# Ursolic acid inhibits proliferation and reverses drug resistance of ovarian cancer stem cells by downregulating ABCG2 through suppressing the expression of hypoxia-inducible factor-1α *in vitro*

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**Abstract.** Hypoxia in tumors is closely related to drug resistance. It has not been verified whether hypoxia-inducible factor-1α (HIF-1α) or ABCG2 is related to hypoxia-induced resistance. Ursolic acid (UA), when used in combination with cisplatin can significantly increase the sensitivity of ovarian cancer stem cells (CSCs) to cisplatin, but the exact mechanism is unknown. The cell growth inhibitory rate of cisplatin under different conditions was evaluated using Cell Counting Kit-8 (CCK-8) in adherence and sphere cells (SKOV3, A2780, and HEY). The expression of HIF-1α and ABCG2 was tested using quantitative PCR, western blotting, and immunofluorescence under different culture conditions and treated with UA. Knockdown of HIF-1α by shRNA and LY294002 was used to inhibit the activity of PI3K/Akt pathway. Ovarian CSCs express stemness-related genes and drug resistance significantly higher than normal adherent cells. Under hypoxic conditions, the ovarian CSCs grew faster and were more drug resistant than under normoxia. UA could inhibit proliferation and reverse the drug resistance of ovarian CSC by suppressing ABCG2 and HIF-1α under different culture conditions. HIF-1α inhibitor YC-1 combined with UA suppressed the stemness genes and ABCG2 under hypoxic condition. The PI3K/Akt signaling pathway activation plays an important functional role in UA-induced downregulation of HIF-1α and reduction of ABCG2. UA inhibits the proliferation and reversal of drug resistance in ovarian CSCs by suppressing the expression of downregulation of HIF-1α and ABCG2.

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## Introduction

Ovarian cancer is the fifth common cause of cancer-related deaths in women, and the rate of mortality is the highest in all the gynecologic malignancies. The high rates of mortality in women with ovarian cancer are due to its late diagnosis, with approximately three-fourths of patients diagnosed with advanced disease (1). Although in recent years, with the advances in chemotherapy, the treatment for ovarian cancer has shown significant improvement with consequent reduction in the rate of mortality, the treatment is susceptible to chemotherapeutic drug resistance, in particular the emergence of multidrug resistance (MDR). The 5-year rate of survival of patients with ovarian cancer is only 27% (2).

A very few subgroups of tumor cells have the ability to self-renew, differentiate, and form secondary/tertiary tumors after serial xenotransplantation into immune-compromised animal models and are called cancer stem cells (CSCs) or cancer-initiating cells (3). With the development of CSCs, ovarian CSCs have been isolated from ovarian solid tumors, ovarian cell lines, and ovarian cancer ascites (4). Increasing number of studies have demonstrated that CSCs are closely associated with drug resistance. ATP-binding cassette (ABC), subfamily G, member 2 (ABCG2), is highly expressed in various stem cell populations, and has become one of the stem cell markers (5,6). Furthermore, ABCG2 is an important MDR transporter, which can efflux various chemotherapeutic drugs and may contribute to drug resistance of cancer cells (7-10).

Current evidence suggests that ABCG2 gene transcription is regulated by a number of trans-acting elements including hypoxia-inducible factor 1α (HIF-1α), estrogen receptor, and peroxisome proliferator-activated receptor. Among these, HIF-1α, a master transcription factor that regulates hypoxia-responsive genes, has been recognized to play a critical role in tumor, metastasis, and chemoradiation resistance (11-15).

Ursolic acid (UA; 3β-hydroxy-urs-12-en-28-oic acid) is a naturally-derived pentacyclic triterpene acid widely present in medicinal and other plants (16). UA has a number of biological properties including antioxidation, anti-inflammation, anticancer, and hepatoprotection (17-19). However, the exact mechanism through which the anticancer and reversal of multidrug resistant properties occurs remains unclear. Therefore, major improvements are required in the development of safe and effective method for the reversal of multidrug resistance.

## Materials and methods

*Cells and cell culture*. Ovarian cancer cell line SKOV3 was obtained from the International Peace Maternity and Child Health Hospital (Shanghai, China). This cell line had been used in our previous study which was cooperating with International Peace Maternity and Child Health Hospital and approved by the Ethics Committee of the International Peace Maternity and Child Health Hospital (20). The HEY and A2780 cells were purchased from the Shanghai Cell Collection (Shanghai, China) (http://www.cellbank.org.cn). All cells were maintained in RPMI-1640 (Hyclone, Logan, UT, USA), supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) at 37˚C.

Ovarian cancer sphere culture: Single cancer cells were-plated in the cell culture dish (100x200 mm style; Corning, NY, USA), which had been treated with poly-(2-hydroxyethyl methacrylate (poly-HEMA) (Sigma, St. Louis, MO, USA), for continuous suspension culture. The cells were maintained in embryonic stem (ES) medium (serum-free Dulbecco's modified Eagle's medium-F12; Hyclone), supplemented with 10 ng/ml basic fibroblast growth factor (bFGF; Gibco), 5 $\mu$g/ml insulin (Sigma), 1 mM L-glutamine (Sigma), 10% knockout serum replacement and penicillin/streptomycin (1000 U/ml and 100 mg/ml; Invitrogen, Carlsbad, CA, USA) (21). Stem cells grown under these conditions formed non-adherent spherical clusters. To induce the hypoxic environment, the HERA cell $CO_2$ incubator (Thermo, Germany) was chosen and maintained with 1% $O_2$, 5% $CO_2$, and 94% $N_2$.

*Quantitative PCR*. Total RNAs were extracted using the RNeasy extraction kit (Qiagen, Zürich, Switzerland). After reverse transcription of RNA to cDNA, quantitative polymerase chain reaction (qPCR) using the converted cDNA as template was performed in triplicate using SYBR Green PCR Master Mix. PCR was performed using initial denaturation at 95˚C for 2 min, followed by 40 cycles for 10 sec at 95˚C and 30 sec at 60˚C. The threshold cycle (CT) values of each sample were used in the post-PCR data analysis. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control for mRNA-level normalization. The following primers were used: CD44 F: ACCCCATCCCAGACGAAGACAGTC, R: GGGATGAAGGTCCTGCTTTCCG; NANOG F: GCA AAAAAGGAAGACAAGGTCC, R: CCTTCTGCGTCA CACCATTG; OCT-4 F: CGAAGAGAAAGCGAACCAGT ATC, R: AGAACCACACTGGACCACATC; ABCG2 F: GGT TTCCAAGCGTTCATTCAAA, R: TAGCCCAAAGTAAAT GGCACCTA; HIF-1α F: CCACAGGACAGTACAGGATG, R: TCAAGTCGTGCTGAATAATACC; GAPDH: F: GGT GGTCTCCTCTGACTTCAACA, R: CCAAATTCGTTGT CATACCAGGAAATG.

*Cell viability assay*. For cell viability assay using Cell Counting Kit-8 (CCK-8), cells were seeded onto 96-well plates at $1x10^5$ cells/well and the medium was treated with the drug (cisplatin-DDP, UA) for 48 h. After 2 h of incubation with culture medium containing the CCK-8 reagent (Tongji, Tokyo, Japan), the absorbance was read at 450 nm using a microplate enzyme-linked immunosorbent assay reader.

*Apoptosis assay using Annexin V staining*. Apoptotic cells were quantified using an Annexin V-APC/7-aminoactinomycin D (7-AAD) kit (Becton, Dickinson and Co., San Jose, CA, USA) and detected using flow cytometry according to the manufacturer's protocol. After treatment with UA and/or DDP, respectively, cells were resuspended in 100 $\mu$l 1X binding buffer and incubated with 5 $\mu$l Annexin V-APC and 5 $\mu$l 7-AAD for 15 min in the dark. After staining, 400 $\mu$l of 1x binding buffer was added to the cells, and samples were analyzed using flow cytometry. Cells in the early stage of apoptosis stained only-positive for Annexin V, while those in the late stage stained positive for both Annexin V and 7-AAD.

*Western blots*. Whole-cell lysate for sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and cells were washed twice with phosphate-buffered saline containing protease inhibitors. The protein content was determined using the bicinchoninic acid protein assay using a commercial kit (BSA Protein Assay Reagent; Merck & Co., White House Station, NJ, USA). The following antibodies were used: rabbit anti-CD44 antibody, rabbit anti-Nanog antibody, mouse anti-OCT-4 antibody, rabbit anti-p-AKT-308 antibody, rabbit anti-p-AKT-492 antibody, rabbit-AKT antibody, rabbit-PI3K antibody (all Cell Signaling Technology, Beverly, MA, USA), mouse anti-HIF-1α antibody (Novus Biologicals LLC, Littleton, CO, USA), rabbit anti-ABCG2 antibody, rabbit-anti-P-gp antibody (all Abcam, Cambridge, MA, USA). Equal loading was confirmed using GAPDH. Densitometric analysis was performed using the Scion Imaging software (Scion Corp., Tokyo, Japan), using GAPDH as a control for each sample.

