

SPOCK2 Expression Correlates with Non-Small Cell Lung Cancer Proliferation, Invasion, and Tumorigenesis

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Research

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Abstract

Purpose: Lung cancer is one of the leading causes of death worldwide. Sparc (osteonectin), cwcv, and Kazal-like domains proteoglycan 2 (SPOCK2) play important roles in the development and progression of various types of human cancers. However, the role of SPOCK2 in non-small-cell lung cancer (NSCLC) is unclear. Hence, we aimed to elucidate the role of SPOCK2 in NSCLC.

Methods: Real-time PCR and immunohistochemistry were used to study the relationship between SPOCK2 expression and the clinicopathology of NSCLC. SPOCK2 expression was modulated using siRNA (knockdown) or pcMV6-myc-DDK-SPOCK2 (overexpression). The invasion and migration abilities of NSCLC cells were assessed using a Transwell chamber assay, and cell proliferation was studied using the MTT and colony formation assays. Furthermore, axilla and tail vein inoculation using nude mice helped elucidate the function of SPOCK2 *in vivo*. The expression of related proteins was analyzed using western blotting. SPOCK2 methylation was detected using real-time PCR following the treatment of cells with decitabine.

Results: The mRNA and protein levels of SPOCK2 were lower in NSCLC tissues than in adjacent normal lung tissues. SPOCK2 overexpression inhibited cell proliferation, migration, and invasion in A549, H1299 cells, and nude mice. Notch and Erk signaling pathways were inhibited by SPOCK2, whereas the Hippo pathway was activated. SPOCK2 was methylated in NSCLC cell lines. SPOCK2 expression was negatively correlated with NSCLC progression.

Conclusion: Taken together, our findings indicate that SPOCK2 methylation may result in its low expression, and the levels of SPOCK2 methylation may be associated with poor prognosis in NSCLC.

1 Introduction

Lung cancer is a major public health issue (Mao et al. 2016) and a leading cause of cancer-related deaths globally (Nasim et al. 2019). Approximately 85–90% of lung cancer cases involve non-small cell lung cancer (NSCLC) (Siegel et al. 2016). Various genetic and dynamic epigenetic alterations have been reported to be associated with the initiation and progression of lung cancer.

Some signaling pathways, such as Notch (Braune and Lendahl 2016; Leonetti et al. 2019), MAPK (Roskoski 2018; Wee and Wang 2017), and Hippo (Harvey et al. 2013; Liu et al. 2018) signaling pathways, regulate the occurrence and development of tumors by interacting to form a network. Cell proliferation, migration, and invasion are considered critical biological behaviors in tumorigenesis and development. Alterations in various signaling pathways have considerable or negligible effects on these biological behaviors.

During the past decades, several small molecular inhibitors, which significantly improve the overall prognosis of lung cancer, have been introduced clinically (Lovly 2015; Reck 2013). Epigenetic differences among patients are considered a critical determinant of poor response to drug therapy (Chen 2015).

Among the epigenetic changes associated with human cancers, DNA methylation is most widely studied (Mehta et al. 2015). The role of altered DNA methylation in cancer was identified in 1983. Within 30 years of this discovery, DNA methylation inhibitors were developed to clinically treat a variety of cancers, such as myelodysplastic syndrome (MDS). Furthermore, our understanding of the epigenetic changes associated with lung cancer has improved greatly in the past two decades (Duruisseaux and Esteller 2018). Currently, DNA methylation has been widely accepted as an important biomarker in the clinical management of lung cancer as DNA methylation provides useful information that enables early diagnosis, staging, prognosis, and therapy-response prediction (Fleischhacker et al. 2013).

The Sparc/osteonectin, cwcv, and Kazal-like domains proteoglycan 2 (*SPOCK2*) gene encodes a 424-amino-acid long protein containing a Kazal, SPARC_EC, follistatin-like, and calcium-binding domains. SPARC is a multifunctional glycoprotein and extracellular matrix protein that regulates the interaction of the extracellular matrix, binds collagen and fibronectin to endothelial cells, and inhibits cell proliferation. Piersigilly et al. (2016) reported various potential biomarkers for bronchopulmonary dysplasia, including *SPOCK2*, using “omics” technologies. Furthermore, Hadchouel et al. (2011) showed that the *SPOCK2* mRNA levels markedly increase during the alveolar stage of lung development in new-born rat lungs, and exposure to hyperoxia results in higher *SPOCK2* expression than observed in air-exposed controls. Using gene difference analysis, Helena et al. showed that when AT2 cells are isolated, cultured, and stimulated to differentiate into AT1 cells, *SPOCK2* expression is increased. Lastly, Ren et al. (2011a, 2014b) showed that epigenetic inactivation caused by the methylation of the *SPOCK2* promoter is associated with malignant transformation of ovarian endometriosis. Tumorigenesis is a process of dedifferentiation. Hence, in this study, we hypothesized that *SPOCK2* might be methylated in lung cancer and play a role in the development of NSCLC.

2 Materials And Methods

2.1 Patients and specimens

Two hundred and sixty-two cases of NSCLC (157 men and 105 women; age, 34–78 years old; operated between 2013 and 2014) were randomly selected from the Pathology Archive of the First Affiliated Hospital of China Medical University for immunohistochemistry, and 40 samples of NSCLC tumor and paracancerous lung tissue from operations in 2015 were randomly selected for real-time PCR and 16 samples of NSCLC tumor and paracancerous lung tissue from operations in 2015 were randomly selected for western blotting. None of the patients whose data were subjected to the pathological correlation analysis had received preoperative chemotherapy or radiotherapy. Clinicopathological information was obtained from patient records. This study was approved by the Medical Research Ethics Committee of China Medical University, and informed consent was obtained from all patients (AF-SOP007-1.1-01). The animal care and experimental protocols were approved by the Medical Research Ethics Committee of the China Medical University (CMU2020406).

