

FSH inhibits ovarian cancer cell apoptosis by up-regulating survivin and down-regulating PDCD6 and DR5

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Abstract

Ovarian epithelial cancer is the leading cause of death among gynecological malignancies. FSH may increase the risk of ovarian malignancy and play an important role in ovarian carcinogenesis. Our previous studies showed that FSH increases the expression of VEGF through survivin. In this study, the function and mechanism of FSH in ovarian cancer were further explored. We found that FSH promoted proliferation and prevented apoptosis of ovarian cancer cells by activating survivin through the SAPK/JNK and PI3K/AKT pathways. FSH also down-regulated the expression of programmed cell death gene 6 (PDCD6) and death receptor 5 (DR5), two molecules required for induction of apoptosis. RNA interference was applied to knock down survivin and PDCD6 expression, and we found that the blockage of survivin reversed the effects of FSH on apoptosis and proliferation, whereas knock down of PDCD6 enhanced these effects. The expression of DR5, cyclin D1, and cyclin E correlated with survivin expression, but PDCD6 did not. Using immunohistochemical staining, we further showed that ovarian serous cystadenocarcinoma samples had higher expression of survivin than did benign ovarian cystadenoma and borderline cystadenoma samples ($P < 0.01$). Furthermore, survivin expression in the ovarian serous cystadenocarcinoma specimens was correlated with disease stage ($P < 0.05$). Our results suggest that FSH promotes ovarian cancer development by regulating the expression of survivin, PDCD6, and DR5. Greater understanding of the molecular mechanisms of FSH in ovarian epithelial carcinogenesis and development will ultimately help in the development of a novel targeted therapy for ovarian cancer.

Endocrine-Related Cancer (2011) 18 13–26

Introduction

Currently, ovarian epithelial cancer is the leading killer among all gynecological malignancies (Ozols *et al.* 2004). Ovarian epithelial cancer is more common in individuals with elevated GnRH including FSH and LH, such as postmenopausal women or women who have received treatment to induce

ovulation (Venn *et al.* 1999, Brekelmans 2003). Conversely, reduced risk of ovarian cancer is associated with a history of multiple pregnancies, breastfeeding, oral contraceptive use, and estrogen replacement therapy, all of which are related to lower levels of and reduced exposure to FSH and LH (Gnagy *et al.* 2000).

Survivin, a newly identified member of the inhibitor of apoptosis protein family, is capable of regulating both cell proliferation and apoptosis. Survivin is regulated in a cell cycle-dependent manner; its expression is markedly increased in the G2/M phase and is associated with mitotic spindle microtubules, centromeres, and intracellular mid-bodies (Li *et al.* 1998). Survivin inhibits cell death induced by a variety of apoptotic stimuli, including Fas/Fas ligand (FasL), caspases, and anticancer drugs (Ling *et al.* 2004). Overexpression of survivin confers cytoprotection against a variety of apoptotic stimuli, whereas a decrease in survivin expression or function causes spontaneous apoptosis of cancer cells or sensitizes the cells to apoptotic stimuli (Altieri 2003a,b). Survivin is overexpressed in 60 cancer cell lines and most human tumor types, including lung, breast, stomach, liver, bladder, and ovarian cancers. Overexpression of survivin has also been associated with increased cancer metastasis and decreased patient survival (Altieri 2003a,b). Our previous studies indicated that FSH increases the expression of VEGF through survivin, which is activated by the PI3K/AKT signaling pathway (Huang *et al.* 2008).

Apoptosis is a cell-suicide mechanism that plays a crucial role in development and homeostasis. Members of the TNF family of cytokines, including FasL and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and their receptors, are critically involved in the apoptotic process. TRAIL can bind two apoptosis-inducing receptors, TRAIL-R1 (death receptor 4, or DR4) and TRAIL-R2 (death receptor 5, or DR5), to initiate the apoptotic machinery in cancer cells (Oldenhuis *et al.* 2008). Programmed cell death gene 6 (PDCD6), also known as apoptosis-linked gene-2 (ALG-2), encodes a 22 kDa Ca^{2+} -binding protein that is required for programmed cell death in response to various apoptotic agents, including FasL (Vito *et al.* 1996).

In this study, we investigated the molecular mechanism by which FSH promoted ovarian cancer cell growth and prevented apoptosis in cell culture and in mouse xenografts. Our data indicated that coordinate regulation of survivin, cyclin D1, and cyclin E contributed to cell cycle progression and proliferation, whereas down-regulation of PDCD6 and DR5 expression appeared to play a role in controlling apoptosis. Importantly, the FSH-induced down-regulation of DR5 expression was mediated through survivin, implicating a novel role for survivin in regulating apoptosis. Furthermore, we examined the expression of survivin in ovarian tumor tissues and found that survivin was overexpressed in ovarian serous cystadenocarcinoma. Hence, survivin could be a new target for the treatment of ovarian cancer.

Materials and methods

Ovarian cancer cell lines and human tissue samples

The ovarian serous cancer cell line, SKOV-3, and ovarian clear-cell cancer cell line, ES-2, were obtained from the American Type Culture Collection (Manassas, VA, USA). Both cell lines were cultured in RPMI 1640 media (Gibco BRL) containing 10% FCS (Sijiqing Co., Shanghai, China) in 5% CO_2 at 37 °C.

Sixty-five samples of paraffin-embedded ovarian tumor tissue (15 ovarian benign serous cystadenomas, 13 borderline ovarian serous cystadenomas, 25 ovarian serous cystadenocarcinomas, and 12 ovarian clear-cell carcinomas) were collected from patients who had undergone surgery from January 2001 to December 2002 in the Obstetrics and Gynecology Hospital of Fudan University in Shanghai, China. None of the patients had a history of other neoplasms or had undergone radiotherapy, chemotherapy, hormone replacement therapy, immunotherapy, or any other therapy before surgery. In addition, clinical characteristics, such as age, disease stage, and tumor grade, were considered for enrollment. The histological diagnosis and tumor grade were based on the 1986 International Federation of Gynecology and Obstetrics classification system. All of the tissue samples were obtained with the patients' informed consent using protocols and procedures approved by the Institutional Review Board at the Obstetrics and Gynecology Hospital of Fudan University.

Reagents and preparations

FSH, the SAPK/JNK inhibitor SP600125, the PI3K/AKT inhibitor LY294002, 5-bromo-20-deoxyuridine (BrdU), and an anti-BrdU antibody were purchased from Sigma Chemical Co.; Opti-MEM (a modification of Eagle's minimum essential media) was purchased from Gibco BRL; a GAPDH monoclonal antibody and anti-rabbit and anti-mouse IgGs conjugated with HRP were purchased from Kangchen Bioengineering Corporation (Shanghai, China); an anti-pJUN (Ser73) antibody, an anti-cJUN antibody, an anti-pAKT (Ser473) antibody, an anti-AKT antibody, an anti-pCREB (Ser133) antibody, an anti-CREB antibody, an anti-pI κ B (Ser32) antibody, an anti-I κ B antibody, and an anti-DR5 antibody were purchased from Cell Signaling Technology (Danvers, MA, USA); an anti-survivin monoclonal antibody was purchased from Abcam, Inc. (Cambridge, MA, USA); fluorescence-labeled goat anti-mouse IgGs and 4',6-diamidino-2-phenylindole (DAPI) were purchased

from Molecular Probes (Eugene, OR, USA); anti-cyclin D1 and anti-cyclin E antibodies were purchased from Neomarker (Fremont, CA, USA); an anti-DR4 antibody was purchased from Epitomics, Inc. (Burlingame, CA, USA); an anti-PDCD6 antibody was purchased from Proteintech Group, Inc. (Chicago, IL, USA); MTT assay kit was purchased from Biotium, Inc. (Hayward, CA, USA). Lipofectamine 2000 transfection reagent was purchased from Invitrogen, and the Annexin V-FITC apoptosis detection kit I was purchased from BD Pharmingen (San Diego, CA, USA).

