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Effects of shRNA-mediated knockdown of SPOCK1 on ovarian cancer growth and metastasis

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Abstract

Ovarian cancer is one of the three most common gynecological malignant tumors. The mortality rate of ovarian cancer is high because of the insidious disease onset and the lack of effective methods for early diagnosis. In this study, we assessed the potential of SPOCK1 as a significant biomarker for ovarian cancer development. We determined that the expression of SPOCK1 was evidently high in ovarian cancer tissues and the cell lines OVCAR3 and SKOV3. The knockdown of SPOCK1 by specific shRNA significantly inhibited cell proliferation and colony formation in both OVCAR3 and SKOC3 cells. In a xenograft model of ovarian cancer, the mice implanted with SPOCK1 knockdown SKOV3 cells exhibited a slower tumor growth rate. The dissected tumors also weighed less in the SPOCK1-depleted mice group. Furthermore, the knockdown of SPOCK1 evidently inhibited the wound recovery process. Concomitantly, cell migration was inhibited by up to 67% after the knockdown of SPOCK1 in OVCAR3 cells and 75% in SKOV3 cells. Invasion capability also decreased by up to 80% in OVCAR3 cells and 83% in SKOV3 cells after SPOCK1 knockdown. Moreover, the knockdown of SPOCK1 caused a decrease of its known target, i.e., matrix metalloproteinase-2. Interestingly, it also reduced phosphorylated ERK and AKT. These data suggest that SPOCK1 is a potential biomarker that promotes ovarian cancer growth and metastasis. The biological behavior of SPOCK1 in ovarian cancer may be related to the ERK and AKT signaling pathways.

Key words: SPOCK1, shRNA, growth, metastasis, ovarian cancer.

Introduction

Ovarian cancer, which is one of the three most common gynecological malignancies, ranks third in incidence rate. Over 70% of patients exhibit an advanced stage when diagnosed because of the insidious disease onset and the lack of effective methods for early detection (1). At present, the three major therapeutic strategies for ovarian cancer treatment include surgery, chemotherapy, and radiotherapy; however, most patients relapse shortly after surgery or develop resistance to chemotherapy drugs, and only approximately 30% of patients survived for a longer period (2). The early diagnosis of ovarian cancer should significantly reduce mortality. Molecular therapy has been constantly suggested as a promising strategy to develop novel therapeutics for cancer treatments (3). Thus, discoveries of novel targets that are critically involved in cancer development are highly important.

SPARC/osteonectin, cwcv, and kazal-like domains proteoglycan 1 (SPOCK1, also known as testican) is a member of the matricellular glycoprotein family that belongs to a novel Ca^{2+}-binding proteoglycan family. Other members of this family include SPARC, testican2, and testican3. All members share similar structural bases, which contain N-terminus, follistatin-like domain, and C-terminus, and are widely recognized to be involved in cell cycle progression, apoptosis, adhesion, and migration (4,5). Among these members, SPARC has been well documented and implicated in human tumor biology (5). Interestingly, the roles of SPOCK1 in human tumorigenesis have been recently recognized and analyzed because of structural similarity.

SPOCK1 was initially isolated from the testis (6) and was later observed to be expressed in other tissues, including the brain (7), cartilage (8), vascular endothelium (9), myoblasts (10), fibroblasts (11), lymphocytes (12), neuromuscular junction (13), and blood (14). The wide expression of SPOCK1 in human tissues suggests its crucial roles at the systemic level and under physiological conditions. However, SPOCK has been recently determined to be aberrantly expressed in various types of cancers and to exert pathological roles in human tumorigenesis. The overexpression of SPOCK1 has been observed in cancers, including gastrointestinal neuroendocrine carcinoma (15), prostate cancer (16), hepatocellular carcinoma (17), lung cancer (18), and glioblastoma (19). Studies have demonstrated that SPOCK1 plays critical roles in prostate cancer recurrence, hepatocellular carcinoma progression, glioblastoma invasion, and lung cancer cell epithelial–mesenchymal transition (16-19). The inhibition of matrix metalloproteinase-2 (MMP-2) activation is a well-documented pathway in which SPOCK1 exhibits its biological activity (8,20,21). However, the expression of SPOCK1 is also strictly controlled. Chromodomain helicase/adenosinetriphosphatase DNA binding protein 1-like (CHD1L) and transforming growth factor-β (TGFβ) are identified as upstream regulators of *SPOCK1* to maintain SPOCK1 at a favorable expression level for cancer development (17,18). These reports suggest that SPOCK1 may exert potential tumor-promoting effects in various cancers. The regulatory machinery that controls SPOCK1 expression is highly crucial because of the biological activities of SPOCK1 in human tumorigenesis.

Despite increasing evidence that proves that SPOCK1 is a key mediator of human cancer development, the role of SPOCK1 in gynecological oncology remains lar-

gely unknown, particularly in ovarian cancer. Recently, the well-known SPOCK1 target gene *MMP-2* has been reported to be a key factor in mediating cyclicendometrial menstrual breakdown and the onset of menstrual bleeding (22). In addition, genome-wide association analysis has also identified *SPOCK1* as a novel gene that underlies age at menarche (23). Evidence strongly indicates a close link between SPOCK1 expression and gynecological diseases. Hence, this study aims to examine whether SPOCK1 plays a role in human ovarian cancer growth and metastasis and to determine the underlying mechanism by which SPOCK1 contributes to ovarian cancer development and progression.

