FSH enhances the proliferation of ovarian cancer cells by activating transient receptor potential channel C3

Xiang Tao¹, Naiqing Zhao², Hongyan Jin¹,³, Zhenbo Zhang⁶, Yintao Liu¹,³, Jian Wu⁴, Robert C Bast Jr⁵, Yinhua Yu¹,³ and Youji Feng¹,⁶

¹Department of Gynecology, Obstetrics and Gynecology, Hospital of Fudan University, Shanghai 200011, China
²Department of Biostatistics, Shanghai Medical College, Fudan University, Shanghai 200032, China
³Shanghai Key Laboratory of Female Reproductive Endocrine Related Diseases, Shanghai 200011, China
⁴Department of Pathology, Gongli Hospital, Shanghai 200135, China
⁵Department of Experimental Therapeutics, M. D. Anderson Cancer Center, The University of Texas, Houston, Texas 77030, USA
⁶Department of Obstetrics and Gynecology, Shanghai First People’s Hospital of Jiao Tong University, Shanghai 200080, China

Correspondence should be addressed to Y Feng or Y Yu
Emails fengyj4806@sohu.com; yinhuay@gmail.com

Abstract
Recent studies have suggested that FSH plays an important role in ovarian epithelial carcinogenesis. We demonstrated that FSH stimulates the proliferation and invasion of ovarian cancer cells, inhibits apoptosis and facilitates neovascularisation. Our previous work has shown that transient receptor potential channel C3 (TRPC3) contributes to the progression of human ovarian cancer. In this study, we further investigated the interaction between FSH and TRPC3. We found that FSH stimulation enhanced the expression of TRPC3 at both the mRNA and protein levels. siRNA-mediated silencing of TRPC3 expression inhibited the ability of FSH to stimulate proliferation and blocked apoptosis in ovarian cancer cell lines. FSH stimulation was associated with the up-regulation of TRPC3, while also facilitating the influx of Ca²⁺ after treatment with a TRPC-specific agonist. Knockdown of TRPC3 abrogated FSH-stimulated Akt/PKB phosphorylation, leading to decreased expression of downstream effectors including survivin, HIF1-α and VEGF. Ovarian cancer specimens were analysed for TRPC3 expression; higher TRPC3 expression levels correlated with early relapse and worse prognosis. Association with poor disease-free survival and overall survival remained after adjusting for clinical stage and grade. In conclusion, TRPC3 plays a significant role in the stimulating activity of FSH and could be a potential therapeutic target for the treatment of ovarian cancer, particularly in postmenopausal women with elevated FSH levels.

Key Words
- ovarian epithelial cancer
- follicle-stimulating hormone (FSH)
- transient receptor potential channel C3 (TRPC3)
- cell proliferation
- prognosis

Introduction
Ovarian cancer is the sixth most common cancer and the fifth leading cause of cancer-related death among women in developed countries. Ovarian epithelial cancer (OEC) accounts for ~90% of all ovarian malignancies
(Berek & Natarajan 2007). However, the precise mechanism of OEC development remains largely unknown. To date, several hypotheses have been proposed to explain the aetiology of ovarian cancer. The well-known fact that early menarche and late menopause increase the risk of ovarian cancer (Franceschi et al. 1991) led to the hypothesis that suppression of ovulation may be an important factor in ovarian cancer development. Another extensively studied hypothesis is the ‘gonadotrophin theory’, which proposes that excessive levels of gonadotrophins after menopause or premature ovarian failure may play a role in the development and progression of OEC (Biskind & Biskind 1944, Cramer & Welch 1983, Vanderhyden 2005, Choi et al. 2007). Approximately, 2–3 years after menopause, the levels of FSH and LH are particularly high, reaching almost ten to 20 times (50–100 mIU/ml) the levels observed in women of reproductive age for FSH and three to four times (20–50 mIU/ml) the levels of LH (Chakravarti et al. 1976, Choi et al. 2007). The majority of women with OEC are present at this stage (Howlader et al. 2011). FSH expression levels in OEC patients have been correlated with clinical outcome. FSH expression levels in the ascites of ovarian cancer patients corresponded with patient survival (Chen et al. 2009). The highest gonadotrophin concentrations are observed in the cyst fluid from malignant ovarian tumours (Thomas et al. 2008). These observations suggest that FSH may play an important role in ovarian cancer carcinogenesis. However, not all studies have supported this theory. One study found no association between circulating gonadotrophin levels and ovarian cancer risk (Arslan et al. 2003), and one study reported that higher levels of circulating FSH decreased the risk of developing ovarian cancer (McSorley et al. 2009). Therefore, the relationship between FSH and ovarian cancer remains inconclusive, and further studies are needed.

Gonadotrophins bind to their specific receptor and activate downstream signalling pathways including PI3K/Akt and MAPK cascades, thereby regulating cell growth, apoptosis and metastasis in ovarian cancer (Biskind & Biskind 1944, Choi et al. 2005, 2006). Our group has found that FSH stimulates the proliferation and invasion of ovarian cancer cells, inhibits apoptosis, facilitates neovascularisation and increases the expression of VEGF by up-regulating the expression of survivin, which is activated by the PI3K/Akt signalling pathway (Huang et al. 2008, 2011). Studies from other groups have also revealed that FSH enhances Notch 1 expression (Park et al. 2010), promotes prostaglandin E2 production (Lau et al. 2010) and activates ERK1/2 signalling in a calcium- and PKCδ-dependent manner (Mertens-Walker et al. 2010).