FSH stimulation

SKOV-3 and ES-2 cells were plated at 4×10^4 or 4×10^5 and 1×10^4 or 1×10^5 cells per well respectively onto 96-well or 6-well plates. Twenty-four hours after plating, RPMI 1640 without serum was replaced, and the cells were serum-starved for 18 h. The cells were then stimulated with FSH at 40 mIU/ml for different time periods (up to 120 min for signaling or up to 24 h for protein expression); PBS was used as a control. Transfected cells (described in the transfection section below) were also starved for 18 h and then stimulated with FSH at 40 mIU/ml for another 24 h. The cells were then harvested, and the proteins were extracted for western blot analysis.

Cell proliferation and DNA synthesis analyses

Cell proliferation and DNA synthesis were evaluated by MTT assay and BrdU incorporation respectively. After FSH stimulation, 10 μ l MTT solution was added to each well. The cell culture medium and MTT solution were mixed by gently tapping the side of the tray or shaking it briefly on an orbital shaker. The cells were incubated at 37 °C for 4 h. The medium was then removed, and 200 μ l dimethyl sulfoxide was added to each well to dissolve the formazan, which was aided by pipetting the liquid up and down several times. To obtain the sample signal, absorbance was measured on an ELISA plate reader with a test wavelength of 570 nm and a reference wavelength of 630 nm. After FSH stimulation, 20 μ M BrdU was added, and the cells were incubated at 37 °C for 1 h. Cells were then fixed in 3.5% paraformaldehyde for 30 min at 4 °C, washed, and treated with 2 M HCl for 1 h at 37 °C. Immediately after the acid wash, 0.1 M borate buffer was added to the cells for 30 min at room temperature. The cells were then treated with 0.1% Triton X-100 for 2 min at room temperature and incubated in 10% normal goat serum for 1 h before being incubated overnight at 4 °C with an anti-BrdU antibody. Finally, the cells were treated with fluorescence-labeled goat anti-mouse IgG

at room temperature for 2 h. PBS was used as a negative control. DAPI was used for staining nuclei specifically. The mean percentage of cells positive for BrdU was determined in at least ten random fields at a magnification of 400 \times under an Olympus DX51 fluorescence microscope (Japan).

Cell cycle and apoptosis analyses

Cell cycle distribution was determined by measuring the cellular DNA content by flow cytometry. Cells were trypsinized, washed in PBS, fixed in 70% ice-cold ethanol at 4 °C overnight, washed with PBS again, and stained with 100 ml of 50 mg/l propidium iodide at 37 °C for 30 min. Annexin V-FITC apoptosis detection kit I was used to identify apoptotic and viable cells following the manufacturer's instructions. The percentage of early apoptotic (FITC-positive and propidium iodide-negative) cells was calculated from the data generated by flow cytometry.

Western blot analysis

Cell lysates were collected from cultures and quantified using the BCA (Ji Kai Co., Shanghai, China) method. Following 10, 12, and 15% SDS gel electrophoresis, 30 μ g protein lysates was separated from the gel and transferred to a nitrocellulose filter. The membranes were sealed with PBS containing 5% defatted milk for 1 h at room temperature, sealed with a primary antibody overnight at 4 °C, and mixed with a secondary antibody for 1 h the next day at 37 °C; GAPDH was used as a loading control. The protein bands were visualized with the Amersham ECL Plus system (GE Healthcare, Piscataway, NJ, USA). Images were scanned by Hewlett-Packard 5200C (China) with HP PrecisionScan software v2.02 and quantified using NIH image Version 1.61.

Transfection assay for RNAi

We purchased the cJUN siRNAs, J1 and J2, and negative siRNA (neg-siRNA) from Cell Signaling Technology. The following survivin-specific RNAi plasmids were obtained: pR-suv1 (5'-GCATCTCTA-CATTCAAGAA-3'), pR-suv2 (5'-GAAGCAGTTT-GAAGAATTA-3'), and pR-suv3 (5'-AGGAAACC-AACAATAAGAA-3'); the following PDCD6-specific RNAi plasmids were obtained: pR-pd1 (5'-GACAG-GAGUGGAGUGUAU-3'), pR-pd2 (5'-GGAGTG-GAGTGATATCAGA-3'), pR-pd3 (5'-GTTCCACGA-CATCCTCATT-3'), and an RNAi negative plasmid, pR-neg (5'-TTCTCCGAACGTGTACGT-3'). These constructs were purchased from Kangchen.

Transfection of plasmids (pR-suv1, pR-suv2, pR-suv3, pR-pd1, pR-pd2, pR-pd3, and pR-neg) or siRNA (cJUN siRNAJ1, siRNAJ2, and neg-siRNA) was performed with Lipofectamine 2000 following the manufacturer's instructions. PBS and non-targeting siRNA (pR-neg or neg-siRNA) were used as controls. Briefly, the day before transfection, 4×10^5 SKOV-3 cells or 1×10^5 ES-2 cells per well were plated onto 6-well plates and grown for 1 day to reach 80–85% confluence. The shRNA vector (5 μ g) and Lipofectamine 2000 were each diluted in 250 μ l of serum-free Opti-MEM and incubated for 5 min at room temperature, then combined at a ratio of 1:2. Two milliliters of this combination were added to each well. The cells were then incubated for 48 h, with or without FSH stimulation, and harvested for western blot analysis.

Animal tissue array

Using a Beecher Instruments tissue array device (Silver Spring, MD, USA), animal tissue microarrays were constructed from core biopsies of mouse-transplanted tumors arising from SKOV-3 and ES-2 cells. Each array contained 36 samples of paraffin-embedded tissue consisting of three groups of transplanted tumors that had been treated with low-dose FSH (3 IU/day), high-dose FSH (10 IU/day), or physiological saline. For each tissue sample, two tissue cylinders 1.5 mm in diameter were taken from each block.

Immunohistochemical staining for survivin and DR5

Survivin expression and DR5 expression were detected in the animal tissue array sections. Survivin expression was also investigated in 65 samples of ovarian tissue. Tissue sections were deparaffinized, rehydrated, and subjected to antigen retrieval by heating in target antigen retrieval solution. Polyclonal antibodies against human survivin (1:100) and DR5 (1:500) were used for immunohistochemical detection. Immunoreactive sites were visualized using Envision kits (DAKO), and sections were counterstained with hematoxylin. The negative controls were performed by omitting the primary antibody or using isotype-matched IgG.

Immunostaining was reviewed by two independent evaluators. Survivin staining was mainly localized in the cytoplasm, whereas DR5 was concentrated in the nucleus. Immunohistochemical reactivity for survivin and DR5 was graded in a semi-quantitative manner according to the percentage of positive tumor cells (0, 0%; 1, <20%; 2, 20–50%; 3, >50%) and the intensity of staining (0, none; 1, weak; 2, moderate; 3, strong).