Materials and methods

Specimens and cell lines

One hundred and twenty cases of ovarian cancer tissues and their adjacent non-cancerous tissues were collected from patients who underwent ovariectomy atTaizhou Central Hospital.None of these patients received chemotherapyor radiotherapy prior to surgical operation. Use of these samples for research purposes was approvedby an Institutional Review Board at Taizhou Central Hospital.All cases were diagnosed with ovarian cancer by two experienced pathologists. Fourovarian cancer cell lines CoC1, CaoV-3, OVCAR3, and SKOV3 werepurchased from the American Type Culture Collection (ATCC) andmaintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, CA) containing 10% fetal bovine serum (FBS) and 100 U/ml penicillin/streptomycin(Sigma, St. Louis, MO). Cell culture medium was refreshed every two days.

Immunohistochemisty (IHC) analysis

Paraffin-embedded tissues were cut into 4-μm sections. Sections were then subjected to deparaffinization/rehydration protocols. After that, sections were antigen retrieved in a microwave at 100°C for 9 minutes in 0.1M citric acid buffer (PH 6.0), and then incubated with primary antibodies at 4°C overnight. After two rounds of PBS washes, secondary antibody was added to the slides for incubation at room temperature for 1h. Slides were then developed in 0.05% diaminobenzidine(DAB) containing 0.01% hydrogen peroxidase. For each case, the IHC staining of SPOCK1 was graded as negative (-), weakly positive (+), moderately positive (++), or strongly positive (+++) according to the intensity and positive percentage.Representative images were photographed under a light microscopy (Leica, Wetzlar, Germany).

Western blot analysis

Prior to sample loading, total proteins from cultured cells were harvested when growth confluence reached 80% and quantified using a BCA kit (Pierce, Rockford, IL, USA). An equal amount of 30μg protein was loaded to each lane in a 12% SDS-PAGE gel and later transferred to a nitrocellulose membrane. Primary antibodies were then incubated with the membrane at 4°C overnight. The membrane was washed three times for 10 min in TBS with 0.1% Tween-20 and then incubated with a secondaryantibody at room temperature for 1h.Immunoreactivity was determinedusing an enhanced chemoluminescent autoradiography kit (Amersham, Pittsburgh, PA), according to the manufacturer's instruction. GAPDH was synchronously developed for control of loading.

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

Total RNAs from human samples or cell lines were extracted using a Trizol reagent(Invitrogen, CA). The concentration of extracted RNAs was measured using a NanoDrop ND-1000 Spectrophotometer (Agilent, CA).For quantification of mRNA, total RNAs were immediately reverse-transcribed into cDNA usingSuperScript III First-Strand Synthesis System (Takara, Shiga, Japan). The real-time qPCR was performed in an ABI prism 7500 real-time system using the SYBR Premix Ex TaqTM Perfect Real Time (TaKaRa, Shiga, Japan).*β-actin* was used as the internal control.Specific primers used here were as follows:
SPOCK1, Forward sequences: 5'- CCCGTTACTGCCGGTGATTA-3' Reverse sequences: 5'-CCAGGTCTGGACAAGCTGAG-3' *β-actin*, Forward sequences: 5'-GTGGACATCCGCAAAGAC-3'Reverse sequences: 5'- AAAGGGTGTAACGCAACTA-3'
Relative mRNA was determined as previously described (24).

shRNA and transfection

Specific shRNA against *SPOCK1* was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). For negative control, a non-specific shRNA was also provided which was short for "NC" throughout this study. Both specific and non-specific shRNAs express greenfluorescence protein (GFP) which is easily detected under a fluorescent microscopy. Alipofectamine 2000 was used for transfection of shRNAs under the instructions of manufacturer. Transfection efficiency was first assessed by detecting the GFP and later confirmed by both western blot and qRT-PCR analyses.

MTT assay

3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H-tetrazolium bromide (MTT) assay was performed for evaluation of cell viability. Briefly, ovarian cancer cells transfected with specific SPOCK1 shRNA (shRNA group) or non-specific shRNA (NC group) were seeded in a 96-well plate at an initial density of 8×10^3 cells/well and cultured in DMEM mediumbefore proceeding to tests. Cell viability was evaluated for a consecutive of 5 days. On each monitored day, Cells from each group were added with a total of 10μl/well MTT solution, and further incubated at 37°C for 4h to form crystals. After 4h incubation, cell proliferation rate was determined by detecting the crystal absorbance of each well at 490nm under a microplate reader (Bio-Tek, USA). Values for the latter four days were normalized to that on day 1, respectively. Results were obtained from three-independent tests with each test in triplicate.

