

# Gonadotropins promote human ovarian cancer cell migration and invasion via a cyclooxygenase 2-dependent pathway

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**Abstract.** It is generally accepted that ovarian cancer is associated with local elevation of gonadotropins (FSH and LH), with repeated ovulation and accompanying expression of inducible cyclooxygenase 2 (COX2). However, the roles of gonadotropins and the concomitant elevation of COX2 in the development of ovarian cancer have not been fully characterized. Herein, we report that excessive FSH/LH exposure did not induce proliferation in ovarian cancer cell lines but significantly promoted cell migration and invasion. Moreover, FSH/LH treatment rapidly upregulated COX2 expression within 24 h, whereas COX1 expression remained unchanged. Further results showed that enhancement of epithelial-mesenchymal transition (EMT) and upregulation of matrix metalloproteinase (MMP)2 and MMP9 contributed to the stimulatory effect of gonadotropins on cell migration and invasion; these effects were sufficiently blocked by a selective COX2 inhibitor. In conclusion, the present study suggests that gonadotropin-induced migration and invasion in ovarian cancer may be caused by EMT and MMP upregulation via a COX2-dependent pathway.

## Introduction

Ovarian cancer is the leading cause of death among all gynecological malignancies. Despite advancements in the surgical and

systemic treatment of ovarian cancer, the 5-year survival rate remains low (1). The etiology of ovarian cancer also remains largely unclear. Some evidence has indicated that endocrine factors may influence the tumorigenesis of ovarian cancer (1,2). Ovarian cancer is more common in patients with elevation of the gonadotropins follicle stimulating hormone (FSH) and luteinizing hormone (LH), such as postmenopausal women or women who have received treatment for the induction of ovulation (3-5). Additionally, reduced risk for ovarian cancer is associated with multiple pregnancies, breastfeeding, and oral contraceptive use, which are all associated with lower levels of and reduced exposure to gonadotropins (1). Moreover, the concentrations of both FSH and LH in cysts and the tumor fluid of ovarian cancer are greater than in borderline tumors, benign tumors, and functional cysts of the ovary (6). The expression of FSH receptor (FSHR) and LH receptor (LHR) and the growth-stimulating effect of their ligands in normal and neoplastic ovarian surface epithelium (OSE) and ovarian cancer cells have been demonstrated (2,4,7-9), although some reports are controversial (10,11). Recently, some studies reported that treatment of ovarian cancer cells with gonadotropins *in vitro* enhances tumor angiogenesis and cell adhesion (2,12-14). Together, these observations suggest a crucial role for gonadotropins in the development and progression of ovarian cancer.

Cyclooxygenases (COXs) are a family of myeloperoxidases that catalyze the biosynthesis of prostaglandins (PGs) from arachidonic acid. COX1 is constitutively expressed in most tissues and plays a role in various physiological functions, whereas COX2 is transiently inducible by stimuli such as cytokines, growth factors, mitogens, tumor promoters and hormones and regulates inflammation, differentiation, mitogenesis, and angiogenesis (15-18). A number of studies have revealed that COX2 plays a critical role in the ovulatory process, whereas repeated ovulations were suggested to cause neoplastic transformation of the ovarian epithelium (4,9,19,20). COX2 is rarely found in normal OSE but is present in pre-malignant ovarian inclusion cysts and is expressed in most ovarian cancer cells (21,22). Additionally, to some extent, COX2 is an independent prognostic factor for overall survival in patients with ovarian cancer (23,24).

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*Abbreviations:* COXs, cyclooxygenases; PGs, prostaglandins; FSH, follicle stimulating hormone; LH, luteinizing hormone; FSHR, FSH receptor; LHR, LH receptor; OSE, ovarian surface epithelium; MMPs, matrix metalloproteinases; EMT, epithelial-mesenchymal transition

*Key words:* gonadotropins, COX2, migration and invasion, epithelial-mesenchymal transition, matrix metalloproteinases, ovarian cancer

However, the roles of gonadotropins and of the concomitant elevation of COX2 in ovarian cancer development and progression have not been fully characterized. Therefore, we conducted the present study to examine the effect of gonadotropins on metastasis-related proteases and the invasiveness of ovarian cancer cells, thus to identify the key regulator of the gonadotropin pathway in ovarian cancer cells.

