Research Square

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Inhibition of Sphingosine Kinase 2 Down-Regulates ERK/c-Myc Pathway and Reduces Cell Proliferation in Human Epithelial Ovarian Cancer

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Abstract

Background: Epithelial ovarian cancer (EOC) is the leading cause of death from female cancers. In our previous study, Sphingosine kinase 2 (SphK2) inhibitor was shown to display anti-EOC activities. The purpose of this study was to further evaluate the expression characteristics and clinical significance of SphK2 in EOC, and to explore the roles and underlying mechanisms of SphK2 in EOC cell survival.

Methods: SphK2 expression was examined by Immunohistochemistry and western blot, and its clinical implications and prognostic significance were analyzed. Cellular proliferation assay and mouse xenograft model was established to confirm the roles of SphK2 in vitro and in vivo. Cell cycle analysis, apoptosis assay and western blot were performed to examine cell cycle progression and apoptosis rate. Gene set enrichment analysis (GSEA) and western blot was used to investigate the downstream signaling pathways related to SphK2 function.

Results: SphK2 expression level was shown to be associated with stage, histological grade, lymph node metastasis and ascite status. More importantly, high SphK2 expression level was a prognostic indicator of overall survival and relapse-free survival. Moreover, knockdown of SphK2 arrested cell cycle progression and inhibited the proliferation of EOC cells both in vitro and in vivo. Furthermore, ERK/c-Myc, the key pathway in EOC progression, was important for SphK2-mediated mitogenic action in EOC cells.

Conclusion: Our findings provided the first evidence that SphK2 played a crucial role in EOC proliferation by regulating ERK/c-Myc pathway. SphK2 may serve as a prognostic marker and potential therapeutic target in EOC.

Background

Epithelial ovarian cancer (EOC), the most lethal gynecologic malignancy, is the leading cause of death from female cancers [1]. The majority of EOC patients are first diagnosed at late stages because the early-stage disease is nearly asymptomatic, which causes significantly poor prognosis and high mortality [2]. Surgery combined with platinum/taxane chemotherapy represents the first-line treatment for EOC. Unfortunately, most patients, who initially respond to the treatment, eventually develop chemoresistance [3]. Thus, development of a novel therapeutic strategy is needed. Unlike traditional cytotoxic chemotherapy, targeted therapy inhibits cancer growth by interfering with specific molecules needed for cancer progression, which may become a new hope for EOC therapy [4, 5]. An understanding of the underlining molecular mechanisms associated with EOC progression is therefore critical to identify new targets for new targeted therapy.

The roles of sphingolipids in cell biology and cell fate have been explored for several decades. There is accumulating evidence demonstrating that disturbed sphingolipid metabolism may contribute to cancer initiation and progression and present an exploitable target for cancer therapy [6]. Sphingosine kinases (SphKs), ceramide, sphingosine, transmembrane transporters, and sphingosine-1-phosphate (S1P) are the key players in sphingolipid metabolic pathway. Among these, much attention has been paid to SphKs

because their catalytic activity lies at the critical juncture in regulating sphingolipid metabolism. SphKs exhibit two isoforms, SphK1 and SphK2. SphK1 has emerged as an important and critical signaling enzyme because it involves in many aspects of cancer progression, such as proliferation, angiogenesis, metastasis and chemoresistance [7–9]. Consistent with this, we found previously that the expression level of SphK1 was significantly increased in EOC tissues and was associated with EOC metastasis and angiogenesis [10, 11]. Although the two isoforms of SphKs (SphK1 and SphK2) share high sequence similarity, they differ significantly in distribution, regulation and function. The role of SphK2 in cell survival is controversial, and both pro-apoptotic and pro-proliferative functions have been suggested. Initially, SphK2 was recognized as a pro-apoptotic protein because SphK2 over-expression inhibited the growth and promoted the apoptosis of cancer cells [12, 13]. However, it was subsequently found to be pro-survival as SphK2 down-regulation or inhibition suppressed the growth of tumor cells [14, 15]. Until now, much is still unknown or controversial about SphK2.

Recently, our research group found that treatment with an orally active and specific SphK2 inhibitor, ABC294640, significantly inhibited EOC cell proliferation and increased its apoptosis [16]. This result suggested SphK2 might be a potential target for EOC therapy. However, there is no available data on SphK2 expression patterns in EOC tissues and its clinical significance. Moreover, the molecular mechanisms of SphK2 in EOC growth remain largely unknown. This study was aimed to evaluate the expression characteristics and clinical significance of SphK2 in EOC, and to explore the roles and underlying mechanisms of SphK2 in EOC cell survival.

Materials And Methods

Reagents and antibodies

Antibodies against SphK2, c-Myc and GAPDH were purchased from Abcam. Antibodies against ERK1/2, phosphor-ERK1 (Thr202/Tyr204)/ERK2 (Thr185/Tyr187), cyclin D1, phosphor-Rb were purchased from Cell Signaling Technology. U0126 was ordered from Sigma-Aldrich.

Tissue specimens

Tissue specimens were collected from the patients without preoperative chemotherapy, including 5 normal ovarian tissues and 50 primary epithelial ovarian cancer (PEOC) tissues (stage I-II 20 cases, stage III-IV 30 cases). All patients signed the informed consent approved by Institutional Review Board of Shanghai Jiaotong University.

Cell lines and culture conditions

Human EOC cell line SKOV3 and OVCAR3 were obtained from American Type Culture Collection. SKOV3 was routinely cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and 1% antibiotics. OVCAR3 was routinely cultured in RPMI-1640 medium (Invitrogen) supplemented with 20% fetal bovine serum, 0.01 mg/ml insulin (Sigma-Aldrich) and 1% antibiotics. A2780 was purchased

from the China Center for Type Culture Collection and routinely cultured in DMEM supplemented with 10% fetal bovine serum and 1% antibiotics. Immortalized ovarian surface epithelial (IOSE) cell line was a gift from Prof. MW-Y Chan (National Chung Cheng University, Taiwan) and cultured in DMEM supplemented with 10% fetal bovine serum and 1% antibiotics.

