

# Ssrp1 Promotes Lung Cancer Progression By Blocking The Wnt Pathway and Is Negatively Regulated By Mirna-28-5p

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## Research

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# Abstract

**Background:** Structure-specific recognition protein 1 (SSRP1) plays important roles in the development of various tumors. Numerous reports have described the effects of microRNAs (miRNAs) on lung cancer apoptosis, metastasis and proliferation. However, the relationship between SSRP1 and miRNAs in the development of lung cancer remains unclear. Therefore, the purpose of our study was to explore the functions of SSRP1 in the occurrence and development of lung cancer.

**Methods:** First, we analyzed the expression of SSRP1 in human lung cancer tissues and normal tissues, and the relationship between SSRP1 gene expression and overall survival through the UALCAN and GEPIA browsers. Second, we conducted experiments *in vivo* and *in vitro* to demonstrate the roles and mechanisms of SSRP1 in the occurrence and development of lung cancer and its relationship with miR-28-5p.

**Results:** Our study demonstrated over-expression of SSRP1 in tissue sections from patients with lung cancer and in lung cancer cell lines, as validated by bioinformatics analysis. The over-expression of SSRP1 was found to be associated with lung cancer development and low overall survival rates. Silencing of SSRP1 by siRNA inhibited lung cancer proliferation, migration and invasion by blocking the WNT signaling pathway *in vitro* and *in vivo*. We also verified SSRP1 was negatively regulated by *miR-28-5p* as predicted by a variety of miRNA-related databases. Further studies showed that *miR-28-5p* mediated suppression of SSRP1 inhibited lung cancer cell proliferation.

**Conclusion:** Therefore, our data suggested that SSRP1 promotes lung cancer progression by blocking the WNT pathway and is negatively regulated by *miRNA-28-5p*.

## Background

Lung cancer has the highest morbidity and mortality rate among cancers in China and worldwide [1], and severely endangers human health. In early phases, lung cancer is mostly asymptomatic, and most patients are in advanced stages when they are diagnosed, thus resulting in a 5-year overall survival rate of only approximately 16% [2]. The mechanisms underlying lung cancer are complicated, and the exact molecular mechanisms are not completely clear [3-5]. Therefore, exploring the molecular mechanisms underlying the occurrence, development, invasion and metastasis of lung cancer, and providing novel ideas and new targets for further treatment of lung cancer has become a research hotspot.

Structure-specific recognition protein-1 (SSRP1) is a subunit of the facilitates chromatin transcription (FACT) complex, which is involved in DNA replication, transcription and repair. SSRP1 is associated with the cell differentiation stage. SSRP1 is highly expressed in highly proliferative and undifferentiated cells [6]. The expression of SSRP1 is significantly higher in multiple types of human tumor cells than in normal cells, including non-small lung cancer, gliomas, osteoblasts and hepatocellular cancer [7-10]. In several cancers, high expression of SSRP1 is associated with high-grade, less-differentiated and metastasized tumors, and SSRP1 has consequently been considered a potential marker of poor prognosis and an anti-cancer target for inhibiting tumors [11, 12]. Our previous study has also found that *SSRP1* knockdown

significantly inhibits proliferation, invasion and migration, and promotes apoptosis in colorectal tumors [13]. Josephine et al. have reported that inhibition of SSRP1 decreases resistance to Erlotinib in non-small cell lung cancer [7]. The crucial role of SSRP1 in tumors prompted us to determine whether SSRP1 might participate in the development of lung cancer.

MicroRNAs (miRNAs) are 18–25 nt endogenous non-coding RNAs that down-regulate gene expression by binding the 3' untranslated regions (UTRs) of target mRNAs and subsequently inhibiting translation [14]. MiRNAs participate in cellular processes including proliferation, apoptosis and tissue morphogenesis [15, 16]. Many miRNAs have been shown to regulate the expression of genes with tumor-associated functions, and to play important roles in promoting or inhibiting tumorigenesis [17]. *MicroRNA-28-5p* (*miR-28-5p*) has been verified to have tumor suppressor activity in several types of tumors, such as hepatocellular carcinoma, prostate cancer and natural killer/T-cell lymphoma [18-20]. Moreover, Cheng Wang et al. have found that *miR-28-5p* suppresses human renal carcinoma cell lines' proliferation and migration [21]. Liang Ma et al. have shown that *miR-28-5p* inhibits the migration of breast cancer cells [22]. Studies have shown that the miR-28-5p/CAMTAN2 axis regulates colorectal cancer progression through the WNT/ $\beta$ -catenin signaling pathway [23], and SSRP1 is regulated negatively by *miR-28-5p* [24]. However, the relationship and role of *miR-28-5p*, SSRP1 and WNT/ $\beta$ -catenin signaling in the development of lung cancer were not elucidated. In this study, we aimed to explore the expression and the detailed functions and channels of SSRP1 in the proliferation, migration and invasion of lung cancer, and to identify the upstream miRNA regulating SSRP1 in lung cancer, to assess whether SSRP1 might be used as a criterion for the diagnosis and prognosis of lung cancer patients.

## Materials And Methods

### Bioinformatics

The analysis of bioinformatic data on the expression level of SSRP1 in human lung cancer and corresponding normal tissues and the relationship between SSRP1 gene expression and overall survival rate in patients with lung cancer were assessed through the UALCAN (<http://ualcan.path.uab.edu/index.html>) and GEPIA (<http://gepia.cancer-pku.cn/index.html>) browsers.

### Cell culture and human tissue specimens

The human lung cancer cell lines A549, NCI-H446, H1299 and PC9, murine cells (LLC) and the normal human lung epithelial cell B2B were obtained from the Department of Respiratory Medicine, First Hospital of Jilin University (Changchun, Jilin, China) and Basic Medical School of Jilin University (Changchun, Jilin, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 5.5 mmol/l D-glucose and supplemented with 10% FBS (BI, Israel), 100 U/ml penicillin and 100 mg/ml streptomycin. The cells were cultured in a 37 °C incubator with 5% CO<sub>2</sub>. Five tissue samples from lung cancer patients (mean age, 58.2±6.85 years, ranging from 49 to 68; three female patients and two male patients) were collected at the First Hospital of Jilin University

from September 2018 to May 2019. Patients receiving radiotherapy or chemotherapy were excluded from our study. Patients whose tissue samples were used in this research had provided written informed consent under an IRB-approved protocol. This study was approved by the local institutional review board of the First Hospital of Jilin University. The study methodologies conformed to the standards set by the Declaration of Helsinki.

### **Transient transfection**

The siRNA was purchased from Genepharma (Suzhou, China). The sequences of siRNAs were as follows: siSSRP1-1, sense, 5'-GCCAUGUCUACAAGUAUGATT-3' and antisense, 5'-UCAUACUUGUAGACAUGGCTT-3'; siSSRP1-2, sense, 5'-CCCAGAAUGGUGUUGUCAATT-3' and antisense, 5'-UUUGACAACACAUUCUGGGTT-3'; and negative control, sense, 5'-CACGCAGAACGTGAACACC-3' and antisense, 5'-GGCAGTAGATAACGTGAGGGA-3'. NCI-H446 and H1299 cells were transfected with siRNA in 96-well plates or 6-well plates for 48–72 h. The Interferin transfection agent (Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used according to the manufacturer's protocol.

### **Cell viability, proliferation and colony formation assays**

NCI-H446 and H1299 cells were plated on 96-well plates at a concentration of  $1 \times 10^4$  cells/well to determine cell viability with a Cell Counting Kit-8 (CKK-8; MCE, New Jersey, USA) at 24, 48 and 72 h after transfection. The OD450 was measured with a FLUO star Omega reader (BMG LABTECH, Ortenberg, Germany). Cell growth was assessed by counting at 24, 48, and 72 h after transfection at a concentration of  $5 \times 10^4$  cells/well in six-well plates. A total of 100 cells/well were cultured in six-well plates for colony formation assays. At 5–7 days after transfection, the colonies were fixed with 4% paraformaldehyde and then dyed with 0.2% crystal violet. Finally, colony formation was measured with an IX71 inverted fluorescence microscope (Olympus, Shinjuku, Tokyo, Japan).

### **Cell cycle analyses**

Cells were collected with 0.25% trypsin and washed twice with cold PBS, then fixed with 70%–75% frozen ethanol at  $-20^\circ\text{C}$  for 1 h or fixed overnight at  $4^\circ\text{C}$ . Subsequently, cells were washed once with cold PBS and then resuspended in 200–500  $\mu\text{l}$  cold PBS. Then, 20  $\mu\text{l}$  RNase A solution was added, and samples were incubated in a  $37^\circ\text{C}$  water bath for 30 min. Finally, 400  $\mu\text{l}$  PI stain solution (BestBio, Shanghai, China) was mixed gently and incubated for 30–60 min at  $4^\circ\text{C}$  in the dark. The results were detected with an Accuri C6 flow cytometer (Becton Dickinson, Franklin Lakes, New Jersey, USA).

