



## Original Research

## CPT2 down-regulation promotes tumor growth and metastasis through inducing ROS/NF $\kappa$ B pathway in ovarian cancer

Xiaohong Zhang, ConceptualizationMethodologyData curationInvestigationWriting-Original draft preparation<sup>a,#</sup>, Zhen Zhang, Data curationInvestigation<sup>b,#</sup>, Shujuan Liu, Conceptualization and Methodology<sup>a,#</sup>, Jia Li, VisualizationInvestigation (in vitro cell lines studies)<sup>a</sup>, Liying Wu, VisualizationInvestigation (in vitro cell lines studies)<sup>a</sup>, Xiaohui Lv, Investigation (animal studies)<sup>a</sup>, Jia Xu, Investigation (animal studies)<sup>a</sup>, Biliang Chen, Writing- Reviewing and Editing<sup>a,\*</sup>, Shuhua Zhao, Writing-Original draft preparation<sup>a,\*</sup>, Hong Yang, SupervisionWriting- Reviewing and Editing<sup>a,\*</sup>

<sup>a</sup> Department of Gynaecology and Obstetrics, Xijing Hospital, Fourth Military Medical University, Xi'an 710032, China.

<sup>b</sup> Department of stomatology, Shaanxi Provincial People's Hospital, Xi'an 710068, China.

## ARTICLE INFO

## Keywords:

Carnitine palmitoyltransferase 2  
Growth  
Metastasis  
Glycolysis  
Ovarian cancer

## ABSTRACT

**Background:** Carnitine palmitoyltransferase 2 (CPT2) is a rate-limiting enzyme involved in fatty acid  $\beta$ -oxidation (FAO) regulation. Recently, it has been increasingly recognized that lipid metabolism dysregulation is closely implicated in tumorigenesis. However, the involvement of CPT2 in the progression of cancer is still largely unclear, especially in ovarian cancer (OC).

**Methods:** In the present study, CPT2 expression and its clinical significance were determined in OC tissues and cells. The biological functions and molecular mechanisms of CPT2 in OC growth and metastasis were determined by *in vitro* and *in vivo* assays.

**Findings:** We found that CPT2 was frequently down-regulated in primary ovarian serous carcinomas, which is significantly correlated with poor survival of ovarian cancer patients. Functional experiments revealed that CPT2 inhibited OC cell growth and metastasis via suppression of G1/S cell cycle transition and epithelial to mesenchymal transition (EMT), as well as induction of cell apoptosis. Mechanistically, suppression of ROS/NF $\kappa$ B signaling pathway by increasing fatty acid oxidation-derived NADPH production was involved in the anti-tumorigenic functions of CPT2 in OC cells.

**Interpretation:** Altogether, our findings demonstrate that CPT2 functions as a potential tumor suppressor in OC progression. CPT2 may serve as a novel prognostic marker and therapeutic target in OC.

## Research in context

**Evidence before this study** Carnitine palmitoyltransferase 2 (CPT2) is a rate-limiting enzyme in fatty acid  $\beta$ -oxidation (FAO). Recently, it has been increasingly recognized that lipid metabolism dysregulation is closely implicated in tumorigenesis. However, the involvement of CPT2 in the progression of cancer is still largely unclear, especially in ovarian cancer (OC).

**Added value of this study** We demonstrated that CPT2 was frequently down-regulated in primary ovarian serous carcinomas, which is significantly correlated with poor survival of ovarian cancer patients.

Functional experiments revealed that CPT2 inhibited OC cell growth and metastasis via suppression of G1/S cell cycle transition and epithelial to mesenchymal transition (EMT), as well as induction of cell apoptosis. Mechanistically, suppression of ROS/NF $\kappa$ B signaling pathway by increasing fatty acid oxidation-derived NADPH production was involved in the anti-tumorigenic functions of CPT2 in OC cells.

**Implications of all the available evidence** CPT2 functions as a potential tumor suppressor in OC progression and may serve as a novel prognostic marker and therapeutic target in OC.

\* Corresponding author.

E-mail addresses: [cbxjh@fmmu.edu.cn](mailto:cbxjh@fmmu.edu.cn) (B. Chen), [zhaoshuhua5044@sina.com](mailto:zhaoshuhua5044@sina.com) (S. Zhao), [yanghongdoc@163.com](mailto:yanghongdoc@163.com) (H. Yang).

# These authors contributed equally to this work.

## 1. Introduction

Alteration of lipid metabolism has emerged as a phenotype common to most human carcinomas [1,2]. Cancer cells show continuous *de novo* lipogenesis to provide membrane building blocks and substrates, as well as lipid signaling molecules required for rapid proliferation of cancer cells [3,4]. With most studies focusing on fatty acid synthesis (FAS), the relevance of fatty acid oxidation (FAO) to cancer has received less attention [3,5]. During recent years, the critical role of FAO in cancer development and progression has been increasingly recognized [6–8]. FAO can provide tumor cells with required ATP, NADPH and metabolic intermediates for cell survival and growth [9].

Carnitine palmitoyltransferase system (CPTs) are mitochondrial located enzymes involved in FAO regulation through mediating the first step of fatty acid transport from cytoplasm into mitochondria for oxidation [10,11]. CPT family is made up of two separate proteins, including CPT1 located in the outer membrane and CPT2 located in the inner membrane of mitochondrial. To date, three tissue-specific isoforms of CPT1 have been identified, including the liver isoform CPT1A, muscle isoform CPT1B and brain isoform CPT1C. Nevertheless, CPT2 is an ubiquitous protein [11]. A large number of studies have reported the over-expression of CPT1 and its tight association with tumor progression in many types of human cancers [12,13]. In addition, two recent studies also have demonstrated a critical role of CPT2 in the tumorigenesis of hepatocellular carcinoma [14,15]. However, the expression and biological effect of CPT2 in other types of cancer remains largely unknown, including ovarian cancer.

In the present study, we analyzed the expression profile, clinical significance, biological effects and the underlying molecular mechanisms of CPT2 in ovarian cancer (OC).

## 2. Materials and methods

### 2.1. Ovarian cancer cell lines and tissue samples

Four OC cell lines (A2780, ES2, SKOV3 and HEY) and one immortalized ovarian cell line IOSE80 were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and maintained in DMEM cell culture medium containing 10% fetal bovine serum (HyClone) at 37 °C under 5% carbon dioxide. All cell lines have been authenticated using STR profiling within the last three years.

In addition, fresh ovarian malignant tissues and matched adjacent non-tumor tissues were collected from 121 patients who underwent surgery at the First Affiliated Hospital of Fourth Military Medical University. The Ethics Committee of the Fourth Military Medical University approved the study documents and the use of archived cancer tissues and written informed consent was also obtained from each patient.

### 2.2. Knockdown and forced expression of target genes

CPT2 was knocked-down in ovarian cancer cells by two small interference RNAs (siRNAs) with following sequences: siCPT2#1: 5'-CCAGTGAGAACCGAGACAT-3'; siCPT2#2: 5'-TCTTGAATGATGGCCAGTT-3'. A scramble form (siCtrl) was used as a negative control: 5'-GCACTACCAGAGCTAACTCAGATAGTACT-3'. For CPT2 over-expression in ovarian cancer cells, CPT2 were amplified by PCR reaction and cloned into expression vector. All transfections were applied with lipofectamine 2000 (Life Technologies) according to the manufacturer's protocols.

### 2.3. RNA extraction and real-time PCR analyses

Trizol reagent was added to extract total RNA from ovarian cancer cells. Then, random primers were used for reverse transcription of RNA into cDNA. A SYBR Green master mixture (Applied Biosystems)

was used for PCR analysis. The primer sequences were provided in the supplementary Table 2.

### 2.4. Western blot

Ovarian cancer cells were lysed with RIPA buffer and equal amount of the protein extracts were resolved through SDS–polyacrylamide gel electrophoresis. Then, protein was transferred to PVDF membranes. After blocked with 5% milk, the membranes were probed with primary antibodies directed against human CPT2 or  $\beta$ -actin and then with a peroxidase-conjugated secondary antibody (Proteintech). The reactions were finally visualized by an enhanced chemiluminescence (GE, Fairfield, CT, USA). The primary antibodies used are provided in supplementary Table 3.

