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IARS2 silencing induces non-small cell lung cancer cells proliferation inhibition, cell cycle arrest and promotes cell apoptosis

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The purpose of this study was to investigate the potential role of Ileucyl-tRNA synthetase (IARS2) silencing in non-small cell lung cancer (NSCLC). The silencing of *IARS2* in H1299 cells and A549 cells were performed by lentivirus encoding shRNAs. The efficiency of *IARS2* silencing was detected by quantitative real time PCR and western blot. The effects of *IARS2* silencing on cell growth, cell apoptosis, cell cycle and cell colony formation ability were assessed by cells counting, MTT assay, flow cytometer analysis and soft agar colony formation assay, respectively. Compared with negative control group, *IARS2* was significantly knockdown by transfection with lentivirus encoding shRNA of *IARS2*. The IARS2 silencing significantly inhibited the cells proliferation and cells colony formation ability, induced cell cycle arrest at G1/S phase and promoted cell apoptosis. *IARS2* silencing induced NSCLC cells growth inhibition, cell cycle arrest and promoted cell apoptosis. These results suggest that IARS2 may be a novel target for the treatment of NSCLC.

Key words: IARS2 silencing, NSCLC, cell proliferation, cell apoptosis, cell cycle

Non-small cell lung cancer (NSCLC) is the leading cause of cancer-related mortality worldwide, which accounts for 85% of lung cancer and carries a 5-year survival rate of 15% [1]. Recently, there has been increasing optimism that gene therapy may be utilized as an approach to develop novel anti-cancer treatment modalities. However, the field of gene therapy is still in its infancy with rare major successes being reported yet in the treatment of cancer patients [2]. Therefore, exploring effective gene target for the therapy of NSCLC is still necessary.

Aminoacyl-tRNA synthetases (ARSs) are housekeeping enzymes essential for protein synthesis. These synthetases perform an integral step in the initiation of protein synthesis by catalyzing ligation of specific amino acids to their cognate tRNAs, which is a crucial determinant to maintain the fidelity of protein synthesis [3]. In mammalian, the activity of ARSs are often controlled by forming a complex and interacting with multifunctional Aminoacyl tRNA synthetase complex-interacting multifunctional proteins (AIMPs), such as AIMP1/p43, AIMP2/p38, and AIMP3/p18. Recently, accumulated evidences have demonstrated that besides their essential role of catalytic activities, ARSs and AIMPs are also involved in RNA processing and trafficking, apoptosis, rRNA synthesis, angiogenesis and inflammation [4-6]. Moreover, several lines of evidence indicate that the non-canonical functions of ARSs and AIMPs are associated with the development of cancer or other severe diseases [7, 8]. Many studies have found that some ARSs are highly expressed in kinds of tumors and play roles in triggering or suppressing the hallmarks of cancer [7, 9]. For instance, Met-tRNA synthetase exhibits a higher specific activity in human colon cancer [10]. It has been reported that over-expression of leucyl-tRNA synthetase (LARS1) is prominent in lung cancer and knockdown of LARS1 inhibits the cells growth and migration [11].

Ileucyl-tRNA synthetase 2 (IARS2) is a nuclear gene encoding mitochondrial ARS. It has been reported that mutation in the *IARS2* is presented in patients with cataracts, growth hormone deficiency with short stature, partial sensorineural deafness, and peripheral neuropathy or with leigh syndrome [12]. The variant in *IARS2* is a pathogenic mutation and a primary cause of the above abnormal phenotypes. However, Paul

Mazarisa *et al.* have found that compared with the expression in long-term survivors of glioblastoma (GBM), an increased expression of IARS2 is found in short-term survivors of GBM [13], which indicates that the high expression of IARS2 is a risk factor for the GBM. Mitochondrial IARS has been reported to increase in hereditary nonpolyposis colorectal cancer (HNPCC) and Turcot syndrome [14]. In human ovarian cancer cells, expression of IARS2 could be inhibited by fenretinide (4-HPR), and the latter could induce cancer cell growth inhibition and apoptosis [15]. All the above results suggest that IARS2 may be involved in the development of cancer. However, the potential role of IARS2 in NSCLC is not clear.