Immunohistochemistry

Immunostaining was performed and scored as previously described [10]. The primary antibody used was anti-SphK2 (1:100, Abcam). Staining index (SI) was calculated as staining intensity score × proportion score. The protein expression level was considered to be high when score was >3, and low when score was ≤3.

Western blot analysis

Cells were harvested after the indicated treatments. The protein concentration was determined using BCA reagent. Western blotting was performed as previously described [17].

Transient (siRNA) and stable (shRNA) transfection

The siRNAs specifically targeting human ERK1 (5'-CAUGAAGGCCCGAAACUACUU-3'), ERK2 (5'-GCGCUUCAGACAUGAGAACUU-3'), and the scrambled control siRNA (5'-AAUUCUCCGAACGUGUCACGU-3') were synthesized by GenePharma. siRNA duplexes were transfected using Lipofectamine (Invitrogen) according to the manufacturer's protocol. The shRNA lentiviral packaging plasmid specifically targeting human SphK2 (5'-AACCUCAUCCAGACAGAACGA-3') and the non-targeting negative control plasmid (5'-AAUUCUCCGAACGUGUCACGU-3') were constructed by GenePharma. Cell lines were transduced with lentiviral vectors at multiplicity of infection (MOI) = 5. To establish the cells stably down-regulating SphK2, transfected cells were selected by culturing in puromycin. Single colonies of stable transfectants were isolated and expanded.

Real-time RT-PCR

RNA was extracted by TRIzol Reagent. The mRNA levels were measured by SYBR Green RT-PCR and then calculated by $2^{-\Delta\Delta Ct}$ method. Primers were as follows: SphK2, 5'-GGTTGCTTCTATTGGTCAATCC-3' (forward) and 5'-GTTCTGTCGTTCTGTCTGGATG-3' (reverse); GAPDH, 5'-TGCACCACCAACTGCTTAGC-3' (forward) and 5'-GGCATGGACTGTGGTCATGAG-3' (reverse) [17].

Cellular proliferation assay

Cell proliferation was assessed using WST-1 (Roche) assay as previously described [18]. Briefly, the indicated cells were seeded into 96-well plates and the cell number was measured at 24, 48, 72 and 96 h. At each time point, WST-1 assay reagent was added into each well and cultured at 37℃ for 2 h. The supernatant from each well was then collected for measurement of absorbance at 450 nm. There is a direct correlation between the cell number and the absorbance at 450 nm in the current study.

Animal studies

The animal experiments were performed according to the Laboratory Animal Guidelines provided by Shanghai Jiao Tong University School of Medicine. The experimental protocols were approved by the Institutional Animal Care and Use Committee of Shanghai Jiao Tong University School of Medicine. All the experiments and methods were performed in accordance with the relevant guidelines and regulations. Mice were purchased from the Chinese Academy of Sciences. To establish subcutaneous transplantation models, female BALB/c nu/nu mice aged 5-6 weeks (8 mice in each group) were subcutaneously injected with 2×10^6 EOC cells stably transfected with indicated plasmids. Tumor volumes were calculated twice a week using the following formula: V = (largest diameter × small diameter × depth) ×p /6. 28 days after injection of EOC cells, the mice were sacrificed and the weight of tumors was measured.

Cell cycle analysis

Cells were trypsinized, fixed in 70% ice-cold ethanol overnight, and stained with propidium iodide at room temperature for 45 minutes. The cellular DNA content was analyzed by flow cytometry.

Apoptosis assay

The Annexin V-FITC/PI apoptosis detection kit (BD, USA) was used to identify apoptotic cells by flow cytometry following the manufacturer's instructions.

Microarray analysis

Tumor RNA was prepared with RNeasy Plus Mini Kit (Qiagen) according to manufacturer’s protocol. Total RNA was subjected to microarray analysis with Agilent Whole Human Genome Oligo Microarray.

Statistical analysis

Statistical analyses were performed by using the SPSS software. c^2 test was used to analyze the correlations between SphK2 expression and the clinicopathologic features of EOC. Kaplan–Meier curves and log-rank tests were used to analyze the survival data. The values were presented as the mean ± SD and were analyzed by t-test. A *P* value less than 0.05 was considered significant.

Results

SphK2 expression patterns in primary EOC tissues

SphK2 protein expression was examined in 50 primary epithelial ovarian cancer (PEOC) and 5 normal ovarian tissue specimens by immunohistochemical staining. Representative photographs of SphK2 immunostaining were shown in Fig. 1A. 62% (31/50) of the PEOC specimens showed high SphK2 expression and 38% (19/50) showed low expression. In contrast, none of the normal ovarian tissue specimens exhibited high SphK2 expression. Consistent with the findings from tissue specimens, the

expression of SphK2 was significantly elevated in all four EOC cell lines examined compared to IOSE cells, an immortalized non-tumorigenic human ovarian surface epithelial cell line (Fig. 1B). We further examined the relationship between SphK2 expression levels in PEOC and clinic-pathological characteristics (Table 1). High SphK2 expression level was associated with higher FIGO stage ($p = 0.001$), higher histological grade ($p = 0.049$), lymph node metastasis ($p = 0.001$) and ascites ($p = 0.012$), which indicated that SphK2 expression was linked to the oncogenic behavior of EOC. However, we observed no correlation between SphK2 levels and age and histologic subtypes.

