

# Retinoic acid receptor $\alpha$ expression exerts an anti-apoptosis effect on PC12 cells following oxygen-glucose deprivation

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**Abstract.** It has been established that the primary form of neuron death following hypoxic ischemic brain damage is apoptosis. Imbalances in the expression of genes in the B-cell lymphoma 2 (Bcl-2) family located in the mitochondrion, and in the expression of their encoded proteins, are key events in the mitochondrial apoptotic pathway, which lead to damage of cellular structure and function. The present study aimed to explore the regulatory effect of retinoic acid receptor  $\alpha$  (RAR- $\alpha$ ) on the apoptosis of PC12 cells induced by oxygen-glucose deprivation (OGD) in the retinoic acid signaling pathway. Recombinant adenovirus RAR- $\alpha$  small interfering RNA (Ad-siRAR- $\alpha$ ) was used to transduce PC12 cells, and the efficiency of RAR- $\alpha$  expression inhibition was detected by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). An empty adenovirus vector was transfected in PC12 cells, which were used as the control. Flow cytometry with Annexin V-propidium iodide (PI) and fluorescence probe JC-1 staining was used to detect the apoptosis rate and mitochondrial transmembrane potential (MMP), respectively, of PC12 cells after transduction with Ad-siRAR- $\alpha$ . Furthermore, the expression levels of key genes in the RAR- $\alpha$  and mitochondrial apoptotic pathway, Bcl-2 and Bcl-2-associated protein (Bax) were analyzed by RT-quantitative (q)PCR and western blot analysis. RAR- $\alpha$  mRNA expression was observed to be decreased in PC12 cells following OGD-induced injury, and this decrease can be

reversed by 4  $\mu$ mol/l ATRA treatment. After 36 h transfection with Ad-siRAR- $\alpha$ , RAR- $\alpha$  gene expression was significantly inhibited compared with the control ( $P < 0.05$ ). The results of Annexin V-PI, fluorescence probe JC-1 staining and flow cytometry demonstrated that the apoptosis rate significantly increased and MMP significantly decreased in OGD-induced PC12 cells following transduction with Ad-siRAR- $\alpha$  compared with the control (both  $P < 0.05$ ). RT-qPCR and western blot analysis indicated that Bax expression was significantly increased and Bcl-2 expression was significantly decreased in PC12 cells transduced with Ad-siRAR- $\alpha$  after OGD-induced injury at the mRNA and protein level ( $P < 0.05$ ). In conclusion, Ad-siRAR- $\alpha$  transduction could promote apoptosis in OGD-induced PC12 cells. This suggests that the expression of Bax and Bcl-2 in the mitochondrial apoptosis signaling pathway is, at least in part, mediated by RAR- $\alpha$  expression, thereby indicating that RAR- $\alpha$  expression exerts an anti-apoptotic effect on OGD-damaged PC12 cells.

## Introduction

Hypoxic ischemic brain damage (HIBD) occurs primarily in newborns, and is characterized as a brain lesion caused by a number of factors during the perinatal period (1,2). It is a brain injury syndrome that causes neonatal death, mental retardation in children and epilepsy (1,2). HIBD is a common central nervous system disease in neonatal infants (2). Currently, 25% of children who survive moderate-to-severe HIBD will have permanent neurological deficits. At present, there is no accepted treatment method for HIBD, and the mechanism of the disease remains to be elucidated, thus the disease often has a poor prognosis (3). Therefore, it is critical that an effective neuroprotective therapy is developed.

It has previously been demonstrated that retinoic acid (RA), which is a key metabolite of vitamin A (VA), and its derivatives regulate the apoptosis and proliferation of tumor cells (4). The PC12 cell line consists of rat pheochromocytoma cells, the main secretion products of which are catecholamines, including dopamine and norepinephrine (5). PC12 cell membranes contain nerve growth factor receptor and the cells exhibit typical nerve cell characteristics; as such, they are used as an *in vitro* model for the study of numerous nervous system diseases, including Parkinson's disease and Alzheimer's

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*Abbreviations:* HIBD, hypoxic ischemic brain damage; VA, vitamin A; RAR- $\alpha$ , retinoic acid receptor  $\alpha$ ; ATRA, all-trans retinoic acid; MMP, mitochondrial transmembrane potential; RNAi, RNA interference; OGD, oxygen-glucose deprivation

*Key words:* recombinant adenovirus retinoic acid receptor  $\alpha$  small interfering RNA, retinoic acid receptor  $\alpha$ , oxygen-glucose deprivation, retinoic acid, anti-apoptotic action

disease (5,6). It has been established that the main form of neuron death following HIBD is apoptosis (7). Imbalances in the expression of genes in the B-cell lymphoma 2 (Bcl-2) family located in the mitochondrion, and in the expression of their encoded proteins, are key events in the mitochondrial apoptotic pathway (8) that lead to damage of cellular structure and function. A previous study on HIBD in rats indicated that there is an association between the level of VA nutrition and the repair of the nervous system following injury (9). The mechanism primarily functions through the regulation of RA receptor  $\alpha$  (RAR- $\alpha$ ) nuclear receptors, thereby affecting  $Ca^{2+}$  levels in nerve cells and affecting learning and memory functions (9,10).

The protective effects of all-trans RA (ATRA) on oxygen-glucose deprivation (OGD) damage may be partly mediated by regulation of the mitochondrial apoptotic pathway, and promotion of the repair of nerve cells (10). RNA interference (RNAi) technology may be used to efficiently degrade the endogenous target gene mRNA, which is homologous with small interfering RNA (si)RNA, and silence the expression of target genes, in order to study the function of specific target genes (11). In the present study, recombinant adenovirus RAR- $\alpha$ -siRNA (Ad-siRAR- $\alpha$ ) was used to inhibit RAR- $\alpha$  expression, in order to investigate whether ATRA exerts anti-apoptotic effects through the RAR- $\alpha$  pathway. Furthermore, the present study aimed to explore the regulatory effect of RAR- $\alpha$  on the apoptosis rate of OGD-induced PC12 cells.

