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ORIGINAL RESEARCH

# Knockdown of USP8 Inhibits the Growth of Lung Cancer Cells

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**Purpose:** Lung cancer is the deadliest tumor in the world. This study aimed to investigate the effection of USP8 on the proliferation and growth of NSCLC cells.

**Methods:** The proliferation, migration, invasion, cell cycle progression, and apoptosis of A549 and H1299 cells were evaluated with CCK8, colony formation, scratch, transwell, and flow cytometry experiments. Furthermore, the expression of cell cycle- and apoptosis-related proteins was detected by western blot.

**Results:** Knockdown of USP8 inhibited the proliferation, migration, invasion, and cell cycle progression of A549 and H1299 cells, and promoted the apoptosis. The results of western blot indicated that knockdown of USP8 down-regulated the expression of Cyclin D1, CDK4, CDK6, p-AKT, and Bcl2, and up-regulated the expression of Bax.

**Conclusion:** Knockdown of USP8 inhibited the proliferation of human lung cancer cells by regulating cell cycle- and apoptosis-related proteins. USP8 may be a therapeutic target for lung cancer.

**Keywords:** apoptosis, lung cancer, PI3K/AKT pathway, proliferation, USP8

## Introduction

Lung cancer is the deadliest tumor in the world.[1] In the early stages of tumor, patients usually have no typical clinical symptoms, causing nearly 75% of the patients to delay treatment. Despite continuous improvements in diagnosis and treatment methods, the recovery rate of advanced lung cancer is still very low, with a 5-year survival rate of less than 15%.[2] According to relevant statistics, more than 1.1 million people succumb to lung cancer every year worldwide, of which non-small cell lung cancer (NSCLC) accounts for 85%. With the in-depth study of NSCLC, there is a new direction of NSCLC therapy, which is molecular targeted therapy.

Deubiquitinating enzymes (DUBs) mediate the reversal of protein ubiquitination, and play an important role in the development of human malignant tumors. Ubiquitin-specific proteases (USPs) are the member of the DUBS superfamily and are good targets for cancer treatment.[3,4] USP7 and USP2a have been reported to mediate the deubiquitinate of MDM2, which leads to the degradation of p53, while USP7 and USP10 can directly deubiquitinate p53 under specific conditions.[5,6] USP15 also can target MDM2, which affects the stability of both p53 and the T-cell transcription factor, NFATc.[7] USP8 has been reported to be involved in the deubiquitination and stability regulation of

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a variety of clinical cancer targets, such as CDC 25,[8] ERBB 2,[9] NRDP1, and EGFR.[11,12] In this study, we will explore the specific functions of USP8 in NSCLC.

# Materials and Methods

## Cell Culture

The cell lines A549 and H1299 (CAS, Shanghai, China) were cultured with DMEM medium contained with 10% fetal bovine serum (FBS), 0.1 mg/mL streptomycin and 100 U/mL penicillin, and incubated with 5% $CO_2$ at 37°C.

## Transfection

The cells in the logarithmic stage were replaced with a new complete culture medium before 2 h of transfection. The USP8 interfere sequence of siRNA1 was 5′-TCAAGCAACAGCAGGATTA-3′; siRNA2 was 5′-CACTGGAACCTTTCGTTAT-3′. 5 μL lipofectamine 6,000 (TMO, Waltham, MA, USA) was added to 125 μL serum-free medium, and 5 μL siRNA1 or siRNA2 was dissolved into another 125 μL medium at room temperature. Mix and let stand for 20 min at room temperature. Then, the liposome mixture was mixed with the siRNA mixture and added to the cells in the 6-well-plate for 24 h. Then, the medium was changed, and subsequent experiments were carried out after 24 h. The NC group was transfected with negative control siRNA, and the interfere sequence was 5′-TTCTCCGAACGTGTCACGT -3′.