A combined grade of immunohistochemical positivity (0, negative; 2–4, weak positive; 5–6, strong positive) was calculated for each tumor by adding the individual grades for percentage of tumor cells (0–3) and intensity of staining (0–3).

In vivo experiments

Female athymic Balb/c nude mice (Shanghai Cancer Institute, China) aged 4–6 weeks were used in the ovarian tumor xenograft model. The nude animals were housed individually in ventilated cages and kept isolated from normal mice. Exponentially growing SKOV-3 or ES-2 cells were subcutaneously injected into the right flank of mice (5×10^6 cells/mouse). After injection, the nude mice were divided into three groups; each group of eight to ten mice was treated with low-dose FSH (3 IU/day), high-dose FSH (10 IU/day), or physiological saline. The weights of the nude mice were recorded every 3 days. After 30 days of treatment, mice were euthanized, and the serum FSH concentration and the weight of tumors were determined. All experiments were approved by the ethics committee for Animal Experimentation of the Institute of Shanghai Tumor Institute according to institutional guidelines.

Statistical analysis

Results for all the experiments were analyzed using χ^2 analysis or a Student's *t*-test (χ^2 analysis for positive rate comparison and Student's *t*-test for the other experiments). The means and s.d.s from at least three independent experiments are calculated. Differences were considered significant at $P < 0.05$. The 5-year survival curve was analyzed using the Kaplan–Meier product-limit method. SPSS 11.0 software (SPSS, Inc., Chicago, IL, USA) was used to perform statistical analyses. The half maximal effective concentration (EC50) was calculated by Hill equation.

Results

FSH stimulates ovarian cancer cell proliferation *in vitro* and *in vivo*

To verify the effect of FSH on cancer cell growth, DNA synthesis and cell proliferation were assessed using MTT assays and BrdU incorporation. As shown in Fig. 1A, the MTT values in SKOV-3 cells significantly increased with FSH stimulation and reached a peak at 20 mIU/ml. ES-2 cells are very sensitive to FSH stimulation; the MTT values in ES-2 cells significantly increased upon FSH stimulation, reaching a peak at

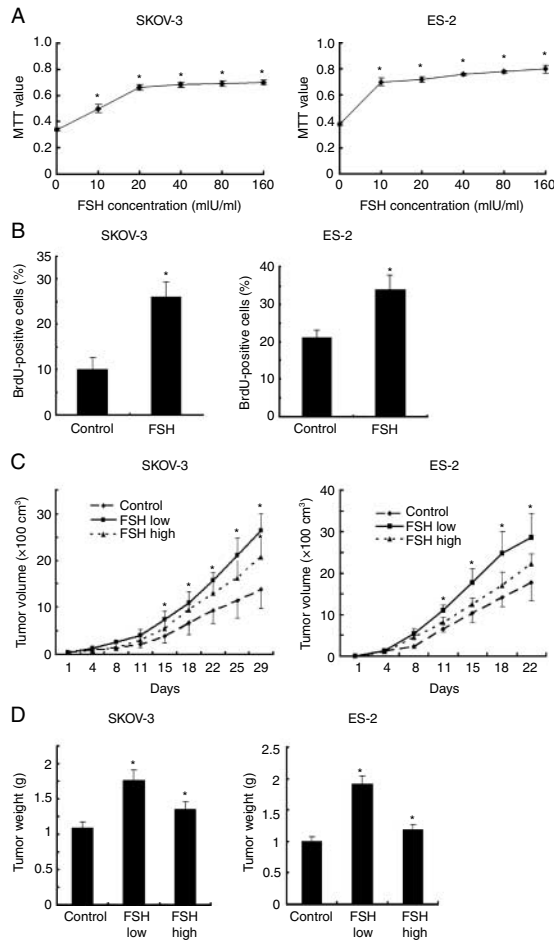


Figure 1 FSH stimulated ovarian cancer cell proliferation *in vitro* and *in vivo*. (A) FSH stimulated proliferation of SKOV-3 and ES-2 cells in MTT assays. * $P < 0.05$, FSH-treated versus control. (B) Effect of FSH on BrdU incorporation in SKOV-3 and ES-2 cells. BrdU incorporation was measured by co-staining with DAPI and BrdU in SKOV-3 and ES-2 cells. Data represent the mean \pm s.d. of positive cells from three independent experiments. Each experiment was performed in triplicate. * $P < 0.05$, FSH-treated versus control. (C) The growth curve of SKOV-3 and ES-2 xenograft tumors treated with low-dose FSH (3 IU/day) or high-dose FSH (10 IU/day). * $P < 0.05$, FSH-treated versus control, data represent the mean \pm s.d. of eight to ten mice per group. (D) The weights of SKOV-3 and ES-2 xenograft tumors treated with low-dose FSH (3 IU/day) or high-dose FSH (10 IU/day). * $P < 0.05$, FSH-treated versus control (eight to ten mice per group).

10 mIU/ml. The EC₅₀ is 17.04 mIU/ml for SKOV-3 cells, and EC₅₀ is < 10 mIU/ml for ES-2 cells. As we reported before (Huang *et al.* 2008), the stimulation conditions of 40 mIU/ml FSH produced the most obvious effects; we used this dose to conduct further experiments for evaluating FSH-stimulated effects in SKOV-3 and ES-2 cells.

In agreement with the MTT assay, FSH also significantly increased BrdU incorporation in SKOV-3

and ES-2 cells compared with control cells (Fig. 1B). After 48 h of 40 mIU/ml of FSH stimulation, $26 \pm 3.38\%$ (mean \pm s.d.) of SKOV-3 cells were BrdU-positive, whereas $10 \pm 2.75\%$ of SKOV-3 cells incorporated BrdU without FSH stimulation. In ES-2 cells, $34 \pm 3.74\%$ of cells were BrdU-positive after 40 mIU/ml of FSH stimulation for 24 h, whereas $21 \pm 2.13\%$ of ES-2 cells incorporated BrdU without FSH stimulation. These results indicate that FSH promotes proliferation in SKOV-3 and ES-2 cells.

To determine whether FSH affected ovarian tumor growth *in vivo*, SKOV-3 or ES-2 cells were subcutaneously injected into nude mice. The nude mice were treated with low-dose FSH (3 IU/day), high-dose FSH

