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SPOCK1 is a novel transforming growth factor-β target gene that regulates lung cancer cell epithelial-mesenchymal transition

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ABSTRACT

Lung cancer is the leading cause of cancer related death worldwide and the prognosis is still poor with 5-year survival of approximately 15%. Metastasis is the leading cause of death by cancer. Recent researches have demonstrated that epithelial-to-mesenchymal transition (EMT) plays a key role in the early process of metastasis of cancer cells. Here, we identified that SPARC/osteonectin, cwcv and kazal-like domains proteoglycan 1 (SPOCK1) is a novel transforming growth factor-β1 (TGF-β) target gene that regulates lung cancer cell EMT. TGF-β has been reported as a major inductor of EMT. We observed that the expression of SPOCK1 in lung cancer tumor tissues is significantly higher than matched normal lung tissues. Moreover, the expression of SPOCK1 was also significantly higher in metastasis tumor tissues than non-metastasis tumor tissues. Levels of SPOCK1 mRNA were increased among patients with shorter disease-free survival times, indicating the potential role of SPOCK1 in lung cancer progression and metastasis. Silencing SPOCK1 expression with endoribonuclease-prepared small interfering RNA (esiRNA) in lung cells inhibits lung cancer cell growth, colony formation and invasion *in vitro*. Interestingly, ectopic expression of SPOCK1 in epithelial lung cancer cells induced EMT with increased expression of the mesenchymal marker Vimentin and decreased expression of epithelial marker E-cadherin. We also found that the expression of SPOCK1 was increased under treatment of TGF-β, indicating that SPOCK1 is a novel downstream target of TGF-β. Taken together, our study showed that SPOCK1 is a novel metastasis related biomarker in lung cancer and may be new diagnostic and therapeutic target for lung cancer.

1. Introduction

Lung cancer is the leading cause of cancer-related death, both in the United States and worldwide; however, the prognosis is still poor with a 5-year survival rate of approximately 15% [1]. Despite improvements in early diagnostic modalities, around 50% of lung cancer patients present with local or systemic advanced disease [2]. Moreover, the majority of cancer patients die of metastasis rather than the primary disease.

Recent studies have found that epithelial-to-mesenchymal transitions (EMT) play a key role in the early process of tumor metastasis. EMTs are trans-differentiation programs that are required for tissue morphogenesis during embryonic development [3,4]. The EMT process can be regulated by a number of cytokines and growth factors, such as transforming growth factor (TGF)-β, whose expressions are dysregulated during malignant tumor progression [5].

At the transcriptional level, a diverse list of embryonic transcription factors, such as E-cadherin repressing Zinc finger proteins Snail (SNAI1), and Slug (SNAI2), ZEB1 (δEF1) and ZEB2 (SIP1), the basic helix-loop-helix proteins TWIST1 and TWIST2, and the winged-forkhead transcription factor FOXC2, can be potently activated by TGF-β, resulting in epithelial-to-mesenchymal transitions. However, other EMT core signature makers, such as SPOCK1, have never been reported in lung cancer cells. Even less is known about the function and mechanism by which SPOCK1 contributes to the growth and progression of lung cancer cells.

SPOCK1 encodes a matricellular glycoprotein family member. Other members of this family include SPARC, TESTICAN-2 and TESTICAN-3. Among these, SPARC has been well documented; it has been demonstrated to regulate proliferation, apoptosis, adhesion and cell–matrix interaction in various cancers [6]. In view of the similarity between SPOCK1 and SPARC in structure, it is of great interest to investigate the role of SPOCK1 in the development and progression of cancer. More interestingly, a number of studies

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have demonstrated that SPOCK1 plays a critical role in prostate cancer recurrence, glioblastoma invasion and HCC progression [7–9], indicating that SPOCK1 may also be involved in the invasion of lung cancer. Here, we reported that the silencing of SPOCK1 can inhibit the lung cancer cell growth rate and colony formation in soft agar. Furthermore, the silencing of this protein decreases the invasion ability of lung cancer cells. We also found that the inductor of EMT–TGF-β treatment can induce the expression of SPOCK1, which indicates that SPOCK1 may play a role in epithelial-to-mesenchymal transition in lung cancer.