### **Apoptosis analyses**

NCI-H446 and H1299 cells were plated on six-well plates at a concentration of  $1 \times 10^5$  cells/well. Transfection experiments were performed the following day. Cells were harvested after 48 h and washed twice with PBS. Cells were resuspended in 400  $\mu\text{l}$   $1\times$  buffer, then stained with 5  $\mu\text{l}$  Annexin-V-FITC and 5  $\mu\text{l}$  PI (50  $\mu\text{g}/\text{ml}$ ) with an Annexin V FITC Apop Dtec Kit (BD Biosciences, San Jose, CA, USA) in the dark for

15 min at room temperature, and detected with an Accuri C6 flow cytometer (Becton Dickinson, Franklin Lakes, New Jersey, USA).

### **Cell migration and invasion assays**

Cell migration assays were conducted with Transwell chambers (Corning Costar, Cambridge, MA, USA) with an 8-mm pore size. The lower chamber was filled with DMEM containing 20% FBS, and the upper chamber contained cells resuspended in serum-free DMEM, at a concentration of  $5 \times 10^4$  cells per well. After cultivation under 5% CO<sub>2</sub> for 48 h at 37 °C, the bottom surfaces of the polycarbonate membranes in the upper chamber were wiped with cotton swabs to remove residual cells, and cells were counted visually under an IX71 inverted fluorescence microscope (Olympus, Shinjuku, Tokyo, Japan) after staining with 0.1% crystal violet dye. Matrigel (BD Biosciences, San Jose, CA, USA) was also used in the Transwell chambers before cells were cultured in the invasion assays. Cell migration and invasion were determined by counting five random fields under an optical microscope. The data are presented as mean  $\pm$  standard deviation.

### **Western blot analyses**

Cells and tissues were harvested and lysed in RIPA buffer for 30 min at 4–8 °C. Proteins were detected and quantified with a BCA protein assay kit (Thermo Fisher Scientific, Inc., MA, USA). Protein samples (30–50  $\mu$ g) were separated with 12% SDS-PAGE and transferred to polyvinylidene fluoride membranes. Nonspecific binding was blocked with 5% low-fat milk with Tween-20 for 2 h at room temperature. Subsequently, the membranes were incubated overnight at 4 °C with the following primary antibodies: anti-SSRP1 (Proteintech, Chicago, IL, USA), anti-p21 (Proteintech, Chicago, IL, USA), anti-CyclinD1 (Proteintech, Chicago, IL, USA), anti-GSK3 $\beta$  (Proteintech, Chicago, IL, USA), anti-p-GSK3 $\beta$  (Ser9) (Proteintech, Chicago, IL, USA), anti- $\beta$ -catenin (Proteintech, Chicago, IL, USA), anti-Lamin B1 (Proteintech, Chicago, IL, USA), anti- $\beta$ -actin (Proteintech, Chicago, IL, USA) and anti-cleaved caspase3 (Cell Signaling Technology, Beverly, Massachusetts, USA). Lamin B1, encoded by the LMNB1 gene, is composed of a two-dimensional matrix of proteins located next to the inner nuclear membrane, which has been highly conserved during evolution. Therefore, Lamin B1 is often used as a nuclear protein internal control. The membranes were scanned for statistical analysis by enhanced chemiluminescence with a gel image processing system (Tanon, Shanghai, China).

### **Nuclear extraction**

A nuclear and cytoplasmic protein extraction kit was purchased from Beyotime (Beyotime Biotechnology Inc., Nantong, China). Specific experimental steps were performed according to the manufacturer's protocol.

### **Animal experiments**

The study was approved by the Animal Research Ethics Committee of Jilin University. The siRNA used *in vivo* was synthesized by Genepharma (Suzhou, China) and dissolved in PBS buffer. The dose of siRNA in nude mice was 0.5 mg/kg.

Ten BALB/c male nude mice (18–22 g) were purchased from Beijing Vital River Laboratory Animal Technology and housed under a 12/12 h light/dark cycle in an air-conditioned room at  $22 \pm 2$  °C, and were given free access to food and water. All animal experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Scientific Investigation Board of the College of Basic Medicine, Jilin University. Ten nude mice were randomly divided into two groups equally (five mice per group). A 100  $\mu$ l subcutaneous injection containing  $1 \times 10^6$  NCI-H446 cells was delivered into the upper right back in mice in each group. When the tumor size reached 3–5 mm, siSSRP1 or negative control (NC, used with isodose PBS) was inoculated into the xenograft tumor by multi-point injection every 3 days. Tumor volume was calculated with the following formula:  $V = (\text{length}) \times (\text{width})^2/2$ . After 28 days, mice were killed by intraperitoneal administration of barbiturates (240 mg/kg) followed by cervical dislocation. Tumor tissue was removed and frozen at  $-80$  °C for protein analysis or fixed in 4% paraformaldehyde for hematoxylin-eosin (H&E) staining and immunofluorescence staining.

### **H&E staining and Immunohistochemistry**

Tissues were fixed in 4% paraformaldehyde solution for at least 4 h at room temperature, and this was followed by dehydration, dipping in wax, paraffin embedding and cutting into sections. The sections were then H&E stained. For immunohistochemistry, sections were incubated with serum or BSA for 30 min at room temperature, and then were incubated with diluted primary antibody for 2 h and then with secondary antibody. The samples were observed under a BX53 fluorescence microscope (Olympus, Shinjuku, Tokyo, Japan).

### **Online searches of miRNA-related databases**

The miRNA-related databases used in this research were as follows: TargetScan (<http://www.targetscan.org/>), DIANA-microT (<http://www.microrna.gr/microT>), miRDB (<http://www.mirdb.org/>), miRWalk (<http://www.ma.uni-heidelberg.de/apps/zmf/mirwalk/>) and starBase (<http://starbase.sysu.edu.cn/>).

### **Luciferase reporter assays**

After co-transfection of fluorescein reporter vector and *miR-28-5p* mimics into HCT15 cells for 48 h, detection of luciferase activity was performed with a Dual Luciferase Reporter Gene Assay Kit (Beyotime Biotechnology Inc., Nantong, China) according to the manufacturer's instructions.

### **Statistical analyses**

Data analysis was performed on at least three independent experimental groups, and the results are expressed as mean  $\pm$  standard deviation. Statistical analyses were performed in SPSS version 17.0. Comparison of the two sets of data was performed with unpaired Student's t-test. One-way ANOVA was followed by Dunnett's test to compare more than two sets. Differences were considered significant at  $*p < 0.05$  and  $**p < 0.01$ , vs. the NC group.

## Results

### **SSRP1 expression is upregulated in lung cancer and is associated with overall survival rate**

To determine the role of SSRP1 in lung cancer, we analyzed the expression of SSRP1 in human tumor tissues from the online UALCAN browser and found that SSRP1 expression was upregulated in both human lung squamous cell carcinoma (LUSC) and lung adenocarcinoma (LUAD) samples (Fig. 1A and 1B,  $p < 0.01$ ), compared with normal samples. The GEPIA browser also showed that the mean expression level of SSRP1 was higher in LUAD and LUSC than in the corresponding normal tissues (Fig 1C,  $p < 0.01$ ). Overall survival rate analysis showed that lung cancer patients with high expression of SSRP1 showed a low overall survival rate, as compared with patients with low expression of SSRP1 (Fig. 1D,  $p < 0.05$ ). Immunohistochemistry results showed that the SSRP1 levels were higher in grade I-IV lung cancer than normal lung tissues. Furthermore, the SSRP1 levels in high-grade lung cancer were significantly higher than those in lower grade lung cancer (Fig. 1E). Moreover, western-blotting indicated that SSRP1 was up-regulated in a panel of human lung cancer cell lines (A549, NCI-H446, H1299, PC9) and murine cells (LLC), as compared with normal human lung epithelial cells (B2B), and the effects were significant in NCI-H446 and H1299 cell lines (Figs. 1F and 1G). According to the statistical data, we chose the NCI-H446 and H1299 cell lines for subsequent experiments. The results indicated that SSRP1 is overexpressed in lung cancers, thus suggesting that SSRP1 might be associated with the development of lung cancer.