## Fluorescence Quantitative PCR

The Ultrapure RNA Kit (CWBIO, Beijing, China) was used to extract total RNA from cells. The Reverse Transcription Reaction Kit (CWBIO) was used to synthesize cDNA. On an H-4800 Real-Time PCR System, the real-time fluorescence quantitative PCR was carried out to detect the expression of USP8 with the primers (Genewiz Company, Beijing, China) of USP8 (5′-CTGCTGTGGCTTCAGTTCCT-3′ and 5′-GGAAATAATCCTGCTGTTGCTTGA-3′). The PCR thermal cycling conditions were as follows: 40 cycles of 5 min at 95°C, 30 s at 95°C and 45 s at 60°C with a final extension 72°C for 30 min. The expression of USP8 was calculated by the $2^{-\Delta CT}$ method.

## Western Blot

After 48 h of transfection, RIPA lysate (CwBio, Beijing, China) was added to extract protein, and BCA kit (CwBio, Beijing, China) was used to determine the protein concentration. Then, the cell lysate was heated at 95°C for 5 min. SDS polyacrylamide gel electrophoresis was used to separate protein samples. 20 μL protein samples were added into the vertical electrophoresis tank, then transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with 5% skimmed milk at room temperature for 1 h, and incubated at 4°C with a primary antibody overnight, and then incubated with a secondary antibody for 1 h. After flushing, the membrane was developed with ECL developer (B500024, Proteintech, Chicago, IL, USA). The gray value was scanned by quality one software, and GAPDH was used as an internal reference control. The relative protein expression levels were calculated by objective protein/internal reference.

Antibodies, including anti-USP8 (27,791-1-AP, 1:1,000), CDK4 (11,026-1-AP, 1:1,000), CDK6 (14,052-1-AP, 1:1,000), anti-GAPDH (10,494–1-AP, 1:5,000), and HRP sheep anti-rabbit/mouse (1:5,000) were ordered from PTG Company (Bellevue, WA, USA); anti-Bcl-2 (ab32124, 1:1,000), Bax (ab32503, 1:1,000), Cyclin D1 (ab134175, 1:1,000) were ordered from Abcam (Cambridge, United Kingdom).

## CCK8 Assays

After transfected with 24 h, the cells were trypsinized. Approximately 2,000 cells were seeded into each well of 96-well-plate. 10 μL CCK8 reagent was added into each well every 24 h, and the absorbance (OD) value of each well at 450 nm was detected. Then, the proliferation curves were drawn.

## Scratch Test

The migration of A549 and H1299 cells was analyzed by scratch test. After digestion, the cells were plated in the 6-well plates, and cultured overnight to make the cells confluent. A sterile pipette tip was used to make scratches in the cell layer, and the sloughed cells were washed off with PBS. The cell migration was recorded every 24 h. Image J was used to process the results and the

migration area of the cells in the NC group and experimental groups were compared.

## Transwell Assay

The invasion of A549 and H1299 cells was detected by Matrigel-coated Transwell chamber (Millipore, Billerica, MA, USA). A total of $5 \times 10^4$ cells were added to the upper chamber, and 500 μL complete medium was added to the lower chamber. After 24 h, the upper chamber cells were washed with PBS and wiped clean with a cotton swab. The invaded cells on the membrane were fixed with 4% paraformaldehyde for 30 min and stained with 0.1% crystal violet for 20 min. Then, count and take pictures under a microscope.

## Cell Apoptosis and Cycle Flow Cytometry

The apoptosis was evaluated with the flow cytometric analysis. After 24 h of transfection, the complete medium was replaced with FBS-free medium to starve the cells. After starving for 24 h, collect the cells and adjust the cell density to $1–5 \times 10^6$ cells/mL. 100 μL cell suspension and 5 μL of annexin V/FITC was added into 5 mL flow tube, and incubate at room temperature in dark for 5 min. Then, on-board detection was carried out after added 10 μL PI dye and 400 μL PBS. The flow Jo software analyzed and processed the flow results.

The cell cycle was detected after being immobilized in ice ethanol for 24 h. The subsequent treatment was the same as apoptosis detection. The cells were stained with PI. The flow Jo software was used to analyze and process the flow results.

