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S-Phase Cell Cycle Arrest, Apoptosis, and Molecular Mechanisms of Aplasia Ras homolog Member I–Induced Human Ovarian Cancer SKOV3 Cell Lines

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Objective: Aplasia Ras homolog member I (*ARHI*) is associated with human ovarian cancer (HOC) growth and proliferation; however, the mechanisms are unclear. The purpose of this study was to investigate *ARHI* effects in HOC SKOV3 cells.
Methods: We transfected SKOV3 cells with PIRES2-EGFP-ARHI and measured growth inhibition rates, cell cycle distribution, apoptosis rates, and expression of P-STAT3 (phosphorylated signal transduction and activators of transcription 3) and P-ERK (phosphorylated extracellular signal regulated protein kinase).
Results: Our data showed significant inhibition of growth, significantly increased S-phase arrest and apoptosis rates, and reduction of P-STAT3 and P-ERK1/2 expression levels.
Conclusions: We propose the mechanism may involve *ARHI*-induced phosphorylation of ERK1/2 and STAT3 protein kinases, thereby blocking proliferation signaling pathways, to induce HOC SKOV3 apoptosis.

Key Words: Ovarian neoplasms, *ARHI*, SKOV3, STAT3, Autophagy

Abbreviations: ARHI-aplasia Ras homolog member I, ERK-extracellular signal regulated protein kinase, GAPDH-glyceraldehyde phosphate dehydrogenase, GFP-green fluorescence protein, IR-inhibitory rate, OD-optical density, P-STAT3-phosphorylated STAT3, STAT-signal transduction and activators of transcription

Received July 9, 2013, and in revised form January 7, 2014.
Accepted for publication January 7, 2014.

(*Int J Gynecol Cancer* 2014;24: 629–634)

Tumorigenesis is a complex multistep and multifactorial biological process involving the activation of multiple oncogenes and inactivation of tumor suppressor genes; however, studies on the effects and mechanisms of these genes in ovarian cancer are limited and not well connected. Although it is known that changes in the expression of oncogenes and tumor suppressor genes, such as *p21*, *Ras*, *c-myc*, *p53*, *RB*, *p27*, and *p16, BRCA1* in human ovarian cancer (HOC), are linked to tumor progression, further studies are required to determine the effect and specific mechanisms on the occurrence and development of HOC. This will include exploring interactions between genes, the clinicopathologic correlation between gene

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This project was supported by the Medical Science and Technology Development Foundation, Nanjing Department of Health of China (no. YKK10037).
The authors declare no conflicts of interest.

ISSN: 1048-891X
DOI: 10.1097/IGC.0000000000000105

expression products and HOC, and the potential of altering expression of these genes and gene products for treating patients with HOC.

The *ARHI* gene (aplasia Ras homolog member I, also known as *DIRAS3* or *NOEY2*) was first discovered by The University of Texas M. D. Anderson Cancer Center, United States. It is a member of the Ras superfamily and encodes a small GTP-binding protein. *ARHI* is highly expressed in normal human tissues, including mammary glands and ovaries, and heart, liver, pancreas, and brain. Conversely, it is reported to be down-regulated in tumor tissues including breast, HOC, and prostate cancers.[1–3] The possible mechanisms underlying abnormal expression of *ARHI* in tumors may involve abnormal methylation, loss of heterozygosity, and low expression levels of acetylated histones.[4–7] Depletion of histone deacetylases 1, 3, and 11 not only significantly increased the ARHI promoter activity of the transfected reporter but also activated the transcription of the endogenous ARHI gene.[8] Some studies by Yu et al[9] and Lu et al[10] revealed ARHI is down-regulated by transcriptional mechanisms that involve E2F1 and E2F4, as well as by the loss of RNA binding proteins that decrease the half-life of ARHI mRNA. Mutation of the putative E2F binding site in the ARHI promoter reversed this inhibitory effect and significantly increased ARHI promoter activity.