*Immunofluorescence staining*. Cells were seeded onto glass slides and fixed with 4% paraformaldehyde, permeabilized with Triton X-100, and subsequently incubated with the monoclonal mouse anti-ABCG2 antibody and mouse anti-HIF-1α (1:50) overnight at 4˚C. Samples were incubated with goat anti-mouse immunoglobulin G antibody (Biyuntian Biological, Nanjing, China), and cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; Biyuntian Biological). Images were captured using a 80i laser confocal microscope (Nikon, Tokyo, Japan). Images were further digitally processed for contrast enhancement using Adobe Photoshop.

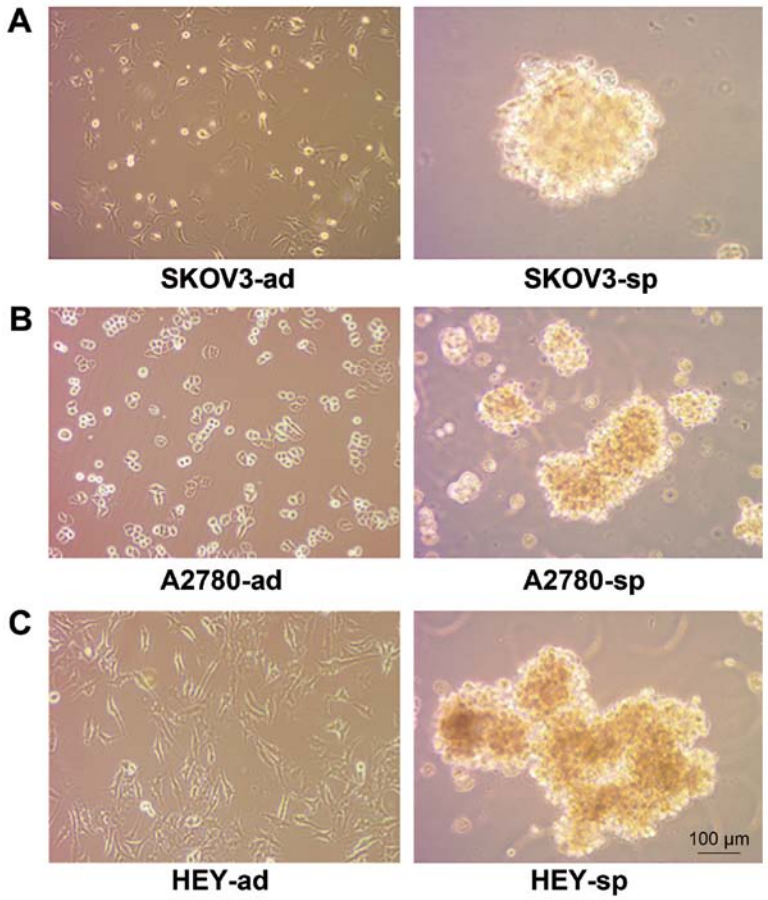


Figure 1. The morphology of tumor spheres. (A) SKOV3 cells were maintained in RPMI-1640 with 10% FBS (SKOV3-ad, SKOV3-adherence) and in ES medium (SKOV3-sp, SKOV3-spheres). (B) A2780 cells were maintained in RPMI-1640 with 10% FBS (A2780-ad, A2780-adherence) and in ES medium (A2780-sp, A2780-spheres) (100e). (C) HEY cells were maintained in RPMI-1640 with 10% FBS (HEY-ad, HEY-adherence) and in ES medium (HEY-sp, HEY-spheres). Scale bars represent 100 $\mu$M.

*Plasmid DNA amplification, extraction, and purification*. The plasmids were transformed into DH5a competent cells, extracted, and purified according to the plasmid extraction kit instructions (Tiangen, Beijing, China). The concentration of the obtained plasmid DNA was measured using spectrophotometry, and the absorbance of the plasmid was measured at 260 nm. The $OD_{260}$ titration was between 1.8 and 2.1, indicating that there was no contamination in the plasmid DNA.

*Plasmid transfection*. Cell culture plate was prepared using 40% cell density and 30 $\mu$l serum-free RPMI-1640 was added to a plastic tube. Plasmid DNA (1 $\mu$g) was added to the tube and mixed by pipetting. HilyMax was added to prepare DNA ($\mu$g): HilyMax ($\mu$l) = 1:3 and mixed by pipetting, and the tube was incubated at room temperature for 15 min. DNA-HilyMax complex was added to the cell culture well. The plate was incubated at 37˚C in a $CO_2$ incubator for 4 h. Later the medium was changed and incubation was continued for 48 h.

*Statistical analysis*. All data are represented as mean ± standard deviation. Statistical differences between two datasets were compared using Student's t-test; nonparametric data were compared using the Mann-Whitney U test. SPSS Statistics 21 was used for the statistical analysis.

## Results

*Ovarian CSC sphere culture*. Ovarian CSC sphere culture and single cancer cells (including SKOV3, A2780, HEY), which have been found to have stemness characteristics when serum-free suspension culture (21-23) when plated onto the cell culture dish, were maintained in ES medium. Stem cells grown under these conditions formed nonadherent spherical clusters. The formation of sphere cells could be observed on the third day and the sphere cells matured on the seventh day (Fig. 1).

*Ovarian CSCs express stemness-related genes and drug resistance significantly higher than normal adherent cells*. Ovarian cancer cells were maintained in RPMI-1640 with 10% FBS and ES medium, and the expression of stemness-related genes (CD44, CD133, Nanog, Oct-4, and ABCG2) was detected (24-30). The qPCR showed that the expression of CD44, CD133, Nanog, Oct-4, and ABCG2 in stem cell phenotype of SKOV3 sphere cells was much higher than those in the SKOV cells (Fig. 2A). The expression levels of these stemness genes in HEY and A2780 sphere cells were much higher than in the normal adherent cells (Fig. 2A-C). To investigate whether ovarian CSCs have significantly higher drug resistance than normal adherent cells, different concentrations of cisplatin (Sigma) were used to treat cells, and after 48 h half