### 2.5. Immunohistochemistry staining

An IHC detection kit was used for immunohistochemistry staining. Firstly, paraffin-embedded tissue slides were deparaffinized, rehydrated and treated with H<sub>2</sub>O<sub>2</sub> and hot citrate buffer. Then, primary antibodies were added to the slides and incubated at 4 °C for 10 h followed by secondary antibody incubation at 28 °C for 2 h. Hematoxylin was used for counterstaining of the nuclei. Finally, the results were scored based on the percentage of positive staining and their intensities.

CPT2 staining was scored (0–12 points) based on the percentage and intensity of positive staining. The median IHC staining score was chosen as the cutoff for distinguishing CPT2 low and CPT2 high OC patients.

### 2.6. MTS and colony formation assays

Cell proliferation was evaluated using the MTS assay (Promega, G3581). OC cells were plated into 96-well cell culture plates (#020096, Xinyou Biotech, Hangzhou, China) at a density of 2000 cells/well. Twenty  $\mu$ l of MTS (0.2%)-PMS solution was added into each well at 0, 24, 48, 72, 96 and 120 h and the absorbance at 490 nm was measured after 2 h incubation.

To evaluate the ability of cell colony formation, 1,000 ovarian cancer cells were plated into 6-well plates. After culture for two weeks, colonies were stained with crystal violet for 15 min and their numbers were counted.

### 2.7. Flow cytometry analysis for cell cycle and apoptosis

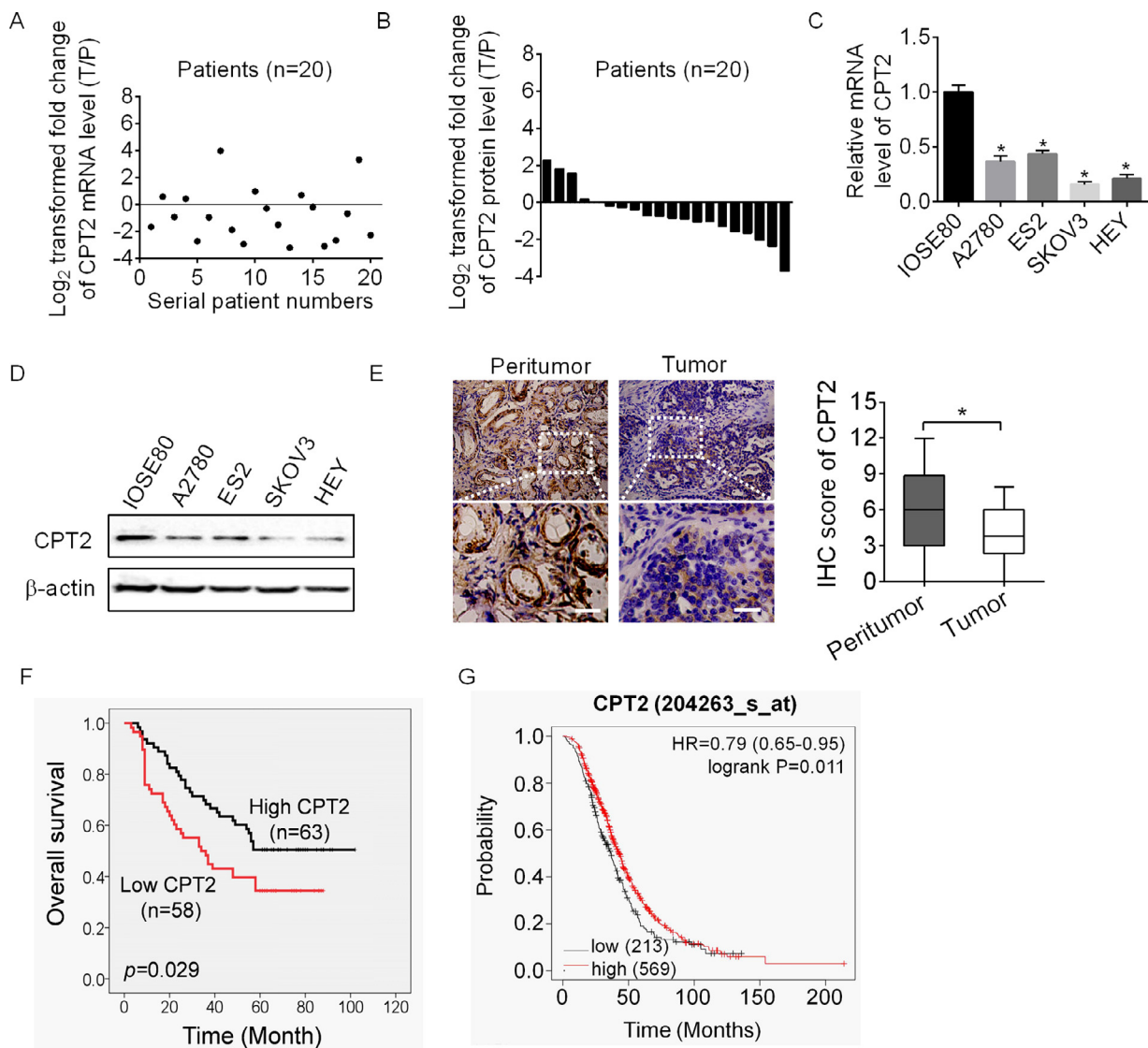
For determination of cell cycle distribution, cold phosphate buffered saline was used for washing ovarian cancer cells. After fixed in cold ethanol (70%), ovarian cancer cells were incubated in RNase and propidium iodide (PI) solutions. Then, the population of cells in each phase was evaluated by flow cytometry.

Ovarian cancer cell apoptosis was also evaluated by flow cytometry using a double staining apoptosis kit (F6012, US Everbright Inc). An *in situ* cell death detection kits (Roche) was used for TUNEL staining assay. Results were evaluated with a fluorescence microscope.

### 2.8. Wound-healing and matrigel invasion assays

For a microscope wound-healing migration assay, ovarian cancer cells were plated into 6-well plates and cultured to about 95% confluency. Then, a scratch was placed in the monolayer cells with a sterile pipette tip. The cells were observed at 0 and 48 h after the scraping. The average percentage of the gap closure in each group was compared.

The invasion of ovarian cancer cells was evaluated by matrigel invasion assay. Briefly, the transwell chamber was coated with matrigel basement membrane matrix on the upper surface (BD Biosciences). Then, a total of  $1 \times 10^5$  ovarian cancer cells were seeded into the top chamber wells and cultured in a serum-free medium for 48 h. The penetrated cells on the lower side of the filter were then fixed with



**Fig. 1.** CPT2 expression was frequently down-regulated in OC cells and contributes to worse survival in OC patients. (A and B) CPT2 expression evaluation by qRT-PCR and western blot analysis in tumor and peritumor tissues from 20 OC patients. (T, tumor; P, peritumor). (C and D) CPT2 expression was determined in four OC cell lines A2780, ES2, SKOV3 and HEY, and one nonmalignant ovarian cell line IOSE80. (E) IHC staining of CPT2 in OC tumor and peritumor tissues from 121 OC patients. Scale bar, 20  $\mu$ m. (F) Analysis for overall survival of OC patients according to the IHC staining results of CPT2. (G) Bioinformatic prognostic significance analysis of CPT2 expression level based on the online web Kaplan–Meier Plotter. \* $P < 0.05$ .

paraformaldehyde and stained with crystal violet. At last, the results were evaluated with a microscope.

