

MicroRNA-137 regulates hypoxia-induced retinal ganglion cell apoptosis through Notch1

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Abstract. The apoptosis of retinal ganglion cells (RGCs) is a hallmark of several optic neuropathies. MicroRNAs (miRNAs) are recently identified regulators of various biological processes. However, the role of miRNAs in regulating RGC apoptosis remains largely unknown. We herein aimed to demonstrate that miR-137 acts as a hypoxia-responsive gene in RGCs that is downregulated under hypoxic conditions. It was observed that overexpression of miR-137 markedly aggravated hypoxia-induced cell apoptosis, whereas inhibition of miR-137 effectively protected RGCs against hypoxia-induced apoptosis. Hypoxia induced Notch1 expression and signaling activation, while blocking Notch signaling significantly aggravated hypoxia-induced cell apoptosis. Further data revealed that the pro-survival Akt signaling pathway was involved in miR-137-Notch signaling pathway-mediated RGC protection. Knockdown of Notch significantly reversed the effect of anti-miR-137 on RGC protection and Akt signaling activation. In addition, blocking Akt signaling also significantly abrogated the protective effect of anti-miR-137 on hypoxia-induced cell injury. Overall, the results of the present study demonstrated that miR-137 targets Notch1 expression, revealing a novel link between miR-137 and Notch signaling, and suggesting that a miR-137/Notch1 axis may serve as a potential molecular target for the treatment of hypoxia-induced retinal diseases.

Introduction

Retinal ganglion cells (RGCs) are important neurons in the retina that transmit visual signals from the retina to the

brain (1,2). RGCs are particularly susceptible to acute, transient and mild systemic hypoxic stress that induces their apoptosis or necrosis (3). The loss of RGCs in a number of ocular pathologies, such as retinal detachment, leads to irreversible visual injury (1,2). Hypoxia is the critical pathological factor for several optic neuropathies, and hypoxia-induced apoptosis of RGCs results in progressive vision loss (4-6). Detachment of the retina causes retinal ischemia/reperfusion and hypoxia, resulting in apoptosis of RGCs (7), which is irreversible; thus, protecting RGCs against hypoxia-evoked cell apoptosis is crucial for treating hypoxia-induced retinal diseases.

The Notch signaling pathway plays an important role in various cellular processes, including cell proliferation and apoptosis, and is involved in numerous pathological processes (8,9). There are four Notch receptors (Notch1-4) and five Notch ligands, including Jagged1, Jagged2, Delta-like 1 (Dll1), Dll3 and Dll4 (10). The binding of Notch receptors and ligands results in cleavage of the Notch intracellular domain (NICD) that translocates into the nucleus to activate the transcription of Notch target genes, such as Hes1 and Hey1 (11,12). The Notch signaling pathway plays an important role in the development of the liver, kidney, eye, heart and skeleton (13). Notch signaling has been implicated in the regulation of an increasing number of stem cells in several different tissues (14). Aberrant activation of the Notch signaling pathway has been observed in various cancers (15). The Notch signaling pathway is extensively involved in regulating cell proliferation, apoptosis and differentiation through crosstalk with other signaling pathways (16). Recent studies indicated that Notch signaling participates in several hypoxia-induced pathological processes (17,18). Notch signaling has also been shown to protect the myocardium (19), liver (20) and cerebrum (21) from ischemia/reperfusion- and hypoxia-induced injury. However, the role of Notch signaling in hypoxia-induced cell apoptosis of RGCs has not been extensively investigated.

A group of small non-coding RNAs, referred to as microRNAs (miRNAs), have drawn significant attention over the past few decades, due to their negative regulatory effects on gene expression. miRNAs consist of ~22 nucleotides and suppress gene expression by targeting the 3'-untranslated region (3'-UTR), leading to translation inhibition (22-24). Thus, miRNAs are involved in the regulation of a wide range of cellular processes, such as stress response, cell survival and apoptosis (22). Several studies have demonstrated that

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Abbreviations: RGCs, retinal ganglion cells; miRNAs, microRNAs; NICD, Notch intracellular domain; 3'-UTR, 3'-untranslated region; GSI, γ -secretase inhibitors

Key words: retinal ganglion cells, miR-137, Notch1, hypoxia, apoptosis

targeting specific miRNAs may be a novel strategy for inhibiting apoptosis of RGCs (25-27). However, the precise mechanisms of miRNAs in regulating RGC apoptosis have not been fully elucidated.

miR-137 is a neuron-associated miR that is abundantly expressed in the brain and regulates neural differentiation and maturation (28-30). Recently, miR-137 was reported to be a hypoxia-responsive miRNA in mouse brain cells exposed to hypoxic conditions (31). However, whether miR-137 plays a role in RGCs exposed to hypoxia remains unknown. In the present study, the role of miR-137 and the involvement of Notch signaling in hypoxia-induced apoptosis of RGCs was investigated.

Materials and methods

Cell culture. The rat retinal ganglion cell line RGC-5 and human embryonic kidney 293 (HEK-293) cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (both from Gibco-BRL; Thermo Fisher Scientific, Rockville, MD, USA) and 1/100 streptomycin/penicillin (Sigma-Aldrich; Merck KGaA, St. Louis, MO, USA). Cells were routinely maintained in a culture chamber containing 5% CO₂ at 37°C.