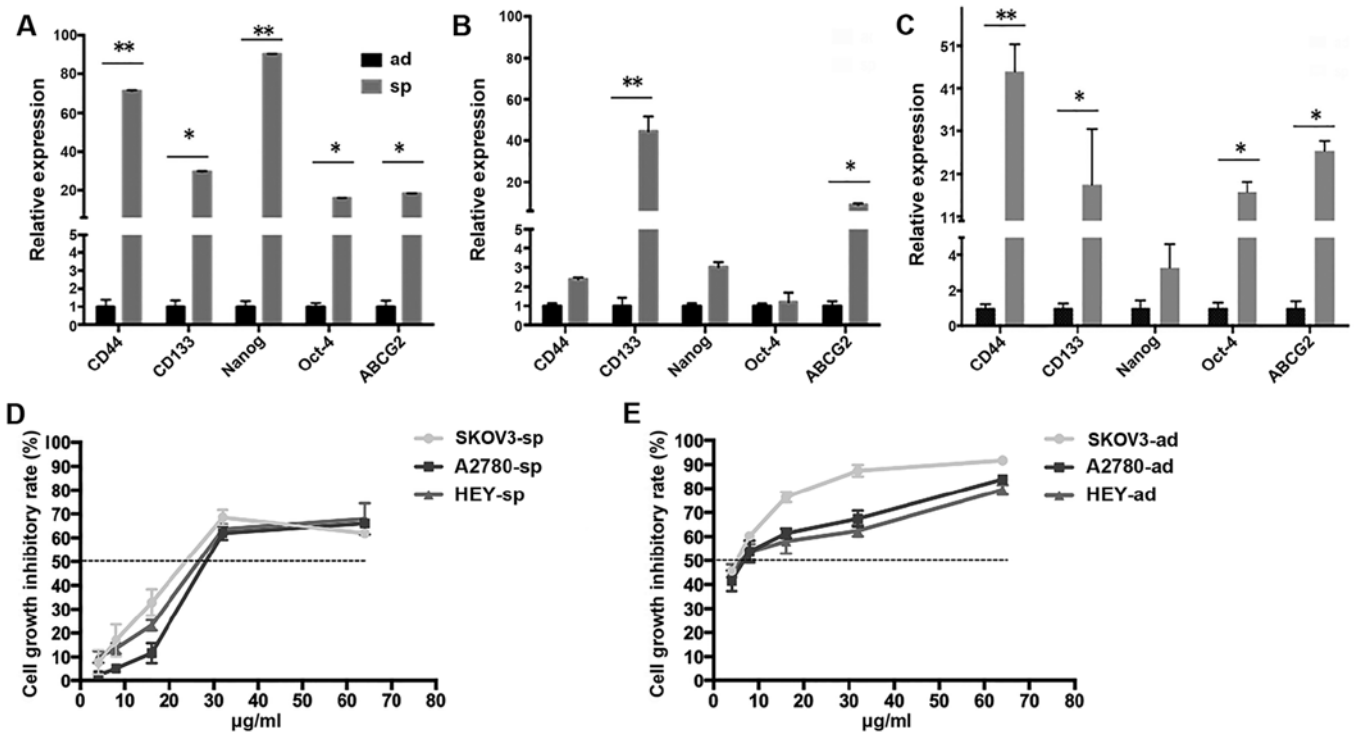


Figure 2. Ovarian cells have characteristics of stem cells. (A-C) Quantitative PCR shows that under stem cell-selective conditions, SKOV3-sp, HEY-sp, and A2780-sp cells overexpressed stemness genes CD44, CD133, Nanog, Oct-4, and ABCG2 compared with SKOV3-ad ($^{*}$P<0.05, $^{**}$P<0.01). (D) The sphere cells showed stronger drug resistance as compared with the adherence cells after treatment with DDP (0, 4, 8, 16, and 32 $\mu$g/ml). (E) The cell growth inhibitory rate of SKOV3-ad, A2780-ad and HEY-ad with DDP (0, 4, 8, 16, and 32 $\mu$g/ml).

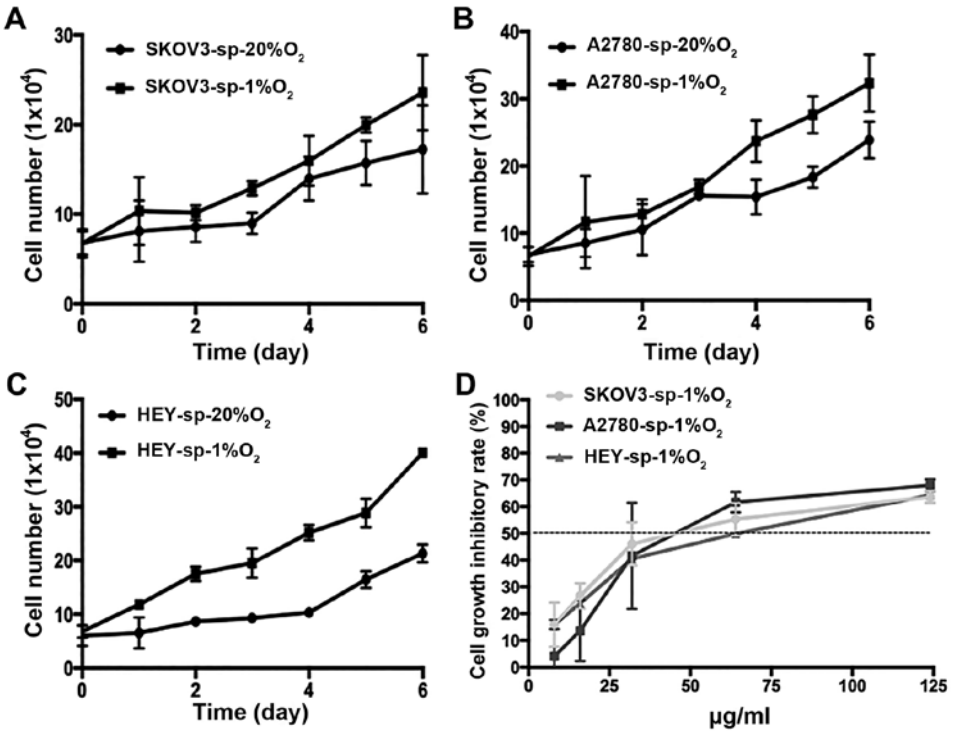


Figure 3. Cell number of ovarian sphere cells under different conditions. (A-C) SKOV3-sp, A2780-sp, and HEY-sp cells under hypoxic conditions grew faster as compared with under normoxia. (D) The $IC_{50}$ of SKOV3-sp, A2780-sp, and HEY-sp increased significantly under hypoxic conditions when treated with DDP (4, 8, 16, 32, and 64 $\mu$g/mL). $IC_{50}$, half maximal inhibitory concentration.

maximal inhibitory concentration ($IC_{50}$) of DDP was analyzed using CCK-8. As shown in Fig. 2D, the median $IC_{50}$ values of SKOV3-sp, A2780-sp, and HEY-sp were 28.223, 35.414, and 30.031 $\mu$g/ml, respectively. The $IC_{50}$ values of SKOV3-ad,

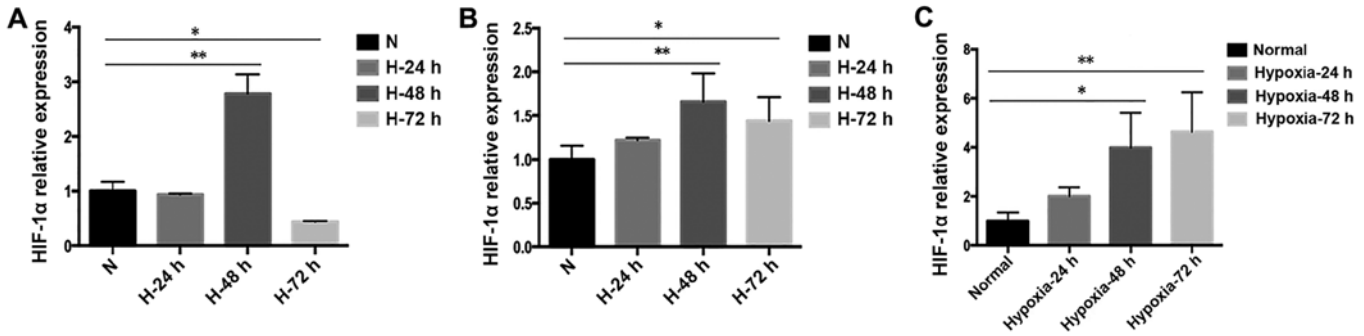


Figure 4. Expression of HIF-1α at different time points. (A) SKOV3-sp cultured under normal (20% $O_2$, N) and hypoxic conditions for 24 h (H-24 h), 48 h (H-48 h), and 72 h (H-72 h). (B) HEY-sp cultured under N and H-24 h, H-48 h, and H-72 h. (C) A2780-sp cultured under N and H-24 h, H-48 h, and H-72 h. The experiment was repeated thrice ($^{*}$P<0.05, $^{**}$P<0.01).

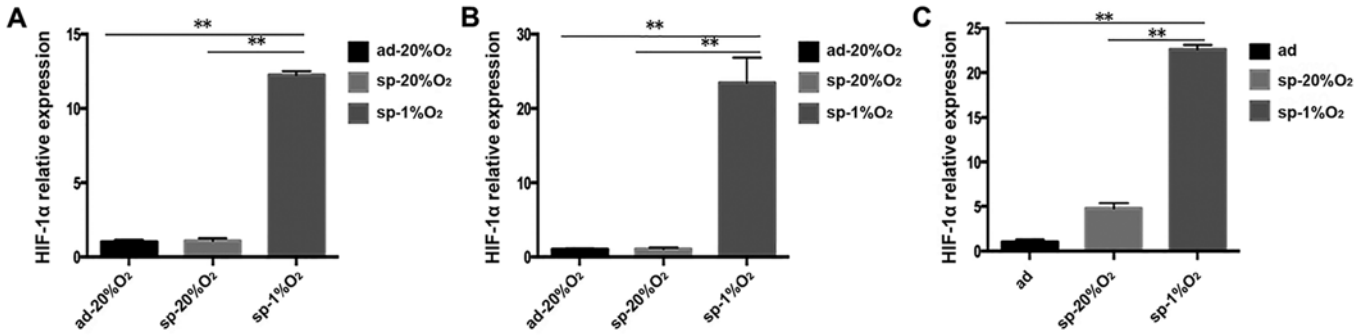


Figure 5. Expression of HIF-1α after 48 h hypoxia culturing (A) SKOV3-ad and SKOV3-sp were cultured under normal conditions. At the same time, SKOV3-sp was cultured under normoxic and hypoxic conditions for 48 h. (B) HEY-ad and HEY-sp were cultured under normal conditions. At the same time HEY-sp was cultured under normoxic and hypoxic conditions for 48 h. (C) A2780-ad and A2780-sp were cultured under normal conditions. At the same time, A2780-sp was cultured under normoxic and hypoxic conditions for 48 h. The experiment was repeated thrice ($^{*}$P<0.05, $^{**}$P<0.01).

