

# Inactivation of AKT, ERK and NF- $\kappa$ B by genistein derivative, 7-difluoromethoxyl-5,4'-di-n-octylygenistein, reduces ovarian carcinoma oncogenicity

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**Abstract.** Cancer stem cells (CSCs) have central functions in cancer formation and development. Aberrant expression of AKT, ERK and NF- $\kappa$ B signaling pathways have been reported in several types of CSCs. Phytochemicals from dietary compounds possess anti-CSC properties, and have been characterized as promising therapeutic agents for the prevention and treatment of many types of cancers. We previously showed that the newly synthesized genistein derivative, 7-difluoromethoxyl-5,4'-di-n-octylygenistein (DFOG), can inhibit the self-renewal ability of ovarian cancer stem cells (OVCSLCs). In the present study, we further assessed whether various signaling pathways are regulated by DFOG. We found that spheroids derived from the SKOV3 cell line possessed OVCSLC properties and DFOG efficiently inhibited the stemness of the OVCSLCs. In addition, the suppression of spheroid and colony formation by DFOG was associated with inhibition of AKT and ERK1/2 protein phosphorylation, and NF- $\kappa$ B activity in OVCSLCs from the SKOV3 cells. Importantly, DFOG inhibited the oncogenicity of the OVCSLCs by activation of FoxO3a and/or inactivation of FoxM1 by the targeting of multiple pro-survival (AKT and ERK1/2) and proinflammatory (NF- $\kappa$ B) pathways, providing a new avenue for the treatment of ovarian carcinoma in humans.

## Introduction

Several signaling pathways are crucial in orchestrating cancer stem cell (CSC) activity. Dysregulation of these pathways has been implicated in the maintenance and function of CSCs. A study by Sunayama *et al* showed that simultaneous blocking of phosphoinositide 3-kinase (PI3K)/AKT and extracellular signal-regulated kinase (ERK1/2) signaling promoted glioblastoma cancer stem-like cell (CSLC) differentiation, suppressing tumorigenicity by activating FoxO3a, which is commonly phosphorylated by AKT and ERK for signal transduction (1). Jacobsen *et al* reported that deregulation of Foxo3a and NF- $\kappa$ B/Rel is associated with malignancy in SCID temperature-sensitive form of Abelson mouse leukemia virus pre-B cells (2). Our previous studies demonstrated that FoxO3a inactivation and/or FoxM1 activation are essential for oncogenicity as well as stemness in ovarian cancer stem-like cells (OVCSLCs) derived from established ovarian cancer cell lines (3,4). However, whether FoxO3a can integrate these pro-survival and pro-inflammatory pathways to induce tumorigenicity in OVCSLCs remains to be investigated.

Numerous epidemiological studies have substantiated the efficient anticancer properties of dietary components in vegetables and fruits (5). These bioactive and non/low-toxic phytochemicals are considered promising candidates for cancer intervention (5,6). Genistein, 5,7,4'-trihydroxyisoflavone, a major soybean compound, possesses antitumor properties (7,8). However, genistein has poor bioavailability due to its low solubility in both organic solvents and water (9). Notably, the introduction of HCF<sub>2</sub> or CF<sub>3</sub> into the genistein molecule improves the anticancer activities of genistein derivatives (9). Furthermore, the newly synthesized genistein derivative, 7-difluoromethoxyl-5,4'-di-n-octylygenistein (DFOG), was found to induce apoptosis in ovarian and gastric carcinomas (3,4). DFOG acts as an inhibitor of CSCs or tumor-initiating cells by the activation of FoxO3a and/or inactivation of FoxM1 (3,4). Nevertheless, whether DFOG inhibits oncogenicity in OVCSLCs by activating FoxO3a and/or inactivating FoxM1 through targeting of multiple pro-survival

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(AKT and ERK1/2) and pro-inflammatory (NF- $\kappa$ B) pathways is unclear.

In the present study, we demonstrated that DFOG suppressed *in vitro* spheroid and colony formation on soft agar of OVCSLCs obtained from SKOV3 cells. Mechanistically, DFOG exhibited effects similar to those of the PI3K inhibitor (LY294002), MEK inhibitor (U0126) and NF- $\kappa$ B inhibitor (PDTC), simultaneously. The suppressive activity of DFOG on spheroid formation in serum-free medium (SFM) and colony formation on soft agar were dependent on FoxO3a and FoxM1 protein levels. These results indicate that DFOG may be potentially used for the treatment of human ovarian cancer.

## Materials and methods

**Reagents.** Invitrogen Life Technologies (Shanghai, China) supplied Dulbecco's modified Eagle's medium (DMEM) and DMEM/F12, trypsin-EDTA, fetal bovine serum (FBS) and penicillin-streptomycin. Monoclonal antibodies raised in mice against human anti- $\beta$ -actin were manufactured by Sigma-Aldrich (St. Louis, MO, USA) (catalog no. A2066). Rabbit polyclonal antibodies targeting CD44, ALDH1, CD133 and FoxO3a were obtained from Abcam Co. (Cambridge, MA, USA) (catalog nos. ab24504, ab9883, ab19898 and ab53287, respectively). Monoclonal antibodies against FoxM1 (C-20) and NF- $\kappa$ Bp65 raised in rabbits were manufactured by Santa Cruz Biotechnology, Inc. (Beverly, MA, USA) (catalog nos. sc-502 and sc-8008). Primary antibodies against phospho-AKT (Ser473), AKT, p-ERK (Thr202/Tyr204), phospho-FoxO3a (Ser253) and ERK1/2 were manufactured by Cell Signaling Technology (Danvers, MA, USA) (catalog nos. #9171, #9272, #9101, #9466 and #9102).

The pHBad-U6-GFP, pHBad-U6-GFP-shFOXO3a and pHBad-U6-GFP-shFOXM1 plasmid packaging adenoviral particles were obtained from Hanbio Biotechnology Co. Ltd. (Shanghai, China) (2.0 ml,  $1 \times 10^{11}$  PFU/ml). Pyrrolidone dithiocarbamate, ammonium salt (PDTC), LY294002 and U0126 were purchased from Sigma-Aldrich.

**Cell culture and sphere formation assay.** Human ovarian carcinoma SKOV3 cells were obtained from the Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM containing 10% FBS.

Sphere formation was assessed in serum-free culture medium containing antibiotics, growth factors, vitamin B27, and the N2 supplement (Invitrogen), following instructions from the manufacturer. Cell seeding was performed at  $10^4$  cells/well in 6-well ultra-low attachment plates (Corning, Corning, NY, USA).

Spheroids were obtained by centrifugation (200  $\times$  g) and trypsin-EDTA digestion, followed by mechanical disruption. Single cells were washed and transferred into SFM for sphere induction. Second-generation spheroids were used as ovarian cancer stem-like cells (OVCSLCs).

