Original Article
Stable silencing of ROR1 regulates cell cycle, apoptosis, and autophagy in a lung adenocarcinoma cell line

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Abstract: Lung cancer has the highest mortality and recurrence rate among cancers in the world. Receptor tyrosine kinase-like orphan receptor 1 (ROR1) has been widely recognized for its role in promoting the growth and metastasis of lung cancer, but its comprehensive role and molecular mechanisms in regulating cell cycle, apoptosis, and autophagy remain unclear. In this study, a series of ROR1-stably silenced monoclonal clones from lung adenocarcinoma cell lines PC9, PC9erlo, and NCI-H1975 were successfully selected and confirmed by qRT-PCR, western blot, and flow cytometry, and used as cell models in the following assays. Our study clearly shows that blocking ROR1 significantly downregulates cell cycle-inducing molecules such as CDK4 and Cyclin E1, and anti-apoptotic molecules such as Bcl-XL and Bcl-2, while it markedly upregulates pro-apoptotic molecules such as Bak, Caspase-3, and Caspase-7, which extends our previous observation on the molecular mechanism of ROR1-mediated tumor growth in lung adenocarcinoma. Our data also show that silencing ROR1 promotes autophagy since the key molecules involved in autophagy including ATG7, ATG12, BNIP3L, LC3A, LC3B, and NBS1 were up-regulated. We further screened key phosphokinase signaling pathways downstream of ROR1 in lung adenocarcinoma by a human phospho-kinase array. Our data indicate that blocking ROR1 could deactivate Akt, then activate GSK-3α/β by de-phosphorylation, and finally deactivate mTOR. In this way blocking ROR1 could effectively regulate the cell cycle, apoptosis, and autophagy in lung cancer.

Keywords: ROR1, lung adenocarcinoma, apoptosis, cell cycle, autophagy

Introduction

Lung cancer remains the leading cause of cancer morbidity and mortality [1]. In 2018, 2.1 million new lung cancer cases were reported, accounting for nearly a fifth (18.4%) of cancer deaths [2]. Although until now ten types of standard therapeutic options including surgery, radiotherapy, chemotherapy, targeted therapy, and immunotherapy [3-7] are available for patients with lung cancer and shown exciting treatment efficacy in small portion of patients, especially those with late-stage metastasis, the overall 5-year survival rate is still poor, which impels more study on the molecular and pathogenetic mechanisms of lung cancer [8].

Oncogene receptor tyrosine kinase-like orphan receptor 1 (ROR1) is expressed during embryonic development [9, 10], but found to be over-expressed in several types of hematologic neoplasms including chronic lymphocytic leukemia (CLL) [11, 12] and acute lymphoblastic leukemia (ALL) [13], and a variety of solid tumors such as breast cancer [14-16], colorectal carcinoma [17], ovarian carcinoma [18], melanoma [19, 20], as well as lung adenocarcinoma [21, 22]. ROR1 has been widely known for its vital roles in promoting the growth and metastasis of tumor, inducing drug-resistance and enhancing apoptosis-resistance, which makes it a feasible target for tumor therapy [23, 24], but little is known about its roles and downstream signaling cascades in regulating the cell cycle and autophagy in lung adenocarcinoma.

Autophagy is a cell mechanism by which cellular components are subjected to the organized degradation and recycling, which has raised great interest in its role in tumorigenesis [25,

26]. Moreover, increasing studies have shown that cell cycle, apoptosis and autophagy are closely linked and may positively and negatively regulate each other [27], so it is interesting to know whether ROR1 plays a role in regulating the cell cycle and autophagy. Based on our previous work which showed that ROR1 protein is widely expressed in both tissues and cell lines of lung adenocarcinoma, and that silencing ROR1 with small interfering RNA (siRNA) can induce tumor cell death and apoptosis [22, 23, 28], here in this study we continue to investigate the underlying molecular mechanism of ROR1 in regulating the cell cycle and autophagy in ROR1-stably silenced lung adenocarcinoma cell lines.

Materials and methods

Cell Lines and cell culture

Three human lung adenocarcinoma cell lines were used in our study: the erlotinib-sensitive cell line PC9 was kindly provided by Dr. Jun Zhang of Shanghai Pulmonary Hospital; the primary erlotinib-resistant cell line NCI-H1975 was obtained from the stem cell bank of Chinese Academy of Sciences. Those two cell lines were cultured in RPMI-1640 medium (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS, Transgen, Beijing, China) and 1% penicillin/streptomycin (HyClone, Logan, UT, USA). The acquired erlotinib-resistant cell line PC9erlo was established in our laboratory as reported before [29] and cultured in complete RPMI-1640 culture medium containing 2.5 µM erlotinib to maintain drug resistance. All the cells were incubated at 37°C in a 95% humidified air incubator (Panasonic, Ehime, Japan) containing 5% CO_2.

Infection with lentivirus-mediated small-hairpin RNA (shRNA)

The packaged lentiviruses containing shRNA with a specific human ROR1 targeting sequence [23, 25, 26] (Sense 5'-GUACUGCGAUGAAA-CUUCATT-3'; antisense 5'-UGAAGUUUCAUCGC-AGUACGG-3') (HBLV-H-ROR1-shRNA1-GFP-PU-RO, Lv-shROR1, titer 1 × 10⁸ TU/mL) or non-related shRNA (HBLV-GFP-PURO, Lv-shCon, titer 2 × 10⁸ TU/mL) were purchased from HanBio Biotechnology Co. (Shanghai, P. R. China). Lung adenocarcinoma cell lines PC9, PC9erlo, and NCI-H1975 were infected with

Lv-shROR1 or Lv-shCon as below: cells were seeded in 96-well plates at a density of 3 × 10³ cells/well and infected with lentiviruses at various multiplicity of infection (MOI) (PC9 MOI 10, PC9erlo MOI 10 and NCI-H1975 MOI 20) with 5 µg/mL polybrene for 24 h. 1.5 µg/mL puromycin was used 48 h later to maintain the selection pressure. The efficiency of lentivirus infection was determined by green fluorescent protein (GFP) fluorescence intensity measured under inverted fluorescence microscope (Nikon ECLIPSE Ti, Tokyo, Japan). Monoclonal cells (clones) were selected by finite dilution method, and PC9 and NCI-H1975 clones were subcultured in complete RPMI-1640 culture medium containing 1.5 µg/mL puromycin, and PC9erlo clones were subcultured in complete RPMI-1640 culture medium containing 2.5 µM erlotinib and 1.5 µg/mL puromycin.