Table 1
Clinicopathological features of ovarian tissue with regard to the relative expression of SphK2

Parameters	No. cases	SphK2 expression		χ^2	*P* value
		low	high		
Age(years)					
≤Mean	21	10	11	1.422	0.233
Mean	29	9	20		
FIGO stage					
I-II	20	13	7	10.314	0.001 *
III-IV	30	6	24		
Histologic grade					
I	7	5	2	3.861	0.049 *
II-III	43	14	29		
Histology					
Serous	42	13	29	5.675	0.059
Mucinous	3	2	1		
Endometrioid	5	4	1		
Lymph node metastasis					
Absent	13	10	3	11.297	0.001 *
Present	37	9	28		
Ascites					
Negative	18	11	7	6.376	0.012 *
Positive	32	8	24		
*, $P < 0.05$					

Correlations Between Sphk2 Expression Level And Survival Analysis

The prognostic value of SphK2 was analyzed by comparing the overall survival (OS) and relapse free survival (RFS) of patients with high and low SphK2 expression. Kaplan-Meier analysis showed that patients with high SphK2 expression had a significantly lower postoperative 5-year OS and a significantly

lower postoperative RFS than patients with low SphK2 expression (Fig. 1C and 1D) ($P < 0.01$). Together, these data suggested that SphK2 was a potentially useful predictor for the outcome of EOC.

SphK2 is required for EOC cell growth in vitro and in vivo

To assess the importance of SphK2 in EOC cells, we performed shRNA-based knockdown experiments in human EOC cell lines, which resulted in efficient reduction in SphK2 mRNA and protein levels compared to the control cells transfected with a negative control vector (Fig. 2A-2B). Proliferation assays revealed that SKOV3 cells subjected to SphK2 knockdown by shRNA were strongly inhibited in cell growth compared to controls (Fig. 2C-2D). To investigate whether SphK2 also has a role in EOC progression, we used a subcutaneous transplanted model of ovarian cancer in nude mice. The nude mice were subcutaneously injected with SphK2-downregulated SKOV3 cells or control cells, and tumor growth was measured after one month. As shown in Fig. 2E-2G, the SphK2-downregulated cell tumors were much smaller than the control cell tumors. Together, these results showed that SphK2 is critical for EOC cell growth *in vitro* and *in vivo.*

Sphk2 Controls Cell Cycle Progression Of Eoc Cells

To investigate the cellular mechanisms by which SphK2 was required for ovarian cancer cell growth, we used SKOV3 to analyze the effects of SphK2 down-regulation on cell cycle and apoptosis. Compared to the control cells, shRNA-mediated SphK2 inhibition resulted in an accumulation of SKOV3 in the G0/G1 phase with a reduction of cells in S phase (Fig. 3A). Moreover, subsequent western blot analysis indicated that SphK2 down-regulation reduced the levels of Cyclin D1 and phosphor Rb (Fig. 3B). In contrast to the strong effects on cell cycle progression, SphK2 down-regulation did not affect the apoptotic rate of ovarian cancer cells (Fig. 3C). Together, these data suggested that SphK2 inhibition in ovarian cancer cells induces cell cycle G0/G1 arrest.

SphK2 inhibition in ovarian cancer cells induces an expression profile linked to MAPK signaling pathway and MYC targets

To investigate the underlying molecular mechanisms whereby SphK2 is required for the growth of EOC cells, we performed RNA-Seq analysis of ovarian cancer SKOV3 cells transfected with shRNA targeting SphK2. Heatmap clustering of the top 100 upregulated or downregulated genes (Fig. 4A) showed a high degree of reproducibility between triplicates, and a significant differentiation between the SphK2 knockdown cells and the cells transfected with a scramble control. Gene set enrichment analysis (GSEA) was used to analyze the SphK2-regulated gene signatures. The Kyoto encyclopedia of genes and genomes (KEGG) pathway analysis revealed that genes deregulated upon SphK2 suppression were enriched for gene sets such as MAPK signaling pathway (Fig. 4B-4C). Consistent with the link to MAPK pathway, GESA analysis revealed significant associations with the gene set involving ERK MAPK targets (Fig. 4D). Intriguingly, the results of GESA showed the expression changes in response to SphK2

inhibition were most significantly correlated with the gene signature of two individual subgroups of MYC targets data sets (Fig. 4E-4F).

Sphk2 Down-regulation Induces C-myc Inhibition Partly Through Erk1/2 Pathway

ERK1/2 pathway is one of the best-characterized members of the MAPK family. Moreover, it has been demonstrated to play essential roles in cell cycle progression and the survival of cells [19]. Thus, we detected the expression level of phosphorylated ERK1/2 protein by western blot and confirmed that SphK2 inhibition down-regulated the ERK1/2 pathway activation (Fig. 5A). C-Myc, the most widely studied gene in MYC family, is involved in cell cycle regulation, cell proliferation and differentiation [20, 21]. Down-regulation of c-Myc after SphK2 blockage was also confirmed by western blot (Fig. 5A). C-Myc has been reported to be an inducible gene of ERK [22]. To determine whether the down-regulation of c-Myc was partly through ERK1/2 pathway in EOC cells, we investigated the effect of ERK1/2 blockage on c-Myc expression. As expected, ERK1/2 blockage by U0126, a specific inhibitor of ERK1/2 pathway, significantly inhibited c-Myc expression (Fig. 5B). Moreover, ERK1/2 knockdown also resulted in the suppression of c-Myc (Fig. 5C). These results suggested that SphK2 down-regulation induced c-Myc inhibition partly through ERK1/2 pathway.