2.2. Immunohistochemistry

Immunohistochemistry was performed in accordance with methods described previously (Xu et al. 2018) using paraffin sections of 262 samples of clinical NSCLC. Anti-SPOCK2 rabbit polyclonal antibody (1:100; Sigma, St. Louis, MO, USA) was used to probe the samples at 4°C for 18 h. Single-blinded scoring of SPOCK2 staining intensity was performed as follows: 0 (no staining), 1 (weak staining), 2 (moderate staining), and 3 (high staining), and the percentage of cells stained was scored in the following manner: 0 (0%), 1 (1–30%), 2 (31–70%), and 3 (71–100%). These two scores for each tumor sample were multiplied to obtain a final score ranging from 0 to 9. Tumor samples with scores ≥ 6 were considered SPOCK2-positive, and those with scores ≤ 6 were considered SPOCK2-negative.

2.3 Quantitative real-time PCR

RNA/cDNA preparation and the quantitative real-time PCR assays were performed as described previously (Xu et al. 2018). The primer sequences used were as follows: SPOCK2- Forward: 5'-GGGATTGAGCACCCCTGTACT-3', Reverse: 5'-CTAGCTGTCCAACAGGCAGA-3'; β-actin- Forward: 5'-ATAGCACAGCCTGGATAGCAACGTAC-3', Reverse: 5'-CACCTTCTACAATGAGCTGCGTGTG-3'.

2.4 Cell culture and treatment

The lung cancer cell lines A549, H1299, Calu1, H292, H460, H661, and SK-MES-1 were purchased from the Cell Bank of the China Academy of Sciences (Shanghai, China), and normal bronchial epithelial HBE cells were procured from American Type Culture Collection (ATCC; Manassas, VA, USA). A549, H1299, Calu1, H292, H460, and H661 cells were cultured in RPMI 1640 medium (Gibco, Waltham, MA, USA), SK-MES-1 cells were cultured in minimal essential medium (Gibco) containing 1.5 g/L NaHCO₃ and 0.11 g/L sodium pyruvate, and HBE cells were cultured in DMEM (Gibco) containing 1.5 g/L NaHCO₃. All media were supplemented with 10% FBS (CLARK Bioscience, Richmond, VA, USA). The cells were maintained in a 5% CO₂ humidified incubator at 37°C.

A549 and H1299 cells were transfected using Lipofectamine 3000 (Invitrogen, Waltham, MA, USA) according to the manufacturer's instructions. The cells were transfected with SPOCK2-specific siRNA or scrambled control siRNA (GenePharma, Shanghai, China) for 48 h to generate the SPOCK2 knockdown model, whereas for SPOCK2 overexpression, the cells were transfected with a pCNA3.0-myc-DDK-SPOCK2 plasmid or the corresponding empty pCNA3.0-myc-DDK vector.

For the demethylation studies, the cells were treated with 2 μM decitabine (prepared in DMSO; Sigma-Aldrich) for 24 h; the control cells were treated with the same volume of DMSO.

2.5 Immunocytochemistry

Cells were prepared for ICC and the immunocytochemistry as described previously (Xu et al. 2018). Samples were first probed with anti-SPOCK2 rabbit polyclonal antibody (1:100; Sigma) at 4°C for 18 h and then with FITC-labeled goat anti-rabbit IgG (1:100, Zsbio, Beijing, China) at 25°C for 2 h.

2.6 Western blotting

Cell were prepared for western blotting as described previously (Xu et al. 2018). All the primary antibodies used are shown in Table 1.

Table 1
List of antibodies used for western blotting in the study

Antibody name	Source	Catalog number	Host	Dilution
SPOCK2	Sigma-Aldrich	HPA030464	Rabbit	1:500
GAPDH	Beyotime	AF0006	Mouse	1:1000
RhoA	Cell Signaling Technology Inc.	2117	Rabbit	1:500
RhoC	Cell Signaling Technology Inc.	3430	Rabbit	1:500
Rock1	Cell Signaling Technology Inc.	4035	Rabbit	1:500
MMP2	Cell Signaling Technology Inc.	40994	Rabbit	1:500
MMP9	Cell Signaling Technology Inc.	13667	Rabbit	1:500
CyclinD1	Cell Signaling Technology Inc.	2978	Rabbit	1:500
p21	Cell Signaling Technology Inc.	2947	Rabbit	1:1000
c-myc	Cell Signaling Technology Inc.	13987	Rabbit	1:500
E-Cadherin	Cell Signaling Technology Inc.	3195	Rabbit	1:500
Jagged1	Cell Signaling Technology Inc.	2620	Rabbit	1:500
Notch1	Cell Signaling Technology Inc.	3608	Rabbit	1:500
NICD	Wanlei Bio	wl03097a	Rabbit	1:1000
DLL4	Cell Signaling Technology Inc.	96406	Rabbit	1:500
HES1	Cell Signaling Technology Inc.	11988	Rabbit	1:500
MEK	Cell Signaling Technology Inc.	4694	Rabbit	1:500
p-ERK1/2	Cell Signaling Technology Inc.	4370	Rabbit	1:500
p-JNK	Proteintech	80024	Rabbit	1:500
p-p38	Cell Signaling Technology Inc.	4511	Rabbit	1:500
p-YAP	Cell Signaling Technology Inc.	13619	Rabbit	1:500
β-Catenin	Cell Signaling Technology Inc.	8480	Rabbit	1:500
CTGF	Cell Signaling Technology Inc.	86641	Rabbit	1:500
CyclinE	Cell Signaling Technology Inc.	4129	Mouse	1:500
Anti-mouse IgG	Cell Signaling Technology Inc.	7076	Horse	1:1000
Anti-rabbit IgG	Cell Signaling Technology Inc.	7074	Goat	1:1000

2.7 Cell proliferation and colony formation assays

The cell proliferation and colony formation assays were performed as described previously (Xu et al. 2018).

