Expression of gonadotropin receptor and growth responses to key reproductive hormones in normal and malignant human ovarian surface epithelial cells

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Et al.
Expression of Gonadotropin Receptor and Growth Responses to Key Reproductive Hormones in Normal and Malignant Human Ovarian Surface Epithelial Cells

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ABSTRACT

Epidemiological data have implicated reproductive hormones as probable risk factors for ovarian cancer (OCa) development. Although pituitary and sex hormones have been reported to regulate OCa cell growth, no information is available regarding whether and how they influence normal ovarian surface epithelial (OSE) cell proliferation. To fill this data gap, this study has compared cell growth responses to gonadotropins and sex steroids in primary cultures of human OSE (HOSE) cells with those observed in immortalized, nontumorigenic HOSE cells and in OCa cell lines. Both malignant and normal cell lines/cultures responded equally well to the stimulatory actions of luteinizing hormone and follicle-stimulating hormone and to 17β-estradiol and estrone, although the latter estrogen has a much lower affinity for estrogen receptor than does the former estrogen. In normal HOSE cell cultures/lines, 5α-dihydrotestosterone was found to be more effective than testosterone in stimulating cell growth, but in OCa cell lines, 5α-dihydrotestosterone and testosterone are equally potent. One OCa cell line, OVCA 433, was found to be nonresponsive to androgen stimulation. In general, primary cultures of normal HOSE cells exhibited the greatest hormone-stimulated growth responses (>10-fold enhancement), followed by immortalized HOSE cell lines (4–5-fold enhancement) and by OCa cell lines (2–4-fold enhancement). Interestingly, progesterone (P4), at low concentrations ([10^-11 to 10^-10] M), was stimulatory to HOSE and OCa cell growth, but at high doses ([10^-8 to 10^-6] M), P4 exerted marked inhibitory effects. In all cases, cotreatment of a cell culture/line with a hormone and its specific antagonist blocked the effect of the hormone, confirming specificity of the hormonal action. Taken together, these data support the hypothesis that reproductive states associated with rising levels of gonadotropins, estrogen, and/or androgen promote cell proliferation in the normal OSE, which favors neoplastic transformation. Conversely, those states attended by high levels of circulating P4, such as that seen during pregnancy, induce OSE cell loss and offer protection against ovarian carcinogenesis.

INTRODUCTION

OCa varies widely in frequency among different geographic regions and ethnic groups, with high incidences observed in the Scandinavian, Western Europe, and North American and low incidences found in Asian countries (1). The majority of cases are sporadic, whereas about 5–10% of OCa cases are familial. Although all cell types of the human ovary may undergo neoplastic transformation, the vast majority (80–90%) of benign and malignant tumors are derived from the OSE and its cystic derivatives (2). The origin of OSE could be traced to the mesothelium of the embryonic gonads, or the Mullerian epithelium; therefore, ovarian tumors often resemble those of the fallopian tube, endometrium, and endocervix (2, 3).

Although the etiology of OCa remains poorly understood, evidence is mounting to indicate the involvement of gonadotropins and/or sex hormones in its etiology. Because OCa incidence increases dramatically in women above the age of 45 years and peaks at 10–20 years after menopause, it has been suggested that elevated levels of gonadotropins during this reproductive period are risk factors for the cancer (4–7). The gonadotropin theory is further supported by several case studies reporting development of OCa shortly after ovulation induction with fertility drugs such as clomiphene citrate or gonadotropins (7, 8). It has also been proposed that entrapment of OSE cells in inclusion cysts increases the odds of OSE neoplastic transformation, possibly due to exposure of these cells to a stromal hormonal milieu rich in androgens (2, 9, 10). In support of the androgen theory is the observation that women with polycystic ovary syndrome have a higher risk of developing OCAs, which is likely attributable to the higher levels of androgen present in their circulation. With regard to estrogens, earlier data are in inconclusive in demonstrating a positive relationship between estrogen usage and OCa risk (11–15). However, recent large-scale epidemiological studies (16–18) consistently demonstrate that postmenopausal usage of estrogen elevates OCa incidence in a manner dependent on usage duration. Finally, epidemiological data have established pregnancy, particularly one that occurs in late life, as a protective factor against OCa development (19). These findings, in conjunction with laboratory studies (20, 21) demonstrating induction of apoptosis in OCa cell lines by P4, raise the possibility that progesterins are protective against ovarian carcinogenesis. Taken together, these theories strongly argue for major roles played by reproductive hormones, such as those associated with the female cycle, pregnancy, perimenopause, and postmenopause, in ovarian carcinogenesis.