Therefore, in this study, we performed the RNA interference for IARS2 using lentivirus in NSCLC cells, followed by assessing its effects on cells growth, cell cycle and cell apoptosis. We hope the study can supply new insights for the understanding of IARS2 functions in NSCLC and provide a novel therapy target for the treatment of NSCLC.

Materials and methods

Cell culture. NSCLC cells H1299, A549 and 293T cell lines were purchased from cell research Institute of Chinese Academy of sciences (Shanghai, China). Cells were maintained in Dulbecco's modification of Eagle's medium Dulbecco (DMEM) medium supplemented with 10% fetal bovine serum (FBS) and cultured at 37 °C under 5% CO_2.

IARS2 shRNA design and plasmid construction. IARS2 shRNA lentivirus gene transfer vector pGCSIL-GFP encoding green fluorescent protein (GFP) sequence was constructed by Genechem Co., Ltd, Shanghai, China. The hairpin sequence of the *IARS2* shRNA was CCGG GTA CTT GCA GTC ATC CAT TAA TTC AAG AGA TTA ATG GAT GAC TGC AAG TAC TTT TTG (Genebank accession number: NM_018060). DNA oligos containing the target sequence were chemically synthesized, annealed, and inserted into the expression vector by double digestion with Age I and EcoRI. The ligate was transformed into competent Escherichia coli DH5a cells. The correct transformant was identified by restriction enzyme analysis and DNA sequencing. As a control for *IARS2* shRNA, a corresponding random shRNA sequence (control shRNA) was used. The hairpin sequence of the control shRNA was: CCG GTT CTC CGA ACG TGT CAC GTT TCA AGA GAA CGT GAC ACG TTC GGA GAA TTT TTG. The shRNA-infected cells were regarded as *IARS2*-shRNA group; the cells infected with the control shRNA unrelated to *IARS2* sequence as negative control (NC) group.

Lentivirus production and transduction. Lentivirus-IARS2 shRNA and Lentivirus-NC were produced by plasmid co-transfection of 293T cells, as described previously [16]. The 293T cells were transfected with 0.5μg lentivirus plasmids in opti-MEM1 using 1 μl Lipofectamine 2000 reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). The viral supernatant was harvested 48 h after transfection, passed through 0.45 μm filters and concentrated, and the viral titer was determined. The viral supernatant was added into target cells according to multiplicity of infection with enhanced infection solution (ENi.S) and 5 μg/mL polybrene to obtain stably transfected IARS2 shRNA and IARS2-NC cells. After incubation for 24 h, the cells were observed under a fluorescence microscope in high-power (100×) field (Olympus America, Melville, NY).

Quantitative real time PCR (qPCR). The silencing efficiency of *IARS2* at RNA level was assessed by qPCR after transfection for 48 h. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene served as the internal reference. Total cellular RNA was extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). The cDNA was reversely transcribed using MMLV (Promega, Madison, USA). SYBR Green real time PCR was performed using the Light Cycler 2.0 Real-Time PCR System (Roche Germany) with SYBR Green Real time PCR Master Mix (Toyobo Co., Ltd., Osaka, Japan) according to the manufacturer's instructions. The PCR reaction was initiated with a 15 s pre-denaturation at 95 °C. Amplification was carried out for 45 cycles of denaturation 5 s at 95 °C, annealing 30 s at 60 °C. The primers were designed by Beacon designer 2 and synthetized by Genechem Co., Ltd, Shanghai, China. The primer of *IARS2*: F 5'- TGGACCTCCTTATGCAAACGG-3', R 5'-GGCAACCCATGACAATCCCA-3'. The primer of GAPDH: F 5'-TGACTTCAACAGCGACACCCA-3', R 5'-CACCCTGT TGCTGTAGCCAAA-3'. The expression level was determined by $2^{-\Delta\Delta Ct}$ analysis.