## Statistical Analysis

All data were evaluated by SPSS 18.0 software. The Student's *t*-test was used for comparison between the two groups. P<0.05 was statistically significant.

# Results

## Knockdown of USP8 Inhibits the Proliferation and Growth of Lung Cancer Cells

To explore the role of USP8 in lung cancer cells, we first used RNA interference technology to knock down the expression of USP8 in A549 and H1299 cells. As shown in Figure 1A and B, the transfection of siRNA1 and siRNA2 significantly reduced the mRNA and protein expression of USP8 in the cells (P<0.05). Furthermore, the results of CCK8 and colony formation assays showed that USP8 knockdown significantly inhibited NSCLC cell proliferation and growth. As shown by CCK8 assay, 48 h after siRNA1 or siRNA2 transfection, the OD value of siRNA1 and siRNA2 group was significantly lower than that of the NC group (P<0.05, Figure 1C and D). In addition, colony formation experiments showed that the formed clone numbers of the siRNA transfection group were significantly less than that of the NC group (P < 0.05, Figure 1E and F).

## Knockdown of USP8 Inhibits the Migration and Invasion of Lung Cancer Cells

Subsequently, we tested the effects of USP8 knockdown on the migration and invasion of NSCLC cells through scratch and tranwell experiments. The results showed that the healing area of siRNA1 and siRNA2 groups was significantly lower than that of the NC group both in A549 and H1299 cells ($P < 0.05$, Figure 2A and B). The results of the transwell assays showed that the number of invasive cells of A549 and H1299 cells in the siRNA1 and siRNA2 groups decreased significantly compared with the NC group ($P < 0.05$, Figure 2C and D).

## Knockdown of USP8 Arrests the Cell Cycle Progression of Lung Cancer Cells

The function of USP8 on cell cycle was evaluated by flow cytometric analysis and western blot. As shown in Figure 3A, the transfection of siRNA1 and siRNA2 affected the cell cycle of both A549 and H1299 cells compared with the NC group. Compared with the NC group, the number of cells in the G1-phase of the siRNA1 and siRNA2 groups was significantly increased, while the number of cells in the S-phase was significantly reduced. The expression of Cyclin D1 increases in the early stage of cell cycle. It combines with CDK4 or CDK6 in cells to form Cyclin D1/CDK4 or Cyclin D1/CDK6 complex, which is an important regulatory factor from G1-phase to