According to modern concept of hormonal carcinogenesis (22), endogenous and exogenous hormones enhance cell proliferation and thus enhance the opportunity for the accumulation of random genetic errors and the emergence of malignancy. Previous studies on hormones and OCa were focused primarily on the effects of pituitary and/or sex hormones on OCa cell growth (23–38). To the best of our knowledge, no information is available regarding whether and how key reproductive hormones regulate the growth of normal OSE cells. Answers to these questions are critical to our understanding of hormone-induced tumor initiation in the OSE. To fill this data gap, in this study, we have simultaneously compared the impacts exerted by gonadotropins and key sex steroids on primary cultures of HOSE cells with those observed in immortalized, nontumorigenic HOSE cells (39, 40) and in OCa cell lines (39). Because women are exposed to a great variety of endogenous hormones at wide concentration ranges during their lifetime, we have chosen to study the growth responses of HOSE/OCa cells to the predominant premenopausal estrogen, E2, the major postmenopausal estrogen, E1, the circulating androgen, T, the cellular androgen, DHT, the pregnancy hormone P4, and the gonadotropins FSH and LH at a wide dose range ([10^-11 to 10^-6] M). Direct
cell counting or a surrogate cell proliferation assay was used to quantify cell growth responses, and specific hormone antagonists were used to demonstrate specificity. Semiquantitative RT-PCR was used to demonstrate expression of FSH-R and LH-R in normal HOSE cells for the first time. Our data now show that gonadotropins, estrogens, and androgens are positive regulators of HOSE and OCa cell growth, whereas P4 is a negative regulator for both cell types.

MATERIALS AND METHODS

Primary Cell Cultures and Cell Lines. Four normal primary HOSE cell cultures (HOSE 693, HOSE 770, HOSE 783, and HOSE 785), four immortalized normal HOSE cell lines (HOSE 642, HOSE 301, HOSE 306, and HOSE 12-12), and four OCa cell lines (OVCA 420, OVCA 429, OVCA 432, and OVCA 433) were used in this study. The normal HOSE cell primary cultures, HOSE 693, HOSE 770, HOSE 783, and HOSE 785, were obtained from surface scrapings of normal ovaries removed from a 32-year-old patient with adenocarcinoma of the cervix, a 42-year-old patient with moderately differentiated squamous cell carcinoma of the cervix, a 42-year-old patient with leiomyoma, and a 72-year-old patient with inflamed bladder mucosa, respectively. The immortalized normal HOSE cell lines, HOSE 642, HOSE 301, HOSE 306, and HOSE 12-12, were established by human papillomavirus E6/E7 immortalization (39) of normal HOSE cells obtained from a 46-year-old patient with normal tissue, a 47-year-old patient with endometrioid adenocarcinoma of the ovary, a 53-year-old patient with breast cancer, and 39-year-old patient with ovarian stromal hyperplasia, respectively. OCa cell lines (OVCA 420, OVCA 429, OVCA 432, and OVCA 433) were established cell lines derived from freshly isolated ascites or tumor explants obtained from patients with late-stage serous ovarian adenocarcinomas according to Tsao et al. (39). The epithelial nature of the HOSE cell primary cultures and the HOSE cell lines was verified by immunostaining for K7, K8, K18, and K19 cytokeratins and vimentin as described previously (39). The HOSE cell primary cultures and immortalized cell lines exhibited uniform epithelial-like morphology; immunopositivity for cytokeratins K7, K8, K18, and K19; and immunonegativity for vimentin. The immortalized HOSE cell lines were shown to be nontumorigenic in nude mice and express no CA-125 (39). In addition, they responded to transforming growth factor β-induced growth inhibition (39). In contrast, the OCa cell lines (OVCA 420, OVCA 429, OVCA 432, and OVCA 433) expressed high levels of CA-125 and failed to respond to transforming growth factor β-induced growth arrest (39).