Western blot. The silence efficiency of *IARS2* at protein level was assessed by Western blot. The concentrations of total proteins were measured by a BCA Protein Assay Kit (Pierce, Rockford, USA). Equal amounts (20 μg) of lysis proteins were separated on 10% SDS-PAGE gels. GAPDH was used as an internal loading control. After incubation with the primary antibody of anti-IARS2 (Sigma, st Louis, USA), or anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1: 3,000 at 4 °C overnight, the membranes were probed with secondary antibodies at a dilution of 1: 5,000 at room temperature for 2 h. The signals were detected using enhanced chemiluminescence (ECL) detection kit (Amersham, USA).

Cell counting. The stably transfected *IARS2* shRNA and *IARS2*-NC cells were seeded in the 96 -well plate at a density 2×10^4/ml in triplicate. The living cells with green signal were counted by cellomics HCS scanning software (Thermo, Massachusetts, USA) once a day for 5 days.

MTT assay. Cell viability was determined by using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazoniumbromide (MTT) assay (Sigma, ST. Louis, MO). Briefly, the exponential growth cells were seeded in 96-well plates at a density of 1×10^4 cells/well in triplicate. Every day, MTT (5 mg/ml, Sigma, ST. Louis, MO) was added to each well to a final concentration of 0.5 mg/ml/well and incubated at 37 °C for 4 h. Then, the culture medium was removed and 150 μl DMSO was added to solubilize the formazan product formed by viable cells. The optical density (OD) values were measured at 490 nm by a microplate reader (BioTek Instruments, Winooski, VT).

Soft agar colony formation assay. Cells in exponential growth were harvested using 0.25% trypsin and resuspended to a single-cell suspension of 1×10^3/ml cells in DMEM medium supplemented with 20% FBS. Soft agar assay was performed as previous description [17]. A total of 0.5 ml cells in 0.6% low-melt agarose were plated on the top of existing 1.2% bottom low-melt agarose. After incubation for approximately 2 weeks at 37 °C under 5% CO_2 in a humidified incubator, the numbers of colonies were counted. The colony forming efficiency (%) = colony numbers / cell inoculation numbers ×100%.

Flow cytometric analysis of cell cycle. The stably transfected IARS2 shRNA and IARS2-NC cells were seeded in six plates at a density of 1×10^5 cells/well in triplicate and cultured for 48 h. Then, the cells were harvested, washed twice with phosphate buffered saline (PBS), and then the cellular precipitation was resuspended and fixed in 70% ethanol at 4 °C overnight. After that, the cells were washed twice with PBS, and then the cellular precipitation was resuspended and added into 50 μl Propidium Iodide (PI) solution (50 mg/mL) (Sigma, st Louis, USA). After incubation for 15 min at room temperature in the dark, the cell cycle distribution was detected by flow cytometer (FACS Calibur, BD Biosciences, USA).

Flow cytometric analysis of apoptotic cells using annexin V-APC. The stably transfected *IARS2* shRNA and *IARS2*-NC cells were seeded in six-well plates at a density of 1×10^5 cells/well in triplicate and incubated for 24 h. Cells were harvested, washed twice with cold PBS, resuspended to a single-cell suspension of 1.0×10^6/ml cells in 100 μl staining buffer and successively incubated with 5 μl of Annexin V-APC in the dark for 15 min at room temperature or 30 min at 4°C. Then cell apoptosis was analyzed by a flow cytometer (FACS Calibur, BD Biosciences).

Statistical analysis. Statistical data were expressed as means ± standard deviation (SD). Comparisons among different groups were carried out with Student's t-test. $P < 0.05$ was considered to be statistically significant. All data were analyzed by SPSS 19.0 software.

Results

Analysis of silencing efficiency of IARS2. The results showed that the GFP encoded in the lentivirus transduction