Discussion

In the current study, we found that SphK2 played a crucial role in EOC progression and that knockdown of SphK2 could significantly inhibit the proliferation of the cancer. Specially, our study provided the following new findings: (1) SphK2, up-regulated in ovarian cancer tissues, correlated with a poor prognosis in this deadly disease; (2) SphK2 blockage in EOC cells caused significant inhibition of cell growth and an arrest in the G1/G0 phase; (3) Inhibition of SphK2 down-regulated ERK/c-Myc pathway, which partly mediating SphK2 induced cell cycle arrest.

SphKs exhibit two isoforms, SphK1 and SphK2. SphK1, a key player in cancer progression [23], is elevated markedly in ovarian cancer [11, 24]. However, the analysis of SphK2 expression in EOC has not been previously reported. Here, we showed that the level of SphK2 was elevated in EOC specimens. Notably, the SphK2 level was closely correlated with the well-known prognostic parameters of EOC, including FIGO stage, histological grade, lymph node metastasis and ascites. Moreover, elevated expression level of SphK2 was found to be associated with poor prognosis and was a prognostic factor for predicting OS and RFS. These findings implicated that SphK2 might be a potentially important factor in EOC progression.

The contribution of SphK2 to cancer progression is unclear. Some studies found high-level expression of SphK2 could induce cell cycle arrest and apoptosis [12, 13]. Despite the notion that SphK2 is a pro-apoptotic factor, a number of studies emerged and indicated SphK2 promoted cancer survival. SphK2

knockdown or inhibition has been shown to induce apoptosis in some cancer types [14, 15]. Moreover, in some cancer cell lines, targeting SphK2 even has a more powerful anti-cancer effect than targeting SphK1 [25, 26]. In this study, the relationship between SphK2 levels and the prognosis of EOC patients suggests a role for SphK2 in promoting EOC. Therefore, we investigated the effect of SphK2 down-regulation on the growth of EOC cells *in vitro.* As expected, SphK2 blockage by shRNA significantly inhibited ovarian cancer proliferation. In accordance with the *in vitro* results, SphK2 down-regulation also reduced tumor growth in a mouse ovarian cancer model. These results indicated that SphK2 was associated with EOC progression and raised the possibility that SphK2 might serve as a new target for EOC therapy.

SphK2 inhibition has been reported to be associated with both caspase-dependent and autophagy-dependent cell death [15, 27]. Autophagy is implicated in both cell death and cell survival. Although SphK2 inhibition could induce autophagy in EOC cells, this was not the cause of cell death, a situation similar to that following FTY720 exposure [28]. Moreover, in ovarian cancer, SphK2 down-regulated cells displayed only modest levels of early and late apoptotic cells. Consistent with the lack of apoptosis, genes involved in apoptosis were not changed significantly in SphK2 down-regulated cells (data not shown). In contrast to the modest effects in cell apoptosis, SphK2 depleted cells showed a dramatic accumulation in the G0/G1 phase compared to the controls. Furthermore, SphK2 down-regulation caused the inhibition of Cyclin D1 and phosphor-Rb, several key cell cycle check-point factors. Taken together, our data indicated that the primary cellular mechanism, whereby inhibition of SphK2 prevented cell growth of ovarian cancer cells, is mediated through the inhibition of cell cycle progression, rather than induction of autophagy or apoptosis.

Another new finding in this study is the down-regulation of ERK/c-Myc pathway as a result of SphK2 inhibition. Extensive studies have demonstrated essential roles of ERK1/2 signaling in the survival of ovarian cancer [29–31]. Moreover, c-Myc, an inducible gene of ERK, was reported to be a key mediator of the progression of ovarian cancer [32, 33]. Furthermore, ERK or c-Myc inhibition has been reported to cause a G0/G1 block in the cell cycle of ovarian cancer cells [29, 32]. Therefore, it is possible that inhibition of SphK2 resulted in the repression of ERK/c-Myc pathway and subsequently caused G0/G1 cell cycle arrest, which consequently reduced cell proliferation in human epithelial ovarian cancer. Moreover, it was reported that ERK1 could activate SphK2 by direct phosphorylation in breast cancer [34], which indicated that ERK1 was the upstream activator of SphK2. This report, together with our observation that SphK2 blockage resulted in a significant inhibition of ERK activation, suggest ERK1 might be placed both upstream and downstream of SphK2 signaling and might has a dual role in the initiation and amplification of the SphK2 signaling loop in EOC cells. This speculation needs to be further investigated in the future.

Conclusions

Our study provided the first evidence that SphK2 played a crucial role in EOC proliferation by regulating ERK/c-Myc pathway. High SphK2 expression level was a prognostic indicator of overall survival and

relapse-free survival. Moreover, knockdown of SphK2 inhibited the proliferation of EOC cells both in vitro and in vivo by arresting cell cycle progression. Furthermore, ERK/c-Myc, the key pathway in EOC progression, was important for SphK2-mediated mitogenic action in EOC cells. These data suggested that SphK2 was a prognostic marker and a potential therapeutic target in EOC.

Declarations

Acknowledgments

Not applicable.

Authors' Contributions

LD and WD designed the experiments and wrote the paper. LD, KQS, WJW and YXL conducted the experiments and analyzed the data. All authors read and approved the final version of the manuscript.

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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.

