transfection and/or stimulation with H_2O_2, the culture medium was replaced by TMCM containing 10% CCK-8 solution and cells were incubated at 37°C for an additional 1 h. Finally, the absorbance at 450 nm was detected by using a Microplate Reader (Bio-Rad, Hercules, CA, USA).

Apoptosis Assay

Early-stage apoptotic cells after cell transfection and/or stimulation with H_2O_2 were identified by using the FITC Annexin V/Dead Cell Apoptosis Kit with FITC Annexin V and PI, for Flow Cytometry (Invitrogen, Carlsbad, CA, USA). In brief, after the desired treatments, TM cells were collected and washed in phosphate-buffered saline (PBS). Then, the cells were suspended in the

binding buffer provided by the kit, followed by staining with 5 μL of FITC Annexin V and 100 ng of propidium iodide (PI) according to the manufacturer's instructions. Finally, the stained cells were subjected to a FACS can (Beckman Coulter, Fullerton, CA, USA), and the percentage of apoptotic cells was analyzed using the FlowJo software (Tree Star, San Carlos, CA, USA).

Reactive Oxygen Species (ROS) Assay

Intracellular ROS level in TM cells was measured by using a Reactive oxygen species Assay Kit (E004, Nanjing Jiancheng, Nanjing, China). In brief, after cell transfection and/or stimulation with H_2O_2, the cell culture was discarded, and the cells were washed with PBS two times. Then, the cells were incubated in TMCM containing 10 μM 2,7-dichlorofluorescein diacetate (DCFH-DA) for 30 min at 37°C in the dark. Subsequently, after being rinsed in PBS two times, the cells were collected by trypsin treatment (0.25% trypsin-EDTA) and centrifugation. Cells were washed again using PBS and resuspended in 500 μL PBS, followed by measurements of fluorescent intensity by using a flow cytometer (485 nm excitation, 525 nm emission).

RNA Immunoprecipitation (IP)

RNA IP was performed by using Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Bedford, MA). The wild-type ANRIL or the mutant ANRIL (mutant in the predicted miR-7 binding site) was inserted into a pc-DNA3.1-MS2 plasmid (Addgene, Cambridge, USA). The constructed vectors were co-transfected with pMS2-GFP (Addgene, Cambridge, USA) into the cell by using lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA). Then, the cell lysate was extracted and incubated with magnetic beads, GFP antibody (ab290, Abcam, Cambridge, UK) or IgG (ab190475, Abcam, Cambridge, UK). Reverse transcription-quantitative PCR (RT-qPCR) was then performed to detect signals from miR-7 specifically binding to ANRIL.

RT-qPCR

After cell transfection and/or stimulation with H_2O_2, TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used for the extraction of total RNA according to the manufacturer's instructions. For the quantification of lncRNA ANRIL expression, the One Step SYBR® PrimeScript™ PLUS RT-RNA PCR Kit (TaKaRa Biotechnology, Dalian, China) was used following the supplier's protocol. For quantification of miR-7 expression, cDNA was prepared from 500 ng RNA using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). The thermocycling program of reverse transcription was 30 min at 16°C, 30 min at 42°C, and 5 min at 85°C. Real-time PCR was performed using the TaqMan Universal Master Mix II (Applied Biosystems, Foster City, CA, USA) on a Bio-Rad iQ5 Real-time PCR Detection System (Bio-Rad, Hercules, CA, USA). The temperature profile was 1 cycle at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Relative expression fold of lncRNA ANRIL and miR-7 was analyzed on the basis of the $2^{-\Delta\Delta Ct}$ method[19]. GAPDH and U6 were used as housekeeping genes for relative quantification of lncRNA ANRIL and miR-7, respectively.

Western Blot Analysis

After cell transfection and/or stimulation with H_2O_2, the cells were collected and washed in ice-cold PBS. After centrifugation to the pellet, the cells were suspended in RIPA buffer (Beyotime Institute of Biotechnology, Shanghai, China) supplemented with 1 mM PMSF (Beyotime Institute of Biotechnology, Shanghai, China). The whole cell lysates were centrifuged, and the protein content in the supernatant was measured with the bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA). Proteins were separated by SDS-PAGE and electrotransferred to polyvinylidene difluoride (PVDF) membranes. Then, the PVDF membranes were blocked with 5% non-fat milk and incubated with primary antibody and HRP-conjugated goat anti-rabbit IgG (ab205718, Abcam, Cambridge, UK), successively. Primary antibodies used in this study included anti-Bcl-2 antibody (ab196495), anti-Bax antibody (ab32503), anti-pro caspase-3 antibody (ab90437), anti-cleaved caspase-3 antibody (ab2302), anti-pro caspase-9 antibody (ab32539), anti-cleaved caspase-9 antibody (ab2324), anti-p70S6K antibody (ab186753), anti-phospho (p)-p70S6K antibody (ab59208), anti-mTOR antibody (ab32028), anti-p-mTOR antibody (ab109268), anti-MEK antibody (ab178876), anti-p-MEK antibody (ab194754), anti-ERK antibody (ab115799), anti-p-ERK antibody (ab214036) and anti-β-actin antibody (ab8227; all from Abcam, Cambridge, UK). The proteins in the PVDF membranes were visualized using the chemiluminescence detection (ECL Plus; GE Healthcare Bio-Science, Piscataway, NJ, USA).