## Materials and methods

**Reagents.** Dulbecco's modified Eagle's medium (DMEM), high glucose and DMEM, no glucose were purchased from Beijing Qingdatianyi Biological Technology Co., Ltd. (Beijing, China). Hank's balanced salt solution was purchased from Hyclone (GE Healthcare, Logan, UT, USA). Fetal bovine serum (FBS), human serum (HS) and TrypLE were purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). RNeasy Mini kit was purchased from Qiagen GmbH (Hilden, Germany). PrimeScript™ RT Reagent kit (Perfect Real Time) and the DNA ladder were purchased from Takara Biotechnology Co., Ltd. (Dalian, China). Primers for polymerase chain reaction (PCR) were purchased from Invitrogen (Thermo Fisher Scientific, Inc.). SuperReal PreMix Plus (SYBR Green) was purchased from Tiangen Biotech Co., Ltd. (Beijing, China). ATRA standard was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). ReadyPrep™ Protein Extraction kit (Total Protein) was purchased from Bio-Rad Laboratories, Inc. (Hercules, California, USA). Primary antibodies for Bcl-2 (cat. no. sc-509), Bcl-2-associated X protein (Bax; cat. no. sc-20067) and  $\beta$ -actin (cat. no. sc-47778), and goat anti-rat IgG-HRP secondary antibodies (cat. no. sc-2065) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Goat anti-rabbit FITC-conjugated secondary antibodies (cat. no. TA130021) were purchased from OriGene Technologies, Inc. (Beijing, China). Bovine serum albumin was purchased from Solarbio Science and Technology Co., Ltd (Beijing, China). Annexin V-Propidium Iodide (PI) Apoptosis Detection kit was purchased from BestBio Biotechnology Co., Ltd. (Shanghai, China). JC-1 Mitochondrial Membrane

Potential kit was purchased from Beyotime Institute of Biotechnology (Haimen, China). Earle's balanced salt solution (EBSS) sugar-free culture medium was purchased from Hyclone (GE Healthcare).

**Instruments.** StepOne 2.1 Real-Time PCR system was purchased from Applied Biosystems (Thermo Fisher Scientific, Inc.). Mastercycler® nexus flat eco was purchased from Eppendorf (Hamburg, Germany). A Odyssey® Fc Imaging system (LI-COR Biosciences, Lincoln, NE, USA), BD Accuri™ C6 Plus flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA), and Qubit™ 3 Fluorometer (Thermo Fisher Scientific, Inc.) were also used in the present study.

**Culture of PC12 cells.** PC12 cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). PC12 cells were transferred into high glucose DMEM containing 5% FBS, 10% HS,  $1 \times 10^5$  U/l penicillin and 100 mg/l streptomycin, then incubated in a constant temperature incubator at 37°C, saturated humidity, 5% CO<sub>2</sub> + 95% O<sub>2</sub>; the medium was changed every 2 days. When cells reached 80-90% confluence, they were digested with 1 ml TrypLE solution at 37°C for 16-20 h, and centrifuged at room temperature and 252 x g for 5 min (12). The cell suspension was prepared in the high glucose DMEM medium, and the cell concentration was adjusted to  $5 \times 10^4$  cells/ml and transferred to the culture dish. Passages were proceeded when the adherent cells approached 80% confluence. Cells were divided into four groups: ATRA, OGD, ATRA + OGD and control. A 100 mM stock solution of ATRA was prepared with dimethylsulfoxide (DMSO) and diluted to the indicated final concentration (4  $\mu$ mol/l) with high glucose DMEM prior to adding to the culture medium. To induce OGD injury, the PC12 cell culture medium (high glucose DMEM) was replaced by glucose-free DMEM. The cells were then incubated (95% N<sub>2</sub> and 5% CO<sub>2</sub>) for 4 h at 37°C. Cells in the OGD and ATRA groups were subjected to OGD injury and 4  $\mu$ mol/l ATRA, respectively, for 4 h at 37°C. Cells in the OGD + ATRA group were subjected to OGD injury in glucose-free DMEM containing 4  $\mu$ mol/l ATRA. The control cells were washed with high glucose DMEM and incubated in a normoxic incubator in 100  $\mu$ l DMSO for 4 h.

**Ad-siRAR- $\alpha$  and RFP transduction of PC12 cells.** Ad-siRAR- $\alpha$  was designed and constructed by the present authors. Three different pairs of sequences of siRNA were designed according to the gene sequence of rat RAR- $\alpha$  (NM\_031528.2) provided by GenBank (ncbi.nlm.nih.gov/genbank), as shown in Table I. The adenovirus shuttle plasmid pSES-HUS-s RAR- $\alpha$  gifted by Professor Tong-Chuan He (Molecular Oncology Lab, Department of Orthopaedic Surgery and Rehabilitation Medicine, University of Chicago Medical Center, Chicago, IL, USA) was constructed and recombined with a skeleton plasmid, pAd-Easy-1 (Stratagene; Agilent Technologies GmbH, Waldbronn, Germany), to obtain Ad-siRAR- $\alpha$  recombinant adenovirus in 293 cells (American Type Culture Collection, Manassas, VA, USA) using ViraDuctin™ Adenovirus Transduction Reagent (Cell Biolabs, Inc., San Diego, CA, USA) according to the

Table I. siRAR- $\alpha$  sequences of RAR- $\alpha$  genes.

Name	Sequence (5'-3')
siRAR- $\alpha$	Fwd: ACCAAGGAGTCGGTGCAGAAATTTT Rev: ATTTTCGACCCGACTCCTTGGTTTT
siRAR- $\alpha$ 2	Fwd: AGCAAGTACACTACGAACAATTTT Rev: ATGTTCGTAGTGTACTTGCTTTT
siRAR- $\alpha$ 3	Fwd: ACCTCATCTGTGGAGACCGATTTT Rev: ATCGGTCTCCACAGATGAGTTTT

RAR- $\alpha$ , retinoic acid receptor  $\alpha$ ; siRAR- $\alpha$ , RAR- $\alpha$  small interfering RNA; Fwd, forward; Rev, reverse.

Table II. Primer sequences for polymerase chain reaction analysis of RAR- $\alpha$  expression.

Gene name	Primer sequence (5'-3')
RAR- $\alpha$	Fwd: GACTCCGCTTTGGAATGG Rev: ACTGCTGCTCTGGGTCTCG
$\beta$ -actin	Fwd: GCATAGCCACGCTTGTCTTGAAG Rev: GAACCGCTCATTGCCGATAGTG

RAR- $\alpha$ , retinoic acid receptor  $\alpha$ ; Fwd, forward; Rev, reverse.

manufacturer's protocol. The inhibitory effect of the siRNA was verified by semi-quantitative RT-PCR. pAd-Easy-1 alone was transfected as aforementioned and used as the negative control for the semi-quantitative RT-PCR.