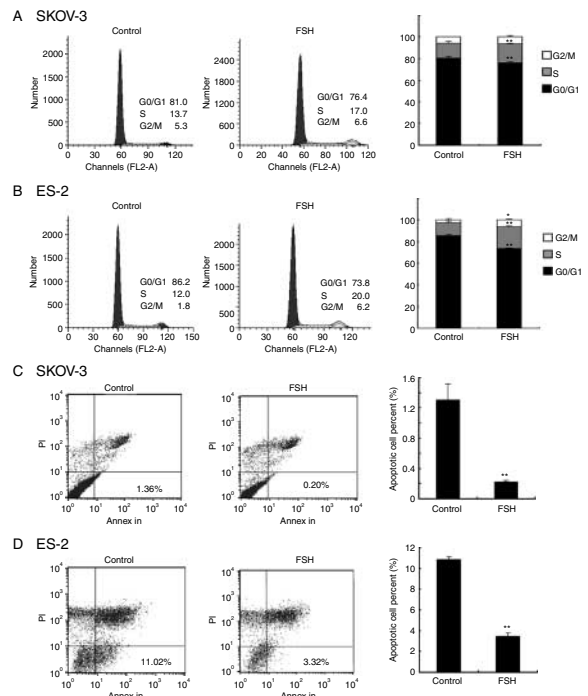


Figure 2 FSH regulated cell cycle progression and suppressed apoptosis. Cell cycle distribution in SKOV-3 (A) and ES-2 (B) cells with or without FSH treatment. The cells were stimulated with 40 mIU/ml of FSH for 48 h in SKOV-3 cells or 24 h in ES-2 cells; PBS was used as a control. Cell cycle distribution was determined by measuring the cellular DNA content by flow cytometry. Histograms show a representative analysis of cell cycle distribution, and bar graphs represent the quantitative analyses from three independent experiments. Each experiment was performed in triplicate. ** $P < 0.01$ or * $P < 0.05$, FSH-treated versus control. FSH suppressed cell apoptosis in SKOV-3 (C) and ES-2 (D) cells. The cells were stimulated with 40 mIU/ml of FSH for 48 h in SKOV-3 cells or 24 h in ES-2 cells; PBS was used as a control. Annexin V-FITC staining was used to detect apoptotic SKOV-3 or ES-2 cells. Flow plots show a representative analysis of FITC-positive cells, and bar graphs represent the quantitative analyses from three independent experiments. Each experiment was performed in triplicate. ** $P < 0.01$, FSH-treated versus control.

(10 IU/day), or physiological saline. The weights of FSH-treated and control mice were not statistically different, but xenograft tumor growth was significantly greater in animals treated with the low dose of FSH compared with the untreated group (Fig. 1C). The serum FSH levels in the SKOV-3 xenograft tumors after treatment with low- or high-dose FSH were 59.56 ± 19.80 and 237.66 ± 26.20 mIU/ml respectively, and these were higher than FSH levels observed without FSH treatment (0.40 ± 0.64 mIU/ml; $P < 0.001$). The serum FSH levels in the ES-2 xenograft tumors treated with low- or high-dose FSH were 58.82 ± 13.00 or 242.98 ± 27.66 mIU/ml respectively, both of which were greater than those observed without FSH treatment (0.00 ± 0.00 mIU/ml; $P < 0.001$). The weights of SKOV-3 xenograft tumors in the low- or high-dose FSH groups were 1.41 ± 0.12 and 1.08 ± 0.09 g respectively, both of which were greater than those observed in the control group (0.87 ± 0.07 g; $P < 0.05$; Fig. 1D). The weights of ES-2 xenograft tumors in the low- or high-dose FSH groups were 1.92 ± 0.12 and 1.19 ± 0.08 g respectively, both of which were greater than those observed in the control group (1.00 ± 0.08 g; $P < 0.05$; Fig. 1D).

FSH regulates the cell cycle and suppresses apoptosis in ovarian cancer cells

To investigate the effect of FSH in regulating cell proliferation, we examined cell cycle progression using flow cytometry (Fig. 2). The cell distribution at the G0/G1 phase with FSH treatment was $76.5 \pm 0.9\%$ in SKOV-3 cells and $73.3 \pm 0.9\%$ in ES-2 cells, which was statistically lower than that of the control group ($80.8 \pm 1.6\%$ in SKOV-3 cells and $85.6 \pm 1.2\%$ in ES-2 cells) ($P < 0.01$). The decrease in G0/G1 phase was accompanied by an increase in cells entering S phase; the cell distribution in the S phase with FSH treatment was $17.2 \pm 0.3\%$ in SKOV-3 cells and $20.1 \pm 1.4\%$ in ES-2 cells, which was statistically higher than that of the control group ($13.6 \pm 1.5\%$ in SKOV-3 cells and $11.9 \pm 1.1\%$ in ES-2 cells) ($P < 0.01$; Fig. 2A and B).

In addition to cell cycle regulation, FSH treatment resulted in a decrease in apoptosis in SKOV-3 and ES-2 cells, as detected by Annexin V-FITC staining. The percentage of cells undergoing apoptosis with FSH treatment was $0.22 \pm 0.02\%$ in SKOV-3 cells and $3.43 \pm 0.35\%$ in ES-2 cells, which was statistically

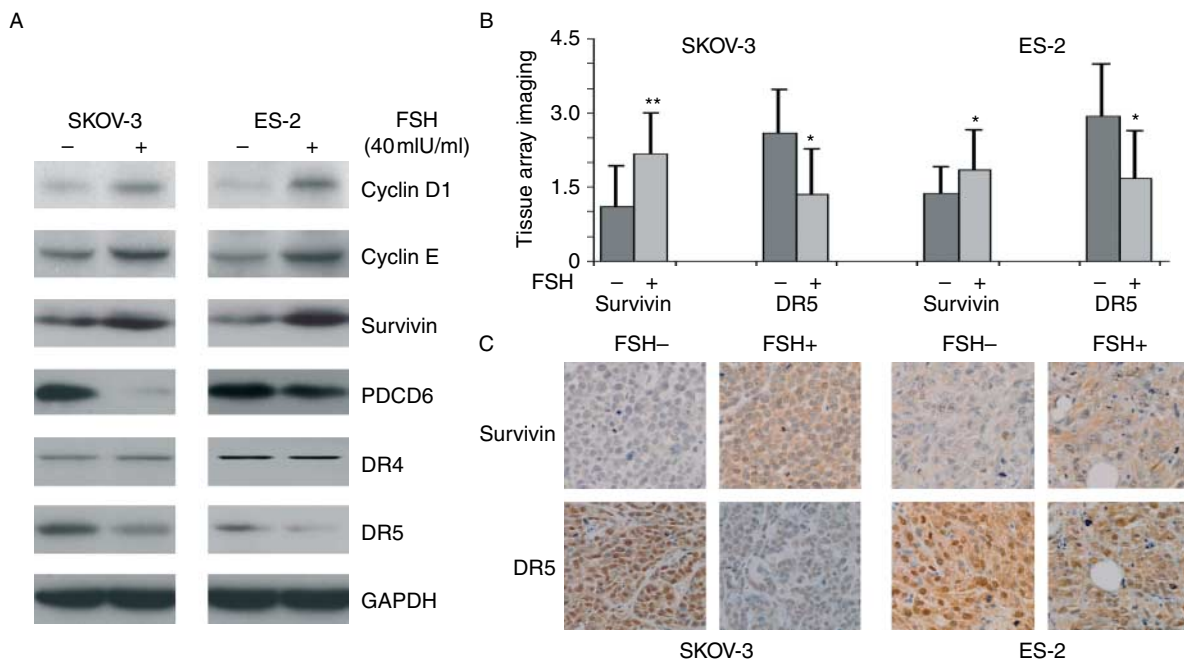


Figure 3 FSH increased the expression of survivin and decreased the expression of PDCD6 and DR5 *in vitro* and *in vivo*. (A) SKOV-3 and ES-2 cells were stimulated with 40 mIU/ml of FSH for 48 h in SKOV-3 cells or 24 h in ES-2 cells; PBS was used as a control. Cyclin D1, cyclin E, survivin, DR4, DR5, and PDCD6 were detected by western blot. (B) Survivin and DR5 expression in SKOV-3 and ES-2 xenograft tumors with and without FSH treatment. Sections from the animal xenograft tissue array were examined by immunohistochemical staining, and semi-quantitative analysis was performed as described in the Materials and methods. ** $P < 0.01$ or * $P < 0.05$, FSH-treated versus untreated, data represent the mean \pm s.d. of eight to ten mice per group. (C) Representative images of survivin and DR5 expression in SKOV-3 and ES-2 xenograft tumors with and without FSH treatment.

less than that of the control group ($1.30 \pm 0.10\%$ in SKOV-3 cells and $10.84 \pm 0.25\%$ in ES-2 cells) ($P < 0.01$; Fig. 2C and D).