Hypoxia treatment. Cells were cultured in a multi-gas incubator (Thermo Fisher Scientific, Shanghai, China) containing 5% O₂, 90% N₂ and 5% CO₂, and incubated at 37°C. After washing twice with deoxygenated serum-free DMEM, cells from each treatment were incubated in the hypoxic incubator for 24, 48 and 72 h. Cells cultured under normoxic conditions were used as control.

Quantitative polymerase chain reaction (qPCR) analysis. Briefly, total RNA was extracted using the miRNeasy mini kit (Qiagen, Dusseldorf, Germany) according to the recommended protocols. cDNA was synthesized using M-MLV reverse transcriptase (BioTeke, Beijing, China) or miScript reverse transcription kit (Qiagen). qPCR was performed using SYBR-Green PCR Master Mix (for mRNA amplification) or TaqMan miRNA reverse transcription kit (for miRNA amplification) (Applied Biosystems, Carlsbad, CA, USA). The primers used were as follows: miR-137 forward, 5'-gcgcgcttatgcttaagaatac-3' and reverse, 5'-gtgcagggtccgaggt-3'; U6 snRNA forward, 5'-ctcgcttcggcagcaca-3' and reverse, 5'-aacgcttcacgaatttgcgt-3'; Notch1 forward, 5'-atgactgccaggaacaac-3' and reverse, 5'-gtccagccattgacacacac-3'; Hes-1 forward, 5'-agccaactgaaaacacctgatt-3' and reverse, 5'-ggactttatgattagcagtg-3'; Hey1 forward, 5'-ccgcttcgttctgcctggt-3' and reverse, 5'-tgctgcctgtgagggtgtgc-3'; and β -actin forward, 5'-gtggggcgc cccaggcaca-3' and reverse, 5'-cttccttaatgtcacgcacgatttc-3'. β -actin (for mRNA) and U6 snRNA (for miRNA) were used as internal control genes. Relative gene expression was calculated using the 2^{- $\Delta\Delta C_q$} method (32).

Cell transfection. The miR-137 mimics (5'-uuauugcuuaagaauacgcuagtt-3'), miR-137 inhibitor (anti-miR-137, 5'-cuacgcguuuuuuagcauaa-3' with 2'-O-methyl modification) and their negative controls (miR-NC or anti-miR-NC) were synthesized

by GenePharma (Shanghai, China). The miRNAs and Notch1 siRNA (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) were transfected into cells using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. The transfections were incubated for 24 h and then subjected to hypoxic conditions for another 24 h.

Cell viability. The 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and lactate dehydrogenase (LDH) assays were used to assess cell viability. For the MTT assay, cells were seeded into 96-well plates (5x10³ cells/well) overnight. Thereafter, the cells were transfected with miR-137 mimics, anti-miR-137 or Notch1 siRNA for 24 h under normoxic conditions, followed by 24 h under hypoxic conditions. Following incubation, 20 μ l of MTT stock solution (Sigma-Aldrich; Merck KGaA) were added to each well and incubated for 4 h. The medium was discarded and the formazan crystals were dissolved by addition of dimethyl sulfoxide (200 μ l/well; Sigma-Aldrich; Merck KGaA). After gently shaking for 15 min, the optical density (OD) value at 490 nm was measured using a microplate reader (Bio-Tek Instruments; Winooski, VT, USA). The LDH assay was performed using a cytotoxicity LDH assay kit (BioVision, Milpitas, CA, USA). Briefly, cells were seeded into 96-well plates with treatments as described above. Subsequently, 0.2% Triton X-100 was added to lyse the cells, followed by centrifugation at 250 x g for 2 min at 4°C. A total of 100 μ l of the supernatant from each well were added to a new 96-well plate; then, 100 μ l of working solution was added to each well and incubated for 30 min in the dark. The reaction was stopped by adding 50 μ l of stop solution. The OD value at 490 nm was determined using a microplate reader. The lysis ratio was calculated as follows: (Experimental release-spontaneous release)/(maximum release-spontaneous release) x 100%.

Apoptosis assay. Cell apoptosis was detected with the caspase-3 activity assay using a commercial kit (BioVision). Briefly, after treatment, cells were harvested, lysed in ice-cold cell lysis buffer and centrifuged at 10,000 x g for 1 min. Protein concentration was measured in the resultant supernatant. Subsequently, 50 μ l of cell lysis buffer was added to 100 μ g protein and incubated with 50 μ l of reaction buffer and 5 μ l of 4 mM DEVD-pNA substrate at 37°C for 2 h. The OD value at 405 nm was determined using a microplate reader.

Western blot analysis. Proteins were extracted from the cells using a protein extraction kit (Applygen Technologies, Beijing, China). The protein concentration was measured using the Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA, USA). A total of 25 μ g proteins from different groups were separated by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Temecula, CA, USA). The membranes were then blocked by 3% non-fat milk at 37°C for 1 h. The membranes were incubated at 4°C overnight with primary antibodies. The membranes were washed thrice with TBST and then incubated with goat anti-rabbit horseradish peroxidase-labeled secondary antibodies (sc-2004; 1:5,000; Santa Cruz Biotechnology, Inc.) at 37°C for 1 h. The bands were visualized using an enhanced