Ethics approval and consent to participate

The animal experiments were performed according to the Laboratory Animal Guidelines provided by Shanghai Jiao Tong University School of Medicine. The experimental protocols were approved by the Institutional Animal Care and Use Committee of Shanghai Jiao Tong University School of Medicine.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

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Figures

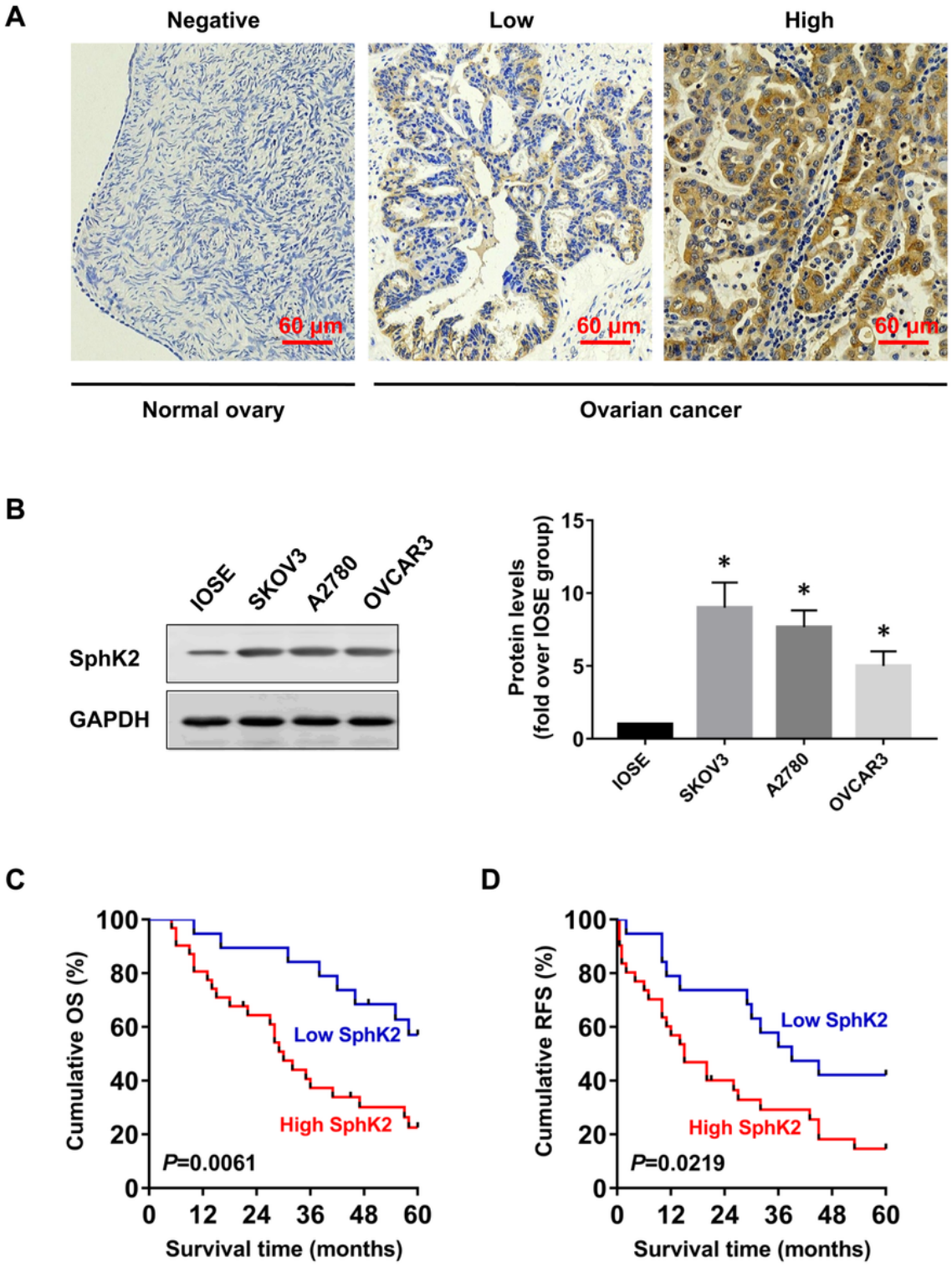


Figure 1

SphK2 over-expression in human ovarian cancer and its prognostic significance. (A) Immunohistochemical staining of SphK2 in normal ovary and ovarian cancer tissues. The scale bar represents 60 μm. (B) Western blot analysis of SphK2 in Immortalized ovarian surface epithelial (IOSE) cell line and ovarian cancer cell lines. (C and D) Kaplan-Meier curves showing overall survival (OS) and relapse free survival (RFS) of ovarian cancer patients.