### 2.9. In vivo tumor growth and metastasis assays

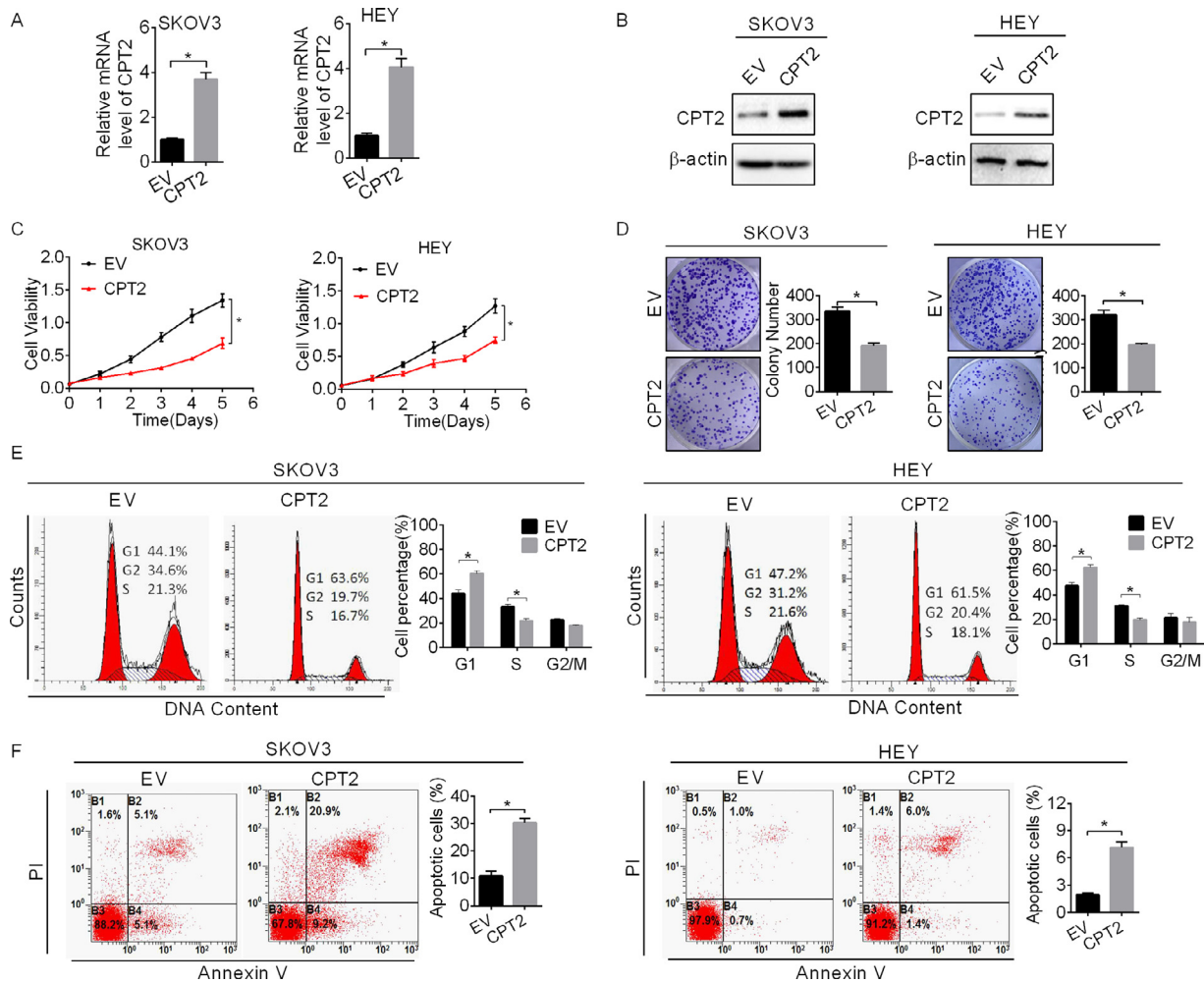
Ovarian cancer cells ( $1 \times 10^7$  cells per mice) with stable knockdown of CPT2 in 100  $\mu$ l PBS were injected subcutaneously into the flanks of 5-week-old male Balb/c nude mice (six mice per group). Tumor size was measured every week for five weeks. At the end of the experiment, the mice were sacrificed and the primary tumors were carefully removed and their weights were measured. For in vivo metastasis analysis, ovarian cancer cells were injected into the tail vein of 5-week-old male Balb/c nude mice (six mice per group) to allow metastatic colonization in the lungs. The mice were sacrificed 45 days post injection and metastasis nodules formed in lungs were counted. All animal experimental procedures were approved in accordance with the Institutional Animal Care and Use Committee of the Fourth Military Medical University.

### 2.10. Detection of cellular reactive oxygen species (ROS)

A fluorescent probe DCFH-DA (Beyotime Biotechnology, S0033) was used for ROS level detection following to the manufacturer's protocols. Briefly, 10 mM fluorescent probe DCFH-DA in serum free medium was added to OC cells in each group. Flow cytometry was used for fluorescence determination after culture for 20 min at 37 C.

### 2.11. Fatty Acid $\beta$ -Oxidation detection

Ovarian cancer cells ( $1.5 \times 10^6$  cells/well) with different levels of CPT2 were cultured in 6-well plate. Then, 1 ml of HBSS containing 2  $\mu$ Ci [9, 10(n)- $^3$ H] oleic acid (Amersham Pharmacia Biotech, Italy) and 0.5 mg fatty acid-free BSA was added to the cells and cultured for 12 h. The  $^3$ H $_2$ O in aqueous phase was collected and extracted. After that, scintillation solution was added and radioactivity was detected using a L6500 scintillation counter (Beckman Coulter, Brea, CA).



**Fig. 2.** Over-expression of CPT2 suppressed OC growth by inducing arrest of G1–S cell cycle and cell apoptosis. (A and B) Expressions of CPT2 at mRNA and protein levels were determined in SKOV3 and HEY cells (EV, empty vector; CPT2, expression vector encoding CPT2). (C) Cell proliferation was determined by MTS assay in SKOV3 and HEY cells transfected with CPT2 expression (CPT2) or empty (EV) vectors. (D) Colony formation was determined in SKOV3 and HEY cells transfected with CPT2 expression (CPT2) or empty (EV) vectors. (E) Cell cycle distribution was analyzed in SKOV3 and HEY cells transfected with CPT2 expression (CPT2) or empty (EV) vectors. (F) Cell apoptosis analysis in SKOV3 and HEY cells transfected with CPT2 expression (CPT2) or empty (EV) vectors. \* $P < 0.05$ .

## 2.12. Statistical analysis

All data were shown as the means  $\pm$  SEM. Student's *t*-tests was used for comparisons between two groups, while one-way ANOVA was used for comparisons between multiple groups. For the measurement of the association between variables, Spearman correlation was performed. Additionally, Kaplan–Meier overall and recurrence-free survival curves were used for prognostic analysis.