2.8 Cell migration and invasion analysis

The cell migration and invasion assays were carried out in 24-well Transwell chambers containing inserts with a pore size of 8- μm (Costar, Washington, DC, USA). For the invasion assay, the inserts were coated with 100 μL of Matrigel (1:9 dilution; BD Bioscience). Cells were trypsinized 24 h after transfection, and 5×10^4 cells resuspended in 100 μL of a medium supplemented with 2% FBS were transferred to the upper Transwell chamber, whereas 600 μL of the medium supplemented with 10% FBS was added to the lower chamber. After incubation for 24 h, cells on the upper membrane surface were removed with a cotton tip, and those that passed through the membrane were fixed with polyformaldehyde and stained with hematoxylin. The number of migrated/invasive cells was counted in 10 randomly selected fields under a microscope at high magnification. All experiments were repeated at least three times independently under identical conditions.

2.9 Tumor formation in nude mice

All experiments with nude mice were performed according to the experimental animal ethics guidelines of China Medical University. The animal care and experimental protocols were approved by the Medical Research Ethics Committee of China Medical University (CMU2020406).

Four-week-old female BALB/c nude mice were procured from Slac (Shanghai, China). The mice were housed in a laminar-flow cabinet under specific pathogen-free conditions for 1 week before the experiments. Each mouse was transplanted either subcutaneously in the right axilla with 1×10^7 tumor cells (SPOCK2-transfected H1299 cells or the corresponding vector-transfected control cells) resuspended in 0.2 mL of sterile phosphate-buffered saline (PBS) or subjected to tail vein injection with 1×10^6 tumor cells resuspended in 0.1 mL of PBS. The mice were executed four and eight weeks after axilla and tail vein injection, respectively, and autopsies were performed to examine tumor growth and dissemination. A portion of tissue from the tumor and each organ was fixed in 4% formaldehyde (Sigma) and embedded in paraffin. Serial 4 μm thick sections were cut and stained with hematoxylin and eosin. The stained sections were examined microscopically.

2.10 Gene set enrichment analysis (GSEA)

GSEA was performed using GSEA v2.2.2 (www.Broadinstitute.org/gsea). The SPOCK2 expression level was stratified into low and high categories to annotate the phenotype, and we used lung cancer-related gene sets from KEGG and GO. All other parameters were set based on their default values.

2.11 Statistical analysis

SPSS 16.0 software was used for data analysis. The correlations between SPOCK2 expression and clinicopathological features were determined using the Chi-square test, and the differences between cell

groups were analyzed using paired *t*-tests. Results with a two-sided *P* value < 0.05 were considered significant.

3 Results

3.1 Clinical significance of SPOCK2 downregulation

Analysis of SPOCK2 protein expression by immunocytochemistry indicated that SPOCK2 is localized in both the cytoplasm and the nuclei of A549, H1299, Calu1, H292, H460, H661, and SK-MES-1 cell lines and normal bronchial epithelial HBE cells (Fig. 1A).

Overall, 40 pairs of clinical lung cancer tissues and corresponding normal lung tissue samples were collected for RNA extraction. SPOCK2 mRNA expression levels were detected by real-time quantitative PCR. SPOCK2 mRNA expression levels in the lung cancer tissues were significantly lower than those in the corresponding normal lung tissues (single sample paired *t*-test, *P* = 0.0176) (Fig. 1B).

Western blotting showed that SPOCK2 protein expression was lower in 14 of the 16 lung cancer tissues than in the matched normal tissues (Fig. 1C). Immunohistochemical analysis in 262 patients showed that SPOCK2 was expressed at low levels in clinical lung cancer tissues, whereas in normal bronchi and alveoli, SPOCK2 was expressed at high levels. In the correlation analysis (cross-table analysis) with clinicopathological factors, SPOCK2 expression did not correlate with age (*P* = 0.242) or sex (*P* = 0.319), but was positively correlated with histological type (*P* = 0.045) and differentiation (*P* = 0.000), and inversely correlated with tumor size (*P* = 0.000), lymph node metastasis (*P* = 0.000), and p-TNM stage (*P* = 0.000). Statistical analyses are shown in Table 2. The data from SPOCK2 differential expression analyses are shown in Fig. 1D. Immunohistochemistry of 156 clinical samples showed that low SPOCK2 expression is associated with poor prognosis (Fig. 1E).

Table 2
Association of SPOCK2 expression with clinical and pathological characteristics of 262 NSCLC patients

Clinicopathological characteristics	Total N	SPOCK2-positive	SPOCK2-negative	Pvalue
Age (years)				
≤ 60	148	62	86	
> 60	114	42	72	0.242
Gender				
Male	157	60	97	
Female	105	44	61	0.319
Histological type				
Squamous cell carcinoma	108	50	58	
Adenocarcinoma	154	54	100	0.045
Differentiation				
Well-Moderate	83	57	26	
Poor	179	47	132	0.000
Tumor size (cm)				
≤ 3	123	64	59	
> 3	139	40	99	0.000
Lymph node metastasis				
Negative	137	69	68	
Positive	125	35	90	0.000
TNM stage				
I	101	56	45	
II-III	161	48	113	0.000

3.2 SPOCK2 methylation correlates with poor prognosis

Given the methylation of *SPOCK2* in endometrial carcinoma (Ren et al. 2011a, 2014b), we analyzed whether the low expression of *SPOCK2* in NSCLC is also associated with methylation. First, we analyzed the methylation level of *SPOCK2* in 830 NSCLC and 74 normal lung tissue samples using the sample information hosted in the TCGA. The expression level of *SPOCK2* was negatively correlated with its

methylation level (Fig. 1F). The methylation level of *SPOCK2* in tumor samples was significantly higher than that in normal lung tissues (Fig. 1G). A high level of *SPOCK2* methylation was associated with poor prognosis of NSCLC (Fig. 1H).