These cell lines were cultured and maintained at 37°C in a 5% CO₂ humidified atmosphere in medium 199 (Sigma Chemical Co., St. Louis, MO) and MCDB 105 (1:1; Sigma Chemical Co.) supplemented with 10% FCS (Sigma Chemical Co.), 100 units/ml penicillin (Sigma Chemical Co.), and 100 μg/ml streptomycin (Sigma Chemical Co.) under 5% CO₂. Normal and malignant cells grown in this medium after two or more passages exhibited uniform epithelial-like morphology.

Cell Proliferation Assay. Cell lines or primary cultures cultured in medium 199/MCDB 105 (1:1) were harvested when they reached ≥ 80% confluence, washed twice in PBS, and then plated into the wells of 96-well microculture plates at a density of 1000 cells/well in medium containing 10% activated charcoal (Sigma Chemical Co.)/dextran-70 (Pharmacia)-treated FBS. Forty-eight h after cell plating, the medium was replaced with the same medium containing either human FSH (Calbiochem, San Diego, CA; purity, 99%; contamination with growth factors, <1%), human LH (Calbiochem; purity, 99%; contamination with growth factors, <1%), E₂ (Sigma Chemical Co.), E₃ (Sigma Chemical Co.), T (Sigma Chemical Co.), or P₄ (Sigma Chemical Co.). To study the synergistic action of FSH and E₂ on cell growth, cells were cultured with a combination of E₂ and FSH. Steroids were solubilized in absolute ethanol. The exposure concentrations ranged from 10⁻²⁰ to 10⁻⁸ m for each hormone. The final concentration of ethanol in the medium was 0.1%. The control cells were exposed to ethanol vehicle without the testing hormone. The cells were treated with hormones for 5 days, with a fresh addition of hormone to ensure stable bioavailability. Because DHT was metabolized rapidly, cells were subjected to DHT treatment every 4 days. Hormone receptor antagonists were added 30 min prior to the addition of hormone to ensure receptor occupancy. Hormone receptor antagonists were added 30 min prior to the addition of hormone to ensure receptor occupancy. Hormone receptor antagonists were added 30 min prior to the addition of hormone to ensure receptor occupancy.

An equal amount of total RNA (1–3 μg) from the cellular total RNA sample was reverse-transcribed to cDNA using the GeneAmp RNA PCR kit (Perkin-Elmer, Foster City, CA). Aliquots (1–2 μl of 50 μl) of cDNA were subjected...
to hot-start PCR using AmpliTaq Gold DNA polymerase (Perkin-Elmer). The enzyme was activated by preheating the reaction mixtures at 95°C for 6 min before thermal cycling. This protocol was chosen to minimize nonspecific product amplification. Initially, to determine the conditions under which PCR amplification was considered significant at

\[ P < 0.05. \]

Statistical Analyses. Statistical analysis was carried out using ANOVA, followed by Tukey’s post hoc test. Values are presented as the mean ± SD and are considered significant at \( P < 0.05. \)

RESULTS

Transcripts of FSH-R and LH-R Are Expressed in Normal and Malignant HOSE Cells. The expression of FSH-R mRNA and LH-R mRNA in normal and malignant HOSE cells was investigated by semiquantitative RT-PCR. RT-PCR analyses of total cellular RNA prepared from four primary cultures of normal HOSE cells, four immortalized normal HOSE cell lines, and four OCa cell lines revealed that transcripts of FSH-R and LH-R were present in all cell cultures/lines (Fig. 1A and C). Relative FSH-R mRNA expression levels in the four OCa cell lines were higher than those found in normal HOSE cells in primary cultures or in immortalized cell lines (Fig. 1B). Conversely, relative LH-R mRNA expression levels in normal HOSE cell cultures/lines were higher than those observed in OCa cell lines (Fig. 1D). Nonetheless, the differences in receptor expression levels between normal and malignant HOSE cell lines were not dramatic.

Because we have used four different cell lines in each group, a representative cell line from each group (primary cultures, immortalized normal HOSE cells, and OCa cells) is shown in Figs. 2–5. In addition to the representative cell lines, any cell line that showed divergence in response to hormones compared with the other cell lines in the group is shown under the respective group.