Studies have shown that *ARHI* inhibits cell growth in HOC and that loss of *ARHI* expression contributes to the formation of HOC.[11] When *ARHI* re-expression was promoted using demethylation factors and histone deacetylase inhibitors in different tissues, tumor growth rates were reduced, and apoptosis increased.[12] Furthermore, several studies have found that continuous expression of *ARHI* in HOC epithelia was associated with expression of cyclin-dependent kinase inhibitor p21 (WAF1/CIP1)[1] and extension of tumor-free survival time. Bao et al[13] directly injected the *ARHI* adenovirus vector into human breast tumor in nude mice and found that the tumor volume was significantly reduced.

Although these reports demonstrated the possibility of tumor treatment by targeting the *ARHI* gene, the mechanism remains unclear. To address this, we did some research; according to our preliminary experiment, we found 3 ovarian cancer cell lines show low expression of ARHI in 9 ovarian cancer cell lines including HOSE, ES2, SKOV3, A2780, 3AO, OVCAR3, HO8910, HO8910PM, and CAOV3. They are SKOV3, OVCAR3, and CAOV3. We reconstructed the PIRES2-EGFP-ARHI plasmid and transfected it into HOC SKOV3 cells with low *ARHI* gene expression levels. We then investigated the effects and molecular mechanisms of *ARHI* on cell proliferation in HOC SKOV3 cells and changes to the signal transduction pathways of *ARHI* protein products. This study has provided an experimental basis for new approaches in the development of HOC therapies.

MATERIALS AND METHODS

Reagents

Fetal bovine serum was purchased from Hyclone (Logan, Utah), Lipofectamine 2000 from Invitrogen (Carlsbad, CA), Tripure Isolation Reagent from Roche Molecular Biochemicals (Mannheim, Germany), GAPDH (glyceraldehyde phosphate dehydrogenase) antibody from Abmart (Shanghai, China), and phospho-Stat3 (Tyr705) (D3A7) XP rabbit monoclonal antibody. Phospho-p44/42 mitogen-activated protein kinase (MAPK) (Erk1/2) (Thr202/Tyr204) (197G2) rabbit monoclonal antibody were all purchased from Cell Signaling Technology (Beverly, MA). Primer synthesis was performed by Sangon Biological Engineering Technology & Service Co (Shanghai, China). The cell cycle detection kit, cell lysis solution, and cell counting kit 8 (CCK-8) were from Beyotime (Shanghai, China). DNA marker, T4 DNA ligase, and Taq enzyme were all from Takara (Dalian, China). Horseradish peroxidase goat anti–rabbit immunoglobulin G antibody was purchased from Abgent (San Diego, CA), DNA rapid purification kit was from Omega Bio-Tek (Norcross, GA).

Construction of the Eukaryotic Expression Vector

The *ARHI* gene sequence was obtained from GenBank (EMBL). The primers were designed based on the coding regions of the *ARHI* gene as follows: sense, 5′-*CCGGAATTC* ATGGGTAACGCCAGCTT-3′; antisense, 5′-*CGCGGATCC*TC ACATGATTATGCACTTGT-3′ (the underlined GAATTC and GGATCC sequences are the *Eco*RI and *Bam*HI recognition sites, respectively). The amplified fragment was 690 base pairs. Polymerase chain reaction (PCR) was performed using pcDNA3.0-ARHI as the template (kindly donated by Dr D. B. Badgwell, at The University of Texas M. D. Anderson Cancer Center, Houston, TX). The PCR products were separated by electrophoresis on a 1.5% agarose gel, followed by ethidium bromide staining. The resulting PCR fragments and plasmid were digested with *Bam*HI and *Eco*RI. The fragments of interest were recovered from the agarose gel, purified, and ligated by T4 DNA ligase to express the PIRES2-EGFP-ARHI plasmid. The ligation mixtures were used for transformation of *E. coli* cells. The positive recombinant products were selected on LB agar plates using 100 μg/mL ampicillin and confirmed by PCR and DNA sequencing.

