SPARCL1 suppresses the proliferation and migration of human ovarian cancer cells via the MEK/ERK signaling

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Abstract. Ovarian cancer is the most lethal gynecological malignancy worldwide and is one of the five leading causes of cancer-associated mortality in women. There is an urgent requirement to obtain a greater understanding of the molecular mechanism underlying ovarian cancer progression in order to identify novel drug targets and biomarkers. Secreted protein acidic and rich in cysteine-like protein 1 (SPARCL1) has been suggested as a candidate tumor suppressor in various types of human cancers. However, the potential role of SPARCL1 for ovarian cancer has not yet been clearly established. In the present study, lower protein expression levels of SPARCL1 were detected in ovarian cancer tissues when compared with adjacent normal tissues. Overexpression of SPARCL1 significantly suppressed the proliferation and migration of cells from the ovarian cancer cell line SKOV-3, whereas knockdown of SPARCL1 significantly increased cell growth and migration. Furthermore, the results revealed that SPARCL1 overexpression significantly suppressed the activation of the mitogen-activated protein kinase kinase (MEK)/extracellular signal-related kinase (ERK) signaling pathway. Collectively, these results indicated that SPARCL1 may suppress the proliferation and migration of ovarian cancer cells by down-regulating signaling via the MEK/ERK pathway.

Introduction

Ovarian cancer is the most lethal gynecological malignancy and the fifth leading cause of cancer-associated deathin women (1,2). Ovarian cancers are responsible for more than half of the female genital tract cancers caused deaths, with more than 2 million new cases being diagnosed every year (1,3,4). Despite significant advances in surgery and chemotherapy, the long-term survival rate of patients with ovarian cancers is still very low (3). Thus, there is clearly an urgent need to unravel the molecular mechanisms of ovarian cancer progression for an improved treatment outcome.

Secreted protein acidic and rich in cysteine-like protein 1 (SPARCL1) is a member of the SPARC family, which is associated with various biological behaviors including osteoblast differentiation and development of cancers. Previous studies have suggested SPARCL1 as a potential tumor suppressor (5-7). Low SPARCL1 expression is associated with the development and progression of cervical cancer (8). Zhao *et al* (5) detected that SPARCL1 was significantly down-regulated in human osteosarcoma cell lines and clinical tissue samples. Interestingly, a recent study revealed that SPARCL1 was strongly down-regulated by 6.4-fold in 594 ovarian cancer cases compared with eight normal ovaries and was suspected to contribute to tumor invasiveness (9). However, the effect of SPARCL1 on ovarian cancer and its underlying mechanisms remain elusive.

The present study aimed to investigate the role of SPARCL1 in ovarian cancer. In this study we are the first to evaluate the expression levels of SPARCL1 in human ovarian cancer tissues and the specific effects of SPARCL1 on ovarian cancer cell proliferation and migration, and to explore possible mechanisms of action.

Materials and methods

Clinical tissues. The present study was approved by the Medical Ethics Committee of the Third Affiliated Hospital, Xinjiang Medical University (Urumqi, China). Signed written informed consent was obtained from all subjects. Twenty ovarian cancer tissues and paired adjacent normal tissues were collected during surgery from ovarian cancer patients without prior chemotherapy or radiotherapy. Disease status had been confirmed in all specimens by pathological diagnosis.

Immunohistochemistry (IHC). IHC staining was performed using the standard immunoperoxidase staining procedure as previously reported (10-12), to detect SPARCL1 expression in paraffin-embedded specimens. The slides of 3 μm thickness were incubated overnight with polyclonal rabbit anti-SPARCL1 antibody (1:100; Abcam, Cambridge, UK). After a secondary antibody was applied for 30 min at room temperature, the slides were stained with diaminobenzidine (DAB) and hematoxylin. The samples were blinded during

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the independent assessment of immunostaining intensity by three senior pathologists. Each section was photographed and semi-quantitative analyzed using computerized image analysis (Image J; National Institutes of Health, Bethesda, MD, USA).