FSH and LH Are Equally Potent in Stimulating Normal and Malignant HOSE Cell Growth. The effects of a 5-day treatment with FSH or LH at a dose range between \( 10^{-11} \) and \( 10^{-6} \) μg on the proliferation of normal and malignant HOSE cells were investigated. FSH and LH enhanced cell proliferation in primary cultures of normal HOSE cells (HOSE 693, HOSE 770, HOSE 783, and HOSE 785), in immortalized normal HOSE cell lines (HOSE 642, HOSE 301, HOSE 306, and HOSE 12-12), and in OCa cell lines (OVCA 420, OVCA 429, OVCA 432, and OVCA 433) compared with cell growth in the absence of hormonal stimulation. A representative cell line from primary cultures (HOSE 770), immortalized normal HOSE cells (HOSE 642), and OCa cell lines (OVCA 420) is shown in Fig. 2A. The hormone-induced cell growth exhibited a clear dose dependency, and both gonadotropins were found to be equally potent in stimulating cell growth in all cell cultures/lines. However, in the immortalized HOSE 12-12 cell lines, FSH might be more effective than LH in stimulating cell growth (Fig. 2A). Although gonadotropin significantly enhanced cell growth of all normal and cancerous HOSE cell cultures/lines, normal HOSE cells in primary cultures exhibited the best responses (8–14-fold increases), followed by those displayed in
PKA signaling pathway, cell cultures/lines were treated with FSH or 
adotropin-stimulated cell growth is mediated via a receptor-triggered 
cognate receptors and activate a stimulatory G-protein that leads to an 
appearance of two cell lines in a group are shown under the representative cell line. Primarily 
HOSE (HOSE 693, HOSE 770, HOSE 783, and HOSE 785), immortalized 
HOSE (HOSE 642, HOSE 301, HOSE 306, and HOSE 12-12), and malignant OCa (OVCA 420, OVCA 429, OVCA 432, and OVCA 433) cells were cultured at a density of (A) 1000 
cells/well in a 96-well plate in medium 199:MCDB 105 supplemented with 10% FBS (heat-inactivated, charcoal-stripped FBS) and 100 units/ml penicillin-streptomycin for MTT assay. 
After preincubation for 48 h, the cells were treated with different concentrations (10 
M) of FSH (○) or LH (□) for 5 days. The cell growth was assessed by MTT assay 
as described in “Materials and Methods.” The absorbance of wells not exposed to hormones was arbitrarily set as 1, and FSH- and LH-treated cell growth was expressed as the fold 
increase compared with the control. The representative cell line from primary HOSE (HOSE 770), immortalized HOSE (HOSE 642), and malignant OCa (OVCA 420) is shown. The 
immortalized HOSE line (HOSE 12-12) that showed divergence from the other lines in the group is shown under the representative cell line. B, to confirm the specificity of FSH and 
LH, 2 × 10^5 cells/T-25 flask were cultured alone (□) or cotreated with 10^{-5} M of either FSH or LH (●) and two doses of the PKA inhibitor H89 (10^{-5} M, ■, 10^{-6} M) for 5 days. 
The control cells were treated with vehicle. After 5 days, the number of cells was counted. Treatment of cells with FSH and LH induced proliferation of cells in a dose-dependent 
manner. Cotreatment with PKA blocker H89 abolished the response of normal HOSE cells to gonadotropins. The data are shown as the mean of two experiments with triplicate samples 
and represent the mean ± SD. *, P < 0.05.

immortalized normal HOSE cell lines (5–7-fold increases) and in OCa 
cell lines (3–4-fold increases; results not shown).

It is now well accepted that gonadotropins interact with their 
cognate receptors and activate a stimulatory G-protein that leads to an 
induction of cAMP, followed by activation of PKA and subse-
quent biological responses. To ascertain whether the observed go-
adotropin-stimulated cell growth is mediated via a receptor-triggered 
PKA signaling pathway, cell cultures/lines were treated with FSH or 
LH (at 10^{-8} M) for 5 days in the presence or absence of a PKA-
selective antagonist, H89 (at either 10^{-5} or 10^{-6} M). Exposure of cells 
to H89 abolished the gonadotropin-induced cell growth enhancement 
in normal and malignant HOSE cell cultures/lines in a manner 
dependent on the dose of the PKA antagonist (Fig. 2B). Furthermore, 
H89 by itself had no effect on cell growth.