Statistical Analysis

Experiments were performed in triplicate with three repeats. Data are presented as the mean ± standard deviation (SD). Statistical analysis was performed using GraphPad Prism 5 software (GraphPad, San Diego, CA, USA). The comparison was evaluated using the unpaired two-tailed *t*-test, one-way analysis of variance (ANOVA) or multiple *t*-tests. ANOVA was conducted and was followed by the Tukey post-hoc test. $p < 0.05$ was considered a significant difference.

Results

Stimulation With H2O2 Induced Oxidative Injury in Human TM Cells

Oxidative injury of human TM cells was induced by stimulation with H_2O_2. Cells were stimulated with increasing doses of H_2O_2, and CCK-8 results showed that cell viability was significantly reduced by 100 μM H_2O_2 ($p < 0.05$), 200-300 μM H_2O_2 ($p < 0.01$), and 400 μM H_2O_2 ($p < 0.001$, Figure 1A), as compared to the control group. Since cell viability was dropped by more than 50% when the concentration of H_2O_2 was 200 μM, the oxidative injury of TM cells was induced by 200 μM H_2O_2 in subsequent experiments. Cell apoptosis and ROS generation were analyzed afterward to evaluate whether the oxidative injury was successfully induced. In Figure 1B, the percentage of apoptotic cells in the H_2O_2 group was dramatically higher than the control group ($p < 0.001$). Likewise, H_2O_2 stimulation up-regulated pro-apoptotic Bax expression while down-regulated anti-apoptotic Bcl-2 expression and expression of cleaved caspase-3 and cleaved caspase-9 was observed in the H_2O_2-treated cells (Figure 1C). The results indicated that H_2O_2 stimulation elevated cell apoptosis. Finally, we also found that ROS level in the H_2O_2 group was remarkably higher than the control group ($p < 0.001$, Figure 1D). the results collectively illustrated that oxidative injury of human TM cells was successfully induced after H_2O_2 stimulation.

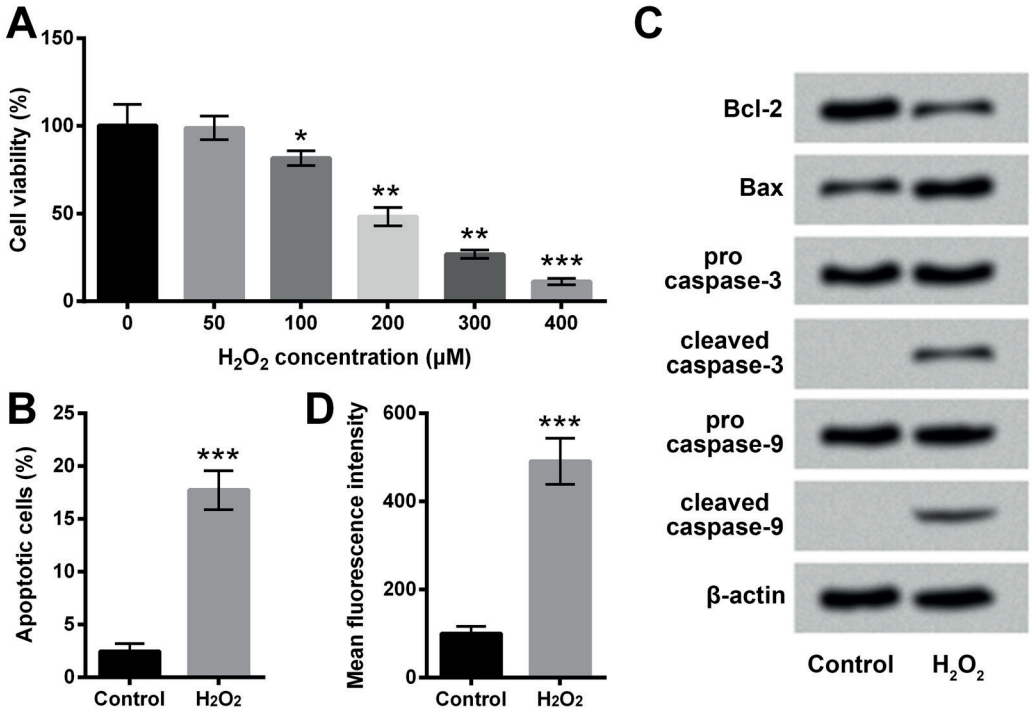

Figure 1. Stimulation with H_2O_2 induced oxidative injury in human TM cells. Human TM cells were stimulated with increasing doses of H_2O_2 for 24 h. ***A,*** Cell viability was measured by the CCK-8 assay. The cells were stimulated with 200 μM H_2O_2 for 24 h, and non-treated cells were acted as control. ***B,*** Percentage of apoptotic cells was determined by flow cytometry assay. ***C,*** Expression of proteins associated with apoptosis was evaluated by Western blot analysis. ***D,*** ROS generation was estimated by DCFH-DA staining. Data are presented as the mean ± SD of at least three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