The canonical transient receptor potential channel C3 (TRPCs), a family of non-selective cation channels mainly permeated by Ca2⁺, can be involved in the calcium influx and downstream pathways, regulating cell survival, proliferation and carcinogenesis by intracellular translocation induced by hormones and growth factors (Kanzaki et al. 1999, Smyth et al. 2006, Goel et al. 2010). The human TRPC family includes six subtypes, including subtypes 1–7 but excluding 2 (Abramowitz & Birnbaumer 2009), of which many are proposed to be associated with several types of malignancies such as TRPC6 in prostate cancer (Thebault et al. 2006), gastric cancer (Cai et al. 2009) and glioblastoma (Chigurupati et al. 2010) and TRPC1 in breast cancer (El Hiani et al. 2009). A recent report from Ding et al. demonstrated that TRPC6 plays an essential role in glioma development via regulation of the G2/M phase transition (Ding et al. 2010). Our collaborative works have revealed that TRPC3 plays an important role in ovarian cancer cell proliferation in vitro and in vivo (Yang et al. 2009).

Our gene expression array data demonstrate that TRPC3 expression levels increase following stimulation with FSH. Therefore, we hypothesised that TRPC3 may be involved in the FSH-dependent pathway of OEC cell proliferation. Here, we investigated whether TRPC3 plays a role in FSH-induced ovarian cancer cell proliferation. We also examined TRPC3 expression levels in ovarian cancer tissue samples and tested possible correlations with clinical outcome for ovarian cancer patients.

Materials and methods

Cell lines and tissue sections

The human OEC cell lines SKOV-3, ES-2 and HEY were obtained from the M. D. Anderson Cancer Center. Ninety paraffin-embedded OEC tissue sections were retrieved from Shanghai First People’s Hospital of Jiao Tong University. Nineteen samples of normal ovaries from non-malignant patients in the perimenopausal period, 20 samples from serous cystadenomas, and 15 samples from borderline serous tumours were obtained from the Obstetrics and Gynecology Hospital of Fudan University and Gongli Hospital. All patient samples were surgically resected tissues collected between 2003 and 2008. Diagnoses were confirmed independently by two pathologists. All tissue samples were obtained with the informed consent of the patient according to the protocols and
procedures approved by the Institutional Review Boards of the three hospitals. All patients were followed regularly, with the follow-up time ranging from 3 to 8 years.

**Cell culture and siRNA transfection**

OEC cell lines were cultured as described previously (Huang et al. 2008). TRPC3 ON-TARGETplus SMARTpool siRNA (siTRPC3) and siGLO Non-Targeting siConTROL siRNA (siNON) were purchased from Dharmacon (Lafayette, CO, USA). The siTRPC3 pool contained four specific siRNAs targeting TRPC3. The cells were transfected with siRNA using DharmaFECT 1 reagent (Dharmacon) for SKOV-3 cells and DharmaFECT 3 reagent (Dharmacon) for HEY and ES-2 cells according to the manufacturer’s instructions. Control samples (siCon) were treated with the same reagents except that the siNON siRNA was used instead of siTRPC3.

**Determination of the specificity of anti-TRPC3 antibody**

Anti-TRPC3 antibody was purchased from Abcam Co. (Cambridge, MA, USA). In order to determine the specificity of the antibody, HEY and ES-2 cells were transfected with Myc-tagged human wild-type TRPC3 or control vector (kindly provided by Prof. Yizheng Wang) by Lipofectamine 2000 (Invitrogen). The cell lysates were harvested 48 h after transfection and western blotted with anti-TRPC3 and anti-Myc tag antibodies (Cell Signaling Technology, Danvers, MA, USA). ES-2 cell lysates were western blotted and detected with anti-TRPC3 antibody or the antibody pre-mixed with the antigenic peptide (14 amino acids near the N-terminal of human TRPC3 protein, synthesised by Shenggong Biotech, Shanghai, China) for 1 h. Transfected HEY and ES-2 cells were also performed immunofluorescent staining for TRPC3 by the protocol indicated below and captured with Olympus BX-51 fluorescence microscope (Olympus Corporation, Japan). Paraffin-embedded mouse heart tissue was used as a positive control for immunofluorescent tests (indicated by the vendor’s manufacture).

**SRB cell proliferation assay**

FSH from a human pituitary was purchased from Sigma Chemical Co. (F4021). The cells were plated into 96-well plates at a concentration of 2000 cells/well for SKOV-3 cells and 1000 cells/well for HEY and ES-2 cells; the cells were subsequently incubated for 24 h following siRNA transfection as described earlier. After overnight starvation in Opti-MEM medium, FSH was added to the medium, and the cells were incubated for an additional 48 h. The plates were then routinely processed with SRB staining as described previously (Zou et al. 2011).

**Real-time quantitative RT-PCR**

The total cellular RNA was extracted from cells using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. cDNA was synthesised from 2 μg RNA using a RT Kit (TOYOBO Co. Ltd., Osaka, Japan). The transcription levels were quantified using real-time quantitative PCR with a Prism 7000 System (Applied Biosystems, Inc.). For each reaction, 10 ng cDNA was added to 25 μl reaction mixture containing 12.5 μl 2×SYBR Green PCR Master Mix from the SYBR Premix Ex Taq Kit (TAKARA BIO Inc., Shiga, Japan) and 300 nM of each TRPC3 primer (forward, 5′-CATTACCTCCACCTTT-CAGTC-3′; reverse, 5′-AGTGCTTGGCCTGTTGTCTT-3′). The GAPDH gene (forward, 5′-GAAGGTGAAGGTCG-GAGTC-3′; reverse, 5′-GAAGATGGTATGGGGATTTTC-3′) was selected as an endogenous control to normalise variations in the total RNA. We calculated mRNA levels using the comparative Ct method normalised to human GAPDH.