## Materials and methods

**Cell lines, drugs, inhibitors, and ovary tissues.** The ovarian cancer cell lines SKOV3 and HO8910 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The SKOV3 cell line was cultured in McCoy's 5A medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, Gibco), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco) in a 5% CO<sub>2</sub> humidified atmosphere at 37°C. The HO8910 cell line was cultured in RPMI-1640 (Gibco) under the above-mentioned culture conditions. Recombinant human FSH (75 IU) and LH (75 IU) were purchased from Merck Serono (Darmstadt, Germany). NS-398, a selective cyclooxygenase-2 (COX-2) inhibitor, was purchased from Beyotime (Shanghai, China). In this study, 10 µmol/l of NS-398 was used, which specifically inhibits COX2 enzyme activity but has no effect on COX1 activity (25).

The ovary tissues, used as positive controls for FSHR and LHR detection, were obtained from patients of ovarian cancer undergoing surgical excision. The investigation has been conducted in accordance with the ethical standards and according to the Declaration of Helsinki and has been approved by the ethics committee of the China-Japan Friendship Hospital. Written informed consent was obtained from each patient.

**Immunocytochemical staining.** SKOV3 and HO8910 cells were seeded on 18-mm glass coverslips in a 6-well culture plate and cultured for 48 h. Coverslips were washed twice with PBS and fixed in ice-cold acetone for 5 min. After the cells were washed twice with PBS, they were permeabilized with 0.1% Triton X-100 in PBS for 5 min at room temperature. The coverslips were then washed with PBS and further incubated with normal goat serum (Boster, Wuhan, China) for 20 min at room temperature to block non-specific binding. The cells were then incubated with FSHR antibody (cat. PB1120; 1:200, Boster) or LHR antibody (cat. A01120; 1:500, Boster) at 4°C overnight. Rabbit IgG (cat. ab27478, Abcam, Cambridge, UK) was used as isotype control instead of primary antibody. The binding of the primary antibodies was visualized using a ChemMate Detection kit (cat. SV0002; Boster). The coverslips were lightly counterstained with Mayer's hematoxylin for 30 sec.

**Cell viability assay.** SKOV3 and HO8910 cells were seeded in 96-well plates at a density of 1000 cells/well and incubated for 24 h. To examine the effect of FSH and LH on the viability of SKOV3 and HO8910 cells, the cells were treated with 100 and 500 mIU/ml FSH and LH alone or in combination. The levels of gonadotropins were chosen because 100 mIU/ml are commonly achieved during ovarian hyperstimulation (COH) and in 2-3 years after menopause (10,26), while 500 mIU/ml is based on previous references (11,27). Cell viability was measured

Table I. Specific primer sequences for RT-PCR and qRT-PCR.

Gene name	Primer sequences (5'-3')
FSHR <sup>a</sup>	F: TATGACTCAGGCTAGGGGTC R: ATAAGGAACCTGGGGACTGT
LHR <sup>a</sup>	F: CACCCCGATGTGCTCCTGAA R: TAGAGTGATGACGGTGAGGG
COX1	F: CGCTCCAACCTTATCCCCAGTCCCC R: CTTTAGGCACAGAGGGCAGAATACG
COX2	F: TCAAGTCCCTGAGCATCTACGGTT R: CTGTTGTGTTCCCGCAGCCAGATT
E-cadherin	F: ACAATGCCGCCATCGCTTAC R: AACTCTCTCGGTCCAGCCCA
Slug	F: GCCCATTAGTGATGAAGAGGAAA R: AGCCCAGAAAAAGTTGAATAGGTC
Vimentin	F: AAGCAGGAGTCCACTGAGTA R: GCTTCAACGGCAAAGTTCTC
MMP2	F: AACTACGATGATGACCCGAA R: CTCCTGAATGCCCTTGATGT
MMP9	F: GTCTTCCAGTACCGAGAGAAAGCCT R: CAGGATGTCATAGGTCACGTAGCCC
GAPDH	F: CTTAGCACCCCTGGCCAAG R: GATGTTCTGGAGAGCCCCG

<sup>a</sup>Primer sequences for RT-PCR. F, forward; R, reverse.

using an MTS cell proliferation kit (Promega, Madison, WI, USA) per the manufacturer's instructions. Assays were performed in triplicate and repeated three times.

**Annexin V-FITC staining.** Cell apoptosis was evaluated using an Annexin V-FITC apoptosis kit (eBioscience, San Diego, CA, USA) according to the manufacturer's instruction. In brief, SKOV3 and HO8910 cells were cultured in 10-cm plates, and treated with 500 mIU/ml FSH and LH for 24 h. After that, cells were collected and washed with ice-cold PBS, and then resuspended in 100 µl of binding buffer, containing 5 µl of each Annexin V-FITC and propidium iodide (PI) solution. The samples were then examined using flow cytometer (FACSCalibur, BD Biosciences, Franklin Lakes, NJ, USA).