FSH increases expression of survivin, cyclin D1, and cyclin E and decreases expression of PDCD6 and DR5

To elucidate the mechanism of FSH-enhanced cell growth in ovarian cancer cells, we measured the expression of cyclin D1, cyclin E, and survivin, three important molecules involved in cell proliferation. Western blot analysis revealed an increase in cyclin D1, cyclin E, and survivin expression after 40 mIU/ml of FSH treatment (Fig. 3A and Supplementary Figure 1, see section on supplementary data given at the end of this article). Because FSH inhibited apoptosis in ovarian cancer cells, we also examined whether FSH controlled the expression of apoptosis-related molecules. We found that FSH administration decreased the expression of PDCD6 and DR5, which mediate apoptosis through the FasL and TRAIL pathways respectively. In contrast, the expression of DR4, another TRAIL death receptor, was not changed (Fig. 3A and Supplementary Figure 1, see section on supplementary data given at the end of this article).

Using animal xenograft tissue arrays, we validated our observations *in vivo* and found that the tumors from the FSH-treated groups had higher expression of survivin and lower expression of DR5 compared with the tumors from the untreated group. Although the changes are modest (Fig. 3B), we got statistical difference between FSH-treated groups versus untreated groups (each group contained eight to ten mice), thus further confirming the interaction of FSH–survivin–DR5 (Fig. 3B and C).

FSH regulation of survivin and PDCD6 is necessary for its effects on proliferation and apoptosis

As shown in Fig. 4A and B, and Supplementary Figure 2, see section on supplementary data given at the end of this article, transfection with pR-suv2, one of the three survivin RNAi constructs, selectively silenced the expression of survivin; whereas transfection with the PDCD6 RNAi constructs pR-pd1 or pR-pd2 silenced the expression of PDCD6 effectively. Both pR-suv2 and pR-pd1 were then used to specifically knock down the expression of survivin and PDCD6 respectively. Western blot analyses showed that knock down of survivin attenuated the FSH-induced increase in survivin, whereas a blockade of PDCD6 further inhibited the expression of PDCD6

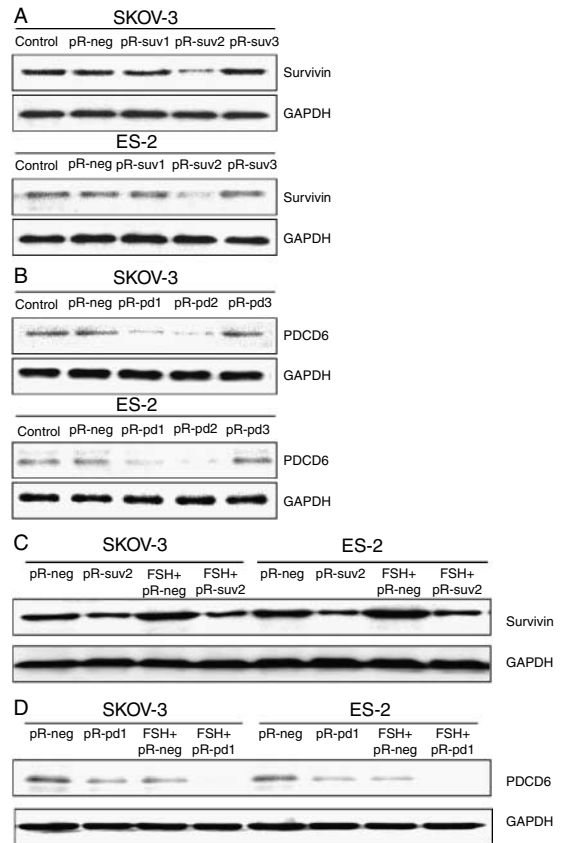


Figure 4 Selective silencing of survivin and PDCD6 expression in SKOV-3 and ES-2 cells by RNAi. (A and B) Different constructs of survivin and PDCD6 RNAi were verified. Transfections were performed with reagent only (control), a negative control plasmid (pR-neg), recombinant plasmids of survivin RNAi (pR-suv1, pR-suv2, and pR-suv3), and recombinant plasmids of PDCD6 RNAi (pR-pd1, pR-pd2, and pR-pd3). Expression of survivin and PDCD6 was determined by western blot. GAPDH was used as a loading control. (C and D) RNAi blocked FSH effects on survivin and PDCD6 expression. The plasmids pR-suv2 and pR-pd1 were selected to knock down the expression of survivin and PDCD6 respectively. After transfection, cells were treated with or without FSH. Transfection with pR-neg was used as a negative control. GAPDH was used as a loading control.

induced by FSH (Fig. 4C and D, and Supplementary Figure 2, see section on supplementary data given at the end of this article).

We then investigated whether survivin RNAi and PDCD6 RNAi could block the effects of FSH on proliferation and apoptosis. As shown in Fig. 5, survivin RNAi reversed the effects seen with FSH treatment in SKOV-3 and ES-2 cells, with cells exhibiting increased apoptosis and decreased proliferation ($P < 0.05$; Fig. 5A and B, and Supplementary Figure 3, see section on supplementary data given at the end of this article). Conversely, PDCD6 RNAi