**Western blot analysis**

Western blotting was performed as described previously (Huang et al. 2008). The primary antibodies used include the following: rabbit anti-TRPC3 (Abcam), rabbit anti-p473 serine Akt (Cell Signaling Technology), rabbit anti-Akt (Cell Signaling Technology), rabbit anti-survivin (R&D Systems, Minneapolis, MN, USA), mouse anti-VEGF (Cell Signaling Technology), rabbit anti-HIF1-α (Cell Signaling Technology), and mouse anti-GAPDH (Sigma-Aldrich Co.). The signal intensities were evaluated using densitometry and semi-quantified using the ratio between the intensity of the protein of interest to that of GAPDH in each experiment. Each experiment was repeated at least three times.

**Cell cycle assay**

The cells were transfected with siRNA as described earlier. The cells were synchronised by serum starvation for over 12 h and were then cultured in medium with 10% FBS for 24 h. The cells were collected and fixed in 70% ethanol overnight at 4 °C, washed with PBS and treated with 500 μg/ml propidium iodide (PI) solution (Dingguo, Shanghai, China) containing 10 μg/ml RNaseA.
(Sigma–Aldrich Co.) for 30 min at room temperature in the darkness. A cell cycle analysis was performed using a FACSCalibur machine (BD Biosciences, Franklin Lakes, NJ, USA) with a phycoerythrin emission (PE) signal detector (FL2); the data were subsequently analysed with Modfit 3.0 Software (Verity Software, Inc., Topsham, ME, USA). The data are presented as a proliferation index (1 – percentage of cells in G0/G1 phase).

**Apoptosis assay**

The cells were transfected with TRPC3 siRNA as described earlier and starved overnight before incubation with FSH for an additional 48 h. Cisplatin was added at a final concentration of 5 μg/ml for 12 h before harvesting. The cells were trypsinised, washed twice with PBS, and resuspended in 1× binding buffer (Invitrogen). After incubation with Annexin V–FITC and PI staining solution (Invitrogen) at room temperature in the darkness for 15 min, the stained cells were analysed immediately using flow cytometry. The signal of Annexin V–FITC was measured with the PE signal detector (FL2), and PI was detected using the FITC signal detector (FL1), and PI was measured with the PE signal detector (FL2). The population of Annexin V (+)/PI (−) cells represents early apoptotic cells.

**Immunocytofluorescence**

SKOV-3, HEY and ES-2 cells were trypsinised and plated on coverslips the day following FSH treatment and were continuously incubated for 48 h. The adherent cells were washed twice with PBS, fixed with 4% paraformaldehyde at 4 °C for 30 min, and then washed again with PBS. After incubation with goat serum blocking buffer (Mingrui, Shanghai, China) for 30 min at room temperature, the coverslips were incubated with rabbit anti-TRPC3 (1:100) at 4 °C for 24 h. The cells were washed three times with PBS and incubated with FITC-conjugated goat anti-rabbit secondary antibody (1:200 dilution, Millipore, Billerica, MA, USA) at 37 °C for 1 h in the darkness. The slides were then washed with PBS and counterstained with 4′,6-diamidino-2-phenylindole (DAPI). The cells were imaged using a confocal microscope (Leica TCS SP5, Germany). The membrane and cytoplasmic fractions of ES-2 cells treated as described earlier were separated according to the manual of the Subcellular Protein Fractionation Kit for Cultured Cells (Thermo Scientific, Rockford, IL, USA). Na+/K+-ATPase was used as the marker for membrane. Antibody against Na+/K+-ATPase was purchased from Thermo Scientific.

**Intracellular calcium imaging**

The cells were cultured, transfected with either siCon or siTRPC3 and then incubated with FSH in a glass-bottomed petri dish for 48 h. Next, the cells were stained with 1 mM Fluo-3 AM fluorescent dye (DOJINDO Laboratories, Kumamoto, Japan) in RPMI 1640 medium in the darkness at 37 °C for 30 min and washed in HBSS buffer (120 mM NaCl, 6 mM KCl, 2 mM CaCl2, 2 mM MgCl2, 12 mM glucose and 10 mM HEPES, pH 7.4) three times before detection. The dishes were placed on a flow irrigating system. Fluorescence was induced with 50 μM 1-oleoyl-2-acetyl-sn-glycerol (OAG) and dynamically recorded by a confocal microscope (Leica TCS SP5) with excitation at 340 and 380 nm every 4 s and emission measured at 510 nm. The changes in [Ca2+]i were monitored as the average intensity of the living cells with a high-power field (objective lens 63×).