**Cell migration and invasion assays.** SKOV3 and HO8910 cells were grown in 6-well plates at a density of 5×10<sup>5</sup> cells/well to nearly confluence and replaced the growth medium with serum-free medium for 5-h incubation to arrest cell growth. Then the cell monolayer was scraped with a pipette tip to generate scratch wounds. After the cells were washed three times to remove cell debris, they were cultured in serum-free medium containing 500 mIU/ml FSH/LH and 10 µmol/l NS-398 alone or in combination. Images were obtained, and the widths of the gaps were measured and analyzed at 24 h.

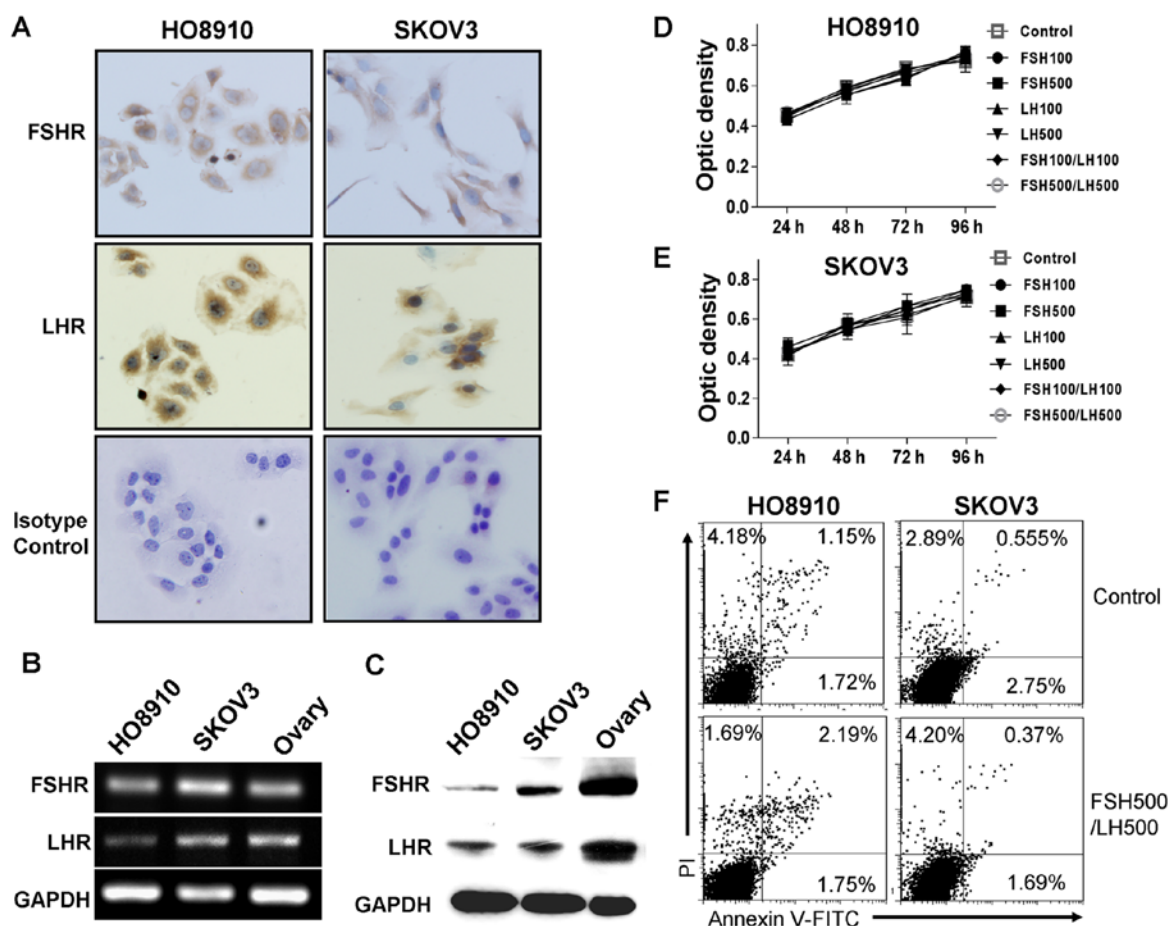


Figure 1. FSHR and LHR were expressed in SKOV3 and HO8910 cells but did not affect cell proliferation and apoptosis. Expression of FSHR and LHR was examined by immunocytochemical staining (A), RT-PCR (B), and western blotting (C). HO8910 and SKOV3 cells were treated with FSH and LH (0, 100, and 500 mIU/ml) alone or in combination, but no changes in proliferation (D and E) and apoptosis (F) were observed.