E_2 and E_1 Are Equally Effective in Stimulating Normal 
and Malignant HOSE Cell Growth. When increasing concentrations 
(10^{-11} to 10^{-6} M) of E_1 or E_2 were added to primary cultures of 
normal HOSE cells (HOSE 639, HOSE 783, HOSE 785, and HOSE 
770; HOSE 770, representative cell line shown in Fig. 3A), immor-
talized normal HOSE cell lines (HOSE 642, HOSE 301, HOSE 306, 
and HOSE 12-12; HOSE 642, representative cell line shown in Fig. 
3A), and OCa cell lines (OVCA 420, OVCA 429, OVCA 432, and 
OVCA 433; OVCA 420, representative cell line shown in Fig. 3A), a 
dose-dependent increase in cell growth was observed in cell cultures 
challenged with an estrogen. An approximately 10–14-fold increase 
in cell growth was noted in primary cultures of normal HOSE 
cells exposed to the highest concentration (10^{-6} M) of E_1 or E_2 (results 
not shown). In contrast, both estrogens at this dose only induced a 6-fold 
increase in cell growth in immortalized normal HOSE cell lines and a 
3–4-fold increase in cell growth in OCa cell lines (results not shown). 
E_1 and E_2 were equally effective in enhancing cell proliferation in 
all cell lines studied, with the exception of HOSE 12-12 cells, which 
responded better to E_1 than to E_2 (Fig. 3A). Simultaneous treatment of 
cell cultures/lines with E_2 and FSH induced no additive effect on 
enhancement of cell growth (results not shown).

When normal and malignant HOSE cells were exposed to a 5-day 
treatment with 10^{-6} M E_1 in the presence or absence of ICI 182,780 
(10^{-5} or 10^{-4} M), a marked attenuation in E_2-induced growth 
enhancement was observed in cultures exposed to the antiestrogen (Fig. 
3B). ICI 182,780 is recognized as a pure antiestrogen, and it has been 
shown to inhibit the action of both estrogen receptor-α and estrogen 
receptor-β (44).

Differential Responsiveness of Normal and Malignant HOSE 
Cells to DHT- and T-induced Cell Growth Enhancement. Testos-
terone and DHT significantly stimulated cell growth in primary 
cultures of normal HOSE cells (HOSE 639, HOSE 783, HOSE 785, and 
HOSE 770), immortalized normal HOSE cell lines (HOSE 642, 
HOSE 301, HOSE 306, and HOSE 12-12), and malignant OCa cell 
lines (OVCA 420, OVCA 429, and OVCA 432, but not OVCA 433). 
The responses of HOSE 770, the representative cell line for primary
HOSE cells, HOSE 642, the representative cell line for immortalized HOSE cells, and OVCA 420, the representative cell line for malignant cells, are shown in Fig. 4A. Primary cell cultures of normal HOSE cells (HOSE 770, Fig. 4A) and immortalized normal HOSE cell lines (HOSE 642, Fig. 4A) were more responsive to DHT than T, whereas the OCa cell lines (Fig. 4A) responded equally well to both androgens. Although all of the immortalized normal HOSE cell lines were extra receptive to DHT, HOSE 306 showed a greater sensitivity to DHT (Fig. 4A). The OCa cell line OVCA 433 failed to respond to both T and DHT stimulation (Fig. 4A). An androgen-induced cell growth enhancement was found to be dose dependent (Fig. 4A) and reversible by cotreatment of cells with the antiandrogen 4-hydroxy flutamide (Fig. 4B) in all of the cell lines tested.