Three pairs of different Ad-siRAR- $\alpha$  recombinant adenoviruses were mixed in equal proportions (2  $\mu$ l) and transduced into PC12 cells at 70% confluence, with pSES-HUS adenovirus as the negative control. RFP was incubated with cells treated with ATRA and OGD for 4 h at 37°C. Viral transduction was subsequently verified using fluorescence microscopy by red fluorescence observation, and the fluorescence was quantified by ImageJ software (version 1.51d; National Institutes of Health, Bethesda, MD, USA) under three different fields of view with magnification of x200. PC12 cells were washed with Hank's solution following 36 h of viral transduction at 37°C. Following the transduction, the cells in the siRAR- $\alpha$  + ATRA + OGD group and RFP + ATRA + OGD group were subsequently subjected to OGD injury in glucose-free DMEM containing 4  $\mu$ M ATRA, then incubated (95% N<sub>2</sub> and 5% CO<sub>2</sub>) for 4 h at 37°C. The control cells were washed with high glucose DMEM and incubated in a normoxic incubator in 100  $\mu$ l DMSO for 4 h.

*Detection of inhibition rate of siRAR- $\alpha$  in PC12 cells by semi-quantitative reverse transcription PCR (RT-PCR).* RNA was extracted from all cells using an RNeasy Mini kit and reverse-transcribed into cDNA using PrimeScript™ RT Reagent kit (Perfect Real Time) according to the manufacturers' protocols. PCR amplification was performed using a SuperReal PreMix Plus (SYBR-Green) and specific primers for RAR- $\alpha$  in rats (Table II) under the following

conditions: 94°C For 2 min; 10 cycles at 93°C for 20 sec, 65-55°C for 20 sec (each cycle reduced by 1°C) and 72°C for 20 sec; 28 cycles at 93°C for 20 sec, 56°C for 20 sec and 72°C for 20 sec, and 72°C for 2 min.  $\beta$ -actin was used as the internal control. Finally, RAR- $\alpha$  mRNA expression level in transduced PC12 cells was analysed by separating total RNA in a 1.5% agarose gel with ethidium bromide staining and quantified software (Image Studio™ Lite, version 4.0) embedded in Odyssey® Fc Imager (version 1.0.17; both LI-COR Biosciences).

*Annexin V-PI flow cytometry detection of apoptosis.* PC12 cells were gently rinsed with Hank's solution (2 ml) and the flushing fluid was collected in each test tube. The cells with different treatments were digested with 0.5 ml TrypLE solution at 37°C until 70% of the cells were detached and a glass straw attached to a rubber head was used to blow cells into corresponding test tubes. Tubes were centrifuged at 4°C and 252 x g for 4 min, then the supernatant of each tube was discarded. Annexin V-fluorescein isothiocyanate and PI staining were performed on cell sediments according to the Annexin V-PI kit protocol. The apoptosis rate of each group was detected using a flow cytometer and analyzed using FCS Express Flow Cytometry Lite RUO software (version 6; De Novo Software, Glendale, CA, USA). The incidence of apoptosis for all groups was measured three times.

*Detection of mitochondrial transmembrane potential (MMP) by JC-1.* Cells were collected using the aforementioned method, and the supernatant was discarded following centrifugation. The freshly prepared JC-1 working solution and high glucose DMEM were added to the PC12 cell pellet at a ratio of 1:1. Following mixing, the mixture was incubated in a humidified atmosphere at 37°C, 5% CO<sub>2</sub> + 95% O<sub>2</sub> incubator for 20 min. The mixture was centrifuged for 4 min at 252 x g, 4°C and the supernatant was discarded, followed by washing with 1X PBS twice (8-10 min/wash). The PC12 cells were resuspended in 300  $\mu$ l 1X PBS buffer, and the MMP was measured a flow cytometer and FCS Express Flow Cytometry Lite RUO software (version 6). The above experiments were repeated three times.

*RT-quantitative (q)PCR detection of Bax and Bcl-2 expression in PC12 cells with OGD-induced injury following Ad-siRAR- $\alpha$  transduction.* Following OGD-induced injury, PC12 cells were washed with 2 ml Hank's solution and subjected to mRNA extraction according to the protocol of the RNeasy Mini kit. The extracted RNA was added to RQ1 RNase-free DNase (Promega Corporation, Madison, WI, USA) to remove DNA contamination. Following the detection of RNA concentration using the Qubit RNA HS Assay kit and Qubit 3 Fluorometer according to the manufacturer's protocol, the RNA was reverse transcribed into cDNA using the PrimeScript Reverse Transcription kit (reaction conditions: 37°C for 15 min, then 85°C for 5 sec), and the obtained cDNA was diluted 10 times with water and preserved at -20°C. qPCR was performed to detect the mRNA expression level of Bax and Bcl-2 in a PCR system using SuperReal PreMix Plus (SYBR Green). The thermocycling conditions were as follows: 95°C for 3 min, 45 cycles of 95°C for 20 sec, 55°C for 20 sec

and 72°C for 20 sec, then 65°C for 5 sec with  $\beta$ -actin as the internal reference gene. PCR primers were designed using Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA, USA). The specific primer sequences are shown in Table III. Band intensity values for Bcl-2 and Bax were normalized to those of  $\beta$ -actin and the relative expression was calculated using the  $2^{-\Delta\Delta C_q}$  method (13).

**Western blot analysis of protein expression in PC12 cells with OGD-induced injury following Ad-siRAR- $\alpha$  transduction.** PC12 cells were washed twice with PBS and protein was extracted according to the protocol of ReadyPrep Protein Extraction kit (Total Protein) kit. The concentration was determined by bicinchoninic acid assay, and equal amounts of protein (25  $\mu$ g/lane) were separated by 12% SDS-PAGE. The proteins were transferred to polyvinylidene fluoride membranes and blocked for 1 h with 5% bovine serum albumin TBS-Tween-20 (TBST) solution at room temperature. The TBST solution was used to wash the membrane three times (8-10 min/wash), and membranes were incubated in TBST solution with primary antibodies directed against Bcl-2, Bax and  $\beta$ -actin (all 1:1,000) overnight at 4°C. Following three washes of the membrane in TBST solution (8-10 min/wash), horseradish peroxidase-labeled secondary antibodies (goat anti-rabbit, 1:500; goat anti-rat, 1:800) were added and incubated for 1 h at room temperature. Pierce™ ECL Plus Western Blotting Substrate (Thermo Fisher Scientific, Inc.) was used to visualize the bands, which were quantified using Image Studio Lite software (version 4.0).