## 3. Results

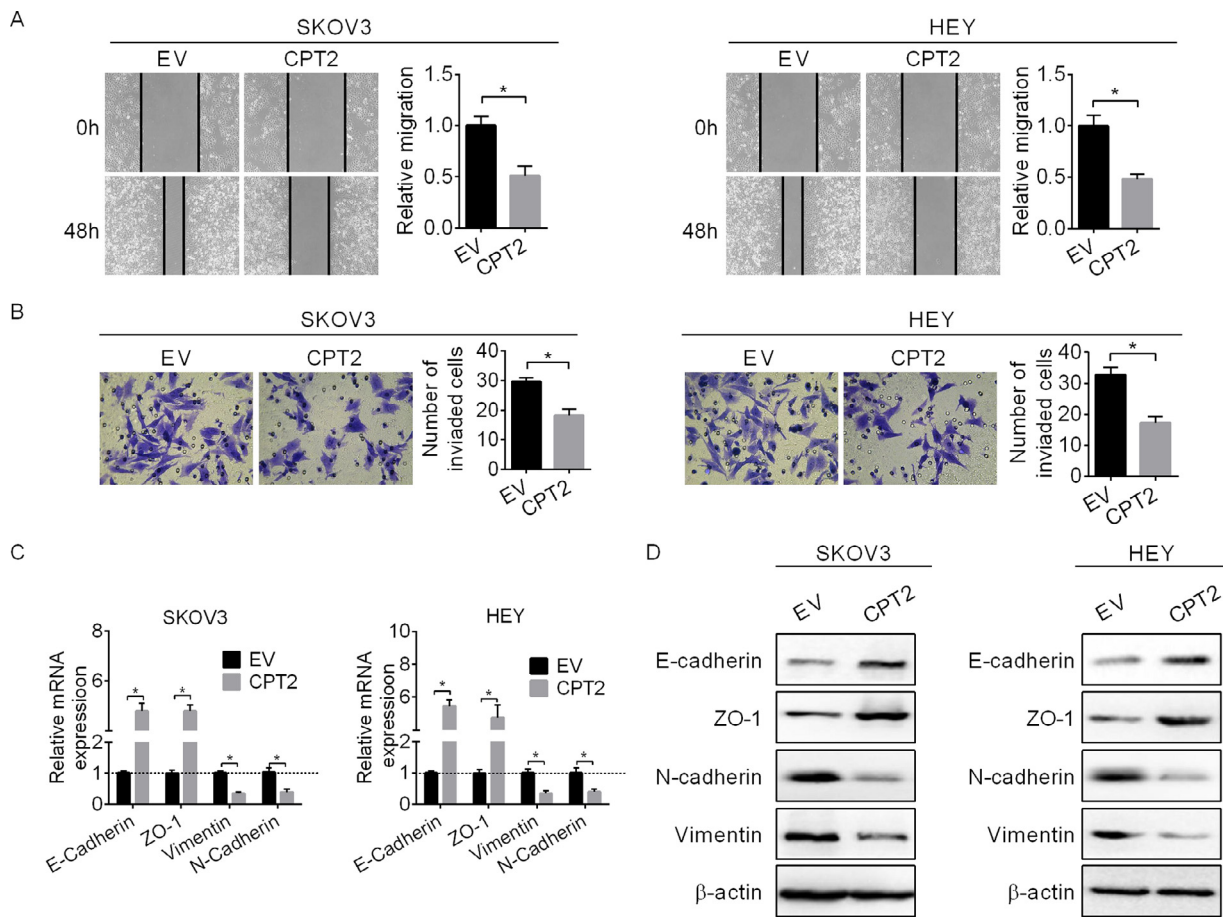
### 3.1. CPT2 expression was frequently down-regulated in OC and contributes to worse prognosis for patients with OC

To determine the expression pattern of CPT2 in ovarian cancer (OC), quantitative real-time PCR (qRT-PCR) and western blot analyses were applied in tumor and peritumor tissues for 20 OC patients. Results show that CPT2 expression level is significantly decreased in OC tumor tissues compared with peritumor tissues (Fig. 1A, 1B and S1). A consistent expression pattern of CPT2 was also observed in OC cell lines, showing that CPT2 expression is significantly lower in four OC cell lines than in nonmalignant ovarian cell (Fig. 1C and 1D).

To further evaluate the expression pattern and clinical significance of CPT2 in OC, immunohistochemical staining (IHC) was applied in tissues from 121 OC patients. CPT2 expression was significantly down-regulated in tumor tissues of OC as compared with their adjacent normal tissues (Fig. 1E). Although no significant association between CPT2 expression and the FIGO stage was observed (Supplementary table 1), patients with low CPT2 expression level had significantly poorer overall survival (OS) than those with high CPT2 expression level (Fig. 1F) as revealed by Kaplan Meier-plotter analysis, which was further supported by the bioinformatic prognostic significance analysis based on the online web Kaplan–Meier Plotter [16] (Fig. 1G).

### 3.2. Over-expression of CPT2 suppressed OC growth by inducing G1–S cell cycle arrest and cell apoptosis

The significantly down-regulation of CPT2 in OC tissues prompted us to explore its potential tumor-suppressive function in OC cells. CPT2 expression was firstly over-expressed in SKOV3 and HEY cells that expressed low CPT2 (shown in Fig. 1C and 1D). The successful over-expression of CPT2 was confirmed by qRT-PCR and western blot analyses (Fig. 2A and 2B). The results of MTS cell viability and colony formation assays indicated that CPT2 over-expression obviously decreased



**Fig. 3. Over-expression of CPT2 suppressed OC cell migration and invasion through inhibition of epithelial-mesenchymal transition (EMT).** (A) Cell migration was determined by wound healing assay in SKOV3 and HEY cells transfected with CPT2 expression (CPT2) or empty (EV) vectors. (B) Cell invasion was determined by transwell matrigel invasion assay in SKOV3 and HEY cells transfected with CPT2 expression (CPT2) or empty (EV) vectors. (C and D) The levels of EMT markers in SKOV3 and HEY cells transfected with CPT2 expression (CPT2) or empty (EV) vectors were determined. \* $P < 0.05$ .

the growth abilities of SKOV3 and HEY cells when compared with control cells (Fig. 2C and 2D). Both suppressed cell cycle progression and increased apoptosis could contribute to decreased OC cell growth. We thus explored the role of CPT2 in cell cycle progression and apoptosis in OC cells. CPT2 over-expression resulted in a significant cell cycle arrest at G1/G0 phase in SKOV3 and HEY cells (Fig. 2E). Cell apoptosis analysis showed significantly more apoptotic cells in SKOV3 and HEY cells with CPT2 over-expressed than those in control cells (Fig. 2F). Collectively, these results indicate that CPT2 inhibited OC growth through inducing G1-S cell cycle arrest and cell apoptosis.

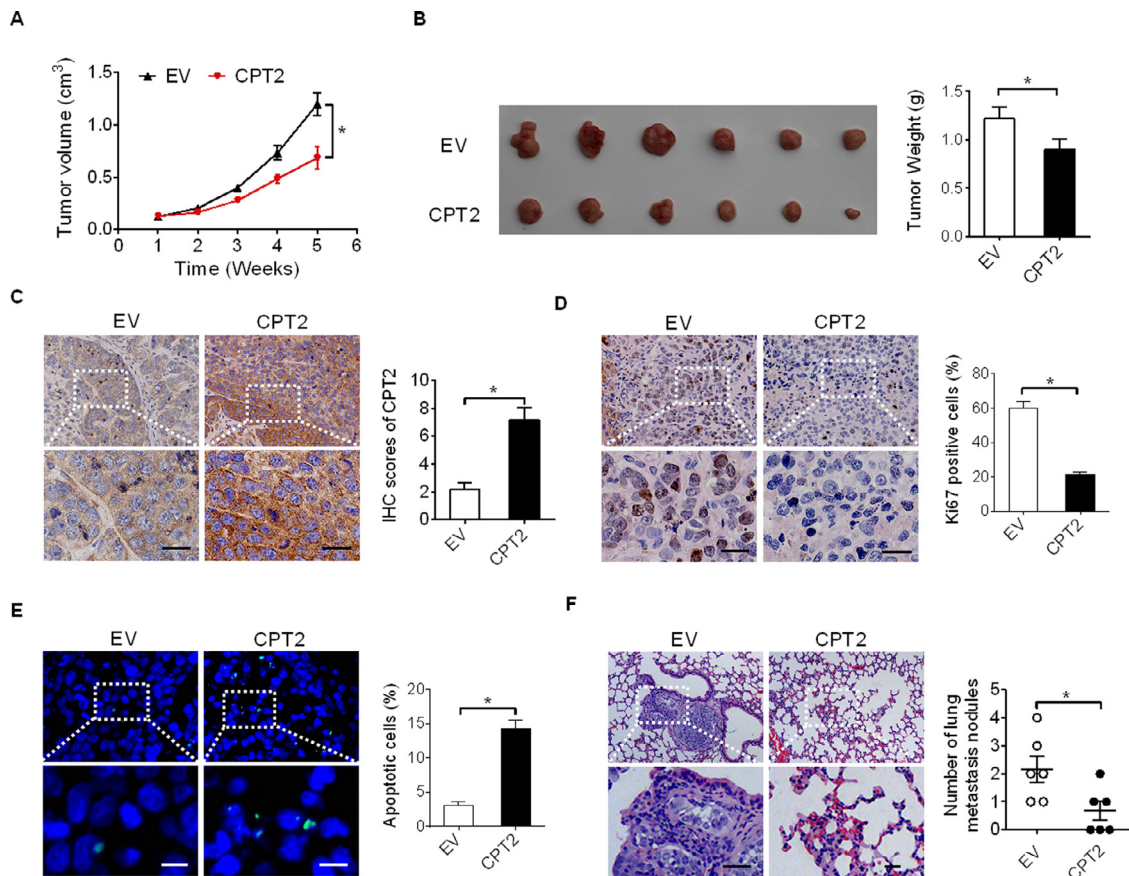
### 3.3. Over-expression of CPT2 suppressed OC cell migration and invasion through inhibition of epithelial-mesenchymal transition (EMT)