**Immunohistochemistry**

The methods for immunohistochemical staining have been well described (Cheng et al. 2009). Briefly, the slides were placed in 3% hydrogen peroxide for 5 min to block endogenous peroxidase activity. Antigen retrieval was achieved using treatment in EDTA buffer at 99 °C for 30 min. After blocking with goat serum for 15 min, the sections were incubated in primary antibody overnight at 4 °C and washed twice in a PBS solution. The sections were then incubated in biotin-conjugated secondary antibody (Thermo Fisher Scientific, Inc.) for 30 min and then in streptavidin peroxidase (Invitrogen) for 30 min. A DAB Kit (Sigma Diagnostics) was used for chromogen detection. The primary antibodies were replaced by rabbit serum as a control. The staining intensity in epithelial cells was evaluated on the following scale: 0 for a negative stain, 1 for weak positivity, 2 for median positivity and 3 for strong positivity. The area containing positive cells was scored as 0–100%. Next, the expression score (ES) was calculated as the intensity of positivity multiplied by the positive area. The ES of each section was ranked and the median was calculated as the cut-off point for which an ES above or equal to this cut-off value was considered as high expression, while an ES below the cut-off point was considered low expression (Sun et al. 2009, Shimoyamada et al. 2010).

**Statistical analysis**

SPSS (version 16.0) was applied for data analyses. Either a t-test or an ANOVA was utilised to compare the differences
in the mean among groups when the data displayed approximately normal distribution and homogeneity in variance; otherwise, the Wilcoxon’s rank sum test was utilised to perform the analysis. The Spearman’s correlation was utilised to analyse the tendency between TRPC3 and clinical characteristics. The general association test was performed using either the Pearson $\chi^2$ or Fisher’s exact test for categorical data. The survival curves were estimated using the Kaplan–Meier method, and the comparison of the survival curves was performed using either the Log-rank test or the Cox regression model. A $P$ value $\leq 0.05$ (two-sided test) was considered significant.

**Results**

**Testing the specificity of anti-TRPC3 antibody**

In the beginning, we determined the specificity of the antibody against TRPC3 in the application of western blot and immunofluorescence. As shown in Supplementary Figure 1A, see section on supplementary data given at the end of this article, the antibody recognised the overexpressed TRPC3 protein in ovarian cancer cells, HEY and ES-2, which were transfected with Myc-tagged human wild-type TRPC3. It was confirmed by simultaneously expressed Myc protein at the same migration positions. We further verified the specificity of the antibody in recognising endogenous TRPC3 in the ES-2 cell lysates, which could be blocked by the synthesised antigenic peptide (Supplementary Figure 1B). Moreover, the specificity of the antibody in immunofluorescence was confirmed by recognising more signals from the exogenic expressed TRPC3 of transfected HEY and ES-2 cells than non-transfected ones (Supplementary Figure 1C) and also by positively stained paraffin-embedded mouse heart tissue, which is instructed by the vendor (Supplementary Figure 1D).

**FSH up-regulated TRPC3 expression in ovarian cancer cells**

Based on our gene expression array data, we observed a 2.4- to 2.8-fold increase in TRPC3 expression following stimulation of OEC cell lines with FSH. To confirm this result, three OEC cell lines including the serous cystadenocarcinoma lines SKOV-3 and HEY and the clear cell ovarian cancer line ES-2 were utilised in the following in vitro experiments. Although different pathological subtypes can display quite different gene expression patterns, all three of these cell lines showed almost the same reaction pattern as that of FSH stimulation. Of the three OEC cell lines, the ES-2 cell line was the most sensitive to FSH stimulation; however, the ES-2 cell line did not respond at times to the lower doses of FSH, while the other two cell lines did. The cells were treated with different concentrations of FSH ranging from 0 to 40 mIU/ml for different intervals ranging from 12 to 48 h, and the expression levels of TRPC3 mRNA and protein were analysed using quantitative real-time RT-PCR and western bloting. The TRPC3 amplicons were verified through sequencing. The increases in TRPC3 were shown to be both time and dose dependent in the three cell lines, with optimal mRNA expression observed using 40 mIU/ml FSH for 24 h (Fig. 1A, B and C). Under these conditions, FSH increased TRPC3 mRNA expression levels by 6.0-, 4.0- and 41.9-fold in the SKOV-3, HEY and ES-2 cell lines respectively compared with the PBS control. Accordingly, we used western blot analysis to examine the TRPC3 protein levels, which indicated that the maximum stimulating dosage of FSH was a concentration of 40 mIU/ml (Fig. 1D and E).

**Knockdown of TRPC3 attenuated FSH-induced proliferation and resistance to chemotherapy in ovarian cancer cells**

To clarify the role of TRPC3 in mediating the FSH-induced stimulation of OEC, we utilised the Dharmacon ON-Target plus siRNA pool to specifically knock down TRPC3 expression (siTRPC3). TRPC3 protein levels decreased by 68.7 and 48.1% in HEY and ES-2 cells respectively (Fig. 2A and B). Cell proliferation was evaluated using SRB assays. TRPC3 knockdown resulted in modest inhibition of cell proliferation compared with siCon controls in the absence of FSH. Incubation with siTRPC3 significantly reduced the proliferative effect of FSH in HEY and ES-2 cell lines ($P<0.05$; Fig. 2C and D); we found greater differences over a longer time period (Fig. 2E and F).

A fluorescence-activated cell sorting (FACS) analysis of the cell cycle indicated an increased proliferation index (i.e. the percentage of cells in all phases excluding the G0/G1 phases) following FSH treatment (a minor tendency in HEY cells, a significant difference in ES-2 cells). The stimulatory effects of FSH were partially diminished by TRPC3 knockdown in the HEY and ES-2 cell lines ($P<0.05$, compared with control siRNA; Fig. 2G and H; Supplementary Figure 2, see section on supplementary data given at the end of this article).