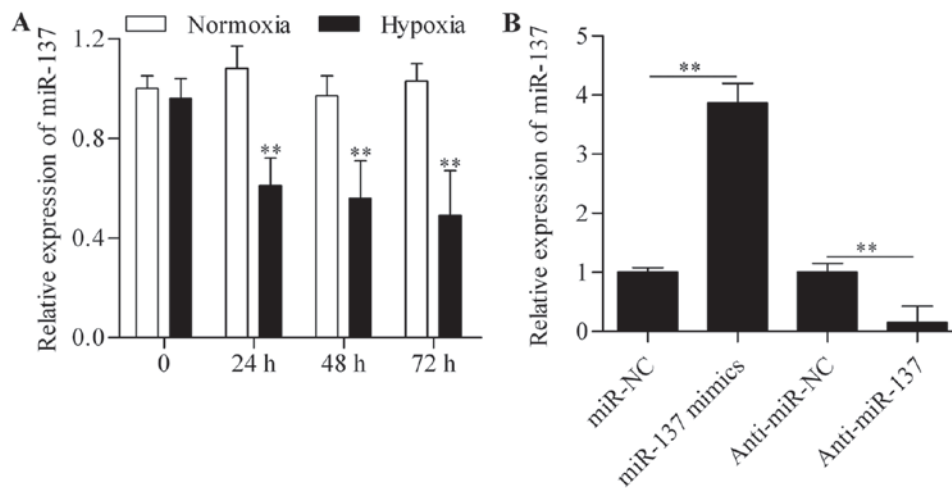


Figure 1. miR-137 is downregulated in the RGC-5 retinal ganglion cell line under hypoxic conditions. (A) Quantitative polymerase chain reaction (qPCR) analysis of miR-137 expression level in RGC-5 cells subjected to hypoxic conditions for 24, 48 and 72 h. Cells cultured under normoxic conditions were used as control. n=3, **P<0.01 vs. normoxia. (B) qPCR analysis of miR-137 expression level in RGC-5 cells transfected with miR-137 mimics or anti-miR-137 for 24 h. n=3, **P<0.01. NC, negative control.

chemiluminescence kit (Pierce, Rockford, IL, USA) and intensity was quantitatively analyzed using Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, MD, USA). Rabbit polyclonal primary antibodies including anti-Notch1 (sc-9170; 1:200) anti-PTEN (sc-9145; 1:250), anti-Akt (sc-8312; 1:500), anti-pAkt (sc-135650; 1:200) and anti- β -actin (sc-7210; 1:500) were purchased from Santa Cruz Biotechnology, Inc. Rabbit monoclonal anti-NICD (no. 3608; 1:1,000) was purchased from Cell Signaling Technology (Danvers, MA, USA).

Dual-luciferase reporter assay. The 3'-UTR of Notch1, containing the miR-137 binding site, was amplified and then cloned into pmirGLO Dual-Luciferase miRNA Target Expression Vectors (Promega, Madison, WI, USA). For reporter assays, cells were seeded into 24-well plates and co-transfected with miR-137 mimics and pmirGLO-Notch1 3'-UTR constructs using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific). After a 48-h incubation, cells were lysed and firefly and *Renilla* luciferase activity was detected using the Dual-Luciferase Assay system (Promega).

Statistical analysis. Quantitative data are presented as mean \pm standard deviation. Statistical analyses were performed by Student's t-test or one-way analysis of variance using SPSS software, version 11.5 (SPSS, Inc., Chicago, IL, USA). A P-value <0.05 was defined as statistically significant.

Results

miR-137 affects the survival of RGC-5 cells exposed to hypoxic conditions. To investigate whether miR-137 is a hypoxia-responsive miRNA in RGC-5 cells, the expression levels of miR-137 were measured by qPCR. The results revealed that the expression of miR-137 was significantly suppressed in RGC-5 cells subjected to hypoxic conditions (Fig. 1A), indicating that miR-137 may play a role in RGC-5 cells following hypoxia. To further investigate this role, gain- and loss-of-function experiments were performed. RGC-5 cells were transfected with

miR-137 mimics or anti-miR-137 to overexpress or silence miR-137, respectively. On qPCR analysis, miR-137 expression was found to be markedly increased in cells transfected with miR-137 mimics and significantly decreased by anti-miR-137 transfection (Fig. 1B). The effect of miR-137 overexpression or silencing on hypoxia-induced cell injury was next analyzed. Results from the MTT and LDH assays revealed that miR-137 overexpression significantly aggravated hypoxia-induced cell injury, whereas suppression of miR-137 markedly reversed the inhibitory effect of hypoxia on cell viability (Fig. 2A and B). To verify the role of miR-137 in RGC-5 cell survival following hypoxia, hypoxia-induced apoptosis was analyzed using a caspase-3 assay. RGC-5 cells subjected to hypoxia exhibited higher level of caspase-3 activity, indicating hypoxia-induced cell apoptosis (Fig. 2C). As expected, the caspase-3 activity was further upregulated by miR-137 overexpression, but was significantly downregulated by miR-137 silencing (Fig. 2C). In addition, the role of miR-137 on cell viability of RGCs was further examined under normal conditions. The results demonstrated that miR-137 overexpression significantly inhibited cell viability, while miR-137 suppression promoted viability of RGCs (Fig. 2D). These results suggest that suppression of miR-137 is conducive to sustaining viability of RGC-5 cells under hypoxic conditions.