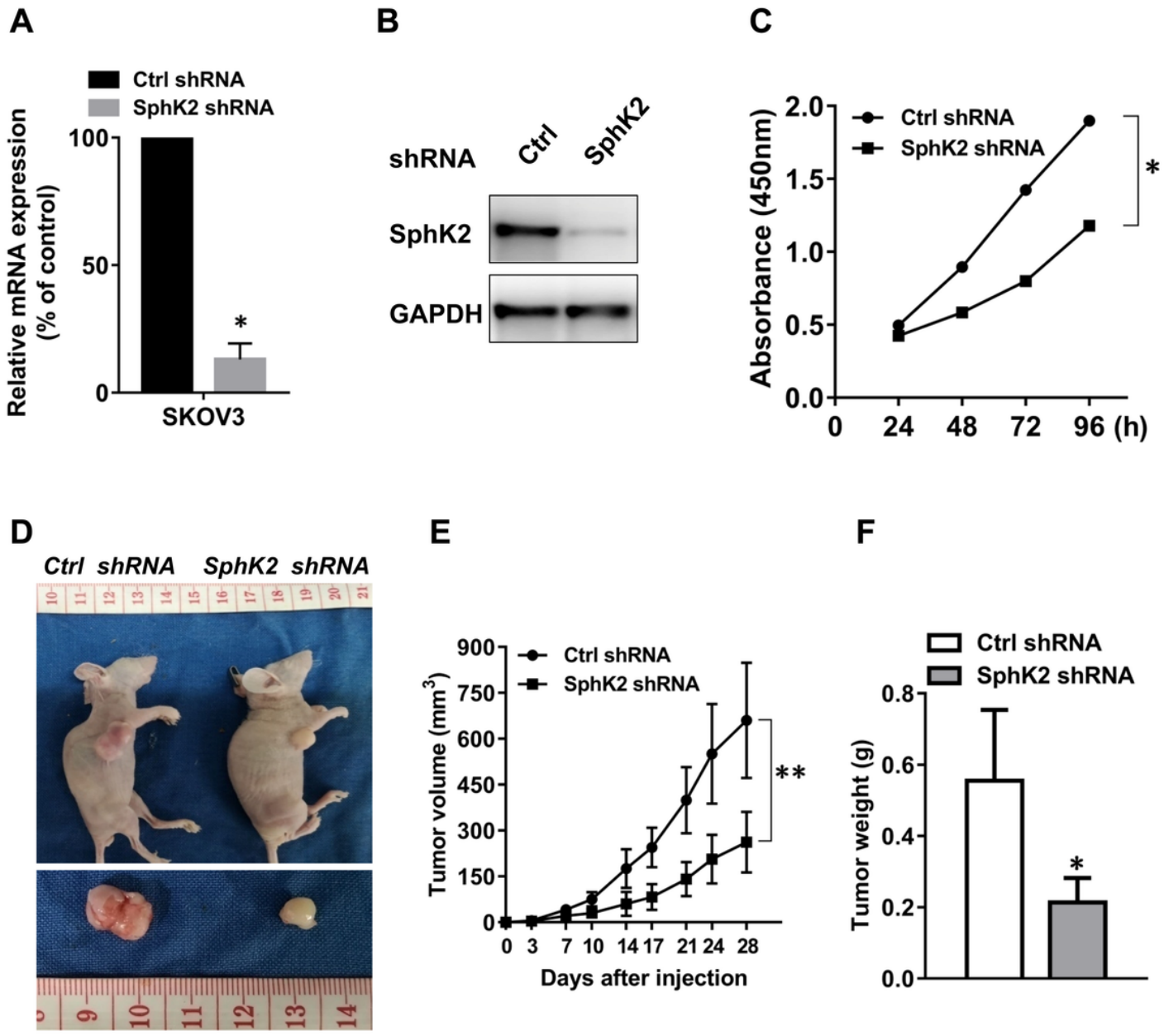


Figure 2

SphK2 inhibition suppresses the growth of EOC cells in vitro and in vivo. (A) SKOV3 cells are transfected with control shRNA or SphK2 shRNA. Expression of SphK2 mRNA levels are determined by PCR and normalized to GAPDH mRNA. (B) Protein levels of SphK2 are determined by western blot. (C) A significant reduction in cell growth rate in the SphK2 shRNA group is observed by the Cellular proliferation assay in ovarian cancer cells. (D) Representative images of tumors in subcutaneous ovarian cancer xenograft model. Mean tumor volume (E) and weight (F) in the SphK2 shRNA group are significantly reduced compared with the control group. *p < 0.05, **p < 0.01.