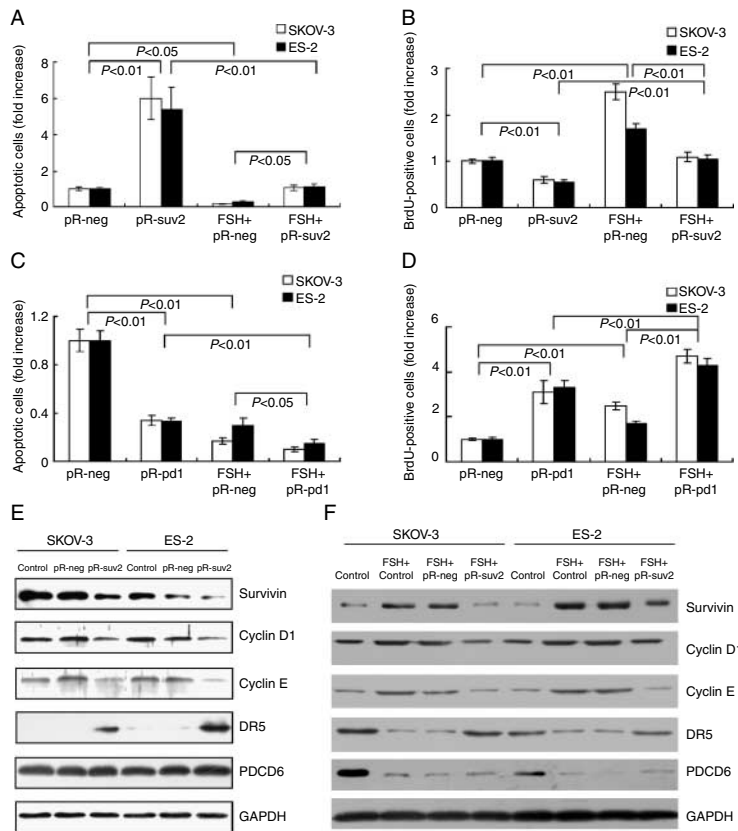


Figure 5 Survivin or PDCD6 RNAi blocked FSH effects on cell proliferation and apoptosis. (A and C) Survivin or PDCD6 RNAi blocked FSH effects on apoptosis. The plasmids pR-suv2 and pR-pd1 were selected to knock down the expression of survivin and PDCD6 respectively. After transfection, cells were treated with or without FSH, stained with Annexin V-FITC, and analyzed by flow cytometry. Transfection with pR-neg was used as a negative control. $P < 0.05$ or $P < 0.01$. (B and D) Survivin or PDCD6 RNAi blocked FSH effects on cell proliferation. The cells were treated as in (A) and (C). Percentages of BrdU-positive cells were calculated based on counts in at least ten random fields at $400\times$ magnification. $P < 0.05$ or $P < 0.01$. (E) The expression of several cell cycle regulators and death receptors was altered by treatment with survivin RNAi. SKOV-3 and ES-2 cells were transfected with pR-suv2, and transfection with pR-neg was used as a negative control. Cyclin D1, cyclin E, survivin, DR5, and PDCD6 were detected by western blot. GAPDH was used as a loading control. (F) Survivin RNAi blocked FSH effects on cell cycle regulator and death receptor expression. SKOV-3 and ES-2 cells were transfected with pR-suv2 and treated with FSH. Transfection with pR-neg was used as a negative control. Cyclin D1, cyclin E, survivin, DR5, and PDCD6 were detected by western blot. GAPDH was used as a loading control.

enhanced FSH effects, resulted in decreased rates of apoptosis, and increased proliferation in SKOV-3 and ES-2 cells ($P < 0.05$; Fig. 5C and D, and Supplementary Figure 3, see section on supplementary data given at the end of this article).

Survivin regulates the expression of cyclin D1, cyclin E, and DR5

To understand the role of survivin in regulating gene expression during FSH stimulation, we used RNAi to knock down survivin expression in SKOV-3 and ES-2 cells. We observed that transfection of SKOV-3 and ES-2 cells with pRNAT-suv2 specifically down-regulated survivin expression and also

down-regulated cyclin D1 and cyclin E expression. When survivin was down-regulated, DR5 expression was increased, but PDCD6 expression was not affected (Fig. 5E and Supplementary Figure 3, see section on supplementary data given at the end of this article). After stimulation with FSH, survivin siRNA blocked the FSH-induced increase in cyclin D1 and cyclin E and the FSH-induced decrease in DR5, whereas it had no effect on the FSH-induced decrease in PDCD6 (Fig. 5F and Supplementary Figure 3, see section on supplementary data given at the end of this article). These data suggest that FSH stimulates survivin expression and plays a major role in regulating cyclin D1, cyclin E, and DR5.

FSH stimulates survivin and inhibits PDCD6 through the PI3K/AKT and SAPK/JNK signal transduction pathways

We found that FSH stimulation of SKOV-3 and ES-2 cells significantly increased the expression of pAKT and pJUN, but had no effect on pCREB (PKA downstream messenger) or pI κ B (NF- κ B downstream messenger) (Fig. 6A and B, and Supplementary Figure 4, see section on supplementary data given at the end of this article). To confirm that both signal transduction pathways were involved, we pretreated SKOV-3 or ES-2 cells with the pAKT inhibitor, Ly294002, or the pJUN inhibitor, Sp600125, for 30 min; FSH was then added for 48 h in SKOV-3 cells or 24 h in ES-2 cells. Survivin expression was not changed by Ly294002 or Sp600125 treatment only, but markedly declined by Ly294002 or Sp600125 pretreatment followed by FSH (Fig. 6C and D, and

Supplementary Figure 4, see section on supplementary data given at the end of this article). These data indicate that FSH up-regulates survivin and decreases PDCD6 expression by activating the SAPK/JNK and PI3K/AKT pathways in SKOV-3 and ES-2 cells. Moreover, cJUN siRNA blocked the expression of cJUN and pJUN and reversed the effects of FSH on survivin and PDCD6 expression in SKOV-3 cells and ES-2 cells (Fig. 6E and F, and Supplementary Figure 4, see section on supplementary data given at the end of this article).

Survivin is overexpressed in human ovarian cancer tissues

To investigate the clinical significance of survivin expression in ovarian cancer, we examined its expression in ovarian serous cystadenoma, ovarian

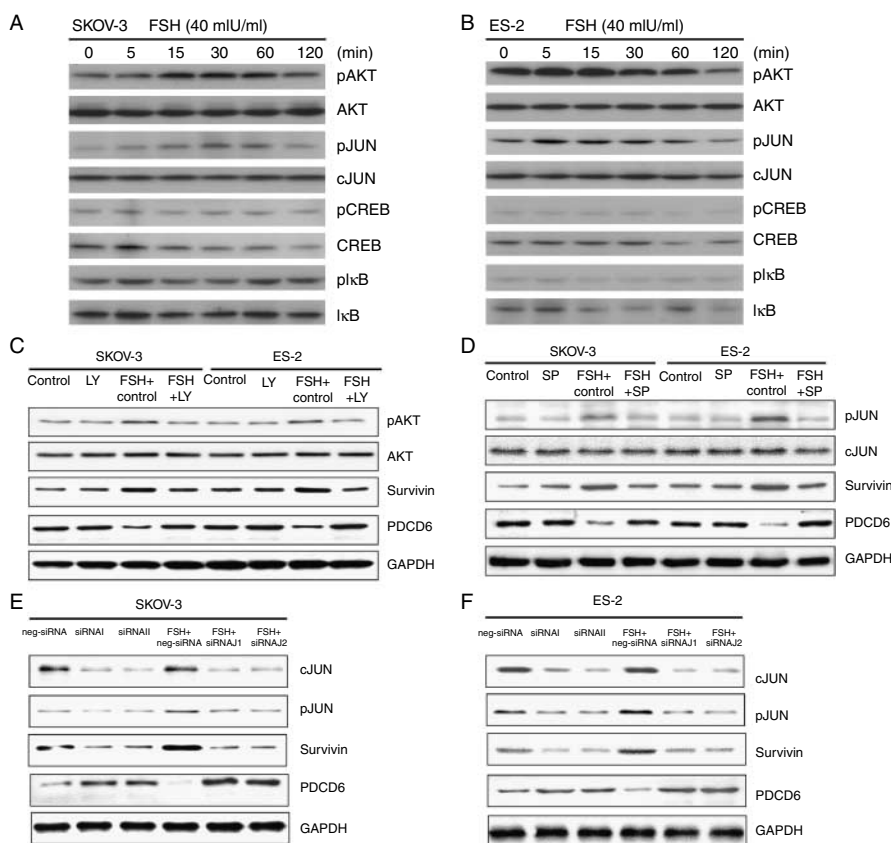


Figure 6 FSH stimulated survivin expression through the PI3K/AKT and SAPK/JNK signal transduction pathways. FSH stimulated pAKT and pJUN expression in (A) SKOV-3 cells and (B) ES-2 cells with different kinetics but did not affect pCREB and pI κ B expression. The molecules pAKT, AKT, pJUN, cJUN, pCREB, CREB, pI κ B, and I κ B were detected by western blot. (C and D) The PI3K/AKT inhibitor LY294002 (LY) and the SAPK/JNK inhibitor SP600125 (SP) reversed the effects of FSH on survivin and PDCD6 expression in SKOV-3 and ES-2 cells. The molecules pAKT, AKT, pJUN, cJUN, survivin, and PDCD6 were detected by western blot. GAPDH was used as a loading control. (E and F) siRNA of cJUN (siRNAJ1 and siRNAJ2) blocked the expression of cJUN and pJUN, and reversed the effects of FSH on survivin and PDCD6 expression in SKOV-3 and ES-2 cells. Transfection with negative siRNA (neg-siRNA) was used as a negative control. The molecules cJUN, pJUN, survivin, and PDCD6 were detected by western blot. GAPDH was used as a loading control.