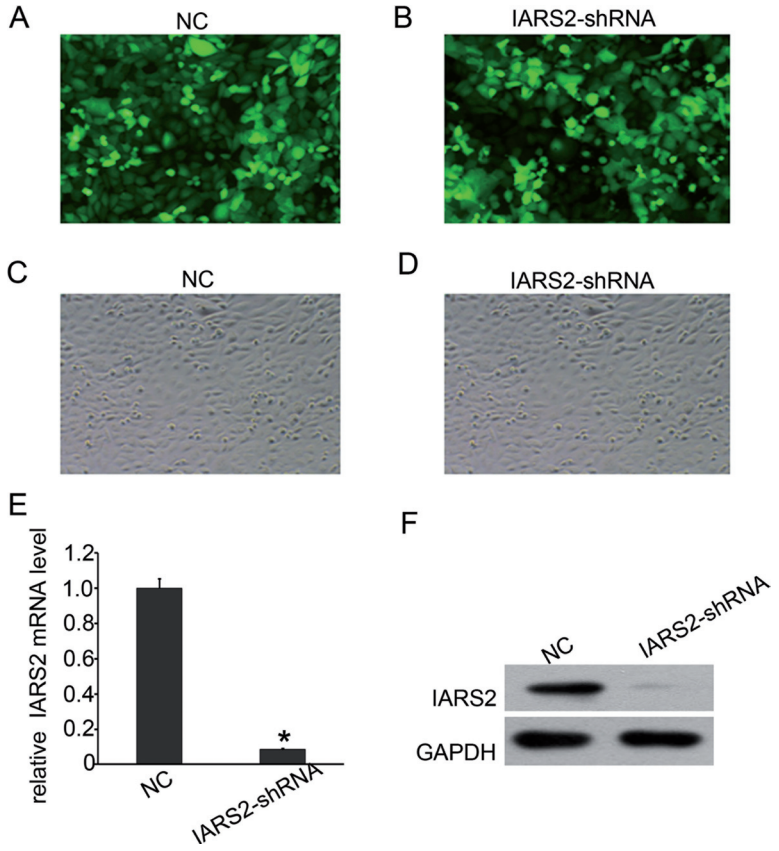


Figure 1. The *IRAS2* silencing in H1299 cells. A and B Photographs of H1299 cells in *IARS2*-siRNA and NC groups taken after cell sorting under fluorescent microscope (100×); C and D Photographs of H1299 cells s in *IARS2*-siRNA and NC groups taken after cell sorting under a light microscope (100×). E qPCR analysis on the efficiency of *IRAS2* silencing at mRNA level. F Western blot analysis on the efficiency of *IRAS2* silencing at protein level. * indicates $P < 0.05$ *vs.* NC group.

vector was highly expressed in H1299 cells, which suggested that the cells were successfully infected with the *IARS2* shRNA (Figure 1A-D). The silencing efficiency of *IARS2* at mRNA level and protein level were detected by qPCR and western blot, respectively. The results showed in Figure 1 E, the *IARS2* mRNA level in H1299 cells was 0.012 ± 0.003 which was significantly decreased compared with that of the NC group (1.001 ± 0.046, $P < 0.05$). Consistently, *IARS2* at protein level in H1299 cells (Figure 1 F) was significantly lower than that in NC group.

The effects of IARS2 silencing on cell proliferation. The effects of *IARS2* silencing on cell proliferation were assessed by cell counting and MTT assay. As the cell counting results showed that *IARS2* silencing significantly inhibited the H1299 cell proliferation compared with control group (Figure 2 A). Especially, on the fifth day, the proliferation folds in NC group was 11.09 ± 0.39, which was obviously higher than that in *IARS2*-shRNA group (2.18 ± 0.13, $P<0.05$). Similarly, the results of MTT assay displayed that the H1299 cell growth inhibition was significantly observed in *IARS2* shRNA group. The most significantly inhibitory effect was observed on the fifth day when the proliferation folds in NC group was 5.347 ± 0.161, while the proliferation folds in *IARS2* silencing group was 3.463 ± 0.112 at day 5 (Figure 2 B, $P<0.05$). Meanwhile, MTT assay of *IARS2* silencing in A549 cells also showed a notable cells growth inhibition in *IARS2* shRNA group (3.590 ± 0.031) compared with that in NC group (4.582 ± 0.031, $P<0.05$) (Additional file 1).