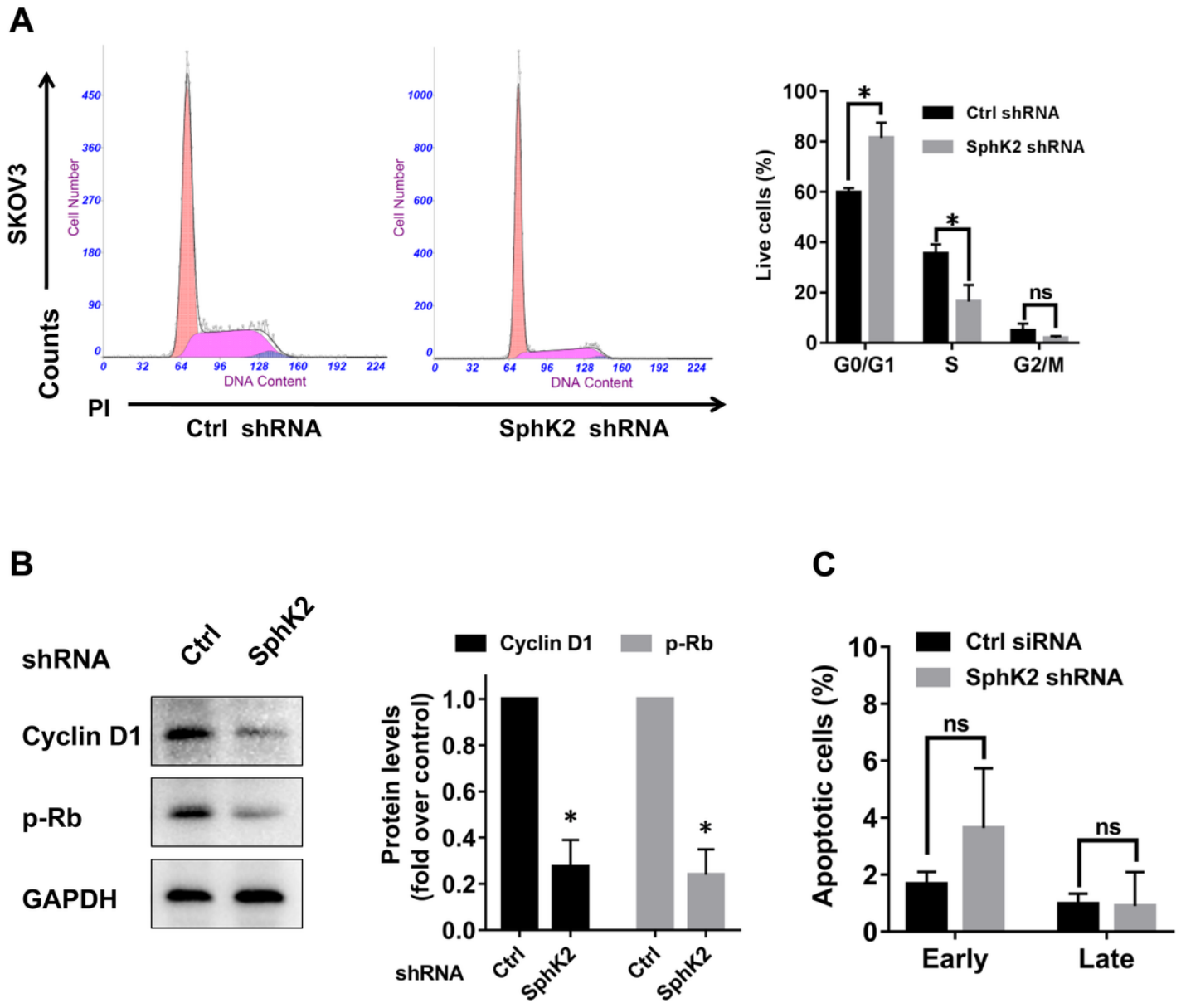
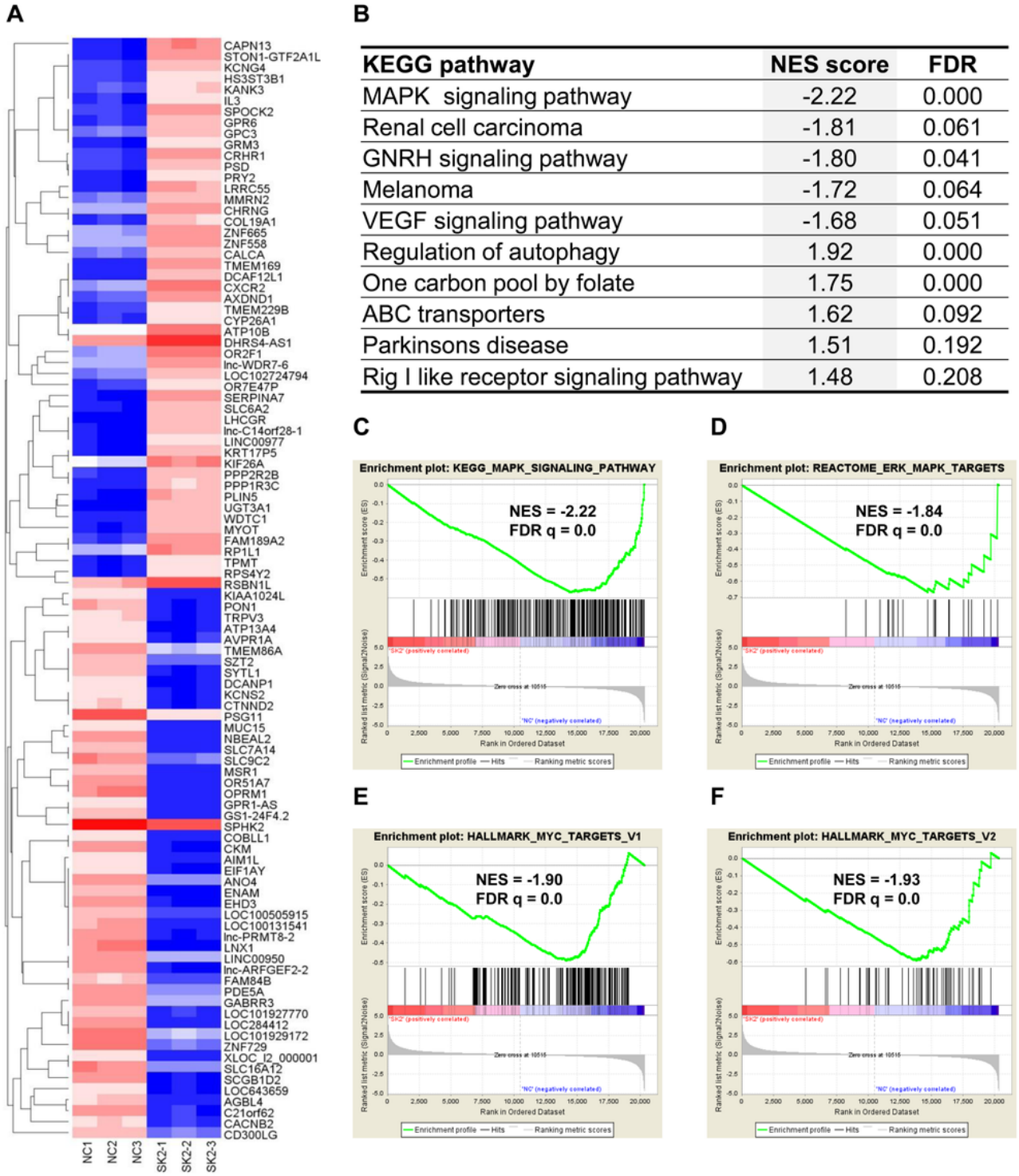


Figure 3

SphK2 inhibition in EOC cells causes an arrest in the G0/G1 phase of the cell cycle. (A) Representative flow cytometry charts of SKOV3 cells transfected with SphK2 shRNA or Ctrl shRNA and stained by propidium iodide (PI). The percentage of cells in each cell cycle population was quantificated. (B) Western blot analysis of Cyclin D1 and p-Rb in ovarian cancer cells transfected with SphK2 shRNA or Ctrl shRNA. Right panels show that densitometric analysis of Cyclin D1 and p-Rb (normalized to GAPDH) is reduced in SKOV3. (C) Bar graphs showing the percentage of apoptotic cells of SKOV3 transfected with SphK2 shRNA or Ctrl shRNA. *p < 0.05, **p < 0.01.