### **SSRP1 inhibition represses lung cancer cell proliferation**

To explore the function of SSRP1 in lung cancer, we used RNAi technology to silence the expression of SSRP1 in lung cancer cells and observed whether SSRP1 might affect lung cancer progression.

First, SSRP1 protein levels in NCI-H446 and H1299 cells significantly decreased (Figs. 2A and 2B) after SSRP1 siRNA transfection for 48 h. CCK8 assays demonstrated that SSRP1 siRNA did not decrease the cell viability of normal human lung epithelial cells (B2B) (Fig. 2C). Compared with that of lung cancer cells transfected with negative control (NC), the viability of NCI-H446 and H1299 cells transfected with SSRP1 siRNA at OD 450 nm showed different degrees of decline after siRNA transfection for 24, 48 and 72 h (Figs. 2D and 2E). In addition, the number of NCI-H446 and H1299 cells (Figs. 2F and 2G) decreased significantly after SSRP1 siRNA transfection on the basis of cell counting assays. Likewise, colony formation assays showed that the colony formation ability of NCI-H446 and H1299 cells (Figs. 2H and 2I) was significantly inhibited after SSRP1 siRNA transfection. These data revealed that blocking the expression of SSRP1 represses the proliferation of lung cancer cells.

## **SSRP1 affects the cell cycle and apoptosis in lung cells**

To further explore the regulatory role of SSRP1 in lung cancer cell proliferation, we examined cell cycle and apoptosis in lung cancer cells by flow cytometry. NCI-H446 and H1299 cells treated with SSRP1 siRNA for 48 hours showed significant G0/G1 phase arrest, as compared with cells treated with NC (Fig. 3A). Simultaneously, the percentage of apoptotic cells in NCI-H446 and H1299 cells transfected with SSRP1 siRNA was markedly higher than that in the NC group (Figs. 3B and 3C). Thus, knockdown of *SSRP1* inhibited lung cancer cell cycle progression from G0/G1 phase to S phase and induced lung cancer cell apoptosis.

## **Downregulation of SSRP1 inhibits migration and invasion of lung cancer cells**

We additionally explored the effects of SSRP1 on migration and invasion in lung cancer cells. Transwell assays showed that downregulation of *SSRP1* with SSRP1 siRNA transfection significantly impaired migration (Figs. 4A and 4B) and invasion (Figs. 4C and 4D) in both NCI-H446 and H1299 cells. Therefore, SSRP1 silencing inhibited migration and invasion of lung cancer cells.

## **SSRP1 inhibition blocks lung cancer growth in vivo**

Next, we used the NCI-H446 cells to construct a lung cancer xenograft model to explore the role of SSRP1 in lung cancer *in vivo*. The xenograft model construction and administration method were as shown in Fig. 5A. Animal experiment results revealed that the tumor size and volume in siSSRP1 group were much smaller than those in the NC group (Figs. 5B and 5C). However, the body weight did not show a significant difference between the groups (Fig. 5D), thus indicating that SSRP1 siRNA did not cause any damage to the bodies of the mice.

Immunohistochemistry showed that the expression of SSRP1 in the siSSRP1 group was markedly downregulated (Fig 5E). As an index of cell proliferation, proliferating cell nuclear antigen (PCNA) expression was inhibited in the siSSRP1 group (Fig 5E), compared with the NC group. Bcl-2 in the siSSRP1 group was markedly downregulated (Fig 5E), and the expression of Bax in the siSSRP1 group was clearly upregulated (Fig 5E), as compared with that in the NC group. MMPs play a key role in matrix remodeling [25] and are associated with tumor invasion [26]. We found that the expression of matrix metalloproteinase (MMP)-2 and MMP-9 was inhibited in the siSSRP1 group. However, no organ toxicity was observed by H&E staining (Fig 5F).

Furthermore, *SSRP1* silencing increased the expression of the cell cycle regulator p21 and the pro-apoptotic factor Bax, but decreased the expression of Cyclin D1 and Bcl2 in NCI-446 and H1299 cells (Figs. 5G and 5H). The results above demonstrated that SSRP1 inhibition blocked lung cancer growth both *invitro* and *in vivo*.

## **SSRP1 affects lung cancer cell activity by inhibiting the WNT signaling pathway**

The WNT pathway is considered the classical signaling pathway regulating tumor proliferation and metastasis [27]. To determine whether the WNT signaling pathway participates in SSRP1 regulation of lung cancer growth, we used western blotting to detect alterations of critical proteins in the WNT signaling pathway. Western blotting showed that *SSRP1* knockdown inhibited expression of p-GSK3 $\beta$  and  $\beta$ -catenin, which are critical proteins in the WNT signaling pathway (Figs. 6A and 6B), in total protein extract from NCI-446 and H1299 cells, and p-GSK3 $\beta$  and  $\beta$ -catenin were also downregulated in nucleoprotein (Figs. 6C and 6D) from NCI-446 and H1299 cells. In addition, protein expression of p-GSK3 $\beta$  and  $\beta$ -catenin was inhibited in the siSSRP1 group in the xenograft model of lung cancer (Figs. 6E and 6F). Therefore, SSRP1 silencing may block proliferation and promote apoptosis by blocking the WNT signaling pathway.

### **Upregulation of SSRP1 in lung cancer is associated with miR-28-5p**

MiRNAs are associated with the initiation and progression of cancer [28]. We previously confirmed that SSRP1 expression affects lung cancer progression. To investigate whether miRNAs regulate SSRP1 and whether SSRP1 affects the development of lung cancer, we used four miRNA-related databases to predict miRNAs that might regulate SSRP1 (Fig. 7A). The predictive results revealed that SSRP1 miRNA contains a potential *miR-28-5p* seed sequence within its 3'UTR (Fig. 7B). To further confirm whether SSRP1 is the target gene of *miR-28-5p*, we generated a luciferase expression plasmid containing the wild-type or mutant SSRP1 3'UTR (report-SSRP1-wt and report-SSRP1-mut, respectively). Luciferase activity results indicated that *miR-28-5p* overexpression suppressed report-SSRP1-wt-expressed luciferase, whereas report-SSRP1-mut was unaffected under the same conditions (Fig. 7C). To determine whether *miR-28-5p* negatively regulates SSRP1, we constructed *miR-28-5p* mimics and inhibitors (Figs. 7D and E) and used them to transfect NCI-446 and H1299 cells. When the expression of *miR-28-5p* was upregulated, the expression of SSRP1 was inhibited in both cell lines (Figs. 7F and G). In addition, the expression of *miR-28-5p* was downregulated, and the expression of SSRP1 was upregulated in both cell lines (Figs. 7H and I). In conclusion, SSRP1 is negatively regulated by *miR-28-5p* in lung cancer.

### **MiR-28-5p inhibits lung cancer proliferation by regulating the expression of SSRP1**

To further determine whether *miR-28-5p* participates in the progression of lung cancer by regulating SSRP1 expression, we used *miR-28-5p* mimic and inhibitor to detect the influence of *miR-28-5p* on NCI-446 cell proliferation. Compared with that of cells transfected with NC miRNA, the cell viability of NCI-446 cells at 450 nm OD decreased after *miR-28-5p* mimic transfection for 48 and 72 h (Fig. 8A)—results opposite from those observed for the *miR-28-5p* inhibitor (Fig. 8B). In addition, the cell numbers and colony formation ability of NCI-446 cells decreased significantly after *miR-28-5p* mimic transfection (Figs. 8C, 8D and 8E) and increased after *miR-28-5p* inhibitor transfection (Figs. 8F, 8G and 8H). These results showed that *miR-28-5p* inhibits the progression of lung cancer by inhibiting the expression of SSRP1.

## **Discussion**

FACT, a heterodimer consisting of two subunits, SSRP1 and suppressor of Ty16, plays a crucial role in transcription and DNA replication, recombination and repair [29, 30]. SSRP1 is thought to be involved in the development of several cancers and to have different roles in different cancers. SSRP1 knockdown increases the sensitivity of HCC cells to doxorubicin, 5-Fluorouracil and cisplatin, and SSRP1 is negatively regulated by *miR-497* in hepatic cancer [8]. Reports have indicated that SSRP1 silencing inhibits colorectal cancer proliferation, migration and invasion via the AKT pathway, and the lncRNA LOC101927746 acts as a competing endogenous RNA that suppresses *miR-584-3p* and then activates its target gene SSRP1, thus inhibiting the growth of colorectal cancer [13, 31]. However, SSRP1 inhibition suppresses the activity of the phosphorylation of the MAPK signaling pathway and consequently blocks the proliferation and metastasis of glioma cells [10]. FACT is also both a marker and a target of aggressive breast cancer cells [32]. In non-small cell lung cancer, knockdown of SSRP1 decreases cancer cell growth and overcomes resistance to Erlotinib by modulating the nuclear factor-kappa B signaling pathway [7]. However, the detailed function of SSRP1 in the lung cancer proliferation, metastasis and invasion remains unclear.