2. Materials and methods

2.1. Patients, specimens and cell lines

NSCLC samples (40 samples) and their adjacent non-tumor lung tissues were collected from patients who underwent lobectomy at Drum Tower Hospital Affiliated to Nanjing University (Nanjing, China). None of these patients received preoperative chemotherapy or radiotherapy. The samples used in this study were approved by the Committees for Ethical Review of Research Involving Human Subjects at the Drum Tower Hospital Affiliated to Nanjing University. The mean age of the 40 NSCLC patients included in this study was 58 years (range 32–78). The median follow-up period was 32.4 months (range 2–87.6). A total of 55% (22/40) of NSCLC patients were smokers or ex-smokers. Normal lung tissues were obtained from the lung that had undergone surgery (without any lung cancer disease). The NSCLC cell lines A549 and H1975 were obtained from the American Type Culture Collection (ATCC) and cultured in Dulbecco's modified Eagle's medium (DMEM) medium (Invitrogen, CA) with 10% FBS and 100 U/ml penicillin/streptomycin (Sigma, St. Louis, MO).

2.2. Real-Time quantitative reverse transcription PCR (RT-qPCR)

Total RNA from cell lines was extracted using TRIzol reagent (Invitrogen, CA). The concentration of isolated total RNA was measured by a NanoDrop ND-1000 Spectrophotometer (Agilent, CA). For mRNA detection, the total RNA was reverse-transcribed using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, CA). The qPCR was performed using the SoFast™ EvaGreenH Supermix (Bio-Rad). HPRT1 was used as the internal control.

2.3. MTS assays

A549 and H1975 cells were transfected with the esiRNA of SPOCK1 (esiSPOCK1) or esiControl (Sigma) using Lipofectamine RNAiMAX (Invitrogen, life Technologies, Carlsbad, CA, USA). 24 h after transfection, transfected cells were seeded into 96-well plates at a density of 5×10^3 per well (100 μl). For the MTS assay, the CellTiter 96® AQueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI, USA) was used following the manufacturer's instructions. Briefly, 2 h before each of the desired time points (1–5 days), 20 μl of the MTS reagent was added into each well and cells were incubated at 37 °C for around 2 h. The absorbance was detected at 490 nm using a Wallac Victor 1420 Multilabel plate reader. All experiments were repeated three times.

2.4. Soft agar colony formation assay

Single transfected A549 and H1975 cells (1×10^4) were plated onto a 6-well ultra-low attachment plate (Corning, Corning, NY) in serum-free DMEM-F12, supplemented with 2 mM L-glutamine, 1% sodium pyruvate, 1% MEM nonessential amino acids, 100 μg/ml penicillin, and 100 U/ml streptomycin supplemented with 20 ng/ml epithelial growth factor and 10 ng/ml fibroblast growth factor-2 (Invitrogen). After 2–3 weeks of culture, the number of spheres (diameter >40 μm) was manually counted in three randomly selected fields at a magnification of 40× under an inverted fluorescence microscope. This assay was performed in triplicate in three independent experiments.

2.5. In vitro Matrigel invasion assay

The cell invasiveness was assessed with the use of BioCoat Matrigel Invasion Chambers (BD Biosciences, Bedford, MA). Medium with 10% FBS was added to the lower chamber as a chemoattractant. Then, equal numbers of transfected A549 and H9175 cells were resuspended in 500 μl serum-free medium and seeded into the rehydrated insert. After 24 h of incubation at 37 °C, non-invading cells on the upper surface of the Matrigel membrane were gently removed with a cotton-tipped swab. The cells were then fixed with 100% methanol and stained with 1% toluidine blue (Sigma). The stained invasive cells on the lower surface of the membrane were photographed under an inverted light microscope (40× objective) and quantified by manual counting in three randomly selected areas. This experiment was performed in duplicate in three independent experiments.

2.6. Immunofluorescence analysis

The stable cells were seeded in the BD Falcon™ 8-well CultureSlide and incubated with primary antibodies against E-cadherin, or Vimentin and then incubated with Alexa Fluor® 594 Goat Anti-Rabbit IgG (Invitrogen). The culture slides were counterstained with Hoechst 33342 and imaged with a confocal laser-scanning microscope (Carl Zeiss). Data were processed with Adobe Photoshop 7.0 software for analysis.

