

# Lentivirus-mediated RIG-I knockdown relieves cell proliferation inhibition, cell cycle arrest and apoptosis in ATRA-induced NB4 cells via the AKT-FOXO3A signaling pathway *in vitro*

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Received April 12, 2016; Accepted March 16, 2017

DOI: 10.3892/mmr.2017.6858

**Abstract.** Retinoic acid inducible gene I (RIG-I) is upregulated during all-trans retinoic acid (ATRA)-induced terminal granulocytic differentiation of NB4 acute promyelocytic leukemia (APL) cells. However, the function and mechanism of RIG-I in NB4 cells remains to be fully elucidated. In the present study, lentivirus-mediated RIG-I-knockdown was used to investigate the proliferation, cell cycle and apoptotic processes of ATRA-induced NB4 cells *in vitro* using an MTT assay and flow cytometry, respectively. The roles of RIG-I and the AKT-FOXO3A signaling pathway were investigated using western blot analysis. The results showed that the ATRA-induced expression of RIG-I was specifically and effectively knocked down at the mRNA and protein levels by lentivirus mediated RIG-I short hairpin RNA. In addition, silencing of RIG-I reduced the ATRA-induced inhibition of NB4 cell proliferation, cell cycle arrest and apoptosis. Further investigations indicated that with ATRA-induced expression of RIG-I, levels of phosphorylated (p)AKT-Thr308 and pForkhead Box (FOX) O3A-Thr32 were decreased, the expression levels of cell cycle arrest protein p27 and the apoptotic protein, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), directly transcribed by FOXO3A were increased. By contrast, following the knockdown of ATRA-induced expression of RIG-I, the levels of pAKT-Thr308 and pFOXO3A-Thr32 were increased, and the protein expression levels of p27 and TRAIL were decreased. Taken together, these results showed that the knockdown of RIG-I reduced

the inhibition of cell proliferation, cell cycle arrest and apoptosis in the ATRA-induced NB4 cells via the AKT-FOXO3A signaling pathway.

## Introduction

Acute promyelocytic leukemia (APL) is a specific type of acute myeloid leukemia (AML), which exhibits an aggressive clinical course. Patients with APL are prone to hemorrhage, infection and concurrent disseminated intravascular coagulation, and have a high rate of mortality. APL is treated with all-trans retinoic acid (ATRA) in conjunction with other approaches, including therapeutic regimens, have shown promising results, and there are examples of the successful treatment of tumors using agents, which induce cellular differentiation (1). Retinoic acid inducible gene I (RIG-I) was originally identified in the ATRA-induced terminal granulocytic differentiation of the NB4 APL cell line (1-3). In a previous study in 2004, RIG-I was cloned in double-stranded RNA-induced innate immunity. Structural analysis revealed that RIG-I is a RNA helicase enzyme with a DExD/H box motif consisting of 925 amino acid residues, with two CARD domains at the N terminus and one RNA helicase domain at the C terminus (4). Following these observations, investigations of RIG-I as an important pattern recognition receptor in the innate immune response have become increasingly popular (5-9).

Although the role of RIG-I in the anti-virus innate immune response has been clearly defined, it was originally identified using a system, which did not use extraneous virus, namely during the ATRA-induced terminal granulocytic differentiation of APL cells, suggesting it may have other inherent activities. Analysis performed using RIG-I gene-knockout mice showed that RIG-I was an essential negative regulatory factor of myelogenous hyperplasia (3). Additionally, RIG-I was shown to promote the differentiation of leukemia cells by stimulating activity of the signal transducer and activator of transcription 1 signaling pathway (10). Previous studies have also indicated that RIG-I attenuates the proliferation of U937 AML cells by inhibiting the AKT-mammalian target of rapamycin (mTOR) pathway (11). In addition to mTOR, the Forkhead Box (FOX)O3A transcription factor is another important AKT downstream target protein, which assists in the control of cell proliferation, differentiation, apoptosis and cell cycle. FOXO3A is located in the nucleus, and when

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**Key words:** lentivirus, short hairpin RNA, retinoic acid inducible gene I, proliferation, cell cycle, apoptosis, phosphoinositide-3 kinase/Akt, Forkhead box O3A

combined with DNA, induces the protein expression of p27, p130-Rb2 and cyclinD1/2 (cell-cycle regulation), and the expression of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and B cell lymphoma-2-interacting mediator of cell death in apoptosis. When AKT is phosphorylated, the Thr32 and Ser253 phosphorylation sites of FOXO3A are directly phosphorylated by AKT. FOXO3A then interacts with 14-3-3 proteins, leading to the transfer of FOXO3A from the nucleus into the cytoplasm. Therefore, the retention of FOXO3A in the cytoplasm inhibits its associated transcription, and results in cell proliferation and survival (12). It has been reported that ATRA induces APL cell differentiation and apoptosis by activating the transcription factor, FOXO3A (13). The above-mentioned studies suggest a potential association between the expression of RIG-I during ATRA-induced terminal granulocytic differentiation of the NB4 APL cell line and the AKT-FOXO3A signaling pathway.