Plate colony formation assay

Both OVCAR3cells and SKOV3 cells were evenly seeded into 6-well plates, respectively, at an initial density of 8×10^3 cells/well. Cells were then transfected with SPOCK1 shRNA (shRNA group) or the non-specific

shRNA (NC group). After 24 hours growth, the supernatant was removed and cells were allowed to growth under normal conditions with the medium refreshed every two days. Ten days later, colonies were observed in each well. Each plate was thenadded with 4% paraformaldehyde before proceeding to stain with 0.1% crystal violet (Sigma-Aldrich, Shanghai, China). After washing, the plates were air-dried, and stained colonies were photographed using a microscope (Leica, Wetzlar, Germany).Colonies for each group were counted and averaged from three independent tests.

Soft agar colony formation assay

Both OVCAR3 and SKOV3 cells (1×10^4) from shRNA group or NC group were platedonto a 6-well ultra-low attachment plate (Corning)(Corning, NY)in serum-free DMEM medium containing 100 U/ml streptomycin, 100μg/ml penicillin,2mM L-glutamine, 1% sodium pyruvate, and 1% MEM nonessential amino acidssupplemented with10ng/ml fibroblast growthfactor-2 and 20ng/ml epithelial growth factor (Invitrogen, Shanghai). After 20 days of culture, the number ofspheres with diameter over 40μm was counted in five randomlyselected fields at a magnification of 40×under an invertedfluorescence microscope. Each assay was performed in triplicate and repeated at least three times.

Wound-healing assay and Boyden chamber assay

Cells with distinct treatments were plated on 6-wellplates to allow fornormal growth. For wound healing assay, smooth woundswere made with a sterile pipette tip. Wound recovery was observed at indicated time points after wounds were made. Cell migration and invasion assays were carried out using Boyden chambers (6.5mm diameter and 8μm pores)containing polycarbonatemembranes. Chambers were pre-treated with culture medium. Except for using Matrigelto assess the cell invasiveness, protocols for both migration and invasion assay were basically the same. Briefly, cells were serum-starved for 24h and latertrypsinized and counted. Cells (1×10^6) were thenre-suspended in100μl serum-free medium and seededinto the upperchamber. 600μl of DMEM medium with 10% FBS wasadded to the lower chamber. After 12h of incubation at 37°C, noninvasivecells on the upper surface of the Matrigel membrane weregently removed with a cotton-tipped swab. The migrated cells on the lower surface of the membrane were thenstained with Giemsa for 10 minutes at room temperature.The stained cells were photographed under an inverted light microscopeand manuallyquantified by counting the cell numbers in three randomlyselected fields. Each assay was repeated three times with each one in duplicate.

Mouse xenograft models of ovarian cancer

Ten female athymic nude mice ($BALB/c^{nu/nu}$) (six-week-old) were purchased from the Model Animal Research Center ofNanjing University (Nanjing, China). Mice were randomized and assigned to each group (n=5per group). SKOV3 Cells (1×10^6) that stably expressing SPOCK1 shRNA (shRNA group) or non-specific shRNA (NC group) were injected subcutaneously into the left flank of each mouse. Tumor diameters were measured every four days and tumor volumes (TV) were calculated by using the function: $TV=L\times W^2/2$ where L and W represent the longer and smaller diameter, respectively. On day 28, all mice were sacrificed and tumors were dissected. Our protocol for animal experiments was approved by the Ethics Committee from Taizhou Central Hospital. And all efforts were made tominimize suffering.

Statistical analysis

Data are expressed as the mean ± standard deviation (SD). The datastatistics were analyzed using the SPSS 12.0 software.The one-way analysis of variance (ANOVA) orthe Student's *t* test was used to analyze the statistical differences. A Spearman correlation was employed to analyze the association between SPOCK1 expression and ovarian cancer clinical variables in Table1. A *p*value<0.05 were considered statisticallysignificant.

Table 1. Association between Spock1 expression and common clinicopathological parameters in 120 cases of ovarian cancer.

Variables	Total	Spock1 expression		*P* value
		-/+	++/+++	
Age				
≤50 yrs	42	16	26	0.8443
>50 yrs	78	28	50	
Histology				
Serous	85	25	60	0.3363
Nonserous	35	19	16	
Nodal metastasis				
Negative	68	33	35	0.0023*
Positive	52	11	41	
Differentiation				
Well/moderate	50	16	34	0.4435
Poor	70	28	42	
TNM staging				
I/II	36	20	16	0.007*
III/IV	84	24	60	

*$P<0.05$

Results

SPOCK1 is highly expressed in ovarian cancer and correlates with ovarian cancer malignancy

To assess the expression level of SPOCK1 in ovarian cancer tissues, we randomly selected 15 cases of ovarian cancer and their paired adjacent tissues. The total RNA in each case was extracted and analyzed using the quantitative real-time polymerase chain reaction (qRT-PCR) technique (Figure 1A). Some of the 15 ovarian cancer tissues exhibited lower mRNA levels of SPOCK1 compared with the non-cancerous tissues. However, the average mRNA level of SPOCK1 in the ovarian cancer tissues was approximately twice higher than that in the non-cancerous tissues. Concomitantly, we determined that SPOCK1 protein was only selectively and highly expressed in the ovarian cancer tissues, although SPOCK1 could also be slightly detected in the adjacent normal tissues (Figure 1B). The high expression level of SPOCK1 in the ovarian cancer tissues compared with that in the normal tissues was also confirmed by Western blot analysis (Figure 1C). In addition, we also examined the protein level of SPOCK1 in four ovarian cancer cell lines. SPOCK1 was observed to be differentially expressed in the four cell lines, with the highest expression recorded in SKOV3 cells and OVCAR3 cells (Figure 1C). These data demonstrate the high expression of SPOCK1 in ovarian cancer.