**Statistical analysis.** SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA) was used for one-way analysis of variance to compare multiple groups, with Student-Newman-Keuls analysis as a post-hoc test for multiple comparisons. A Student's t-test was used to compare differences between two groups. Experimental data are expressed as the mean  $\pm$  standard deviation.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Evaluation of Ad-siRAR- $\alpha$  transduction.** When the Ad-siRAR- $\alpha$  recombinant adenovirus was transduced by Ad-siRAR- $\alpha$  for 36 h, it was identified that >70% of the cells exhibited red fluorescence under fluorescent microscope observation (Fig. 1). This indicated that PC12 cells had been transduced with Ad-siRAR- $\alpha$ .

**Expression of RAR- $\alpha$  mRNA in PC12 cells following transduction with Ad-siRAR- $\alpha$ .** Semi-quantitative RT-PCR results indicated that the expression of RAR- $\alpha$  mRNA was significantly decreased in PC12 cells following transduction with Ad-siRAR- $\alpha$  compared with the control group, which was transfected with adenovirus blank vector ( $P < 0.05$ ; Fig. 2). This suggested that Ad-siRAR- $\alpha$  was able to effectively suppress RAR- $\alpha$  gene expression.

**Expression of RAR- $\alpha$  in PC12 cells following transduction with Ad-siRAR- $\alpha$  and OGD-induced injury.** As presented in Fig. 3, the results of semi-quantitative RT-PCR indicated

Table III. Primer sequences for polymerase chain reaction analysis of apoptosis-related gene expression.

Gene name	Primer sequence (5'-3')
Bax	Fwd: ATTTCCAGACACCGAGGG Rev: TAAGCCAAATGTAGCAAGG
Bcl-2	Fwd: CGGGAGAACAGGGTATGA Rev: CAGGC TGG AAGGAGAAGAT
$\beta$ -actin	Fwd: GCATAGCCACGCTTGTCTTGAAG Rev: GAACCGC TCATTGCCGATAGTG

Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; Fwd, forward; Rev, reverse.

that RAR- $\alpha$  mRNA expression was significantly increased in PC12 cells following exposure to 4  $\mu$ mol/l ATRA ( $P < 0.05$ ), whereas the expression of RAR- $\alpha$  in OGD-injured cells was significantly decreased compared with the control group cells ( $P < 0.05$ ). However, compared with the control group, no significant difference was observed in the expression of RAR- $\alpha$  mRNA in OGD-induced PC12 cells treated with 4  $\mu$ mol/l ATRA. Similarly, RAR- $\alpha$  mRNA expression level in the PC12 cells transduced with Ad-siRAR- $\alpha$  was significantly lower compared with the group that was transduced with adenovirus vector ( $P < 0.05$ ). These results indicate that OGD-induced injury down-regulated the RAR- $\alpha$  mRNA expression level and the Ad-siRAR- $\alpha$  vector was successfully transduced.

**Apoptosis rate of PC12 cells following transduction with Ad-siRAR- $\alpha$  and OGD-induced injury.** As shown in Fig. 4, the apoptosis rate of PC12 cells following transduction with Ad-siRAR- $\alpha$  and OGD-induced injury was significantly higher compared with the adenovirus-transfected OGD-induced injury group ( $P < 0.05$ ). This indicates that down-regulation of RAR- $\alpha$  can induce the apoptosis of PC12 cells.

**Effect of Ad-siRAR- $\alpha$  on MMP in PC12 cells following OGD-induced injury.** JC-1 is an ideal fluorescent probe for the detection of MMP, and the decrease of MMP is a landmark event in the early stage of mitochondrial apoptosis (14). When MMP is normal, JC-1 forms a polymer in the mitochondrial matrix, producing red fluorescence. When MMP decreases, JC-1 becomes a monomer, resulting in green fluorescence (15). In the present study, it was investigated whether different expression levels of RAR- $\alpha$  were able to influence MMP in PC12 cells with OGD-induced injury. It was identified that in PC12 cells transduced with Ad-siRAR- $\alpha$ , the detection rate of green fluorescence was significantly increased ( $P < 0.05$ ; Fig. 5), indicating a decrease in MMP compared with the control. In Fig. 5A, the images indicate the MMP at a certain time, and cannot reveal the trend changes in membrane potential. The two axes in the figure represent the green fluorescence detected by the FL1-H channel, and the green fluorescence detected by the FL2-H channel. The detection rate of green fluorescence indicates the degree of decrease in MMP. MMP was decreased in the two groups and no red fluorescence was observed.

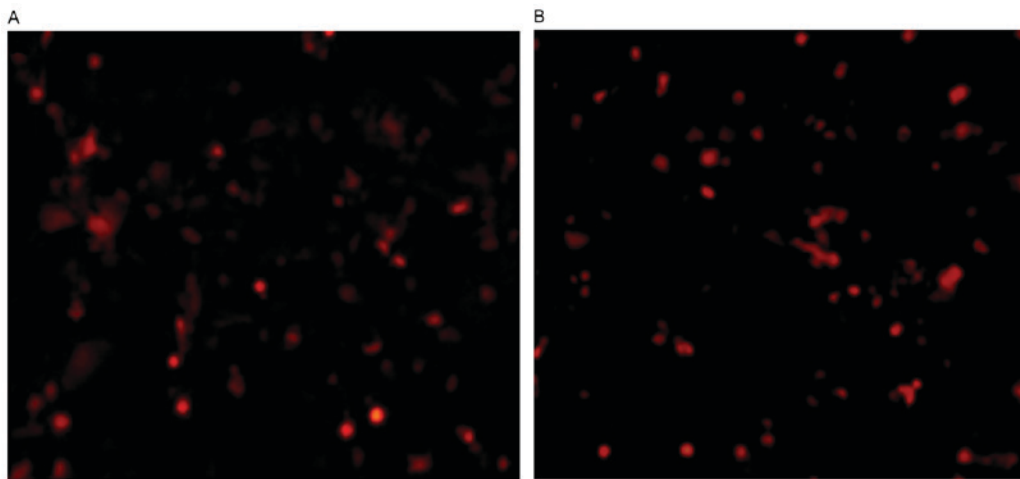


Figure 1. Morphological observation of PC12 cells following transduction with Ad-siRAR- $\alpha$  using RFP. (A) siRAR- $\alpha$  + ATRA + OGD group. (B) RFP + ATRA + OGD group. Magnification,  $\times 200$ . RAR- $\alpha$ , retinoic acid receptor  $\alpha$ ; siRAR- $\alpha$ , RAR- $\alpha$  small interfering RNA; Ad-siRAR- $\alpha$ , recombinant adenovirus siRAR- $\alpha$ ; ATRA, all-trans retinoic acid; OGD, oxygen-glucose deprivation; RFP, red fluorescent protein.