Notch1 is a direct target of miR-137. To elucidate the molecular mechanism of miR-137 in regulating RGC-5 cell survival, bioinformatics analyses were conducted to identify genes that contain putative target sites in their 3'-UTR sequences. Interestingly, Notch1 was found to be a potential target of miR-137 and the binding sites were conserved from human to rats (Fig. 3A). To validate this prediction, the direct interaction between miR-137 and Notch1 3'-UTR was examined using a dual-luciferase reporter assay. The results revealed that miR-137 mimics significantly decreased the luciferase activity of the pmirGLO reporter vector containing the wild-type 3'-UTR of Notch1 (WT) (Fig. 3B). This inhibitory effect was not observed in the pmirGLO reporter vector carrying Notch1

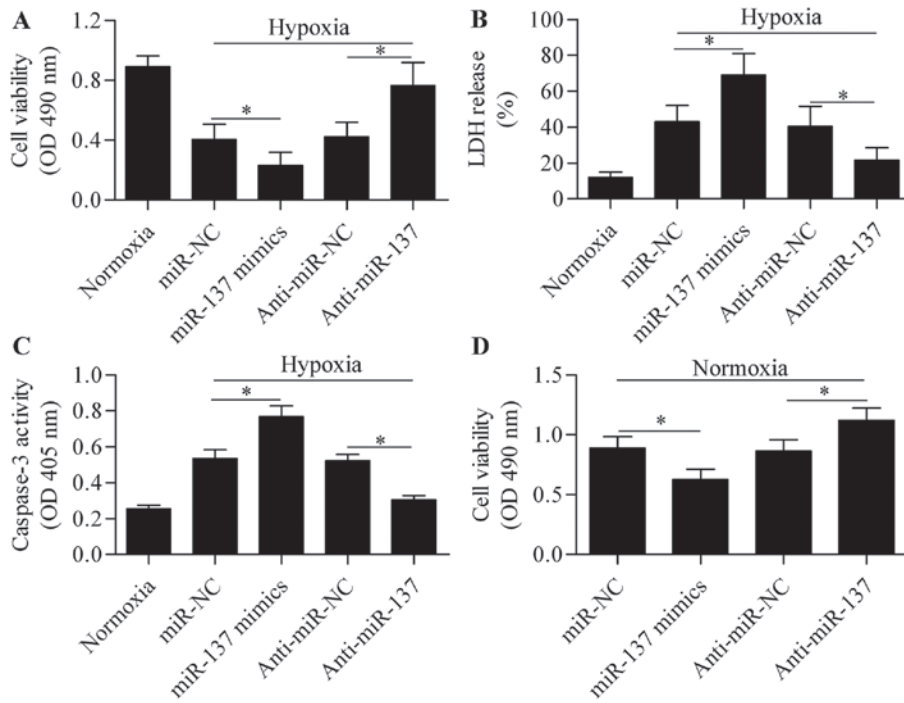


Figure 2. Suppression of miR-137 protects RGC-5 retinal ganglion cells from hypoxia-induced cell injury. Cells were transfected with miR-137 mimics or anti-miR-137 for 24 h under normoxic conditions and then subjected to hypoxic conditions for another 24 h. Hypoxia-induced cell injury was detected by (A) MTT and (B) lactate dehydrogenase (LDH) assays. n=3, *P<0.05. (C) Hypoxia-induced cell apoptosis was examined by caspase-3 activity. n=3, *P<0.05. (D) The effect of miR-137 on cell viability of RGC-5 cells was detected by MTT assay. RGC-5 cells were transfected with miR-137 mimics or anti-miR-137 for 48 h under normoxic conditions. n=3, *P<0.05. NC, negative control.