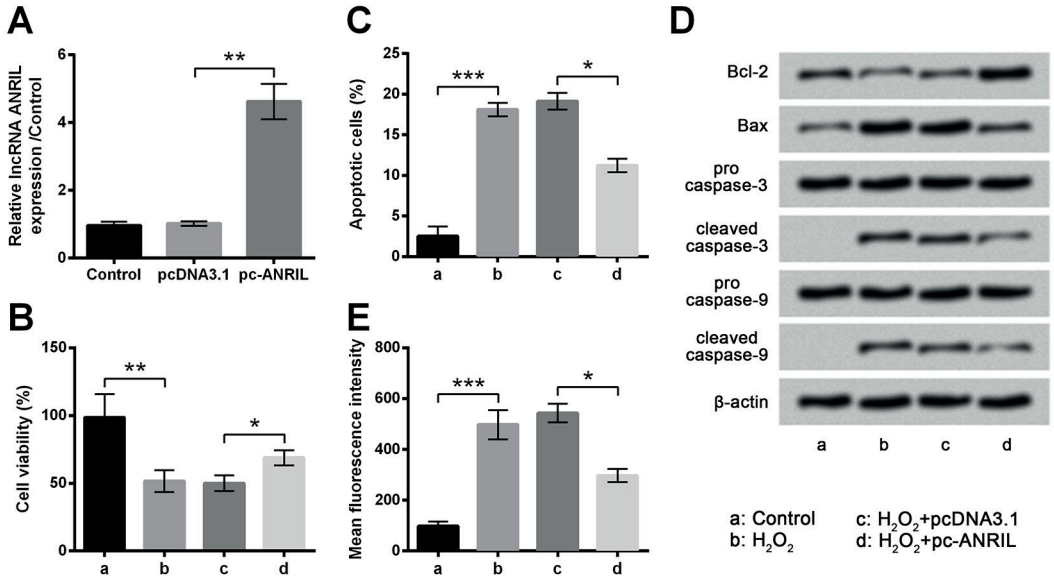

Figure 2. LncRNA ANRIL attenuated H_2O_2-induced TM cell injury. Human TM cells were transfected with pcDNA3.1 or pc-ANRIL, and untransfected cells were acted as control. ***A,*** Expression of lncRNA ANRIL was analyzed by RT-qPCR. Cells transfected with pcDNA3.1 or pc-ANRIL and untransfected cells were stimulated with 200 μM H_2O_2 for 24 h. Non-treated cells were acted as control. ***B,*** Cell viability was measured by the CCK-8 assay. ***C,*** Percentage of apoptotic cells was determined by flow cytometry assay. ***D,*** Expression of proteins associated with apoptosis was evaluated by Western blot analysis. ***E,*** ROS generation was estimated by DCFH-DA staining. Data are presented as the mean ± SD of at least three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Overexpression of LncRNA ANRIL Attenuated H_2O_2-Induced TM Cell Injury

Effects of lncRNA ANRIL on the oxidative injury of TM cells were analyzed. After cell transfection, lncRNA ANRIL level in cells transfected with pc-ANRIL was markedly higher than the cells transfected with pcDNA3.1 ($p < 0.01$), suggesting that lncRNA ANRIL was overexpressed successfully after cell transfection (Figure 2A). Then, transfected and untransfected cells were stimulated with 200 μM H_2O_2 for 24 h, and non-treated cells were acted as control. The CCK-8 assay showed that lncRNA ANRIL overexpression significantly elevated cell viability of H_2O_2-treated cells ($p < 0.05$, Figure 2B). Flow cytometry assay showed that lncRNA ANRIL overexpression observably reduced the percentage of apoptotic cells in H_2O_2-treated cells ($p < 0.05$, Figure 2C). Meanwhile, lncRNA ANRIL overexpression could up-regulate Bcl-2 expression while down-regulating the expression of Bax, cleaved caspase-3 and cleaved caspase-9 compared to the H_2O_2 + pcDNA3.1 group (Figure 2D). Besides, the ROS level in cells overexpressing lncRNA ANRIL was significantly lower than pcDNA3.1-transfected cells after H_2O_2 stimulation ($p < 0.05$, Figure 2E). The results collectively indicated that lncRNA ANRIL overexpression could attenuate H_2O_2-induced oxidative injury in TM cells.

Overexpression of LncRNA ANRIL Down-Regulated MiR-7 Expression

Effects of lncRNA ANRIL overexpression on the miR-7 level were testified. In Figure 3A, the miR-7 expression level in cells overexpressing lncRNA ANRIL was significantly lower than the pcDNA3.1-transfected cells ($p < 0.01$), indicating that lncRNA ANRIL might down-regulate miR-7 expression in TM cells. To further reveal the regulatory relationship between lncRNA ANRIL and miR-7, anti-MS2 RNA IP was performed. RT-qPCR data in Figure 3B showed that the ANRIL RNA IP in MS2-ANRIL group was significantly enriched for miR-7 compared to the MS2-ANRIL-mut group ($p < 0.01$). These results evidenced that lncRNA ANRIL down-regulated miR-7 expression might be via directly binding with miR-7.