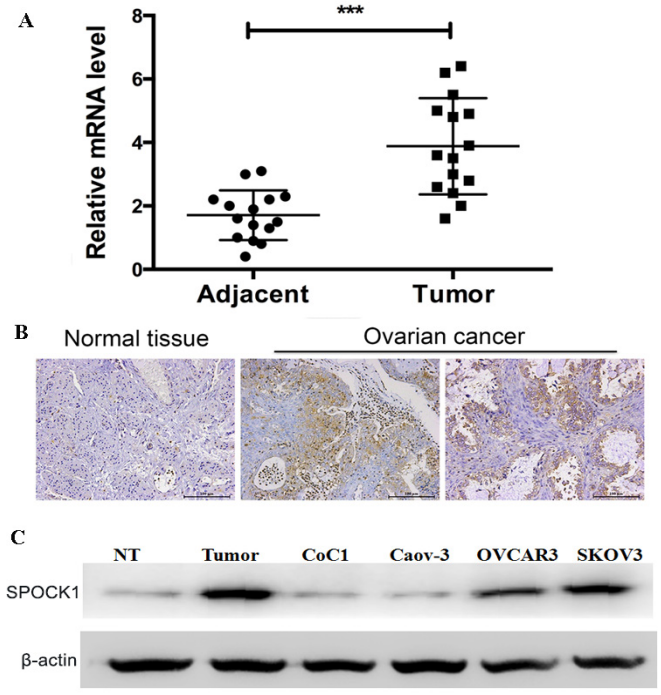

Figure 1. SPOCK1 is highly expressed in ovarian cancer. (A) qRT-PCR analysis of the mRNA level of SPOCK1 in 15 cases of ovarian cancer and their paired adjacent non-cancerous tissues. ***, p<0.0001. (B) Immunohistochemical analysis of the protein level of SPOCK1 in the ovarian cancer tissues and the adjacent non-cancerous tissues. Representative images are shown in this figure. SPOCK1 positive staining was observed in the cancer tissues but not in the non-cancerous tissues. (C) Western blot analysis of the protein level of SPOCK1 in the ovarian cancer tissues and four ovarian cancer cell lines. The protein level of SPOCK1 was highly expressed in the cancer tissues. This level was also differentially expressed in the four cell lines, with the highest levels observed in SKOV3 cells and OVCAR3 cells.

The higher expression of SPOCK1 in SKOV3 cells and OVCAR3 cells indicate that the two cell lines are optimal for our subsequent analysis.

In addition, we analyzed the association of SPOCK1 expression with the clinical variables in 120 cases of ovarian cancer. Spearman correlation analysis revealed that the expression of SPOCK1 was positively correlated with the nodal metastasis status (p=0.0023) and TNM staging (p=0.007) of the patients. No significant association between SPOCK1 expression and other clinical variables, including onset age, histological classification, and tumor differentiation, was observed (Table 1). The conclusive observation suggests that the expression of SPOCK1 correlates with ovarian cancer malignancy.

Knockdown of SPOCK1 inhibits ovarian cancer cell growth in vitro

To assess the role of SPOCK1 in the cell growth of ovarian cancer, specific shRNA against SPOCK1 was used to knock down the expression of SPOCK1. SPOCK1 exhibited higher expression in SKOV3 cells and OVCAR3 cells. Thus, these two cell lines were selected for the transfection of specific shRNA. Transfection efficiency was initially confirmed under fluorescent microscopy (Figure 2A). In addition, the mRNA level of SPOCK1 in either SKOV3 cells or OVCAR3 cells decreased by up to 75% after transfection with SPOCK1 shRNA (Figure 2B). Similarly, the protein level of SPOCK1 significantly decreased in response to SPOCK1 shRNA transfection in both SKOV3 cells