Cell Culture and Transfection

SKOV3 cells were purchased from Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China) and were maintained in RPMI 1640 media supplemented with 10% fetal bovine serum, penicillin 100 U/mL, and streptomycin 100 U/mL at 37°C in a humidified 5% CO_2 atmosphere. The cells were seeded in 6-well plates at a density of 34×10^5 cells per well in 2 mL of antibiotic-free medium and incubated for 1 day before transfection at 90% to 95% confluence. Transfection was performed by first diluting 2 μL plasmid and 5 μL Lipofectamine 2000 per well in serum-free medium to a final volume of 250 μL, gently mixing, and incubating at room temperature for 5 min. This was followed by further gentle mixing and incubation at room temperature for 20 minutes. The cells were washed with serum-free medium before the diluted plasmid/Lipofectamine complex was added to the 6-well plates for 4 to 6 hours, after which the complex was replaced with normal cell culture medium. The

cells were visualized 24 hours after transfection under a Nikon Eclipse Fluorescence microscope (Nikon, Japan).

In Vitro Cell Growth Assay

The SKOV3 cells were divided into the following 4 groups: cells transfected with PIRES2-EGFP-ARHI plasmid (treated group), cells transfected with PIRES2-EGFP plasmid (positive control group), cells without transfection (negative control group), and a blank control without cells. Cells were passaged at the logarithmic growth phase, trypsinized, and adjusted to 5×10^4 cells/mL. The cells were then seeded in 96-well plates in 100 μL 10% fetal bovine serum and cultured for 24, 48, 72, 96, and 120 hours at 37°C in a humidified 5% CO_2 atmosphere. Each experimental group included 6 wells, and all experiments were repeated in triplicate. After incubation for 4 hours under the same condition, 10 μL CCK-8 reagent was added to each well. The optical density value of each well was measured using a microculture plate reader at a wavelength of 450 nm. The growth inhibitory rate (IR) was calculated using the following formula:

$$IR = ([\text{ OD cell control well}]-[\text{ OD experimental well}])/[\text{ cell control well}]) \times 100\%.$$

Apoptosis Assay

The SKOV3 cells were divided into the following 3 groups: PIRES2-EGFP-ARH–transfected group (treated), PIRES2-EGFP–transfected group (positive control), and cells without transfection (negative control). Each experimental group included 4 wells. The cells were harvested after 48 and 72 hours, and cell apoptosis was detected using Cell Cycle and Apoptosis Analysis Kits (Haimen, China), following the manufacturer's instructions.

Western Blotting

The SKOV3 cells were grouped as described above. Aliquots of cell lysates containing 50 μg of proteins were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. The membranes were blocked with TBST (Tris-buffered saline and Tween 20) buffer (10 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 0.05% Tween 20) containing 5% skimmed milk; incubated with rabbit polyclonal antibodies to phospho-Stat3, p44/42 MAPK (ERK1/2 [extracellular signal regulated protein kinase 1/2]), or phospho-p44/42 MAPK (ERK1/2) at 4°C overnight; and followed by the addition of horseradish peroxidase–linked anti–rabbit immunoglobulin G. The bands were visualized by electrochemiluminescence, and the intensity of each Western blot band was analyzed using Quantity One software (BioRad).

Statistical Analysis

SPSS version 13.0 software was used for statistical analyses. Comparisons of growth IRs and apoptotic rates were performed by χ^2 test, and protein relative expression levels were compared by Student t test. $P < 0.05$ was considered statistically significant.

RESULTS

Confirmation of PIRES2-EGFP-ARHI Plasmid Transfection

The sequencing results confirmed that the *ARHI* gene was successfully inserted into PIRES2-EGFP. The nucleotide sequence is as follows:

ATGGGTAACGCCAGCTTTGGCTCCAAGGAACAG
AAGCTGCTGAAGCGGTTGCGGCTTCTGCCCGCCCTG
CTTATCCTCCGCGCCTTCAAGCCCCACAGGAAGATCA
GAGATTACCGCGTCGTGGTAGTCGGCACCGCTGGTGT
GGGGAAAAGTACGCTGCTGCACAAGTGGGCGAGCGG
CAACTTCCGTCATGAGTACCTGCCGACCATTGAAAAT
ACCTACTGCCAGTTGCTGGGCTGCAGCCACGGTGTGC
TTTCCCTGCACATCACCGACAGCAAGAGTGGCGACG
GCAACCGCGCTCTGCAGCGCCACGTTATAGCCCGGG
GCCACGCCTTCGTCCTGGTCTACTCAGTCACCAAGAA
GGAAACCCTGGAAGAGCTGAAGGCCTTCTATGAGCT
ATCTGCAAGATCAAAGGTAACAACCTGCATAAGTTCC
CCATCGTGCTGGTGGGCAATAAAAGTGATGACACCCA
CCGGGAGGTGGCCCTGAATGATGGTGCCACCTGTGC
GATGGAGTGGAATTGCGCCTTCATGGAGATTTCAGCC
AAGACCGATGTGAATGTGCAGGAGCTGTTCCACATG
CTGCTGAATTACAAGAAAAAGCCCACCACCGGCCTC
CAGGAGCCCGAGAAGAAATCCCAGATGCCCAACACCA
CTGAGAAGCTGCTTGACAAGTGCATAATCATGTGA