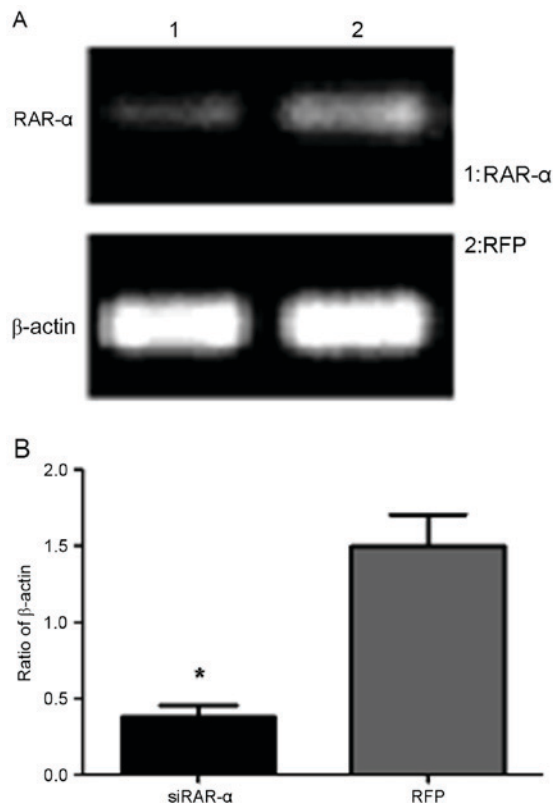


Figure 2. Levels of RAR- $\alpha$  mRNA expression in PC12 cells following recombinant adenovirus siRAR- $\alpha$  transduction. (A) Semi-quantitative results of reverse transcription polymerase chain reaction. (B) RAR- $\alpha$  mRNA expression relative to  $\beta$ -actin. \* $P < 0.05$  vs. RFP group. RAR- $\alpha$ , retinoic acid receptor  $\alpha$ ; siRAR- $\alpha$ , RAR- $\alpha$  small interfering RNA; RFP, red fluorescent protein.

*Effect of Ad-siRAR- $\alpha$  on Bax and Bcl-2 mRNA expression in PC12 cells following OGD-induced injury.* The flow cytometry results described above indicated that Ad-siRAR- $\alpha$  was able to significantly increase the apoptosis rate in OGD-induced PC12 cells. Therefore, the current study aimed to explore whether Ad-siRAR- $\alpha$  also affected the expression of Bax and

Bcl-2, which are key molecules in apoptosis. As presented in Fig. 6, Ad-siRAR- $\alpha$  significantly increased the Bax mRNA expression level ( $P < 0.05$ ) and significantly decreased the Bcl-2 mRNA level ( $P < 0.05$ ) compared with the control group of RFP + ATRA + OGD.

*Effect of Ad-siRAR- $\alpha$  on Bax and Bcl-2 protein expression in PC12 cells following OGD-induced injury.* As presented in Fig. 7, the protein expression of apoptosis factor Bax in the siRAR- $\alpha$  + ATRA + OGD group was significantly higher compared with the RFP + ATRA + OGD group ( $P < 0.05$ ), whereas the expression of anti-apoptotic Bcl-2 protein was significantly lower compared with the control group ( $P < 0.05$ ). These results were consistent with the mRNA levels in Fig. 6. These results suggest that siRAR- $\alpha$  promotes apoptosis in OGD-induced PC12 cells, and that OGD-induced apoptosis is, at least in part, mediated by RAR- $\alpha$  expression.

## Discussion

RA serves a key function in embryonic development, particularly in neural system development (16,17). It is the primary active substance of VA in the body, which acts via regulating downstream gene transcription through signal transduction of RARs (16,17). RARs may be divided into three subtypes:  $\alpha$ ,  $\beta$  and  $\gamma$ . RARs and retinoid X receptors (RXRs) typically form heterodimers, which may combine with RA response elements of target genes to directly regulate the transcription of target genes (including HOX, surfactant protein B and forkhead box P3) (18). RXRs and RARs may also form homodimers between themselves, or form homodimers combined with peroxisome proliferator-activated receptor, thyroid hormone receptor, vitamin D receptor, actin-related protein 1 or nerve growth factor IB (16,18). As the central nervous system develops, the RA signaling pathway is active in numerous brain tissues (19), and different RA receptors express specific functions in specific regions (20). Previous studies have demonstrated that during the embryonic development of the nervous system,

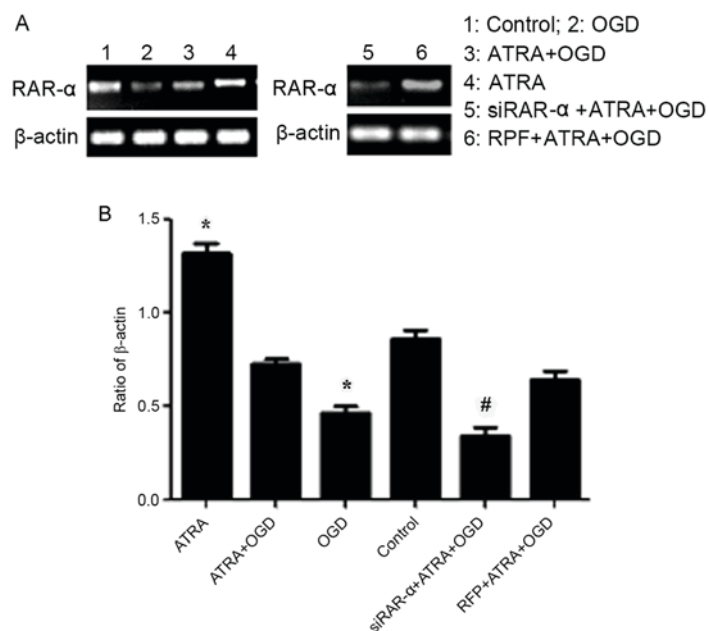


Figure 3. Levels of RAR- $\alpha$  mRNA expression in PC12 cells following recombinant adenovirus siRAR- $\alpha$  transduction and OGD-induced injury. (A) Semi-quantitative results of reverse transcription polymerase chain reaction. (B) RAR- $\alpha$  mRNA expression relative to  $\beta$ -actin. \* $P < 0.05$  vs. control group; # $P < 0.05$  vs. RFP + ATRA + OGD group. RAR- $\alpha$ , retinoic acid receptor  $\alpha$ ; siRAR- $\alpha$ , RAR- $\alpha$  small interfering RNA; ATRA, all-trans retinoic acid; OGD, oxygen-glucose deprivation; RFP, red fluorescent protein.