2.7. Western blotting

A549 cells transfected with pcDNA3-SPOCK1 or transfected with control cells were washed twice with PBS and solubilized in radioimmunoprecipitation assay lysis buffer. The supernatants, which contained the whole-cell protein extracts, were obtained after centrifugation of the cell lysates at 12,000*g* for 10 min at 4 °C. Heat-denatured protein samples (20 μg per lane) were resolved by SDS–poly-acrylamide gel electrophoresis (PAGE) and transferred to an Immobilon-P membrane (Millipore, Bedford, MA). The membrane was incubated for 60 min in PBS containing 0.1% Tween 20 and 5% skim milk to block nonspecific binding, followed by incubation for 1 h at room temperature with a primary antibody. The membrane was washed three times for 10 min in PBS with 0.1% Tween 20 and then incubated for 1 h with a secondary antibody. The membrane was washed thoroughly in PBS containing 0.1% Tween 20, and the bound antibody was detected with the use of enhanced chemiluminescence detection reagents (Amersham Biosciences) according to the manufacturer's instructions.

2.8. Statistical analysis

Data are presented as the mean ± standard deviation. The data were analyzed using the SPSS Windows version 12.0 software. Statistical analyses were done by analysis of variance (ANOVA) or Student's *t* test. *P* values <0.05 were considered statistically significant.

3. Results

3.1. SPOCK1 is significantly upregulation in lung cancer and associated with metastasis and survival

To investigate the clinical significance of SPOCK1 in NSCLC, expression of SPOCK1 mRNA in 40 examples of NSCLCs (tumor and corresponding non-tumor tissues) was compared by qRT-PCR. The relative expression level of SPOCK1 was significantly higher in tumor tissues compared with their non-tumor counterparts ($P < 0.001$, paired Student's t-test; Fig. 1A). In another study, SPOCK1 was found to be significantly associated with advanced clinical stage and metastasis [8]. Thus, we detected the expression of SPOCK1 in metastatic and non-metastatic tissue of 20 paired NSCLC patients. Interestingly, we found that NSCLC patients who developed metastasis after lobectomy demonstrated a significantly higher expression level of SPOCK1 than those without metastasis ($P < 0.0001$, Student's t-test, Fig. 2B), which implies that SPOCK1 may play a role in metastasis. More intriguingly, overexpression of SPOCK1 was significantly correlated with shorter disease-free survival (DSF) of patients ($P = 0.0024$; Fig. 2C).