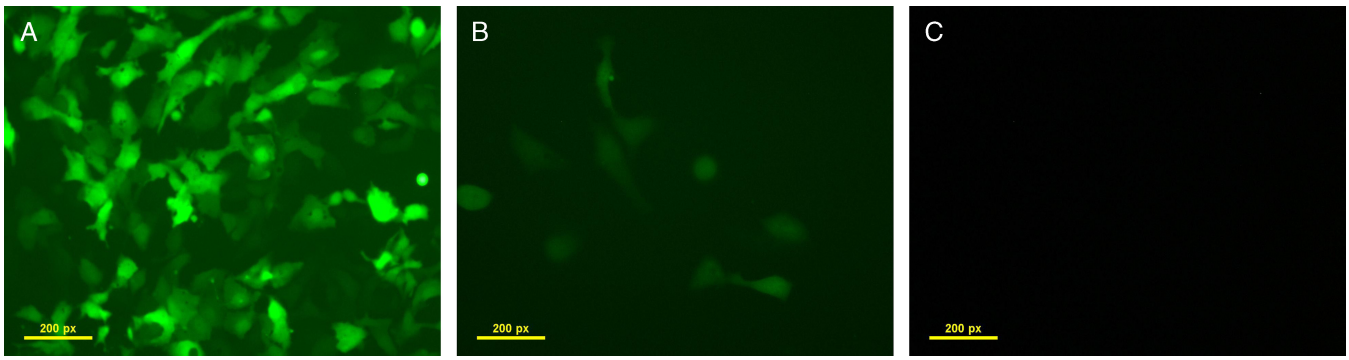

FIGURE 1. Green fluorescence protein expression 48 hours after transfection: (A) PIRES2-EGFP-ARHI-SKOV3–treated group; (B) PIRES2-EGFP-SKOV3 plasmid control group; (C) untransfected SKOV3 cells negative control group. The high level of GFP in the treated group compared with the control groups confirms successful transfection.

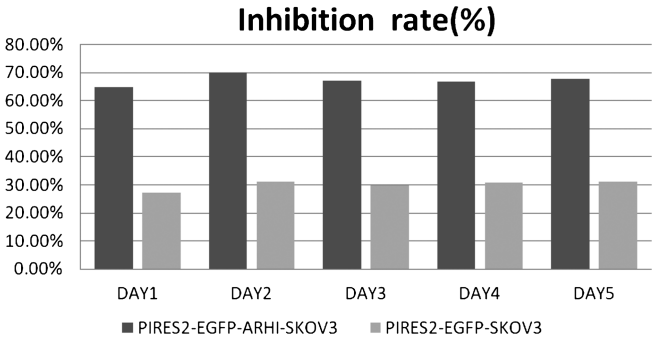

FIGURE 2. The effect of ARHI on cell proliferation in SKOV3, measured by CCK-8 assays, shows that ARHI significantly inhibits growth of SKOV3 cells.

ARHI Protein Expression Levels in SKOV3 Cells

The micrographs (Figs. 1A-C) showed a high level of green fluorescence protein (GFP) in PIRES2-EGFP-ARHI-SKOV3 cells (Fig. 1A) compared with a low level in PIRES2-EGFP-SKOV3 cells (Fig. 1B); no green fluorescence was observed in untransfected SKOV3 cells (Fig. 1C). The high level of GFP in the treated group compared with the control groups confirms successful transfection.