We continued to evaluate the effects of CPT2 on the migration and invasion of OC cells. Wound healing assay revealed that cell migration was significantly suppressed in SKOV3 and HEY cells when CPT2 was over-expressed compared with control cells (Fig. 3A). Matrigel invasion assay also showed significantly impaired invasiveness in SKOV3 and HEY cells when CPT2 was over-expressed compared with control cells (Fig. 3B). To explore the potential underlying mechanisms of the suppressive-role of CPT2 in OC cell migration and invasion, the expression levels of molecular markers involved in Epithelial-mesenchymal transition (EMT), a crucial program for the metastasis of epithelial tumors through reduced cell-cell contact and increased cell motility [17], were determined by qRT-PCR and western blot analyses. CPT2 over-expression significantly

up-regulated the epithelial markers of E-cadherin and ZO-1, while down-regulated the mesenchymal markers of N-cadherin and Vimentin in SKOV3 and HEY cells (Fig. 3C and 3D). Together, these findings indicate that CPT2 inhibited OC migration and invasive mainly through inhibition of EMT.

### 3.4. Over-expression of CPT2 attenuates tumor growth and metastasis in vivo

We further investigated the effect of CPT2 over-expression on OC tumor growth *in vivo*. Stable CPT2 over-expression or control SKOV3 cells (Figure S2A and S2B) were subcutaneously injected into the right flanks of nude mice. As shown in Fig. 4A, the growth of tumors in CPT2 over-expression group was significantly slower as compared with those in the control group. Consistently, the size and weight of tumors from CPT2 over-expression group were significantly reduced when compared with the control group (Fig. 4B). In addition, IHC analysis indicated significantly increased CPT2 level in CPT2 over-expression group as compared with the control group (Fig. 4C), indicating that the tumor growth suppressive-role was exerted by forced expression of CPT2 (Fig. 4C). Consistent with the *in vitro* results, significantly fewer proliferating and more apoptotic cells were observed in CPT2 over-expressed xenografts when compared with the controls, while apoptotic cells (Fig. 4D and 4E). Moreover, the *in vivo* tail vein metastasis assay indicated significantly fewer metastatic nodules in the lungs from CPT2 over-expression group than those from control group (Fig. 4F).



**Fig. 4.** Over-expression of CPT2 attenuates OC growth and metastasis in vivo. (A) The growth rates of xenografts developed from SKOV3 cells with CPT2 stably over-expressed or control cells. (B) Tumor size and weight were evaluated. (C) IHC staining for CPT2 expression in xenografts established from SKOV3 cells with CPT2 stably over-expressed or control cells. Scale bars, 20  $\mu\text{m}$ . (D) IHC analysis of Ki-67 expression in xenografts developed from SKOV3 cells with CPT2 stably over-expressed or control cells. Scale bars, 20  $\mu\text{m}$ . (E) TUNEL assay for cell apoptosis analysis in xenografts established from SKOV3 cells with CPT2 stably over-expressed or control cells. Scale bars, 5  $\mu\text{m}$ . (F) Lung metastatic foci and incidence in each group were shown. Scale bars, 10  $\mu\text{m}$ . \* $P < 0.05$ .

### 3.5. Knockdown of CPT2 increased OC growth and metastasis

To provide further evidence for the suppressive-role of CPT2 in OC cell growth and metastasis, CPT2 expression was down-regulated by RNA interference in A2780 and ES2 cells with relatively high CPT2 expression. The successful down-regulation of CPT2 was confirmed in Fig. 5A and 5B. As shown in Fig. 5C and 5D, CPT2 knockdown markedly enhanced cell viability and clonogenicity in A2780 and ES2 cells. Expectedly, CPT2 knockdown also markedly increased migration and invasion of A2780 and ES2 cells (Fig. 5E and 5F).

### 3.6. CPT2 repressed ROS/NF $\kappa$ B signaling pathway in OC cells through increasing fatty acid oxidation-mediated NADPH production

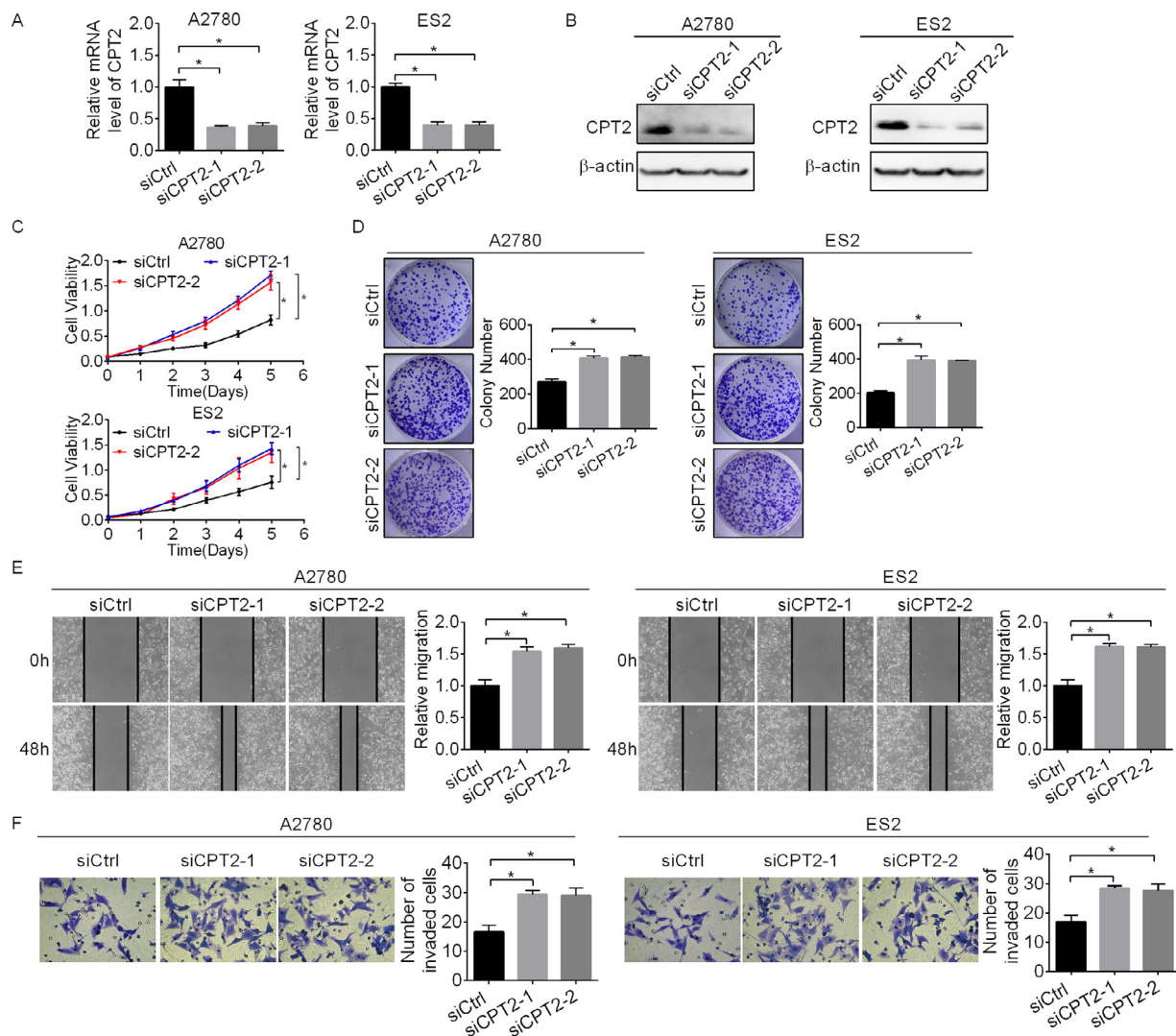
Reactive oxygen species (ROS) has been well established as a central player in tumor progression by activating several oncogenic pathways, mainly including AKT, NF $\kappa$ B, ERK1/2 and Hif-1 $\alpha$ . Considering that fatty acid oxidation (FAO) is a major source of NADPH, which provides redox power to counteract ROS, We thus hypothesized that increased NADPH production by CPT2-regulated FAO could contribute to decreased ROS and subsequent repression of oncogenic signaling and tumor growth and metastasis. To test that, relative FAO rate was firstly accessed using  $^3\text{H}$ -labeled oleic acid as a tracer. As shown in Fig. 6A, CPT2 over-expression significantly promoted FAO in SKOV3 cells, while knockdown of CPT2 markedly decreased FAO in A2780 cells. In addition, the level of NADPH was evaluated in OC cells when CPT2 was over-expressed or knocked-

