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

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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 Ca^{2+} 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

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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 PKA, 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 PKC δ -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'-CATTACCTCCACCTTCAGTC-3'; reverse, 5'-AGTTGCTTGGCTCTGTCTT-3'). The *GAPDH* gene (forward, 5'-GAAGGTGAAGGTCGAGTC-3'; reverse, 5'-GAAGATGGTGATGGGATTTC-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 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 χ^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 ≤ 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 exogenous 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 blotting. 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

Figure 1

FSH stimulates TRPC3 expression at the mRNA and protein levels. Ovarian cancer cell lines SKOV-3 (A), HEY (B) and ES-2 (C) were treated with FSH at different concentrations and different time points, and mRNA was then extracted and analysed using real-time RT-PCR. Each experiment was performed in triplicate. $*P < 0.05$ compared with the no FSH treatment control. (D) A representative western blot is performed on three ovarian cancer cell lines treated with FSH at concentrations ranging from 0 to 80 mIU/ml for 48 h. The total lysates were then extracted, and

immunoblots were subsequently probed with anti-TRPC3 antibodies; GAPDH was used as a loading control. (E) A semi-quantitative analysis of the ratio of TRPC3:GAPDH is shown. The data were calculated as the TRPC3:GAPDH ratios and expressed as fold change relative to the control; the data represent the mean $\pm$ s.d. of three independent western blot assays. $*P < 0.05$ compared with the no FSH treatment control (the no treatment control was set at 1.0).

indicated in Fig. 3A and B, cisplatin inhibited ovarian cancer cell growth with an IC_{50} of 8.9 μ g/ml in HEY and 3.9 μ g/ml in ES-2 cells. A dose of 5 μ g/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 μ M OAG, a TRPC agonist, a rapid influx and subsequent short period of $[Ca^{2+}]_i$ maintenance was detected in control siRNA transfectants with FSH stimulation but not in control siRNA transfectants 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 $\pm$ 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 concentration) 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 $\pm$ s.d. of three independent assays. * $P < 0.05$ or ** $P < 0.01$, compared with siCon control.

Figure 3

TRPC3 knockdown attenuated the effects of FSH on apoptosis in ovarian cancer cells. (A and B) Cisplatin inhibited cell growth in HEY (A) and ES-2 (B) cells. (C and D) The effects of FSH anti-cisplatin-induced apoptosis were blocked by *TRPC3* knockdown in HEY (C) and ES-2 (D) cells. 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 μg/ml. Apoptosis was measured using PI/Annexin V double staining and flow cytometry. The data represent the mean ± 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-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 Ca²⁺ (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-α-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 expression of TRPC3, Akt that was phosphorylated at Ser473 (p473^{Akt}), total Akt, HIF1-α, survivin and VEGF proteins was detected using western blot analysis. Each experiment was performed in triplicate. Figure 6 and Supplementary Figure 4, see section on supplementary data given at the end of this

article shows that with FSH stimulation, the expression levels of TRPC3, p473^{Akt} and the Akt downstream molecules HIF1-α, VEGF and survivin were elevated. Although control siRNA (siCon) brought some undetermined interference to cells, it could be perceived that inhibition of TRPC3 with siRNA partially blocked the FSH-stimulated increase in p473^{Akt}, HIF1-α, VEGF and survivin. Inhibition of Akt by LY294002 suppressed the expression of the downstream molecules HIF1-α, survivin and VEGF, but LY294002 treatment increased TRPC3 expression in ES-2 cells, while not in HEY cells, may be due to intrinsic features of the two cell lines. Consequently, TRPC3 plays a certain role in regulating FSH-induced activation of Akt, thus influencing the expression of HIF1-α, survivin and VEGF.

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

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

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 χ^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 and Fig. 7A). 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,

lymphatic metastasis or clinical stage was not significant (Pearson χ^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 χ^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 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 $[Ca^{2+}]_i$. 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 $[Ca^{2+}]_i$ was determined using the F340:380 ratio and induced with 50 μ M OAG. The upper horizontal bar indicates the period of OAG infusion. The experiment was repeated three times.

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- α -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 Ca^{2+} influx. Knockdown of TRPC3 inhibited the ability of FSH to stimulate proliferation and block

Figure 6

Knockdown of *TRPC3* abrogated the activation of Akt/PKB phosphorylation by FSH stimulation. HEY (A) and ES-2 (B) cells were transfected with either control siRNA (siCon) or *TRPC3* siRNA (siTRPC3) and then treated with FSH. The PI3K inhibitor LY294002 was used as a positive control. The expression levels of phosphorylated Akt (p473^{Akt}), total Akt, HIF1- α , survivin and VEGF were analysed using a western blot analysis. GAPDH was

used as a loading control. Quantification by densitometry (comparing with total Akt for p473^{Akt} or with GAPDH for others; no treatment was set as 1.0) is presented below each blot. Each experiment was performed in triplicate (Supplementary Figure 3); representative immunoblots are shown in A and B.

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⁺]_i 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 carboxyamidotriazole. Carboxyamidotriazole is a cytostatic 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

Figure 7

TRPC3 expression in tissues. (A) The expression levels of TRPC3 in ovarian tissue samples of normal surface epithelium (a), benign (b), borderline serous tumours (c) and malignant serous tumours (d) were detected using immunohistochemistry (bar = 100 μ m). (B) The expression levels of TRPC3 in

malignant ovarian epithelial serous (a), mucinous (b), endometrioid (c) and clear cell (d) tumours were detected by immunohistochemistry (bar = 100 μ m).

low-expression group. 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

Table 1 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 disease-free survival between each parameter.

	Total cases				Serous type			
	P	HR	95% CI for HR		P	HR	95% CI for HR	
			Lower	Upper			Lower	Upper
Age	0.238	1.015	0.990	1.041	0.389	1.015	0.981	1.050
TRPC3 expression	0.003	2.802	1.406	5.586	0.001	4.073	1.753	9.462
Clinical stage	<0.001	6.795	2.985	15.465	0.001	7.069	2.136	23.392
Lymphatic metastasis	<0.001	5.358	2.746	10.457	0.001	3.828	1.754	8.354
Grade	0.002	1.927	1.278	2.905	0.034	1.691	1.041	2.747

HR, hazard ratio.

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.

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

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.

	Total cases				Serous type			
	P	HR	95% CI for HR		P	HR	95% CI for HR	
			Lower	Upper			Lower	Upper
Age	0.228	1.022	0.986	1.059	0.443	1.019	0.972	1.068
TRPC3 expression	0.039	2.866	1.056	7.777	0.039	3.766	1.073	13.226
Clinical stage	0.015	11.977	1.604	89.429	0.034	8.911	1.174	67.668
Lymphatic metastasis	<0.001	11.616	3.426	39.383	0.005	8.540	1.936	37.680
Grade	<0.001	11.616	3.426	39.383	0.137	1.695	0.845	3.398

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 TRPC 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.

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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.

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