In the present study, the lentivirus method was used to knock down the expression of RIG-I in ATRA-induced NB4 cells *in vitro*, and the resulting effects on NB4 cell proliferation, the cell cycle and apoptosis were examined, as were the roles of RIG-I and the AKT-FOXO3A signaling pathway. The results showed that the knockdown of RIG-I reduced cell proliferation inhibition, cell cycle arrest and apoptosis in the ATRA-induced NB4 cells by affecting the AKT-FOXO3A signaling pathway.

## Materials and methods

**Cell culture.** The NB4 APL cell line was grown in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (HyClone Laboratories; GE Healthcare Life Sciences; Logan, UT, USA), 100 U/ml penicillin and 100 U/ml streptomycin (Beyotime Institute of Biotechnology, Shanghai, China), in an incubator set at 37°C and containing 5% CO<sub>2</sub>.

**Lentivirus infection.** Actively proliferating NB4 cells were inoculated in a 12-well culture plate (1x10<sup>5</sup> cells/well) and cultivated in 1 ml of 1640 medium without fetal bovine serum. Subsequently, lentivirus containing green fluorescent protein (GFP) (Shanghai Genepharma Co., Ltd, Shanghai, China) and polybrene (final concentration of 8 µg/ml) were added to each well of NB4 cells, which were then centrifuged at 1,000 g for 90 min at room temperature. Following centrifugation, the cells were placed in a 37°C incubator containing 5% CO<sub>2</sub> for 5 h, following which 10% fetal bovine serum was added. The NB4 cells infected with control (Con)-small interfering (si)RNA (5'-GCTCCCGTGAATTGGAATCCT-3') were designated as LV-shCon cells, and those infected with RIG-I-siRNA lentivirus (5'-GGAATTTGGAACACAGAAATAG-3') were designated as LV-shRIG-I cells. The expression of GFP was detected using flow cytometry at 72 h post-infection.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis.** At 0 and 72 h post-ATRA induction, total cellular RNA was isolated from the NB4, LV-shCon and LV-shRIG-I cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) The extracted RNA was analyzed using electrophoresis, and the concentration of non-degraded RNA

was quantified by measuring optical density values. All samples were reverse transcribed to cDNA using PrimeScript™ RT reagent kit (Takara Biotechnology Co., Ltd., Dalian, China). Reaction systems were established, including RNA (1.0 µg), 5X PrimeScript™ Buffer (2 µl), PrimeScript RT Enzyme Mix I (2 µl), oligo dT Primer (0.5 µl) and random 6 mers (0.5 µl), with RNase-free dH<sub>2</sub>O to make the final volume up to 10 µl. The RT reaction conditions were as follows: 37°C for 15 min followed by 85°C for 5 sec. The primer sequences for qPCR were as follows: RIG-I, sense 5'-GGACGTGGC AAAACAAATCAG-3' and antisense 5'-GCAATGTCAATG CCTTCATCA-3'; β-actin, sense 5'-CTTAGTTGCGTTA CACCCTTTCTTG-3' and antisense 5'-CTGTCACCTTCAC CGTCCAGTTT-3'. The primers and cDNA was prepared using SYBR Green Master mix (Takara Bio, Inc., Otsu, Japan) according to the manufacturer's protocol. The reaction process was performed at 50°C for 2 min, 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec and 60°C for 30 sec, and a final extension step at 72°C for 10 min. All RT-qPCR experiments were detected with an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems; Thermo Fisher Scientific, Inc.). With β-actin as the internal control, the relative gene expression levels of RIG-I were analyzed using the 2<sup>-ΔΔC<sub>q</sub></sup> method (14).

**Western blot analysis.** At 0 and 72 h post-ATRA induction, the cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed in lysis buffer (Solarbio Science and Technology Co., Ltd., Beijing, China) for 30 min. The cell lysate was then centrifuged at 8,000 g for 5 min at 4°C, and the soluble protein fraction was boiled at 100°C for 10 min; following which, the protein concentration was determined using a bicinchoninic acid protein assay kit (Solarbio Science and Technology Co., Ltd.). A 30 µg sample of total soluble protein was separated by electrophoresis on an 8-10% sodium dodecyl sulfate polyacrylamide gel (Solarbio Science and Technology Co., Ltd.) at a constant voltage of 120 V. The separated protein bands were then electrophoretically transferred onto polyvinylidene difluoride membranes (EMD Millipore, Bedford, MA, USA). The membranes were blocked with 5% non-fat milk at room temperature for 1 h, and then immunoblotted at 4°C overnight with the following antibodies: Monoclonal rabbit anti-human RIG-I (cat. no. 4200; 1:2,000; Cell Signaling Technology, Inc, Danvers, MA, USA), polyclonal rabbit anti-human AKT (cat. no. 9272; 1:2,000; Cell Signaling Technology, Inc.), monoclonal rabbit anti-human phospho-AKT (Thr308; cat. no. 4056; 1:500; Cell Signaling Technology, Inc.), polyclonal rabbit anti-human FOXO3A (cat. no. 2497; 1:2,000; Cell Signaling Technology, Inc.), monoclonal rabbit anti-human phospho-FOXO3A (Thr32; cat. no. 2599; 1:1,000; Cell Signaling Technology, Inc.), monoclonal rabbit anti-human p27 (cat. no. 3686; 1:1,000; Cell Signaling Technology, Inc.) and monoclonal rabbit anti-human TRAIL (cat. no. 3219; 1:1,000; Cell Signaling Technology, Inc.). Following immunoblotting, the membranes were incubated with goat anti-rabbit horseradish peroxidase-linked secondary antibody (cat. no. 401315; 1:5,000; EMD Millipore) at room temperature for 1 h. The immunostained protein bands were visualized using SuperSignal West Pico stable peroxide solution (Pierce; Thermo Fisher Scientific, Inc.). Quantitative analysis was performed using Gel-Pro Analyzer software