P4 Exerts Both Stimulatory and Inhibitory Effects on Normal and Malignant HOSE Cell Growth. The effects of P4 on cell proliferation in normal and malignant HOSE cell cultures/lines were investigated over a wide concentration range of 10^{-11} to 10^{-6} M. Results revealed that the steroid could stimulate and inhibit cell growth of normal and malignant HOSE cells depending on the dosage of exposure. All of the primary cell cultures of normal HOSE cells (HOSE 770, HOSE 783, and HOSE 770; HOSE 770 is shown as the representative cell line in Fig. 5A) except HOSE 693 (Fig. 5A) showed stimulation of cell growth when exposed to low concentrations of P4. Exposure to low concentrations (10^{-11} to 10^{-9} M) of P4 induced cell growth enhancement in two immortalized normal HOSE cell lines [HOSE 306 (Fig. 5A) and HOSE 301 (data not shown)], whereas the other two cell lines, HOSE 642 (shown in Fig. 5A) and HOSE 12-12 (data not shown), did not show any increase in cell number. OCa cell lines OVCA 432, OVCA 433, and OVCA 420 (OVCA 420 is the representative cell line shown in Fig. 5A) showed enhancement of cell proliferation in response to low concentrations (10^{-11} to 10^{-9} M) of P4, whereas the OCa cell line OVCA 429 failed to show proliferation of cells in response to low doses of P4 (Fig. 5A). However, when normal and malignant HOSE cell cultures/lines were challenged with higher doses of P4 (10^{-8} to 10^{-6} M), the steroid consistently led to growth inhibition (Fig. 5A, see the representative lines shown for each group). Interestingly, the lowest dose of P4 (10^{-11} M) induced the most cell growth enhancement in responsive cell cultures/lines, whereas the growth-inhibitory effect of P4 was clearly dose dependent, with the higher doses being more effective. Cotreatment of normal and malignant HOSE cells with the progesterin antagonist, RU 38486, at 10^{-5} or 10^{-4} M reversed the growth-inhibitory effects of 10^{-8} M P4 in all cell lines/cultures (Fig. 5B). The latter finding suggests that the antiproliferative effect of P4 on all of the cell cultures/lines is mediated via the P4 receptor.

DISCUSSION

A major goal of this research was to fill a data gap regarding the lack of information on hormonal regulation of normal HOSE cell growth. Additionally, an equally important aim was to generate investigational data to explain epidemiological findings that have implicated hormones as risk factors for OCa. In this investigation, we capitalized on our unique access to normal HOSE cells as primary...
HORMONAL REGULATION OF OSE CELL GROWTH

Fig. 4. Effect of T and DHT on cell proliferation in normal primary HOSE, immortalized HOSE, and malignant cells. Primarily HOSE (HOSE 693, HOSE 770, HOSE 783, and HOSE 785), immortalized HOSE (HOSE 642, HOSE 301, HOSE 306, and HOSE 12-12), and OCAs (OVCA 420, OVCA 429, OVCA 432, and OVCA 433) cell lines were cultured as described in the legend of Fig. 2. After preincubation for 48 h, the cells were treated with different concentrations (10^{-11} to 10^{-6} \text{M}) of T (□) and DHT (○) for 5 days. The cell growth was assessed by MTT assay as described in “Materials and Methods.” The absorbance of wells not exposed to hormones was arbitrarily set as 1, and T- and DHT-treated cell growth was expressed as the fold increase as compared with the control. The representative cell line from primary HOSE (HOSE 770), immortalized HOSE (HOSE 642), and malignant (OVCA 420) cells is shown. The immortalized HOSE cell line (HOSE 306) and the malignant cell line (OVCA 433) that showed divergence from the other lines in their respective groups are shown under the representative cell line. B. to confirm the specificity of T; 2 × 10^{5} cells/T-25 flask were cultured alone (□) or cotreated with 10^{-8} \text{M} T (□□) and two doses of 4-hydroxy flutamide (10^{-3} \text{M}; □□, 10^{-4} \text{M}, □□) for 5 days. The control cells were treated with vehicle. After 5 days, the number of cells was counted. Treatment of cells with T and DHT induced proliferation of cells in a dose-dependent manner in all of the cell lines. Cotreatment with 4-hydroxy flutamide abolished the response of normal HOSE cells to T. The data shown are the mean of two experiments with triplicate samples and represent the mean ± SD. *p < 0.05.