Analysis of Growth Inhibitory Rates in SKOV3 Cells by ARHI

After the cells had been cultured for 24, 48, 72, 96, and 120 hours, the growth IRs were 64.69%, 70.17%, 67.01%, 66.87%, and 67.70%, respectively, in the test group, and were 27.20%, 31.10%, 29.80%, 30.73%, and 31.28%, respectively, in the plasmid control group (Fig. 2). This showed that the growth IR was significantly increased in the test group compared with the plasmid control group ($P < 0.01$).

Influence of ARHI on the Cell Cycle Phase Distribution and Apoptosis Rates

The proportions of S-phase cells and apoptosis rates in SKOV3 cells were determined by flow cytometry. After 48 hours of culture, the mean proportions of S-phase SKOV3 cells in the test, plasmid control, and negative control groups were 64.18%, 38.43%, and 15.15%, respectively, and the mean apoptosis rates were 47.97%, 26.53%, and 9.33%, respectively. After 72 hours of culture, the mean proportions of S-phase SKOV3 cells in the test, plasmid control, and negative control groups were 43.29%, 10.37%, and 10.89%, respectively, and the apoptosis rates were 51.34%, 24.70%, and 4.39%, respectively (Table 1). Data show that the proportions of S-phase cells, and thereby S-phase arrest, were clearly higher in the PIRES2-EGFP-ARHI-SKOV3 cell group compared with the control group at both 48 and 72 hours; the result at 72 hours was much more significant in the test group. In addition, the apoptosis rate was significantly increased in the test group compared with the control group ($P < 0.01$).

Analysis of Protein Expression in SKOV3 Cells by Western Blotting

The Western blot images for P-STAT3 (phosphorylated signal transduction and activators of transcription 3) and P-ERK protein expression for the control and treated groups: SKOV3, PIRES2-EGFP-SKOV3, and PIRES2-EGFP-ARHI-SKOV3, are shown in Figure 3. Compared with GAPDH reference protein, the relative expression levels of P-STAT3 were 1.1473 ± 0.0002, 1.0913 ± 0.0021, and 0.7424 ± 0.0006, respectively, and the relative expression levels of P-ERK were 1.2260 ± 0.0011, 1.1289 ± 0.0018, and 0.5866 ± 0.0013, respectively. In comparison to the 2 control groups, the expression levels of P-STAT3 and P-ERK proteins were significantly lower in the PIRES2-EGFP-ARHI-SKOV3 group ($P < 0.05$).

DISCUSSION

Cell cycle progression is regulated by multiple control points at different phases of the cell cycle; the 3 principal ones being G1/S, G2/M, and at metaphase/anaphase transmission during mitosis. Failure of these control points can lead to abnormal growth or apoptosis. The G1/S check point is the most critical for control of cell proliferation via intracellular and extracellular signals related to transportation and integration of molecules into the nucleus.[14]

By reconstructing the PIRES2-EGFP-ARHI plasmid and successfully transfecting it into HOC SKOV3 cells with low *ARHI* expression levels, as shown by high levels of green fluorescence from the plasmid observed by fluorescent

TABLE 1. Influence of ARHI on the cell cycle phase distribution and apoptosis rate

	n	G0/G1, %	S, %	G2/M, %	AP, %
48 h					
SKOV3 group	4	63.326	15.150	21.520	9.333
PIRES2-EGFP- SKOV3 group	4	49.707	38.425	11.868	26.526
PIRES2-EGFP-ARHI-SKOV3 group	4	29.570	64.182	6.262	47.971
72 h					
SKOV3 group	4	70.511	10.888	18.597	4.387
PIRES2-EGFP- SKOV3 group	4	66.494	11.565	15.411	20.551
PIRES2-EGFP-ARHI-SKOV3 group	4	43.174	43.286	12.041	51.340