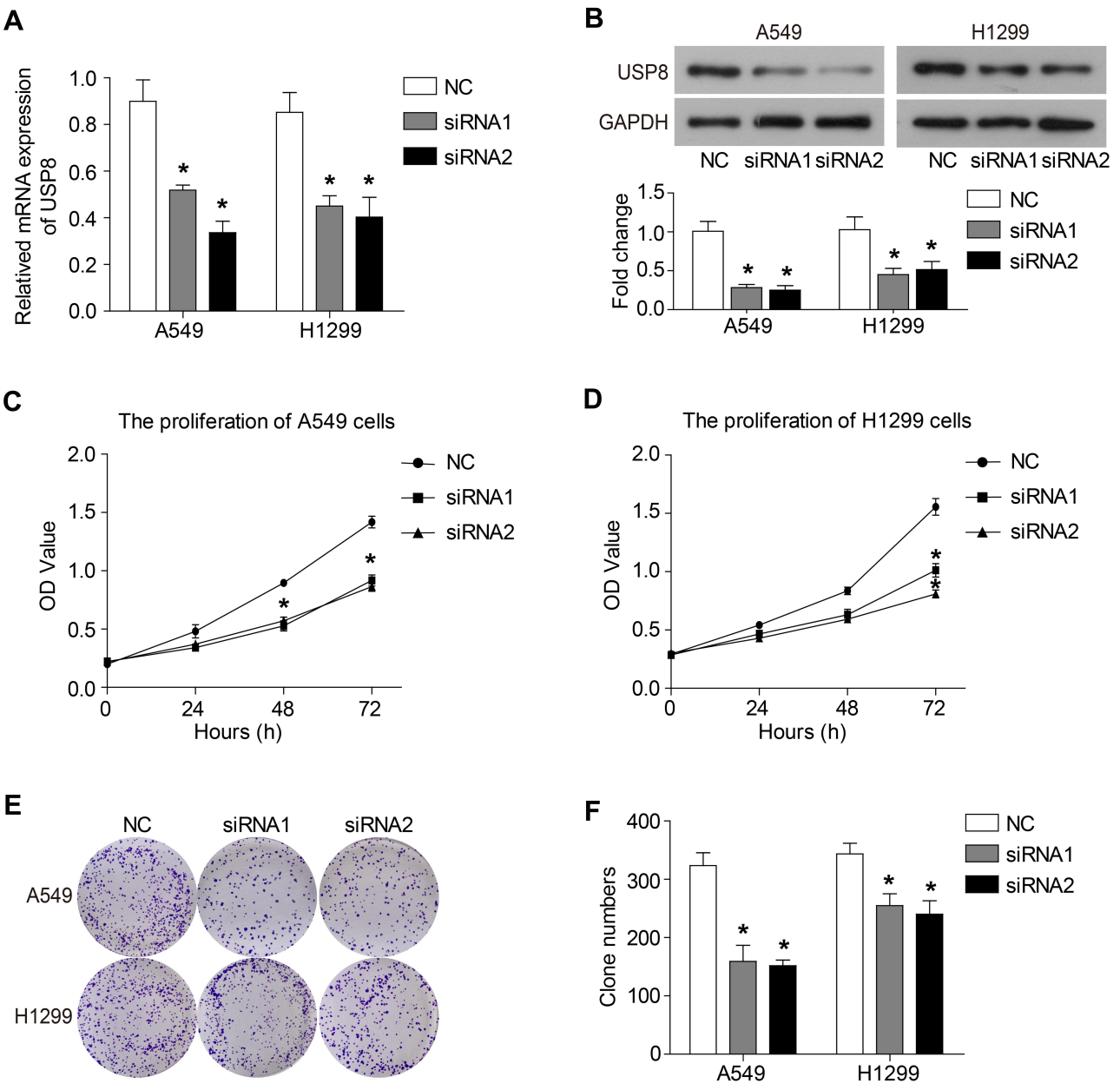


**Figure 1** Knockdown of USP8 inhibited the proliferation of lung cancer cells.

**Notes:** USP8 expression was analyzed by using (**A**) qPCR and (**B**) Wwestern blot in lung cancer cell lines A549 and H1299. The proliferation curves of (**C**) A549 and (**D**) H1299 cells transfected with USP8-specific siRNA were detected by using CCK8. (**E** and **F**) The formed clone numbers of the siRNA transfection groups and NC group wereby using colony formation experiments. Experiments in this figure were all performed in triplicate (*P<0.05).

S-phase.[16] The results of western blot showed that siRNA1 and siRNA2 transfection could significantly down-regulate the expression of cyclin D1, CDK4, and CDK6 both in A549 and H1299 cells (Figure 3B). These results indicated that knockdown of USP8 arrested the cell cycle progression, thereby inhibiting cell proliferation.

## Knockdown of USP8 Promotes the Apoptosis of Lung Cancer Cells

The apoptosis was estimated with flow cytometry, by double-staining with Annexin V and PI. As shown in Figure 4A, the apoptosis rate was significantly increased by transfection of siRNA1 and siRNA2, compared with