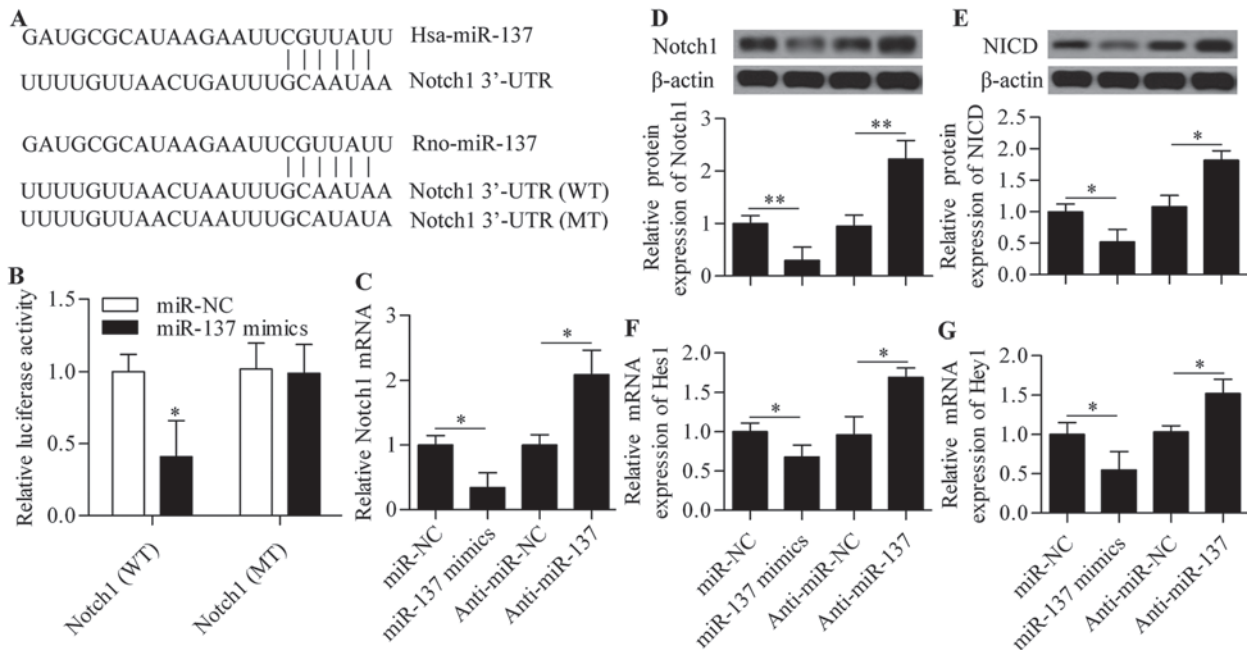


Figure 3. miR-137 targets and regulates the Notch signaling pathway. (A) Diagram of the binding site between miR-137 and Notch1 3'-untranslated region (3'-UTR). Has, *Homo sapiens*; Rno, *Rattus norvegicus*. (B) Luciferase reporter assay of miR-137 and Notch1 3'-UTR. HEK293 cells were co-transfected with miR-137 mimics and pmirGLO-wild-type (WT) Notch1 3'-UTR or pmirGLO-mutated (MT) Notch1 3'-UTR. Luciferase activity was measured after a 48-h incubation. n=3, *P<0.05 vs. miR-NC. (C) Quantitative polymerase chain reaction (qPCR) and (D) western blot analysis of Notch1 mRNA and protein expression, respectively. (E) Western blot analysis of Notch intracellular domain (NICD) protein expression. qPCR analysis of Hes1 (F) and Hey1 (G) mRNA expression. RGC-5 retinal ganglion cells were transfected with miR-137 mimics or anti-miR-137 under normoxic conditions for 24 h and then subjected to hypoxia for 24 h. n=3, *P<0.05 and **P<0.01. NC, negative control.

with mutated 3'-UTR target sites (MT) (Fig. 3B), confirming the specificity of miR-137 for Notch.

The effects of miR-137 on the Notch signaling pathway were detected by qPCR and western blot analysis. Overexpression

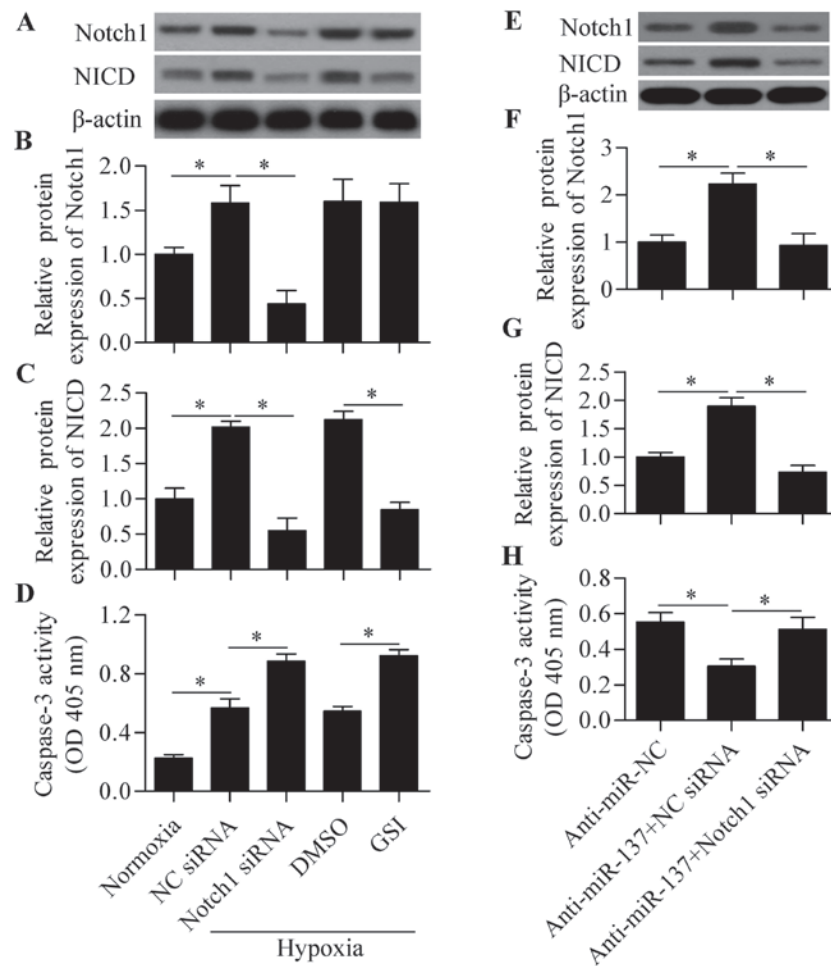


Figure 4. miR-137 regulates hypoxia-induced apoptosis through Notch1. (A) Western blot analysis of Notch1 and Notch intracellular domain (NICD) protein expression levels in RGC-5 retinal ganglion cells treated with Notch1 siRNA or γ -secretase inhibitors (GSI) ($75 \mu\text{M}$) for 24 h and then subjected to hypoxia for 24 h. Relative protein quantification of (B) Notch1 and (C) NICD. (D) Caspase-3 activity assay of RGC-5 cells treated with siRNA or GSI for 24 h and then subjected to hypoxia for 24 h. (E) Western blot analysis of Notch1 and NICD protein expression levels in RGC-5 cells co-transfected with anti-miR-137 and Notch1 siRNA for 24 h and then subjected to hypoxia for 24 h. Relative protein quantification of (F) Notch1 and (G) NICD. (H) Caspase-3 activity assay of RGC-5 cells co-transfected with anti-miR-137 and Notch1 siRNA for 24 h and then subjected to hypoxia for 24 h. $n=3$, $^*P<0.05$. NC, negative control; OD, optical density; DMSO, dimethyl sulfoxide.