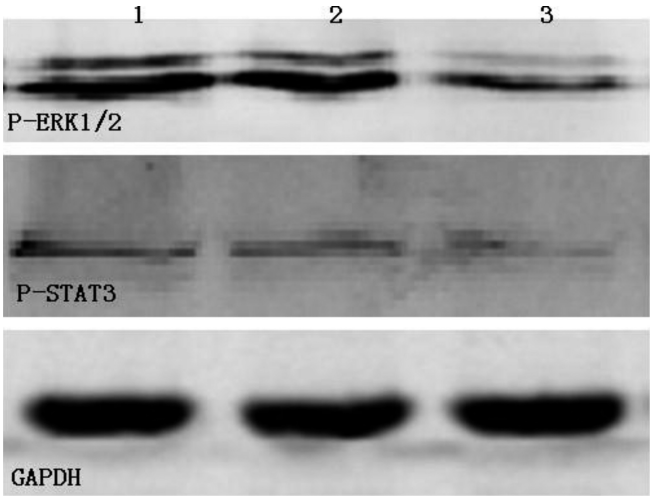

FIGURE 3. Changes in the expressions levels of P-ERK1/2 and P-STAT3 in the different SKOV3 groups: lane 1, protein expression in the untransfected SKOV3 control group; lane 2, protein expression 48 hours after PIRES2-EGFP was transfected into SKOV3 cells; lane 3, protein expression 48 hours after PIRES2-EGFP-ARHI was transfected into SKOV3 cells. The results show that ARHI significantly reduces P-ERK1/2 and P-STAT3 expression levels.

microscopy, in addition, by using a CCK-8 assay to analyze the growth IRs, we observed significantly higher growth inhibition in PIRES2-EGFP-ARHI-SKOV3 cells compared with the control cells; furthermore, flow cytometry showed the proportion of S-phase cells and the apoptosis rates were significantly higher after promoting expression of the *ARHI* gene. These results suggested that *ARHI* inhibited proliferation, arrested the cells at S-phase of the cell cycle, and induced apoptosis in SKOV3 cells. We therefore proposed that the *ARHI* gene is involved in cell cycle regulation in HOC SKOV3 cells, preventing proliferation and inducing apoptosis.

Multiple signaling pathways are involved in cell growth and apoptosis, including the Ras/Raf/MEK/ERK1/2 signaling pathway, which is central for transporting the complex signals received by the cell surface receptors to transcription factors in the nucleus. Extracellular signal regulated protein kinase 1/2 is a key physiological substrate of MEK; MEK1 kinase can cause double phosphorylation of threonine and tyrosine at ERK1/2 residues to activate ERK1/2; activation of ERK1/2 can subsequently lead to phosphorylation of downstream transcription factors, such as p90Rsk kinase, CREB, and c-Myc. Changes in cell proliferation, apoptosis, differentiation, and other critical physiological activities are a consequence of phosphorylation of these transcription factors.[15] Meloche and Pouysségur[16] proposed that ERK1/2, which is a key regulatory factor in mitosis, is also involved in the evolutionary control of the cell cycle. A certain study showed that ARHI plays roles in the EGF-EGFR-Ras-Raf-MAPK/ERK1/2 pathway.[17]

Extracellular signals activate JAK in the JAK-STAT signaling pathway through associated receptors, leading to phosphorylation of specific tyrosine residues on the receptors; these phosphorylate STATs, which are isolated from the receptors, transferred into the nucleus after dimerization, and combined with specific DNA response elements. This initiates transcription at the target genes and promoting synthesis of a variety of proteins and their subsequent biological roles.[18] Studies have shown that inhibiting the JAK-STAT signaling pathway can significantly reduce P-JAK2, P-JAK3, and P-STAT3 expression levels; down-regulate cyclin-D1, which is linked to cell cycle regulation; and up-regulate p16, p21, and p27 expression, thereby inhibiting tumor cell proliferation and growth.[19,20] STAT3, an important member of the STAT family, is found in a variety of tumor tissues and cell lines and is now recognized as an oncogene.[21,22] STAT3 siRNA down-regulates cyclin D1, survivin, and vascular endothelial growth factor expression in HOC cells; inhibits STAT3 and its related genes; inhibits HOC cell growth; and induces apoptosis in vitro. As such, STAT3 has recently become a target for HOC therapies.[23] When the yeast 2-hybrid system was used to search for *ARHI*-interactive proteins by screening the human mammary epithelial cell cDNA gene pool, *ARHI* was found to act with signal transduction proteins and the STAT3 transcription activator, but not STAT1 or STAT5a. Furthermore, when *ARHI* and STAT3 were coexpressed in SKOV3 cells, they did not form a complex in the cytoplasm. In addition, IL-6–induced STAT3 accumulation in the nucleus was blocked. This suggests that by regulating STAT3 phosphorylation, *ARHI* can significantly reduce STAT3 binding to DNA, and thereby reduce the activity of the STAT3-dependent promoter.[24]