Flow cytometry

Cells were collected and washed with phosphate-buffered saline (PBS) at 300 g for 5 min, then cells were incubated with 5 µg/mL chimeric rabbit/human anti-ROR1 monoclonal antibody R12 as reported before [30] or normal human IgG antibodies (Code#: 009-000-003, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 30 min at 4°C. After washing with flow cytometry buffer (PBS+10% FBS), cells were further incubated with HA-Tag (6E2) mouse mAb (Alexa Fluor® 647 Conjugate) (Cat#: 3444, 1:1000, Cell Signaling Technology, Danvers, MA, USA) for 30 min at 4°C. Cells were captured on NovoCyte flow cytometry (ACEA Biosciences, Inc., San Diego, California, USA) and analyzed with FlowJo-V10 software (FlowJo LLC, Ashland, OR, USA). The formula used to calculate the inhibition rate of ROR1 expression level in different cell lines was: [value (Lv-shCon) - value (background)] - [value (Lv-shROR1) - value (background)]/[value (Lv-shCON) - value (background)] × 100% (value is the median fluorescence intensity of ROR1 in different groups).

Western blot analysis

Protein extraction from cell lines was prepared using RIPA lysis buffer (Beyotime Institute of Biotechnology, Hangzhou, China) containing 1% proteinase inhibitor (Millipore, Bedford Park, IL, USA) and 10% phosphatase inhibitor (Roche Diagnostics, Basel, Switzerland) and the con-

centration of protein was analyzed by BCA protein assay kit (Beyotime Institute of Biotechnology, Hangzhou, China). Protein was separated by SDS-PAGE and transferred to 0.45 µM PVDF membrane (Millipore, Bedford Park, IL, USA). After blocking with 5% skim milk at 37°C for 1 h, membranes were incubated with antibodies. The first antibodies used in these studies were ROR1 (D6T8C) (Cat#: 16540, 1:1000), Akt (Cat#: 2920S, 1:2000), P-Akt (S473) (Cat#: 4060S, 1:1000), GSK-3α/β (D7503) (Cat#: 5676S, 1:1000), P-GSK-3α/β (S21/9) (Cat#: 8566S, 1:1000), mTOR (Cat#: 2972S, 1:1000), P-mTOR (Ser2448) (Cat#: 5536S, 1:1000), CDK4 (D9G3E) (Cat#: 12790, 1:1000), Cyclin E1 (D7T3U) (Cat#: 20808, 1:1000), Bcl-XL (54H6) (Cat#: 2764, 1:1000), Bak (D4E4) (Cat#: 12105, 1:1000), Caspase-3 (8G10) (Cat#: 9665, 1:1000), Caspase-7 (D2Q3L) (Cat#: 12827, 1:1000), β-actin (Cat#: HC201, Mouse, 1:1000) (Cell Signaling Technology, Danvers, MA, USA) and Bcl-2 (Cat#: 51-6511GR, 1:500; BD Biosciences, Franklin Lake, New Jersey, USA). The secondary antibodies used in these studies were horseradish peroxidase (HRP)-conjugated anti-rabbit antibody (Cat#: HS101-01, 1:5000) and HRP-conjugated anti-mouse antibody (Cat#: HS201-01, 1:5000; Transgen Biotech Co., Ltd., Beijing, China). Chemiluminescence was detected using enhanced Pierce ECL kit (Foregene Company Limited, Chengdu, China). Images were captured by molecular imager ChemiDoc™ XRS+ Imaging system (Bio-Rad, Laboratories, Hercules, CA, USA) and analyzed by Image Lab 6.0 software (Bio-Rad, Laboratories, Hercules, CA, USA).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Cells were seeded in six-well plates and total RNA was extracted by Trizol reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and transcribed into cDNA by SuperScript™ III reverse transcriptase (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The predesigned ROR1 primers and human GAPDH endogenous reference genes primers used in the study were purchased from Shanghai Bioengineering Co., Ltd. (Shanghai, China) with the primer sequences: ROR1 upstream: 5'-CAGACA-CAGGCTACTTCCAGTGC-3', ROR1 downstream: 5'-CTCCATATAGACGGTGCGGTTGC-3'. The reac-

tion condition of PCR with PowerUp SYBR Green master mix (Thermo Fisher Scientific, Inc., Waltham, MA, USA) was as follows: 94°C for 2 min followed by 35 cycles of denaturation 94°C for 3 s, and annealing/extension 60°C for 30 s. The relative gene expression was normalized to internal GAPDH, and the fold difference in the expression of ROR1 was calculated by Livak method ($2^{-\Delta\Delta Ct}$). All samples were done in triplicate.

Human autophagy array

Protein extraction from cell lines was performed as mentioned for western blot assay and 20 human proteins related to autophagy were semi-quantitatively determined by RayBio® C-Series Human Autophagy Array 1 (Cat#: AAH-ATG-1-8, RayBiotech, Inc., Atlanta, GA, USA) following the protocol provided by the manufacturer. Briefly, 500 µg of cell lysate protein was incubated with specific antibodies precoated on the membrane at 4°C overnight. After washing, the membrane was first incubated with biotinylated antibody cocktail at room temperature (RT) for 2 h, then incubated with HRP-conjugated streptavidin for 1 h at RT. The membrane was washed and chemiluminescence was developed by enhanced Pierce ECL kit (Foregene Company Ltd., Chengdu, China). The density of each spot pixel on the membrane was determined by image lab 6.0 software (Bio-Rad, Laboratories, Hercules, CA, USA) and calculated by AAH-ATG-1 analysis tools provided by RayBiotech, Inc. (Atlanta, USA).