Bioinformatics analysis showed that SSRP1 was over-expressed in both LUSC and LUAD. Moreover, SSRP1 expression was higher in patients with high-grade lung cancer than in patients with low-grade lung cancer and in normal samples. The overall survival rate of patients was negatively correlated with the expression of SSRP1. Meanwhile, the expression of SSRP1 was significantly higher in a series of lung cancer cells, especially in NCI-H446 and H1299 cells, than in normal human lung epithelial cells. These results suggest that SSRP1 might regulate the development of lung cancer, and that SSRP1 overexpression might predict a high clinical grade of lung cancer and low survival rate of lung cancer patients.

To further explore the function of SSRP1 in lung cancer, we silenced SSRP1 by using siRNA to detect the effects of SSRP1 inhibition on lung cancer *in vivo and vitro*. First, we used several methods, including CCK-8, colony formation assays and FACS flow cytometric analysis, to validate that SSRP1 knockdown inhibited cell viability and proliferation and induced apoptosis in NCI-H446 and H1299 cells. Decreased expression of SSRP1 weakened the growth and volume of lung cancer cell xenograft tumors. The immunohistochemistry results in lung cancer cell xenograft tumors showed that SSRP1 silencing inhibited the growth of lung cancer and induced apoptosis *in vivo*. These results indicated that the down-regulation of SSRP1 inhibited lung cancer cell proliferation and stimulated anti-apoptosis function.

Furthermore, we explored the function of SSRP1 in lung cancer invasion and metastasis. NCI-H446 and H1299 cell migration and invasion ability were both inhibited after SSRP1 knockdown. Similarly, the immunohistochemistry results in lung cancer cell xenograft tumors showed that down-regulation of SSRP1 weakened migration and invasion ability *in vivo*. Furthermore, SSRP1 knockdown did not result in toxicity in organs including the heart, liver, spleen, lung and kidney.

On the basis of preliminary results, SSRP1 may have potential value in the lung cancer diagnosis and therapy, and further studies on SSRP1 are urgently needed.

Wnt/ $\beta$ -catenin signaling is an evolutionarily conserved pathway that plays crucial role in embryogenesis, development and homeostasis. When the Wnt pathway is active, Gsk3 is bound to  $\beta$ -catenin and translocates into cell nucleus as a transcription factor. Then the pathway stimulates the expression of downstream genes including *cyclin D1* and *cyclin-dependent kinase inhibitor CDKN1A (p21)*, alters cell cycle progression, and plays a role in tumorigenesis [27, 33]. After wnt/ $\beta$ -catenin signaling is inhibited, the expression of Bcl2 increases and the expression of Bax decreases, and the changes in the expression of the aforementioned genes ultimately promote apoptosis. In this study, we found that blocking *SSRP1* gene expression inhibited not only the expression of p-Gsk3 $\beta$ ,  $\beta$ -catenin, cyclin D1 and p21 in total cell protein, but also the expression of p-Gsk3 $\beta$  and  $\beta$ -catenin in the nucleus in NCI-H446 and H1299 cells. These results showed that Wnt/ $\beta$ -catenin signaling might be activated in lung cancer cells and *SSRP1* inhibition represses lung cancer growth by blocking the WNT signaling pathway, in agreement with findings from a previous study on colorectal cancer [24].

MiRNAs negatively regulate target gene expression by binding the mRNA 3'UTR regions of target genes, which encode proteins and participate in a variety of cellular physiological process in tumors [28]. Numerous studies have suggested roles of miRNAs in anti-tumor therapy [34-36], thus prompting us to speculate that a certain miRNA may regulate the entire process of lung cancer progression by regulating the expression of *SSRP1*. Given the above findings, we used four RNA-related databases to predict the possible binding of miRNAs to *SSRP1* and identified *miR-28-5p* as a miRNA that might regulate *SSRP1* expression. The potential target site of *SSRP1* 3'UTR binding to *miR-28-5p* is located at 335–342. *MiR-28-5p* has been reported to regulate colorectal cancer by inhibiting *SSRP1* expression [24], and *miR-28-5p* inhibits the migration and invasion of gastric cancer cells by suppressing AKT phosphorylation [37]. *MiR-28-5p* was transfected to verify changes in target gene expression in luciferase reporter assays, which revealed that *SSRP1* was a target gene of *miR-28-5p* and was negatively regulated by *miR-28-5p*. Transfection of *miR-28-5p* mimics resulted in the downregulation of *SSRP1* and also inhibited the proliferation of lung cancer cells, thus reflecting the findings of *SSRP1* inhibition by siRNA. However, transfection of *miR-28-5p* inhibitor reversed this effect. Importantly, the diagnostic ability of *miR-28-5p* combined with *SSRP1* was stronger than that of *miR-28-5p* or *SSRP1* alone, and also stronger than that of the tumor markers used in the clinic. These data may provide new potential diagnostic and therapeutic targets for lung cancer in the future and highlight the clinical relevance of this study. However, further research to clarify the molecular mechanism of the interaction between *miR-28-5p* and *SSRP1* is required. Our study demonstrates the roles of *SSRP1*, *miR-28-5p* and the WNT signaling pathway in lung cancer growth.

## Conclusion

In conclusion, our study reported that silencing of *SSRP1* by siRNA inhibited lung cancer proliferation, migration and invasion by blocking the WNT signaling pathway, however, miR-28-5p mediated suppression of *SSRP1*, thus influencing lung cancer growth via the WNT signaling pathway.

# Abbreviations

SSRP1: Structure-specific recognition protein 1; FACT: Facilitates chromatin transcription; miRNAs: MicroRNAs ; UTRs: Untranslated regions; miR-28-5p: MicroRNA-28-5p; DMEM: Dulbecco's modified Eagle's medium ; NC: Negative control; LUSC: Human lung squamous cell carcinoma ; LUAD: Lung adenocarcinoma; PCNA: Proliferating cell nuclear antigen.

# Declarations

## Acknowledgements

Not applicable.

## Authors' contributions

SJ, LP and LZ designed the research. SJ and QW performed the experiments and prepared the images. YJ and BG analyzed the bioinformatic data. SJ, QW, BG, YJ and LW analyzed and interpreted the experimental data. SJ prepared the original manuscript. LP and LZ reviewed and edited the manuscript. All authors read and approved the final manuscript.

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## Availability of data and materials

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researchers.

## Ethics approval and consent to participate

The present study was approved by the Ethical Committee of the First Hospital of Jilin University [grant no: 2017-444]. All patients provided written informed consent.

## Patient consent for publication

Not applicable.

## Declaration of competing interest

The authors declare no potential conflicts of interest.