Cisplatin is often used to treat ovarian cancer and produces objective tumour regression in 70% of patients, primarily by inducing apoptosis in cancer cells. As
indicated in Fig. 3A and B, cisplatin inhibited ovarian cancer cell growth with an IC$_{50}$ of 8.9 μg/ml in HEY and 3.9 μg/ml in ES-2 cells. A dose of 5 μg/ml cisplatin induced more than 10% apoptosis in both HEY and ES-2 cells (Fig. 3C and D) when treated with control siRNA. However, FSH effectively blocked this effect; the apoptotic proportion decreased from 11.78 to 2.81% in HEY cells and from 10.56 to 4.25% in ES-2 cells.

TRPC3 knockdown promoted cell apoptosis and attenuated the anti-apoptotic effect of FSH as the apoptotic fraction increased from 2.81 to 8.83% in HEY cells and from 4.25 to 10.95% in ES-2 cells (Fig. 3C and D; Supplementary Figure 3, see section on supplementary data given at the end of this article).

**FSH enhanced TRPC3 expression in ovarian cancer cells**

A confocal microscope was used to evaluate FSH stimulation effects on TRPC3 protein expression and subcellular localisation in the ovarian cancer cell lines HEY and ES-2 by immunofluorescent staining. We found that TRPC3 was expressed weakly in FSH-untreated cells. When stimulated with FSH, however, TRPC3 intensity increased in both HEY and ES-2 cells (Fig. 4A and B). Through the isolation of the membrane and cytoplasmic fractions of ES-2 cells, we found that TRPC3 expression on the membrane was enhanced more than on the cytoplasm by FSH stimulation (Fig. 4C).

**TRPC3 knockdown blocked the FSH-induced facilitation of calcium influx**

TRPC3 primarily mediates the influx of calcium ions via agonist-stimulating mechanisms. We used confocal microscopy to trace over time the intracellular calcium ([Ca$^{2+}$]$_i$) levels within living ovarian cancer cells. The cells were transfected with either siCon or siTRPC3 and then treated with FSH for 48 h and stained with Fluo-3 AM fluorescent dye immediately before visualisation. With the perfusion of 50 μM OAG, a TRPC agonist, a rapid influx and subsequent short period of [Ca$^{2+}$]$_i$ maintenance was detected in control siRNA transfecants with FSH stimulation but not in control siRNA transfecants without FSH stimulation, thereby suggesting that FSH treatment facilitated intracellular calcium influx.
**Figure 2**

TRPC3 knockdown attenuated the effects of FSH on proliferation in ovarian cancer cells. (A and B) Knockdown of TRPC3 expression by TRPC3 siRNA (siTRPC3). (A) A representative western blot of HEY and ES-2 cells is shown. The cells were transfected with siTRPC3; the total lysates were extracted and the immunoblots were probed with anti-TRPC3 and GAPDH antibodies. The transfectants without siRNA and with non-targeting control siRNA (siCon) were used as controls. (B) A semi-quantitative analysis of the TRPC3:GAPDH ratio was performed. The data represent the mean ± S.D. of three independent western blot assays as in (A). *P<0.05, compared with siCon control. (C and D) The cell growth stimulation effects of FSH (by different concentrations) were reduced by TRPC3 knockdown in HEY (C) and ES-2 (D) cells. The cells were transfected with siTRPC3 and treated with FSH at various concentrations ranging from 0 to 80 mIU/ml for 48 h. The siCon transfectants were used as a control. The cell proliferation rate was detected using the SRB assay. Each experiment was performed in triplicate. *P<0.05, compared with siCon transfectants. (E and F) The cell growth stimulation effects of 40 mIU/ml FSH (at different stimulation times of 0–72 h) were reduced by TRPC3 knockdown in HEY (E) and ES-2 (F) cells. *P<0.05, compared with siCon transfectants. (G and H) The cell cycle changes induced by FSH stimulation were attenuated by TRPC3 knockdown in HEY (G) and ES-2 (H) cells. The cells were transfected with siTRPC3 and either treated with FSH at 40 mIU/ml or left untreated for 48 h. The cell cycle distribution was measured using flow cytometry and is displayed as a proliferation index. The data represent the mean ± S.D. of three independent assays. *P<0.05 or **P<0.01, compared with siCon control.
TRPC3-specific knockdown was associated with a block in the rapid calcium influx. Similar patterns were observed in the three OEC cell lines (Fig. 5A, B and C). The direct perfusion of 40 mIU/ml FSH in OEC cells failed to trigger the influx of Ca\textsuperscript{2+} (data not shown), which suggests that FSH did not directly mediate the activation of TRPC3.

Knockdown of TRPC3 partially abrogated the activation of Akt/PKB phosphorylation by FSH stimulation

Our previous studies have indicated that FSH facilitated angiogenesis via the Akt-HIF1-\(\alpha\)-survivin-VEGF pathway (Huang et al. 2008). Here, we evaluated the relationship between TRPC3 and the Akt/PKB-associated angiogenesis biomarkers. TRPC3 expression was knocked down in ES-2 and HEY cells, which were then treated with FSH and the PI3K-specific inhibitor LY294002. The cells were transfected with siCon or siTRPC3 and either treated with FSH at 40 mIU/ml or left untreated for 48 h. Cisplatin was then added 12 h before harvesting at a final concentration of 5 \(\mu\)g/ml. Apoptosis was measured using PI/Annexin V double staining and flow cytometry. The data represent the mean \(\pm\) S.D. of three independent assays. *P<0.05, compared with siCon control. The no cisplatin treatment groups with or without FSH are presented on the left.