Cell culture. The human ovarian cancer cell line SKOV-3 was purchased commercially from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were grown in RPMI-1640 medium (Sigma, Shanghai, China) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 50 μg/ml streptomycin and 50 IU/ml penicillin, according to the instructions of the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured at 37˚C in a humidified atmosphere of 5% CO_2 and were regenerated every 3 days when they reached 70-90% confluence.

Lentiviral transfection. A lentiviral short hairpin RNA (shRNA) construct targeting SPARCL1 (with targeting sequences to SPARCL1 mRNA as follows: 5'-CCCGACAAA TGCAAGATTATT-3') was obtained from Jikai Corporation (Jikai, Shanghai, China). The oligonucleotides were then phosphorylated, annealed, and cloned into the pLKO.1 (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) following the manufacturer's instructions. The overexpression particles of SPARCL1 were purchased from GenePharma (Shanghai, China). Transfection of SPARCL1 was performed following the manufacturer's instructions.

Cell proliferation. The cell proliferation was assessed using a MTT assay kit (Beyotime Institute of Biotechnology, Haimen, China). To evaluate the effects of SPARCL1 on human ovarian cells, SKOV-3 cells (5,000 cells/well in 100 μl medium) were seeded into 96-well plates on days 1, 2, 3, and 4 post-transfection. After 24 h, the medium was removed, and replaced with 100 μl RPMI-1640 medium without FBS supplemented with 20 μl MTT (5 mg/ml). After incubation for 4 h at 37˚C, the absorbance at 450 nm was determined using a microplate reader (iMark; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Scratch wound healing assay. SKOV-3 cells ($1x10^6$ cells/well) were seeded into 6-well plates and cultured to a 100% confluent monolayer. In each well a wound was induced by scratching a straight line into the cell layer with a 200 μl pipette tip. After 24 h of incubation with RPMI 1640 medium without FBS, images of the wound healing areas were captured and measured with a Leica DM2500 image analysis system (Leica, Mannheim, Germany).

Cell migration assay. The cell migration assay was performed to analyze the effect of SPARCL1 on the migration of SKOV-3 cells. SKOV-3 cells ($2.5x10^4$ cells) were suspended in serum-free medium and seeded into the upper chamber of a transwell plate (Corning Costar, Rochester, NY, USA). RPMI-1640 medium containing 10% FBS was added in the lower chamber. The cells were allowed to migrate for 24 h at 37˚C. Then, the non-migratory cells that remained in the upper chamber were removed, while the cells that had migrated to the lower chamber were fixed and stained with 0.1% crystal violet. Six randomly selected fields were photographed, and the cells per field of view were counted under a light microscope (at x200 magnification).

Western blot analysis. Following treatment, the SKOV-3 cells were harvested and lysed in protein lysis buffer supplemented with a protease inhibitor (Beyotime Institute of Biotechnology). The total protein concentrations were determined using a bicinchoninic acid (BCA) protein assay kit (Bio-Rad Laboratories, Inc.). Equal amounts of protein were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a polyvinylidene fluoride (PVDF) membrane (EMD Millipore, Billerica, MA, USA). After blocking in 5% non-fat dry milk for 2 h at room temperature, the membranes were incubated overnight at 4˚C with primary antibodies specific to GAPDH (1:8,000; Cell Signaling Technology, Inc., Danvers, MA, USA), SPARCL1 (1:1,000; Abcam), p-mitogen-activated protein kinase kinase (p-MEK; 1:1,000; Cell Signaling Technology, Inc.), t-MEK (1:1,000; Cell Signaling Technology, Inc.), p-extracellular signal-related kinase (p-ERK; 1:1,000; Cell Signaling Technology, Inc.), t-ERK (1:1,000; Cell Signaling Technology, Inc.) and MMP-2 (1:1,000; Cell Signaling Technology, Inc.). Then the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:1,000) for 2 h at room temperature. Immunoreactive bands were developed by enhanced chemiluminescence reagents (Pierce; Thermo Fisher Scientific, Inc.) and quantified using Bio-Rad Quantity One software (Bio-Rad Laboratories, Inc.).