down. Our results showed that forced expression of CPT2 significantly increased NADPH level in SKOV3 cells, while CPT2 knockdown resulted in a remarkable decrease of NADPH level in A2780 cells (Fig. 6B). Expectedly, flow cytometry analysis indicated a significantly decrease of ROS level when CPT2 was over-expressed in SKOV3 cells, while knockdown of CPT2 exhibited an opposite effect on ROS level in A2780 cells (Fig. 6C). In addition, forced expression of CPT2 suppressed NF- $\kappa$ B signaling in SKOV3 cells, while had no significant effect on AKT and Hif-1 $\alpha$ . By contrast, CPT2 knockdown in A2780 cells activated NF- $\kappa$ B signaling (Fig. 6D), indicating that CPT2 repressed ROS/NF $\kappa$ B signaling pathway in OC cells.

### 3.7. CPT2 suppressed OC growth and metastasis possibly through suppression of ROS/NF $\kappa$ B signaling pathway

To further test whether the tumor suppressive role of CPT2 is mediated by decreased ROS in OC cells,  $\text{H}_2\text{O}_2$  or NAC were added to regulate the ROS level in SKOV3 and A2780 cells. Treatment with  $\text{H}_2\text{O}_2$  reversed the growth and metastasis of SKOV3 cells repressed by CPT2 over-expression (Fig. 7A-7D). By contrast, treatment with NAC in A2780 cells significantly inhibited the growth and metastasis of A2780 cells promoted by CPT2 knockdown (Fig. 7A-7D). These results collectively indicate that decreased ROS production may account for CPT2-promoted OC growth and metastasis.

A previous study reported that down-regulation of CPT2 promoted tumorigenic activity and metastatic potential of hepatoma cells through



**Fig. 5. Knockdown of CPT2 increased OC growth and metastasis.** (A) CPT2 expression was detected by qRT-PCR analysis in A2780 and ES2 cells (siCPT2, siRNA against CPT2; siCtrl, control siRNA). (B) CPT2 expression was detected by western blot analysis in A2780 and ES2 cells. (C) Cell proliferation was determined by MTS assay in A2780 and ES2 cells. (D) Colony formation was determined in A2780 and ES2 cells. (E) Cell migration was determined by wound healing assay in A2780 and ES2 cells. (F) Cell invasion was determined by ranswell matrigel invasion assay in A2780 and ES2 cells. \* $P < 0.05$ .

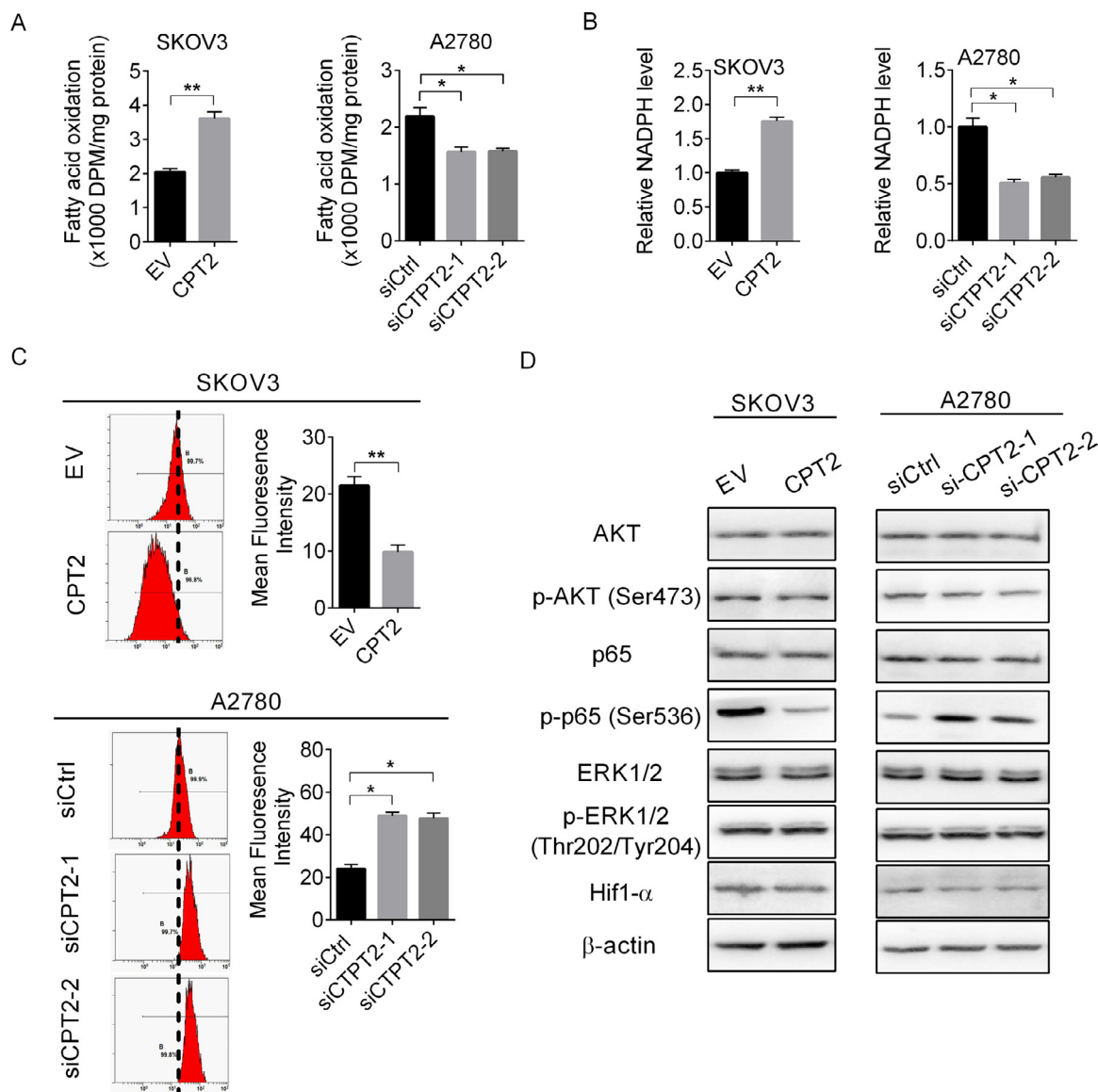
enhancing stearyl-CoA desaturase-1 (SCD1)-mediated lipogenesis [15]. We thus analyzed the expressions of SCD1 when CPT2 was over-expressed or knocked-down in OC cells. As shown in Figure S3A and S3B, no significantly change of SCD1 expression was observed when CPT2 was over-expressed or knocked-down, indicating that CPT2 suppressed OC growth and metastasis may not through SCD1-regulated lipogenesis.