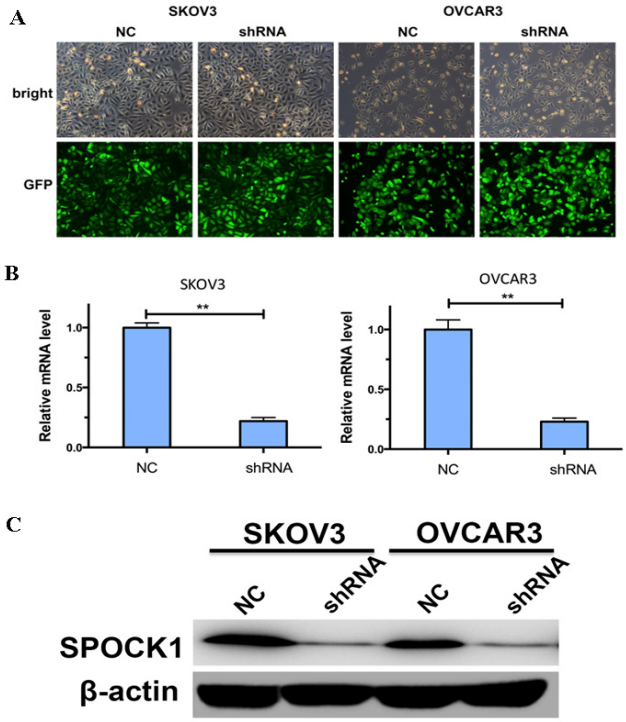

Figure 2. shRNA against SPOCK1 effectively depletes the expression level of SPOCK1 in vitro. (A) Under fluorescent microscopic fields, green fluorescent protein-expressing cells accounted for approximately 80% of the total number of cultured cells in both cell lines. (B) The mRNA level of SPOCK1 in either SKOV3 cells or OVCAR3 cells decreased by up to 75% after transfection with SPOCK1 shRNA. **, p<0.01. (C) shRNA against SPOCK1 also evidently decreased the protein level of SPOCK1 in both SKOV3 cells and OVCAR3 cells.

and OVCAR3 cells. All these pieces of evidence suggest that our synthesized shRNA effectively depletes SPOCK1 in SKOV3 cells and OVCAR3 cells.

Next, we assessed the effects of shRNA-mediated knockdown of SPOCK1 on ovarian cancer cell growth in vitro. MTT assay showed that proliferative rate varied between the negative control (NC) group and the shRNA group since day 3, and the difference became even more significant on day 4. By day 5, the cell number in the shRNA group was approximately 50% of that in the NC group in SKOV3 cells. In OVCAR3 cells, the cell number in the shRNA group was only 40% of that in the NC group (Figure 3A). The plate colony formation assay further showed that the knockdown of SPOCK1 substantially decreased the number of colonies in both cell lines (Figure 3B, left panels). The quantification of colonies determined that only 40 colonies were formed in the shRNA group relative to the 120 colonies in the NC group in SKOV3 cells. Similarly, in OVCAR3 cells, only 30 colonies were formed in the shRNA group relative to the 160 colonies in the NC group (Figure 3B, right panels). Concomitantly, soft agar colony formation assay also indicated that clonogenicity in both cell lines was inhibited after the knockdown of SPOCK1 expression. In the SPOCK1-depleted SKOV3 cells, clonogenicity was only 25% of the control SKOV3 cells, whereas it was 36% of the control cells in the SPOCK1-depleted OVCAR3 cells (Figure 3C). These data demonstrate

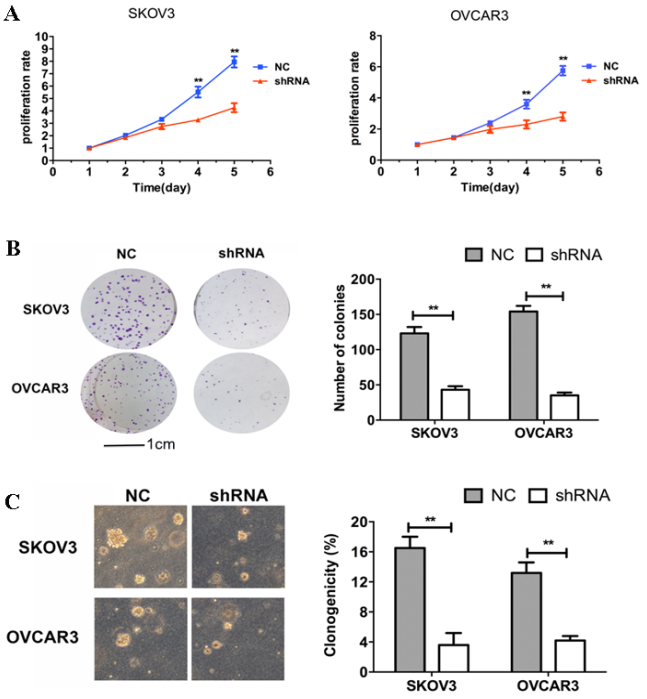

Figure 3. Knockdown of SPOCK1 inhibits ovarian cancer cell growth in vitro. (A) An MTT assay showed that proliferation was significantly inhibited four days after SPOCK1 knockdown in both cell lines. Proliferation was further inhibited in SPOCK1-depleted cells with an extended time interval. (B) Plate colony formation assay indicated that the knockdown of SPOCK1 blocked the formation of colonies in both cell lines. (C) Soft agar colony formation assay demonstrated that clonogenicity in both cell lines was inhibited after the knockdown of SPOCK1 expression. In the SPOCK1-depleted SKOV3 cells, clonogenicity was only 25% of the control cells, whereas in the SPOCK1-depleted OVCAR3 cells, clonogenicity was 36% of the control cells. **, $p<0.01$. Data represent the mean ± SD of three independent experiments.