Single cells with potential for transformation into new spheroids were cultured at 1,000 cells/well in a 24-well plate, to generate new spheroids. Tumor spheroids were counted in 6 day cultures; the efficiency of spheroid formation was expressed as the ratio of the total number of spheroids generated to that of SKOV3 cells seeded, multiplied by 100.

**Colony formation assay.** In the present study, soft agar was used. Medium containing 0.7% agarose was added into a 6-well plate. Then,  $10^4$  cells were seeded/well in medium containing 0.4% agarose (top layer), and incubated for 3 weeks. Routine colony count was carried out on an inverted microscope (Olympus IX53; Olympus, Tokyo, Japan). Three independent experiments were carried out.

**In vivo tumorigenicity experiments.** Balb/c-nu mice aged 4 weeks were purchased from the Animal Institute of the Chinese Academy of Medical Science (CAMS). All animal studies were performed in accordance with the standard protocols approved by the Ethics Committee of The First Affiliated Hospital of Jinan University and the Committee of Experimental Animal Feeding and Management. Mice were randomly divided into 3 groups (4 mice/group) and maintained under standard conditions, according to the standard protocols. Cells were suspended in serum free-DMEM/Matrigel (BD Biosciences) mixture (1:1 volume). Each recipient Balb/c-nu mouse was inoculated subcutaneously with various numbers of SKOV3-derived OVCSLCs ( $1 \times 10^3$ ,  $1 \times 10^4$  and  $1 \times 10^5$  cells) in one flank and the monolayer SKOV3 cells ( $1 \times 10^4$ ,  $1 \times 10^5$  and  $1 \times 10^6$ ) in the other, respectively. Tumorigenicity experiments were terminated 1 month after cell inoculation. Harvested tumors were imaged and weighed immediately. After that, specimens from tumor tissue samples were fixed in 10% neutral buffered formalin, processed in paraffin blocks, and sectioned. The sections were stained with H&E and examined for the histopathology.

**Transduction of shFOXO3a and shFOXMI.** SKOV3 cell-derived OVCSLCs were plated into 24-well culture plates at 40-50% confluency, and incubated overnight. Then, the cells were transduced with the pHBad-U6-GFP or pHBad-U6-GFP-shFOXO3a or pHBad-U6-GFP-shFOXMI plasmid packaging adenoviral particles using an enhanced infection solution (ENi.s; cat. no. REVG0002; GeneChem, Shanghai, China). Following 4 h of transduction, DMEM with 10% fetal calf serum (FCS) was added to replace the transduction medium; this was followed by 48 h of incubation before gene and protein level assessments.

**Western blot analysis.** Cells lysis was performed according to published protocols (10). Monoclonal anti- $\beta$ -actin, anti-NF- $\kappa$ Bp65, anti-FoxM1, anti-phospho-AKT (Ser473) antibodies, and polyclonal anti-CD44, anti-ALDH1A1, anti-CD133, anti-AKT, anti-p-ERK (Thr202/Tyr204), anti-ERK1/2, anti-Phospho-FoxO3a (Ser253) antibodies were used as primary antibodies, for overnight incubation at 4°C. Adequate horseradish peroxidase (HRP) bound secondary antibodies were added for 1 h at ambient temperature; visualization of specific protein bands was carried out using enhanced chemiluminescence, with  $\beta$ -actin employed for normalization.

**Statistical analysis.** Comparisons were conducted by two-tailed Student's t-test. A P-value <0.05 indicated statistical significance.

## Results

**Spheroids reflect SKOV3 cell-derived OVCSLCs.** CSCs are mainly characterized by their capacity to form 3-dimensional

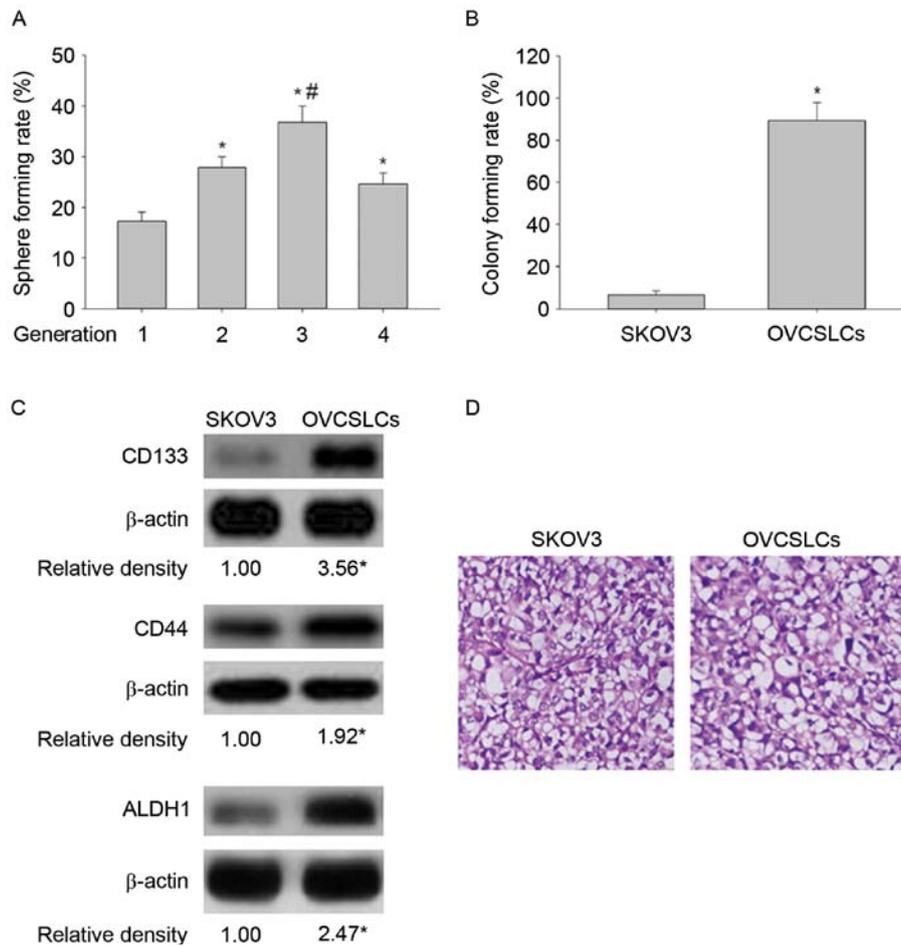


Figure 1. Spheroids display the properties of OVCSLCs in SKOV3 cells. (A) Comparison of spheroid-forming rates in spheroids of different generations (\* $P < 0.05$  compared to primary spheroid culture; # $P < 0.05$  compared to second or fourth spheroid culture). (B) Comparison of colony forming rates and (C) protein levels of stem cell markers in OVCSLCs (second-generation spheroids) and SKOV3 cells in monolayer growth (mean  $\pm$  SD or mean, \* $P < 0.05$  compared to cells in monolayer growth). (D) SKOV3 cell-produced OVCSLCs ( $10^4$ ) or  $10^5$  SKOV3 cells in monolayer growth were subcutaneously injected into male BALB/c-nu mice. After 6 weeks, tumors were obvious in OVCSLC and SKOV3 cell xenotransplantation animals. H&E staining showed similar histology for xenograft tumors from OVCSLCs and SKOV3 cells. Unless otherwise stated, all experiments were performed in triplicate and values are mean  $\pm$  SD.