Our data showed that the expression levels of P-ERK1/2 and P-STAT3 proteins in the Ras/Raf/MEK/ERK1/2 and JAK-STAT3 signaling pathways were significantly reduced 48 hours after *ARHI* transfection. In our study previously, we also measured the expression levels of STAT3 and ERK1/2 and did not find statistically significant. So we proposed that the mechanism by which *ARHI* influences both the Ras/Raf/MEK/ERK1/2 and JAK-STAT3 signaling pathways to inhibit HOC SKOV3 cell proliferation is through regulating *ARHI* gene expression by altering the phosphorylation levels of ERK1/2 and STAT3 protein kinases, thereby blocking the signaling pathway for tumor cell proliferation sufficiently to prevent cell proliferation and induce apoptosis.

A study by Lu et al[25] revealed that the *ARHI* gene maintains HOC cells in transplanted tumors in a resting state; conversely, when the *ARHI* level was decreased, the transplanted tumor grew rapidly, and when *ARHI* expression subsequently recovered to normal levels, the HOC cells died through autophagy. The authors concluded that the *ARHI* gene is a key factor in initiating autophagy in HOC cells; a further study by Mathias et al[26] proposed that this may lead to cell death. An in-depth investigation is required to determine the molecular mechanism for autophagy initiation in the *ARHI* gene, with the aim of developing an application based on *ARHI* for the clinical treatment of HOC.

ACKNOWLEDGMENTS

The authors thank researchers of Jiangsu Institute of Hematology for their help.