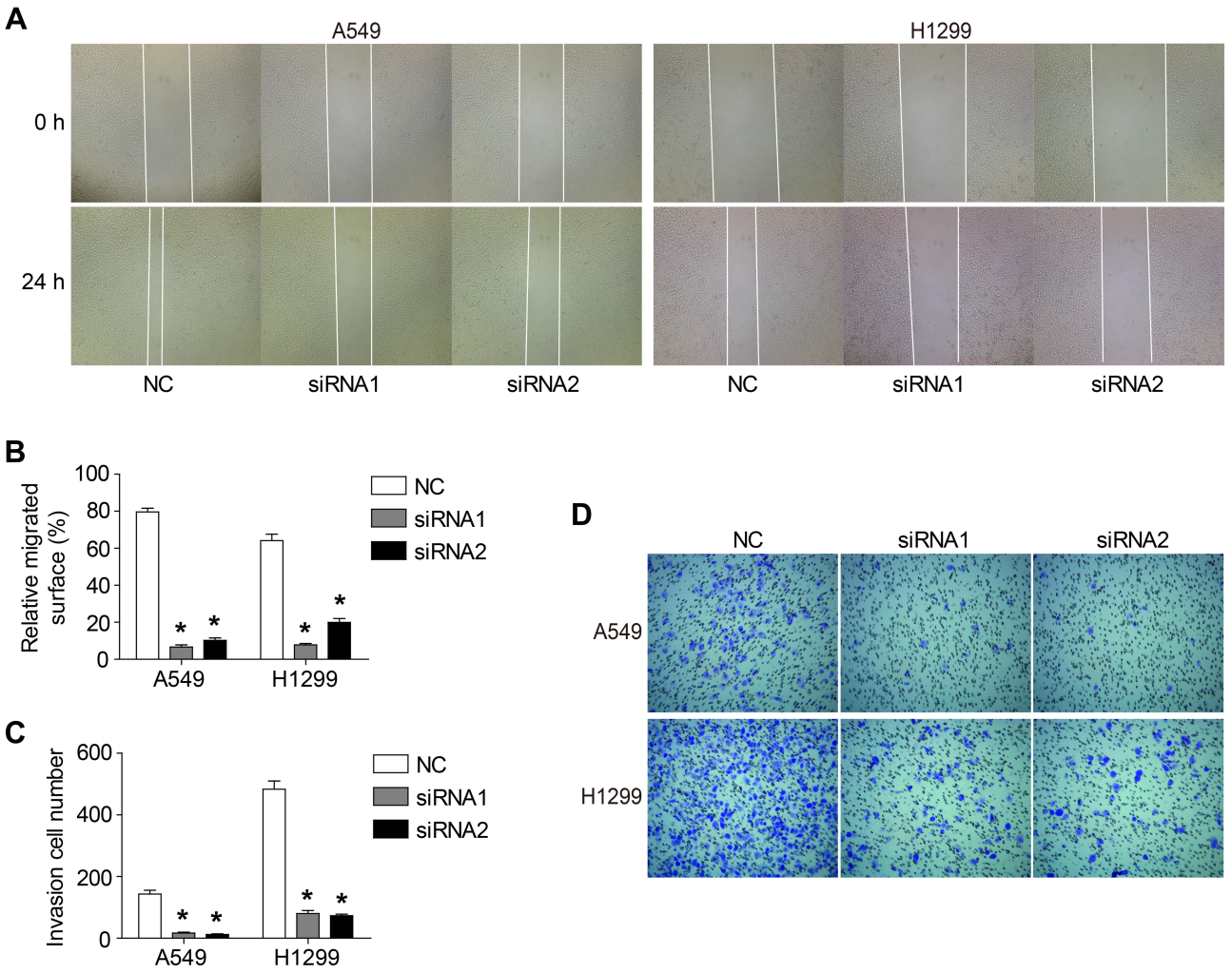


**Figure 2** Knockdown of USP8 inhibited the migration and invasion of lung cancer cells.
**Notes:** Knockdown of USP8 inhibited the migration (**A** and **B**) and invasion (**B** and **C**) of A549 and H1299 cells. Experiments in this figure were all performed in triplicate (*P<0.05).