of miR-137 resulted in a significant decrease of Notch1 mRNA and protein expression, whereas anti-miR-137 transfection caused a marked increase of Notch1 mRNA and protein expression (Fig. 3C and D). Subsequently, the expression levels of NICD, Hes1 and Hey1 were also affected, in that the protein expression of NICD and the mRNA expression of Hes1 and Hey1 were significantly decreased by miR-137 overexpression and increased by miR-137 inhibition (Fig. 3E-G). These results indicate that miR-137 regulates the Notch signaling pathway by targeting Notch1.

miR-137 regulates hypoxia-induced apoptosis through Notch1.

To verify whether the Notch1-mediated signaling pathway was involved in response to hypoxia in RGC-5 cells, the effect of blocking Notch signaling on cell survival under hypoxia was investigated. Compared with normoxia, hypoxic conditions significantly induced the expression of Notch1 and NICD expression (Fig. 4A-C), indicating that hypoxia activated Notch signaling in RGC-5 cells. Next, Notch signaling was blocked by Notch1 siRNA or γ -secretase inhibitors (GSI) to investigate the role of Notch signaling in hypoxia-induced cell apoptosis.

The results revealed that both Notch siRNA and GSI treatment significantly decreased the expression of NICD, indicating a suppressive effect on Notch signaling (Fig. 4A-C). We also demonstrated that blocking Notch signaling significantly aggravated cell apoptosis induced by hypoxia (Fig. 4D). These results indicated that the Notch signaling pathway plays an important role in regulating RGC-5 cell survival in response to hypoxia. To investigate whether miR-137 acts through Notch1, the effect of Notch1 siRNA on anti-miR-137-mediated cell survival was determined. The results demonstrated that knock-down of Notch1 significantly reversed the protective effect of anti-miR-137 on hypoxia-induced cell apoptosis (Fig. 4E-H). Taken together, these results indicate that miR-137 regulates hypoxia-induced apoptosis in RGC-5 cells through Notch1.

PTEN/Akt survival signaling is involved in miR-137-mediated Notch signaling. To further investigate the molecular mechanism by which miR-137 regulates cell survival in response to hypoxia, the effect of miR-137 on PTEN/Akt survival signaling was analyzed. Our results demonstrated that miR-137 overexpression significantly increased the expression of PTEN and decreased