3.3 SPOCK2 inhibits the proliferation of lung cancer cells both *in vitro* and *in vivo*

26. To study the role of *SPOCK2* in NSCLC, we first investigated its expression in various cancer cell lines (Fig. 2A). There was high expression of *SPOCK2* in H661 cells, low expression in Calu1, H292, H460, and SK-MES-1, and moderate expression in A549 and H1299 cell lines. We selected A549 and H1299 to investigate the role of *SPOCK2* in the cell lines. *SPOCK2* was knocked down using siRNA transfection and upregulated by transfection with the pcMV6-myc-DDK-SPOCK2 plasmid. All *in vitro* experiments were carried out under the ideal conditions for ensuring interference and transfection efficiency (Fig. 2B). We analyzed the effect of *SPOCK2* expression on colony formation as a read out of its effect on cell proliferation. In A549 and H1299 cells, *SPOCK2* downregulation promoted colony formation, whereas *SPOCK2* upregulation inhibited colony formation (Fig. 2C). In the MTT assay, *SPOCK2* overexpression inhibited cell proliferation (Fig. 2D). Analysis of the proteins involved in cell proliferation by western blotting indicated that the expression of cyclin D1 (Pestell et al. 1999; Sherr 1996), CDK2 (Morgan 1995), and C-myc (Grandori et al. 2000) was downregulated upon *SPOCK2* overexpression, whereas that of p21 (Pestell et al. 1999) was upregulated; *SPOCK2* downregulation had the opposite effect (Fig. 3E). In subcutaneous tumorigenicity experiments, animals transplanted with A549 and H1299 cells stably transfected with *SPOCK2* exhibited significantly lower tumor volume than those in the control group (Fig. 2F and G).

3.4 SPOCK2 inhibits the migration and invasion of lung cancer cells both *in vitro* and *in vivo*

As *SPOCK2* expression is correlated with lymph node metastasis, we speculated that *SPOCK2* is related to the migration and invasion of lung cancer cells. In both A549 and H1299 cells, Transwell assays showed that *SPOCK2* expression was associated with the promotion of migration (Fig. 3A) and invasion (Fig. 3B). Variations in *SPOCK2* protein levels also affect the expression of proteins involved in migration and invasion (Coussens et al. 2002; McCormack et al. 2013; Totsukawa et al. 2000). The expression of RhoA, Rock1, MMP2, and MMP9 was downregulated, whereas that of RhoC and E-cadherin was upregulated by *SPOCK2* overexpression, whereas *SPOCK2* knockdown caused the opposite effects (Fig. 3C). In nude mice tail vein tumorigenesis experiment, animals transplanted with H1299 cells stably expressing *SPOCK2* exhibited less tumorigenesis in the lung (Fig. 3D-i and E) as well as in the liver (Fig. 3D-ii). Collectively, these findings demonstrate that *SPOCK2* inhibits the migration and invasion of lung cancer cells.

3.5 GSEA of *SPOCK2* in lung cancer

To identify the potential function of SPOCK2, we performed GSEA using TCGA data. Pathways, including “KEGG_MAPK_SIGNALING_PATHWAY,” “KEGG_NOTCH_SIGNALING_PATHWAY,” “KEGG_VEGF_SIGNALING_PATHWAY” (Fig. 4A), “GO_ERK1_AND_ERK2 CASCADE,” “GO_JNK CASCADE,” “GO NOTCH BINDING,” “GO_POSITIVE REGULATION_OF_NOTCH_PATHWAY,” and “GO_HIPPO_SIGNALING” (Fig. 4B), were significant in the SPOCK2 high expression phenotype. In addition, the regulation of non-small cell lung cancer (NSCLC), small cell lung cancer, cell cycle, tight junction, cell adhesion junction, cell-substrate adhesion were reflected in the results of GSEA (Fig. 4A and B).

GSEA showed enrichment of many core elements of these pathways; we integrated and explored the expression and change of these specific pathways. Proteins related to the cell cycle, including CyclinD1, CDK4 (Fig. 2E), and CyclinE1 (Fig. 4E), were downregulated upon SPOCK2 overexpression. Proteins related to cell adhesion, including RhoA, Rock1, MMP2, and MMP9, were downregulated, whereas RhoC and E-Cadherin were upregulated upon SPOCK2 overexpression (Fig. 3C). After SPOCK2 overexpression, Notch1, Jagged1, DLL4, NICD, and HES1 were downregulated (suppression of the NOTCH signaling pathway; Fig. 4C), p-ERK1/2 and p-JNK were downregulated, p-p38 was expressed stably (MAPK pathway; Fig. 4D), p-YAP (Hippo-related protein) was upregulated, and β-catenin, CTGF, and CyclinE were downregulated (Fig. 4E). All changes were reversed in the SPOCK2 interference group in A549 as well as in H1299 cell lines.

3.6 *SPOCK2* methylation in NSCLC

Given the methylation of *SPOCK2* in TCGA data, we treated our NSCLC cell lines with the demethylation drug decitabine (HY-A0004, MCE, NJ, USA) (Gangat et al. 2016; Hagemann et al. 2011; Lai et al. 2018). In each cell line, the *SPOCK2* mRNA levels in the experimental group were higher than those in the control group after decitabine treatment (Fig. 4F). Decitabine is a broad-spectrum anti-methylation drug, which cannot reverse the methylation of *SPOCK2*, but the results can still reflect the methylation of *SPOCK2* in NSCLC cell lines.