Cell invasion was assessed using 24-well Transwell inserts (Corning Inc., Corning, NY, USA) with 8- $\mu$ m pores coated with 1 mg/ml Matrigel (BD Biosciences). Cells in the upper compartment of the chamber were suspended in culture medium with 1% FBS, and the lower chamber contained culture medium with 20% FBS. After cell adhesion, the medium in the upper chamber was replaced with fresh medium containing 1% FBS, 500 mIU/ml FSH and LH, FSH and LH in combination with 10  $\mu$ mol/l NS-398, or NS-398 alone. After a 24-h incubation, the upper surface of the filter was erased carefully with a cotton swab, and then the cells that passed through the Matrigel matrix were fixed and stained with 0.05% crystal violet and counted in 5 random microscopic fields.

**RT-PCR and qRT-PCR.** Total RNA was extracted using TRIzol (Invitrogen) and converted to cDNA using Superscript III reverse transcriptase (Takara, Shiga, Japan). The cDNA was amplified by PCR using Premix Ex Taq™ (Takara). Quantitative real-time PCR (qRT-PCR) was performed using an iQ™ SYBR® Green Supermix kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) on a CFX96 Touch™ Real-time PCR instrument (Bio-Rad Laboratories, Inc.). The primer sets used in the RT-PCR and qRT-PCR are presented in Table I.

**Antibodies and western blots.** Antibodies specific to FSHR (cat. PB1120; 1:200) and LHR (cat. A01120; 1:200) were obtained

from Boster. Antibodies specific to COX1 (cat. ab109025, 1:2000), COX2 (cat. ab52237, 1:1000), E-cadherin (cat. ab76055, 1:500), slug (cat. ab51772, 1:500), and vimentin (cat. ab133260, 1:500) were purchased from Abcam. Antibodies specific to MMP2 (cat. #4022, 1:1000), MMP9 (cat. #3852, 1:1000), and GAPDH (cat. #2118, 1:2000) were obtained from Cell Signaling Technology (Beverly, MA, USA). Protein was prepared from the cells according to the kit manual (Thermo Fisher Scientific, Inc., Waltham, MA, USA). After electrophoresis, the proteins were transferred to polyvinylidene fluoride (PVDF, Merck Millipore, Darmstadt, Germany) membranes and probed with the indicated primary antibodies. Incubation with secondary antibodies of HRP-linked anti-rabbit IgG (cat. #7074, Cell Signaling Technology) or HRP-linked anti-mouse IgG (cat. #7076, Cell Signaling Technology) according to the species reactivity of primary antibodies was performed at room temperature for 1 h. The blots were developed with chemiluminescent substrate (Thermo Fisher Scientific, Inc.), and autoradiography was performed with X-OMAT film (Kodak, Rochester, NY, USA).

**Statistical analyses.** The data are presented as the mean  $\pm$  SD. To assess the statistical significance of differences, an analysis of variance (ANOVA, SPSS software, version 13.0; SPSS Inc., USA) was performed. A P-value of <0.05 was considered to indicate a statistically significant difference.

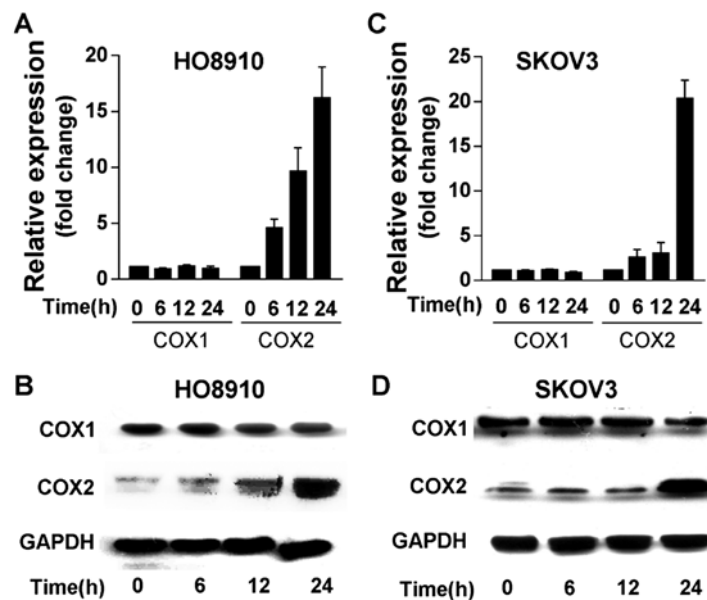


Figure 2. Effect of gonadotropins on COX1 and COX2 expression in ovarian cancer cells. HO8910 (A and B) and SKOV3 (C and D) cells were treated with 500 mIU/ml FSH/LH for 0 to 24 h as indicated. COX1 and COX2 expression was examined with qRT-PCR (A and C) and western blotting (B and D). COX2, but not COX1, was induced by FSH/LH in HO8910 and SKOV3 cells.