A2780-ad, and HEY-ad were 4.910, 7.073, and 6.576 $\mu$g/ml (Fig. 2E), respectively.

It was observed that the $IC_{50}$ of sphere cells is much higher than the normal adherent cells, and sphere cells exhibited higher resistance to chemotherapeutic drugs, with higher rates of survival.

*Under hypoxic conditions, ovarian CSCs grow faster and more drug-resistant than under normoxia*. The SKOV3-sp, A2780-sp, and HEY-sp cells grown under 20% or 1% oxygen at different time points and proliferation were measured by counting the cells (Fig. 3A-C). As shown in Fig. 3A and B, the initial concentrations of SKOV3-sp, A2780-sp, and HEY-sp were $6.7x10^4$, $6.5x10^4$ and $6.0x10^4$, respectively, after 7 days of culture under different conditions. The sphere cells grew faster than under normoxia. In this experiment, it can be observed that hypoxia can promote the proliferation of sphere cells. At the same time, under hypoxic condition, the sphere cells developed more drug resistance than under normoxia. The $IC_{50}$ values of SKOV3-sp, A2780-sp, and HEY-sp were 51.653, 53.889, and 60.774 $\mu$g/mL, respectively (Fig. 3D). Thus, under hypoxic condition, the $IC_{50}$ increased significantly.

*Under hypoxic conditions, the ovarian CSCs exhibit higher stemness than under normal conditions*. Recent advances in cancer research have demonstrated that the enhanced expression and activation of HIFs are frequent in cancer cells during the progression of cancer and is associated with the acquisition of a more malignant behavior, drug resistance, and poor rate of survival in patients with cancer (31-34). Hence the expression of HIF-1α at different time points was tested (Fig. 4). It was observed that when SKOV3-sp, HEY-sp, and A2780-sp cells were cultured under hypoxic condition (1% $O_2$) for 24, 48, and 72 h, the expression of HIF-1 in SKOV3 was higher at 48 h than at 24 or 72 h (Fig. 4A), and the expression of HIF-1α in HEY-sp was the same as SKOV3-sp (Fig. 4B). However, in A2780-sp, it was found that after 72 h hypoxia, the expression of HIF-1α was higher than at 24 or 48 h (Fig. 4C). In subsequent experiments, 48 h was selected as the time point for culture since the expression of HIF was significantly increased in all the three cell strains. When cultured under normal condition, the expression of HIF-1α did not show difference between adherent and sphere cells. The expression of HIF-1α in the HEY-sp, and A2780-sp cells after 48 h under hypoxia was significantly increased and was statistically significant (Fig. 5).

In addition, when the sphere cells were cultured under hypoxic condition for 48 h, in SKOV3-sp cells, the expression of stemness genes CD44, CD133, and Nanog was significantly increased (P<0.05) and the drug resistance gene was apparently more elevated (P<0.01) (Fig. 6A). In HEY-sp cells after 48 h hypoxia culture, the expression of stemness genes Nanog and Oct-4 and drug resistance gene ABCG2 was significantly increased (P<0.01) (Fig. 6B). In the A2780-sp, the expres-

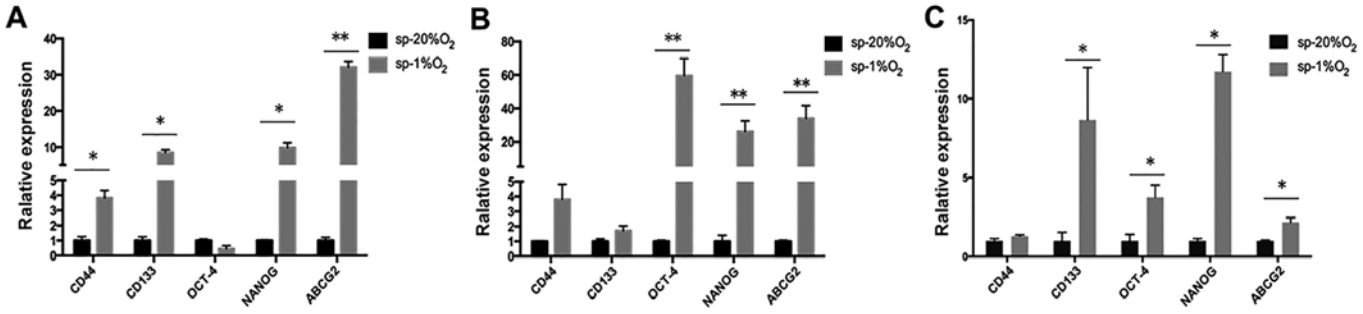


Figure 6. Expression of stemness genes and drug resistance gene under different culture conditions. (A) SKOV3-sp was cultured under normal and hypoxic conditions for 48 h, and then the stemness genes and ABCG2 were tested. (B) After 48 h of hypoxic culture, the expression of stemness genes and ABCG2 of HEY-sp. (C) After 48 h of hypoxic culture, the expression of stemness genes and ABCG2 of A2780-sp ($^{*}$P<0.05, $^{**}$P<0.01).

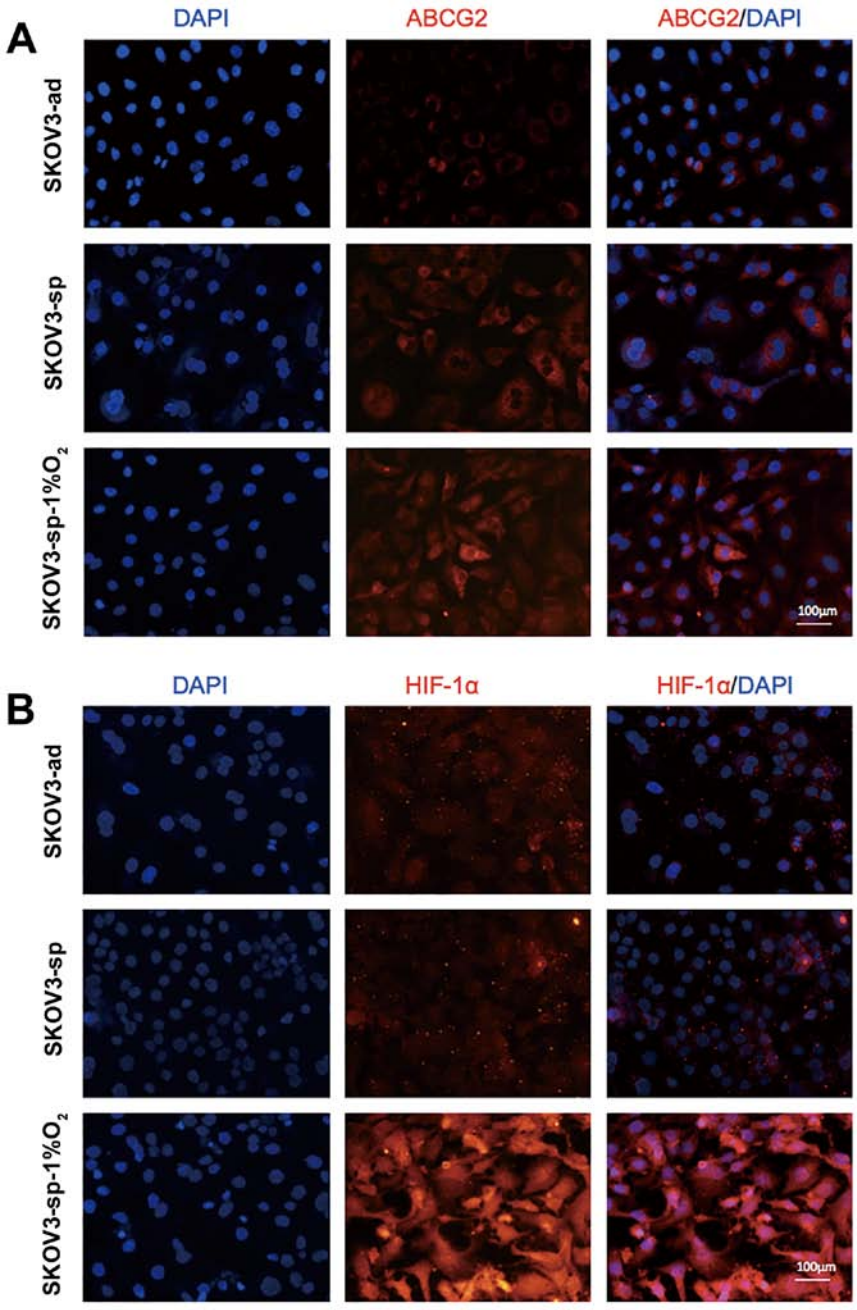


Figure 7. Expression of ABCG2 protein and HIF-1α protein on SKOV3 under different culture conditions using immunofluorescent assay. (A) The expression of ABCG2 under normal or hypoxic condition. Nuclei were stained with DAPI (in blue) and ABCG2 was stained with CY3 (in red). (B) The expression of HIF-1α under normal or hypoxic condition. Nuclei were stained with DAPI (in blue) and the HIF-1α was stained with CY3 (in red). DAPI, 4',6-diamidino-2-phenylindole; HIF, hypoxia-inducible factor.