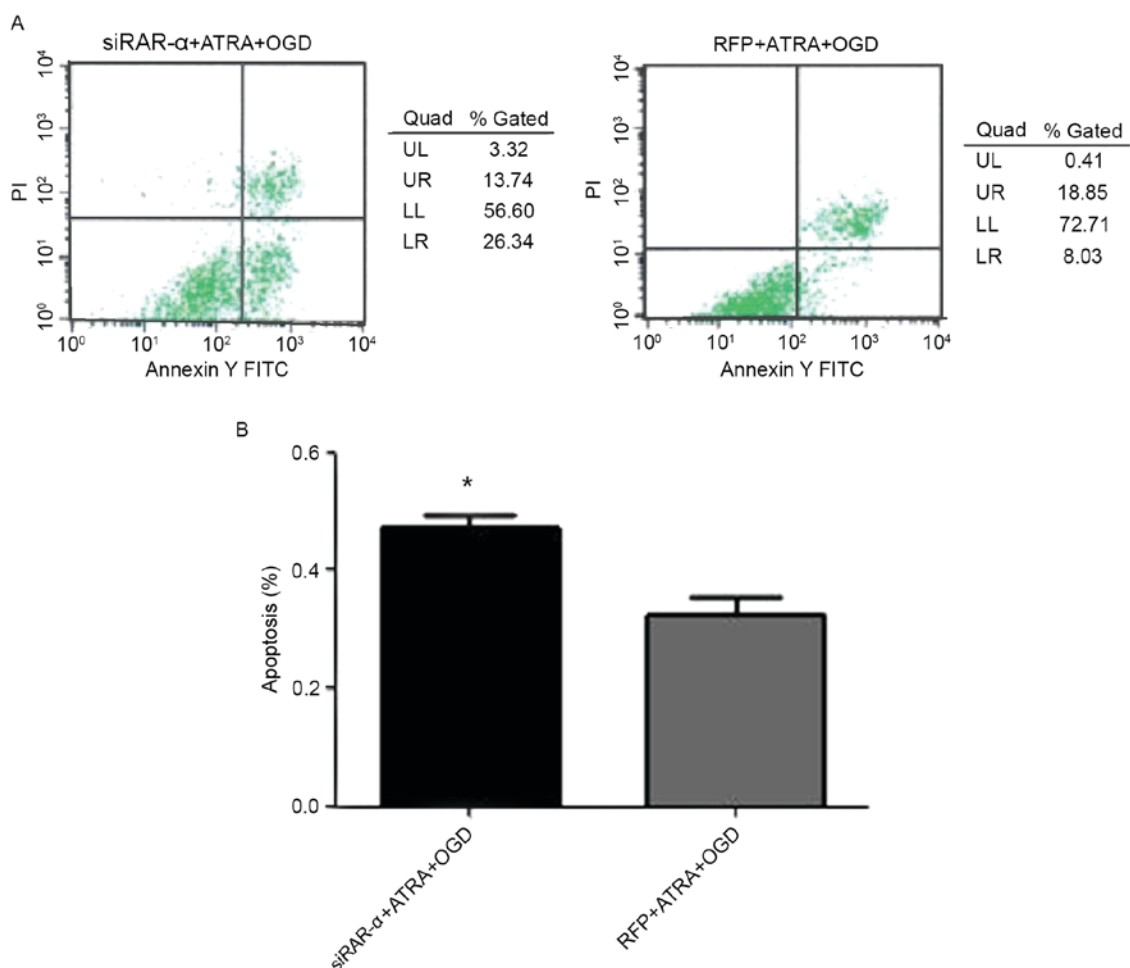


Figure 4. Effect of recombinant adenovirus siRAR- $\alpha$  transduction on the apoptosis rate of PC12 cells following OGD treatment. (A) Apoptosis rate was detected by flow cytometry. (B) Quantification of apoptosis rate. \* $P < 0.05$  vs. RFP + ATRA + OGD group. RAR- $\alpha$ , retinoic acid receptor  $\alpha$ ; siRAR- $\alpha$ , RAR- $\alpha$  small interfering RNA; ATRA, all-trans retinoic acid; OGD, oxygen-glucose deprivation; RFP, red fluorescent protein; FITC, fluorescein isothiocyanate; PI, propidium iodide; UL, upper left; UR, upper right; LL, lower left; LR, lower right.