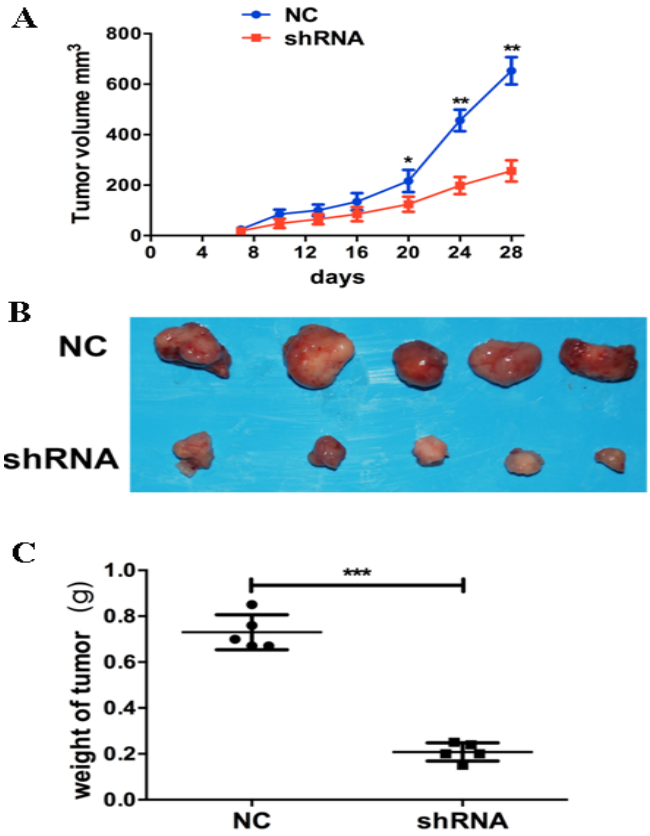

Figure 4. Knockdown of SPOCK1 inhibits tumor growth in a xenograft model of ovarian cancer. (A, B) A xenograft model of ovarian cancer was established, and tumor volume was measured periodically. Tumors from mice from both the NC group and the shRNA group (n=5 per group) began to appear on the seventh day after being injected with SKOV3 cells. In general, the mice in the shRNA group exhibited a smaller tumor volume compared with that of the mice in the control group during the monitored time periods (A) and on day 28 when all the tumors were dissected (B). (C) After all the mice were sacrificed, each tumor was dissected and weighed. The average weight of the tumors from the shRNA group of mice was approximately 0.2 g, whereas that of the NC group of mice was 0.7 g. *, $p<0.05$; **, $p<0.01$; ***, $p<0.0001$.

that the knockdown of SPOCK1 inhibits ovarian cancer cell growth in vitro.

Knockdown of SPOCK1 inhibits tumor growth in a xenograft model of ovarian cancer

Furthermore, we established a xenograft model of ovarian cancer. Tumors were observed in mice from both the NC group and the shRNA group on the seventh day after being injected with SKOV3 cells. The tumors continuously grew as tumor volume enlarged with time. However, the mice in the shRNA group generally exhibited a smaller tumor volume compared with those in the control group (Figures 4A and 4B). After all the tumors were dissected on day 28, each tumor was weighed. The average weight of the tumors from the mice in the shRNA group was approximately 0.2 g, whereas that from the NC group mice was 0.7 g. These data suggest that the knockdown of SPOCK1 significantly inhibits tumor growth in vivo.

Knockdown of SPOCK1 inhibits migration and invasion capabilities

Based on the observations presented in Table 1, we

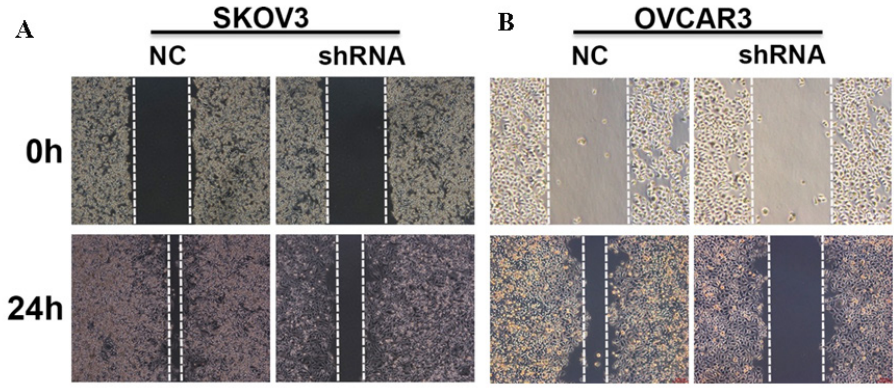

Figure 5. Knockdown of SPOCK1 inhibits the wound healing process in both SKOV3 cells and OVCAR3 cells. Twenty-four after the wounds were inflicted, the control SKOV3 cells and OVCAR3 cells actively recovered the wounds. However, the wounds were still evident and remained largely unrepaired in the SPOCK1-depleted SKOV3 cells and OVCAR3 cells.