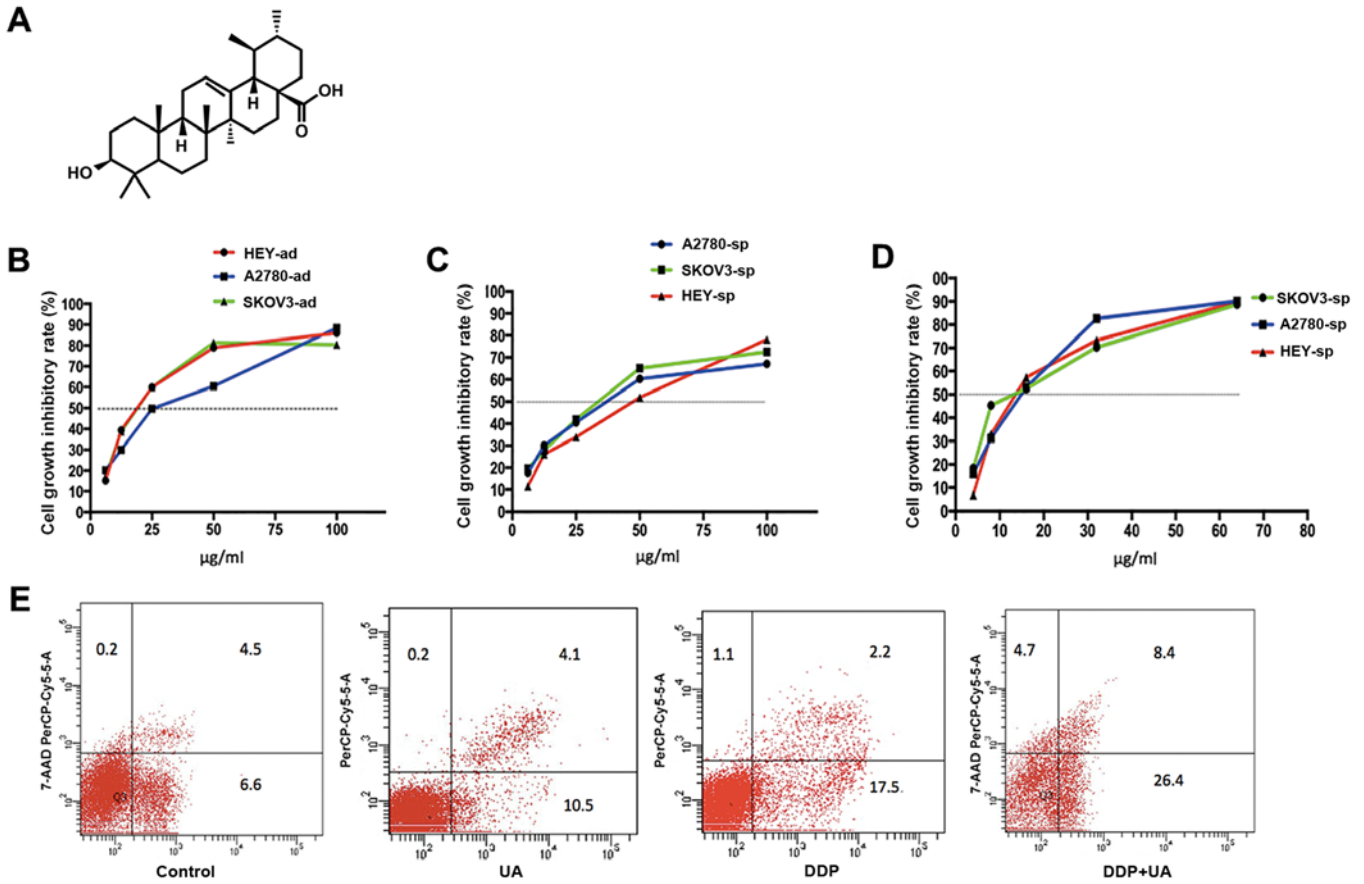


Figure 8. Effects of ursolic acid on proliferation and effect on the sensitivity of cisplatin under hypoxic condition. (A) The chemical structure of ursolic acid. (B) Ursolic acid inhibited the proliferation of SKOV3-ad, HEY-ad, and A2780-ad (6.25, 12.5, 25, 50, and 100 $\mu$g/mL). (C) Ursolic acid inhibited the proliferation of SKOV3-sp, HEY-sp, and A2780-sp (6.25, 12.5, 25, 50, and 100 $\mu$g/ml). (D) The cell growth inhibitory rate when ovarian cells were treated with low-dose ursolic acid ($IC_{10}$) combined with cisplatin (4, 8, 16, 32, and 64 $\mu$g/mL). (E) Apoptosis in SKOV3-sp after treatment with ursolic acid and cisplatin for 48 h under hypoxic condition.

sion of stemness genes CD133, Nanog, and Oct-4 and drug resistance gene ABCG2 was higher than in normal culture ($P<0.05$) (Fig. 6C). It was found that after hypoxia culture, with the increase in HIF-1α, the expression of stemness genes and drug gene ABCG2 was elevated.

To further understand the expression of HIF-1α in ABCG2, the increase in the expression of ABCG2 in SKOV3 cells under hypoxic condition was chosen (Fig. 6), and immunofluorescence staining was performed on SKOV3-ad and SKOV3-sp under different culture conditions. The ABCG2 proteins are mainly found on the plasma membrane (Fig. 7A). It was observed that SKOV3-sp is expressed at higher level than the SKOV3-ad. When SKOV3-sp was cultured under different conditions for 48 h, the sphere cells under hypoxic condition expressed ABCG2 at much higher level than under normal condition (Fig. 7A). At the same time, the expression of HIF-1α between SKOV3-ad and SKOV3-sp did not show any difference, and when the SKOV3-sp was cultured under hypoxic condition, the expression of HIF-1α increased significantly (Fig. 7B).

*UA inhibits the proliferation and enhances the sensitivity of cisplatin in ovarian CSCs*. In this experiment, it was examined whether UA could inhibit the proliferation of ovarian cells and when UA combined with cisplatin can enhance the sensitivity of cisplatin. As shown in Fig. 8B, UA inhibits the adherent and sphere cells. The $IC_{50}$ values at 48 h for SKOV3-ad, A2780-ad, and HEY-ad were 19.370, 25.257, and 19.349 $\mu$g/ml, respectively. The $IC_{50}$ values of SKOV3-sp, A2780-sp, and HEY-sp were 31.669, 36.745, and 39.239 $\mu$g/ml, respectively. Among these, the $IC_{10}$ values of SKOV3-sp, HEY-sp, and A2780-sp were 2.934, 5.359, and 2.557 $\mu$g/ml (Fig. 8C). Less than $IC_{10}$, cell survival was not found to be significantly different from untreated cells. Hence, for cell proliferation experiments, the cells were treated with UA in the concentration range of $IC_{10}$.

To investigate the effect of UA on the sensitivity of cisplatin under hypoxic conditions, the $IC_{10}$ of UA combined with cisplatin for 48 h was chosen, and then analyzed using CCK-8. It can be observed that the $IC_{50}$ values of SKOV3-sp, HEY-sp, and A2780-sp were 12.681, 14.759, and 13.302 $\mu$g/ml. The $IC_{50}$ values were significantly lower than when cisplatin was used alone (Fig. 8D). It can lead to the increase in the sensitivity of cisplatin due to UA under hypoxic condition.

In addition, the low-dose UA ($IC_{10}$) was chosen for combining with median dose of cisplatin to detect cell apoptosis under hypoxic condition. As shown in Fig. 8D, when low-dose UA was used alone, the rate of apoptosis was 14.6% and the cell apoptosis did not change. However, compared to treatment with cisplatin alone, combining cisplatin with UA increased the rate of apoptosis from 19.7 to 34.8% (Fig. 8E).