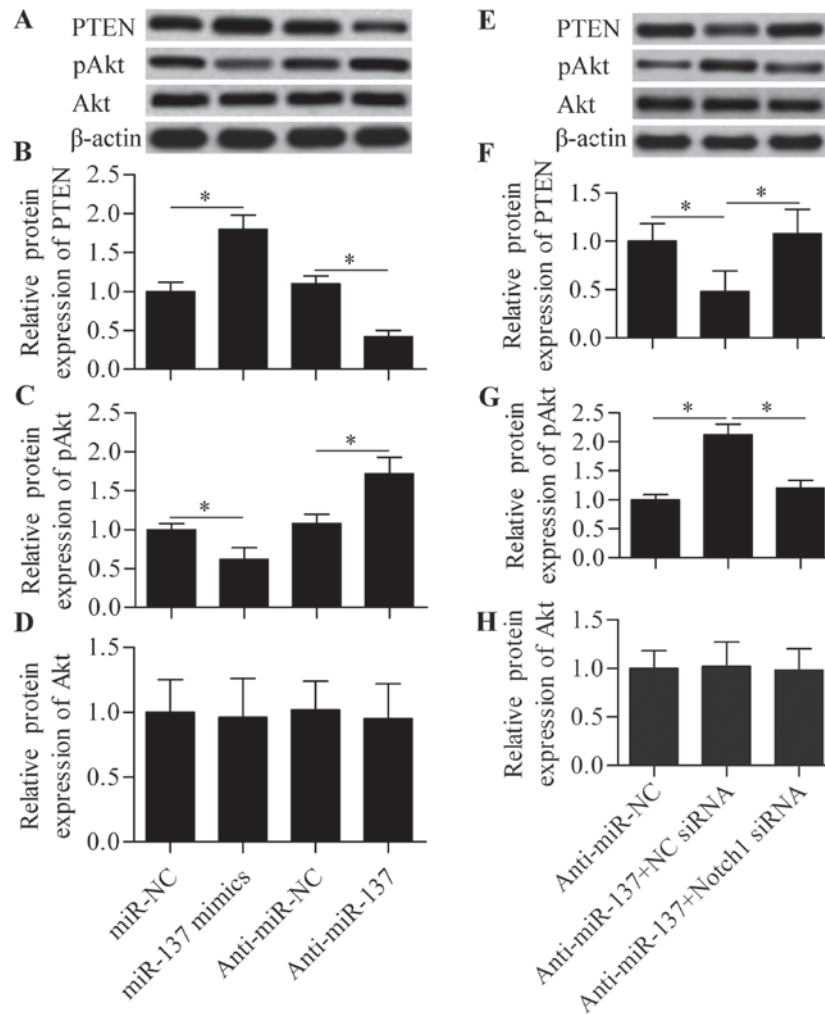


Figure 5. miR-137 regulates PTEN/Akt survival signaling. (A) Western blot analysis of PTEN, total Akt and pAkt protein expression levels in RGC-5 retinal ganglion cells transfected with miR-137 mimics or anti-miR-137. Cells were transfected with miR-137 mimics or anti-miR-137 under normoxic conditions for 24 h and then subjected to hypoxia for 24 h. (B-D) Relative protein quantification of PTEN, pAkt and total Akt expression shown in (A). (E) Western blot analysis of PTEN, total Akt and pAkt protein expression levels in RGC-5 cells co-transfected with anti-miR-137 and Notch1 siRNA. Cells were transfected with anti-miR-137 and Notch1 siRNA under normoxic conditions for 24 h and then subjected to hypoxia for 24 h. Relative protein quantification of (F) PTEN, (G) pAkt and (H) total Akt, from (E). $n=3$, * $P<0.05$. NC, negative control; PTEN, phosphatase and tensin homolog.

the expression of pAkt, whereas anti-miR-137 exerted the opposite effects (Fig. 5A-C). Moreover, the effect of anti-miR-137 was significantly blocked by Notch1 siRNA (Fig. 5E-G). Conversely, these treatments exerted no obvious effect on total Akt expression (Fig. 5D and H). Based on these results, it may be concluded that miR-137 regulates PTEN/Akt survival signaling through Notch1-mediated signaling. Downregulation of miR-137 increases Notch1 expression and activates the expression of Hes1, a negative regulator of PTEN. Then, Hes1 binds to the PTEN promoter and inhibits PTEN expression (33), thus leading to the upregulation of pAkt. To further verify that Akt signaling is involved in the mechanism underlying miR-137 regulation of hypoxia-induced apoptosis, Akt signaling was blocked using the specific inhibitor LY294002 (Beyotime Institute of Biotechnology, Haimen, China), followed by assessment of the effect of anti-miR-137 on cell apoptosis. The results revealed that the protective effect of anti-miR-137 on hypoxia-induced cell injury was significantly abrogated by blocking Akt signaling (Fig. 6A and B). Taken together, these results indicate that PTEN/Akt survival signaling is involved in miR-137-mediated Notch signaling.

Discussion

The present study demonstrated that miR-137 is significantly decreased in RGCs under hypoxic conditions, suggesting that miR-137 is a hypoxia-responsive miR. The downregulation of miR-137 may protect RGCs against hypoxia-induced apoptosis, whereas overexpression of miR-137 may aggravate cell apoptosis. These results suggest that miR-137 may serve as a potential target for inhibiting RGC apoptosis. Notch1 was identified as a novel target gene of miR-137. The underlying mechanism was associated with Notch signaling, which was shown to participate in hypoxia-induced cell apoptosis. These data indicate that miR-137 may serve as a promising molecular target for the treatment of optic neuropathy.

Various studies have demonstrated that miR-137 induces cell apoptosis and inhibits cell proliferation in a number of cancer cells (34-36). The overexpression of miR-137 significantly improves the inhibition rate and apoptosis rate in multiple myeloma cells treated with dexamethasone (37). Downregulation of miR-137 inhibits oxidative stress-induced

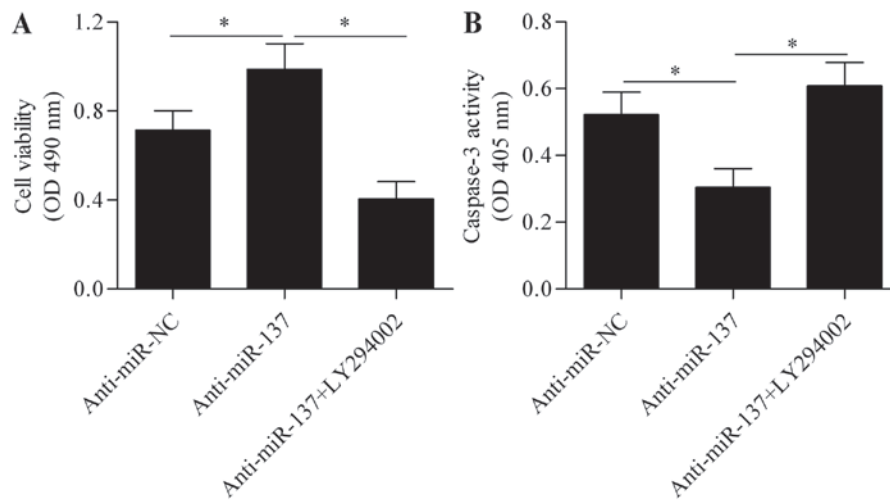


Figure 6. Blocking Akt abrogates the protective effect of anti-miR-137. RGC-5 retinal ganglion cells were transfected with anti-miR-137 in the presence of 10 μ M Akt inhibitor LY294002 under normoxic conditions for 24 h and subjected to hypoxia treatment for another 24 h. (A) Cell viability and (B) cell apoptosis were detected using the MTT and caspase-3 activity assays, respectively. n=3, *P<0.05. NC, negative control; OD, optical density.

cardiomyocyte apoptosis (38). Similarly, downregulation of miR-137 may protect cells against hypoxia-induced apoptosis (31). These findings suggest that miR-137 is a positive regulator of cell apoptosis and may function as a critical regulator in various pathological processes. The role of miR-137 in neurons has been widely investigated. miR-137 is expressed in the brain and regulates neural stem cell proliferation and differentiation (28), as well as neuronal maturation (30). Downregulation of miR-137 has also been found in Alzheimer's disease (39). However, whether miR-137 is involved in regulating RGC apoptosis is currently unknown. We herein demonstrated that miR-137 was downregulated in RGCs under hypoxic conditions, and suppression of miR-137 was able to protect RGCs against hypoxia-induced apoptosis. Our results further support the hypothesis that miR-137 is a hypoxia-responsive miR. Li *et al* (31) demonstrated that the downregulation of miR-137 under hypoxic conditions increased the expression of mitophagy receptors FUNDC1 and NIX, protecting cells from hypoxia-induced apoptosis by activating mitophagy (31). Therefore, the downregulation of miR-137 in response to hypoxia may be a self-protective mechanism of cells adapting to these conditions.