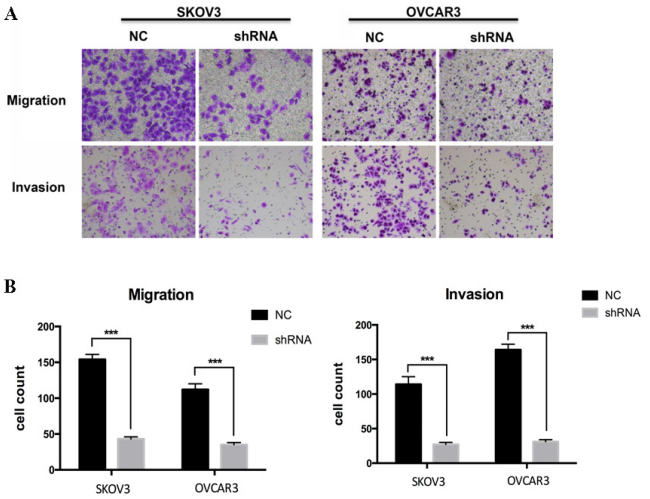

Figure 6. Knockdown of SPOCK1 inhibits cell migration and invasion capabilities. (A) The cells that migrated to the lower surface of the membrane were stained with Giemsa for 10 minutes. Representative images were photographed and shown. (B) By counting the migrated cells, cell migration was determined to be inhibited by up to 67% after the knockdown of SPOCK1 expression in OVCAR3 cells and by up to 75% in SKOV3 cells. Invasion capability was also decreased by up to 80% in OVCAR3 cells and 83% in SKOV3 cells after SPOCK1 knockdown. ***, $p<0.001$. Data represent the mean ± SD of three independent experiments.

hypothesized that SPOCK1 may play a critical role in ovarian cancer metastasis. To this end, we initially conducted wound healing assay to assess the effects of shRNA-mediated knockdown of SPOCK1 on ovarian cancer cell migration. Both cell lines actively recovered the wound 24 h after the wound was inflicted. In fact, the wound was nearly closed by the control SKOV3 cells at 24 h. However, SPOCK1-depleted cells appeared to be blocked from achieving this process. The wounds were still observed at 24 h (Figure 5). Next, we performed transwell migration assay and invasion assay. Inhibited migration capability in the wound healing assay was also confirmed by the findings of the transwell migration assay (Figure 6A). Moreover, the results of Giemsa staining determined that invasiveness in SPOCK1-depleted cells was also blocked (Figure 6A). Cell migration was inhibited by up to 67% after the knockdown of SPOCK1 expression in OVCAR3 cells and by up to 75% in SKOV3 cells. Invasion capability was also reduced by up to 80% in OVCAR3 cells and 83% in SKOV3 cells after SPOCK1 knockdown. These observations support the assumption that the expression of SPOCK1 is positively correlated with ovarian cancer metastasis.

Effects of SPOCK1 knockdown on the ERK and AKT signaling pathways

To explore the potential mechanisms that contribute to SPOCK1-mediated ovarian cancer malignancy, we detected the phosphorylation levels of ERK and AKT signaling that were frequently modulated in tumorigenesis. MMP-2, a well-known target of SPOCK1, was also detected. As expected, the expression of MMP-2 was decreased by the knockdown of SPOCK1, which presented a natural outcome. Interestingly, we found that the phosphorylation levels of ERK (p-ERK) and AKT (p-AKT) also decreased in response to SPOCK1 depletion. However, the total levels of ERK and AKT were barely changed (Figure 7). This finding indicates that the ERK and AKT pathways may be involved in SPOCK1-mediated biological activities in ovarian cancer.

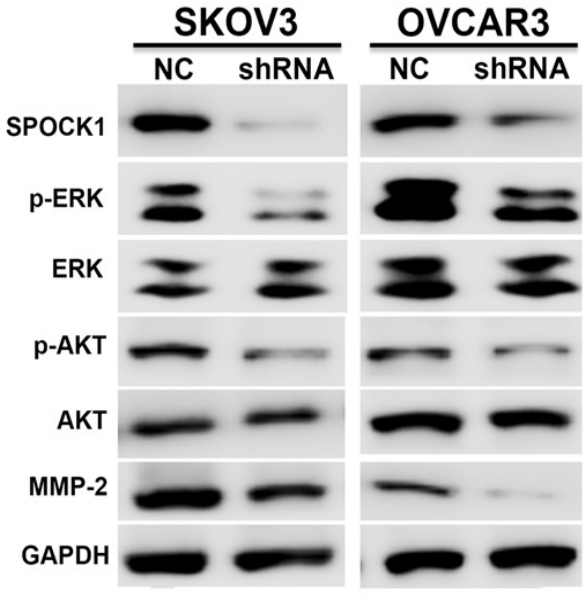

Figure 7. The phosphorylation levels of ERK and AKT signaling are decreased in response to SPOCK1 knockdown in SKOV3 cells and OVCAR3 cells. The expression of MMP-2 (a known downstream target of SPOCK1) was expectedly decreased by the knockdown of SPOCK1. The phosphorylation levels of ERK (p-ERK) and AKT (p-AKT) were also reduced and their total levels barely changed in response to SPOCK1 depletion.