Human phospho-kinase array

The relative levels of protein phosphorylation were determined by proteome profiler human phospho-kinase array kit (Cat#: ARY003B, R&D Systems, Minnesota, USA). Protein extraction was performed as mentioned in western blot assay. Briefly, 400 µg of cell lysate protein was incubated with the microarrays which have been precoated with antibodies against 43 kinase phosphorylation sites and 2 related total proteins at 4°C overnight. After washing, the microarrays were incubated with biotin-labeled antibodies at RT for 2 h, then microarrays were incubated with HRP-conjugated streptavidin at RT for 30 min on a rocking platform. Chemiluminescence and Density of spot pixels was measured as mentioned in a human autophagy array.
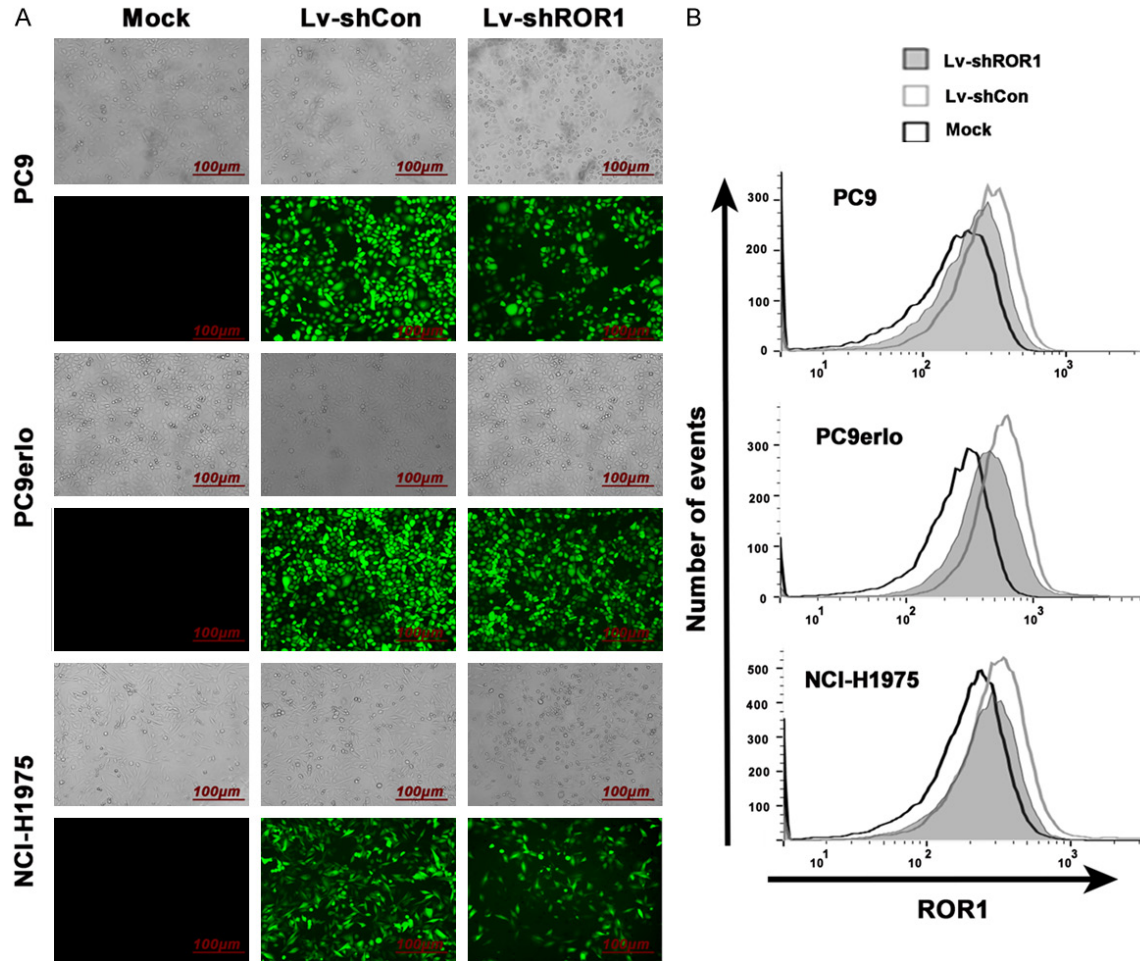
Figure 1. Silencing of ROR1 with lentivirus-mediated shRNA in lung adenocarcinoma cell lines. The infection efficiency was demonstrated by GFP fluorescence intensityunder fluorescence microscope (A) and the relative expression levels of ROR1 were analyzed by flow cytometry (B). Mock, cells cultured with RPMI-1640 culture medium with 10% FBS alone; Lv-shCon, cells infected with lentivirus containing non-related shRNA; Lv-shROR1, cells infected with lentivirus containing ROR1-specific shRNA.

Statistical analysis

Quantitative data were expressed as mean ± SEM of at least three independent experiments. Data were analyzed by one-way ANOVA (LSD method) or Student t test using Graphpad prism 7.0a software (San Diego, CA, USA). A value of $P < 0.05$ was considered significant.

Results

Silencing ROR1 with lentivirus-mediated shR-NA in lung adenocarcinoma cell lines

In our previous study, we investigated the role of ROR1 by transiently blocking ROR1 expression by siRNA. Here we aimed to establish ROR1-stably-silenced clones from PC9, PC9-erlo, and NCI-H1975 cell lines, and study the

long-term effect of ROR1 blocking in lung adenocarcinoma. Our data showed that cells were successfully infected with Lv-shROR1 and Lv-shCon as demonstrated by GFP expression under a fluorescence microscope (**Figure 1A**). Meanwhile, the ROR1-blocking efficacy of Lv-shROR1 was verified by flow cytometry with inhibition rates of 55% in PC9, 50% in PC9erlo, and 35% in NCI-H1975, respectively (**Figure 1B**).