It was recently suggested that the Notch signaling pathway is important in the response to extracellular stress, particularly ischemic or hypoxic stresses. Yu and Song (40) reported that Notch1 signaling suppressed hypoxia-induced cardiomyocyte apoptosis by inhibiting the cell apoptosis pathway. Pei *et al* (41) demonstrated that Notch1 signaling protected cardiomyocytes against ischemia/reperfusion injury by reducing oxidative/nitrative stress. Ischemic preconditioning and postconditioning activates Notch signaling, promoting cardioprotection (42,43). Certain drugs, such as relaxin and TNF- α inhibitor, protect cardiomyocytes from ischemic or hypoxic damage by activating Notch signaling (44,45). Electroacupuncture pretreatment and isoflurane preconditioning attenuated cerebral ischemia-reperfusion injury through activation of the Notch signaling pathway (21,46). Furthermore, Notch signaling is also involved in hepatic and intestinal ischemia/reperfusion (20,47). Overall, these find-

ings indicated that Notch signaling activation was protective for cells against ischemic or hypoxic conditions; however, the role of Notch signaling in RGCs exposed to hypoxia remained unknown. In the present study, we demonstrated that Notch signaling was activated in response to hypoxia in RGCs and that blocking Notch signaling with Notch1 siRNA or Notch inhibitor significantly aggravated cell apoptosis induced by hypoxia. These findings confirm a prosurvival signaling role for Notch in response to extracellular insults.

Of note, Notch1 has been found to be the target gene of miR-137. Luciferase reporter assays demonstrated that miR-137 directly targets the 3'-UTR of Notch1. Overexpression of miR-137 inhibits Notch1 expression, whereas suppression of miR-137 increases expression and activates Notch signaling. Therefore, we hypothesized that the downregulation of miR-137 by hypoxia may contribute to activation of Notch signaling to protect cells. Our data demonstrated that knock-down of Notch1 significantly blocked the protective effect of anti-miR-137, suggesting that miR-137 acted through targeting Notch1. Therefore, targeting miR-137 is a feasible method for modulating Notch signaling. Regulation of Notch signaling by miRNAs is becoming more widely investigated. In fact, several miRNAs, including miR-34a/c (48-50), miR-139-5p (51) and miR-200b (52), have been reported to be capable of modulating Notch signaling by targeting Notch1. To the best of our knowledge, this study is the first to report a direct association between miR-137 and Notch signaling, providing a novel molecular target for modulating Notch signaling.

Akt signaling is another important prosurvival signaling pathway for survival in RGCs (53). In the present study, activation of Notch signaling by downregulation of miR-137 was found to inhibit the expression of PTEN, the suppressor of Akt signaling, and thus increased the protein expression of pAkt. It has been demonstrated that the target gene of Notch signaling, Hes1, may bind to the PTEN promoter and inhibit PTEN expression (33). The activation of Akt signaling may be a major mechanism through which Notch signaling exerts prosurvival effect. In agreement with our findings, a recent study reported that miR-137 inhibited the phosphorylation of

AKT and promoted cell apoptosis in response to dexamethasone in multiple myeloma cells (37). In addition, Cheng *et al* revealed that miR-137 functioned as a tumor suppressor through targeting cyclooxygenase-2, which subsequently suppressed the activation of the Akt signaling pathway in gastric cancer cells *in vitro* and *in vivo* (54). In the present study, we found that miR-137 regulated the Akt signaling pathway through modulating Notch1. Of note, Li *et al* demonstrated that miR-137 regulated cell apoptosis through regulation of FUNDC1 and NIX-mediated mitophagy (31). However, all these findings support the hypothesis that miR-137 is an important regulator of cell apoptosis. However, miR-137 may have diverse function targets in different cell types in response to different stimuli.

The role of miRNAs in regulating RGC apoptosis remains poorly understood. Kong *et al* reported that suppression of miR-100 protected RGCs against oxidative stress-induced apoptosis by targeting the insulin-like growth factor-1 receptor (IGF1R) (25). Upregulation of miR-96 reduces RGC apoptosis by targeting caspase-2 (55). Suppression of miR-134 was also found to protect RGCs against oxidative stress-induced apoptosis (26). Most recently, Kang *et al* (27) revealed that overexpression of miR-26a protected RGCs against oxidative stress-induced apoptosis by inhibiting PTEN and activating Akt signaling. These studies indicated that miRNAs may be novel tools for preventing RGC apoptosis. The present study demonstrated that miR-137 regulates RGC apoptosis in response to hypoxia through targeting Notch1 and Notch signaling, suggesting that a miR-137/Notch1 axis has the potential to be used as a molecular target for treatment of hypoxia-induced retinal diseases.

Acknowledgements

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