Overexpression of LncRNA ANRIL Alleviated H_2O_2-Induced Injury Via Down-Regulating MiR-7

We then studied whether lncRNA ANRIL affected H_2O_2-treated TM cells via regulation of

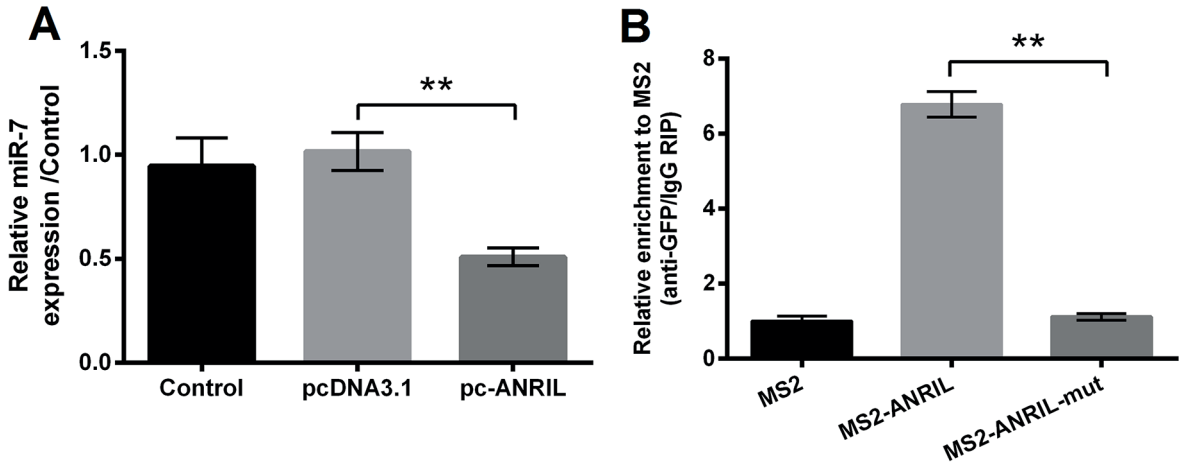

Figure 3. LncRNA ANRIL down-regulated miR-7 expression. ***A,*** Human TM cells were transfected with pcDNA3.1 or pc-ANRIL, and untransfected cells were acted as control. Expression of miR-7 was analyzed by RT-qPCR. ***B,*** Human TM cells were co-transfected with pMS2-GFP and MS2, MS2-ANRIL or MS2-AMRIL-mut. RNA IP was performed to pull down the miRNAs associated with lncRNA ANRIL. Data are presented as the mean ± SD of at least three independent experiments. **, $p < 0.01$.

miR-7. In Figure 4A, miR-7 level in cells transfected with miR-7 mimic was prominently higher than cells transfected with scramble miRs ($p < 0.01$), suggesting that miR-7 level could be elevated after cell transfection. Then, the co-transfected cells or untransfected cells were stimulated with H_2O_2 for 24 h, and non-treated cells acted as control. The results showed that lncRNA ANRIL overexpression-induced alteration of cell viability, apoptosis. The ROS generation was notably reversed by miR-7 overexpression in H_2O_2-treated cells, as evidenced by a significant reduction of cell viability ($p < 0.05$, Figure 4B), enhancement of apoptotic cells ($p < 0.05$, Figure 4C), down-regulation of Bcl-2 and up-regulation of Bax, cleaved caspase-3 and cleaved caspase-9 (Figure 4D), and an increase of ROS generation ($p < 0.05$, Figure 4E), as compared to the H_2O_2 + pc-ANRIL + Scramble group. The results indicated that lncRNA ANRIL might attenuate oxidative injury of TM cells through down-regulating miR-7.

Overexpression of LncRNA ANRIL Activated the mTOR and MEK/ERK Pathways Via Regulation of miR-7

The possible involved signaling pathways were finally investigated. In Figure 5A-5B, we found that the phosphorylation levels of p70S6K, mTOR, MEK, and ERK were all dramatically decreased after H_2O_2 stimulation compared with the control group ($p < 0.01$ or $p < 0.001$). Those significant decreases were all dramatically reversed by lncRNA ANRIL overexpression compared with the H_2O_2 + pcDNA3.1 + Scramble group (all $p < 0.001$). Moreover, the effects of lncRNA ANRIL overexpression were all significantly bated by miR-7 overexpression relative to the H_2O_2 + pc-ANRIL + Scramble group. The results suggested that ANRIL might activate the mTOR and MEK/ERK pathways via regulation of miR-7.

Discussion

IOP, the major risk factor for progression of glaucoma, is closely associated with TM cells. Although the potential involvements of the INK4 locus at chromosome 9p21 have been reported in glaucoma, the effects of lncRNA ANRIL which was encoded from the INK4 locus on TM cells remain (remains) unclear. In this study, we reported for the first time that lncRNA ANRIL could attenuate oxidative injury of human TM cells induced by H_2O_2. We also found that miR-7 expression was down-regulated by lncRNA ANRIL overexpression, and miR-7 overexpression could reverse the effects of lncRNA ANRIL on H_2O_2-treated TM cells. Furthermore, we also found that lncRNA ANRIL could activate the mTOR and MEK/ERK pathways, possibly through down-regulating miR-7 expression.

Previous studies[20,21] have indicated that there is a relationship between elevated IOP and impairment of mitochondrial dynamics in the glau-

comatous retina. Oxidative stress is an important pathophysiological mechanism in mitochondrial dysfunction and glaucoma[22]. Moreover, the oxidative injury of TM cells has been reported to obstruct aqueous humor outflow and thereby to elevate IOP[23]. Therefore, we focused on the effects of lncRNA ANRIL on the oxidative injury of human TM cells, aiming to explore the potential role of lncRNA ANRIL in the progression of glaucoma. In the literature by Kuespert et al[24], oxidative stress in human TM cells was induced by the stimulation with H_2O_2. In the present study, cell viability was reduced, and apoptosis was enhanced after H_2O_2 stimulation. Meanwhile, H_2O_2-induced en-