(version 4.5; Media Cybernetics, Inc., Rockville, MD, USA).  $\beta$ -actin was used as a loading control.

**Detection of cell proliferative activity.** The NB4, LV-shCon and LV-shRIG-I cells were seeded into separate wells of a 96-well plate ( $1 \times 10^4$  cells/well). ATRA was added to each well ( $1 \mu\text{M}$  final concentration), and the plate was incubated at conditions of  $37^\circ\text{C}$  and 5%  $\text{CO}_2$ . Following incubation for 12, 24, 48 and 72 h, respectively,  $20 \mu\text{l}$  of 0.5% MTT solution was added to each well. The cells were then cultured under conventional conditions for 4 h, following which the culture solution was centrifuged at 300 g for 5 min at room temperature and the supernatant removed. Subsequently,  $150 \mu\text{l}$  of DMSO was added to each well, which was then oscillated for 10 min. Following oscillation, a microplate reader was used to measure the absorbance of each well at a wavelength of 490 nm. A cell proliferation curve was created with time on the X-axis and the A490 value on the Y-axis.

**Cell cycle analysis using flow cytometry.** At 0 and 72 h post-ATRA induction, the NB4, LV-shCon, and LV-shRIG-I cells were fixed in ice-cold 70% ethanol at a density of  $1 \times 10^5$  cells/ml, and then treated with ribonuclease ( $200 \mu\text{g/ml}$ ) for 30 min at  $37^\circ\text{C}$ . Propidium iodide was subsequently added to the solution. The DNA content in each aliquot of cells was then quantitated using flow cytometry (FACSCalibur; BD Biosciences; Franklin Lakes, NJ, USA). The resultant data were analyzed using CellQuest software (version 5.1; BD Biosciences) software set on 10,000 events.

**Analysis of apoptosis using flow cytometry.** Cellular apoptosis was analyzed according to the manufacturer's protocol using an apoptosis detection kit (BD Biosciences). At 0 and 72 h following ATRA induction, the NB4, LV-shCon, and LV-shRIG-I cells were washed with cold PBS and resuspended in 1X binding buffer at a concentration of  $1 \times 10^6$  cells/ml. Subsequently,  $100 \mu\text{l}$  of each solution was transferred into a 5 ml culture tube, to which  $5 \mu\text{l}$  of PE Annexin V and  $5 \mu\text{l}$  7-AAD were added. The tubes were then gently vortexed and incubated for 15 min at room temperature in the dark. Following incubation,  $400 \mu\text{l}$  of 1X binding buffer was added to each tube, and the cells were analyzed using flow cytometry (FACSCalibur; BD Biosciences).

**Statistical analysis.** All assays were performed independently in triplicate and results are expressed as the mean  $\pm$  standard deviation. Data were analyzed using Student's t-test in SPSS software (version 19.0; IBM Corp., Armonk, NY, USA).  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**ATRA-induced expression of RIG-I in NB4 cells is knocked down by LV-mediated shRIG-I.** At 72 h post-LV infection, the intensity of the expression of GFP in the LV-shCon and LV-shRIG-I cells was measured using flow cytometry. The LV-shCon and LV-shRIG-I cells were  $>80\%$  positive for GFP, indicating high infection efficiencies (Fig. 1A). The background expression of RIG-I in the NB4 cells was low, and ATRA was capable of inducing the expression of RIG-I.

To validate RIG-I-knockdown in the LV-infected NB4 cells, the induction of RIG-I in the NB4, LV-shCon and LV-shRIG-I cells at the mRNA level was analyzed using RT-qPCR analysis 72 h following ATRA induction. The results showed that, compared with the expression of RIG-I at 0 h, the mRNA expression levels of RIG-I were significantly upregulated in the NB4 and LV-shCon cells at 72 h post-ATRA induction ( $P < 0.01$ ; Fig. 1B). However, at the same time-point, the mRNA expression of RIG-I in the LV-shRIG-I cells had decreased by 78 and 82%, compared to expression in the NB4 and LV-shCon cells, respectively, ( $P < 0.01$ ; Fig. 1B). The results of the western blot analysis showed that, compared with the expression at 0 h, the protein expression levels of RIG-I in the NB4 and LV-shCon cells were significantly upregulated 72 h following ATRA induction ( $P < 0.01$ ; Fig. 1C and D). Compared with its expression in NB4 and LV-shCon cells, the protein expression of RIG-I in the LV-shRIG-I cells was significantly knocked down at 72 h ( $P < 0.01$ ; Fig. 1C and D). These results suggested that the LV-mediated shRNA had successfully infected the NB4 cells, and that the expression of RIG-I in the ATRA-induced NB4 cells had been effectively knocked down by RIG-I-specific shRNA at the mRNA and protein levels.