Establishment of ROR1-stably-silenced clones from lung adenocarcinoma cell lines

The ROR1-stably-silenced clones were screened from cell lines PC9, PC9erlo, and NCI-H19-75 which have been infected with Lv-shROR1 through the finite dilution method. In total, 13 clones (4 clones from PC9, 4 clones from
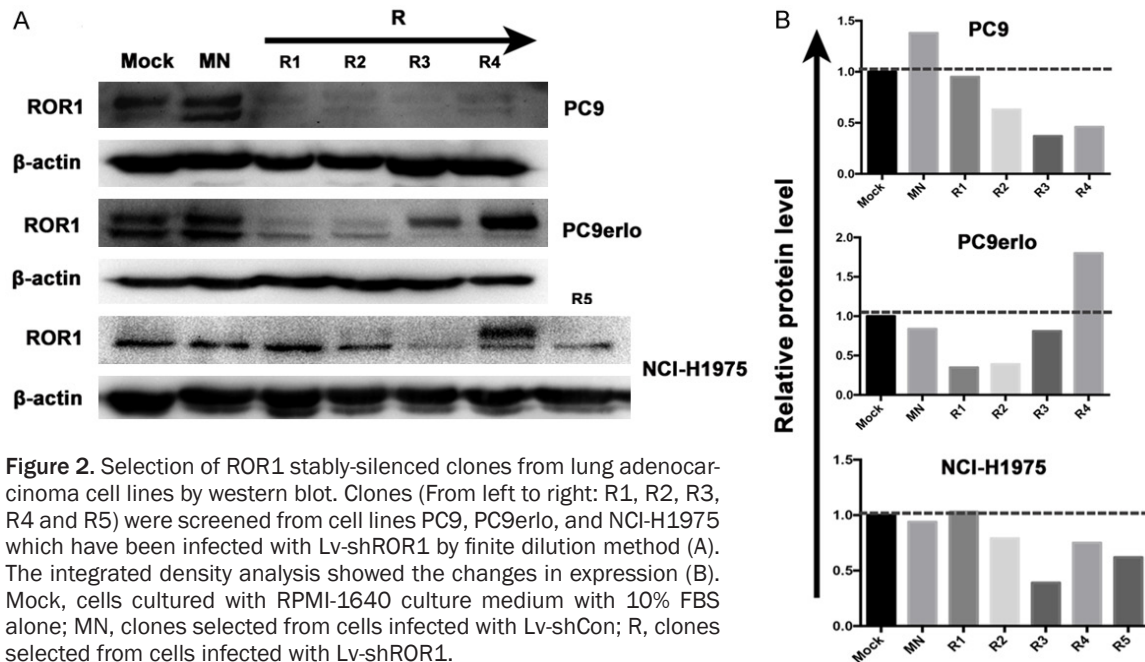
Figure 2. Selection of ROR1 stably-silenced clones from lung adenocarcinoma cell lines by western blot. Clones (From left to right: R1, R2, R3, R4 and R5) were screened from cell lines PC9, PC9erlo, and NCI-H1975 which have been infected with Lv-shROR1 by finite dilution method (A). The integrated density analysis showed the changes in expression (B). Mock, cells cultured with RPMI-1640 culture medium with 10% FBS alone; MN, clones selected from cells infected with Lv-shCon; R, clones selected from cells infected with Lv-shROR1.

PC9erlo, and 5 clones from NCI-H1975) were selected, expanded, and prepared for identification. Firstly, the protein expression level of ROR1 was detected by western blot which indicated that the ROR1-inhibition rates were higher in clones R3 and R4 from PC9, clones R1 and R2 from PC9erlo, and clones R3 and R5 from NCI-H1975 (**Figure 2**). Next, those six clones (Clones R3 and R4 from PC9, clones R1 and R2 from PC9erlo, and clones R3 and R5 from NCI-H1975) were selected and we further detected ROR1 mRNA and protein expressions by qRT-PCR (**Figure 3B**) and flow cytometry assay (**Figure 3C**), respectively. Our results showed that compared with clones infected with Lv-shCon (MN), the inhibition rates of surface ROR1 in clones R3 and R4 from PC9 were 65% and 89%, respectively, clones R1 and R2 from PC9erlo were 80% and 80%, respectively, and clones R3 and R5 from NCI-H1975 were 42% and 33%, respectively. The data from qRT-PCR also had similar results which further confirmed that we successfully established clones with ROR1 stably-silenced from lung adenocarcinoma cell lines. The fluorescence images of the six clones are shown in **Figure 3A**.

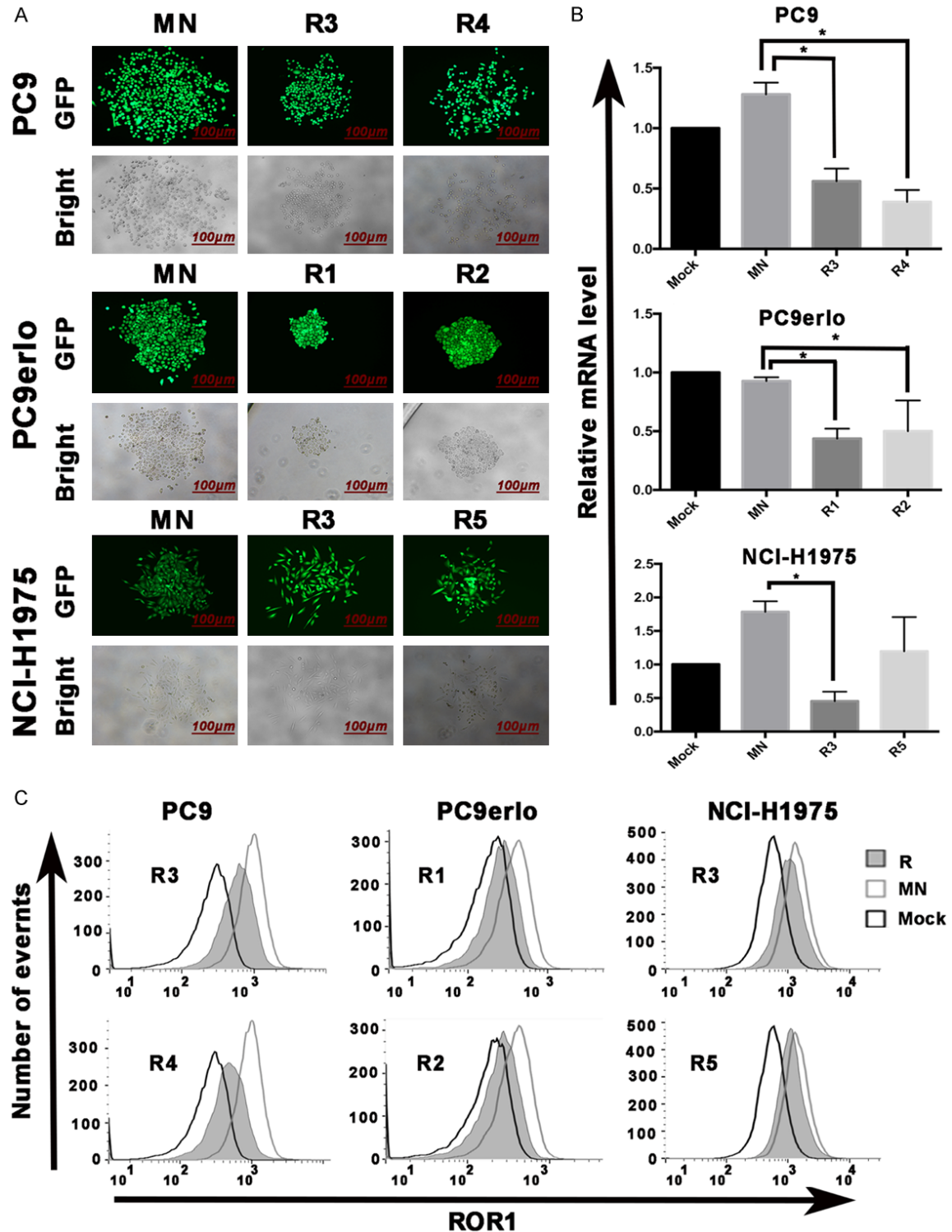*Silencing ROR1 downregulates the expression of cell cycle-related molecules*