the NC group. Western blot was used to analyze the expression of apoptosis-related proteins. Anti-apoptotic Bcl2 and pro-apoptotic Bax are the key factors that initiate apoptosis through mitochondrial. After transfection with siRNA1 or siRNA2, the expression of Bcl2 was reduced, while the expression of Bax was increased significantly both in A549 and H1299 cells (Figure 4B), indicating that knockdown of USP8 can induce apoptosis of lung cancer cells. Furthermore, we analyzed the phosphorylation level of AKT by western blot. The results showed that USP8 knockdown inhibited the ratio of p-AKT/AKT in A549 and H1299 cells (Figure 4B). These results demonstrated that knockdown of USP8 promoted cell apoptosis of lung cancer cells by regulating the PI3K/AKT pathway.

## Discussion

Lung cancer has become a serious life-threatening disease. In this study, we analyzed the effects of USP8 expression on the biological functions of lung cancer cells. The data showed that knockdown of USP8 inhibited the proliferation and promoted the apoptosis of lung cancer cells. Kim et al report that USP8 can specifically bind to stratifin (SFN) protein in lung adenocarcinoma cells, and knockdown of USP8 or SFN gene leads to down-regulation of tumor cell proliferation and up-regulation of apoptosis.[13]

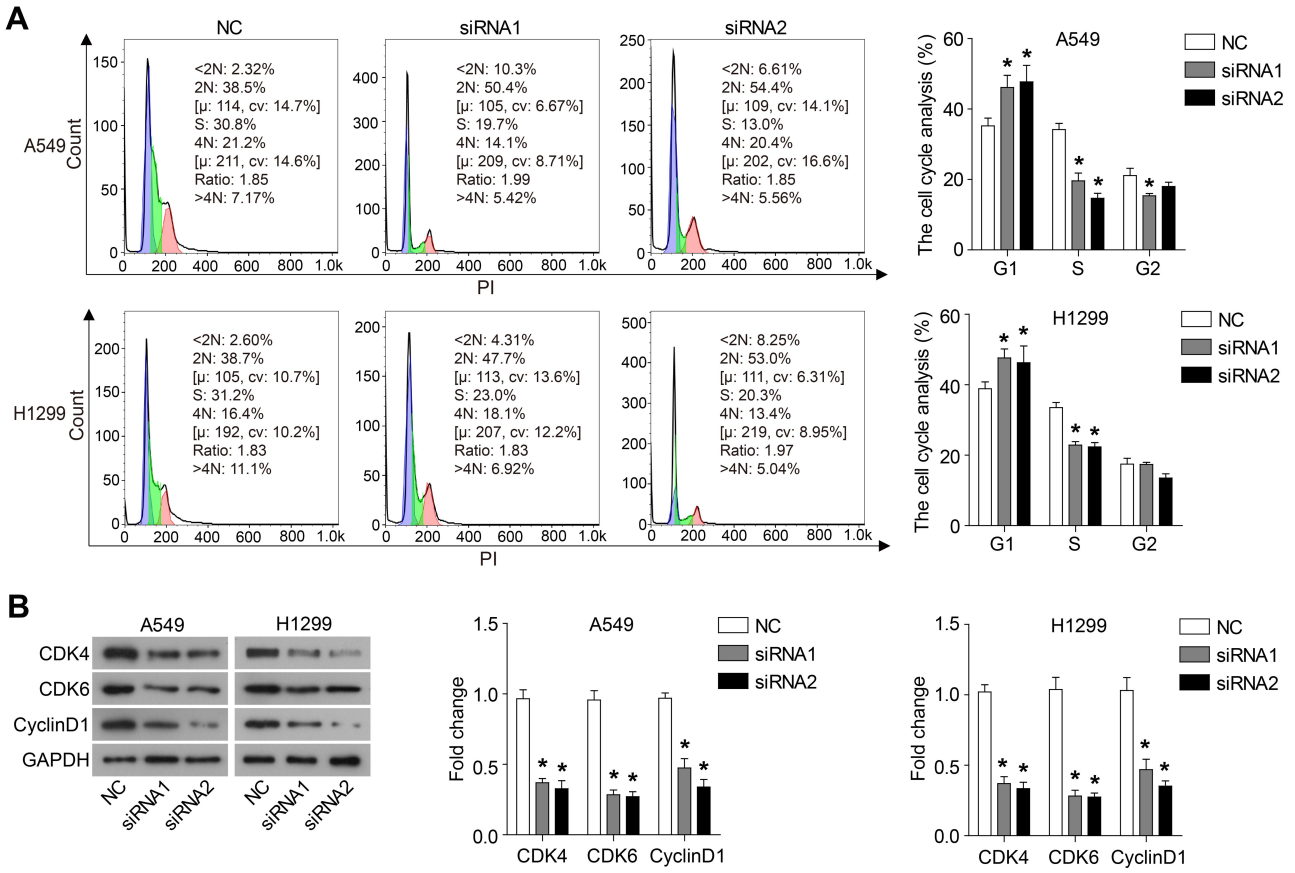


**Figure 3** Knockdown of USP8 inhibited the cell cycle progression of lung cancer cells.
**Notes:** (**A**) Flow cytometric analysis was performed to estimate the cell cycle of A549 and H1299 cells. (**B**) The expression of cycle-related protein was analyzed by Western blot. Experiments in this figure were all performed in triplicate (*P<0.05).

Our research results are basically the same. In addition, studies have shown that USP8 knockdown or inhibitors can significantly reduce the viability of gefitinib-resistant and -sensitive NSCLC cells by reducing the expression of receptor tyrosine kinase (RTK).[14,15]