4 Discussion

In this study, we revealed the relationship between *SPOCK2* and the clinicopathology of NSCLC. A high expression of *SPOCK2* was found to be negatively correlated with tumor size, lymph node metastasis, and pTNM stage, whereas low *SPOCK2* expression indicated poor prognosis. Furthermore, we found that *SPOCK2* can negatively regulate the proliferation, migration, and invasion of NSCLC cells both *in vitro* and *in vivo*.

It has been previously reported that *SPOCK2* expression increases during the transformation from AT2 to AT1 cells (Johansson et al. 2014); thus, *SPOCK2* plays a positive regulatory role in the process of relative stem cell differentiation into mature cells. During tumorigenesis, a process involving immature cells, we found that *SPOCK2* expression was low. Furthermore, low *SPOCK2* expression correlated with

differentiation. Thus, we showed that SPOCK2 has the ability to inhibit cell proliferation, migration, and invasion in NSCLC.

The results of GSEA are consistent with our experiments. SPOCK2 acts as a tumor suppressor gene in NSCLC by downregulating p-ERK1/2, p-JNK, and Notch signaling pathways and promoting the Hippo signaling pathway. These signaling pathways interact to form a network, which can jointly affect the proliferation, migration, and invasion of NSCLC by acting on target proteins, i.e., CyclinD1, CDKs, p21, RhoA, E-Cadherin, and MMPs. These findings indicate the significance of SPOCK2 in NSCLC.

However, the reason for the low expression of SPOCK2 in NSCLC is still unknown. We thus explored the level of *SPOCK2* methylation using TCGA. The methylation level of *SPOCK2* in NSCLC was significantly higher than that in the normal lung tissues, and hypermethylation of *SPOCK2* has been shown to be associated with poor prognosis. This high methylation level, i.e., low *SPOCK2* level, corresponds to our results, indicating that low SPOCK2 mRNA and protein expression was associated with poor prognosis.

Decitabine is an effective demethylation agent used in the treatment of MDS (Gangat et al. 2016). The introduction of decitabine to inhibit methylation strongly suggested the methylation of SPOCK2. However, it remains unclear how SPOCK2 methylation occurs. Whether the elimination of methylation can reverse the occurrence and development of NSCLC is also worthy of follow-up study.

In summary, our findings indicate that SPOCK2 plays a critical role in regulating the progression of NSCLC through the inhibition of cell proliferation, migration, and invasion both *in vitro* and *in vivo*. This inhibition may be counteracted by the methylation of SPOCK2 in NSCLC.

Declarations

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Disclosure Statement

The authors have no conflict of interest.

Availability of data and material Author Contributions

YX designed the study, conducted experiments, acquired and analyzed data, and wrote the manuscript. CW, JJ, YZ, XJ, HS, and HR conducted the experiments and acquired data. XQ was responsible for the conception and supervision of the study and wrote the manuscript. All authors corrected drafts and approved the final version of the manuscript.

Ethics approval

This study was approved by the Medical Research Ethics Committee of China Medical University. The animal care and experimental protocols were approved by the Medical Research Ethics Committee of the China Medical University (CMU2020406).

Consent to participate

Informed consent was obtained from all patients.

Consent for publication (include appropriate statements)