Table I. Tumorigenicity experiments of OVCSLCs and SKOV3 cells in BALB/c-nu mice.

Cells <sup>a</sup>	Inoculum amount	Tumor incidence	Latency period (days)
SKOV3	$1 \times 10^3$	0/4	-
	$1 \times 10^4$	0/4	-
	$1 \times 10^5$	2/4	42
OVCSLC	$1 \times 10^3$	3/4	25
	$1 \times 10^4$	4/4	16
	$1 \times 10^5$	4/4	8

<sup>a</sup>SKOV3, SKOV3 cells in monolayer growth; OVCSLC, second-generation spheroids derived from the SKOV3 cell line.

spheroids, and tumorsphere formation assay via SFM culturing is widely used in their isolation and enrichment *in vitro*. Under SFM culture conditions, most cancer cells undergo apoptosis, whereas only a small proportion form tumorspheres. These

subpopulations of cancer cells are believed to have CSC characteristics such as self-renewal ability and unlimited differentiation. Our group and other investigators have demonstrated that the spheroids of established ovarian cancer cell lines and transplanted human ovarian cancer possess the characteristics of OVCSLCs (3,4,11-14). In the present study, we also identified the characteristics of OVCSLCs in spheroids obtained from SKOV3 cells. We showed that the second-generation spheroids had the highest self-renewal potential (Fig. 1A). We further demonstrated that second-generation spheroids had a higher colony formation rate compared with cells in monolayer growth (Fig. 1B). In addition, the results obtained using western blotting revealed that second-generation spheroids had elevated amounts of CSC-related markers (CD133, CD44 and ALDH1), compared with SKOV3 cells in monolayer growth (Fig. 1C). Significantly, second-generation spheroids displayed more powerful carcinogenicity than cells of the SKOV3 cell line *in vivo* (Fig. 1D and Table I). These results demonstrated that spheroids derived from the SKOV3 cell line possessed OVCSLC properties, such as higher oncogenicity *in vitro* and *in vivo*, and overexpression of 'stemness' biomarkers. For this reason, second-generation spheroids were used as OVCSLCs in the subsequent experimental studies.

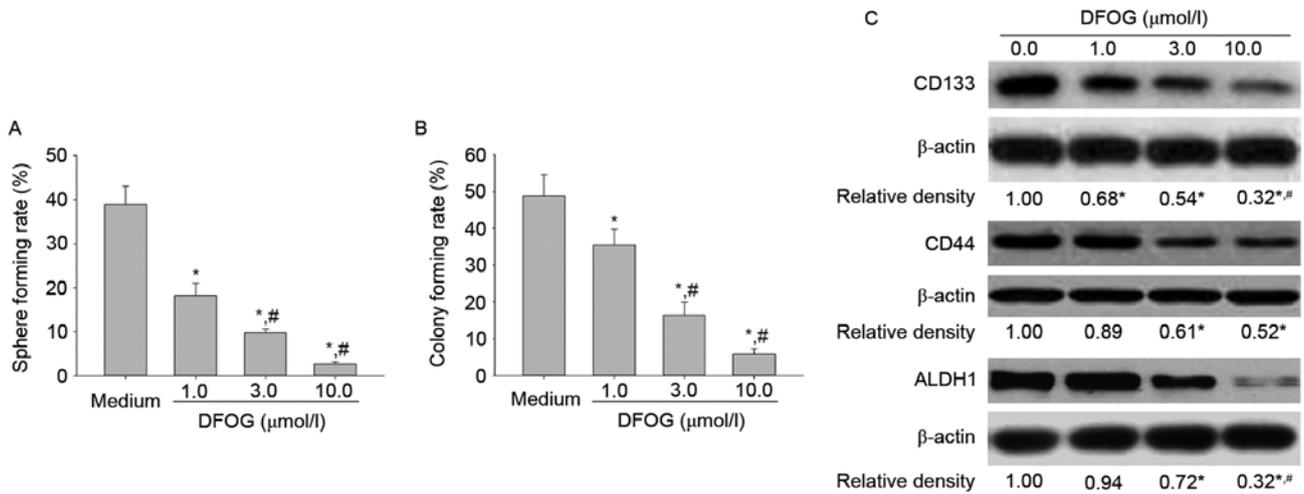


Figure 2. DFOG inhibits the stemness of OVCSLCs derived from SKOV3 cells. DFOG reduced (A) spheroid and (B) colony formation rates, in a concentration-dependent manner (mean ± SD, n=3; \*P<0.05 compared to the vehicle control group; #P<0.05 compared to 1 μmol/l DFOG treatment). (C) DFOG decreased the expression of stem cell markers (mean, n=3; \*P<0.05 compared with the vehicle control group; #P<0.05 compared with 1 μmol/l DFOG treatment).

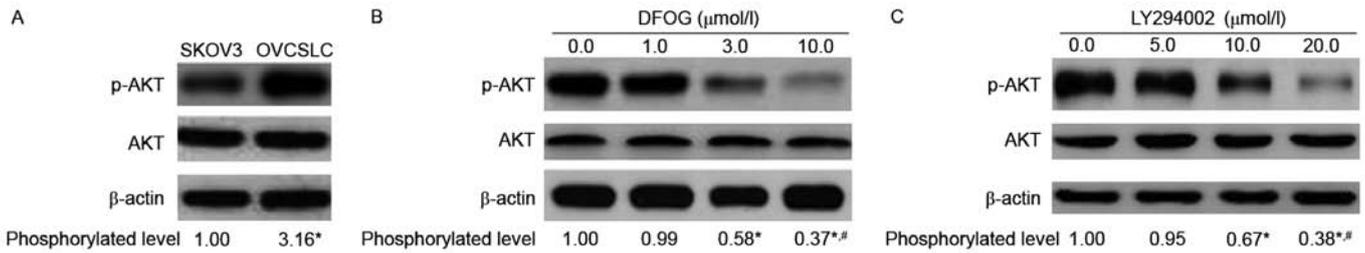


Figure 3. DFOG inhibits AKT activity of OVCSLCs from SKOV3 cells. (A) Phosphorylated AKT levels in OVCSLCs were higher than those of SKOV3 cells in monolayer growth (mean, n=3; \*P<0.05 compared to cells in monolayer growth). (B) DFOG reduced the expression of the p-AKT protein in OVCSLCs from SKOV3 cells (mean, n=3; \*P<0.05 compared to the vehicle control group; #P<0.05 compared to 1 μmol/l DFOG treatment). (C) LY294002 reduced phosphorylation level of AKT in OVCSLCs from SKOV3 cells (mean, n=3; \*P<0.05 compared to the vehicle control group; #P<0.05 compared to 5.0 μmol/l LY294002 treatment).