Discussion

Ovarian cancer is a serious threat to females worldwide. According to a recent cancer research in the United States, 22,280 ovarian cancer patients were newly diagnosed in 2012; among them, 15,500 died. The high mortality rate of ovarian cancer makes this type of cancer the fifth highest cause of cancer deaths among females (approximately 6% of all cancer deaths) (1). A similar situation is observed in Europe (25). Among European women aged 55–74 years, the five-year relative survival rate for ovarian cancer is 37.1% (26), which will otherwise increase to 90% if tumors are detected at localized stages; this finding implies the importance of early detection in reducing ovarian cancer mortality rate (27). Molecular therapy has been constantly promoted as a means to reduce ovarian cancer mortality rate because most cancers initiate with the aberrant expression of specific biomarkers.

In the present study, we assessed the roles of SPOCK1 in the cell growth and metastasis of ovarian cancer. SPOCK1 has been previously reported to be involved in prostate cancer recurrence, hepatocellular carcinoma progression, glioblastoma invasion, and lung cancer cell epithelial–mesenchymal transition (16-19). All these previous studies suggest the different roles of SPOCK1 in human tumorigenesis. Our clinicopathological data demonstrated that the high expression of SPOCK1 was closely associated with ovarian cancer malignancy, such as nodal metastasis and TNM staging. This finding indicates that SPOCK1 may be a critical factor for ovarian cancer growth and metastasis. Hence, we depleted SPOCK1 expression in ovarian cancer cell lines. The knockdown of SPOCK1 significantly inhibited cell proliferation and colony formation and suppressed tumor growth in vivo in a xenograft model of ovarian cancer. These data strongly suggest the tumorigenic function of SPOCK1 in ovarian cancer. Aside from demonstrating the tumorigenic roles of SPOCK1, our study showed that the knockdown of SPOCK1 significantly inhibited cell migration and invasion capabilities. These conclusive findings support the observation that the expression of SPOCK1 correlates well with metastasis and TNM staging in clinicopathological studies. The aggressive behavior of SPOCK1 in ovarian cancer demonstrates its potential as a biomarker for detecting ovarian cancer malignancy.

Identifying SPOCK1 as a key mediator of ovarian cancer development is not incidental. In fact, two studies have reported that SPOCK1 is involved in the pathophysiological development of gynecological conditions (22,23), which implies the possible roles of SPOCK1 in gynecological tumors such as ovarian cancer. Our work confirms this hypothesis and will hopefully lead to a more extensive investigation of the roles of SPOCK1 in other gynecological tumors, including endometrial cancer and cervical cancer.

Interestingly, the phosphorylation levels of ERK and AKT decreased in response to SPOCK1 depletion in both ovarian cancer cell lines. Raf/MEK/ERK and PI3K/Pdk1/AKT signaling have been widely implicated in promoting cell growth and suppressing the apoptotic machinery after being activated by phosphorylase (28-30). Thus, at least two major mechanisms can be involved in SPOCK1-mediated ovarian cancer progression. First, SPOCK1 may inhibit cell apoptosis through the ERK and AKT pathways. Apoptosis inhibition is one of the major mechanisms that contributes to cancer development and ultimately leads to the expansion of neoplastic cells (31). SPOCK1 has been demonstrated to exert anti-apoptotic effects in hepatocellular carcinoma by activating the AKT pathway, and thus, promoting cancer metastasis (17). SPOCK1 also stimulates gallbladder cancer proliferation and metastasis by activating the AKT pathway (32). In fact, previous reports have indicated that the ERK and AKT pathways are classic signaling pathways whose activation by phosphorylation induces cell growth and stimulates Bax-mediated pro-apoptosis (33). The decreased phosphorylation levels of ERK and AKT caused by the knockdown of SPOCK1 may imply that SPOCK1 modulates the ERK and AKT pathways, and thus, exhibits aggressive behavior in ovarian cancer. Meanwhile, the MMP-mediated degradation of the extracellular matrix and the basement membrane is an important proteolytic event in the tumor cell invasion of surrounding tissues, vascular infiltration, and extravasation (34). SPOCK1 can contribute to the progression of ovarian cancer by regulating MMPs. For example, the modulation of MMP-2 was observed in the present study. SPOCK1 increased MMP-9 expression and its activity to promote cell invasiveness in hepatocellular carcinoma (17). When taken together, these two mechanisms may be critically involved in SPOCK1-mediated ovarian cancer growth and metastasis. However, further research should still be conducted.

To our knowledge, our work is the first to report that SPOCK1 is highly expressed in ovarian cancer. The high expression of SPOCK1 positively correlates with ovarian cancer malignancy. Although the knockdown of SPOCK1 inhibits its tumorigenic function and metastasis, SPOCK1 may serve as a significant biomarker for the malignant progression of ovarian cancer. Utilizing specific shRNA or developing novel inhibitors against SPOCK1 may be potential strategies to prevent and treat ovarian cancer.

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