## Results

*Gonadotropins do not induce proliferation in ovarian cancer cells despite the expression of FSHR and LHR in these cells.* Gonadotropins are considered to play a pivotal role in neoplastic conversion and growth potential via their receptors FSHR and LHR during the tumorigenesis of ovarian cancers (9). In this study, we first showed the expression of FSHR and LHR in SKOV3 and HO8910 cells with immunocytochemical staining, RT-PCR, and western blotting (Fig. 1A-C). Then, we determined the effect of gonadotropins on the growth of SKOV3 and HO8910 cells. To our surprise, neither FSH nor LH stimulated the growth of ovarian cancer cells at either tested concentration (100 and 500 mIU/ml). Furthermore, the combination of FSH and LH also failed to increase the viable cell numbers as compared to prior treatment (Fig. 1D and E). In addition, no significant variations in the ratio of cell apoptosis were observed in either cell line after treatment with 500 mIU/ml FSH and LH for 24 h (Fig. 1F).

*Gonadotropins induce COX2 upregulation in ovarian cancer cells.* It is well documented that COX2 is an inducible, immediate early gene that, in addition to inflammatory stimuli, participates in the process of malignant cell transformation, while COX1 is constitutively expressed in most tissues (15). In our study, COX2 was rapidly induced after treatment with 500 mIU/ml FSH combined with 500 mIU/ml LH for 6 h and was steadily upregulated up to 24 h after continued treatment in HO8910 cells (Fig. 2A and B). In SKOV3 cells treated with FSH and LH, the expression of COX2 in SKOV3 cells increased slowly and then elevated sharply at 24 h (Fig. 2C and D). In both cell lines, COX1 maintained stable expression levels with or without FSH and LH treatment (Fig. 2). These results demonstrate that COX2 is selectively upregulated by FSH and LH in SKOV3 and HO8910 cells.

*Gonadotropins promote ovarian cancer cell migration and invasion.* To further investigate the role of excessive exposure to gonadotropins in the development and progression of ovarian cancer, the migration and invasion activities of SKOV3 and HO8910 cells with or without gonadotropin treatment was analyzed using wound-healing and Transwell assays, respectively. At 24 h after the scratch injury, the SKOV3 cells treated with 500 mIU/ml FSH and LH were able to cover 95.3% of the scratch, whereas the control cells only covered 54%. Importantly, NS-398, a specific COX2 inhibitor, blocked SKOV3 cell migration even in the presence of FSH and LH, which resulted in only 29% coverage of the scratch (Fig. 3). Similarly, in HO8910 cells, 500 mIU/ml FSH and LH significantly promoted cell migration compared to the control (73.3% compared with 38%), while NS-398 attenuated the effect of gonadotropins (18.3%) (Fig. 3). The invasion assays also showed that treatment with 500 mIU/ml FSH and LH significantly promoted ovarian cancer cell invasion, whereas cell invasion was effectively blocked by NS-398 (Fig. 4). As low level of COX2 expressed in ovarian cancer cells without FSH/LH stimulation, NS-398 treatment alone also, to some extent, showed inhibition effects in both cell migration and invasion. Collectively, these results demonstrated that the combination of FSH and LH notably enhanced ovarian cancer cell migration and invasion *in vitro*, but these effects were markedly inhibited by NS-398.

*Gonadotropin-induced cell migration and invasion occur due to COX2-dependent enhancement of epithelial-mesenchymal transition (EMT) and upregulation of MMP2 and MMP9.* EMT enables polarized epithelial cells to lose adherence, thus enhancing the migratory capacity and acquirement of invasive properties in these cells. In our study, the results showed that treatment with FSH and LH led to ongoing downregulation of E-cadherin and simultaneous upregulation of slug and vimentin in SKOV3 cells (Fig. 5). Furthermore, FSH and LH