#### 4. Discussion

Lipid metabolism dysregulation is closely implicated in tumorigenesis. In this study, we found that the expression of CPT2, a rate-limiting enzyme in FAO, was frequently down-regulated at both mRNA and protein levels in ovarian cancer (OC) cell lines and primary tumor tissues, which suggests that CPT2 down-regulation in OC cells may occurs at genetic or transcriptional levels. However, another group has recently reported that CPT2 was epigenetically suppressed by nkx2-8 [18]. Accordingly, nkx2-8 and several other post-translational factors may also contribute to CPT2 down-regulation in OC cells, which still needs further confirmation. Down-regulation of CPT2 was significantly associated

with poor survival for patients with OC. Consistently, previous studies in HCC also have reported that CPT2 was down-regulated in tumor tissues of HCC, which was significantly associated with tumor histological differentiation [14,15]. In contrast, the expressions of CPT1 subunits CPT1A and CPT1C were shown to be over-expressed and function as potential oncogenes in several types of human malignancies, including colorectal [19], papillary thyroid [20] and lung [21] cancers. These contradictions may be explained by the fact that different localization of CPT (CPT1 in the outer and CPT2 in the inner membranes of mitochondrial) may play distinct roles in different tumor types, which still needs further investigations.

Significantly down-regulation of CPT2 in OC cells suggests that CPT2 may play a potential tumor suppressor role against ovarian carcinogenesis. Accordingly, biological functions of CPT2 in human OC cell lines were investigated by gain- and loss-of-function in vitro and in vivo studies. Forced expression CPT2 decreased cell growth in SKOV3 and HEY cell lines, while CPT2 silencing enhanced OC cell growth in A2780 and ES2 cells. In addition, we found that CPT2 over-expression also suppressed OC cell migration and invasion, while CPT2 knockdown markedly enhanced OC cell migration and invasion. Consistent with



**Fig. 6.** CPT2 repressed ROS/NF $\kappa$ B signaling pathway in OC cells through increasing fatty acid oxidation-mediated NADPH production. (A)  $^3$ H-labeled oleic acid for determination of fatty acid oxidation in SKOV3 with CPT2 over-expressed or A2780 cells with CPT2 knocked-down. (B) Determination of intracellular NADPH level in SKOV3 and A2780 cells. (C) Determination of ROS level by flow cytometry in SKOV3 and A2780 cells. (D) The effects of CPT2 over-expression or knockdown on the activations of AKT, NF $\kappa$ B, Erk1/2 and Hif-1 $\alpha$  were determined by western blot analysis.

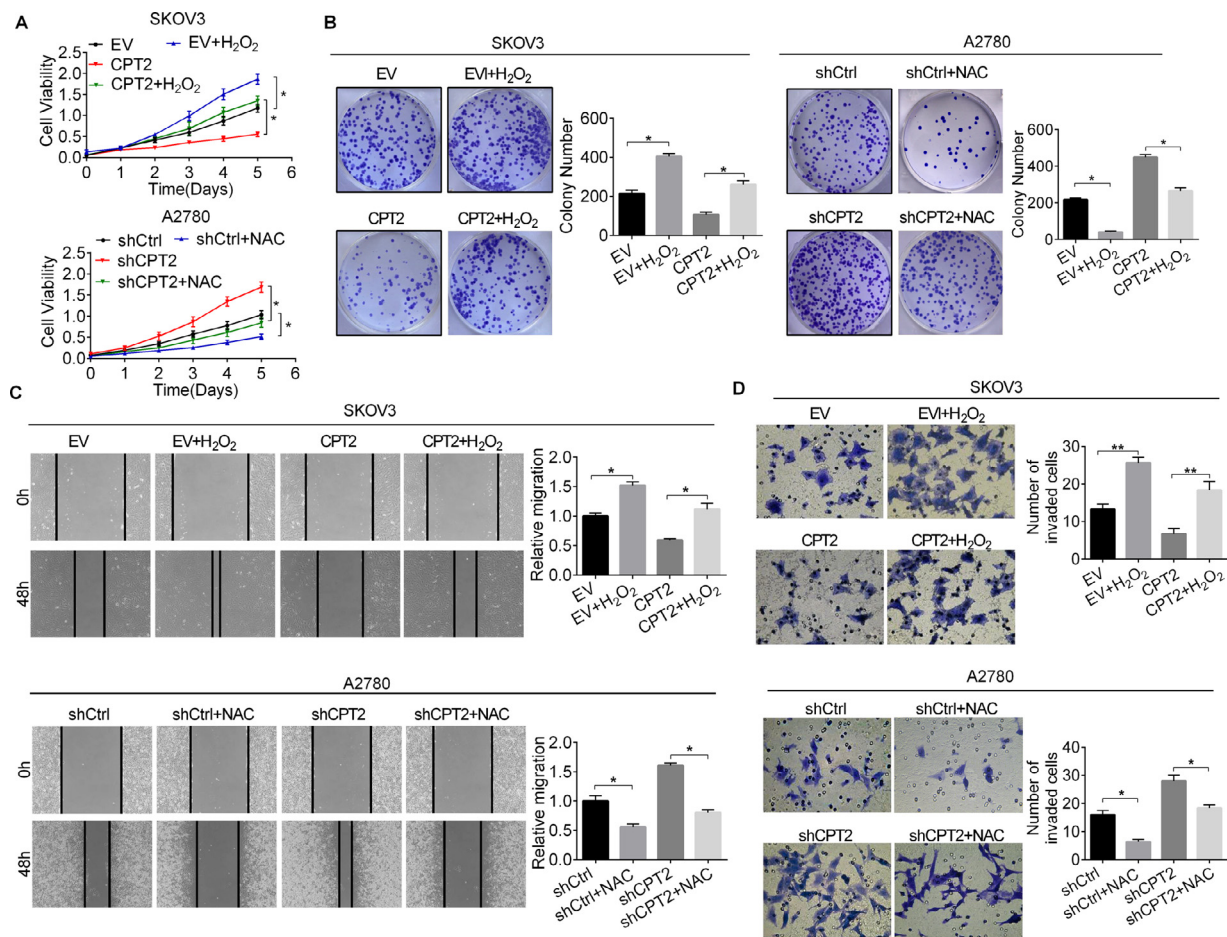
our results from OC cells, a previous study in hepatocellular carcinoma (HCC) also reported that CPT2 silencing enhanced the tumor growth and metastatic capacities of hepatoma cells [15]. Besides, it also has shown that CPT2 silencing induced chemoresistance to cisplatin in HCC cells, indicating that CPT2 may contribute to stress-induced cell death [15]. However, the effect of CPT2 on tumor cell apoptosis remains unexplored. Here, we demonstrated that CPT2 significantly promoted cell apoptosis in OC. In contrast to CPT2, both CPT1A and CPT1C have been shown to play a promotive role in tumor growth and metastasis in colorectal cancer cell and papillary thyroid carcinomas [19,20]. Targeting CPT1A improved the therapeutic effects of radiotherapy in nasopharyngeal carcinoma [22]. Collectively, these findings indicate that CPTs dysfunction plays crucial promotive roles in tumor progression.