The effects of IARS2 silencing on colony formation. Colony formation ability is considered as a key characteristic of cancer cells. The colony formation assay was performed to assess whether *IARS2* silencing could repress oncogenic growth of H1299 cells. The results of colony formation assay showed that compared with NC group (13.8%), the colony forming efficiency was significantly reduced in *IARS2* silencing group (1%) (Figure 2 C-D, $P<0.05$). Similarly, a significant decrease of colony formation efficiency was observed in *IARS2* shRNA group of A549 cells (10.6%) compared with that in NC group (23.0%, $P<0.05$) (Additional file 2) .

The effects of IARS2 silencing on cell cycle and cells apoptosis. The results of H1299 cell apoptosis analysis presented that the proportion of apoptosis cells was significantly increased in the *IARS2* shRNA group compared with that in NC group (8.08% *vs.* 6.21%, $P < 0.01$) (Figure 3 A-C). The results showed that compared with NC group (G0/G1 phase: 42.197 ± 0.877%, S phase: 56.433 ± 0.871%), the silencing of *IARS2* induced a remarkable accumulation of cells in G0/G1 phase (57.390 ± 0.686%, $P<0.05$) and further a significant decrease of cells in S phase (38.797 ± 0.686%, $P<0.05$) (Figure 3 D-F). The above results suggested that

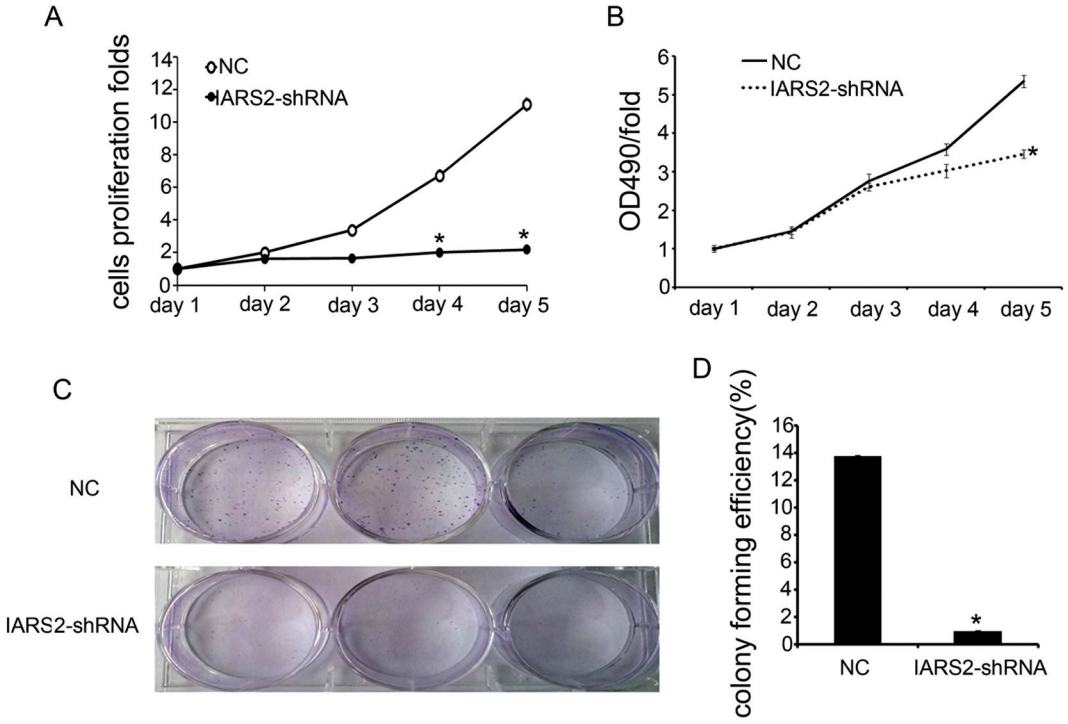


Figure 2. Inhibition of cell growth and cell colony formation ability by IRAS2 silencing in H1299 cells. A Cell counting analysis on the effects of *IRAS2* silencing on cell growth. Cell proliferation folds= number of *IARS2*-siRNA cells/number of control cells. B MTT assay analysis on the effects of *IRAS2* silencing on cell growth. OD490/fold= OD490 value of any time point/OD490 value of control group at day1. C, D Soft agar colony formation assay analysis on the effects of *IRAS2* silencing on the cell colony formation ability. * indicates $P < 0.05$ *vs.* NC group.