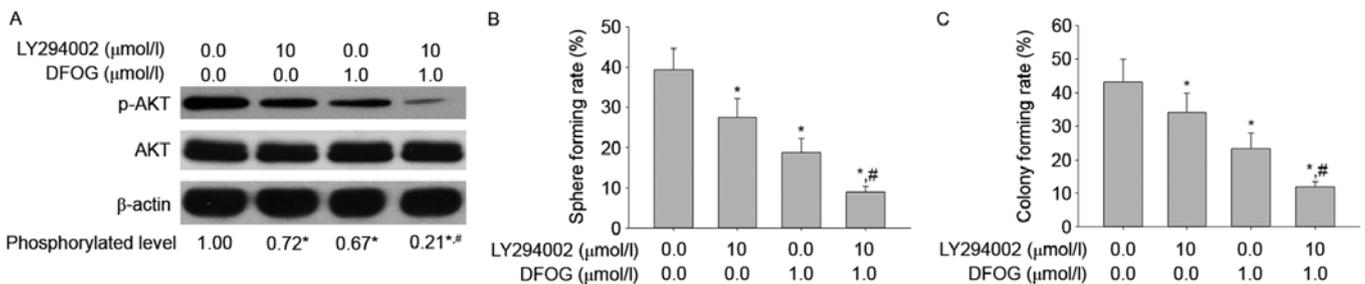


Figure 4. Effects of combined treatment with DFOG and LY294001 on p-AKT protein expression, and spheroid and colony formation in OVCSLCs from SKOV3 cells. (A) Both 10 μmol/l LY294002 and 1 μmol/l DFOG decreased the expression of p-AKT, with the latter having a greater inhibitory effect. However, the combination of both inhibitors further decreased p-AKT levels (mean, n=3; \*P<0.05 compared to the vehicle control group; #P<0.05 compared to 10 μmol/l LY294002 or 1 μmol/l DFOG treatment alone). (B and C) Both 10 μmol/l LY294002 and 1 μmol/l DFOG reduced (B) spheroid and (C) colony formation rates. Combination of both inhibitors further suppressed spheroid and colony formation (mean ± SD, n=3; \*P<0.05 compared to the vehicle control group; #P<0.05 compared to 10 μmol/l LY294002 or 1 μmol/l DFOG treatment alone).

DFOG inhibits the characteristics of SKOV3 cell-produced OVCSLCs. Previous studies revealed the novel genistein analogue DFOG induces apoptosis of various cancer cell lines (9,15). DFOG inhibited the proliferation of CSCs or tumor-initiating cells (3,4). Therefore, we next sought to examine the inhibitory effects of DFOG on stemness of

OVCSLCs from SKOV3 cells. In the present study, we found that DFOG dose-dependently reduced spheroid (Fig. 2A) and colony (Fig. 2B) formation rates. Furthermore, our data provided evidence that DFOG displayed a concentration-dependent downregulation of CSC-related proteins (CD133, CD44 and ALDH1) (Fig. 2C). The current findings suggest that DFOG

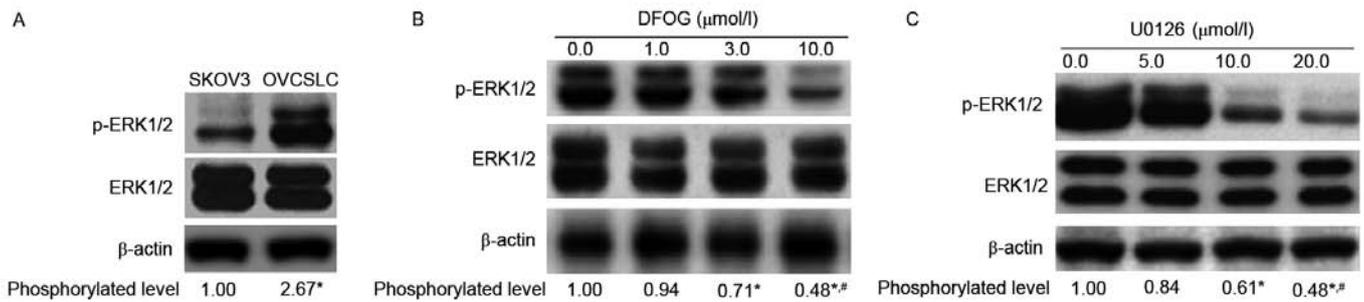


Figure 5. DFOG inhibits ERK1/2 activity of OVCSLCs from SKOV3 cells. (A) The phosphorylation levels of the ERK1/2 protein in OVCSLCs were higher than those of SKOV3 cells in monolayer growth (mean, n=3; \*P<0.05 compared with cells in monolayer growth). (B) DFOG reduced p-ERK1/2 protein amounts in OVCSLCs from SKOV3 cells (mean, n=3; \*P<0.05 compared to the vehicle control group; #P<0.05 compared to 1 μmol/l DFOG treatment). (C) U0126 reduced phosphorylation level of ERK1/2 in OVCSLCs from SKOV3 cells (mean, n=3; \*P<0.05 compared to the vehicle control group; #P<0.05 compared to 5.0 μmol/l U0126 treatment).

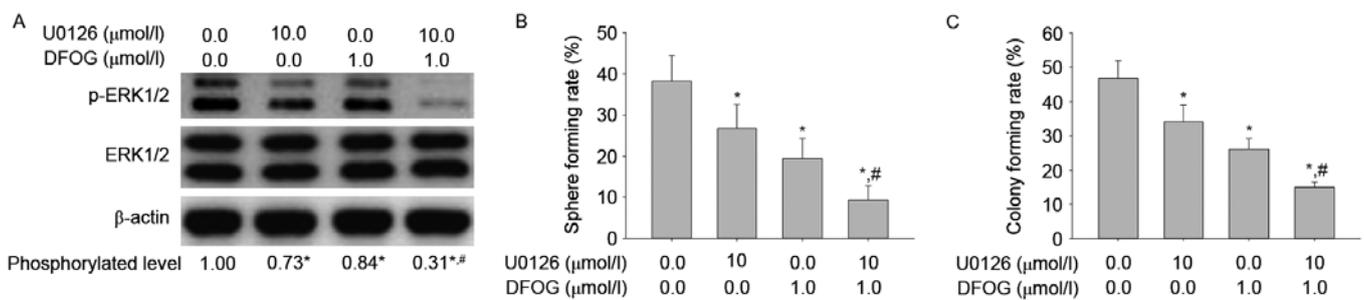


Figure 6. Effects of combined DFOG and U0126 on p-ERK1/2 protein expression, and spheroid and colony formation in OVCSLCs from SKOV3 cells. (A) Both 10 μmol/l U0126 and 1 μmol/l DFOG decreased p-ERK1/2 levels. Combination of both inhibitors further decreased p-ERK1/2 levels (mean, n=3; \*P<0.05 compared to the vehicle control group; #P<0.05 compared to 10 μmol/l U0126 or 1 μmol/l DFOG treatment alone). (B and C) Both 10 μmol/l U0126 and 1 μmol/l DFOG reduced (B) spheroid and (C) colony formation rates. Combination of both inhibitors further suppressed spheroid and colony formation (mean ± SD, n=3; \*P<0.05 compared to the vehicle control group; #P<0.05 compared to 10 μmol/l U0126 or 1 μmol/l DFOG treatment alone).

can efficiently inhibit stemness in OVCSLCs from SKOV3 cells.