Statistical analysis. All experiments were conducted at least three times. Data were presented as the mean ± standard deviation. The results between groups were statistically analyzed by Student's t-test or one-way analysis of variance with Bonferroni's post hoc test using SPSS 23.0 software (IBM Corp., Armonk, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

SPARCL1 was downregulated in clinical ovarian cancer specimens. The expression levels of SPARCL1 in ovarian cancer tissues and adjacent normal tissues was determined by IHC using SPARCL1 specific antibodies. As shown in Fig. 1A, SPARCL1 was predominantly localized to cytoplasm of the adjacent normal tissues, whereas there was none or low level staining observed in ovarian cancer tissues. After quantification we found that significantly lower levels of SPARCL1 were detected in ovarian cancer tissues than in adjacent normal tissues ($P<0.05$; Fig. 1).

SPARCL1 inhibited SKOV-3 cell proliferation. Western blotting analysis verified the successful up- or down-regulation of SPARCL1 expression levels in SKOV-3 cells after lentiviral transfection (Fig. 2). The results of the MTT assay revealed a significantly lower proliferation level in the SPARCL1 overexpression group compared with the other groups. After knock-down of SPARCL1, the proliferation rate increased significantly (Fig. 3A).

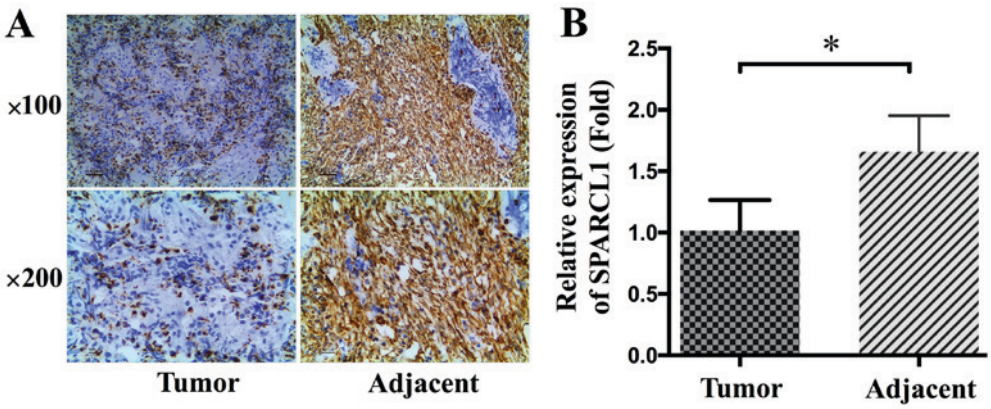

Figure 1. SPARCL1 is downregulated in ovarian cancer clinical specimens. (A) Representative immunohistochemical staining images of ovarian cancer tissues and paired adjacent normal tissues (magnification, x100 and x200 as indicated). (B) Relative quantitative comparison of SPARCL1 expression in ovarian cancer tissues and paired adjacent normal tissues. *P<0.05, as indicated. SPARCL1, secreted protein acidic and rich in cysteine-like protein 1.