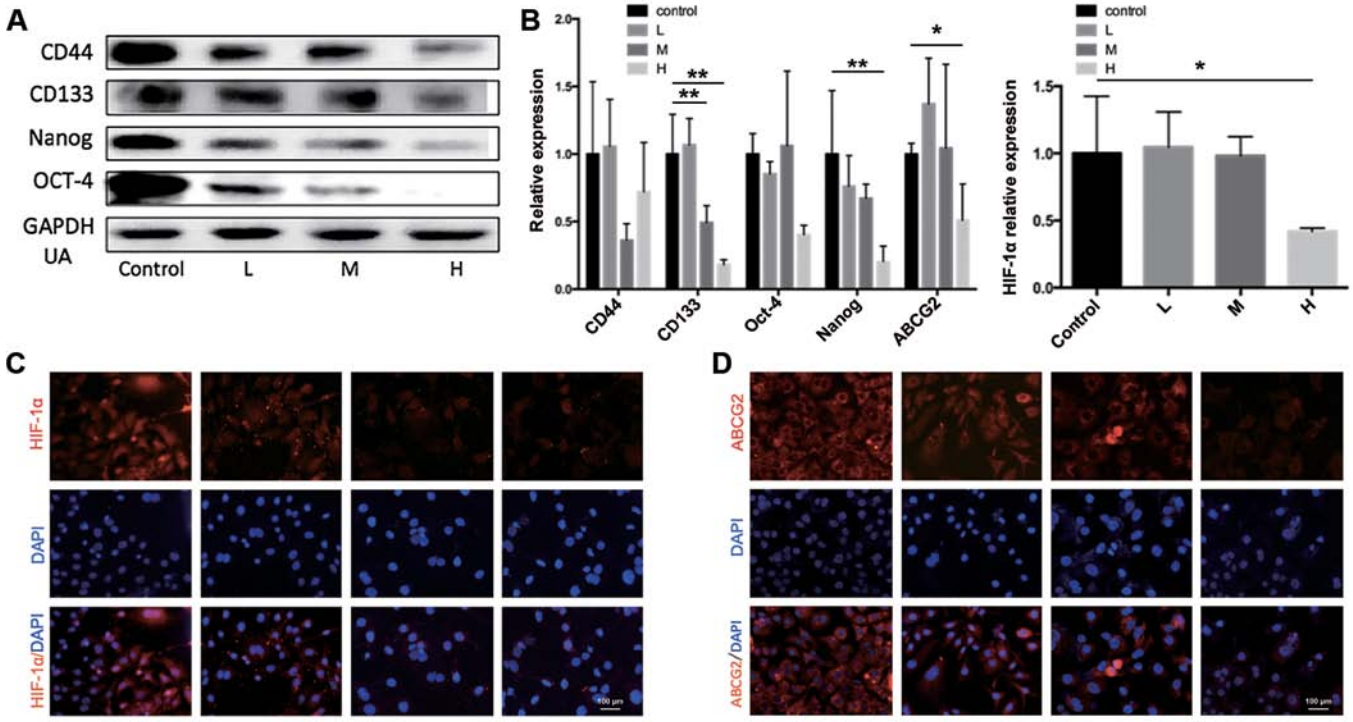


Figure 9. Ursolic acid inhibits ABCG2 and HIF-1α in SKOV3-sp under hypoxic condition. (A) SKOV3-sp cells were treated with different concentrations of ursolic acid (3, 10, and 30 $\mu$g/mL) for 48 h and CD44, CD133, Nanog, and OCT-4 were detected using western blot analysis. (B) Under hypoxic condition, SKOV3-sp cells were treated with ursolic acid (3, 10, and 30 $\mu$g/mL), mRNAs of ABCG2 and HIF-1α were detected using quantitative PCR. $^{*}$P<0.05, $^{**}$P-values represent that ursolic acid group is different compared with control group under hypoxia condition. (C) Immunofluorescence stain detected the expression of HIF-1α in SKOV3-sp after treatment with ursolic acid for 48 h under hypoxic condition. (D) Immunofluorescence stain detected the expression of ABCG2 in SKOV3-sp after treatment with ursolic acid for 48 h under hypoxic condition. DAPI, 4',6-diamidino-2-phenylindole; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HIF, hypoxia-inducible factor; PCR, polymerase chain reaction.

*UA inhibits proliferation and reverses drug resistance of ovarian CSC by suppressing ABCG2 and HIF-1α under different culture conditions*. In this experiment, it was observed that the ovarian CSCs were more drug resistant under hypoxic condition, and the expressions of ABCG2 and HIF-1α were significantly increased simultaneously. The expression of ABCG2 has been proved to be closely associated with drug resistant cancer stem cells (35-38). Here, 3, 10, and 30 ml ($IC_{10}$, $IC_{25}$, and $IC_{50}$) were chosen to treat SKOV3-sp cells for 48 h to explore whether UA inhibit the expression of ABCG and HIF-1α. It was observed that under normal condition, different concentrations of UA could inhibit the stemness gene CD44, CD133, Nanog, and OCT-4 in a dose-dependent manner (Fig. 9A). Later, the mRNA of ABCG2 and HIF-1α in SKOV3-sp treated with UA under hypoxic condition was tested. As shown in Fig. 9B, under hypoxic condition UA could suppress the expression of stemness genes CD44, CD133, Nanog, and Oct-4. High doses of UA could significantly inhibit ABCG2 and HIF-1α. Immunofluorescence staining on SKOV3-sp under hypoxic condition was performed, and SKOV3-sp was treated with UA (3, 10, and 30 $\mu$g/ml for 48 h). In Fig. 9C and D, it shows an increase in the concentration of UA, the expression of HIF-1α gradually reduced under hypoxic condition. The following conclusions were reached: UA could suppress the expression of ABCG2 and HIF-1α under hypoxic conditions and in a dose-dependent manner.

*HIF-1α inhibitor YC-1 combined with UA suppressed the stemness gene and ABCG2 under hypoxic condition*. YC-1, which is 3-(5'-hydroxymethyl-2'-furyl)-1-benzyl-indazole, is a treatment for circulatory disorders, the inhibition of platelet aggregation, and vasoconstriction by inhibiting the soluble guanylate cyclase drugs. In 2001, Chun *et al* found that YC-1 inhibited the activity of HIF-1 (39) in breast cancer (40) and liver cancer (41), and was found to have the anti-tumor effect. To investigate whether inhibition of expression of HIF-1α by YC-1 under hypoxic condition in SKOV3-sp cells is correlated with apoptosis, stemness, and drug-resistant gene ABCG2, the concentrations of YC-1 selected were 6, 14, and 32 $\mu$M ($IC_{10}$, $IC_{25}$, and $IC_{50}$ for 48 h) was selected (Fig. 10A). It was found that with an increase in the concentration of YC-1, the expression of HIF-1α was gradually reduced after treatment with YC-1 for 48 h under hypoxic condition (Fig. 10B). Later low-dose YC-1 (6 $\mu$M) was used to treat SKOV3-sp for 48 h. As shown in Fig. 10C, when low-dose YC-1 was used, the rate of apoptosis was 25.1%, which was much higher than 7.9% under hypoxic condition.

When YC-1 was combined with UA under hypoxic condition, compared with YC-1 alone or UA, the stemness genes Nanog, OCT-4, CD44, and CD133 were significantly inhibited. In addition, the expression of ABCG2 was significantly decreased (Fig. 10D). These results suggest that after the inhibition of HIF-1α, the expression of ABCG2 was degraded.

*PI3K/Akt signaling pathway activation plays an important functional role in UA-induced downregulation of HIF-1α*