**DFOG reduces the phosphorylation levels of AKT in OVCSLCs from SKOV3 cells.** Numerous studies have reported that genistein abolishes stemness in CSCs or CSLCs via inhibition of AKT phosphorylation and activity (10,16). We thus hypothesized that the novel genistein analogue DFOG-inhibited spheroid and colony formation is associated with AKT inactivation. To test the hypothesis, we used western blotting to analyze the protein expression levels of p-AKT in cells treated with DFOG or not. As expected, elevated amounts of phosphorylated AKT in OVCSLCs were obtained compared with the levels in SKOV3 cells grown in monolayers (Fig. 3A). DFOG effects (Fig. 3B) were similar to those of the PI3K inhibitor LY294002 (Fig. 3C), effectively and concentration-dependently reducing the phosphorylation levels of the AKT protein in SKOV3 cell-produced OVCSLCs.

To confirm that the reduced AKT phosphorylation is involved in oncogenicity maintenance *in vitro* in SKOV3 cell-produced OVCSLCs, we next sought to examine p-AKT levels, and spheroid and colony forming capabilities in OVCSLCs treated with DFOG (1 μmol/l) and/or LY294002 (10 μmol/l). We found that DFOG and LY294002 cooperated to reduce the levels of AKT phosphorylation (Fig. 4A), and attenuate spheroid (Fig. 4B) and colony (Fig. 4C) forming

capabilities in OVCSLCs from the SKOV3 cell line. These results suggest that DFOG-inhibited spheroid and colony formation may be associated with AKT inactivation in OVCSLCs from SKOV3 cells.

**DFOG downregulates p-ERK1/2 expression in OVCSLCs from SKOV3 cells.** ERK kinase signaling is essential for cell proliferation. In healthy cells, the activated signaling pathway leads to progression from G1 to S phase, and is involved in the inactivation of antiproliferative genes. Moreover, inhibition of the ERK pathway reduces the development of CSCs (17). Increasing evidence demonstrates that genistein inhibits self-renewal ability and CSC-related protein expression in various CSCs or CSLCs via inhibition of ERK1/2 phosphorylation (18-21). We next sought to analyze whether DFOG-inhibited spheroid and colony formation is associated with reduced ERK1/2 protein phosphorylation. We found elevated levels of phosphorylated ERK in OVCSLCs compared with the amounts obtained for SKOV3 cells in monolayer growth (Fig. 5A). DFOG (Fig. 5B) showed similar effects to the MEK inhibitor U0126 (Fig. 5C), effectively concentration-dependently reducing the phosphorylation levels of the ERK1/2 protein in OVCSLCs derived from SKOV3 cells.

To determine how DFOG-related reduced ERK1/2 protein phosphorylation affects oncogenicity maintenance *in vitro* in SKOV3 cell-produced OVCSLCs, we next sought to examine

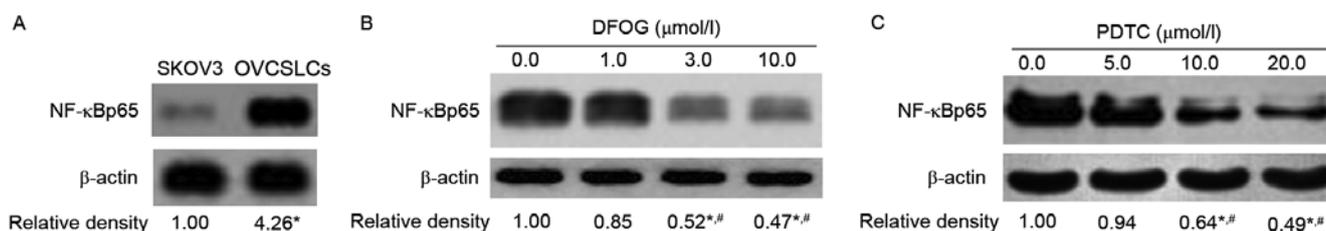


Figure 7 DFOG inhibits NF-κB activity in OVCSLCs from SKOV3 cells. (A) The protein expression levels of NF-κBp65 in OVCSLCs were higher than those of SKOV3 cells in monolayer growth (mean, n=3; \*P<0.05 compared to cells in monolayer growth). (B) DFOG inhibited the protein expression of NF-κBp65 in OVCSLCs from SKOV3 cells (mean, n=3; \*P<0.05 compared to the vehicle control group; <sup>#</sup>P<0.05 compared to 1 μmol/l DFOG treatment). (C) PDTC decreased NF-κBp65 protein levels in OVCSLCs from SKOV3 cells (mean, n=3; \*P<0.05 compared to the vehicle control group; <sup>#</sup>P<0.05 compared to 5.0 μmol/l PDTC treatment).

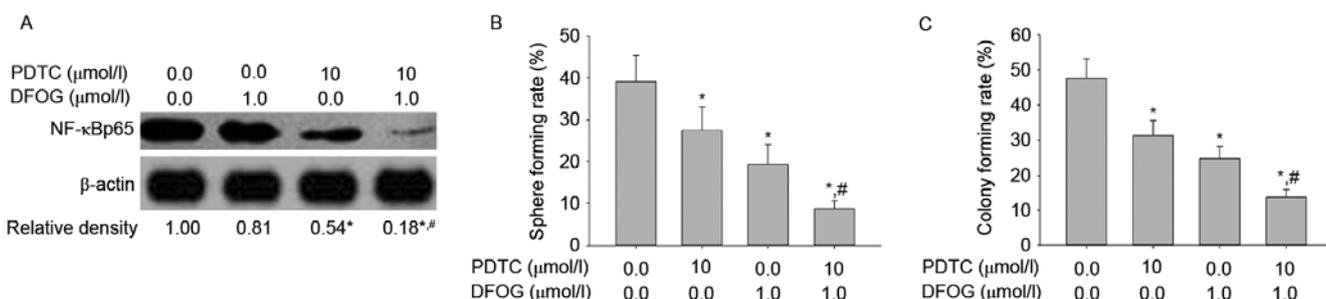


Figure 8. Effects of combined DFOG and PDTC on NF-κBp65 expression, and spheroid and colony formation in OVCSLCs from SKOV3 cells. (A) Both 10 μmol/l PDTC and 1 μmol/l DFOG decreased the expression of NF-κBp65. Combination of both inhibitors further suppressed the expression of NF-κBp65 (mean, n=3; \*P<0.05 compared to the vehicle control group; <sup>#</sup>P<0.05 compared to 10 μmol/l PDTC or 1 μmol/l DFOG treatment alone). (B and C) Both 10 μmol/l PDTC and 1 μmol/l DFOG reduced (B) spheroid and (C) colony formation rates. Combination of both inhibitors further suppressed spheroid formation and colony formation (mean, n=3; \*P<0.05 compared to the vehicle control group; <sup>#</sup>P<0.05 compared to 10 μmol/l PDTC or 1 μmol/l DFOG treatment alone).