TRPC3 expression was associated with a poor prognosis in ovarian cancer patients

Because TRPC3 plays an important role in regulating FSH-related pathways, we further investigated whether TRPC3 expression levels in ovarian tumours correlated with patient clinical outcome. With the consent of the patients, TRPC3 expression levels were measured in a cohort of 50 ovarian cancer patients. The expression levels were determined using quantitative PCR and were correlated with patient survival. A significant correlation was observed between TRPC3 expression and patient survival, with a higher expression level indicating a poorer prognosis. These results suggest that TRPC3 plays a role in the prognosis of ovarian cancer patients.
OEC patients, 90 OEC tissue samples, 19 normal ovarian samples, 20 benign serous tumour samples and 15 borderline serous counterpart samples were selected for investigation into the relationship between TRPC3 protein expression and clinicopathological parameters. The OEC tumours included 63 cases of serous adenocarcinoma, 7 cases of mucinous adenocarcinoma, 9 cases of endometrioid adenocarcinoma and 11 cases of clear cell carcinoma. All 90 ovarian cancer cases had complete follow-up data and were used for prognostic analysis. In these 90 cases, the patient age ranged from 22 to 79 years (54.6 ± 11.7). There were 17 samples from clinical stage I patients, 24 samples from clinical stage II patients and 49 samples from clinical stage III patients. During the observation period, 43 (47.8%) patients relapsed and 22 (24.4%) patients eventually died.

Using immunohistochemistry, we analysed TRPC3 expression in the epithelium of normal ovaries compared with tumour samples. TRPC3 expression levels showed a significant positive correlation with the tumour malignancy (Pearson $\chi^2$ test, $P < 0.001$); the proportion of cells with high TRPC3 expression was substantially higher in malignant tumours than in normal ovarian samples (Supplementary Table 1A, see section on supplementary data given at the end of this article). There were significant differences in the proportion of cells with high TRPC3 expression among the pathological types of malignancies (Fisher’s exact test, $P = 0.050$; Fig. 7B; Supplementary Table 1B). Considering the heterogeneity of tumour origin, the most abundant type of tumour, serous carcinoma, was analysed both independently and together with the other types. Among the 90 ovarian cancer tissue samples, the association between high TRPC3 expression and tumour grade, marker (A and B). (C) Membrane and cytoplasmic fractions were isolated from ES-2 cells treated with FSH or untreated as earlier; a western blot analysis was used to detect TRPC3 expression. Na$^+$/K$^+$-ATPase was used as the marker for membrane. GAPDH was used as a loading control.

lymphatic metastasis or clinical stage was not significant (Pearson $\chi^2$, $P = 0.669$, $P = 0.138$ and $P = 0.534$ respectively; Supplementary Table 2A and B, see section on supplementary data given at the end of this article). A similar pattern was found in the 63 serous ovarian cancer tissues; the association between high TRPC3 expression and tumour grade, lymphatic metastasis or clinical stage was also not significant (Pearson $\chi^2$, $P = 0.220$, $P = 0.159$ and $P = 0.638$ respectively; Supplementary Table 2A and B).

There were no significant differences in the mean patient age between the TRPC3 high-expression group and the TRPC3 low-expression group for the 90 total OEC samples or the 63 cases of serous-type tumours (t-test, $P = 0.739$ and $P = 0.543$ respectively); however, the serum CA125 values were significantly higher in the TRPC3 high-expression group than in the TRPC3 low-expression group for both the 90 total cases and the 63 cases of serous-type tumours (Wilcoxon’s rank sum test, $P = 0.004$ and $P = 0.002$ respectively; Supplementary Table 2C).

Because tumour relapse impacts patient survival, the association of disease-free survival (DFS) and TRPC3 was evaluated. The potential covariables in the multivariate Cox regression model included age, tumour grade, lymphatic metastasis, clinical stage and TRPC3 expression levels. In the 90 ovarian cancer tissue samples, using the Cox model, TRPC3 expression levels, lymphatic metastasis, tumour grade and clinical stage were the important parameters for DFS (Table 1). The hazard ratio (HR) of the high TRPC3 expression group was 2.802 (95% CI: 1.406–5.586; $P = 0.003$), indicating that recurrence in ovarian cancer patients with high TRPC3 expression was significantly earlier than in patients with low TRPC3 expression (Fig. 8A). The follow-up time and survival status

Figure 4
FSH increased the expression of TRPC3 protein in ovarian cancer cells. The ovarian cancer cell lines HEY (A) and ES-2 (B) were treated with 40 mIU/ml FSH for 48 h. The TRPC3 protein was immunofluorescence labelled and imaged using confocal microscopy. DAPI was used as a nuclear staining marker (A and B). (C) Membrane and cytoplasmic fractions were isolated from ES-2 cells treated with FSH or untreated as earlier; a western blot analysis was used to detect TRPC3 expression. Na$^+$/K$^+$-ATPase was used as the marker for membrane. GAPDH was used as a loading control.
was considered as the overall survival (OS). According to the Cox regression analysis, TRPC3 expression levels, lymphatic metastasis, tumour grade and clinical stage were the most important parameters for the OS; the HR of the high TRPC3 expression group was 2.866 (95% CI: 1.056–7.777; \(P = 0.039\); Table 2), indicating that high levels of TRPC3 expression were associated with poor OS (Fig. 8B).