Baykara et al find that the serum USP8 level in NSCLC patients was higher than in healthy individuals.[16] In addition, Sippl et al demonstrate that USP8 is elevated in melanoma and cervical cancers.[17] However, USP8 is down-regulated in breast cancer tissues, compared with that in normal breast tissues, and the high-expression of USP8 is correlated with better clinical features of breast cancer patients.[18] The reasons for the difference among these studies remained to be elucidated. The expression difference of USP8 in NSCLC tissues and normal tissues, as well as the correlation analysis with clinical characteristics of NSCLC patients, are also our future research directions.

One research reports that the inhibition of USP8 down-regulated the Notch signaling pathway via Notch1 intracellular domain (NICD) destabilization, resulting in the retardation of cellular growth, wound closure, and colony-forming ability of breast cancer cell lines.[19] USPs can release bound ubiquitin from target proteins with a high degree of specificity, and participate in a variety of human diseases including cancer, and are rapidly becoming promising targets for drug design.[3,20]

Most importantly, in this study, we discovered the influence of USP8 on cell cycle progression and related proteins. In cell cycle regulation, the regulation point between G-phase and S-phase is the key point of intracellular and extracellular signal transmission, integration, and aggregation to cell nucleus cell proliferation regulation.[21] The normal operation of the cell cycle is regulated by the cell cycle regulatory system, which is composed of cyclin, CDK, and CKI.[22,23] Cyclin D1 is an important regulatory factor from G1-phase to S-phase. Flow cytometry demonstrated that knockdown of USP8 could block A549 and H1299 cells in G1-phase. The results of western blot showed that