borderline serous cystadenoma, ovarian clear-cell carcinoma, and ovarian serous cystadenocarcinoma samples using immunohistochemistry. Our results showed that 33.3% of benign cystadenomas and 46.2% of borderline cystadenomas were positive for survivin expression, but cystadenocarcinoma and clear-cell carcinoma had much higher positive rates at 72.0 and 66.7% respectively ($P < 0.01$; Table 1, Fig. 7A). When compared with cystadenoma samples, cystadenocarcinoma and clear-cell carcinoma samples had much higher expression levels of survivin (Fig. 7B).

In addition, survivin expression levels were higher in stage III–IV tumors than in stage I–II tumors among the serous cystadenocarcinoma specimens ($P < 0.05$; Table 1). However, survivin expression was not correlated with other clinical pathological parameters, such as age, tumor grade, and survival duration in the ovarian serous cystadenocarcinoma specimens (Table 1).

Discussion

The observations that FSH increases the risk of ovarian malignancy and that pregnancies or oral contraceptives protect the ovaries by suppressing FSH secretion led to numerous studies (Brekelmans 2003), including our previous study (Huang et al. 2008) that examined the role of FSH in ovarian carcinogenesis. Collectively, these studies demonstrated that FSH stimulates the growth of normal and immortalized ovarian surface

epithelium (OSE) and ovarian cancer cell lines in a dose- and time-dependent manner (Feng et al. 1996, Zheng et al. 2000, Ji et al. 2004). However, the exact role and mechanism that FSH plays in ovarian cancer development remain unclear. We have recently reported that FSH increases the expression of VEGF through survivin, which is activated by the PI3K/AKT signaling pathway (Huang et al. 2008), suggesting a potential molecular mechanism by which FSH controls tumorigenesis. In the present study, we further demonstrated that FSH promotes ovarian cancer proliferation and suppresses cell apoptosis by upregulating survivin and down-regulating the expression of PCDC6 and DR5, which control cell proliferation and apoptosis respectively. In animal models, FSH also promotes xenografts' tumor growth, increases the expression of survivin, and decreases the expression of DR5, further confirming the observation *in vitro*. The treatment of the mice with lower dose of FSH led to a more significant effect than the treatment with higher dose; the mechanism is not clear yet, it may be related to endogenous hormone secretion and interaction. As we know, different doses of hormone administration may have different effects; further experiments are needed to study FSH dynamics and regulation *in vivo*.

Survivin regulates spindle microtubule formation during mitosis, inhibits apoptosis, and promotes proliferation (Uren et al. 2000, Ai et al. 2006).

Table 1 Immunohistochemical staining of survivin in ovarian tumor tissue samples

	Number of samples			Positive rate (%)	P value
	Total	Survivin–	Survivin+		
Tissue type					
Benign	15	10	5	33.3	
Borderline	13	7	6	46.2	> 0.05 ^a
Cystadenocarcinoma	25	7	18	72.0	< 0.01 ^a
Clear cell carcinoma	12	4	8	66.7	< 0.01 ^a
Patient age (years) ^b					> 0.05
≤ 50	13	2	11	84.6	
> 50	12	1	11	91.7	
Tumor grade ^b					> 0.05
1	2	0	2	100.0	
2–3	23	5	18	78.3	
Disease stage ^b					< 0.05
I–II	7	3	4	57.1	
III–IV	18	2	16	88.9	
Survival duration ^b					
≤ 2 years	5	1	4	80.0	> 0.05 ^c
2–5 years	6	1	5	83.3	> 0.05 ^c
≥ 5 years	14	2	12	85.7	

^a χ^2 analysis, P value comparing with benign cystadenoma.

^bComparing with ≥ 5 years of survival duration.

^cOnly patients from whom the cystadenocarcinoma samples were obtained ($n=25$).

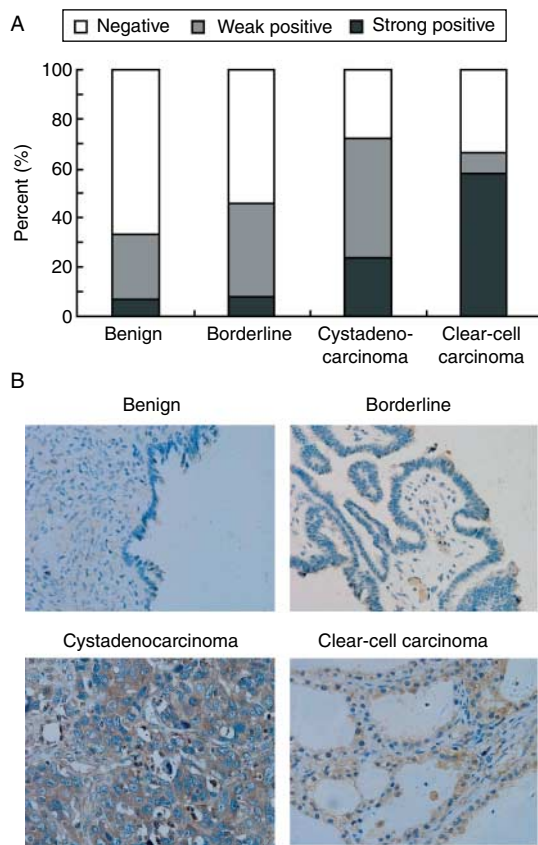


Figure 7 Survivin is overexpressed in ovarian cancer samples. (A) Positive rate of four groups (benign cystadenoma, borderline serous cystadenoma, serous cystadenocarcinoma, and clear-cell carcinoma). (B) Representative histochemical staining of survivin in benign cystadenoma, borderline serous cystadenoma, serous cystadenocarcinoma, and clear-cell carcinoma samples. Original magnification, 200 \times .

The down-regulation of survivin expression by antisense oligonucleotides has been shown to significantly inhibit cell growth and induce apoptosis in melanoma (Grossman *et al.* 2001) and lung cancer (Olie *et al.* 2000). Carvalho *et al.* (2003a,b) first used RNAi to specifically repress survivin in HeLa cells and found that survivin was required for stable checkpoint activation. Although several studies have shown that survivin may be a biomarker for ovarian cancer, and this molecule correlated with clinical pathological parameters (Lin *et al.* 2009), the expression of survivin has been associated with sensitivity to chemotherapy (Xing *et al.* 2008). However, the mechanisms of survivin regulation in ovarian cancer are not yet clear. In the present study, we showed that survivin was increased in ovarian cancer cells *in vitro* and in xenografts *in vivo* by FSH stimulation. FSH promotes survivin expression through two major signal

transduction pathways: the PI3K/AKT and SAPK/JNK pathways. We further demonstrated that ovarian serous cystadenocarcinoma and clear-cell carcinoma samples had much higher levels of survivin expression compared with ovarian benign cystadenoma samples. Moreover, survivin expression in the ovarian serous cystadenocarcinoma specimens was found to correlate with disease stage. Together, these data suggest that FSH-stimulated expression of survivin plays an important role in the development of ovarian cancer.