REFERENCES

1. Rosen DG, Wang L, Jain AN, et al. Expression of the tumor suppressor gene ARHI in epithelial ovarian cancer is associated with increased expression of p21WAF1/CIP1 and prolonged progression-free survival. *Clin Cancer Res*. 2004;10: 6559–6566.
2. Chen Y, Zaman MS, Deng G, et al. MicroRNAs 221/222 and genistein- mediated regulation of ARHI tumor suppressor gene in prostate cancer. *Cancer Prev Res (Phila)*. 2011;4:76–86.
3. Tang HL, Hu YQ, Qin XP, et al. Aplasia Ras homolog member I is downregulated in gastric cancer and silencing its expression promotes cell growth in vitro. *J Gastroenterol Hepatol*. 2012;27:1395–1404.
4. Feng W, Marquez RT, et al. Imprinted tumor suppressor genes ARHI and PEG3 are the most frequently down-regulated in human ovarian cancers by loss of heterozygosity and promoter methylation. *Cancer*. 2008;112:1489–1502.
5. Yu Y, Fujii S, Yuan J, et al. Epigenetic regulation of ARHI in breast and ovarian cancer cells. *Ann N Y Acad Sci*. 2003; 983:268–277.
6. Li J, Cui G, Sun L, et al. STAT3 acetylation-induced promoter methylation is associated with downregulation of the ARHI tumor-suppressor gene in ovarian cancer. *Oncol Rep*. 2013;30:165–170.
7. Zhang L, Liu P, Li H, et al. Effect of histone deacetylase inhibitors on cell apoptosis and expression of the tumor suppressor genes RUNX3 and ARHI in ovarian tumors. *Mol Med Rep*. 2013;7:1705–1709.
8. Feng W, Lu Z, Luo RZ, et al. Multiple histone deacetylases repress tumor suppressor gene ARHI in breast cancer. *Int J Cancer*. 2007;120:1664–1668.
9. Yu Y, Luo R, Lu Z, et al. Biochemistry and biology of ARHI (DIRAS3), an imprinted tumor suppressor gene whose expression is lost in ovarian and breast cancers. *Methods Enzymol*. 2006;407:455–468.
10. Lu Z, Luo RZ, Peng H, et al. Transcriptional and posttranscriptional down-regulation of the imprinted tumor suppressor gene ARHI (DRAS3) in ovarian cancer. *Clin Cancer Res*. 2006;12:2404–2413.
11. Lu Z, Bast RC Jr. The tumor suppressor gene ARHI (DIRAS3) inhibits ovarian cancer cell migration through multiple mechanisms. *Cell Adh Migr*. 2013;7:232–236.
12. Fujii S, Luo RZ, Yuan J, et al. Reactivation of the silenced and imprinted alleles of ARHI is associated with increased histone H3 acetylation and decreased histone H3 lysine 9 methylation. *Hum Mol Genet*. 2003;12:1791–1800.
13. Bao JJ, Le XF, Wang RY, et al. Reexpression of the tumor suppressor gene ARHI induces apoptosis in ovarian and breast cancer cells through a caspase-independent calpain dependent pathway. *Cancer Res*. 2002;62:7264–7272.
14. Skotheim JM, DiTalia S, Siggia ED, et al. Positive feedback of G1 cyclins ensures coherent cell cycle entry. *Nature*. 2008;454:291–296.
15. Chang F, Steelman LS, Lee JT, et al. Signal transduction mediated by the Ras/Raf/MEK/ERK pathway from cytokine receptors to transcription factors: potential targeting for therapeutic intervention. *Leukemia*. 2003;17:1263–1293.
16. Meloche S, Pouysségur J. The ERK1/2 mitogen-activated protein kinase pathway as a master regulator of the G1- to S-phase transition. *Oncogene*. 2007;26:3227–3239.
17. Lu J, Qian JM, Yang H, et al. Inhibitory effect of a Ras homologue member I on pancreatic cancer and its modulation on epithelium growth factor receptor-Ras-Raf-mitogen-activated protein kinase/extracellular regulated protein kinase/1/2 pathway. *Zhongguo Yi Xue Ke Xue Yuan Xue Bao*. 2012;34: 197–201.
18. Krebs DL, Hilton DJ. SOCS proteins: negative regulators of cytokine signaling. 2001;19:378–387.
19. Xiong H, Zhang ZG, Tian XQ, et al. Inhibition of JAK1, 2/STAT3 signaling induces apoptosis, cell cycle arrest, and reduces tumor cell invasion in colorectal cancer cells. *Neoplasia*. 2008;10:287–297.
20. Lin Q, Lai R, Chirieac LR, et al. Constitutive activation of JAK3/STAT3 in colon carcinoma tumors and cell lines: inhibition of JAK3/STAT3 signaling induces apoptosis and cell cycle arrest of colon carcinoma cells. *Am J Pathol*. 2005;167:969–980.
21. Zhao X, Sun X, Li XL. Expression and Clinical Significance of STAT3, P-STAT3, and VEGF-C in small cell lung cancer. *Asian Pac J Cancer Prev*. 2012;13:2873–2877.
22. Badgwell DB, Lu Z, Le K, et al. The tumor-suppressor gene ARHI (DIRAS3) suppresses ovarian cancer cell migration through inhibition of the Stat3 and FAK/Rho signaling pathways. *Oncogene*. 2012;31:68–79.
23. Cai L, Zhang G, Tong X, et al. Growth inhibition of human ovarian cancer cells by blocking STAT3 activation with small interfering RNA. *Eur J Obstet Gynecol Reprod Biol*. 2010;148:73–80.
24. Nishimoto A, Yu Y, Lu Z, et al. A Ras homologue member I directly inhibits signal transducers and activators of transcription 3 translocation and activity in human breast and ovarian cancer cells. *Cancer Res*. 2005;65:6701–6710.
25. Lu Z, Luo RZ, Lu Y, et al. The tumor suppressor gene ARHI regulates autophagy and tumor dormancy in human ovarian cancer cells. *J Clin Invest*. 2008;118:3917–3929.
26. Mathias T, Rosenfeldt KM, et al. The role of autophagy in tumour development and cancer therapy. *Expert Rev Mol Med*. 2009;11:e36.