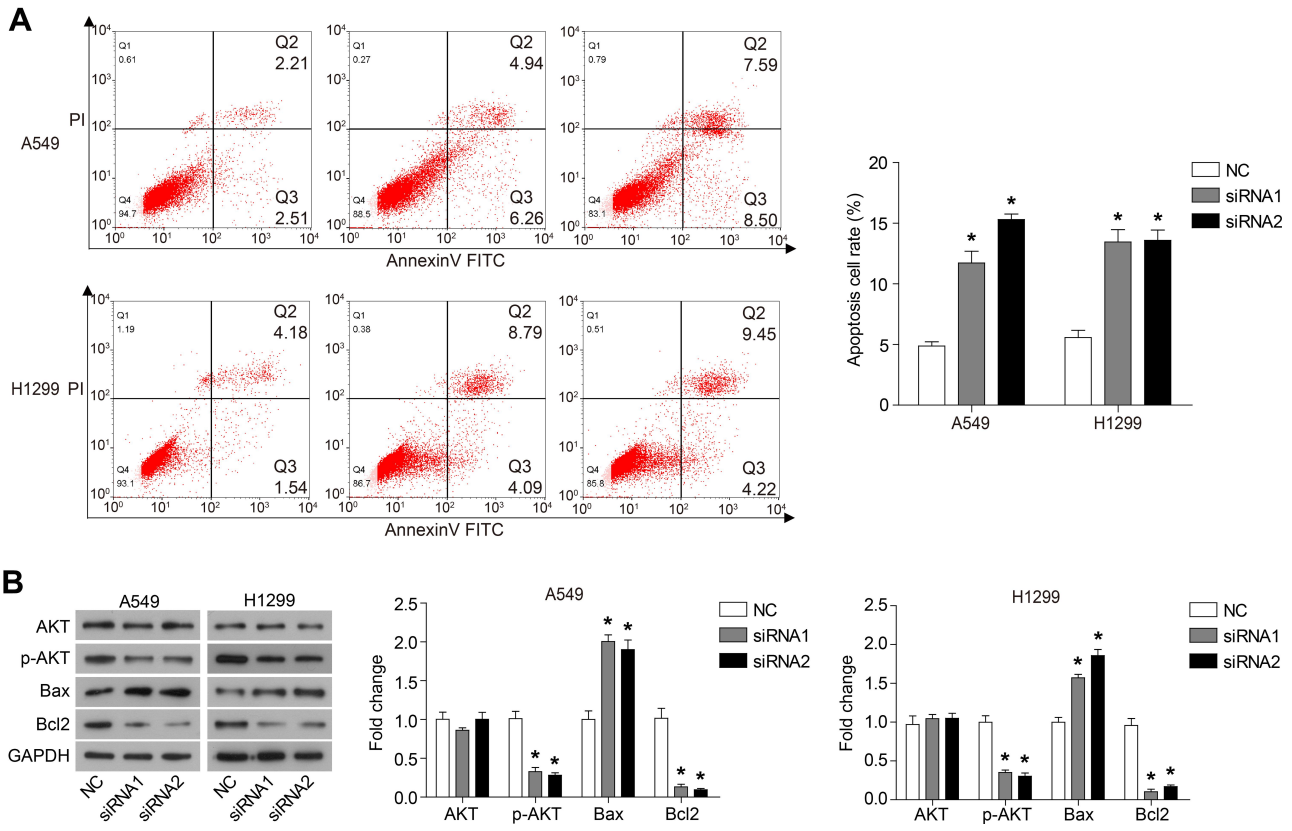


**Figure 4** Knockdown of USP8 induced apoptosis of lung cancer cells.
**Notes:** (**A**) Knockdown of USP8 promoted the apoptosis of A549 and H1299 cells. (**B**) Western blot was performed to analyze the expression of apoptosis-related protein. Experiments in this figure were all performed in triplicate (*P<0.05).

knockdown of USP8 down-regulated the expression of cyclin D1, CDK4, and CDK6. These results indicated that knockdown of USP8 arrested the cell cycle progression from G1-phase to S-phase. Naviglio et al demonstrated that inhibition of USP8 accumulation by antisense plasmid microinjection prevented fibroblasts from entering S-phase in response to serum stimulation.[24] Moreover, the key protein of PI3K/AKT pathway was analyzed by western blot. Knockdown of USP8 down-regulated the expression level of p-AKT, indicating that USP8 knockdown inhibited the cell proliferation by inhibiting the PI3K/AKT pathway.

## Conclusion

Knockdown of USP8 inhibited the proliferation of human lung cancer cells by regulating cyclin- and apoptosis-related proteins.

## Ethical Approval

Not applicable.

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This study did not receive any funding.

## Disclosure

All authors declare that they have no conflicts of interest.

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