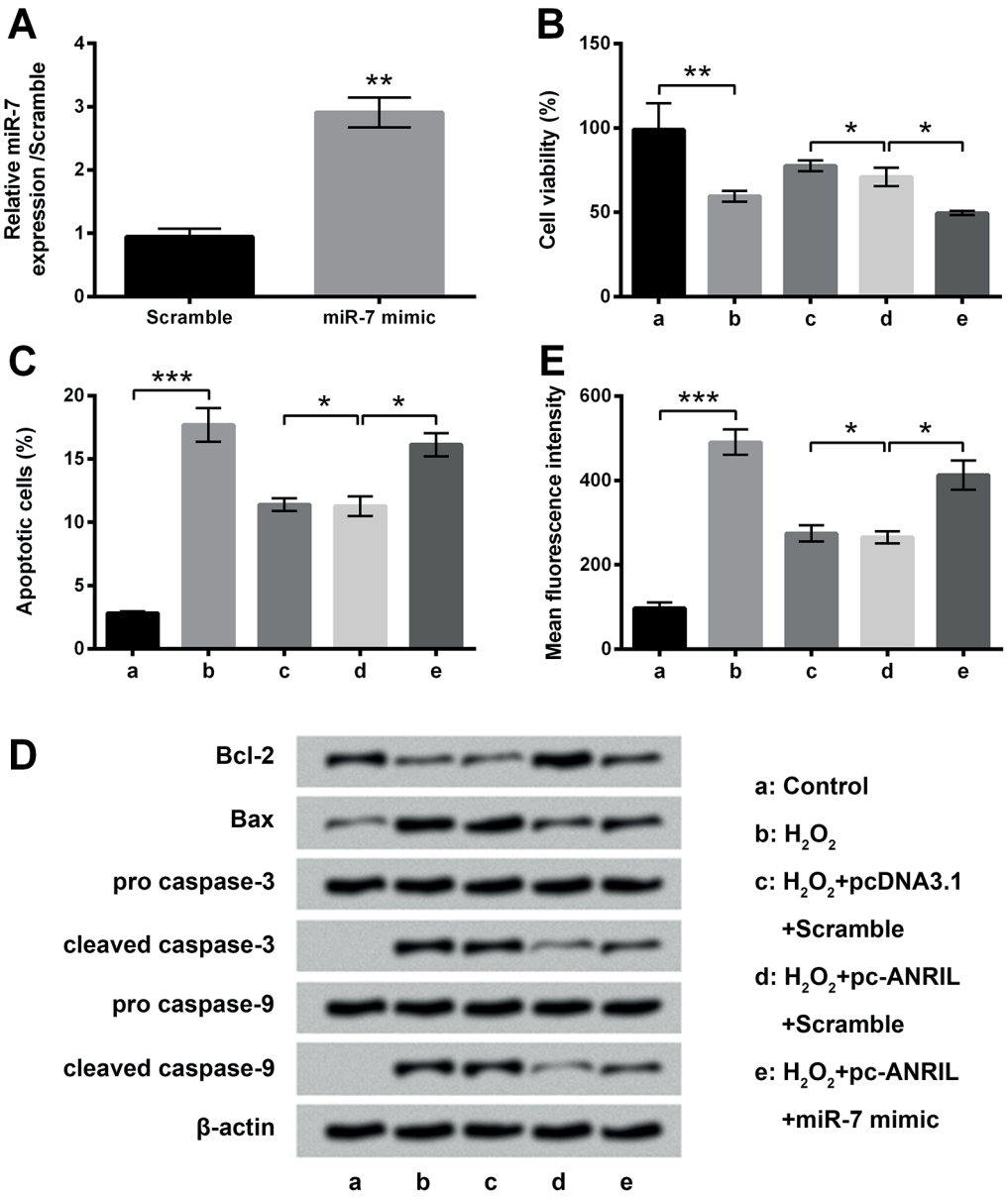

Figure 4. LncRNA ANRIL affected H_2O_2-treated TM cells via regulation of miR-7. Human TM cells were transfected with scramble miRs or miR-7 mimic. ***A,*** Expression of miR-7 was analyzed by RT-qPCR. Cells co-transfected with pc-ANRIL (pcDNA3.1), and miR-7 mimic (scramble miRs) and untransfected cells were stimulated with 200 μM H_2O_2 for 24 h. Non-treated cells were acted as control. ***B,*** Cell viability was measured by the CCK-8 assay. ***C,*** Percentage of apoptotic cells was determined by flow cytometry assay. ***D,*** Expression of proteins associated with apoptosis was evaluated by Western blot analysis. ***E,*** ROS generation was estimated by DCFH-DA staining. Data are presented as the mean ± SD of at least three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