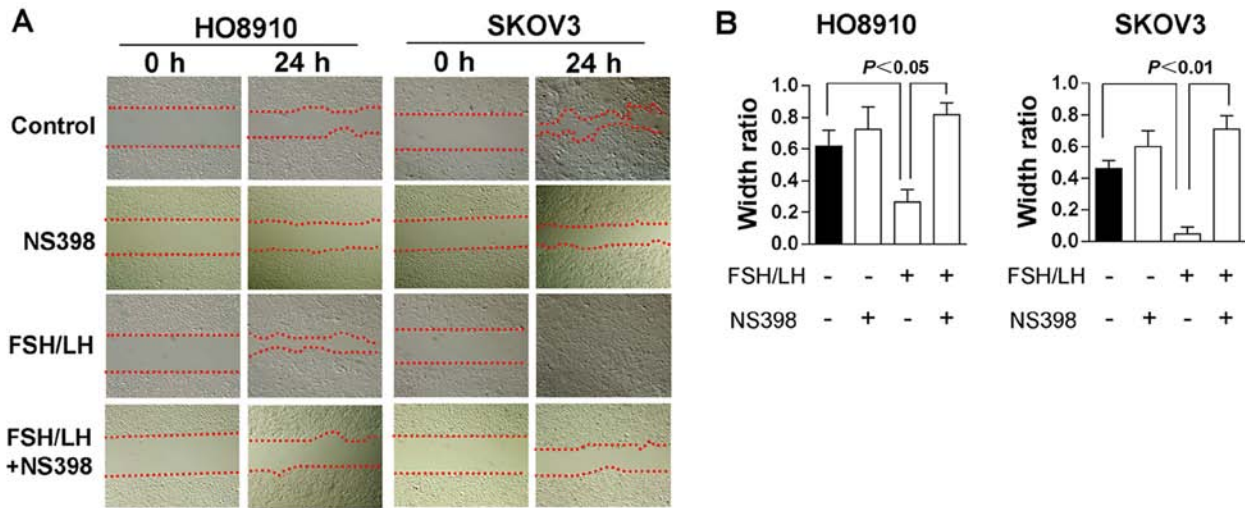


Figure 3. Gonadotropins promote ovarian cancer cell migration, whereas this effect was specifically suppressed by a selective COX2 inhibitor. (A) After scratch injury, HO8910 and SKOV3 cells were treated with 500 mIU/ml FSH/LH for 24 h in the presence or absence of 10  $\mu$ mol/l NS-398, or NS-398 alone. (B) The wound distances were measured, and cell migration was expressed as the average percentage of wound closure compared with time zero.

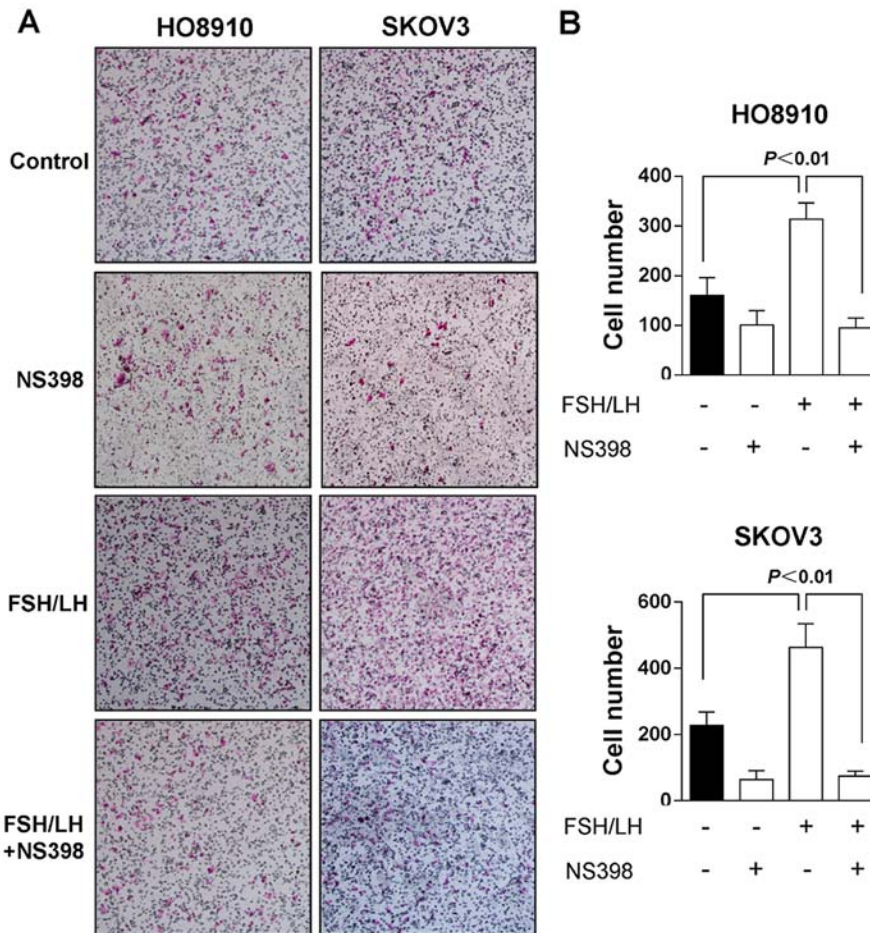


Figure 4. A selective COX2 inhibitor suppresses gonadotropin-induced cell invasion in ovarian cancer cells. (A) HO8910 and SKOV3 cells were treated with 500 mIU/ml FSH/LH for 24 h in the presence or absence of 10  $\mu$ mol/l NS-398, or NS-398 alone. Cells that passed through the Matrigel-precoated filters were stained with 0.05% crystal violet. (B) Cell invasion was quantified by determining the average number of cells per microscopic field.