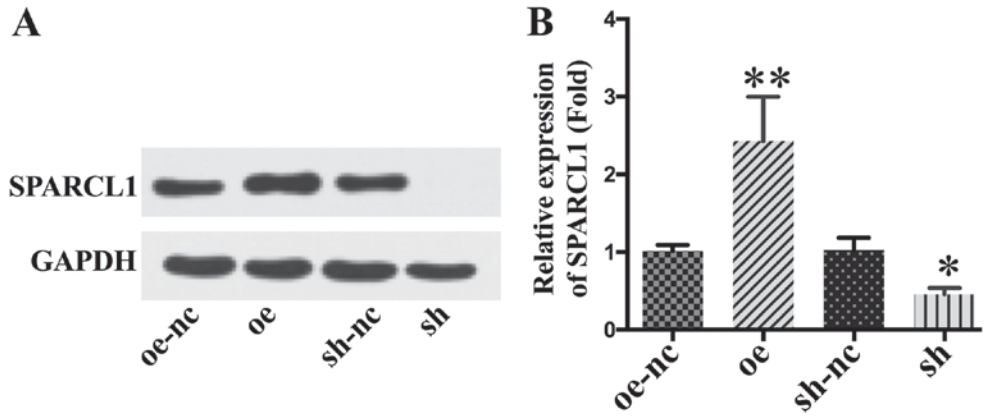

Figure 2. Successful upregulation and downregulation of SPARCL1 expression levels in SKOV-3 cells following lentiviral transfection. (A) Western blot analysis confirmed the upregulation and downregulation of SPARCL1 protein expression by lentiviral infection. (B) Relative quantitative comparison of SPARCL1 expression in SKOV-3 cells. *P<0.05 and **P<0.01 vs. oe-nc. oe, overexpression; nc, normal control; sh, short hairpin RNA; SPARCL1, secreted protein acidic and rich in cysteine-like protein 1.

SPARCL1 inhibits migration of SKOV-3 cells. Relevant photographs of *wound healing assay* were taken immediately after induction of the wound and 24 h later on the following day. SPARCL1-overexpressing SKOV-3 cells had significantly lower levels of wound healing than those of the control group. In contrast, wound healing was significantly enhanced after SPARCL1 knockdown (Fig. 3B and D). In order to further examine the effect of SPARCL1 on human ovarian cancer cell migration, a transwell assay was performed. SPARCL1-overexpression or knockdown was initiated in SKOV-3 cells for 24 h. As shown in Fig. 3C and E, the migration of SKOV-3 cells was significantly inhibited by SPARCL1 overexpression. Furthermore, migration of SKOV-3 cells was significantly enhanced after SPARCL1 knockdown (Fig. 3C and E).

SPARCL1 inhibited the MEK/ERK signaling pathway. To investigate the underlying mechanism of action of SPARCL1 that affected the proliferation and migration of SKOV-3 cells, we investigated different key players of the MEK/ERK signaling pathway, because this pathway has a key regulator function in ovarian cancer cells. As shown in Fig. 4A, western blotting analysis showed that SPARCL1 overexpression led to a significantly reduced expression of activated MMP-2, while SPARCL1 knockdown led to increased expression levels of activated MMP-2. In addition, SPARCL1 overexpression significantly decreased the levels of p-MEK and p-ERK. In contrast, the expression of p-MEK and p-ERK were significantly upregulated in SPARCL1-knockdown cells (Fig. 4A-C).

To examine the mechanism by which SPARCL1 mediated the inhibition of MEK/ERK, a MEK1 specific inhibitor PD98059 (50 μmol/l), was used to inhibit MEK expression. As shown in Fig. 4C, PD98059 significantly inhibited the expression of matured MMP-2. In addition, the phosphorylation of MEK and ERK was blocked by the pretreatment of the cells with PD98059 (Fig. 4C). Furthermore, the results of the wound healing assay showed that the increased migration that was induced by SPARCL1 knockdown were also reduced after PD98059 exposure (Fig. 4D), suggesting that the inhibition effect of SPARCL1 on SKOV-3 cells may be mediated, at least partly, due to the downregulation of MEK/ERK signaling.

Discussion

Although previous studies have revealed a role of SPARCL1 in tumor suppression (5-9), it remains unknown whether SPARCL1 has any effect on ovarian cancer cell proliferation