In this study, we found that FSH stimulation down-regulated several apoptosis-related receptors, such as DR5 and PDCD6. DR5 is one of the two TRAIL death receptors (DRs) and contains an intracellular death domain. DR5 can be activated by TRAILs TNFSF10/TRAIL/APO2L, which allow for the transduction of apoptosis signals (Feng *et al.* 2009, Hori *et al.* 2010). TRAIL, produced in tumors by infiltrating monocytes, was identified as a powerful activator of programmed cell death (apoptosis) in tumor cells while sparing normal cells, and showed little or no overt toxicity when systemically administered to animals (Kruyt 2008). The recombinant form of the DRs and monoclonal antibodies against the TRAIL-DRs induced cell death in a wide variety of tumor cell lines and xenografts and are therefore potential anticancer agents (Oldenhuis *et al.* 2008). Down-regulating DR5 expression in ovarian cancer cells *in vitro* and in xenografts *in vivo* by FSH stimulation may be one of the mechanisms by which FSH inhibits apoptosis. More interestingly, we found that DR5 down-regulation was blocked by survivin siRNA, suggesting that survivin regulates DR5.

PDCD6 (ALG-2) is a calcium-binding protein that belongs to the penta-EF-hand protein family. PDCD6 participates in T cell receptor-, Fas-, and glucocorticoid-induced programmed cell death (Vito *et al.* 1996). PDCD6 was originally discovered as a pro-apoptotic protein in a genetic screen, but data about its function, expression, and localization are inconsistent. La Cour *et al.* (2003) reported that PDCD6 (ALG-2) was up-regulated in hepatomas and lung cancer tissues, thus questioning its previously assumed pro-apoptotic function. Support for PDCD6 (ALG-2) as a pro-apoptotic protein comes from recent work by Mahul-Mellier *et al.* (2006), who found that the part of AIP1/Alix that interacted with PDCD6 (ALG-2) was required for AIP1/Alix-induced cell death. Lee *et al.* (2005) searched a human ovary cDNA library for a novel PDCD6-binding protein using a yeast two-hybrid system. The selected protein was the human death-associated protein kinase 1 (DAPk1), another protein that functions as a positive mediator of apoptosis.

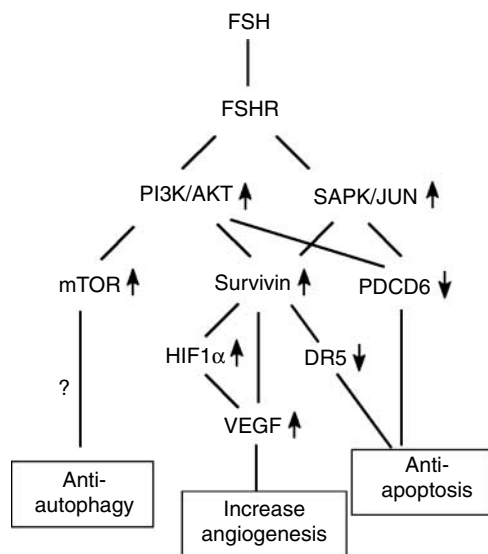


Figure 8 A network model of FSH stimulation in ovarian cancer.

Co-transfection of PDCD6 and DAPk1 cDNA into a tumor cell line accelerated apoptosis via the caspase-3-dependent pathway. In our study, we found that treatment with FSH decreased PDCD6 expression, but this down-regulation was not blocked by survivin siRNA; thus, PDCD6 is not the downstream target of survivin. FSH may directly regulate the expression of PDCD6 through the PI3K/AKT and SAPK/JNK pathways. Further studies to understand the correlation between FSH and death receptors are necessary and will aid in elucidating the mechanisms of FSH regulation in ovarian cancer development.

The actions of FSH on its principal target cells, such as granulosa and theca cells in the ovary, are mediated primarily by its G-protein-coupled transmembrane receptor. Substantial data support the idea that gonadotropins induce cAMP production and activate the PKA pathway when they bind to their receptors. However, in ovarian epithelial cancer cells, we found no alterations in pCREB, which is the PKA downstream second messenger. Gonadotropins may cause activation of PI3K and AKT in other reproductive tissues, including granulosa cells, Sertoli cells, and oocytes (Carvalho *et al.* 2003a,b, Alam *et al.* 2004, Meroni *et al.* 2004), and have also been shown to activate MAPK, PKC, and the interleukin-6/signal transducer and activator of transcription 3 (STAT3) signaling pathway (Ohtani *et al.* 2001, Syed *et al.* 2002, Choi *et al.* 2005). FSH has also been demonstrated to be associated with the NF-κB signaling pathway in granulosa cell apoptosis (Wang *et al.* 2002). However, our present study did not find any change in pIκB,

which is the NF-κB downstream messenger. As for ovarian cancers, we showed previously that the effect of FSH on VEGF may be mediated through the PI3K/AKT signaling pathway. The PI3K signaling pathway plays a role in proliferation, anti-apoptosis, and tumorigenesis in ovarian cancer (Carnero *et al.* 2004). cJUN is a member of the JUN family containing cJUN, JUNB, and JUND, and is a component of the transcription factor, activator protein-1 (AP-1). The activity of cJUN is regulated by phosphorylation at Ser63 and Ser73 through SAPK/JNK (Davis 2000). Aberrant activity of the SAPK/JNK signaling pathway is of great interest. Recent studies have found that it plays an important role in apoptosis (Lee *et al.* 2009, Zhang *et al.* 2009). In this study, we showed that FSH inhibited apoptosis and promoted proliferation by up-regulating survivin and suppressing PDCD6 not only through the PI3K/AKT pathway but also through the SAPK/JNK pathway.

In our previous study, we suggested a model of FSH-VEGF stimulation in ovarian cancer (Huang *et al.* 2008). With the results obtained in the current study, we can supplement this model as shown in Fig. 8. Our ongoing studies indicate that FSH stimulation also inhibits autophagy in ovarian cancer cells (Liu Y, Huang Y, Jin H, Cheng KW, Lu Y, Yu Y & Feng Y unpublished data 2010). Our data suggest that FSH plays an important role in ovarian carcinogenesis, and understanding the molecular mechanisms of FSH will ultimately help to develop an effective ovarian cancer prevention method and to find a novel targeted therapy.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1677/ERC-09-0308>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by National Natural Science Foundation of China (grant numbers 30872755 for Y Feng, 81020108027 for Y Feng, and 81072129 for H Jin); Shanghai Leading Academic Discipline Project (grant number B117 for Y Feng); and Shanghai Health Bureau Youth Research Project (grant number 2008Y008 for J Ding). This research did not receive any specific grant from any funding agency in the public, commercial, or not-for-profit sector.

Acknowledgements

We thank Dr Ming Yao (Shanghai Cancer Institute) for his help in the animal experiments.

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