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Figures

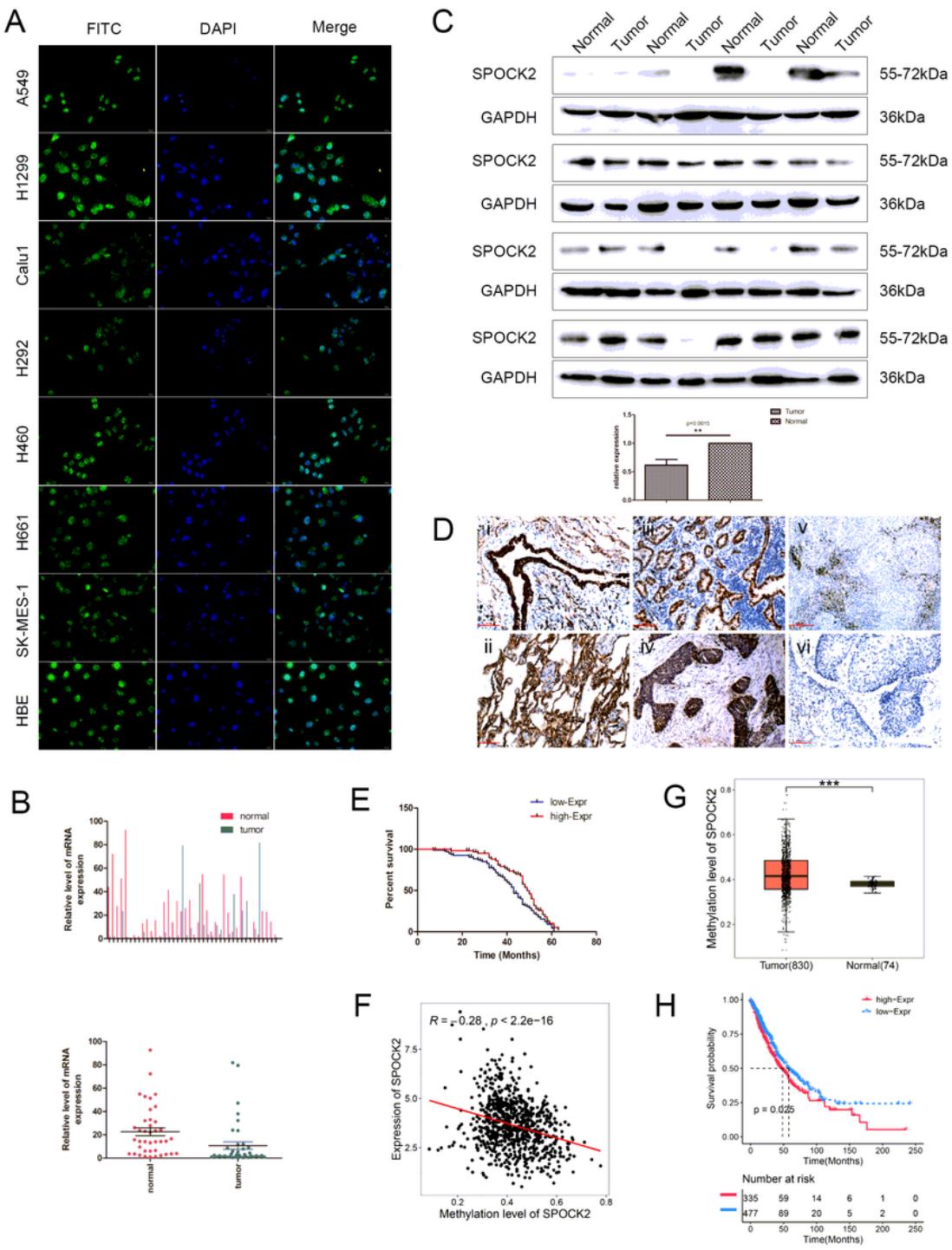


Figure 1

Expression of SPOCK2 in NSCLC is associated with poor prognosis (a) Immunofluorescence assays were performed to detect SPOCK2 localization in NSCLC cell lines and human normal bronchial epithelial cells (b) Relative expression of SPOCK2 mRNA in 40 paired NSCLC samples (red bars) and adjacent normal lung tissue (green bars) (c) SPOCK2 protein expression in 16 lung cancer tissues compared to that in the matched normal tissues (d) SPOCK2 protein expression analyzed by immunohistochemistry in normal

bronchial epithelial cells (i) and alveolar (ii), well-differentiated adenocarcinoma (iii), and squamous cell carcinoma (iv), poorly differentiated adenocarcinoma (v), and squamous cell carcinoma (vi).

Magnification 200 \times (e) Survival of patients with NSCLC with high and low SPOCK2 expression (f) The expression level of SPOCK2 and its methylation level (g) The methylation level of SPOCK2 in tumor samples and normal lung tissues (h) Survival of patients with NSCLC with high and low SPOCK2 methylation