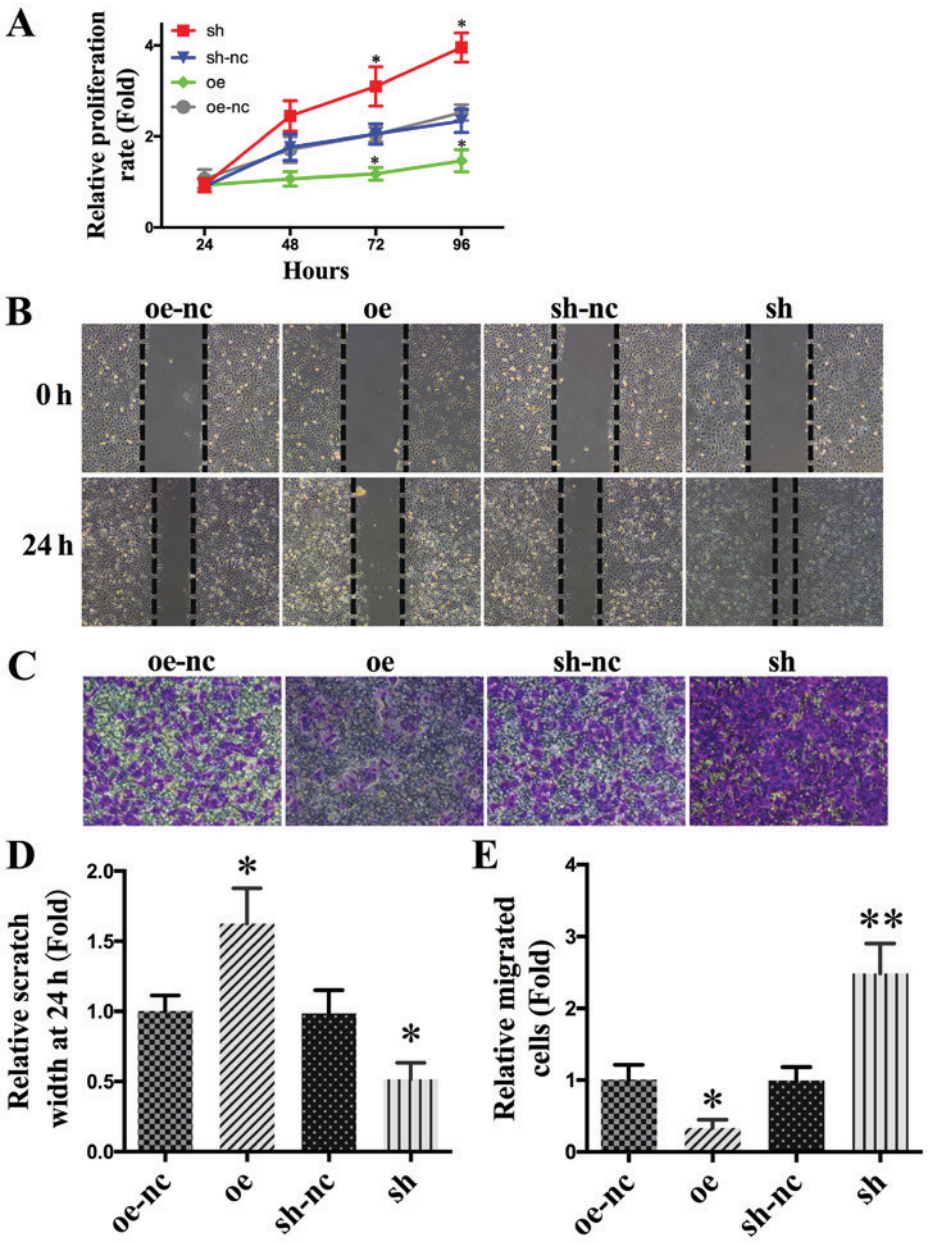

Figure 3. SPARCL1 inhibits SKOV-3 cell proliferation. (A) Cell proliferation was assessed in SKOV-3 cells by MTT assay at 24, 48, 72, and 96 h following lentiviral infection. (B) A wound-healing assay was performed to assess migration in SKOV-3 cells (magnification, x200). (C) Transwell assays were performed to assess migration in SKOV-3 cells (magnification, x200). (D) Relative quantitative comparison of scratch width of the wound healing assay. (E) Relative quantitative comparison of migrated cells in the cell migration assay. *P<0.05 and **P<0.01 vs. oe-nc. oe, overexpression; nc, normal control; sh, short hairpin RNA; SPARCL1, secreted protein acidic and rich in cysteine-like protein 1.

and migration and by which molecular mechanisms. The present study investigated the role of SPARCL1 in ovarian cancer. Our results show that the expression of SPARCL1 in ovarian cancer tissues was significantly lower than in the adjacent normal tissues. SPARCL1 overexpression significantly inhibited the proliferation and migration of SKOV-3 cells. Moreover, the tumor suppressing effect of SPARCL1 might be mediated by inhibiting the MEK/ERK pathway and downregulating the expression of MMP-2.