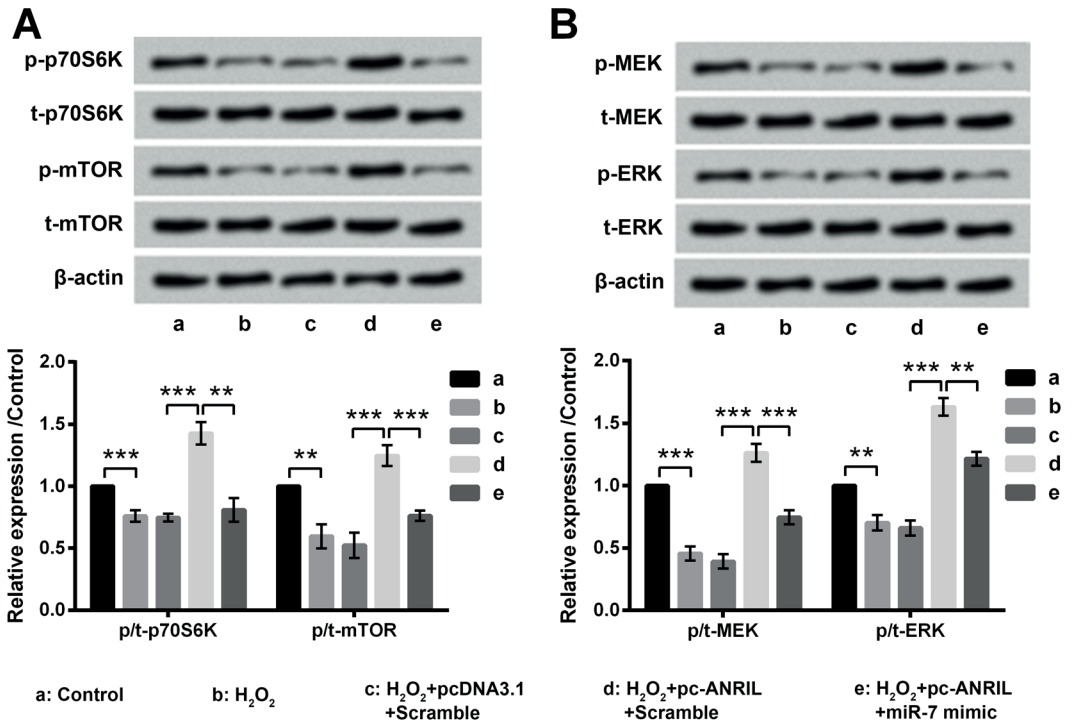

Figure 5. LncRNA ANRIL might activate the mTOR and MEK/ERK pathways via regulation of miR-7. Human TM cells were co-transfected with pc-ANRIL (pcDNA3.1), and miR-7 mimic (scramble miRs) and untransfected cells were stimulated with 200 μM H_2O_2 for 24 h. Non-treated cells were acted as control. Expression of key kinases in the mTOR pathway (***A***) and MEK/ERK pathway (***B***) was measured by Western blot analysis. Data are presented as the mean ± SD of at least three independent experiments. **, $p < 0.01$; ***, $p < 0.001$.

hancements of ROS level illustrated that oxidative stress in TM cells was induced successfully since the elevated intracellular ROS level is an indicator of oxidative stress. Those alterations induced by H_2O_2 could be reduced by lncRNA ANRIL overexpression, indicating that lncRNA ANRIL could attenuate oxidative injury of TM cells. The effects of lncRNA ANRIL on oxidative injury in TM cells were consistent with that reported in PC-12 cells previously[25].

MiRs are single-stranded non-coding RNAs that are involved in numerous biological processes[26]. Recently, a growing number of studies proved that the regulatory role of lncRNA ANRIL was associated with miRs. For example, the proliferation and metastasis of hepatocellular carcinoma cells were suppressed[27] by lncRNA ANRIL silence via regulation of miR-122-5p. Chai et al[28] have reported the oncogenic role of lncRNA ANRIL in oral cancer via regulation of miR-125a. Hence, we focused on the downstream miRs to explore the regulatory mechanism of lncRNA ANRIL. Level of miR-7 is increased in TM cells upon treatment with mechanical stress, which might be a contributor to the progression of glaucoma[29,30]. Therefore, we speculated that if there is a relationship between miR-7 and lncRNA ANRIL in TM cells, the effects of lncRNA ANRIL on TM cells might be explained. Accordingly, we found that miR-7 expression was down-regulated by lncRNA ANRIL overexpression, and miR-7 overexpression could abrogate the effects of lncRNA ANRIL on the oxidative injury of TM cells. This means that the down-regulation of miR-7 induced by lncRNA ANRIL might be an explanation for the protective role of lncRNA ANRIL against oxidative injury. Besides, the anti-growth and pro-apoptotic roles of miR-7 in TM cells were consistent with that in cancer cells[31].

mTOR is a serine/threonine kinase that modulates cell growth and metabolism. mTOR is inhibited under stimulation with H_2O_2, and the phosphorylation of its endogenous substrate such as p70S6K is reduced[32]. The MEK/ERK pathway is the classical anti-apoptotic pathway, and it can promote proliferation in many types of cells[33,34]. Zhu et al[35] have shown that the activation of the MEK/ERK pathway induced by melatonin can abolish the effects

of H_2O_2 on cardiac microvascular endothelial cells. Ran et al[25] also proved that myeloid cell leukemia 1 activated the MEK/ERK pathway and thereby attenuated H_2O_2-induced PC-12 cell injury. Therefore, we studied the alteration of the mTOR and MEK/ERK pathways in TM cells under stimulation with H_2O_2. Consistent with the literature described above, these two pathways were inhibited by H_2O_2 stimulation, and the inhibition was abrogated by lncRNA ANRIL overexpression. Interestingly, miR-7 overexpression reversed the activation of these two pathways induced by lncRNA ANRIL.