**RIG-I knockdown alleviates the inhibition of proliferation and cell cycle arrest induced in NB4 cells by ATRA.** The MTT method was used to analyze the proliferation of NB4, LV-shCon and LV-shRIG-I cells at 12, 24, 48 and 72 h with and without ATRA induction. The results showed that the NB4, LV-shCon and LV-shRIG-I cells had similar rates of proliferation without ATRA induction (Fig. 2A). However, the NB4 and LV-shCon cells, which were induced with ATRA showed significantly reduced proliferation at 72 h post-induction, compared with proliferation in the NB4 and LV-shCon cells, which were not induced with ATRA ( $P < 0.01$ ; Fig. 2A and B). Compared with the ATRA-induced NB4 cells and LV-shCon cells, the ATRA-induced LV-shRIG-I cells exhibited marked proliferation, particularly at 72 h post-induction ( $P < 0.05$ ; Fig. 2B), indicating that RIG-I knockdown alleviated the ATRA-induced inhibition of NB4 cell proliferation. Further analysis of the cell cycle using flow cytometry revealed that, compared with the results obtained at 0 h, NB4 and LV-shCon cells were arrested in the G1 phase at 72 h post-ATRA induction ( $P < 0.05$ ; Fig. 2C). However, at 72 h post-ATRA induction, the percentage of LV-shRIG-I cells in the G1 phase ( $53.28 \pm 4.10\%$ ) had decreased significantly, compared with the percentages of NB4 and LV-shCon cells in the G1 phase ( $73.56 \pm 5.63$  and  $72.04 \pm 5.58\%$ , respectively;  $P < 0.05$ ; Fig. 2C). These results suggested that RIG-I knockdown alleviated ATRA-induced cell cycle arrest of the NB4 cells at the G1 phase.

**RIG-I knockdown inhibits ATRA-induced apoptosis in NB4 cells.** Flow cytometry was used to investigate the effect of RIG-I-knockdown on ATRA-induced apoptosis in NB4 cells. The results showed that, compared with the results obtained at 0 h, the NB4 and LV-shCon cells exhibited signs of apoptosis at 72 h post-induction with ATRA ( $P < 0.01$ ; Fig. 3A and B). By contrast, a significantly lower proportion of LV-shRIG-I cells ( $12.32 \pm 1.10\%$ ) exhibited signs of apoptosis at 72 h post-induction, compared with those in the NB4 and LV-shCon cell groups ( $32.51 \pm 2.39$  and  $30.60 \pm 2.53\%$ , respectively;  $P < 0.01$ ,