We and several other groups have shown that the signal from ROR1 sustains tumor cell proliferation in lung adenocarcinoma. We wondered whether ROR1 also plays a role in regulating the cell cycle, thus enhancing tumor cell growth. Key regulators in the cell cycle were analyzed by western blot in clone R1 from cell line PC9erlo. Compared with clone infected with Lv-shCon (MN), the expression levels of Cyclin E1 and CDK4, two key players in DNA synthesis and G1/S-phase progression, were markedly down-regulated (**Figure 4A**, **4B**) which supported our hypothesis that ROR1 might play an essential role in lung adenocarcinoma growth by upregulation of cell cycle related proteins.

Silencing ROR1 upregulates the expression of pro-apoptotic molecules

Our previous data have shown that silencing ROR1 by siRNA induces tumor cell apoptosis in lung adenocarcinoma [23]. Here, we further assessed the expression levels of pro- or anti-apoptotic molecules in clone R1 from PC9erlo. We found that silencing ROR1 noticeably decreased the expression of anti-apoptotic molecules such as Bcl-2 and Bcl-XL, while it upregulated pro-apoptotic molecules such as Caspase-3, Caspase-7, and Bak (**Figure 4C**, **4D**). Our results reinforced our previous data that ROR1 induces apoptosis-resistance by regulating the activity of pro- or anti-apoptotic players in lung adenocarcinoma cell lines.

Figure 3. Identification of clones with ROR1 stably-silenced by RT-PCR and flow cytometry. The infection efficiency of Lv-shROR1 in selected clones (R3 and R4 from PC9, R1 and R2 from PC9erlo, R3 and R5 from NCI-H1975) was demonstrated by GFP fluorescence intensity under fluorescence microscope (A) and the relative expression levels of ROR1 were analyzed by qRT-PCR (B) and flow cytometry (C), respectively. Asterisks indicate statistically significant differences compared with the non-related shRNA group (*P<0.05). Statistical analyses was performed using one-way ANOVA (LSD method) (R3 and R4 from PC9, R1 and R2 from PC9erlo, R3 and R5 from NCI-H1975 compared with MN). Mock, cells cultured with RPMI-1640 culture medium with 10% FBS alone; MN, clones selected from cells infected with Lv-shCon; R, clones selected from cells infected with Lv-shROR1.
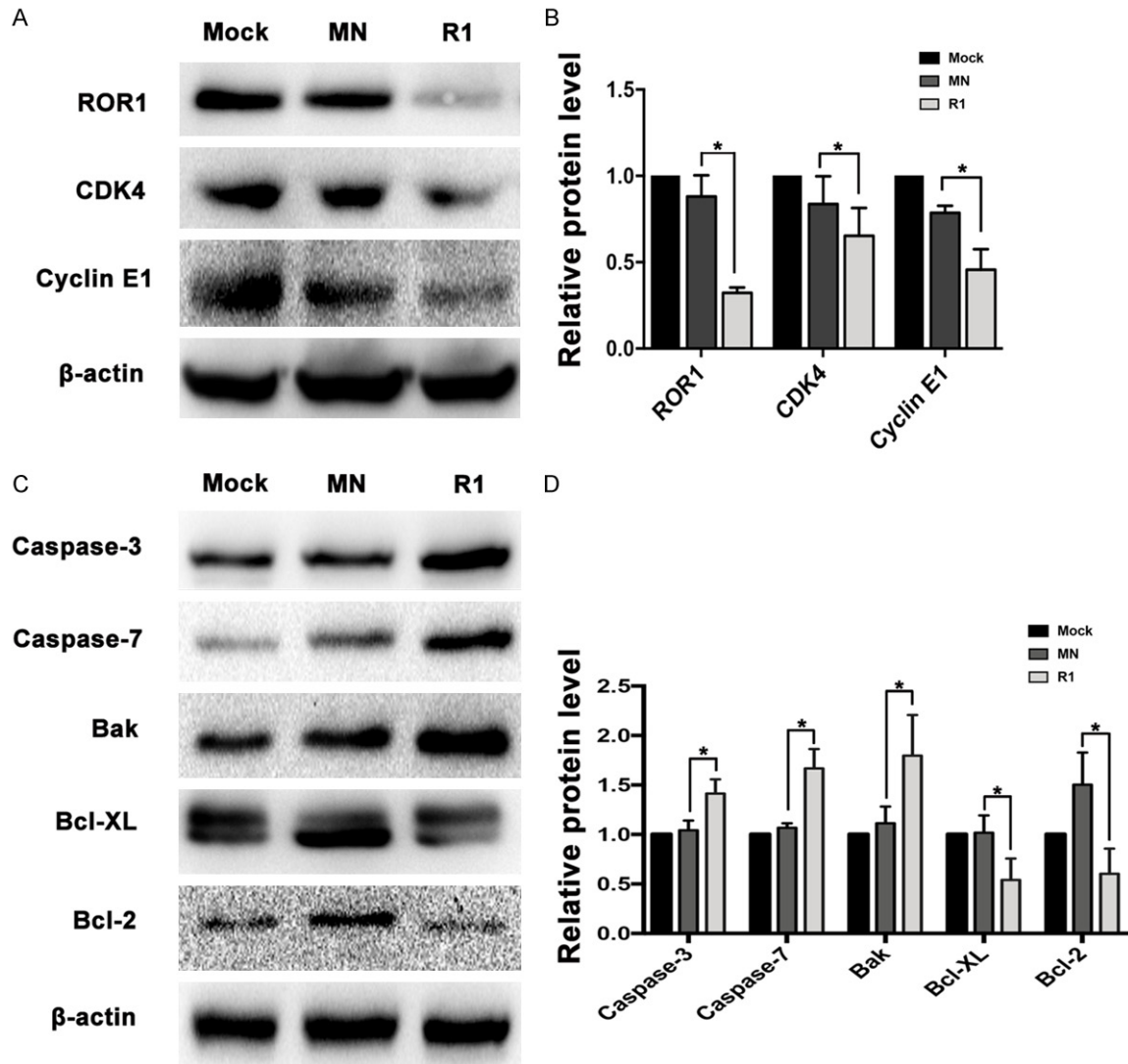
Figure 4. ROR1 regulates cell cycle- and apoptosis-related molecules. The expression levels of ROR1 and cell cycle-related molecules (A) and apoptosis-related molecules (C) and β-actin were detected by western blot. The integrated density analysis showed the changes of expression (B, D). Asterisks indicate statistically significant differences compared with the non-related shRNA group (*P<0.05). Statistical analyses were performed using Student's T test (R1 compared with MN). Mock, cells cultured with RPMI-1640 culture medium with 10% FBS alone; MN, clone selected from PC9erlo cell lines infected with Lv-shCon; R1, clone 1 selected from PC9erlo cell lines infected with Lv-shROR1.