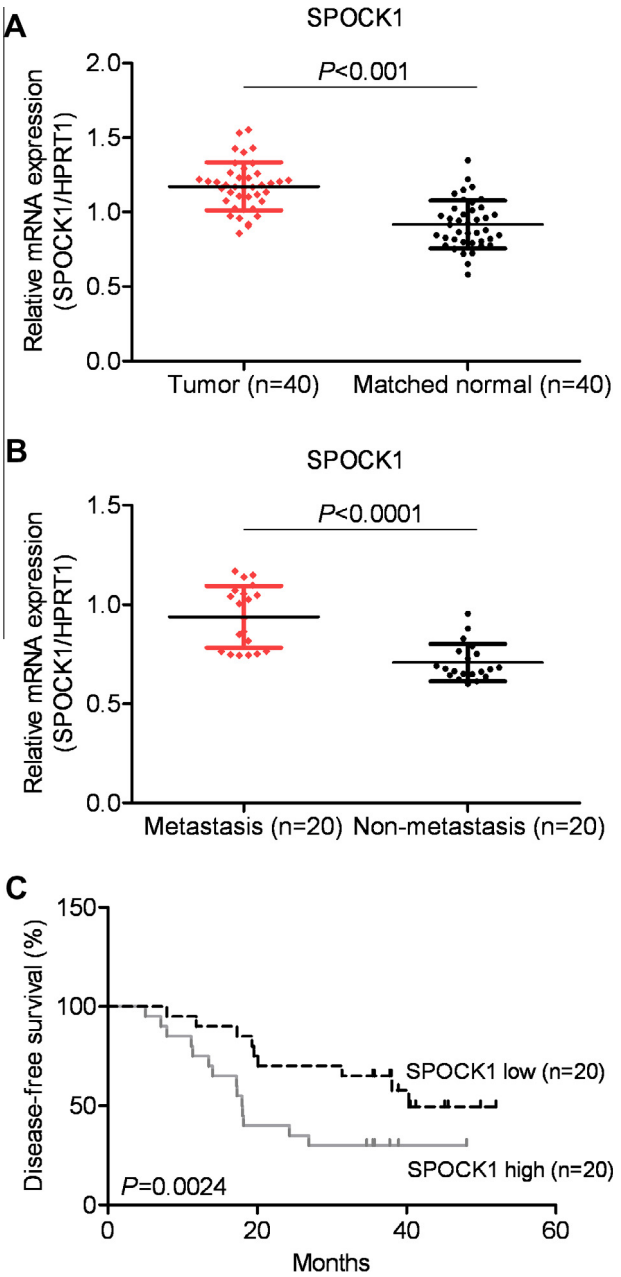

Fig. 1. SPOCK1 is significantly upregulation in lung cancer and associated with metastasis and survival. (A) The expression of SPOCK1 in 40 pairs of lung cancer tissue samples by qRT-PCR analysis. (B) The expression of SPOCK1 in 20 cases of lung cancer tissues with metastasis and non-metastasis by qRT-PCR analysis. (C) The Kaplan–Meier method was used to analyze survival in patients with lung cancer. The average value of SPOCK1 in all 40 samples was chosen as the cut-off point. The probability of patient survival: SPOCK1 high, $n = 20$; SPOCK1 low, $n = 20$ ($P = 0.0024$).

3.2. Silencing of SPOCK1 inhibits lung cancer cell growth rate and colony formation in soft agar

To explore the role of SPOCK1 in tumorigenicity, esiSPOCK1 was transfected into the NSCLC cell lines A549 and H1975. The expression of SPOCK1 in esiSPOCK1-transfected cells was confirmed by qRT-PCR analysis (Fig. 2A and B). The tumorigenic ability of SPOCK1 was assessed by cell proliferation and soft agar assays. Compared with esiControl-transfected cells, esiSPOCK1-transfected cells exhibited decreased growth rates (Fig. 2C and D), and less colony-forming abilities in soft agar (Fig. 2E and F). All of these experiments suggest that the silencing of SPOCK1 inhibits the lung cancer cell growth rate and colony formation in soft agar.

3.3. Silencing of SPOCK1 inhibits lung cancer cell invasion in vitro

To further investigate the potential effects of SPOCK1 inhibition on lung cancer metastasis, an in vitro Matrigel invasion assay was performed in A549 and H1975 cells with SPOCK1 silencing by esiSPOCK1 transfection. The Matrigel invasion assay demonstrated that the invasive capability of A549 and H1975 cells with silencing of SPOCK1 expression was significantly decreased compared with esiControl-transfected A549 and H1975 (Fig. 3A). The invaded cells number was counted and indicated in the Fig. 3B. These results indicate that SPOCK1 silencing significantly abolished lung cancer cell invasion *in vitro*.

3.4. SPOCK1 induces EMT in epithelial lung cancer cells and is a novel target of TGF-β

To identify whether silencing of SPOCK1 could abolish the invasiveness of lung cancer cells via going through EMT process. We further ectopic expression of SPOCK1 in epithelial lung cancer cell, A549. We observed that A549 cells transfected with pcDNA3-control vector displayed cobblestone-like epithelial appearance, while these cells transfected with pcDNA3-SPOCK1 showed spindle-like mesenchymal phenotype (Fig. 4A). Next, we detected the biomarkers of EMT by immunofluorescence analysis and Western blotting. Consistently, we found that A549 transfected with pcDNA-control expressed high level of E-cadherin, which is the characteristic biomarker of epithelial cells. While in A549 transfected with pcDNA3-SPOCK1, Vimentin was highly expressed, indicating the display of mesenchymal phenotype (Fig. 4C–F). Interestingly, we also observed that the expression of SPOCK1 was increased under treatment of TGF-β, indicating that SPOCK1 is a novel downstream target of TGF-β (Fig. 4B). Therefore, SPOCK1 induces EMT in epithelial lung cancer cells and is a novel target of TGF-β.

4. Discussion

SPOCK1 is a proteoglycan protein that was first isolated in human testes and initially called "TESTICAN". It is dysregluated in many organs and tissues, including the brain cartilage, vascular endothelium, lymphocytes, and neuromuscular junctions [10–12].