Reactive oxygen species (ROS) has been well established as a central player in the promotion of tumor growth and metastasis by activating several key oncogenic signaling pathways. Considering that fatty acid oxidation (FAO) is a major source of NADPH, which provides redox power to counteract ROS, we thus hypothesized that increased NADPH

production by CPT2-regulated FAO could contribute to decreased ROS and subsequent suppression of OC growth and metastasis. Our results showed that CPT2-regulated FAO significantly increased NADPH production and subsequently decreased ROS level in OC cells. Furthermore, we found that decreased ROS level possibly contributed to the suppression of OC progression by CPT2. A previous study in hepatocellular carcinoma has revealed that CPT2 suppressed tumor cell growth and metastasis through enhancing stearoyl-CoA desaturase-1 (SCD1)-mediated lipogenesis [15]. However, no significant influence of CPT2 down-regulation on the expression of SCD1 was found in our present study, implying that CPT2 promoted OC growth and metastasis may not through enhancing SCD1-mediated lipogenesis. However, we still cannot rule out the possibility that other important factors such as STAT3 activation, which was shown to be activated by CPT2 in obesity-driven and NASH-driven HCC, [14] could also be involved in the tumor-suppressive property of CPT2 in OC, which still needs further study.

Together, we demonstrate that CPT2 is commonly decreased and plays an important tumor suppressive role in ovarian carcinogenesis





**Fig. 7. CPT2 suppressed OC growth and metastasis possibly through suppression of ROS/NF $\kappa$ B signaling pathway. (A)** Cell proliferation was determined by MTS assay in SKOV3 and A2780 cells treated with 90  $\mu$ M H<sub>2</sub>O<sub>2</sub> or 30 mM NAC (12 h). **(B)** Colony formation assay was applied in SKOV3 and A2780 cells treated with 90  $\mu$ M H<sub>2</sub>O<sub>2</sub> or 30 mM NAC (12 h), respectively. **(C)** Cell migration ability was determined by wound healing assay in SKOV3 and A2780 cells treated with 90  $\mu$ M H<sub>2</sub>O<sub>2</sub> or 30 mM NAC (12 h), respectively. **(D)** Cell invasion ability was determined by transwell matrigel invasion assay in SKOV3 and A2780 cells treated with 90  $\mu$ M H<sub>2</sub>O<sub>2</sub> or 30 mM NAC (12 h), respectively.

mainly through suppression of ROS/NF $\kappa$ B signaling by increasing fatty acid oxidation-derived NADPH production. CPT2 may serve as a novel prognosis biomarker and intervention target for the treatment of OC.

#### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

#### Acknowledgements

This work was supported by the National Natural Science Foundation of China (grants 81972440 and 81972427) and the Joint Project of Colleges and Universities in Shaanxi Province (grant 2020GXLH-Y-009).

#### Data sharing statement

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

#### Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.tranon.2021.101023](https://doi.org/10.1016/j.tranon.2021.101023).

#### References

- [1] E Currie, A Schulze, R Zechner, TC Walther, RV Farese Jr., **Cellular fatty acid metabolism and cancer**, *Cell Metabol.* 18 (2) (2013) 153–161.
- [2] ML Sulciner, A Gartung, MM Gilligan, CN Serhan, D Panigrahy, **Targeting lipid mediators in cancer biology**, *Cancer Metastasis Rev.* 37 (2–3) (2018) 557–572.
- [3] C Corbet, O Feron, **Emerging roles of lipid metabolism in cancer progression**, *Curr. Opin. Clin. Nutr. Metabolic Care* 20 (4) (2017) 254–260.
- [4] Z Li, Y Kang, **Lipid metabolism fuels cancer's spread**, *Cell Metabol.* 25 (2) (2017) 228–230.
- [5] C Cheng, F Geng, X Cheng, D Guo, **Lipid metabolism reprogramming and its potential targets in cancer**, *Cancer Commun.* 38 (1) (2018) 27.
- [6] A Carracedo, LC Cantley, PP Pandolfi, **Cancer metabolism: fatty acid oxidation in the limelight**, *Nat. Rev. Cancer* 13 (4) (2013) 227–232.
- [7] Y Ma, SM Temkin, AM Hawkrige, C Guo, W Wang, XY Wang, X Fang, **Fatty acid oxidation: An emerging facet of metabolic transformation in cancer**, *Cancer Lett.* 435 (2018) 92–100.
- [8] U Harjes, J Kalucka, P Carmeliet, **Targeting fatty acid metabolism in cancer and endothelial cells**, *Critical Rev. Oncol. Hematol.* 97 (2016) 15–21.
- [9] J Xiong, **Fatty acid oxidation in cell fate determination**, *Trends Biochem. Sci.* 43 (11) (2018) 854–857.
- [10] CL Hoppel, **Carnitine and carnitine palmitoyltransferase in fatty acid oxidation and ketosis**, *Federation Proc.* 41 (12) (1982) 2853–2857.
- [11] JP Bonnefont, F Djouadi, C Prip-Buus, S Gobin, A Munnich, J Bastin, **Carnitine palmitoyltransferases 1 and 2: biochemical, molecular and medical aspects**, *Mol. Aspects Med.* 25 (5–6) (2004) 495–520.
- [12] N Casals, V Zammit, L Herrero, R Fado, R Rodriguez-Rodriguez, D Serra, **Carnitine palmitoyltransferase 1C: from cognition to cancer**, *Prog. Lipid Res.* 61 (2016) 134–148.
- [13] H Shao, EM Mohamed, GG Xu, M Waters, K Jing, Y Ma, Y Zhang, S Spiegel, MO Idowu, X Fang, **Carnitine palmitoyltransferase 1A functions to repress FoxO tran-**

- scription factors to allow cell cycle progression in ovarian cancer, *Oncotarget* 7 (4) (2016) 3832–3846.
- [14] N Fujiwara, H Nakagawa, K Enooku, Y Kudo, Y Hayata, T Nakatsuka, Y Tanaka, R Tateishi, Y Hikiba, K Misumi, et al., **CPT2 downregulation adapts HCC to lipid-rich environment and promotes carcinogenesis via acylcarnitine accumulation in obesity**, *Gut* 67 (8) (2018) 1493–1504.
- [15] M Lin, D Lv, Y Zheng, M Wu, C Xu, Q Zhang, L Wu, **Downregulation of CPT2 promotes tumorigenesis and chemoresistance to cisplatin in hepatocellular carcinoma**, *OncoTargets Ther.* 11 (2018) 3101–3110.
- [16] A Nagy, A Lanczky, O Menyhart, B Györfy, **Validation of miRNA prognostic power in hepatocellular carcinoma using expression data of independent datasets**, *Sci. Rep.* 8 (1) (2018) 9227.
- [17] L Li, W Li, **Epithelial-mesenchymal transition in human cancer: comprehensive reprogramming of metabolism, epigenetics, and differentiation**, *Pharmacol. Ther.* 150 (2015) 33–46.
- [18] J Zhu, G Wu, L Song, L Cao, Z Tan, M Tang, Z Li, D Shi, S Zhang, J Li, **NKX2-8 deletion-induced reprogramming of fatty acid metabolism confers chemoresistance in epithelial ovarian cancer**, *EBioMedicine* 43 (2019) 238–252.
- [19] YN Wang, ZL Zeng, J Lu, Y Wang, ZX Liu, MM He, Q Zhao, ZX Wang, T Li, YX Lu, et al., **CPT1A-mediated fatty acid oxidation promotes colorectal cancer cell metastasis by inhibiting anoikis**, *Oncogene* 37 (46) (2018) 6025–6040.
- [20] R Wang, Y Cheng, D Su, B Gong, X He, X Zhou, Z Pang, L Cheng, Y Chen, Z Yao, **Cpt1c regulated by AMPK promotes papillary thyroid carcinomas cells survival under metabolic stress conditions**, *J. Cancer* 8 (18) (2017) 3675–3681.
- [21] K Zaugg, Y Yao, PT Reilly, K Kannan, R Kiarash, J Mason, P Huang, SK Sawyer, B Fuerth, B Faubert, et al., **Carnitine palmitoyltransferase 1C promotes cell survival and tumor growth under conditions of metabolic stress**, *Genes Develop.* 25 (10) (2011) 1041–1051.
- [22] Z Tan, L Xiao, M Tang, F Bai, J Li, L Li, F Shi, N Li, Y Li, Q Du, et al., **Targeting CPT1A-mediated fatty acid oxidation sensitizes nasopharyngeal carcinoma to radiation therapy**, *Theranostics* 8 (9) (2018) 2329–2347.