Silencing ROR1 upregulates the expression of molecules involved in autophagy

Autophagy is an evolutionarily-conserved and genetically programmed lysosomal degradation pathway, but its role in cancer still needs to be clarified. Increasing evidence demonstrates the intimate interactions among the cell cycle, apoptosis, and autophagy. Hence, it is interesting to know whether blocking ROR1 can regulate autophagy. In this study, we screened 20 autophagy-related proteins in clone R1 from PC9erlo by a commercially available human autophagy array kit and found that several play-

ers in the autophagy process such as autophagy-related gene 7 (ATG7), ATG12, LC3A, LC3B, BNIP3L, and NBS1 were relatively upregulated after ROR1 was silenced (**Figure 5**).

Screening of ROR1-mediated phospho-kinase signaling pathways in lung adenocarcinoma cell lines

Although the downstream signal cascades of ROR1 have been reported in several groups including ours [22, 23, 28, 31], the overall screening of key phospho-kinase signaling pathways downstream of ROR1 in lung adenocar-
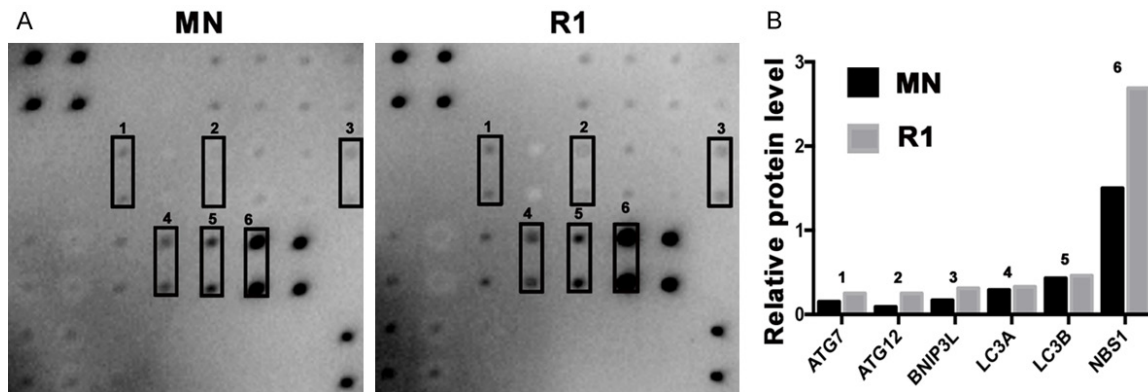
Figure 5. ROR1-silenced induces autophagy. Twenty autophagy-related proteins were screened in clone R1 from PC9erlo by a commercially available human autophagy array kit (A). The relative expression levels of autophagy-related proteins were graphed (B). MN, clone selected from PC9erlo cell lines infected with Lv-shCon; R1, clone 1 selected from PC9erlo cell lines infected with Lv-shROR1.