p-ERK levels, spheroid and colony forming capabilities in OVCSLCs treated with DFOG (1 μmol/l) and/or U0126 (10 μmol/l). We showed that DFOG and U0126 cooperated to reduce the levels of ERK1/2 protein phosphorylation (Fig. 6A), and attenuate spheroid (Fig. 6B) and colony (Fig. 6C) forming capabilities in OVCSLCs from the SKOV3 cell line. These results suggest that DFOG-inhibited spheroid and colony formation may be associated with inhibition of ERK1/2 protein phosphorylation in OVCSLCs from SKOV3 cells.

**DFOG decreases NF-κB activity in OVCSLCs from SKOV3 cells.** Recent studies showed that genistein and its derivatives inhibit the proliferation and invasion of various cancer cells or cancer stem-like cells by inhibiting NF-κB nuclear translocation via IκB signaling (19,22,23). We next sought to assess whether DFOG-inhibited spheroid and colony formation is associated with the inhibition of NF-κB activity. The results obtained by western blot analysis demonstrated an upregulation of NF-κBp65, an active fragment of NF-κB, in OVCSLCs compared with cells in monolayer growth derived from the SKOV3 cell line (Fig. 7A). Both DFOG (Fig. 7B) and PDTC (Fig. 7C), a NF-κB inhibitor, dose-dependently reduced the expression levels of NF-κBp65.

To examine the role of DFOG-related inhibition of NF-κB activity in oncogenicity maintenance *in vitro* in SKOV3 cell-produced OVCSLCs, we next sought to quantify NF-κBp65 levels, and spheroid and colony forming capabilities in OVCSLCs treated with DFOG (1 μmol/l) or PDTC

(10 μmol/l) or both. We found that DFOG and PDTC cooperated to reduce the levels of NF-κBp65 expression (Fig. 8A), and attenuate spheroid (Fig. 8B) and colony (Fig. 8C) forming capabilities in OVCSLCs from the SKOV3 cell line. These results suggest that DFOG-inhibited spheroid and colony formation may be associated with the inhibition of NF-κB activity in OVCSLCs from SKOV3 cells.

**Inhibitory effects of DFOG on oncogenicity *in vitro* depend on FoxO3a expression in OVCSLCs from SKOV3 cells.** Since the FoxO3a function is closely associated with OVCSLC oncogenicity inhibition related to suppressed AKT and/or ERK and/or NF-κB pathway (1,24,25), whether oncogenicity reduction by DFOG *in vitro* depended on FoxO3a expression in OVCSLCs from SKOV3 cells was assessed. Fig. 9A shows elevated expression levels of phosphorylated FoxO3a in OVCSLCs compared with these levels in SKOV3 cells in monolayer growth. DFOG (Fig. 9B) effectively and concentration-dependently reduced the phosphorylation levels of FoxO3a in SKOV3 cell-produced OVCSLCs. In SKOV3 cell-produced OVCSLCs, FoxO3a expression was knocked down by transduction with the FOXO3a shRNA-expressing adenovirus (Fig. 9C). Our results revealed that co-treatment with DFOG and FOXO3a shRNA reduced the expression levels of FoxO3a (Fig. 9D), and synergistically attenuated the spheroid (Fig. 9E) and colony (Fig. 9F) forming capabilities of OVCSLCs from the SKOV3 cell line. Taken together, these data indicate that inhibition of OVCSLC oncogenicity