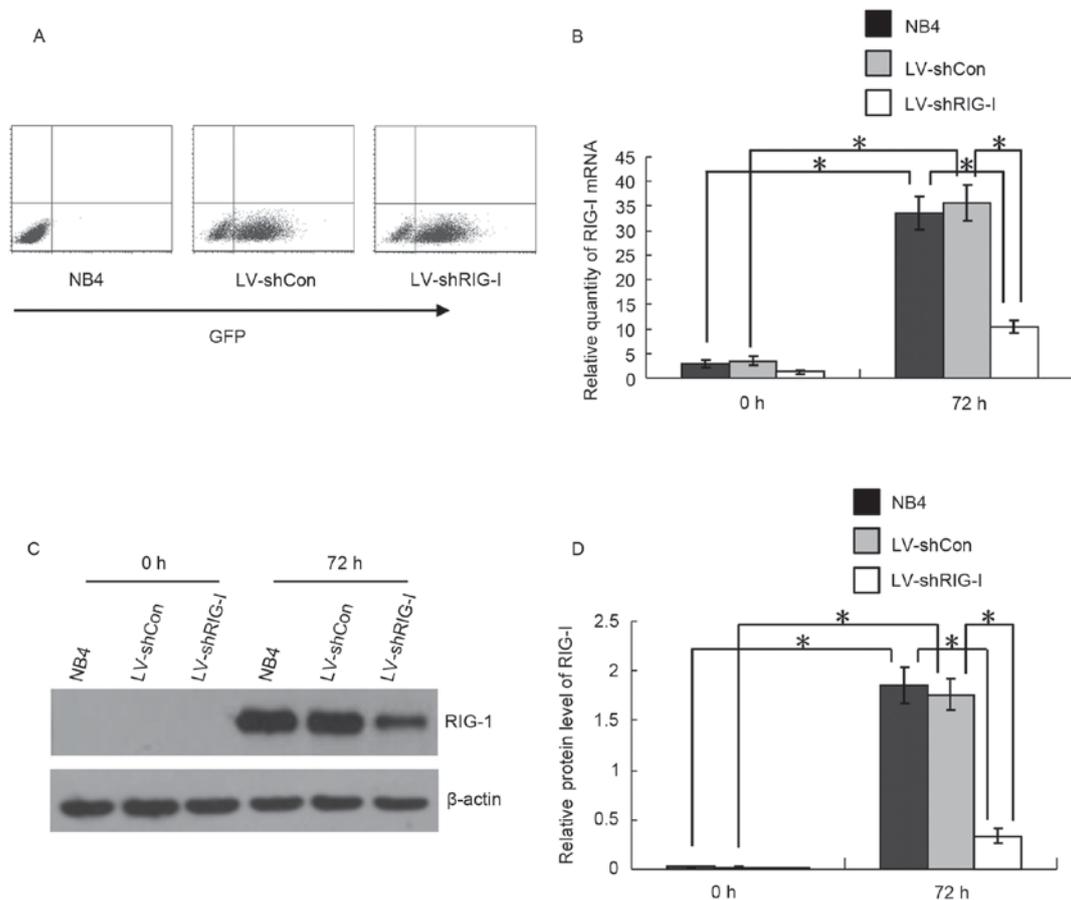


Figure 1. Expression of RIG-I in ATRA-induced NB4 cells is knocked down by LV-mediated shRIG-I. (A) Flow cytometric detection of the expression of GFP in LV-shCon and LV-shRIG-I cells at 72 h post-lentivirus infection. (B) Reverse transcription-quantitative polymerase chain reaction analysis for detection of the RNA expression of RIG-I in NB4, LV-shCon and LV-shRIG-I cells at 0 and 72 h post-ATRA induction (n=3). \*P<0.01. (C) Western blot analysis of the protein expression of RIG-I in NB4, LV-shCon and LV-shRIG-I cells at 0 and 72 h post-ATRA induction. (D) Quantitative analysis of protein expression of RIG-I in NB4, LV-shCon and LV-shRIG-I cells at 0 and 72 h post-ATRA induction (n=3) \*P<0.01. Data are presented as the mean  $\pm$  standard deviation. RIG-I, retinoic acid inducible gene I; ATRA, all-trans retinoic acid; GFP, green fluorescent protein; LV, lentivirus; Con, control; siRNA, small interfering RNA; LV-shCon, NB4 cells infected with LV Con-siRNA; LV-shRIG-I, NB4 cells infected with LV RIG-I-siRNA.

Fig. 3A and B), suggesting that RIG-I-knockdown inhibited the ATRA-induced apoptosis of NB4 cells.

*RIG-I knockdown reduces cell proliferation inhibition, cell cycle arrest and apoptosis in ATRA-induced NB4 cells via the AKT-FOXO3A signaling pathway.* To determine whether RIG-I knockdown reduced the cell proliferation inhibition, cell cycle arrest and apoptosis in ATRA-induced NB4 cells, western blot analysis was used to examine the AKT-FOXO3A signaling pathway in the LV-shCon and LV-shRIG-I cells at 0 and 72 h following ATRA induction. The results showed that, compared with the levels of pAKT-Thr308 at 0 h, the levels of pAKT-Thr308 in the LV-shCon cells at 72 h post-ATRA induction were decreased with RIG-I-induced expression (P<0.01; Fig. 4A and B). Furthermore, pFOXO3A-Thr32 downstream AKT was downregulated (P<0.01; Fig. 4A and C), which resulted in increased expression of protein p27, promoting G1 phase arrest (P<0.01; Fig. 4A and D), and TRAIL, promoting cell apoptosis (P<0.01, Fig. 4A and E), which are directly transcribed by FOXO3A. At 72 h post-ATRA induction, compared with the LV-shCon cells, the LV-shRIG-I cells with RIG-I-knockdown showed increased levels of pAKT-Thr308 (P<0.01; Fig. 4A and B) and pFOXO3A-Thr32 (P<0.01; Fig. 4A

and C), and there was downregulated expression levels of protein p27, (P<0.01; Fig. 4A and D) and TRAIL (P<0.01; Fig. 4A and E). These results showed that the knockdown of RIG-I reduced cell proliferation inhibition, cell cycle arrest and apoptosis in the ATRA-induced NB4 cells via the AKT-FOXO3A signaling pathway.

## Discussion

In the present study, the lentivirus method was used to knock down the gene expression of RIG-I in ATRA-induced NB4 APL cells, and proliferation, cell cycle and apoptosis in the NB4 cells were investigated. The association between RIG-I and the AKT-FOXO3A signaling pathway was also examined. The results showed that the siRNA-mediated knockdown of RIG-I reduced cell proliferation inhibition, cell cycle arrest and apoptosis in the ATRA-induced NB4 cells via the AKT-FOXO3A signaling pathway.