SPARCL1 was found to be downregulated in several human tumors, particularly in non-small cell lung cancer (NSCLC), pancreas and colon carcinomas, osteosarcoma, and prostate cancers (5-7,13-16). Previous studies have demonstrated SPARCL1 to mediate cell detachment of cultured cells *in vitro* and suggested a putative role of SPARCL1 as tumor suppressor (6,7,16). Bendik *et al* (17) found that SPARCL1 was significantly downregulated in nine patients with NSCLC. In another study, Xiang *et al* (7) found that SPARCL1 inhibited the migration and invasion in a prostate cancer cell line both *in vitro* and *in vivo*, suggesting a putative role of SPARCL1 in suppressing prostate cancer metastasis. Naschberger *et al* (18) reported that SPARCL1 is of key importance for the modulation of the tumor microenvironment in colorectal carcinoma, by promoting stromal cell plasticity. Moreover, SPARCL1 was more strongly expressed in highly differentiated tumors than in lowly differentiated ones, which correlated significantly with patient prognosis (19). Similarly, in our study, IHC revealed lower SPARCL1 expression in ovarian cancer

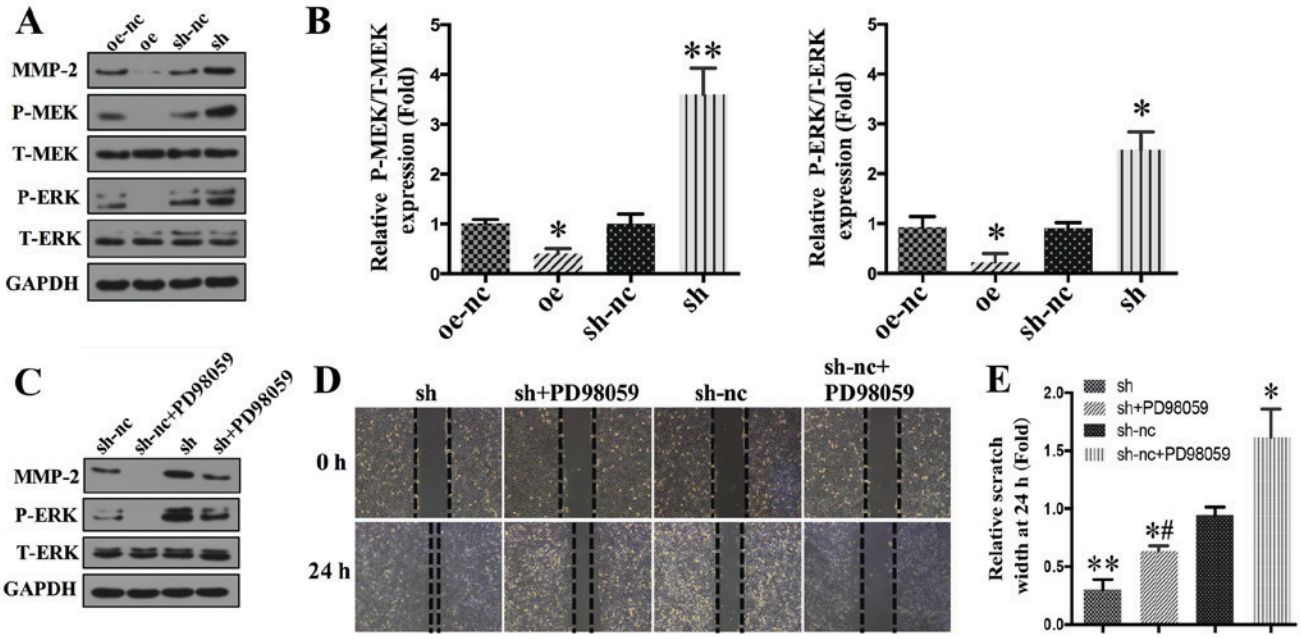

Figure 4. SPARCL1 inhibits the MEK/ERK signaling pathway. (A) The protein levels of MMP-2, p-MEK, MEK, p-ERK and ERK were assessed by western blot analyses. (B) Relative quantitative comparison of the p-MEK and p-ERK protein expression. *P<0.05 and **P<0.01 vs. oe-nc. (C) The effect of SPARCL1-knockdown on p-MEK and p-ERK expression level following the application of PD98059. (D) The effect of SPARCL1-knockdown on SKOV-3 cells migration following the application of PD98059 (magnification, x200). (E) Relative quantitative comparison of scratch width of the wound healing assay. *P<0.05 and **P<0.01 vs. sh-nc group; #P<0.05 vs. sh group. oe, overexpression; nc, normal control; sh, short hairpin RNA; SPARCL1, secreted protein acidic and rich in cysteine-like protein 1; p-, phosphorylated; T-, total; MEK, mitogen-activated protein kinase kinase; ERK, extracellular signal-related kinase; MMP, matrix metalloproteinase.

samples, suggesting that low SPARCL1 expression levels may be associated with a better prognosis. Overexpression of SPARCL1 inhibited the proliferation and migration of human ovarian cancer cells, indicating that SPARCL1 may inhibit the development and metastasis of ovarian cancer.