Conclusions

Preliminarily we explored the protective role of lncRNA ANRIL in human TM cells against oxidative stress as well as the regulatory mechanism. We reported for the first time that lncRNA ANRIL could attenuate oxidative injury of TM cells via down-regulation of miR-7. Moreover, lncRNA ANRIL could activate the mTOR and MEK/ERK pathways through miR-7. The protective effects of lncRNA ANRIL on TM cells might provide innovative strategies for the treatments of glaucoma. More experiments performed in animals are needed in the future to support these conclusions.

Conflict of Interests

The Authors declare that they have no conflict of interests.

References

1) Kimura A, Namekata K, Guo X, Harada C, Harada T. Dock3-NMDA receptor interaction as a target for glaucoma therapy. Histol Histopathol 2017; 32: 215-221.
2) Williams PA, Harder JM, Foxworth NE, Cochran KE, Philip VM, Porciatti V, Smithies O, John SW. Vitamin B3 modulates mitochondrial vulnerability and prevents glaucoma in aged mice. Science 2017; 355: 756-760.
3) Saunders LJ, Russell RA, Kirwan JF, McNaught AI, Crabb DP. Examining visual field loss in patients in glaucoma clinics during their predicted remaining lifetime. Invest Ophthalmol Vis Sci 2014; 55: 102-109.
4) Mac Nair CE, Nickells RW. Neuroinflammation in glaucoma and optic nerve damage. Prog Mol Biol Transl Sci 2015; 134: 343-363.
5) Kim KY, Perkins GA, Shim MS, Bushong E, Alcasid N, Ju S, Ellisman MH, Weinreb RN, Ju WK. DRP1 inhibition rescues retinal ganglion cells and their axons by preserving mitochondrial integrity in a mouse model of glaucoma. Cell Death Dis 2015; 6: e1839.
6) Micheal S, Hogewind BF, Khan MI, Siddiqui SN, Zafar SN, Akhtar F, Qamar R, Hoyng CB, den Hollander AI. Variants in the PRPF8 gene are associated with glaucoma. Mol Neurobiol 2018; 55: 4504-4510.
7) McDonnell F, Irnaten M, Clark AF, O'Brien CJ, Wallace DM. Hypoxia-induced changes in DNA methylation alter RASAL1 and TGFbeta1 expression in human trabecular meshwork cells. PLoS One 2016; 11: e0153354.
8) Heijl A, Leske MC, Bengtsson B, Hyman L, Bengtsson B, Hussein M. Reduction of intraocular pressure and glaucoma progression: results from the early manifest glaucoma trial. Arch Ophthalmol 2002; 120: 1268-1279.
9) Hysi PG, Cheng CY, Springelkamp H, Macgregor S, Bailey JNC, Wojciechowski R, Vitart V, Nag A, Hewitt AW, Hohn R, Venturini C, Mirshahi A, Ramdas WD, Thorleifsson G, Vithana E, Khor CC, Stefansson AB, Liao J, Haines JL, Amin N, Wang YX, Wild PS, Ozel AB, Li JZ, Fleck BW, Zeller T, Staffieri SE, Teo YY, Cuellar-Partida G, Luo X, Allingham RR, Richards JE, Senft A, Karssen LC, Zheng Y, Bellenguez C, Xu L, Iglesias AI, Wilson JF, Kang JH, van Leeuwen EM, Jonsson V, Thorsteinsdottir U, Despriet DDG, Ennis S, Moroi SE, Martin NG, Jansonius NM, Yazar S, Tai ES, Amouyel P, Kirwan J, van Koolwijk LME, Hauser MA, Jonasson F, Leo P, Loomis SJ, Fogarty R, Rivadeneira F, Kearns L, Lackner KJ, de Jong P, Simpson CL, Pennell CE, Oostra BA, Uitterlinden AG, Saw SM, Lotery AJ, Bailey-Wilson JE, Hofman A, Vingerling JR, Maubaret C, Pfeiffer N, Wolfs RCW, Lemij HG, Young TL, Pasquale LR, Delcourt C, Spector TD, Klaver CCW, Small KS, Burdon KP, Stefansson K, Wong TY, Viswanathan A, Mackey DA, Craig JE, Wiggs JL, van Duijn CM, Hammond CJ, Aung T. Genome-wide analysis of multi-ancestry cohorts identifies new loci influencing intraocular pressure and susceptibility to glaucoma. Nat Genet 2014; 46: 1126-1130.
10) Acott TS, Kelley MJ, Keller KE, Vranka JA, Abu-Hassan DW, Li X, Aga M, Bradley JM. Intraocular pressure homeostasis: maintaining balance in a high-pressure environment. J Ocul Pharmacol Ther 2014; 30: 94-101.
11) Vranka JA, Kelley MJ, Acott TS, Keller KE. Extracellular matrix in the trabecular meshwork: intraocular pressure regulation and dysregulation in glaucoma. Exp Eye Res 2015; 133: 112-125.
12) Acott TS, Kelley MJ. Extracellular matrix in the trabecular meshwork. Exp Eye Res 2008; 86: 543-561.
13) Johnson M. 'What controls aqueous humour outflow resistance?'. Exp Eye Res 2006; 82: 545-557.
14) Hajjari M, Salavaty A. HOTAIR: an oncogenic long non-coding RNA in different cancers. Cancer Biol Med 2015; 12: 1-9.
15) Wang Y, Wang J, Wei LJ, Zhu DM, Zhang JS. Biological function and mechanism of lncRNA-MEG3

in Tenon's capsule fibroblasts proliferation: by MEG3-Nrf2 protein interaction. Biomed Pharmacother 2017; 87: 548-554.