cinoma has not been reported yet. In this study, we use a human phospho-kinase array to simultaneously detect the relative phosphorylation levels of 43 human protein kinases in clone R1 from PC9erlo cell lines. We found that after ROR1 blocking, the phosphorylation levels of GSK-3α/β (S21/S9) and Akt123 (S473) were down-regulated (**Figure 6A**, **6B**). We further detected the activity of key molecules in the Akt/GSK-3α/β/mTOR pathway by western blot assay, and found that the phosphorylation levels of GSK-3α/β (S21/S9), Akt1/2/3 (S473), and mTOR (Ser2448) were significantly reduced after ROR1-silencing (**Figure 6C**).
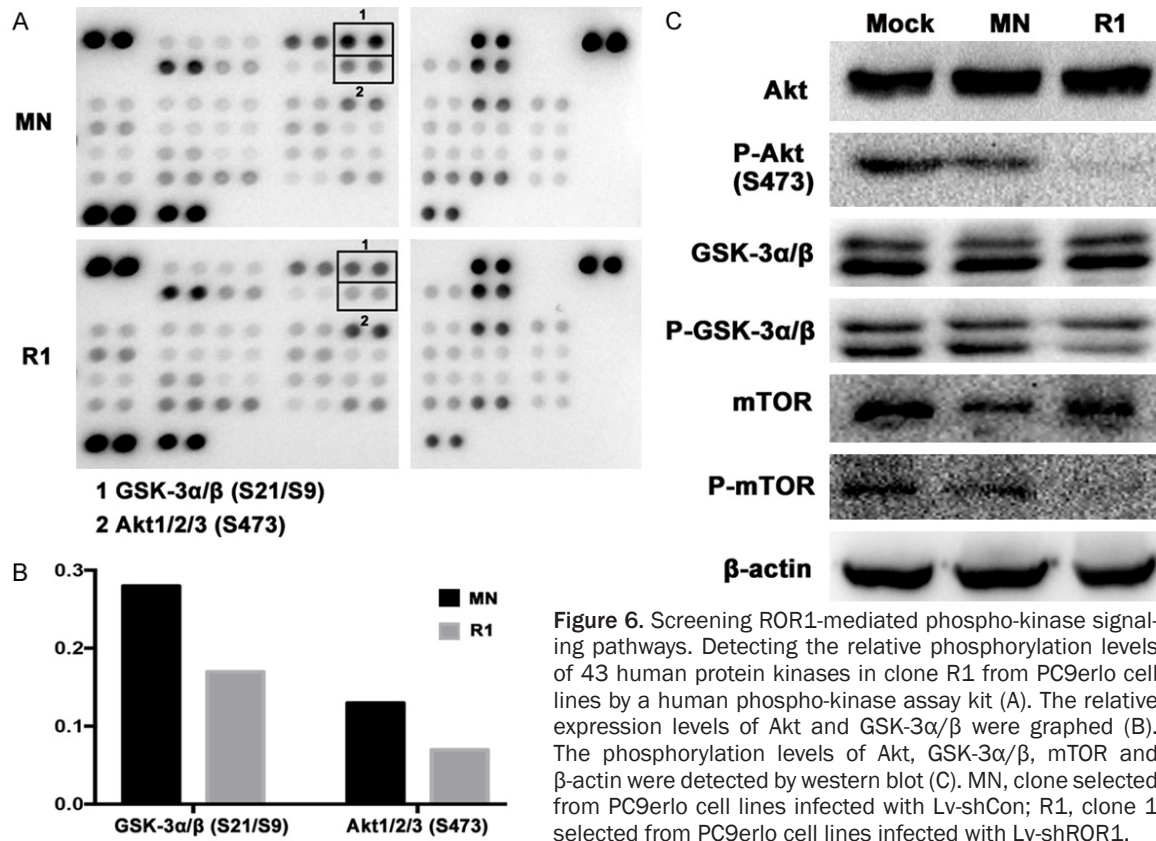
Discussion

Although it is well known that ROR1 plays important roles in tumor growth, metastasis, and drug-resistance, most of those studies employed siRNA strategy to silence ROR1 transiently, thus, not reflecting the long-term effects of ROR1 blocking in tumors [23, 28]. Therefore, in this study we used lentivirus-mediated shRNA instead of siRNA to silence ROR1 permanently and a series of ROR1-stably-silenced clones from lung adenocarcinoma cell lines PC9, PC9erlo, and NCI-H1975 were selected, identified, and used as cell models for follow-up functional study.

Our previous data have demonstrated the cell-proliferating role of ROR1 in lung adenocarcinoma, but little is known about the expression patterns of apoptotic- and cell cycle-related molecules after ROR1 blocking [23]. In this

study, the data clearly showed that pro-apoptotic molecules including Bak, Caspase-3, and Caspase-7, were significantly upregulated, while anti-apoptotic molecules including Bcl-2 and Bcl-XL, were markedly downregulated in ROR1-stably-silenced clones, which extend our previous observations and further explore the molecular mechanism of ROR1-mediated apoptosis-inhibition in lung adenocarcinoma. We also detected the expression levels of key cell cycle regulators and found that the expression levels of CDK4 and Cyclin E1 were significantly reduced after ROR1 blocking. It is well known that CDK4 associates with cyclin D along with its inhibitors. After stimulation by extracellular mitogenic stimuli, the inhibitor will be released and the active complex of CDK4/cyclin D can phosphorylate retinoblastoma protein (Rb), allowing subsequent activation of the Cyclin E/CDK2 complex [32, 33]. Cyclin E/CDK2 further phosphorylates Rb to activate G1/S-phase gene expression. Our study indicates that ROR1 enhances tumor cell proliferation partly by upregulating the activity of CDK4 and Cyclin E1 which sequentially removes the inhibitors involved in transcription of genes required for DNA replication, thereby allowing cell survival and proliferation [34].

Autophagy is a ubiquitous highly conserved pathway in eukaryotic cells whereby cytoplasmic components are catabolically degraded in lysosomes for recycling [25, 26]. Autophagy may function as a tumor-suppressive mechanism during early tumorigenesis [26], but its

Figure 6. Screening ROR1-mediated phospho-kinase signaling pathways. Detecting the relative phosphorylation levels of 43 human protein kinases in clone R1 from PC9erlo cell lines by a human phospho-kinase assay kit (A). The relative expression levels of Akt and GSK-3α/β were graphed (B). The phosphorylation levels of Akt, GSK-3α/β, mTOR and β-actin were detected by western blot (C). MN, clone selected from PC9erlo cell lines infected with Lv-shCon; R1, clone 1 selected from PC9erlo cell lines infected with Lv-shROR1.

role in advanced tumors is dependent on the context [35]. Increasing studies have suggested that autophagy, the cell cycle, and apoptosis are coordinated and reciprocally regulated. It was reported that blocking CDK4/6 by siRNA or chemical inhibitors promotes autophagy in multiple cancer cells [36, 37] and autophagy plays important roles in regulation of cell death, especially apoptosis-signaling pathways [38, 39]. For example, anti-apoptotic Bcl-2 proteins (Bcl-2, Bcl-XL) interact with Beclin-1 to impede autophagy [40-42]. As we have shown above that ROR1 upregulated the cell cycle but downregulated apoptosis, it would be interesting to know whether ROR1 participates in the process of autophagy and how it works in lung tumorigenesis and progression. In this study we first screened 20 human autophagy-related molecules by a commercially available human autophagy array, and found that autophagy-related molecules including ATG7, ATG12, BNIP3L, LC3A, LC3B and NBS1 were mildly or significantly increased in ROR1-stably-silenced cell lines. Although until now we still cannot explain the exact role of ROR1-mediated autophagy in the pathogenesis of lung cancer, its

occurrence is to a certain extent in accordance with ROR1-mediated anti-apoptotic and proliferation-enhancing roles which deserve further study.