KEGG pathway	NES score	FDR
MAPK signaling pathway	-2.22	0.000
Renal cell carcinoma	-1.81	0.061
GNRH signaling pathway	-1.80	0.041
Melanoma	-1.72	0.064
VEGF signaling pathway	-1.68	0.051
Regulation of autophagy	1.92	0.000
One carbon pool by folate	1.75	0.000
ABC transporters	1.62	0.092
Parkinsons disease	1.51	0.192
Rig I like receptor signaling pathway	1.48	0.208

Figure 4

SphK2 suppression induces an expression profile correlating to MAPK signaling pathway and MYC targets. (A) Heatmap of 100 genes with most significant changes in gene expression of SKOV3 cells transfected with SphK2 shRNA or Ctrl shRNA. (B) List of the ten most significant KEGG gene set pathways correlating to gene expression changes resulting from SphK2 knockdown in SKOV3 cells. (C-F) Gene set enrichment analysis (GSEA) shows significant association between SphK2 level and several

gene sets, including MAPK signaling pathway (C), ERK MAPK targets (D), MYC target V1 (E) and MYC target V2 (F).

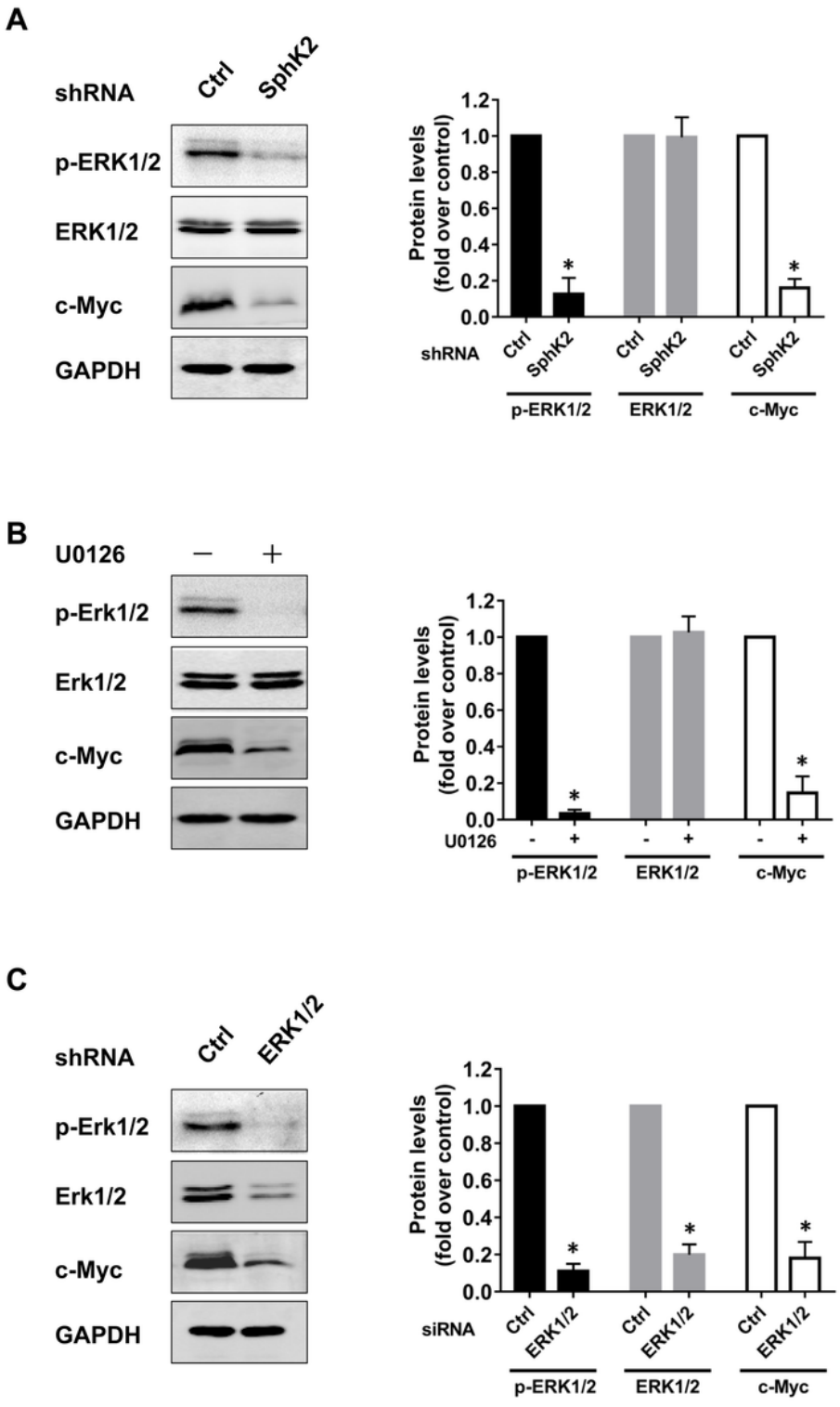


Figure 5

SphK2 blockage induces c-Myc inhibition partly through ERK1/2 pathway. (A) Western blot analysis of p-ERK1/2, ERK1/2 and c-Myc in ovarian cancer cells transfected with SphK2 shRNA or Ctrl shRNA. SphK2 inhibition reduces expressions of p-ERK1/2 and c-Myc not affecting ERK1/2. (B) Pretreatment with

U0126, inhibitor of ERK1/2 pathway, also reduces the expressions of p-ERK and c-Myc in ovarian cancer cells. (C) Western blot analysis of p-ERK1/2, ERK1/2 and c-Myc in ovarian cancer cells transfected with ERK1/2 siRNA or Ctrl siRNA. Right panels show densitometric analysis of p-ERK1/2, ERK1/2 and c-Myc (normalized to GAPDH) corresponding to the bands shown in the Western blots. *$p < 0.05$