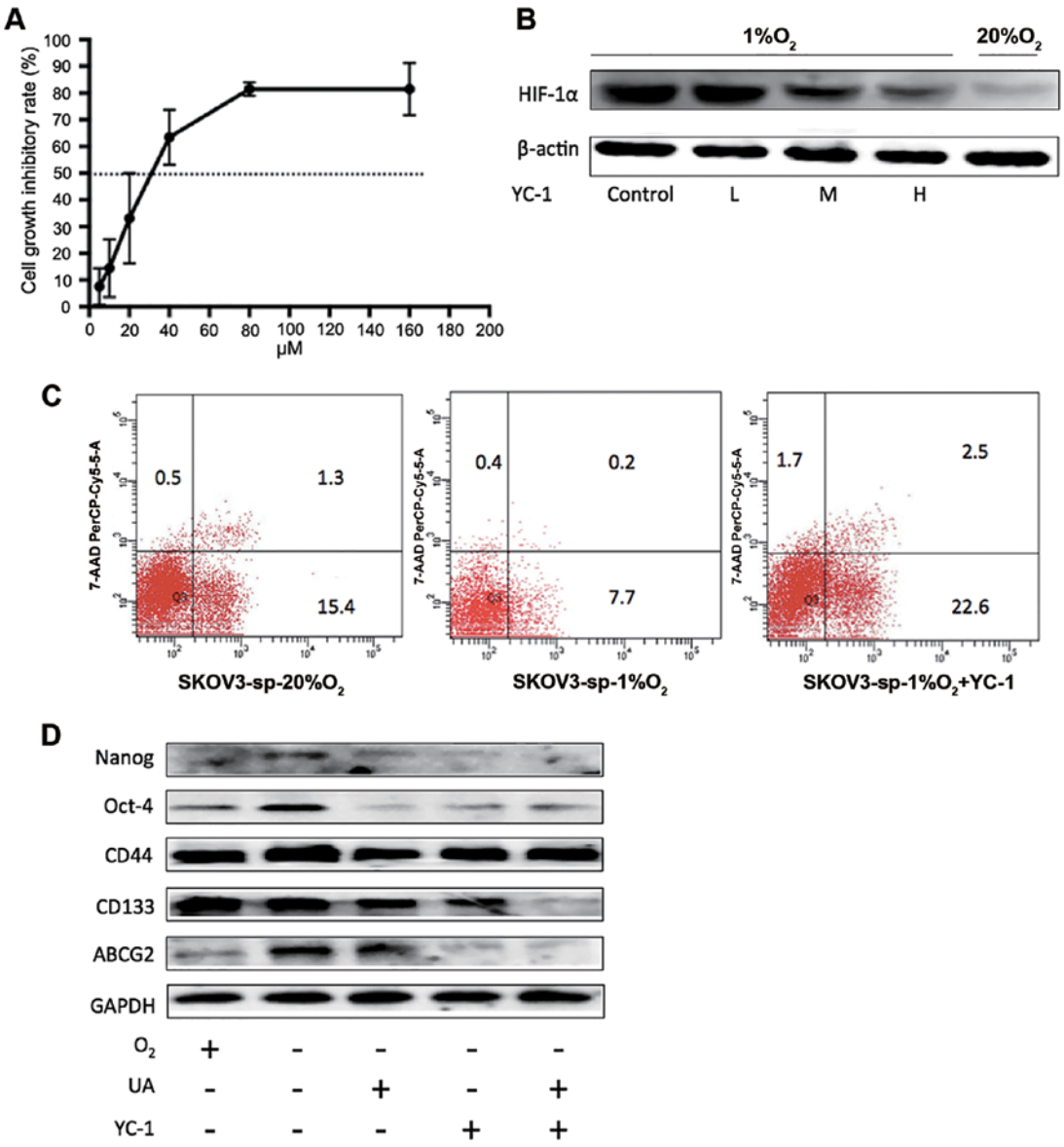


Figure 10. Inhibition of YC-1 on SKOV3-sp under hypoxic condition. (A) SKOV3-sp cells were treated with YC-1 (5, 10, 20, 40, 80, and 160 $\mu$M) for 48 h under hypoxic condition. (B) Different concentrations of YC-1 (6, 14, and 32 $\mu$M) were used to treat SKOV3-sp for 48 h to determine the inhibition on HIF-1α under hypoxic condition. (C) The apoptosis of SKOV3-sp cells after treatment with low-dose YC-1 (6 $\mu$M) under hypoxic condition. (D) The inhibition of YC-1 combined with ursolic acid on the stemness genes and ABCG2 under hypoxic condition. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HIF, hypoxia-inducible factor; UA, ursolic acid.

*and reduction of ABCG2*. Emerging evidence suggests that PI3K/Akt signaling mediates regulation and activation of HIF-1α in various human cancers (42,43). To investigate the relationship between HIF-1α and ABCG2 and whether UA inhibited ABCG2 though downregulation of HIF-1α. The PI3K/Akt signaling pathway, small interfering RNA was used to knockdown the expression of HIF-1α. In this experiment, four sequences were chosen for design, synthesis, and confirmation by sequencing and cloned into pGPU6 vector. The transfection efficiency was detected using western blot and qPCR in SKOV3-ad under hypoxic condition. As shown in Fig. 11A, it can be observed that the positive control shRNA of GAPDH was suppressed obviously and the HIF-1α-Homo-764 site could inhibit the expression of HIF-1α the level of RNA or protein levels. Next the HIF-1α-Homo-764 was chosen to transfect SKOV3-ad and after 48 h the ES medium was changed and the culture dish was treated with poly-HEMA to enrich sphere cells. The transfection efficiency was observed through the fluorescent microscope (Fig. 11B). Under hypoxic condition, no difference was observed between the control and shRNA of NC groups in the expression of HIF-1α, but in the shRNA of HIF-1α of the expression of HIF-1α showed a significant suppression (Fig. 11C). At the same time, the expression of ABCG2 also appeared to be inhibited (Fig. 11D).

To investigate whether the PI3K/Akt signaling was activated under hypoxic condition, the phosphorylation sites (p-Akt-492 and p-Akt-308) of the key protein Akt on the activation time of PI3K/Akt signaling pathway under hypoxic condition were detected. SKOV3-sp was used under hypoxic condition for 0, 0.25, 0.5, 1, 2, 4, 8, and 24 h and then the expression of p-Akt was detected. It was observed that with the increase in hypoxia, the expression of p-Akt-492 and

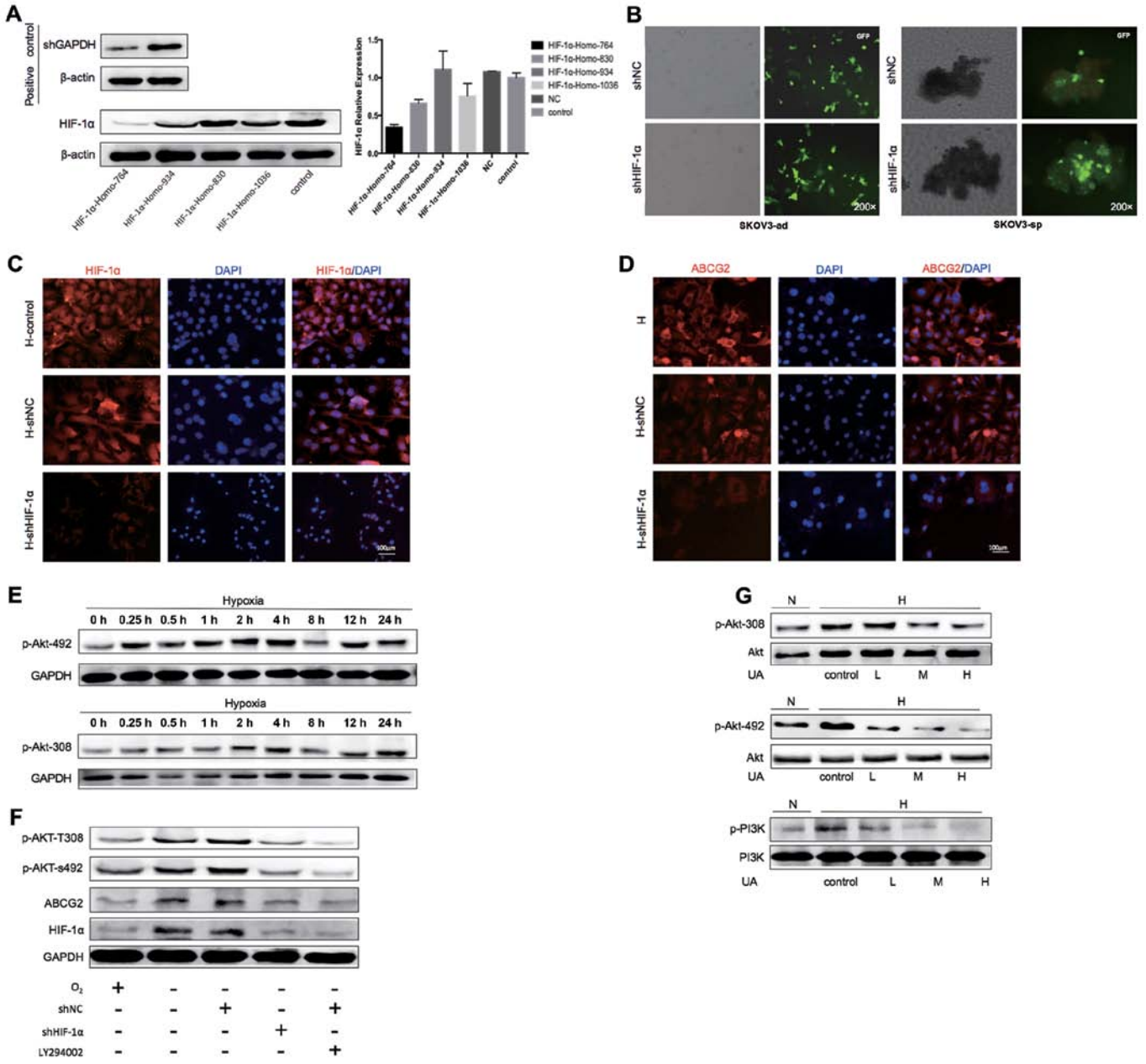


Figure 11. Ursolic acid inhibits ABCG2 though downregulation of HIF-1α. (A) The transfection efficiency was detected using western blot and quantitative PCR in the SKOV3-ad cells under hypoxic condition for 48 h. (B) The transfection efficiency of HIF-1α-Homo-764 site in SKOV3-ad and SKOV3-sp under hypoxic condition for 48 h was detected using a fluorescent microscope. (C) After the knockdown of HIF-1α, the expression of HIF-1α SKOV3-adin SKOV3-sp under hypoxic condition for 48 h was detected using fluorescent microscope. (D) After the knockdown of HIF-1, the expression of ABCG2 in SKOV3-sp under hypoxic condition for 48 h was detected using fluorescent microscope. (E) Western blot detected the activation time of phosphorylated Akt under different durations of hypoxia (0, 0.25, 0.5, 1, 2, 4, 8, 12, and 24 h). (F) Western blot detected the expression of p-Akt-492, p-Akt-308, HIF-1α, and ABCG2 in SKOV3-sp, which were treated with LY294002 or knockdown of HIF-1α under hypoxic condition. (G) Western blot of the expression of p-Akt-492, p-Akt-308, and PI3K after SKOV3-sp was treated with UA (3, 10, and 30 $\mu$g/mL). DAPI, 4',6-diamidino-2-phenylindole; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFP, green fluorescent protein; HIF, hypoxia-inducible factor; PCr, polymerase chain reaction; UA, ursolic acid.