IARS2 knockdown strengthened cell apoptosis and induced cell cycle arrest.

Discussion

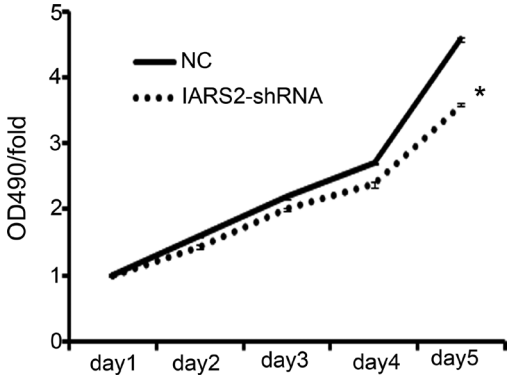
Previous studies are mainly focused on the role of IARS2 in several mitochondrial diseases, however, the potential role of IARS2 in cancers remains an mystery. Therefore, in current research, we investigated the effects of *IARS2* silencing via transfection of *IARS2* shRNA lentivirus vector on cell proliferation, cell colony formation ability, cell cycle distribution and cell apoptosis in NSCLC cells. The results indicated that *IARS2* silencing significantly inhibited the cell proliferation and cell colony formation ability. Moreover, *IARS2* knockdown induced the cell cycle arrest at G1/S phase and promoted cell apoptosis.

In this study, *IARS2* was successfully silenced by transfection with lentivirus encoding shRNA of *IARS2*. The rate of protein synthesis in cancer cells is generally higher than that in normal cells because cancer cells proliferate more extensively and their metabolism is more dynamic than normal cells. The potential role of *IARS2* in NSCLC was assessed on the cells growth of H1299 cells and A549 cells. The cell counting and MTT assay results clearly demonstrated that the silencing of *IARS2* inhibited the cell proliferation.

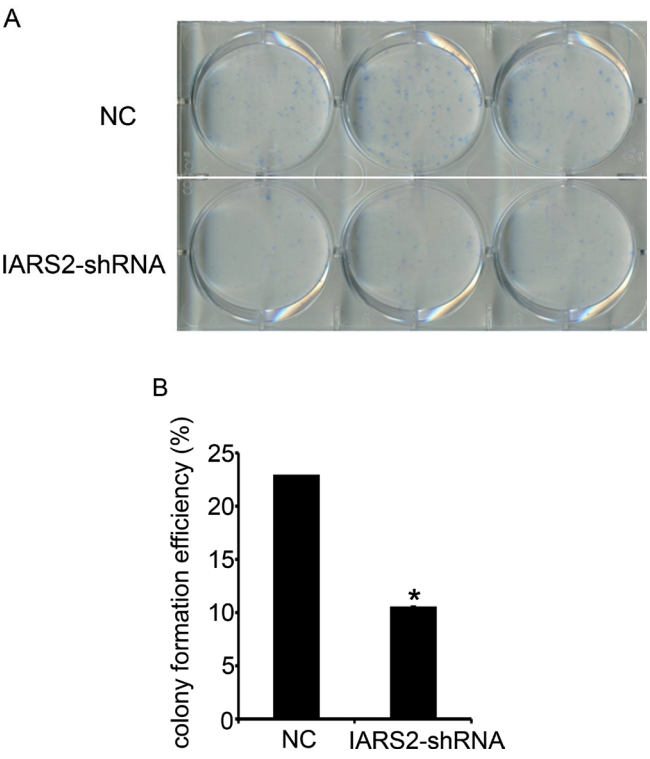
The development of tumors arising predominantly results from deregulated proliferation and a suppression of apoptosis [18, 19]. ARSs have been reported to be related with cell apoptosis. Glutaminyl-tRNA synthetase (QRS) can interact with signal-regulating kinase 1 (ASK1) and block its kinase activity in a glutamine-dependent manner leading to inhibition of cell death [20]. Therefore, we further investigated the effects of *IARS2* silencing on the cell apoptosis and the results indicated that the silencing of *IARS2* enhanced cell apoptosis. These results suggested *IARS2* silencing exhibited antitumor effects including the anti-proliferation and pro-apoptosis effects. It has been established that ARSs play important roles in the mitochondrial protein synthesis which serves the mitochondrial oxidative phosphorylation system [21]. B-cell lymphoma 2 (Bcl-2) and Bcl-2-associated X protein (BAX) proteins are important regulators of mitochondria-dependent cell apoptosis [22, 23]. It is much likely that *IARS2* silencing promoted apoptosis of lung cancer cell partly via regulating Bcl-2 and BAX. Small-molecule Bcl-2 antagonists and BAX agonists have been recommended for lung cancer therapy [24, 25]. Thus, co-targeting IARS2 and Bcl-2 or BAX might hold promise for developing more effective therapy against lung cancer.