RIG-I is upregulated during ATRA-induced terminal granulocytic differentiation of the NB4 cell line of APL cells. Previously, it was found that RIG-I gene-knockout mice had significantly increased numbers of myeloid cells, indicating a degree of differentiation disorder. The percentage of cells

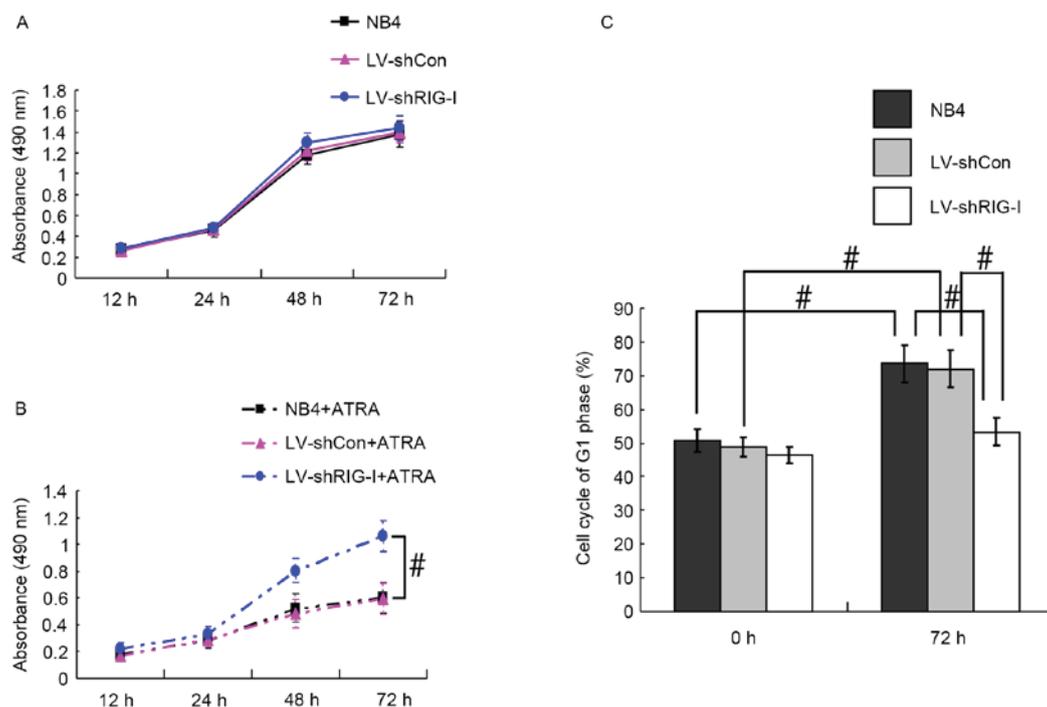


Figure 2. RIG-I knockdown alleviates ATRA-induced reduced proliferation and cell cycle arrest in NB4 cells. (A) MTT detection of NB4, LV-shCon and LV-shRIG-I cell proliferation without ATRA induction at different time points. (B) MTT detection of NB4, LV-shCon and LV-shRIG-I cell proliferation with ATRA induction at different time points (n=3)  $^{\#}P<0.05$ . (C) Flow cytometric detection of NB4, LV-shCon and LV-shRIG-I cells in the G1 phase at 0 and 72 h post-ATRA induction (n=3)  $^{\#}P<0.05$ . Data are presented as the mean  $\pm$  standard deviation. RIG-I, retinoic acid inducible gene I; ATRA, all-trans retinoic acid; LV, lentivirus; Con, control; siRNA, small interfering RNA; LV-shCon, NB4 cells infected with lentivirus Con-siRNA; LV-shRIG-I, NB4 cells infected with lentivirus RIG-I-siRNA. NB4 + ATRA, ATRA-induced NB4 cells; LV-shCon + ATRA, ATRA-induced LV-shCon cells; LV-shRIG-I + ATRA, ATRA-induced LV-shRIG-I cells.

undergoing apoptosis in RIG-I-deficient mice was significantly decreased, whereas the numbers of cells entering their cell cycle were increased, resulting in myeloproliferative disorder, and suggesting that RIG-I is involved in regulating apoptosis and cell cycle (3). Another study showed that, on reducing the expression of RIG-I in HL60 cells, subsequent ATRA-induced cell differentiation, cell cycle arrest and apoptosis were suppressed (15). These findings are analogous with the results of the present study, which showed that RIG-I-knockdown reduced cell proliferation inhibition, cell cycle arrest and apoptosis in ATRA-induced NB4 cells.