stimulated increased expression of MMP2 and MMP9, which indicates that proteolysis, to some extent, plays a role in cell invasion induced by gonadotropins (Fig. 5). Notably, the effects of EMT and MMP expression induced by gonadotropins were

specifically blocked by NS-398. However, neither mRNA nor protein of COX2 expression was inhibited obviously by NS-398 (Fig. 5), which indicated that NS-398 just inhibited the enzyme activity but not the expression of COX2. In addition,

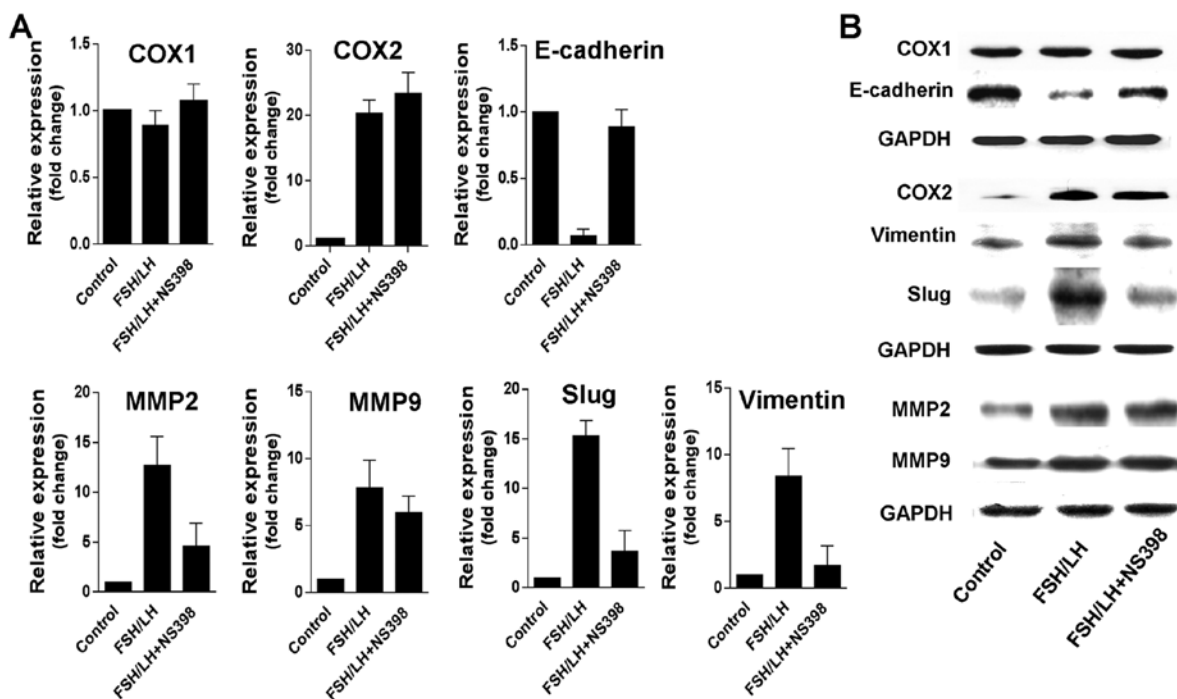


Figure 5. Gonadotropins enhanced EMT and MMP upregulation via a COX2-dependent pathway. SKOV3 cells were treated with 500 mIU/ml FSH/LH for 24 h in the presence or absence of 10  $\mu$ mol/l NS-398. The expression of COX1, COX2, E-cadherin, slug, vimentin, MMP2, and MMP9 was measured by qRT-PCR (A) and western blotting (B).