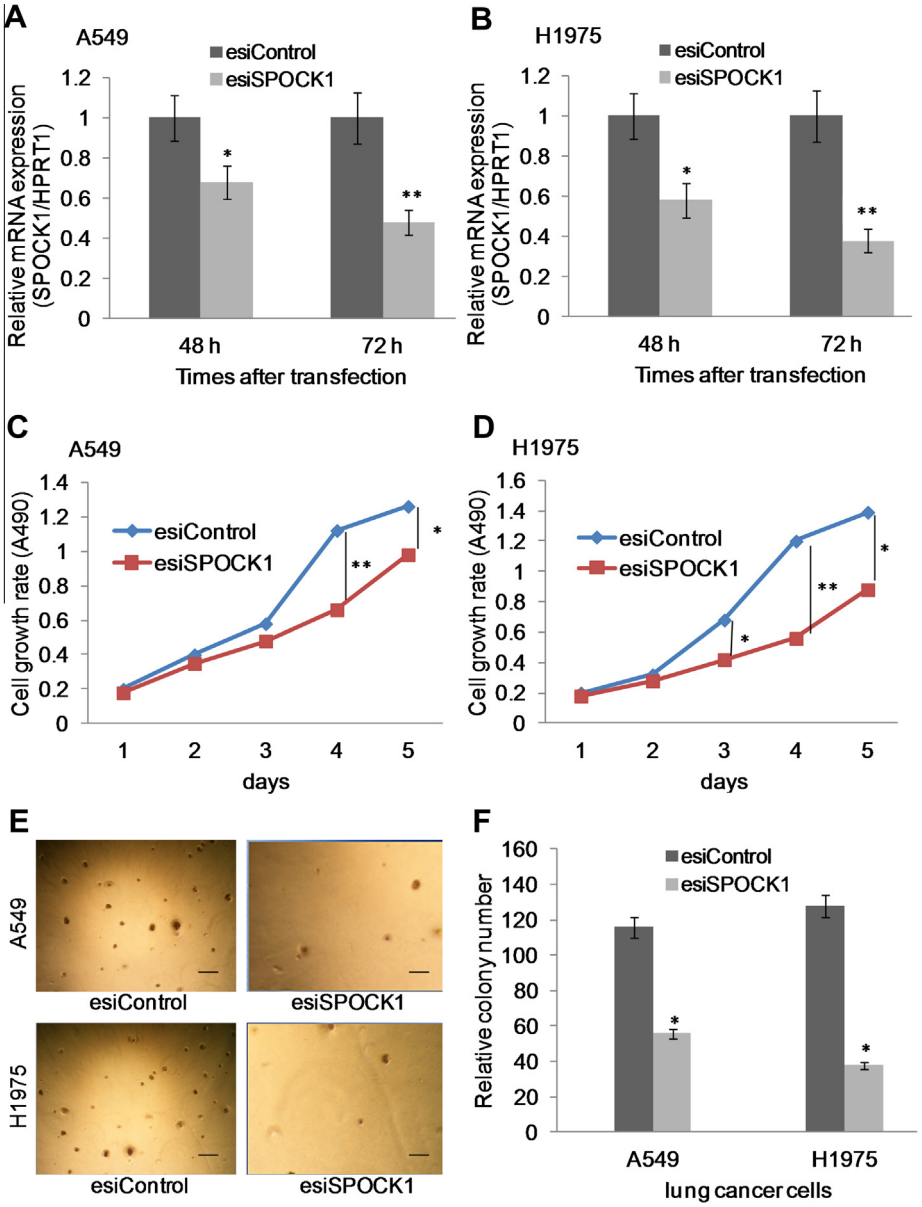

Fig. 2. Silencing of SPOCK1 inhibits lung cancer cell growth rate and colony formation in soft agar. (A and B) The expression of SPOCK1 in A549 (A) and H1975 (B) cells transfected with esi-Control or esiSPOCK1 by qRT-PCR analysis. (C and D) The cell growth rate was detected in A549 and H1975 cells transfected with esiSPOCK1 or esiControl by MTS assay at different time points (1–5 days). (E) The represent image of soft agar colony formation in A549 and H1975 cell transfected with esiSPOCK1 or esiControl. (F) The bar indicates the average number of invaded cells from three independent repeated wells. $^{*}P < 0.05$.