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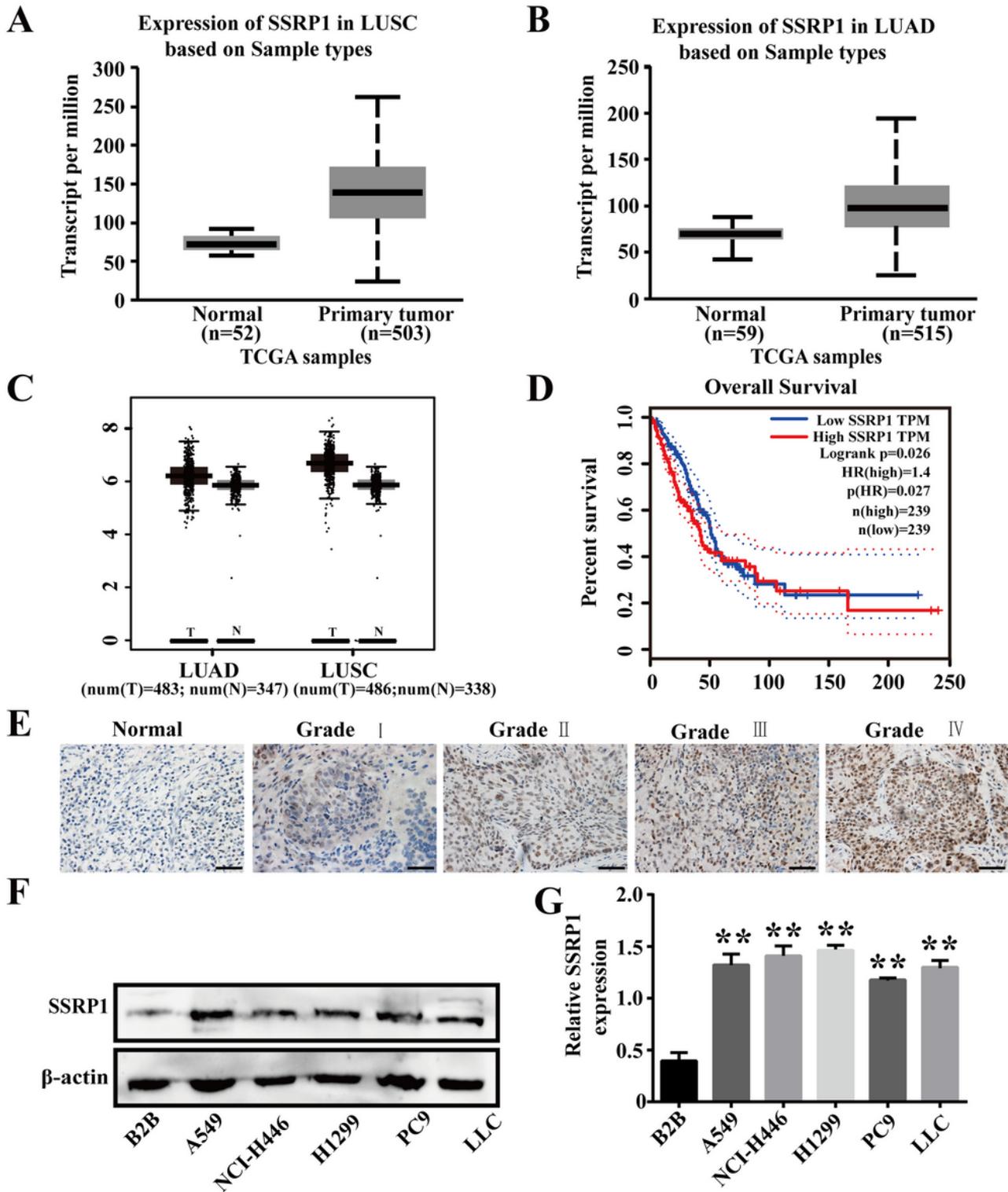
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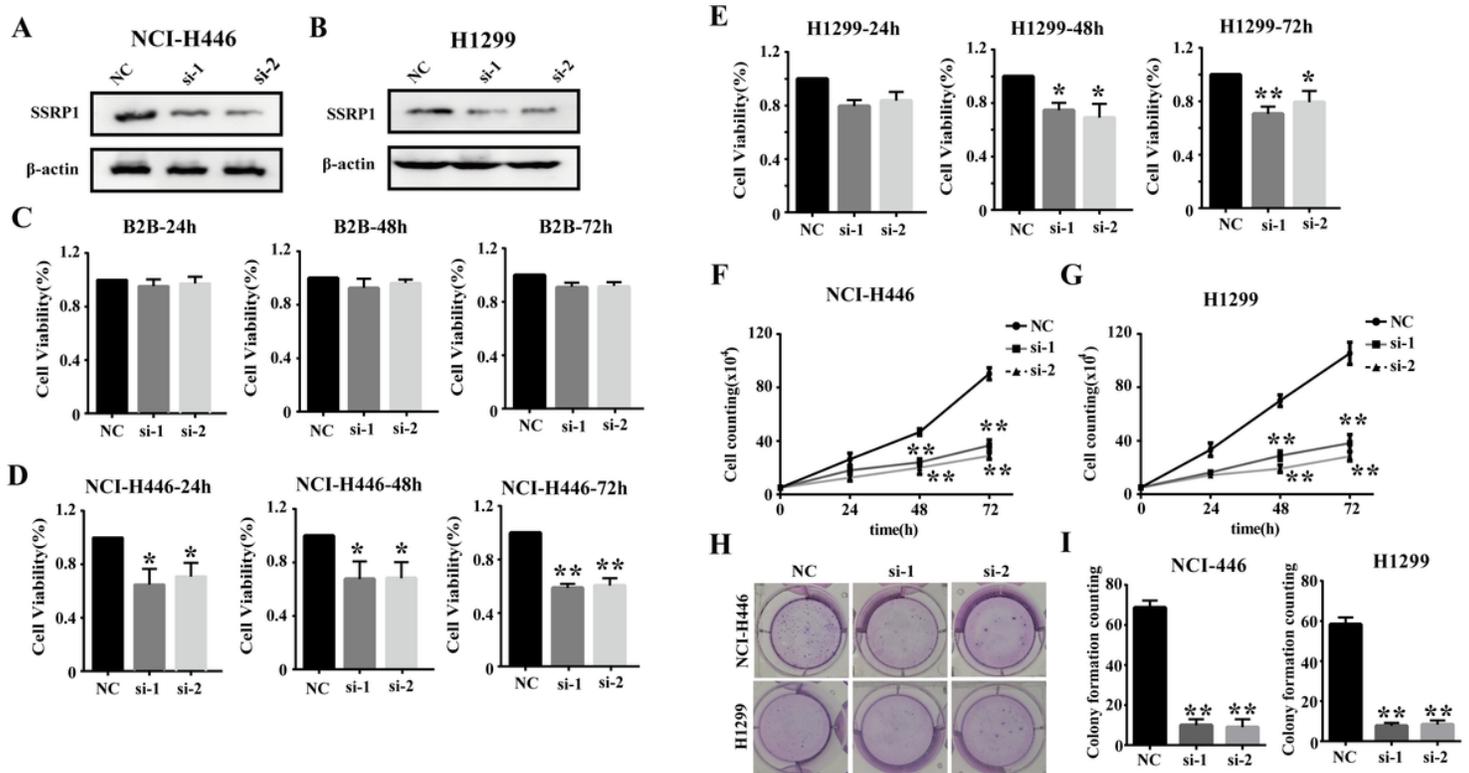
## Figures



**Figure 1**

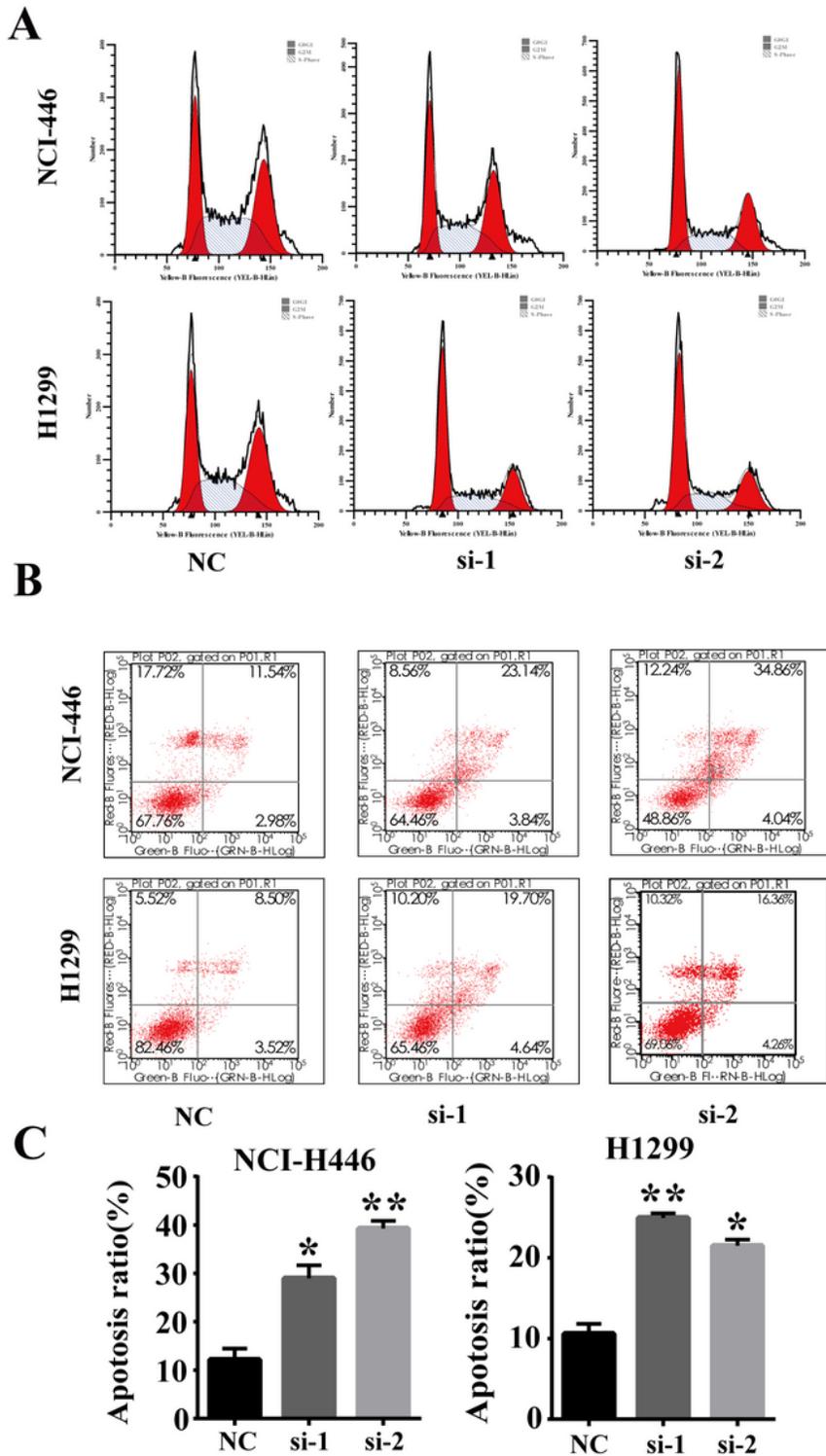
Elevated expression of SSRP1 is correlated with lung cancer progression. (A) SSRP1 expression in normal tissues (n = 52) and lung squamous cell carcinoma (LUSC) tumor tissues (n = 503) from the UALCAN database. (B) SSRP1 expression in normal tissues (n=59) and lung adenocarcinoma (LUAD) tumor tissues (n = 515) from the UALCAN database. (C) SSRP1 expression in normal tissues and LUSC and LUAD tumor tissues from the GEPIA database. (D) Survival rate analyses based on SSRP1 expression in