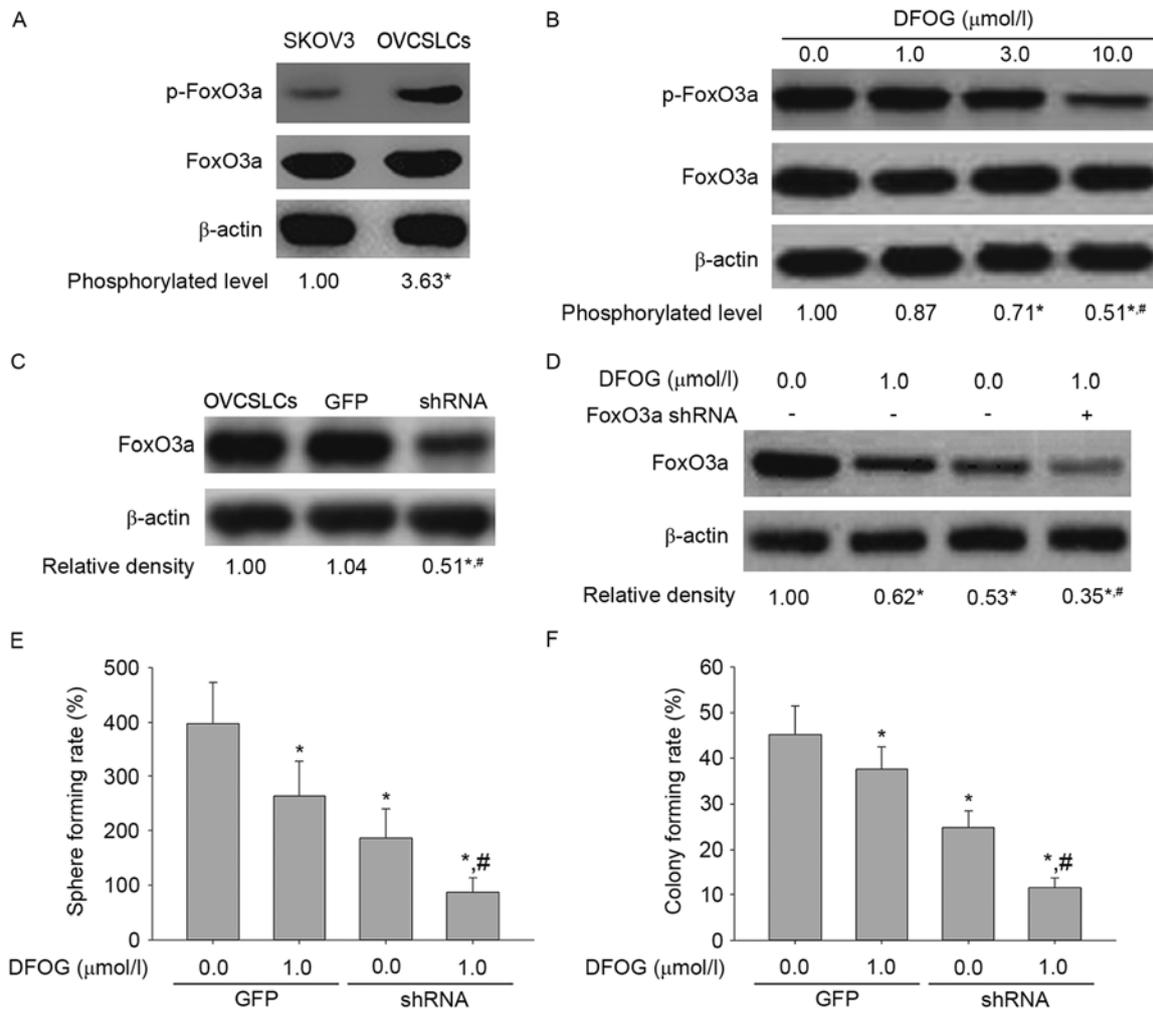


Figure 9. Effects of combined DFOG and FoxO3a shRNA on FoxO3a expression, and spheroid and colony formation in OVCSLCs from SKOV3 cells. (A) Comparison of protein expression of p-FoxO3a in OVCSLCs (second-generation spheroids) and SKOV3 cells in monolayer growth (mean, n=3; \*P<0.05 compared to cells in monolayer growth). (B) DFOG inhibited the protein expression of p-FoxO3a, in a concentration-dependent manner (mean, n=3; \*P<0.05 compared to the vehicle control group; #P<0.05 compared to 1 μmol/l DFOG treatment). (C) FOXO3a gene knockdown decreased the protein expression of FoxO3a (mean, n=3; \*P<0.05 compared to SKOV3 cell-produced OVCSLCs; #P<0.05 compared to OVCSLCs transduced with adenovirus containing the GFP gene). (D) FOXO3a shRNA reduced the expression levels of FoxO3a; Combination of DFOG and FOXO3a shRNA further suppressed the expression of FoxO3a (mean, n=3; \*P<0.05 compared to untreated SKOV3 cell-produced OVCSLCs; #P<0.05 compared to FOXO3a shRNA or 1 μmol/l DFOG treatment alone). (E and F) Both FOXO3a shRNA and 1 μmol/l DFOG reduced (E) spheroid and (F) colony formation rates. Combination of FOXO3a shRNA and 1 μmol/l DFOG further suppressed spheroid and colony formation (mean ± SD, n=3; \*P<0.05 compared to untreated SKOV3 cell-produced OVCSLCs; #P<0.05 compared to FOXO3a shRNA and 1 μmol/l DFOG treatment alone).

*in vitro* mediated by the suppressive effects of DFOG on AKT and/or ERK and/or NF-κB pathways requires FoxO3a expression.

**Oncogenicity reduction by DFOG *in vitro* requires FoxM1 in OVCSLCs from SKOV3 cells.** A study by McGovern *et al* demonstrated ectopically expressed FOXO3a downregulates FOXM1, while FOXO3a silencing increases FOXM1 levels and rescues sensitive breast cancer BT474 cells from gefitinib-related growth inhibition (26). Our results suggested that FoxM1 inhibition by FoxO3a activation induced apoptosis in ovarian cancer cells (27). Furthermore, previous studies demonstrated that FoxM1 inhibition results in altered characteristics of OVCSLCs from SKOV3 cells (4). Importantly, Bao *et al* found that genistein inhibits cell malignancy, in agreement with decreased CD44 and EpCAM levels. Accordingly, we determined that the inhibitory effects of DFOG on onco-

genicity *in vitro* depended on FoxM1 expression in OVCSLCs from SKOV3 cells.

Fig. 10A shows FoxM1 upregulation in OVCSLCs compared with the levels of SKOV3 cells in monolayer growth. DFOG (Fig. 10B) effectively and concentration-dependently reduced the expression of the FoxM1 protein in OVCSLCs derived from SKOV3 cells. In SKOV3 cell-produced OVCSLCs, FoxM1 expression was knocked down by transduction with FoxM1 shRNA-expressing adenovirus (Fig. 10C). Our results also revealed that co-treatment with DFOG and FOXM1 shRNA reduced the expression levels of FoxM1 (Fig. 10D), and synergistically attenuated spheroid (Fig. 10E) and colony (Fig. 10F) forming capabilities in OVCSLCs from the SKOV3 cell line. Taken together, these data indicate that reduced OVCSLC oncogenicity *in vitro* by DFOG through inhibitory effects on AKT and/or ERK and/or NF-κB pathways requires FoxM1 expression.