The association of TRPC3 expression levels with poor DFS and OS remained after adjusting for clinical stage (\(P = 0.001\) for DFS and \(P = 0.048\) for OS; Supplementary Table 3A and B, see section on supplementary data given at the end of this article) or for tumour grade (\(P = 0.001\) for DFS and \(P = 0.032\) for OS; Supplementary Table 3C and D), while the association remained only with poor DFS for lymphatic metastasis (\(P = 0.002\); Supplementary Table 3E). The association was lost with poor OS for lymphatic metastasis (\(P = 0.144\); Supplementary Table 3F), which may be due to an insufficient power for OS analysis; more cases are required for future work.

To avoid the influence of pathological type, we performed the same analysis on DFS and OS with tissue samples from 63 serous cancers and found similar patterns (Fig. 8C and D). The HR of the high TRPC3 expression group was 4.073 (95% CI: 1.753–9.462; \(P = 0.001\)) for DFS and 3.766 (95% CI: 1.073–13.226; \(P = 0.039\)) for OS (Tables 1 and 2). The association of TRPC3 expression levels with poor DFS and OS in serous type also remained after adjusting for clinical stage (\(P = 0.001\) for DFS and \(P = 0.041\) for OS; Supplementary Table 4A and B, see section on supplementary data given at the end of this article) or for tumour grade (\(P = 0.002\) for DFS and \(P = 0.045\) for OS; Supplementary Table 4C and D). However, the association remained only with poor DFS for lymphatic metastasis (\(P = 0.001\); Supplementary Table 4E) but was lost with poor OS for lymphatic metastasis (\(P = 0.079\); Supplementary Table 4F).

**Discussion**

FSH stimulates proliferation and inhibits apoptosis of ovarian cancer cells, although the mechanism and regulation of FSH are not yet clear. Our previous studies have shown that FSH stimulates the Akt-HIF1-\(\alpha\)-survivin-VEGF pathway (Huang et al. 2008, 2011). In this study, we found that TRPC3 is an important molecule that regulates FSH-induced OEC proliferation. We observed that FSH stimulation led to increased TRPC3 protein and mRNA expression levels, facilitating the TRPC3-dependent, agonist-induced \(\text{Ca}^{2+}\) influx. Knockdown of TRPC3 inhibited the ability of FSH to stimulate proliferation and block

---

**Figure 5**

TRPC3 knockdown blocked the FSH-associated effects on calcium influx. A representative image of intracellular calcium influx is shown. The three OEC cell lines SKOV-3 (A), HEY (B) and ES-2 (C) were used to analyse the effect of TRPC3 down-regulation on \([\text{Ca}^{2+}]_i\). The cells were transfected with either control siRNA (siCon) or TRPC3 siRNA (siTRPC3), stimulated with FSH or left untreated for 48 h, and then stained with Fluo-3 AM fluorescent dye before observation. The \([\text{Ca}^{2+}]_i\) was determined using the F340:F380 ratio and induced with 50 \(\mu\)M OAG. The upper horizontal bar indicates the period of OAG infusion. The experiment was repeated three times.
apoptosis of ovarian cancer cells; it also abrogated FSH-induced Akt/PKB phosphorylation, leading to decreased expression of downstream effectors including survivin, HIF1-α and VEGF. We observed that this abrogation is partial, suggesting that TRPC3 may be an indirect regulator of FSH-induced Akt/PKB phosphorylation, and further study is necessary. In this study, we used two ovarian cancer cell lines that belong to the different histological subtypes, HEY is a cystadenocarcinoma cell line, ES-2 is a clear cell carcinoma cell line, and they may show different reaction to FSH stimulation and related pathways. This study supports the findings of Mertens-Walker et al. (2010) and provides evidence that draws a correlation between FSH and ion channel factors, which may open a new area for investigating the function of hormones in gynaecologic cancers.

Ca2+ is a versatile intracellular signalling molecule. It has been demonstrated that Ca2+ is necessary for tumorigenesis and cancer progression (Monteith et al. 2007). Ca2+ influx activates PKB/Akt in both skeletal muscle cells (Lanner et al. 2009) and melanoma cells (Feldman et al. 2010) and also activates the MAPK and JNK/STAT pathways (Hu et al. 2001). Inhibiting the increase in [Ca2+], has an anti-proliferative effect in many cancers. Calcium-related ion channels are the key regulators of Ca2+ influx, which has attracted the attention of researchers of certain types of cancer therapy such as carboxamidotriazole. Carboxamidotriazole is a cyto-static inhibitor of non-voltage-operated Ca2+ channels that has been tested as a potential therapeutic drug for patients with glioblastoma multiforme in phase I and II clinical trials (Murph et al. 2009). TRPCs comprise a group of plasma membrane-localised proteins, which mainly determine intracellular Ca2+ concentrations based on signals from extracellular agonists and levels of cellular Ca2+ store depletion, thereby regulating a large variety of physiological processes. Our clinicopathological analysis revealed that TRPC3 expression was ubiquitous in normal, benign, borderline and malignant epithelia with the tendency of increasing positivity. High levels of TRPC3 expression correlated with poor prognosis and early relapse, with a risk ratio of nearly 3.0 compared with the
Our previous collaborative works showed that the TRPC3 protein levels in human ovarian cancer specimens were greatly increased compared with those in normal ovarian specimens (Yang et al. 2009). This current study has consistently demonstrated the clinical importance of this ion channel factor. TRPC3 expression levels correlated with DFS and OS, and the higher expression group tended to relapse early and had a poor prognosis. In a multivariate analysis, the association with poor DFS and OS remained after adjusting for clinical stage and tumour grade; the association with poor DFS also remained after adjusting for lymphatic metastasis. Although the association with poor OS was lost after adjusting for lymphatic metastasis, it is possible that increasing the number of cases could confirm the prognostic value of TRPC3. The combination of tissue TRPC3 and plasma FSH may provide a more robust marker for prognosis. Regardless of its prognostic significance, TRPC3 could provide a target for therapy. Together with the fact that TRPC3 is an important regulator of FSH, these data strongly suggest that TRPC3 channels are essential for ovarian cancer development and progression.