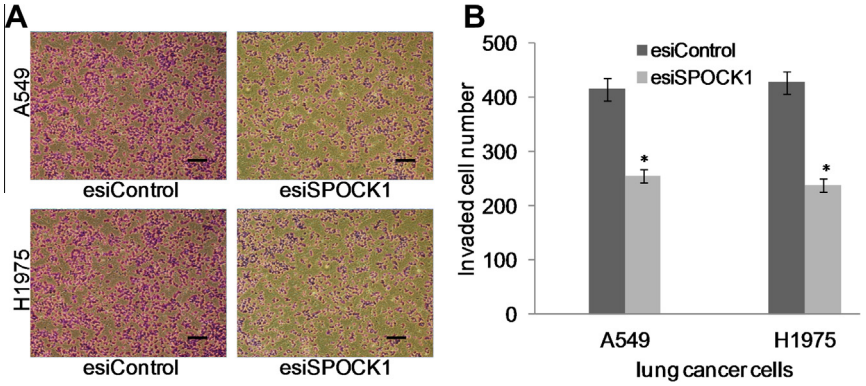

Fig. 3. Silencing of SPOCK1 inhibits lung cancer cell invasion *in vitro*. (A) Image of invasion in A549 and H1975 cells transfected with esi-Control or esiSPOCK1 by transwell Matrige invasion assay. (B) The average invasive cells was counted from three independent repeated wells and shown in the graph. $^{*}P < 0.05$.