The MEK/ERK pathway is one of the key signaling pathways involved in cell survival and proliferation that transmits the signal by phosphorylation of a series of downstream molecules (20-27). In squamous cell carcinoma cells, MEK/ERK signaling has been demonstrated to support cell survival by inducing the anti-apoptotic protein Bcl-2 (28). Previous studies have shown that alteration of the MEK/ERK pathway is also strongly implicated in ovarian cancer pathogenesis (29-37). Al-Ayoubi *et al* (38) reported that signaling via the MEK/ERK pathway helps cells escape anoikis and maintain anchorage-independent growth in several ovarian cancer cell lines. Moreover, several studies have suggested that inhibition of the MEK/ERK pathway disrupts functions essential for ovarian cancer progression (29,35,37). Dang *et al* (39) reported that metformin in combination with cisplatin inhibits cell viability and induces apoptosis of human ovarian cancer cells by inactivating ERK. In another study, Chan *et al* (40) demonstrated that therapies targeting the ERK signaling pathway reduce the aggressiveness of ovarian cancer cells. In our study, SPARCL1 was found to inhibit the expression of p-MEK and p-ERK via the MEK/ERK pathway, which was then rescued by a MEK inhibitor. In line with previous studies, we found that inhibition of MEK/ERK was associated with ovarian cancer cell proliferation and migration. Thus, we conclude that the inhibition effect of SPARCL1 on SKOV-3 cells may be, at least partly, due to the downregulation of MEK/ERK signaling.

To our knowledge, this is the first study that investigates the effect of SPARCL1 on ovarian cancer cells. However, a key limitation of this study is that is an *in vitro* study. Thus, the results of the current study need to be validated by *in vivo* experiments in suitable animal models. Furthermore, there may be additional signal pathways involved apart from the MEK/ERK signaling. Further studies are required to determine possible alternative signal pathways that may also play a role in ovarian cancer progression.

In summary, our results show that SPARCL1 suppresses ovarian cancer cell proliferation and migration by downregulating signaling via the MEK/ERK pathway. These findings suggest that SPARCL1, a possible tumor suppressor, has potential as therapeutic target for developing novel therapeutic strategies in the prevention and intervention of ovarian cancers.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LL conceived and designed the present study. YM and YX performed the experiments, analyzed and interpreted the data, and were also the predominant contributors in the writing of the manuscript. YM made contributions to the interpretation of data and critically revised the manuscript for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Medical Ethics Committee of the Third Affiliated Hospital, Xinjiang Medical University (Xinjiang, China). Signed written informed consent was obtained from all subjects.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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