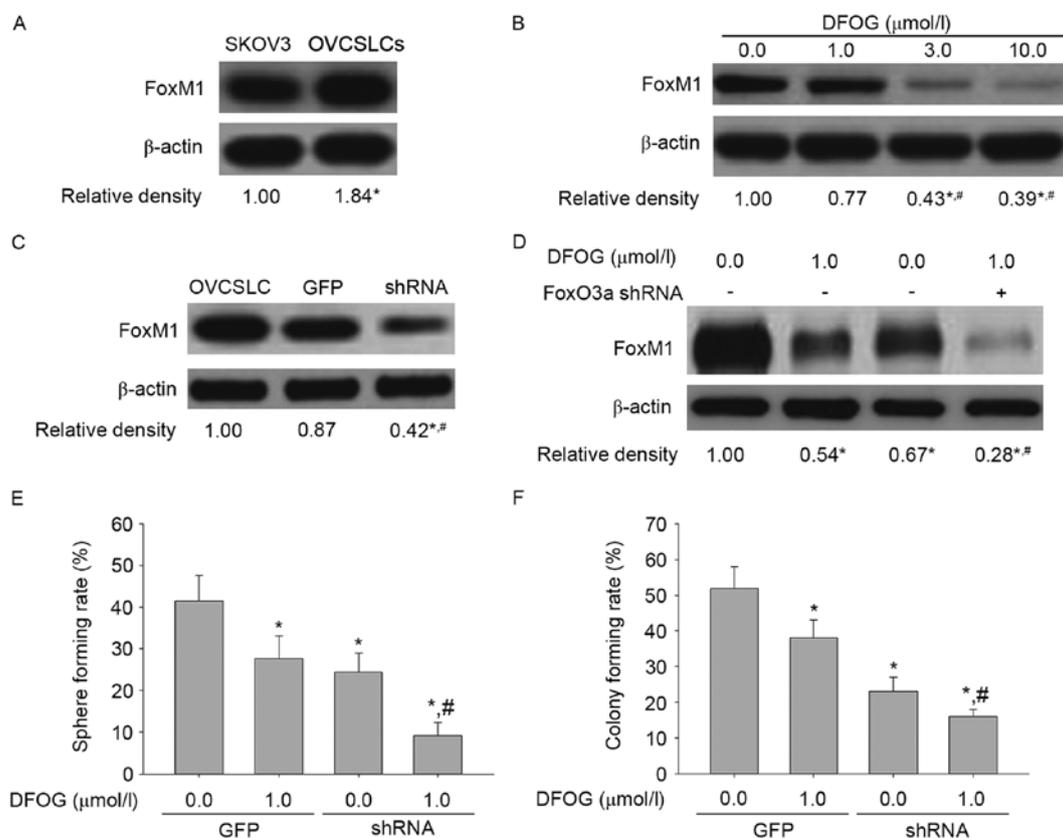


Figure 10. Effects of combined of DFOG and FoxM1 shRNA on FoxM1 expression, and spheroid and colony formation in OVCSLCs from SKOV3 cells. (A) Comparison of protein expression of FoxM1 in OVCSLCs (second-generation spheroids) and SKOV3 cells in monolayer growth (mean, n=3; \*P<0.05 compared to cells in monolayer growth). (B) DFOG concentration-dependently reduced FoxM1 protein amounts (mean, n=3; \*P<0.05 compared to the vehicle control group; <sup>#</sup>P<0.05 compared to 1 μmol/l DFOG treatment). (C) FOXM1 gene knockdown decreased expression of FoxM1 protein (mean, n=3; \*P<0.05 compared to SKOV3 cell-produced OVCSLCs; <sup>#</sup>P<0.05 compared to OVCSLCs transduced with adenovirus containing the GFP gene). (D) FOXM1 shRNA reduced the expression levels of FoxM1. Combination of DFOG and FOXM1 shRNA further suppressed the expression of FoxM1 protein (mean, n=3; \*P<0.05 compared to untreated SKOV3 cell produced OVCSLCs; <sup>#</sup>P<0.05 compared to FOXO3a shRNA or 1 μmol/l DFOG treatment alone). (E and F) Both FOXM1 shRNA and 1 μmol/l DFOG reduced (E) spheroid and (F) colony formation rates. Combination of FOXM1 shRNA and 1 μmol/l DFOG further suppressed spheroid and colony formation (mean ± SD, n=3; \*P<0.05 compared to untreated SKOV3 cell produced OVCSLCs; <sup>#</sup>P<0.05 vs. FOXM1 shRNA and 1 μmol/l DFOG treatment alone).

## Discussion

Cancer is the uncontrolled growth of cells. Numerous signaling pathways regulate cell growth and proliferation (28). Various molecules are associated with tumor development. For example, phosphorylation of AKT, activation of ERK1 and ERK2, and NF-κBp65 proteins lead to cancer cell development (24). In the present study, we ascertained whether a newly synthesized potential anticancer genistein analogue, DFOG, possesses inhibitory effects on p-AKT and p-ERK1/2 expression, and NF-κB activity, as well as on the suppression of oncogenicity *in vitro* in SKOV3-derived OVCSLCs. SKOV3-derived spheroids were therefore used to demonstrate the pharmacological effectiveness of the inhibitors. The other drugs, including LY294002, U0126 and PDTC, whose chemopreventive activities have been demonstrated in previous studies, were compared to DFOG (29). Notably, in the present study, we found that DFOG significantly reduced the levels of phosphorylated AKT. Combination of DFOG with another pharmacological inhibitor LY294002 led to a drastic decrease in p-AKT expression. In addition, the target drug was capable of downregulating phosphorylated ERK1 and

ERK2 proteins in the ERK1/2 signaling pathway. However, DFOG compared with another tumor inhibitor (U0126) was likely to be less effective in diminishing the activation of key proteins involved in cell growth and survival. Nevertheless, simultaneous application of the two anticancer agents resulted in a significant inhibition of phosphorylated ERK1 and ERK2 levels. Finally, the genistein analogue DFOG demonstrated a high potential to decrease the level of the NF-κBp65 protein, which is extremely important in chemopreventive therapies against cancer development. However, based on its antitumor potency, the novel genistein derivative DFOG is considered an agent with activity against different types of cancer (30).

The present study revealed the anticancer activity of DFOG in OVCSLCs. Cancer stem cells (CSCs) are known to maintain and facilitate the formation of cancers. In addition, CSCs drive drug resistance as well as recurrence or relapse (31). As ovarian cancer is resistant to conventional chemotherapies, a new approach to target CSCs is necessary. Compounds containing genistein can be applied against CSCs (32). Thus, in the present study, we determined how an anticancer analogue of genistein, DFOG, affects OVCSLCs. A study by Roy *et al* (33) demonstrated that repressing PI3K/AKT and MEK/ERK pathways

induces FOXO transcription factors. After transduction with the FOXO3a shRNA-expressing adenovirus, we found that the combination of DFOG and FOXO3a shRNA downregulated FoxO3a, synergistically attenuating spheroid and colony forming capabilities of SKOV3 cell-produced OVCSLCs. To integrate the abovementioned tight association of FoxO3a with OVCSLC oncogenicity inhibition related to AKT and/or ERK and/or NF- $\kappa$ B pathway suppression, our data showed that reduced OVCSLC oncogenicity *in vitro* due to the suppressive effects of DFOG on AKT and/or ERK and/or NF- $\kappa$ B pathways requires FoxO3a expression. These results support the viewpoint of Sunayama *et al* (1) that FoxO3a likely has an important function in controlling CSLCs via PI3K/AKT/mTOR and MEK/ERK signaling, thereby implying that tools effectively targeting FoxO3a induction may constitute a viable option for human carcinoma treatment.

McGovern *et al* (26) assessed FOXM1 function and modulation after gefitinib treatment, and demonstrated that gefitinib downregulates FOXM1 through FOXO3a in breast carcinoma. Our previous study demonstrated that DFOG acts as an inhibitor of CSCs or tumor-initiating cells by activating FoxO3a and/or inactivating FoxM1 (4). As demonstrated above, combining DFOG and FOXM1 shRNA reduced the expression levels of FoxM1, and synergistically attenuated the spheroid and colony forming capabilities of OVCSLCs from the SKOV3 cell line. These results indicated that reduced OVCSLC oncogenicity *in vitro* by DFOG through inhibitory effects on AKT and/or ERK and/or NF- $\kappa$ B pathways requires FoxM1 expression.

Overall, DFOG exerts anticancer activity by targeting OVCSLCs. DFOG may be effective in reducing the expression levels of cell regulatory proteins such as p-AKT, p-ERK1/2, and NF- $\kappa$ Bp65. Moreover, the novel genistein derivative prevented spheroid and colony formation in OVCSLCs. These findings provide evidence for DFOG potency in the inhibition of cancer progression. Importantly, the reduced OVCSLC oncogenicity *in vitro* by DFOG through inhibitory effects on AKT and/or ERK and/or NF- $\kappa$ B pathways requires both FoxO3a and FoxM1 expression. Therefore, DFOG may be used as a novel chemotherapeutic drug for ovarian carcinoma.

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