tumor tissues from the GEPIA database. (E) Immunohistochemical technology was used to test SSRP1 expression in normal and different grades of human tumor tissues. (F) SSRP1 expression in a normal human lung epithelial cell (B2B) with a panel of lung cancer cell lines (A549, NCI-H446, H1299, PC9 and LLC), determined via western blotting. (G) Densitometric quantification of SSRP1 protein normalized to  $\beta$ -actin. \* $p < 0.05$ , \*\* $p < 0.01$  vs. the NC group.



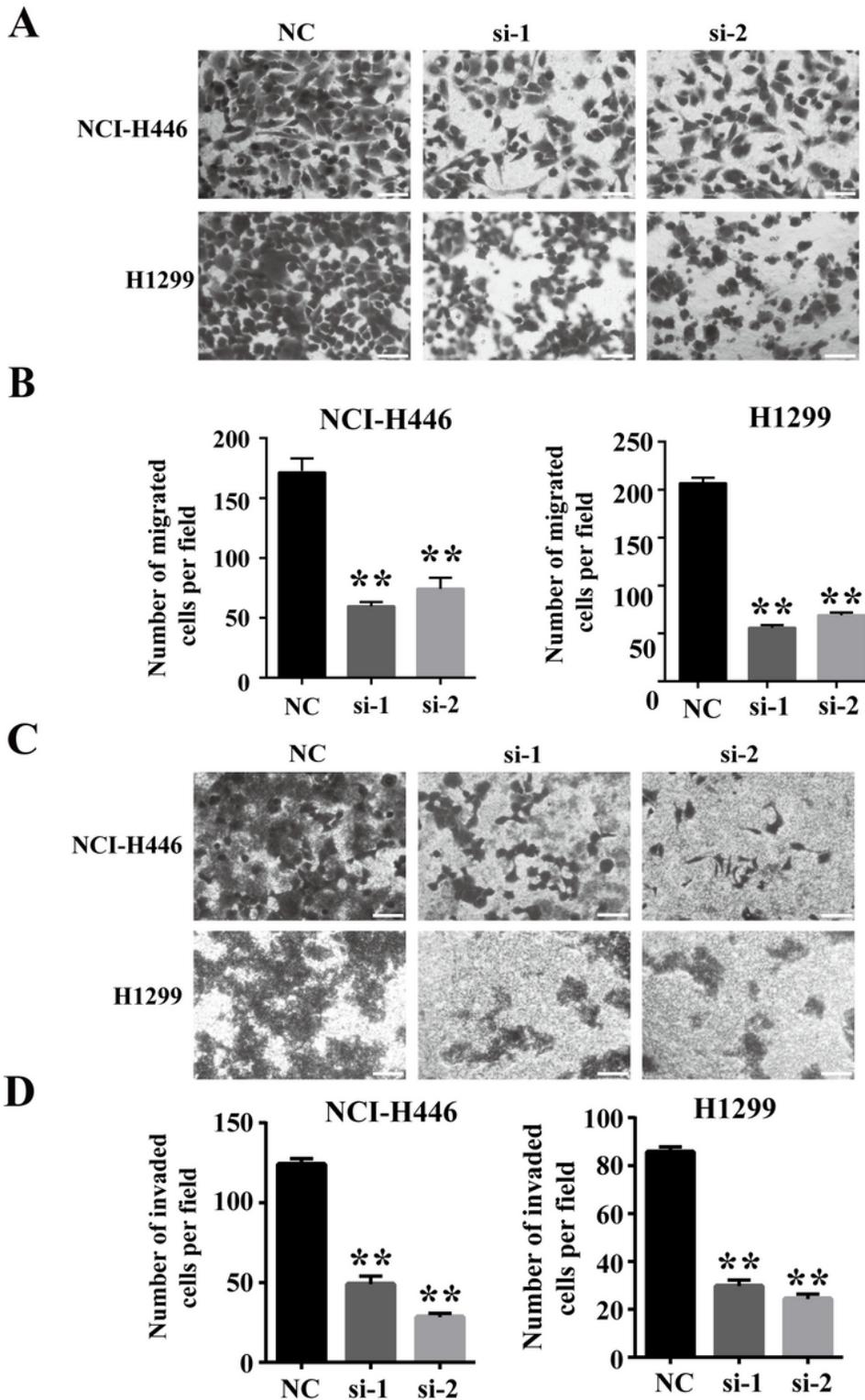
**Figure 2**

SSRP1 silencing suppresses lung cancer cell proliferation. (A-B) SSRP1 expression in NCI-H446 and H1299 cells at 48 h after siRNA transfection. (C–E) Cell viability of B2B, NCI-H446 and H1299 at 24, 48, and 72 h after SSRP1 siRNA transfection. (F–G) Cell counts of NCI-H446 and H1299 at 24, 48, and 72 h after SSRP1 siRNA transfection. (H–I) Colony formation ability of NCI-H446 and H1299 cells after SSRP1 silencing by transfection of siRNA. \* $p < 0.05$ , \*\* $p < 0.01$  vs. NC group.