Translocation events in APL cells typically occur on chromosomes 15 and 17 (16), and result in fusion of the promyelocytic leukemia (PML) gene on chromosome 15 with the retinoic acid receptor  $\alpha$  (RAR $\alpha$ ) gene on chromosome 17, to produce an abnormal PML-RAR $\alpha$  fusion gene (17). Previous studies have shown that ATRA inhibits APL cell proliferation, and promotes cell differentiation and apoptosis by stimulating proteinase dependent PML-RAR $\alpha$  decomposition and consequent PML-nuclear body (PML-NB) formation (18,19). As tumor inhibitors, PML proteins are an important component of PML-NBs. However, the PML-RAR $\alpha$  fusion protein in APL cells damages PML-NBs and inhibits endogenous PML tumor inhibition function. Therefore, PML-RAR $\alpha$  decomposition and PML-NB formation are considered to be key responses, which occur in cells exposed to ATRA (18-20). It has been reported that PML-NBs, protein phosphatase 2A (PP2A), nuclear AKT and the transcription factor, FOXO3A, jointly constitute a tumor inhibitor network. PML proteins have been shown to

specifically collect PP2A into their PML-NBs and then exhibit inhibited nuclear AKT phosphorylation activity, resulting in the deactivation of AKT (21). As the FOXO3A transcription factor can be deactivated by AKT, which is activated by phosphorylation, PML-NB deficiency or its functional deletion activates the phosphorylation of AKT, which results in deficient FOXO3A transcription activity. Sakoe *et al* (13) reported that phosphorylated FOXO3A was located in the cytoplasm of APL-derived NB4 cells and primary patient cells. Following ATRA treatment, the levels of phosphorylated FOXO3A were reduced and FOXO3A entered the nucleus. The mRNA and protein levels of TRAIL were also increased, and transfection with an shRNA oligonucleotide specific for FOXO3A was shown to significantly inhibit differentiation and apoptosis in ATRA-induced NB4 cells. The AKT-FOXO3A signaling pathway is essential in the process of ATRA-induced APL granulocyte differentiation and apoptosis (13). In the present study, ATRA-induced proliferation inhibition, cell cycle arrest and apoptosis of NB4 cells were accompanied by the expression of RIG-I and decreased levels of phosphorylated AKT, resulting in the deactivation of AKT, whereas the levels of phosphorylated FOXO3A regulated by AKT were decreased, leading to its activation. These events led to increased expression levels of the cell cycle arrest protein, p27, and apoptosis protein, TRAIL, which are directly transcribed by FOXO3A. By contrast, following the knockdown of ATRA-induced RIG-I, the levels of phosphorylated AKT increased, AKT was activated, the level of phosphorylated FOXO3A was increased, and FOXO3A was deactivated. The protein expression levels

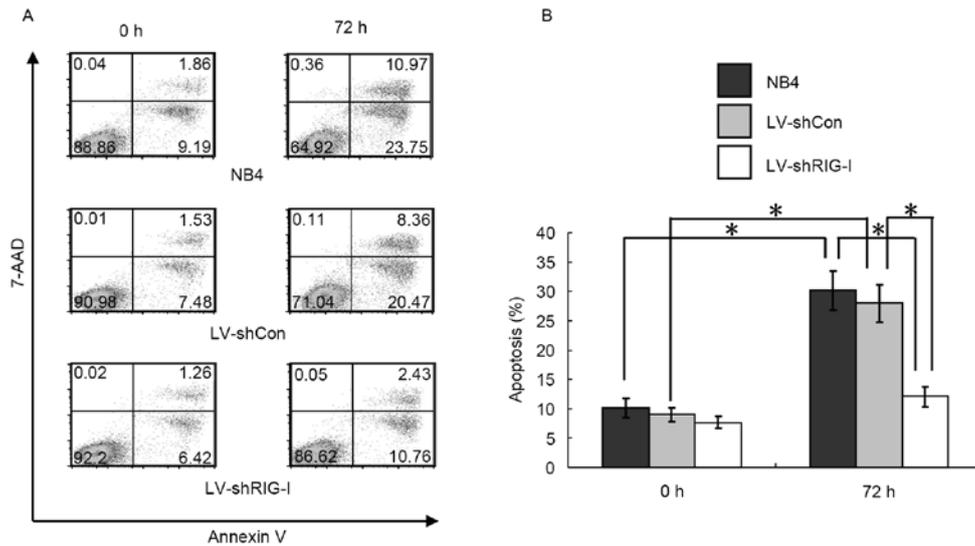


Figure 3. RIG-I knockdown inhibits ATRA-induced apoptosis in NB4 cells. (A) Flow cytometric detection of apoptosis in NB4, LV-shCon and LV-shRIG-I cells at 0 and 72 h post-ATRA induction. (B) Quantitative analysis of apoptosis in NB4, LV-shCon and LV-shRIG-I cells at 0 and 72 h post-ATRA induction (n=3) \*P<0.01. Data are presented as the mean ± standard deviation. RIG-I, retinoic acid inducible gene I; ATRA, all-trans retinoic acid; LV, lentivirus; Con, control; siRNA, small interfering RNA; LV-shCon, NB4 cells infected with lentivirus Con-siRNA; LV-shRIG-I, NB4 cells infected with lentivirus RIG-I-siRNA.