p-Akt-308 increased, reached a peak under hypoxic condition at 4 h and then declined (Fig. 11E). It was assessed that under hypoxic condition for 4 h, AKT was activated. To elucidate whether the PI3K/Akt signaling pathway regulates the expression of ABCG2, HIF-1α affects ABCG2, the PI3K inhibitor LY29004 (44,45). Knockdown of HIF-1α by shRNA were used to investigate HIF-1α causing high expression of ABCG2 under hypoxic condition by the activation of the PI3K/Akt pathway. It was found that under hypoxic condition p-Akt-492, p-Akt-308, HIF-1α, and ABCG2 were significantly increased. The knockdown of HIF-1α and ABCG2 was inhibited, while p-Akt-492 and p-Akt-308 appeared reduced. Treatment with LY29004 resulted in a corresponding reduction in the expression of p-Akt-492 and p-Akt-308. In addition, the expression of phosphorylated Akt was reduced by the treatment with Ly29004, leading to further reduction in HIF-1α and ABCG2 (Fig. 11F).

To investigate whether UA could inhibit the PI3K/Akt pathway, SKOV3-sp was treated with different concentrations of UA for 4 h. A slight decrease in the expression of p-Akt-492

and p-Akt-308 was noted, and the effect was more evident with increasing concentrations, while the expression of PI3K gradually decreased (Fig. 11G). It can be concluded that UA inhibits the activation under hypoxic conditions in the PI3K/Akt signaling pathway.

## Discussion

Ovarian cancer is the most lethal among the gynecologic malignancies as it is diagnosed at an advanced stage in most patients. In spite of success in initial treatment with a combination of surgical debulking and chemotherapy, unacceptably large number of patients (70%) develop terminal, recurrent, chemotherapeutic resistance (46). With the development of CSC theory and assays using markers for the enrichment of CSCs, functional assays have been used to demonstrate CSCs in ovarian cancer. Studies have shown that the CSCs can be identified in tumors by their ability to grow in spheres, which are known as tumor spheres. CSCs from epithelial organs can be expanded as sphere-like cellular aggregates in a serum-free medium containing epidermal growth factor and bFGF (47-50). In these spheres, 4-20% of the cells are stem cells; the others are progenitor cells in various phases of differentiation, which enrich the CSC population by sphere formation and it is applicable to ovarian CSCs (51-53). In this study, the serum-free suspension culture was chosen to enrich the ovarian CSCs. A small population of tumorigenic cells from the ovarian cancer cell lines SKOV3, A2780, and HEY was chosen. Among these sphere cells, it can be observed that the stem cell markers CD44, CD133, Nanog, and OCT-4 were expressed more than the normal cells, and ABCG2, which is widely expressed in various stem cell populations, is highly expressed in sphere cells and is responsible for the maintenance of sphere phenotype (5). As a key MDR transporter, ABCG2 has the capability to efflux various chemotherapeutic drugs and may contribute to drug resistance of cancer cells (54). At the same time, ABCG2, as one of the important stem cell markers, has close association with CSCs. The expression of ABCG2 in stem cells from tumor tissues and tumor cells indicates its important role in stem cell biology. In the present study, the expression of ABCG2 in the SKOV3-sp, A2780-sp, and HEY-sp was found to increase significantly. The ovarian cancer sphere cells increased the cisplatin resistance as compared with the adherent cells.

Hypoxia-induced drug resistance has been observed *in vitro* in breast carcinoma neuroblastoma, and colon cancer (55-57). HIF-1α mediates the cellular response to hypoxia and the master regulators of stem properties (58,59). In the present study, the increase in HIF-1α, and the change in the stemness of ovarian CSCs and the ABCG2 were tested under hypoxic condition. It was found that under hypoxic condition for 48 h in the spheres of SKOV3, A2780, and HEY, in addition to increased HIF, stem genes CD44, CD133, Nanog, Oct-4, and ABCG2 have experienced different degrees of increase. The sphere cells elevated the resistance to cisplatin. Here the following inference was obtained: under hypoxic condition, hypoxia induces drug resistance due to increased HIF-1α and elevated ABCG2.

UA is one of the active compounds in Chinese anticancer herbal medicine. In the present study, it was found that low doses of UA (which was found to be no different from untreated cells and recognized as non-cytotoxic dose) in combination with cisplatin could induce apoptosis significantly as compared to cisplatin alone. Moreover, UA in combination with cisplatin significantly enhanced the cytotoxicity of cisplatin to suppress ovarian CSCs. It could be considered that UA increases the sensitivity of cisplatin under hypoxic condition. Therefore, in the succeeding experiments, the reversal of resistance mechanisms of UA under hypoxic conditions was explored.

In this study, it was found that under hypoxic condition, UA inhibited the expression of HIF-1α and can simultaneously inhibit the expression of resistance gene ABCG2; with the increase in the concentration of the inhibition increasing more obviously. Therefore, it is suspected that UA decreases the expression of HIF-1α to inhibit ABCG2 to reverse the resistance of ovarian CSCs. In this experiment, to elucidate whether the expression of ABCG2 is correlated with the expression of HIF-1α, the HIF-1α inhibitor YC-1 was chosen to treat the SKOV3 sphere cells. It was found that with an increase in the concentration of YC-1, the expression of HIF-1I gradually decreased. Later, the low-dose YC-1 was chosen to treat SKOV3 sphere cells under hypoxic conditions; the rate of apoptosis increased significantly. After treatment with low concentrations of YC-1. At the same time, it was found that after treatment with YC-1, the increasing ABCG2 under hypoxia condition appeared suppressed. When UA was combined with YC-1, the expression of ABCG2 showed a significant decrease.

Under hypoxic condition, enhanced expression of HIF-1α in high tumorigenic cancer stem/progenitor cells sustained the activation PI3K/Akt (60,61). A recent study suggests that the PI3K/Akt signaling mediates the regulation and activation of HIF-1α (43,45). Moreover, excessive activation of PI3K/Akt plays an important role in the chemotherapeutic resistance (62,63). However, whether the PI3K/Akt signaling pathway, which is activated by hypoxia, effects resistance through the regulation of the resistant gene ABCG2 is not clear. Whether the mechanism is impacted by HIF-1α is worth exploring. Hence in the present study, the activation time of p-Akt in SKOV3-sp was tested under hypoxic condition. As a result, it was found that with an increase in hypoxia, the expression of p-Akt-492 and p-Akt-308 was increased, and reached a peak under hypoxia for 4 h and then declined. Later HIF-1α was knocked down by shRNA and the PI3K inhibitor LY294002 was chosen. It was found that inhibiting the PI3K/Akt activity by the inhibitor LY294002 decreased the expression of HIF-1α in A2780-sp cells. Knockdown of HIF-1α, to some extent, can inhibit the expression of phosphorylated Akt. In the succeeding experiment, it was found that when SKOV3-sp is treated with UA under hypoxia it can significantly inhibit the expression of the key protein phosphorylated PI3K and phosphorylated Akt on the PI3K/Akt signaling pathway. The result indicated that under hypoxic condition UA could inhibit the PI3K/Akt signaling pathway activated by the hypoxic condition.

In summary, it was demonstrated that under hypoxic condition UA could inhibit the proliferation and reversal of drug-resistant ovarian CSCs by suppressing the expression of downregulation of HIF-1α and ABCG2. PI3K/Akt signaling

pathway activation plays an important functional role in UA-induced downregulation of HIF-1α and ABCG2 reduction. This study indicates that UA, a compound in traditional Chinese medicine, is a promising agent to reverse drug-resistance in ovarian CSCs.

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