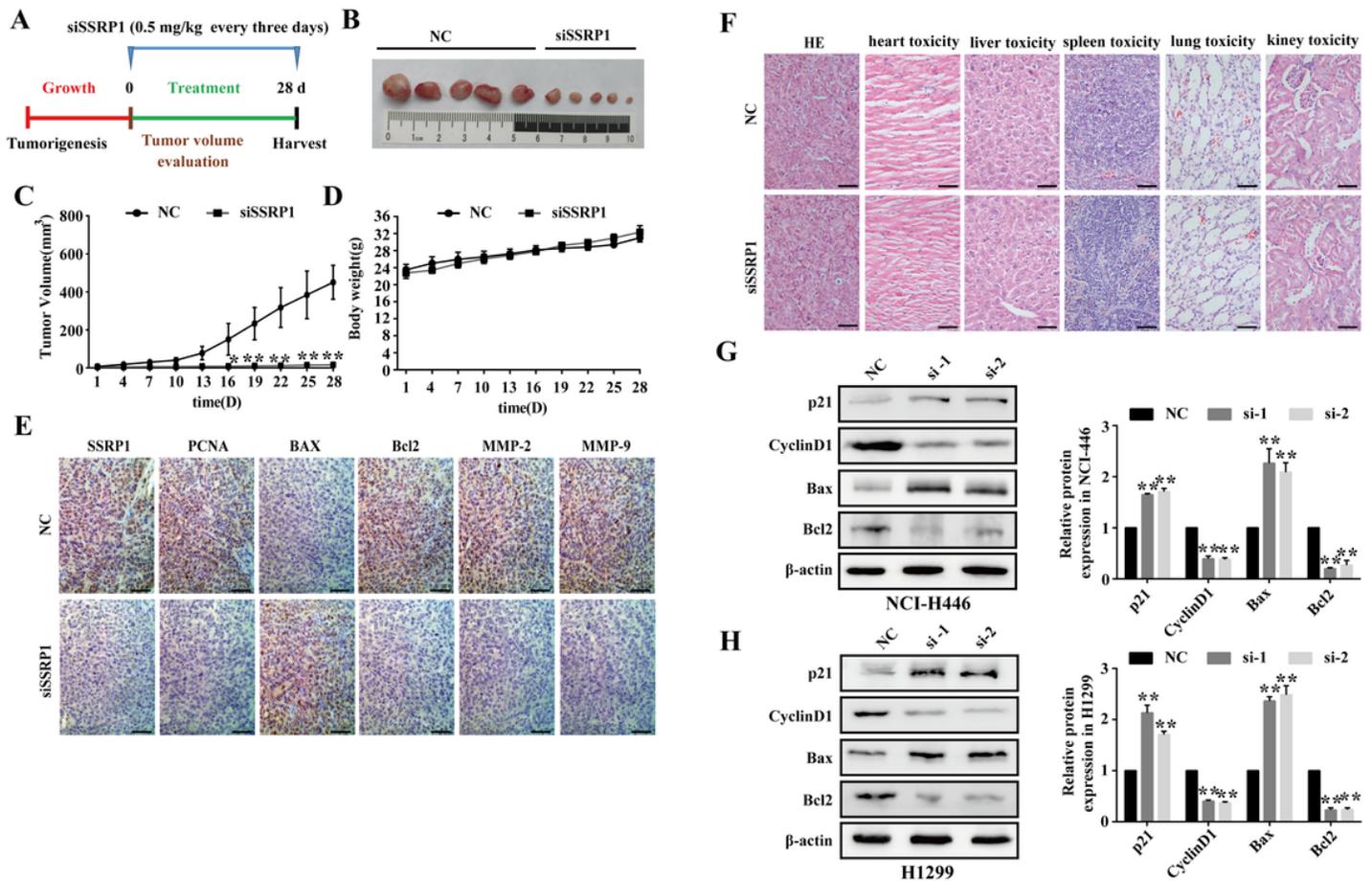
**Figure 3**

SSRP1 silencing affects the cell cycle and apoptosis in lung cells. (A) The effects of SSRP1 silencing on cell cycle progression in NCI-H446 and H1299 cells were detected by flow cytometry analyses. (B) The effects of SSRP1 silencing on cell apoptosis in NCI-H446 and H1299 cells. (C) The apoptosis ratios of NCI-H446 and H1299 cells at 48 h after SSRP1 siRNA transfection. \* $p < 0.05$ , \*\* $p < 0.01$  vs. the NC group.



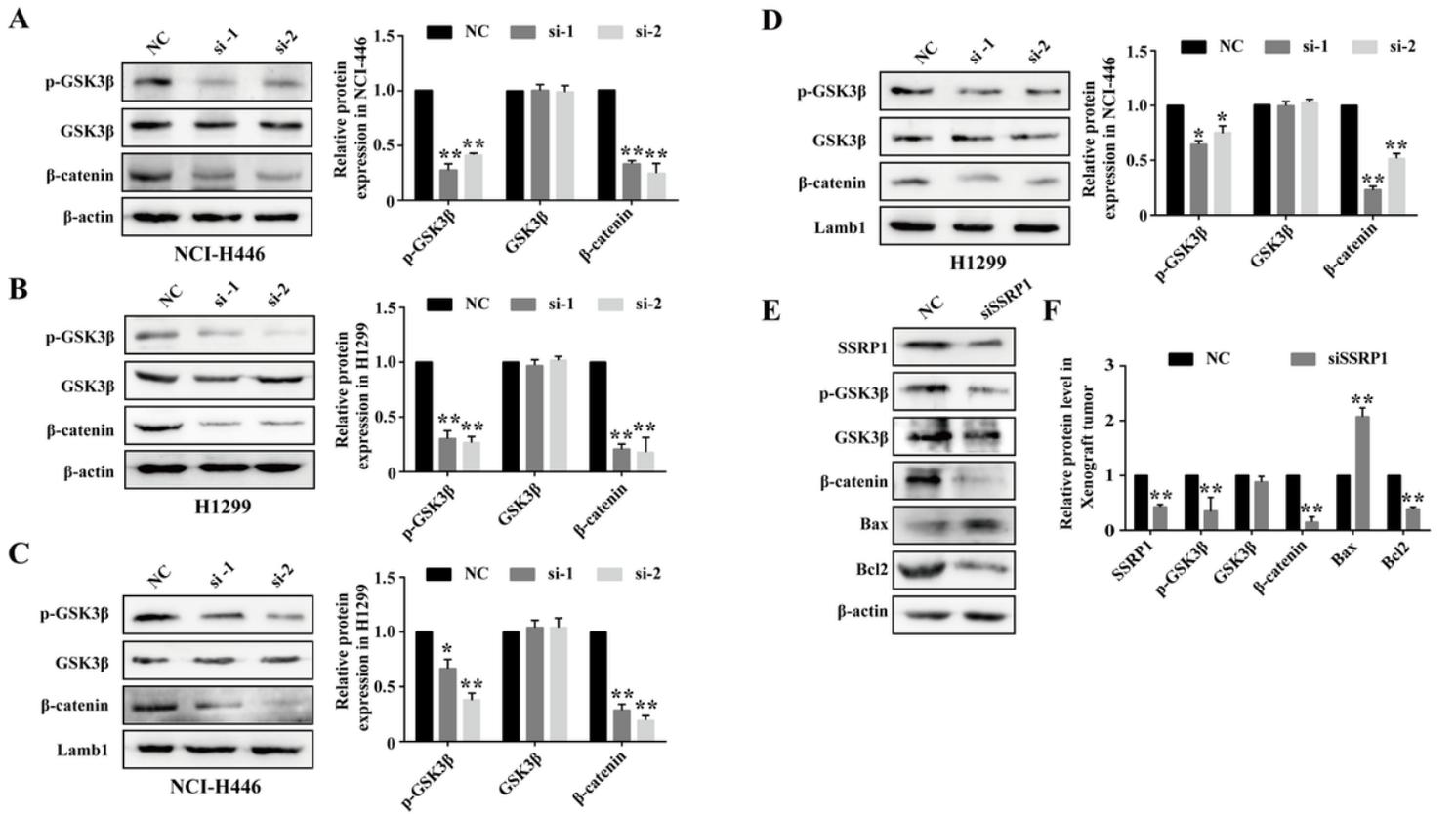
**Figure 4**

SSRP1 silencing suppresses lung cancer cell migration and invasion. (A-B) Transwell assay showing that SSRP1 silencing inhibits the migration of NCI-H446 and H1299 cells. (C-D) Transwell assay showing that SSRP1 silencing inhibits the invasion of NCI-H446 and H1299 cells. \* $p < 0.05$ , \*\* $p < 0.01$  vs. the NC group.