As reported, IARS is the target of Reveromycin A, aminoacyl-tRNA synthetases inhibitor, which can induce the cell cycle arrest at G1 phase [26]. Consistently, the current results also demonstrated that *IARS2* silencing blocked or delayed cell cycle at G1/S phase. The results also suggested that IARS2 might be a novel drug target for the treatment of NSCLC.

In this study, we only investigated the potential role of *IARS2* silencing in NSCLC cells, the underlying molecular mechanisms are the focus of our next work. Both LARS and IARS belong to the class I ARS and are suspected to have similar functions in cancers. LARS has been reported to drive mammalian target of rapamycin (mTORC1) activation and the activation of mTOR pathway plays a significant role in the development, growth and chemo-resistance of NSCLC



Additional file 1 Inhibition of cell growth by *IRAS2* silencing in A549 cells. MTT assay analysis on the effects of *IRAS2* silencing on cell growth * indicates $P < 0.05$ *vs.* NC group.



Additional file 2 Soft agar colony formation assay analysis on the effects of *IRAS2* silencing on the cell colony formation ability in A549 cells. * indicates $P < 0.05$ *vs.* NC group.

[27-29]. These may provide clues for further investigation of IARS2 involved in the development of NSCLC. Moreover, it has been demonstrated that miR194-1 and miR-215 are codified as a cluster within an intron sequence of the gene *IARS2* [30] and that miR-215 can act as an effector as well as a regulator of p53 to suppress cancerogenesis through p21 accumulation and cell cycle arrest [31]. Therefore, we hold an assumption that the *IARS* silencing may influence the expression of miR-215 and further inhibit or promote of some cancer relevant genes. Hence, the typical proteins related to cell proliferation, cell apoptosis and cell cycle such as p53, c-Myc, Bcl-2, and BAX are also considered for further research.

However, the H1299 cells used in this study were p53 deleted, and thus the results also suggested that the cell growth