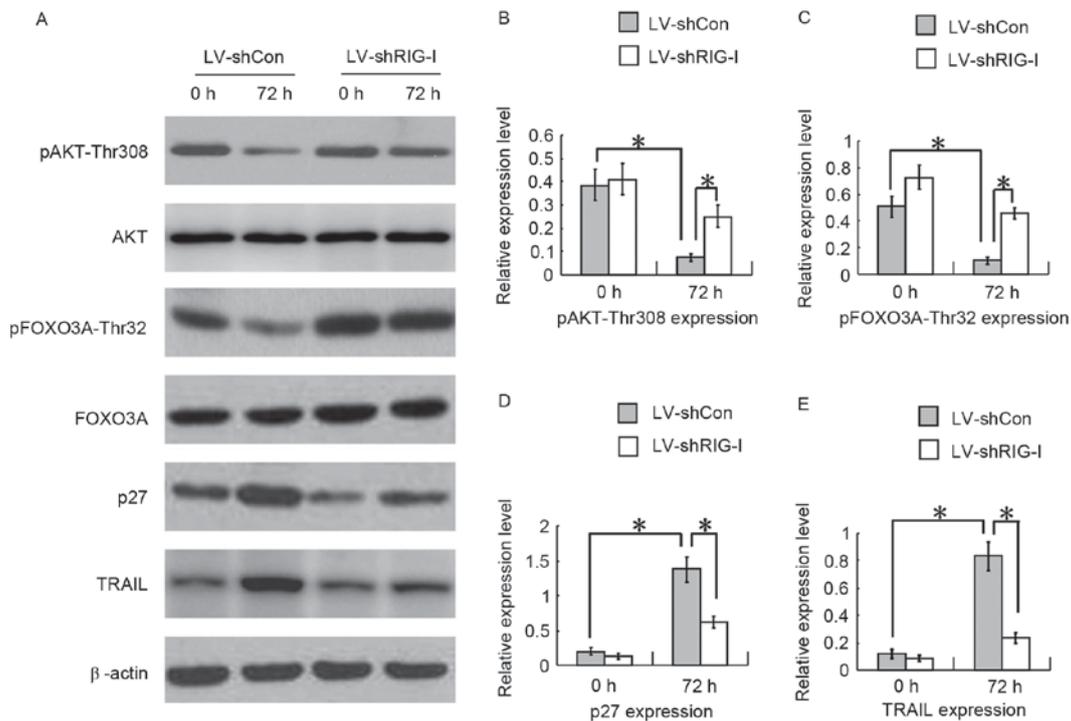


Figure 4. RIG-I-knockdown reduces cell proliferation inhibition, cell cycle arrest and apoptosis in ATRA-induced NB4 cells via the AKT-FOXO3A signaling pathway. (A) Western blot analyses of expression levels of pAKT-Thr308, pFOXO3A-Thr32, p27 and TRAIL in LV-shCon and LV-shRIG-I cells at 0 and 72 h post-ATRA induction. (B) Quantitative analysis of protein levels of pAKT-Thr308 in LV-shCon and LV-shRIG-I cells at 0 and 72 h post-ATRA induction (n=3) \*P<0.01. (C) Quantitative analysis of protein levels of pFOXO3A-Thr32 in LV-shCon and LV-shRIG-I cells at 0 and 72 h post-ATRA induction (n=3) \*P<0.01. (D) Quantitative analysis of protein levels of p27 in LV-shCon and LV-shRIG-I cells at 0 and 72 h post-ATRA induction (n=3) \*P<0.01. (E) Quantitative analysis of protein levels of TRAIL in LV-shCon and LV-shRIG-I cells at 0 and 72 h post-ATRA induction (n=3) \*P<0.01. Data are presented as the mean ± standard deviation. RIG-I, retinoic acid inducible gene I; ATRA, all-trans retinoic acid; LV, lentivirus; Con, control; siRNA, small interfering RNA; LV-shCon, NB4 cells infected with lentivirus Con-siRNA; LV-shRIG-I, NB4 cells infected with lentivirus RIG-I-siRNA.

of p27 and TRAIL transcribed by FOXO3A were decreased, resulting in decreased cell cycle arrest and apoptosis in the ATRA-induced NB4 cells. These findings suggested that RIG-I-knockdown reduced cell proliferation inhibition, cell

cycle arrest and apoptosis of ATRA-induced NB4 cells via the AKT-FOXO3A signaling pathway. Based on the above, it was hypothesized that ATRA-induced proteinase-dependent PML-RAR $\alpha$  decomposition, PML-NB formation and

expression of RIG-I were synergistically involved in the AKT-FOXO3A signaling pathway to inhibit NB4 cell proliferation, arrest cell cycle and promote NB4 cell apoptosis.

In conclusion, RIG-I was shown to be important in the events leading to the inhibition of cell proliferation, arrest of the cell cycle and promotion of apoptosis in ATRA-induced NB4 cells. Lentivirus-mediated RIG-I-knockdown relieved cell proliferation inhibition, cell cycle arrest and apoptosis in the ATRA-induced NB4 cells via the AKT-FOXO3A signaling pathway.

### Acknowledgements

The present study was supported by a grant from the Natural Science Foundation of Tianjin Municipal Committee of Science and Technology (grant no. 13JCYBJC21200).

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