Calcium flux in human ovarian cancers can also be affected by lysophosphatidic acid (LPA), which stimulates the G protein-coupled Edg-4 receptor. LPA is expressed by the majority of ovarian cancers, and high levels are found in ascitic fluid. LPA stimulates proliferation and increases...
resistance to chemotherapy (Conrad et al. 2000, Mikkelsen et al. 2007). It would be of interest to determine whether TRPC3 plays a role in LPA-induced signalling.

The Ca^{2+}-permeable TRPC subfamily comprises seven members (TRPC1–7), and based on the amino acid similarities, the human TRPCs are divided into three subgroups: TRPC1, TRPC3/6/7 and TRPC4/5 (TRPC2 being a pseudogene; Montell 2005). Because of the possibility of heteromultimerisation of TRPCs, the biological activities may involve more than one TRPC, which makes it challenging to identify the function of a single subtype (Flockerzi 2007). Interestingly, we found that TRPC3, TRPC5 and TRPC6 can each be involved in FSH regulation. According to our observations, FSH stimulation may also

**Figure 8**
The expression levels of TRPC3 correlated with prognosis of ovarian cancer patients. The disease-free survival curves are shown for ovarian cancer patients with different levels of TRPC3 expression in 90 samples of ovarian cancer (A) and in 63 samples of ovarian serous cancer (C). The overall survival curves of ovarian cancer patients with different levels of TRPC3 expression in 90 tissue samples of ovarian cancer (B) and 63 samples of ovarian serous cancer (D) are shown.

**Table 2** TRPC3 expression levels correlate with the prognosis of ovarian cancer patients (total 90 cases of ovarian cancer and 63 cases of serous ovarian cancer). Association with overall survival between each parameter.

<table>
<thead>
<tr>
<th>Total cases</th>
<th>Serous type</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TRPC3 expression</strong></td>
<td><strong>TRPC3 expression</strong></td>
</tr>
<tr>
<td>0.039</td>
<td>0.039</td>
</tr>
<tr>
<td>2.866</td>
<td>3.766</td>
</tr>
<tr>
<td>1.056</td>
<td>1.073</td>
</tr>
<tr>
<td>1.005</td>
<td>1.322</td>
</tr>
<tr>
<td>1.695</td>
<td>3.398</td>
</tr>
</tbody>
</table>

HR, hazard ratio.
lead to increased expression of both TRPC5 and TRPC6 and influence the translocation of TRPC5 from the cytoplasm to the cell membrane (data not shown). Future work will address whether other members of the TRCP family are relevant to ovarian cancer and the interrelation between these subtypes.

Recent studies suggest that sufficient RNAi delivery can be achieved to inhibit ovarian cancer xenograft growth (Landen et al. 2006, Mangala et al. 2009). Using RNAi, it may be possible to specifically inhibit individual members of the TRCP family; however, it remains to be determined whether sufficiently specific small molecule inhibitors can be designed. Future studies will evaluate TRPC3 RNAi therapy in ovarian cancer xenograft models. If positive, TRPC3 could provide a novel target for therapy.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/ERC-12-0005.

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 the National Natural Science Foundation of China (grant numbers: 30872755 and 81020108027 to Y Feng and 81072129 to H Jin), the Shanghai Leading Academic Discipline Project (grant number: B117 to Y Feng and H Jin), Shanghai Science and Technologic Committee (grant number: 10JC1413100 to Y Feng), Shanghai International Collaboration Program (grant number: 10410700500 to H Jin) and Shanghai International Collaboration Program (grant number: 10410700500 to H Jin). This work was also supported by funds from the National Cancer Institute (grant number: CA80957 to Y Yu); the National Cancer Institute (grant number: CA80957 to Y Yu); from the M. D. Anderson SPORE in Ovarian Cancer (grant number: NCI P50 CA83639 to R C Bast); the M. D. Anderson CCSG (grant number: CA 80957 to Y Yu); and philanthropic support to H Jin), the Shanghai Leading Academic Discipline Project (grant number: 10JC1413100 to Y Feng), Shanghai International Collabor-

Author contribution statement
X Tao and Z Zhang carried out experiments and prepared the manuscript. N Zhao performed the statistical analysis. H Jin and Y Liu provided support in experiments and helped to prepare the manuscript. J Wu provided tissues and pathologic analysis. R C Bast, Y Yu and Y Feng conceived the study and approved the final manuscript.

Acknowledgements
The authors thank Bin Lai from Department of Neuroscience of Fudan University for help in the experiment of confocal microscopy detection. They also thank Prof. Yizheng Wang of Laboratory of Neural Signal Transduction, Institute of Neuroscience, Shanghai Institutes of Biological Sciences, for providing Myc-tagged human wild-type TRPC3 and control vectors.

References


Received in final form 26 March 2013
Accepted 11 April 2013
Made available online as an Accepted Preprint
11 April 2013