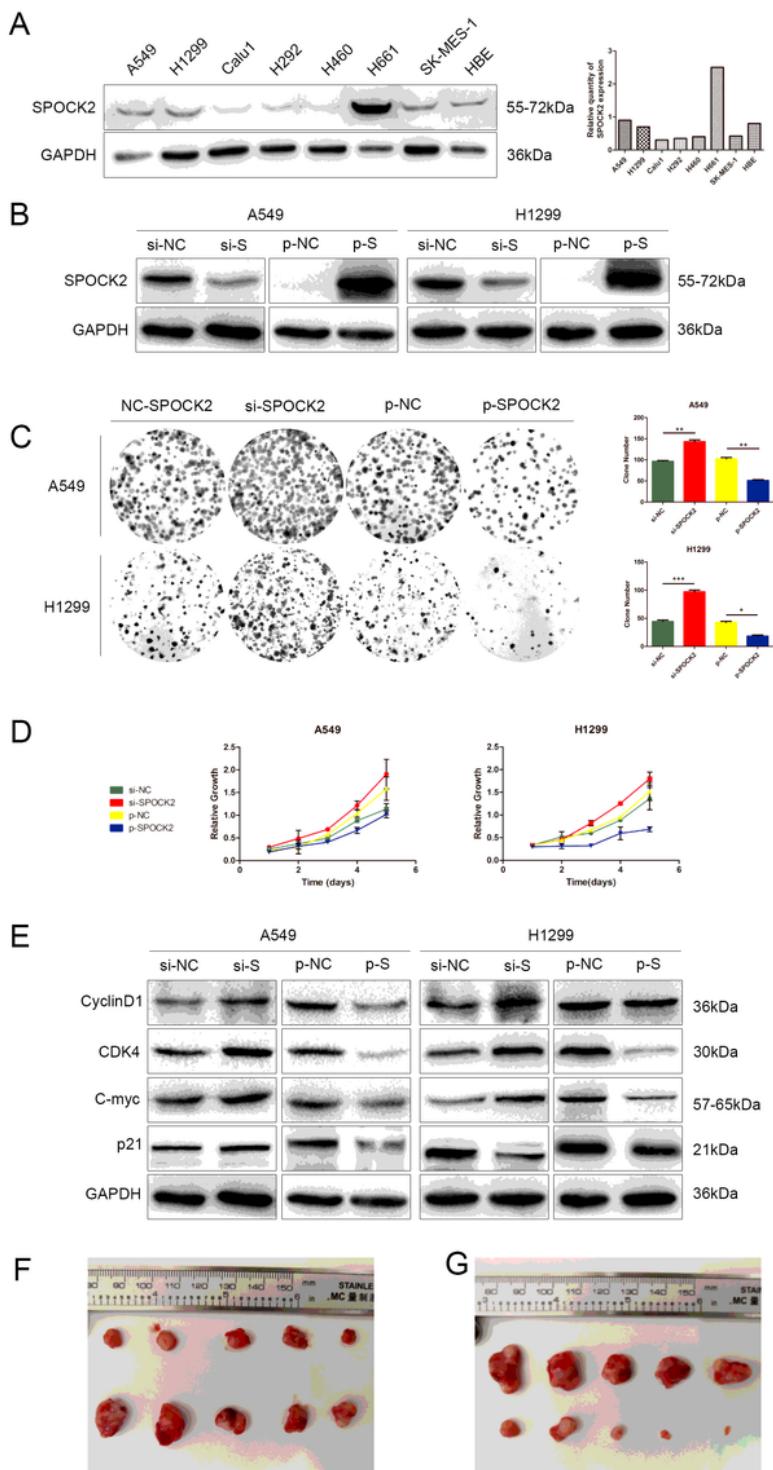


Figure 2

Effect of SPOCK2 expression on NSCLC proliferation (a) SPOCK2 level in HBE cells and seven NSCLC cell lines by western blotting (b) Interference and transfection efficiency of SPOCK2 in A549 and H1299 cell lines (c) Colony formation assay. A549: si-SPOCK2 vs. control siRNA, P = 0.003, p-SPOCK2 vs. control, P = 0.003, H1299: si-SPOCK2 vs. control siRNA, P = 0.000, p-SPOCK2 vs. control, P = 0.022 (d) MTT assay. A549: si-SPOCK2 vs. control siRNA, P = 0.002, p-SPOCK2 vs. control, P = 0.039. H1299: si-SPOCK2 vs. control siRNA, P = 0.0022, p-SPOCK2 vs. control, P = 0.0073 (e) Changes in the expression of cell proliferation-related proteins in A549 and H1299 cells (f) Xenograft tumor volumes were negatively affected by SPOCK2 in A549 cells, the upper row was the SPOCK2(+) group, and the lower row was the control group (g) Xenograft tumor volumes were negatively affected by SPOCK2 in H1299 cells, the upper row was the control group, and the lower row was the SPOCK2(+) group

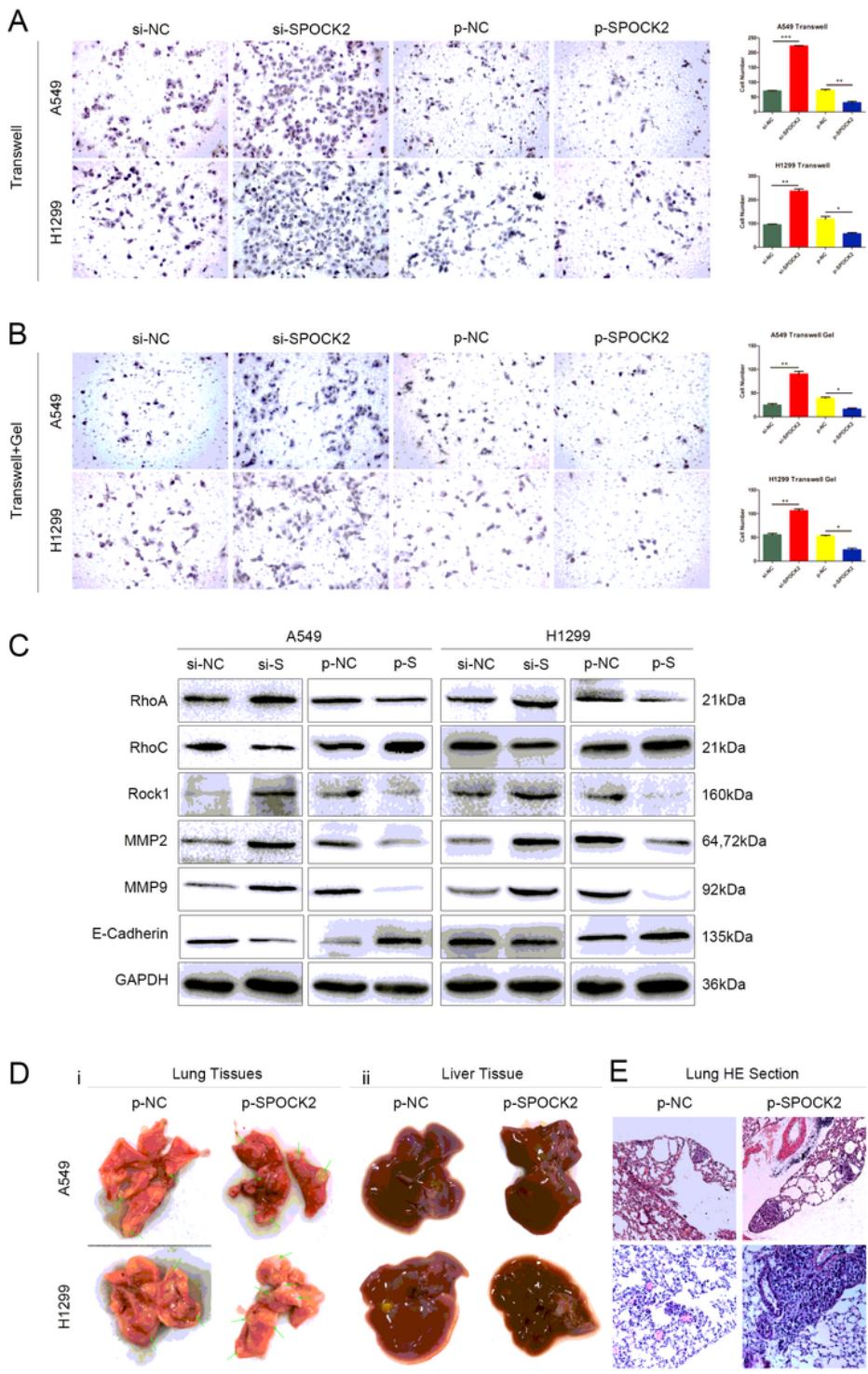
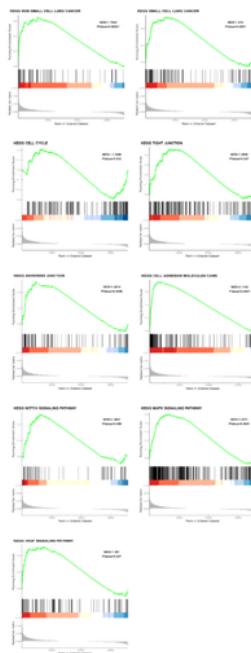