16) Li HB, You QS, Xu LX, Sun LX, Abdul Majid AS, Xia XB, Ji D. Long non-coding RNA-MALAT1 mediates retinal ganglion cell apoptosis through the PI3K/Akt signaling pathway in rats with glaucoma. Cell Physiol Biochem 2017; 43: 2117-2132.

17) Burdon KP, Awadalla MS, Mitchell P, Wang JJ, White A, Keane MC, Souzeau E, Graham SL, Goldberg I, Healey PR, Landers J, Mills RAD, Best S, Hewitt AW, Sharma S, Craig JE. DNA methylation at the 9p21 glaucoma susceptibility locus is associated with normal-tension glaucoma. Ophthalmic Genet 2018; 39: 221-227.

18) Vishal M, Sharma A, Kaurani L, Chakraborty S, Ray J, Sen A, Mukhopadhyay A, Ray K. Evaluation of genetic association of the INK4 locus with primary open angle glaucoma in east indian population. Sci Rep 2014; 4: 5115.

19) Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 2001; 25: 402-408.

20) Ju WK, Kim KY, Lindsey JD, Angert M, Duong-Polk KX, Scott RT, Kim JJ, Kukhmazov I, Ellisman MH, Perkins GA, Weinreb RN. Intraocular pressure elevation induces mitochondrial fission and triggers OPA1 release in glaucomatous optic nerve. Invest Ophthalmol Vis Sci 2008; 49: 4903-4911.

21) Ju WK, Kim KY, Duong-Polk KX, Lindsey JD, Ellisman MH, Weinreb RN. Increased optic atrophy type 1 expression protects retinal ganglion cells in a mouse model of glaucoma. Mol Vis 2010; 16: 1331-1342.

22) Kim KY, Perkins GA, Shim MS, Bushong E, Alcasid N, Ju S, Ellisman MH, Weinreb RN, Ju WK. DRP1 inhibition rescues retinal ganglion cells and their axons by preserving mitochondrial integrity in a mouse model of glaucoma. Cell Death Dis 2015; 6: e1839.

23) Chhunchha B, Singh P, Stamer WD, Singh DP. Prdx6 retards senescence and restores trabecular meshwork cell health by regulating reactive oxygen species. Cell Death Dis 2017; 3: 17060.

24) Kuespert S, Junglas B, Braunger BM, Tamm ER, Fuchshofer R. The regulation of connective tissue growth factor expression influences the viability of human trabecular meshwork cells. J Cell Mol Med 2015; 19: 1010-1020.

25) Li R, Yin F, Guo Y-Y, Zhao K-C, Ruan Q, Qi Y-M. Knockdown of ANRIL aggravates H2O2-induced injury in PC-12 cells by targeting microRNA-125a. Biomed Pharmacother 2017; 92: 952-961.

26) Tüfekci KU, Meuwissen RL, Genç S. The role of microRNAs in biological processes. Methods Mol Biol 2014; 1107: 15-31.

27) Ma J, Li T, Han X. Knockdown of LncRNA ANRIL suppresses cell proliferation, metastasis, and invasion via regulating miR-122-5p expression in hepatocellular carcinoma. J Cancer Res Clin Oncol 2018; 144: 205-214.

28) Chai L, Yuan Y, Chen C, Zhou J, Wu Y. The role of long non-coding RNA ANRIL in the carcinogenesis of oral cancer by targeting miR-125a. Biomed Pharmacother 2018; 103: 38-45.

29) Gonzalez P, Li G, Qiu J, Wu J, Luna C. Role of microRNAs in the trabecular meshwork. J Ocul Pharmacol Ther 2014; 30: 128-137.

30) Banaei-Esfahani A, Moazzeni H, Nosar PN, Amin S, Arefian E, Soleimani M, Yazdani S, Elahi E. MicroRNAs that target RGS5. Iran J of Basic Med Sci 2015; 18: 108-114.

31) Xu K, Chen Z, Qin C, Song X. miR-7 inhibits colorectal cancer cell proliferation and induces apoptosis by targeting XRCC2. Onco Targets Ther 2014; 7: 325-332.

32) Oka SI, Hirata T, Suzuki W, Naito D, Chen Y, Chin A, Yaginuma H, Saito T, Nagarajan N, Zhai P, Bhat S, Schesing K, Shao D, Hirabayashi Y, Yodoi J, Sciarretta S, Sadoshima J. Thioredoxin-1 maintains mechanistic target of rapamycin (mTOR) function during oxidative stress in cardiomyocytes. J Biol Chem 2017; 292: 18988-19000.

33) Kim EK, Choi EJ. Compromised MAPK signaling in human diseases: an update. Arch Toxicol 2015; 89: 867-882.

34) Sun Y, Liu WZ, Liu T, Feng X, Yang N, Zhou HF. Signaling pathway of MAPK/ERK in cell proliferation, differentiation, migration, senescence and apoptosis. J Recept Signal Transduct Res 2015; 35: 600-604.

35) Zhu H, Jin Q, Li Y, Ma Q, Wang J, Li D, Zhou H, Chen Y. Melatonin protected cardiac microvascular endothelial cells against oxidative stress injury via suppression of IP3R-[Ca^{2+}]c/VDAC-[Ca^{2+}]m axis by activation of MAPK/ERK signaling pathway. Cell Stress Chaperones 2018; 23: 101-113.