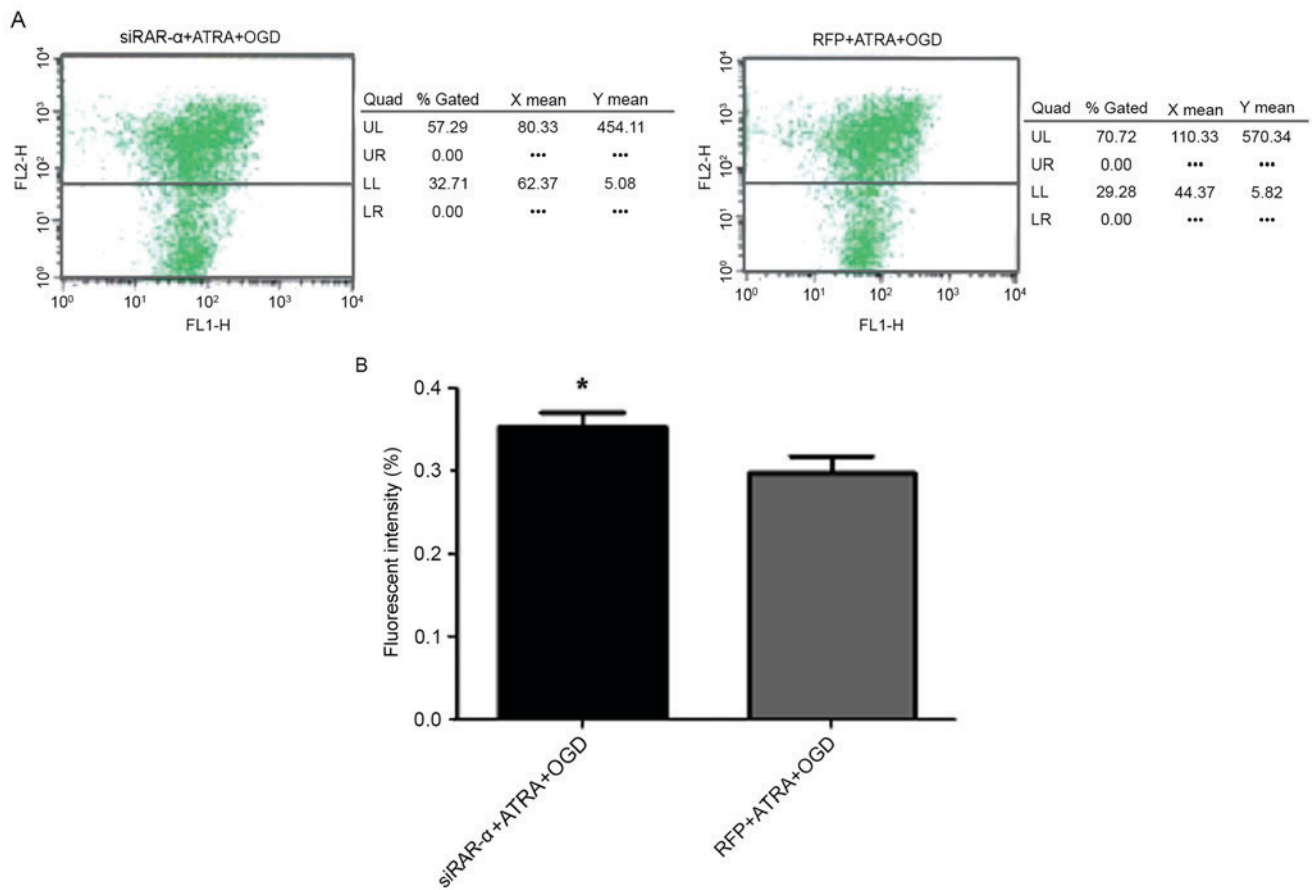


Figure 5. Effect of recombinant adenovirus siRAR- $\alpha$  transduction on MMP in PC12 cells following OGD treatment. (A) Detection of MMP. (B) Quantification of fluorescence in PC12 cells. \* $P < 0.05$  vs. RFP + ATRA + OGD group. MMP, mitochondrial membrane potential; RAR- $\alpha$ , retinoic acid receptor  $\alpha$ ; siRAR- $\alpha$ , RAR- $\alpha$  small interfering RNA; ATRA, all-trans retinoic acid; OGD, oxygen-glucose deprivation; RFP, red fluorescent protein; UL, upper left; UR, upper right; LL, lower left; LR, lower right.

RA is able to associate with corresponding receptors to take part in the division and maintenance of nerve cells (19,21,22). RA is able to activate different receptors, induce neuronal precursor cells to differentiate into specific nerve cells (21), and also serves a key function in the regional distribution of neural plate craniocaudal axis (22). RAR- $\alpha$  is a major RAR expressed during hippocampal development in rats, and serves a key function in learning and memory ability (23).

Previous studies by the present authors have indicated that VA supplementation in rats with marginal VA deficiency (MVAD) are not able to restore the learning and memory ability of rats during postnatal development, indicating that the MVAD from gestation may have irreversible effects on the brain development of newborn rats (24,25). Shinozaki *et al* (26) have previously reported that ATRA may significantly reduce the apoptosis rate of nerve cells in the hippocampus region in rats and that this effect may be weakened by RAR-specific antagonists. Following spinal cord injury in adult rats, exogenous RA is able to activate the RAR- $\beta$ 2 receptor, and stimulate the growth of nerve axons and repair of the peripheral nervous system (27). Furthermore, a study by the present authors on HIBD neonatal rats demonstrated that the VA nutrition level is associated with the repair function of the injured neurons, the primary mechanism of which is to inhibit the activation of  $Ca^{2+}$  in nerve cells through the regulation of

RAR- $\alpha$  nuclear receptors (9). In the present study, it was also observed that OGD-induced injury induced a decrease in RAR- $\alpha$  expression level, whereas ATRA incorporation induced an increase of RAR- $\alpha$ , which indicated that RAR- $\alpha$  may serve a function in the anti-apoptotic effect of ATRA against OGD-induced injury.

RNAi is a phenomenon that inhibits specific gene expression. When double-stranded RNA that is homologous to the endogenous mRNA coding region is introduced into the cell, the mRNA is degraded to cause silencing of gene expression, which is a key method for studying gene function (11). The establishment of highly efficient siRNA has allowed RNAi technology to be widely used in the study of numerous diseases, including Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis (28). In the current study, following transduction with Ad-siRAR- $\alpha$ , RAR- $\alpha$  expression in PC12 cells was significantly inhibited. By using Ad-siRAR- $\alpha$  transduction, flow cytometry analysis indicated that the apoptotic rate of OGD-injured PC12 cells was increased. The MMP also decreased following Ad-siRAR- $\alpha$  transduction. These results indicated that low expression of RAR- $\alpha$  is able to enhance the rate of apoptosis in injured cells, and inhibit the repair function of cells. Previous results have indicated that in the process of neurological damage, RAR- $\alpha$  primarily promotes the proliferation of astrocytes and oligodendrocytes, and serves a key function in the

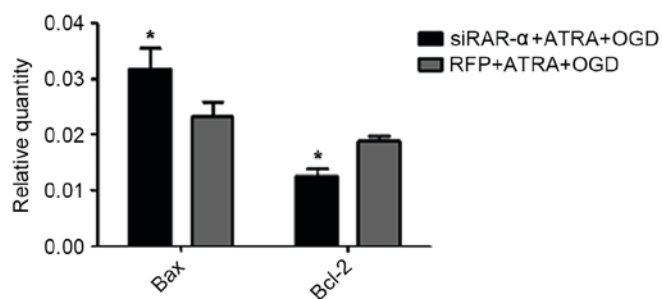


Figure 6. Effect of recombinant adenovirus siRAR- $\alpha$  transduction on Bax and Bcl-2 mRNA expression in PC12 cells after OGD treatment. \* $P < 0.05$  vs. RFP + ATRA + OGD. RAR- $\alpha$ , retinoic acid receptor  $\alpha$ ; siRAR- $\alpha$ , RAR- $\alpha$  small interfering RNA; ATRA, all-trans retinoic acid; OGD, oxygen-glucose deprivation; RFP, red fluorescent protein; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein.

final maturation process (21). Katsuki *et al* (29) previously proposed that RA may enhance the activity of brain-derived neurotrophic factor by upregulating RAR- $\alpha$  expression, thereby protecting against dopaminergic neuronal injury in the mesencephalon, which is consistent with the results of the current study.