Figure 3

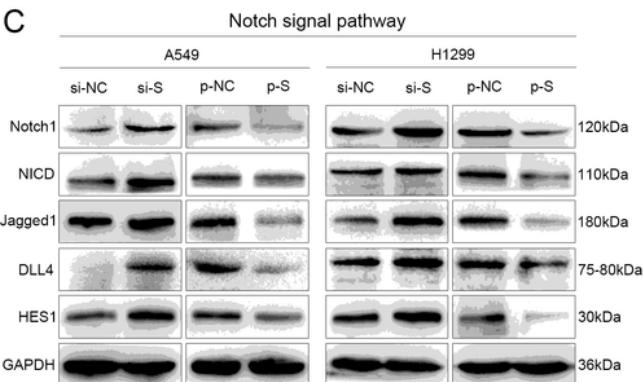
Effect of SPOCK2 expression on the migration and invasion of NSCLC cells (a) Cell migration analyzed by the Transwell migration assay; cells that migrated to the lower chamber were stained with hematoxylin and counted. *P < 0.05; **P < 0.01. A549 migration: A549: si-SPOCK2 vs. control siRNA, P = 0.000, p-SPOCK2 vs. control, P = 0.001, H1299: si-SPOCK2 vs. control siRNA, P = 0.002, p-SPOCK2 vs. control, P = 0.042 (b) Cell invasion analyzed by the Transwell assay. A549: si-SPOCK2 vs. control siRNA, P = 0.005, p-

SPOCK2 vs. control, P = 0.031, H1299: si-SPOCK2 vs. control siRNA, P = 0.002, p-SPOCK2 vs. control, P = 0.031 (c) Expression of SPOCK2 and cell migration- and invasion-related proteins in A549 and H1299 (d) Nude mice tail vein tumorigenesis experiment. Animals transplanted with cells stably expressing SPOCK2 exhibited less tumorigenesis in the lung (i) and liver (ii). (e) HE section of lung tumor, magnification 200 \times

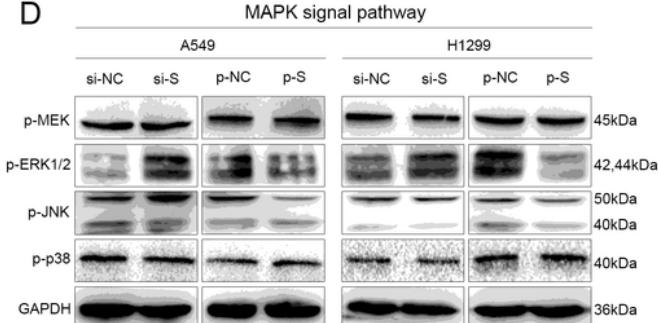
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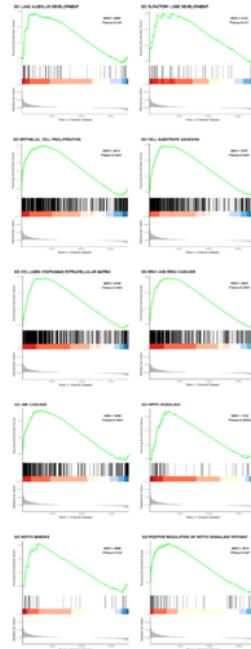
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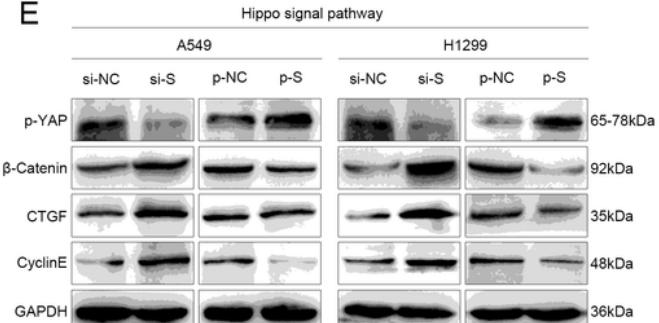
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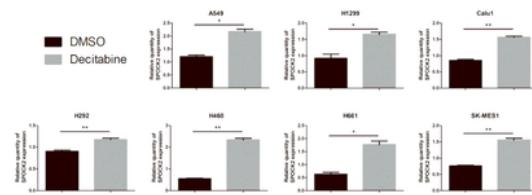


Figure 4

The expression level of SPOCK2 in NSCLC (GSEA) and the expression level after demethylation (a) Figures were derived from a gene set in GSEA showing the expression level of SPOCK2 in the KEGG database (b) Figures were derived from a gene set in GSEA showing the expression level of SPOCK2 in the GO database (c) Expression of SPOCK2 and Notch signaling pathway intermediaries in A549 and H1299 cell lines (d) Expression of SPOCK2 and MAPK signaling pathway intermediaries in A549 and H1299 cell lines (e) Expression of SPOCK2 and Hippo signaling pathway intermediaries in A549 and H1299 cell lines (f) SPOCK2 mRNA increased after decitabine treatment. A549: P = 0.014, H1299: P = 0.0216, Calu1: P = 0.0036, H292: P = 0.0074, H460: P = 0.0013, H661: P = 0.0267, SK-MES-1: P = 0.0025