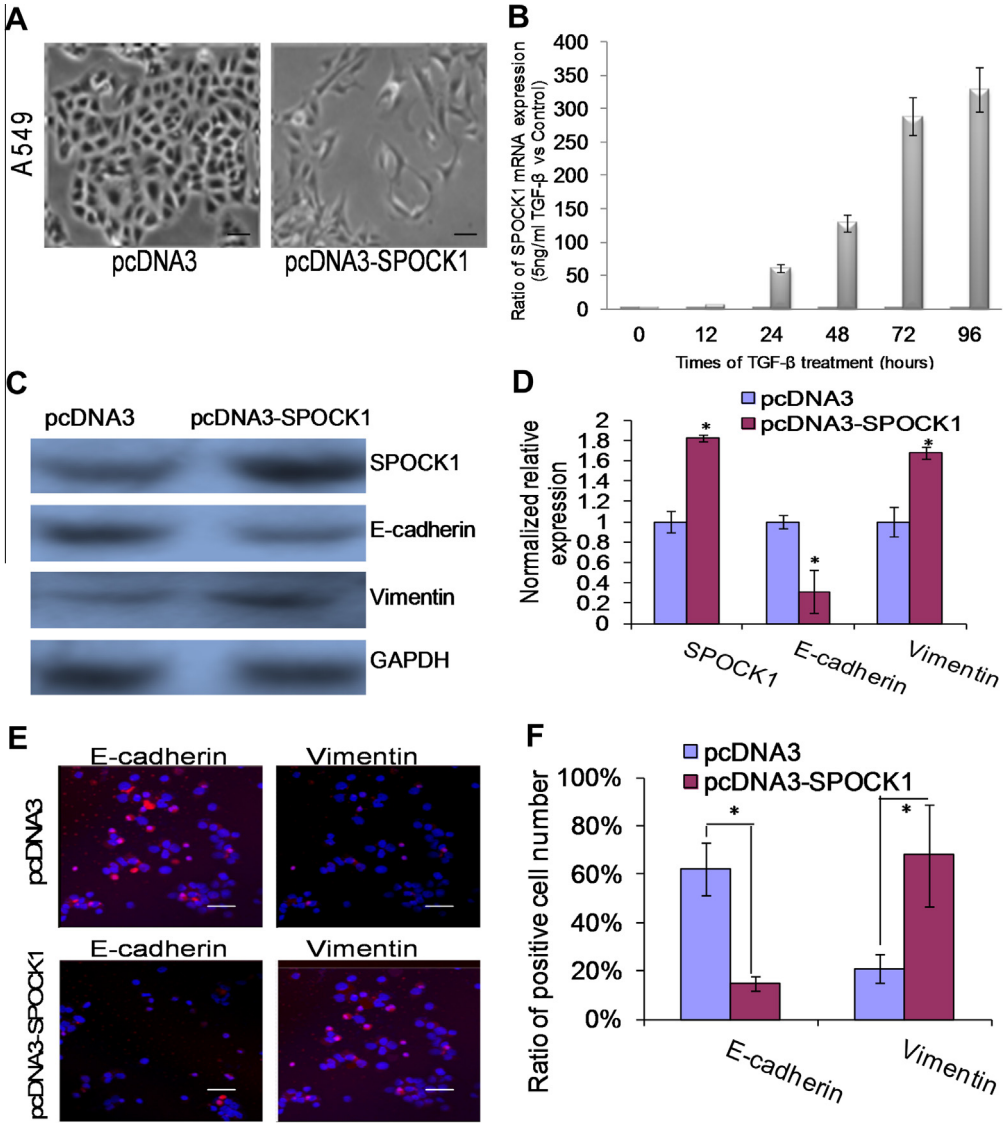

Fig. 4. SPOCK1 induces EMT in epithelial lung cancer cells and is a novel target of TGF-β. (A) Morphological change from an epithelial cobblestone phenotype to an elongated fibroblastic phenotype of A549 cells transfected with pcDNA3-control or pcDNA3-SPOCK1. (B) The expression of SPOCK1 was examined by qRT-PCR at different point of TGF-β (5 ng/ml) or PBS control treatment. (C and D) The protein expression of SPOCK1, E-cadherin and Vimentin in A549 cells transfected with pcDNA3-control or pcDNA3-SPOCK1. (E and F) Expression of E-cadherin, Vimentin, and Hoechst 33342 in A549 cells transfected with pcDNA3-control or pcDNA3-SPOCK1 were analyzed by confocal microscopy. The red signal represents staining for E-cadherin or Vimentin. Nuclear DNA was detected by staining with Hoechst 33342. Scale bar represents 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Recently, SPOCK1 was also identified to be over-expressed in HCCs [8]. In our findings, SPOCK1 was also highly expressed in tumor tissues compared with normal lung tissues, indicating that it may play a role in lung cancer development. Interestingly, the inhibition of SPOCK1 significantly decreased the cancer cell growth capability and colony formation in soft agar, suggesting its role in cancer cell growth and proliferation.

Metastasis is the commonest cause of death in lung cancer patients and a multistep of cellular processes. This process ends at the spread of tumor cells from a primary tumor to a secondary site within the body. It usually involves a variety of complicated molecular and cellular factors related to cell proliferation and migration, the degradation of base membrane and invasion, adhesion and angiogenesis. At the molecular level, the acquisition of genetic and/or epigenetic alterations, along with the cooperation of stromal cells, contributes to successful events of cancer metastasis [13,14]. In vivo, we found that SPOCK1 is significantly associated with metastasis and the silencing of SPOCK1 inhibits both A459 and H1975 cell invasion in vitro, indicating that SPOCK1 is a metastasis-related genetic alteration in lung cancer cells.

Epithelial-to-mesenchymal transition is a crucial step in tumor progression and plays a critical role during cancer invasion and metastasis. During this process, epithelial cells lose their properties and acquire mesenchymal phenotypes. Morphologically, cancer cells usually lose compact cell–cell adhesion and transfer to fibroblast-like morphology during EMT. In addition, mesenchymal phenotype cells present decreased epithelial-related markers such as E-cadherin, and increased mesenchymal-related markers such as Vimentin [15,16]. In the current study, we found that SPOCK1 was associated with metastasis, both in vitro and in vivo. We also found that silencing of SPOCK1 in A549 drives cancers cells to morphologically change to more scattered mesenchymal-like cells. More importantly, the increased expression of Vimentin and decreased expression of E-cadherin suggested that those cells had undergone the EMT change. Our findings indicate that SPOCK1 may drive cells to undergo EMT and result in the metastasis of

cancer cells. More interestingly, we found that TGF-β can induce the expression of SPOCK1, which prompts us speculate that TGF-β/SPOCK1 may be the crucial axis during lung cancer cell metastasis.

Of clinical relevance, we first found that SPOCK1 was significantly up-regulated in lung cancer metastasis more than that in non-metastatic tumor tissues. Also, we observed that after surgery, compared with lung cancer patients with high SPOCK1 expression, those with low SPOCK1 expression had better disease free survival times. Taking these factors together, SPOCK1 may be a novel therapeutic or prognostic biomarker in lung cancer treatment.