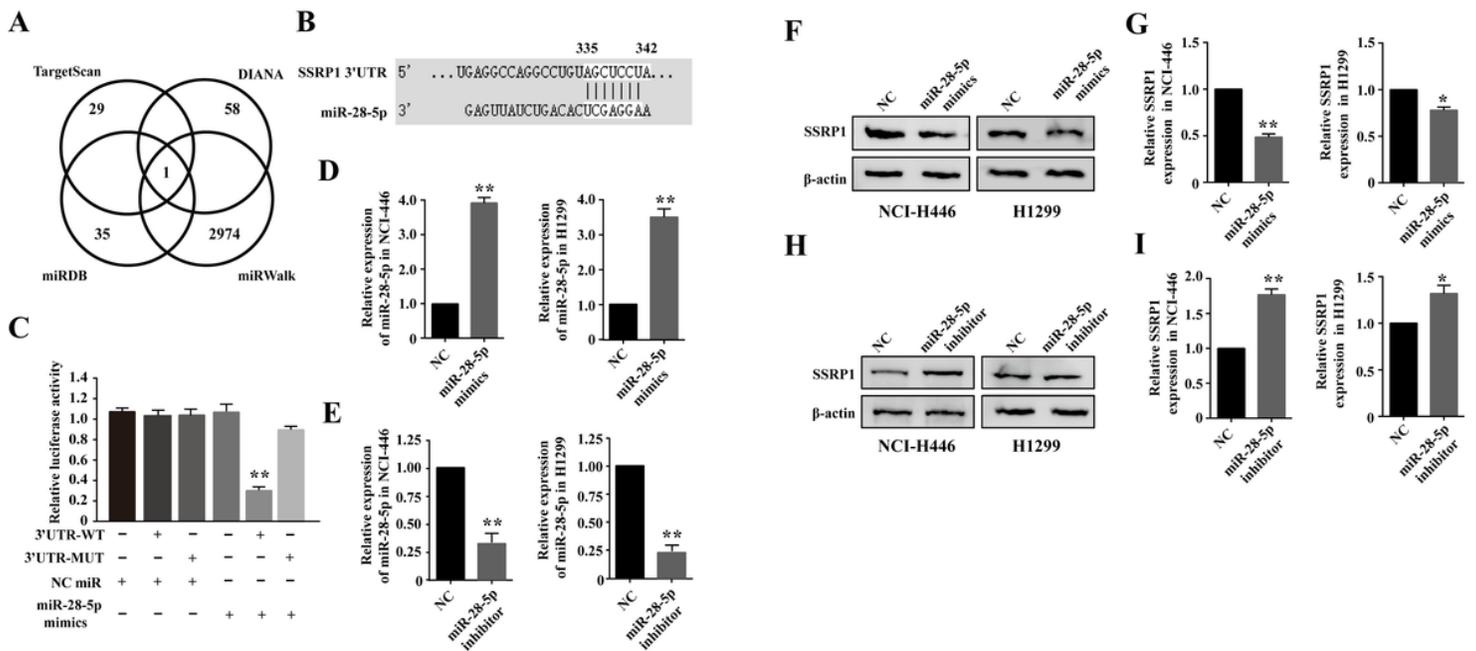
**Figure 5**

SSRP1 silencing suppresses tumor growth in vivo without organ toxicity. (A) Treatment for transplanted xenogeneic models with NCI-H446 cells. (B) Lung cancer tumors after siSSRP1 (SSRP1 siRNA) treatment. (C) Tumor growth curves of the NC group and siSSRP1 (SSRP1 siRNA) group after 28 d treatment. (D) The body weights of the NC group and siSSRP1 (SSRP1 siRNA) group during the treatment. (E) SSRP1, PCNA, Bax, Bcl2, MMP-2 and MMP-9 protein levels, determined by immunohistochemical analysis. (F) H&E staining, showing no organ toxicity after SSRP1 silencing. (G–H) Western blot analyses for protein expression of p21, CyclinD1, Bax and Bcl2 in NCI-H446 and H1299 cells after siRNA transfection. \* $p < 0.05$ , \*\* $p < 0.01$  vs. the NC group.



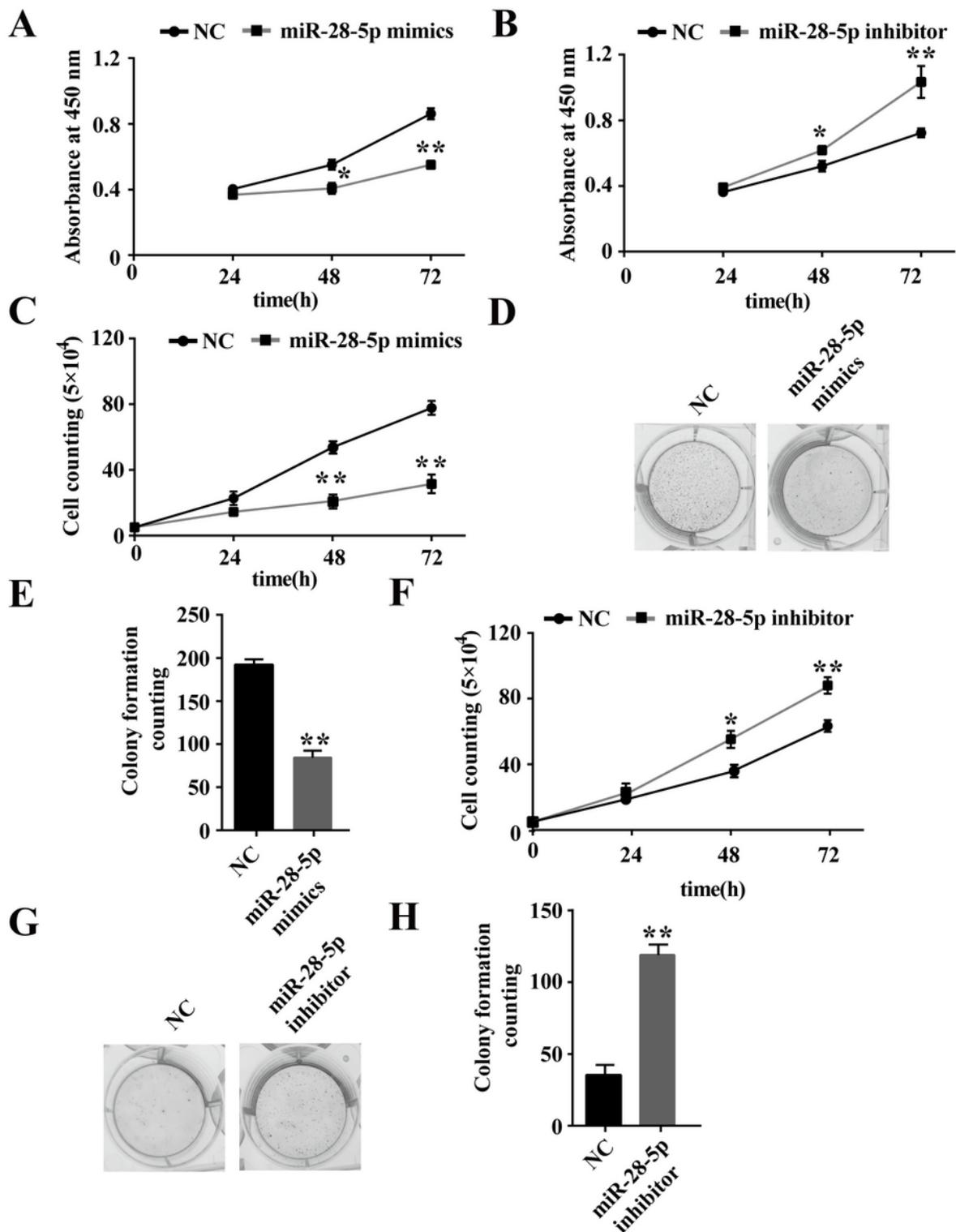
**Figure 6**

SSRP1 silencing affects lung cancer cell activity by inhibiting the WNT signaling pathway. Western blot analyses for cytoplasm protein expression of p-GSK3 $\beta$ , GSK3 $\beta$  and  $\beta$ -catenin in NCI-H446 (A) and H1299 (B) cells. Western blot analyses for nuclear protein expression of p-GSK3 $\beta$ , GSK3 $\beta$  and  $\beta$ -catenin in NCI-H446 (C) and H1299 (D) cells. (E and F) Western blot analyses for protein expression of p-GSK3 $\beta$ , GSK3 $\beta$ ,  $\beta$ -catenin, Bax and Bcl2 in a lung cancer xenograft model \*p < 0.05, \*\*p < 0.01 vs. the NC group.



## Figure 7

Up-regulation of SSRP1 in colorectal cancer is associated with miR-28-5p. (A) MiR-28-5p may regulate SSRP1, according to a Venn diagram, as determined by bioinformatics analyses. (B) The sequence of miR28-5p and the region where the SSRP1 3'UTR may bind miR-28-5p. (C) NCI-H446 cells were co-transfected with luciferase reporter vectors containing wild type or mutant SSRP1 3'UTR (report-SSRP1-wt and report-SSRP1-mut, respectively) and miR-28-5p mimics for 48 h, and the luciferase activity was determined. (D–E) Relative miR-28-5p expression in NCI-H446 and H1299 after transfection with miR-28-5p mimics and inhibitor, determined by qPCR. (F–I) SSRP1 protein levels after transfection with miR-28-5p mimics and inhibitor in NCI-H446 and H1299 for 48 h, as determined by western blot analyses. \* $p < 0.05$ , \*\* $p < 0.01$  vs. the NC group.



**Figure 8**

MiR-28-5p regulates the progression of lung cancer cells by targeting SSRP1. (A–B) Cell viability of NCI-H446 at 24, 48, and 72 h after transfection of miR-28-5p mimics and inhibitor. (C) NCI-H446 cell growth curve in the presence of miR-28-5p mimics. (D, E) Colony formation ability of NCI-H446 cells in the presence of miR-28-5p mimics. (F) NCI-H446 cell growth curve after inhibitor transfection. (G, H) Colony

formation ability of NCI-H446 cells after miR-28-5p inhibitor transfection. \*p < 0.05, \*\*p < 0.01 vs. the NC group.