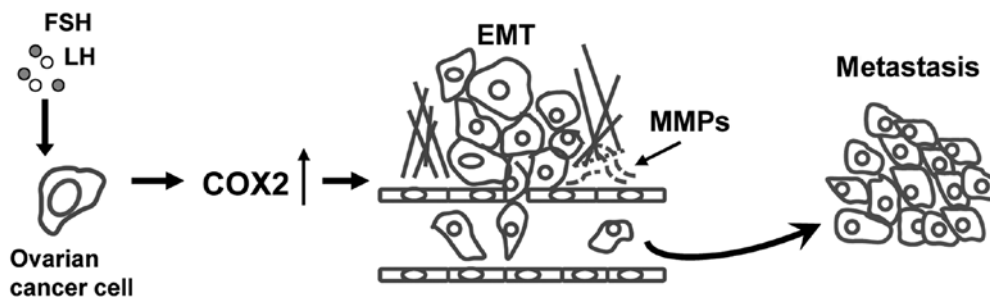


Figure 6. A model of how gonadotropins promote cell migration and invasion via a COX2-dependent pathway in ovarian cancer.

the same changes were observed in HO8910 cells (data not shown). Taken together, the promotion of cell migration and invasion by gonadotropins in ovarian cancer is mostly dependent on the activation of COX2 pathway (Fig. 6).

## Discussion

High levels of gonadotropins during ovulation, loss of gonadal negative feedback during menopause, and premature ovarian failure are all thought to be critical risk factors for ovarian cancer (1-3). FSH and LH levels in the cyst fluid from ovarian malignant tumors are significantly higher than those from benign tumors (6,28). Furthermore, gonadotropins not only induce both ovarian cancer cell proliferation and migration but also stimulate normal OSE proliferation (4,9). In this study, our data showed that the combination of 500 mIU/ml FSH and LH significantly promotes ovarian cancer cell migration and invasion within 24 h compared to cells not exposed to gonadotropins. However, we did not observe proliferation in ovarian

cancer cells treated with 100 and 500 mIU/ml FSH and LH alone or in combination. As previous studies have shown, the association of gonadotropins with the proliferation of OSE and ovarian cancer cells is inconsistent and has been shown to be stimulatory, irrelevant, or even inhibitory (2,11,13). Variations in the concentration of gonadotropin and density of cultured cells and gonadotropin receptors may, to some extent, contribute to these discrepancies.

To date, there is increasing evidence that the expression of COX2 is upregulated in a variety of human cancers, such as colorectal, skin, liver, breast, and lung cancers (29-33). Elevated COX2 expression also has been detected in malignant ovarian tumors, and high levels of COX2 expression are correlated with poor prognosis and decreased overall survival in human ovarian cancer (9,23). Our results showed that excessive exposure to gonadotropins induced significant upregulation of COX2 expression in SKOV3 and HO8910 cells, whereas COX1 expression was unchanged. Moreover, treatment with gonadotropins noticeably promoted cell migration and invasion in

SKOV3 and HO8910 cells, but these effects can be sufficiently blocked by NS-398. Therefore, these data indicate that COX2 plays an essential role in gonadotropin-induced cell migration and invasion.

With further exploration of the mechanisms underlying the above mentioned effects, we demonstrated that treatment with FSH and LH enhanced the expression of MMPs and epithelial-mesenchymal transition in both SKOV3 and HO8910 cells. MMPs are a family of highly homologous, protein-degrading, zinc-dependent enzyme endopeptidases; among the MMPs, MMP2 and MMP9 are notably correlated with tumor invasion and metastasis (34). EMT, a key event in the promotion of neoplastic progression and the development of metastasis, is characterized by multiple molecular changes, including down-regulation of E-cadherin, upregulation of its transcriptional repressors snail and slug, and augmentation of the expression of vimentin, fibronectin, and collagen (35). In our study, inhibition of COX2 by NS-398 restored E-cadherin expression and repressed slug, vimentin, MMP2 and MMP9 expression, which significantly blocked cell migration and invasion induced by gonadotropins. Experimental studies have also shown that COX2 inhibitors block tumor growth and enhance anticancer activity when combined with chemotherapeutic drugs, mainly through antiangiogenic and proapoptotic effects (36,37). Recently, Majumder and his colleagues reported that COX2 upregulation or overexpression in human breast cancer cells induced breast cancer stem cells via EP4/PI3K/AKT/NOTCH/WNT pathway and promoted tumor growth and metastasis in immunodeficient mice, which can be reliably blocked by COX2 inhibitor or EP4 antagonist (38). These data further suggest that COX2 can serve as not only a predictor of disease outcome but also a target for the treatment of ovarian cancers.

In summary, the current study, to a certain extent, demonstrates that gonadotropins promote cell migration and invasion via induction of COX2 expression in ovarian cancer cells, whereas specific inhibitors of COX2 significantly prevent these effects. However, some limitations of this study must be highlighted, such as incomplete elimination of endogenous COX2, an inappropriate method for detection of cell proliferation, lack of enough evidence from other ovarian cancer cell lines and primary cells to further support the work. Thus, to get a more reliable conclusion, further well-designed investigations are needed.

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