Collectively, we have demonstrated that the expression of SPOCK1 is associated with metastasis and the disease free survival time of lung cancer patients. We hypothesize that the TGF-β/SPOCK1 axis may play an important role during the EMT of lung cancer cells and result in the metastasis of cancer cells. These observations support our belief that SPOCK1 is a promising therapeutic target for inhibiting metastasis and is a prognostic biomarker for lung cancer.

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References

[1] A. Jemal, F. Bray, M.M. Center, J. Ferlay, E. Ward, D. Forman, Global cancer statistics, CA Cancer J. Clin. 61 (2011) 69–90.
[2] L. Crinò, W. Weder, J. van Meerbeeck, E. Felip, O.b.o.t.E.G.W. Group, Early stage and locally advanced (non-metastatic) non-small-cell lung cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up, Ann. Oncol. 21 (2010) v103–v115.
[3] A. Singh, J. Settleman, EMT, cancer stem cells and drug resistance: an emerging axis of evil in the war on cancer, Oncogene 29 (2010) 4741–4751.
[4] H. Xia, W.K. Cheung, J. Sze, G. Lu, S. Jiang, H. Yao, X.-W. Bian, W.S. Poon, H.-F. Kung, M.C. Lin, miR-200a regulates epithelial-mesenchymal to stem-like transition via ZEB2 and β-catenin signaling, J. Biol. Chem. 285 (2010) 36995–37004.
[5] B.C. Willis, Z. Borok, TGF-β-induced EMT: mechanisms and implications for fibrotic lung disease, Am. J. Physiol. Lung Cell. Mol. Physiol. 293 (2007) L525–L534.
[6] Y. Huang, J. Zhang, Y. Zhao, W. Jiang, C. Xue, F. Xu, H. Zhao, Y. Zhang, L. Zhao, Z. Hu, SPARC expression and prognostic value in non-small cell lung cancer, Chin. J. Cancer 31 (2012) 541–548.
[7] C. Colin, N. Baeza, C. Bartoli, F. Fina, N. Eudes, I. Nanni, P. Martin, L. Ouafik, D. Figarella-Branger, Identification of genes differentially expressed in glioblastoma versus pilocytic astrocytoma using Suppression Subtractive Hybridization, Oncogene 25 (2005) 2818–2826.
[8] Y. Li, L. Chen, T.H.M. Chan, M. Liu, K.-L. Kong, J.-L. Qiu, Y.-F. Yuan, X.-Y. Guan, SPOCK1 is regulated by CHD1L and blocks apoptosis and promotes HCC cell invasiveness and metastasis in mice, Gastroenterology (2012).
[9] A. Wlazlinski, R. Engers, M.J. Hoffmann, C. Hader, V. Jung, M. Müller, W.A. Schulz, Downregulation of several fibulin genes in prostate cancer, Prostate 67 (2007) 1770–1780.
[10] U. Hartmann, H. Hülsmann, J. Seul, S. Röll, H. Midani, I. Breloy, D. Hechler, R. Müller, M. Paulsson, Testican-3: a brain-specific proteoglycan member of the BM-40/SPARC/osteonectin family, J. Neurochem. (2013).
[11] H.-J. Hausser, R. Decking, R.E. Brenner, Testican-1, an inhibitor of pro-MMP-2 activation, is expressed in cartilage, Osteoarthritis Cartilage 12 (2004) 870–877.
[12] C. Cifuentes-Diaz, P.M. Alliel, F. Charbonnier, S. De La Porte, J. Molgó, D. Goudou, F. Rieger, J.-P. Périn, Regulated expression of the proteoglycan SPOCK in the neuromuscular system, Mech. Dev. 94 (2000) 277–282.
[13] C.L. Chaffer, R.A. Weinberg, A perspective on cancer cell metastasis, Science 331 (2011) 1559–1564.
[14] A. Harlozinska, Progress in molecular mechanisms of tumor metastasis and angiogenesis, Anticancer Res. 25 (2005) 3327–3333.
[15] H. Xia, L.L.P.J. Ooi, K.M. Hui, MicroRNA-216a/217-induced epithelial–mesenchymal transition targets PTEN and SMAD7 to promote drug resistance and recurrence of liver cancer, Hepatology (2013).
[16] H. Xia, K.M. Hui, MicroRNAs involved in regulating epithelial-mesenchymal transition and cancer stem cells as molecular targets for cancer therapeutics, Cancer Gene Ther. 19 (2012) 723–730.