ROR1-mediated downstream signaling cascades in tumors have been reported in several studies including ours [22, 23, 28, 31], but the overall screening of key phosphokinase signaling pathways downstream of ROR1 in lung adenocarcinoma has not been reported yet. In this study, we analyzed the signaling consequences of ROR1-silencing in PC9erlo cells with antibody-based phospho proteomics. We found that the phosphorylation levels of GSK-3α/β (S21/S9) and Akt1/2/3 (S473) were significantly decreased which was further verified in ROR1-knockout cell line PC9erlo by western blot assay. GSK-3 is a serine/threonine kinase which has two isoforms GSK-3α and GSK-3β [43]. GSK-3 was originally demonstrated to play an important role in regulating glycogen synthesis, but recent studies found that GSK-3 also regulates the cell cycle, apoptosis and autophagy [44, 45]. In our previous studies, we have shown that the Akt/mTOR signaling cas-
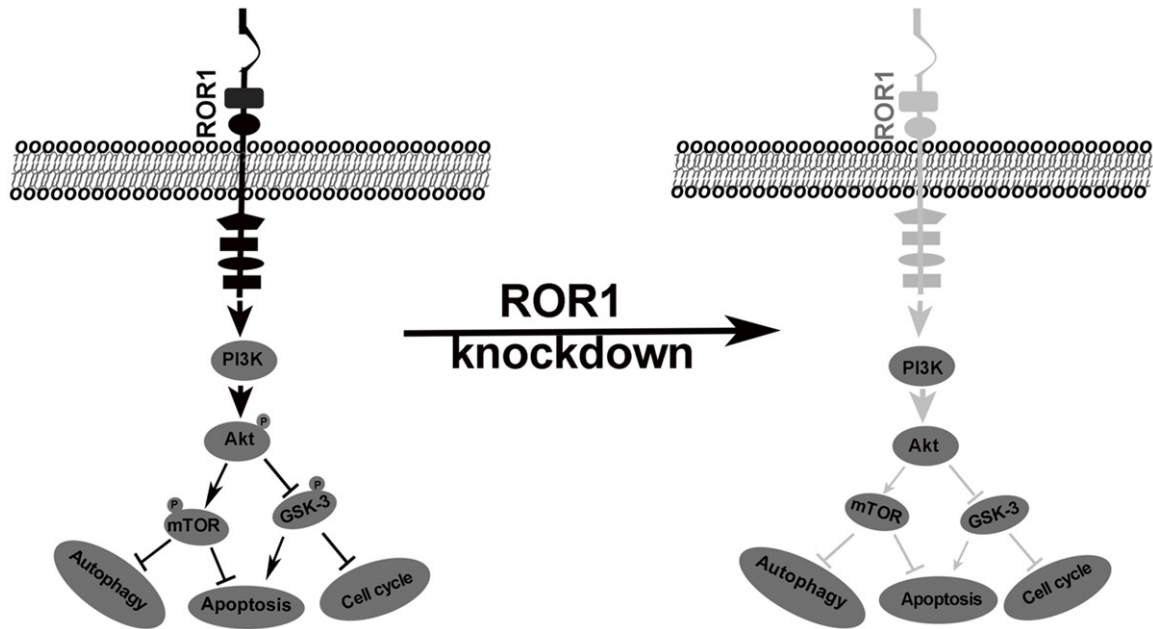
Figure 7. Schematic representation of the proposed mechanism of ROR1 in lung adenocarcinoma cell lines.

cade is one of the key pathways downstream of ROR1. It is well known that mTOR is the most extensively investigated down-regulator of autophagy [46, 47] which could partly explain the autophagy-enhancing role of ROR1-silencing in our observation. Here our data extend our previous study and further indicate that blocking ROR1 could deactivate Akt, then activate GSK-3α/β via de-phosphorylation, and finally deactivate mTOR. In this way blocking ROR1 could effectively regulate cell cycle, apoptosis, and autophagy in lung cancer as shown (**Figure 7**).

In summary, we have successfully established a batch of ROR1-stable-silenced clones with lentivirus-mediated shRNA instead of siRNA from lung adenocarcinoma cell lines PC9, PC9erlo, and NCI-H1975 which provides an effective cell model to comprehensively study the molecular mechanisms of ROR1 in lung adenocarcinoma. We found that ROR1 enhances lung adenocarcinoma growth by regulating the activity of key molecules regulating the cell cycle, apoptosis and autophagy, and that the Akt/GSK-3α/β/mTOR signaling cascade is the central pathway involved in ROR1-mediated pathogenesis which deserves further clarification and at the same time, paves a path for development of small molecules against the key molecules downstream of ROR1 in lung adenocarcinoma.

Disclosure of conflict of interest

None.

Abbreviations

ROR1, receptor tyrosine kinase-like orphan receptor 1; siRNA, small interfering RNA; shRNA, small-hairpin RNA; PBS, phosphate-buffered saline; FBS, fetal bovine serum; DMSO, dimethyl sulfoxide; MOI, multiplicity of infection; GFP, green fluorescent protein; qRT-PCR, quantitative real-time polymerase chain reaction; Akt, protein kinase B; GSK-3, glycogen synthase kinase 3; mTOR, mammalian target of rapamy-

cin; STAT3, signal transducer and activator of transcription 3; ATG, autophagy-related gene; Bcl-2, B-cell lymphoma-2; Bcl-xl, B-cell lympho-ma-extra-large; Caspase, cysteinyl aspartate specific proteinase; Bak, Bcl-2 associated k protein; CDK4, cyclin-dependent kinase 4; HRP, horseradish conjugated peroxidase; NBS1, Nij-megen breakage syndrome 1; BNIP3L, BCL2/adenovirus E1B 19 kDa protein-interacting protein 3-like; LC3, light chain 3.

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