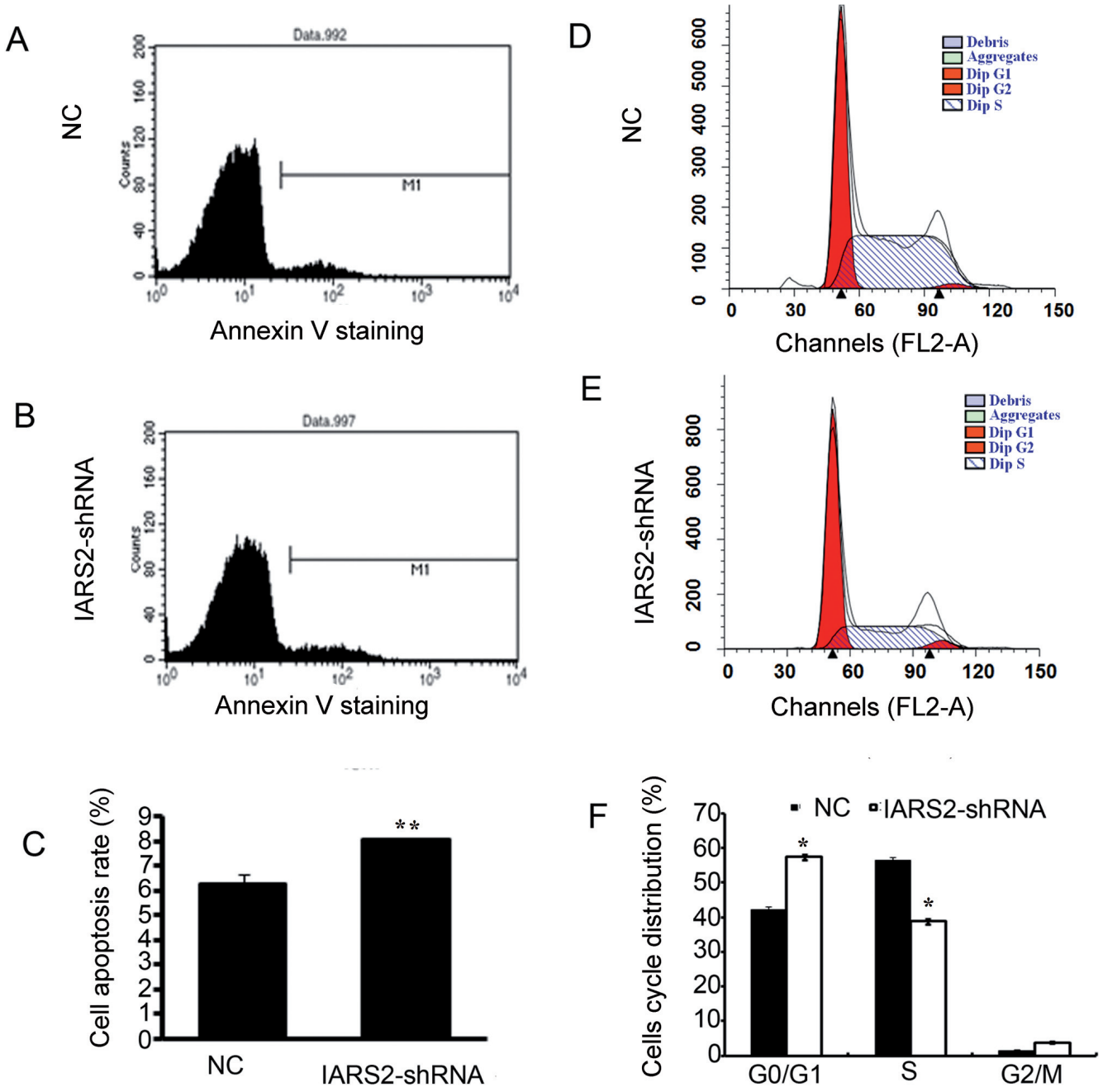


Figure 3. Induction of cell apoptosis and cell cycle arrest at G1/S phase by *IRAS2* silencing in H1299 cells. A, B, C *IRAS2* silencing induces the cell apoptosis. D, E, F *IRAS2* silencing induces an accumulation of cells at G0/G1 phase and a decrease of cells at S phase. * indicates $P < 0.05$ vs. NC group; indicates $P < 0.01$ vs. NC group**

inhibition induced by *IARS2* silencing might be mediated by other genes. Moreover, we also assessed the role of *IARS2* silencing in A549 cells. A549 cells were *Kras* mutant, and the *Kras* mutant has been reported to be a negative predictor of benefit from both adjuvant chemotherapy and anti-EGFR-directed therapies for NSCLC [32]. Moreover, *Kras* has been indicated to be involved in the development of NSCLC [33]. Therefore, the inhibition of cell proliferation caused by *IARS2* silencing in A549 cells indicated that *IARS2* silencing might exhibit an inhibitory role against the malignant tumor cell growth and proliferation induced by *Kras* mutant. These hypotheses provide valuable reference for the further study on the mechanism research of *IARS2* involved in the development of NSCLC.

In conclusion, the results of this study indicated that *IARS2* silencing exhibited antitumor effects on the development of NSCLC by significantly inhibiting cell proliferation, inducing cell cycle arrest at G1/S phase and promoting cell apoptosis. These may provide a novel target for the gene therapy of NSCLC. However, further animal experiments are warranted to confirm the role of *IARS2* in NSCLC and to investigate the possible mechanisms.

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