HIBD injury is typically divided into three phases: Primary cell injury stage, energy recovery stage and late-onset cell injury stage (30). The previous study demonstrated that during the late-onset cell injury stage, mitochondrial dysfunction serves a key function and induces secondary failure of cellular energy metabolism. Bcl-2, which is located on the mitochondrial membrane, is a proto-oncogene that has been identified in lymphoma leukemia cells. Bcl-2 is a highly conserved eukaryotic gene, and is expressed at low levels in normal cells (31). Bax and Bcl-2 are two key apoptosis-regulating genes of the Bcl-2 gene family with opposite functions. Downregulation of Bax and upregulation of Bcl-2 increase the permeability of mitochondrial membrane, which releases cytochrome C from the mitochondria, activates apoptosis-inducible factors and ultimately triggers apoptosis (32). In the current study, it was indicated that ATRA served an anti-apoptosis function by regulating an endogenous apoptosis signaling pathway, and the expression of RAR- $\alpha$  was inhibited by transduction with Ad-siRAR- $\alpha$ . It was also identified that Bcl-2 gene expression was downregulated and Bax gene expression upregulated in the mitochondrial apoptosis signaling pathway, and the results were consistent at the mRNA and protein level. Previous studies have demonstrated that Bcl-2 and Bax are targets of RA signals (33-35). In addition, in thymic tumor cells and pancreatic cancer cells, RA has been demonstrated to induce the upregulation of suppressor gene p53 to increase Bcl-2 expression and inhibit apoptosis; however, the specific mechanism remains unclear (36,37).

In conclusion, ATRA has anti-apoptosis and other protective effects on OGD-induced injury. In the present study, through the method of silencing RAR- $\alpha$  expression in PC12 cells by Ad-siRAR- $\alpha$  transduction, it was demonstrated that the anti-apoptosis effect of ATRA on OGD-induced injury is achieved via increasing the expression of anti-apoptotic factor Bcl-2 and decreasing the expression of pro-apoptotic

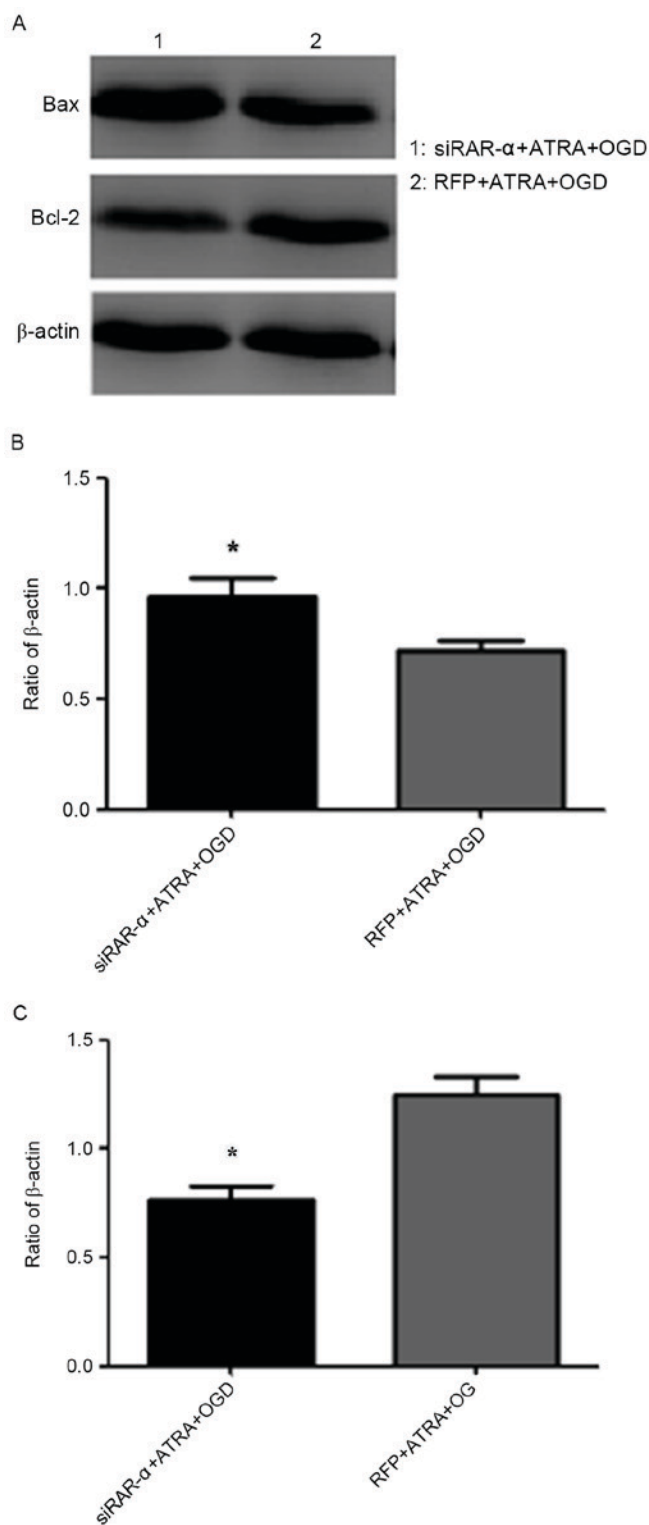


Figure 7. Effect of recombinant adenovirus siRAR- $\alpha$  transduction on Bax and Bcl-2 mRNA expression in PC12 cells after OGD treatment. (A) Western blot analysis of protein expression. (B) Bax protein expression relative to  $\beta$ -actin. (C) Bcl-2 protein expression relative to  $\beta$ -actin. \* $P < 0.05$  vs. RFP + ATRA + OGD. RAR- $\alpha$ , retinoic acid receptor  $\alpha$ ; siRAR- $\alpha$ , RAR- $\alpha$  small interfering RNA; ATRA, all-trans retinoic acid; OGD, oxygen-glucose deprivation; RFP, red fluorescent protein; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein.

factor Bax. This is achieved, at least in part, by inhibiting the mitochondrial apoptosis signaling pathway via upregulation of RAR- $\alpha$  expression.



## Competing interests

The authors declare that they have no competing interests.

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