cultures or immortalized lines to conduct a comparative study to determine cell growth responses induced by gonadotropins and key sex steroids in these cells and in their malignant counterparts. We reported here, for the first time, coexpression of LH-R and FSH-R in normal HOSE cell cultures and immortalized lines. Both gonadotropins (LH and FSH) and the two estrogens (E, and E) were equally potent in enhancing cell growth in normal and malignant HOSE cells. The cellular androgen, DHT, was more effective than the circulating androgen, T, in stimulating the growth of normal HOSE cells in primary cultures, but the two androgens were equally potent in enhancing proliferation of OCA cells. Overall, primary cultures of normal HOSE cells exhibited the greatest responses to gonadotropin-, estrogen-, or androgen-stimulated cell growth when compared with those observed in immortalized HOSE cell lines or in OCA cell lines. Importantly, P at low doses was a promoter, but at higher doses, it was an unvaried growth inhibitor of normal and malignant HOSE cell growth.

Indirect evidence suggests that gonadotropins may have a role in the genesis and promotion of epithelial OCAs (7, 9, 16). The incidence of OCAs peaks 10–20 years after menopause, when gonadotropin levels are elevated. Case studies have reported development of epithelial OCAs in women undergoing fertility treatment, and an increased OCA risk has been reported in association with the use of fertility drugs in population studies (7, 8). A handful of laboratory studies have demonstrated that gonadotropins influence cell growth in some but not all OCA cell lines (23, 25, 26). In early studies (47–50), gonadotropin-binding sites were found in OCA cells. In recent studies (26, 51), transcripts of FSH-R and LH-R were detected in the great majority of ovarian tumors. In this study, we reported coexpression of FSH-R and LH-R transcripts in normal HOSE cells at levels comparable with those found in OCA cell lines. Both FSH and LH, at doses as low as 10^{-11} to 10^{-10} \text{M}, were stimulatory for normal and malignant HOSE cell growth. These doses translate to approximately 20–200 mIU/ml gonadotropin, concentrations that are well within the ranges of circulating FSH and LH reported in women. The circulating levels of FSH and LH in cycling women fluctuate between 10–25 and 18–50 mIU/ml, respectively (52). After menopause, circulating gonadotropins are elevated to levels around 66 mIU/ml for FSH and 23 mIU/ml for LH (53). In our experiments, the effects of FSH and LH on cell growth enhancement were blocked by the selective PKA inhibitor, H89, providing evidence of specificity for the gonadotropin action. When compared over a wide dose range, FSH and LH were found to be equally potent in stimulating normal and malignant HOSE cell growth. The latter finding is clearly in disagreement with a recent study (26) that found FSH and LH to have opposite effects in the growth regulation of two OCA cell lines, AO and 3AO, with FSH as the stimulator and LH as the inhibitor. Interestingly, we found normal HOSE cells in primary cultures to be more responsive to gonadotropin stimulation, producing a 10–14-fold increase in cell growth enhancement, as compared with a 3–5-fold increase in immortalized normal HOSE cell lines and OCA cell lines. This observation suggests that normal HOSE cells are hypersensitive to gonadotropin stimulation and may therefore undergo excessive cell proliferation under a postmenopausal hormonal milieu and be susceptible to malignant transformation. All in all, our findings are in accord with the theory that suggests rising levels of gonadotropins as a risk factor for OCAs and are in disagreement with the hypothesis that high levels of gonadotropins are protective against OCA development (54).
It has become clear with data from recent large case-control studies that OCa risk is significantly increased in postmenopausal women following long-duration ERT (18, 55–61). However, the mechanisms underlying this association have not been established. Findings from our present investigation have provided the first evidence that estrogens directly promote normal HOSE cell growth, which may favor malignant transformation. Interestingly, normal HOSE cells were found to be much more responsive to estrogen stimulation than their malignant transformation. Interestingly, normal HOSE cells were found to be much more responsive to estrogen stimulation than their malignant counterparts. In addition, the major finding that OCa risk is significantly increased in postmenopausal women with declining androgen levels (67) and in postmenopausal estrogen, E1 (62), displayed equal potency as the immortalized or transformed counterparts. In addition, the major finding that OCa risk is significantly increased in postmenopausal women with declining androgen levels (67) and in postmenopausal estrogen, E1 (62), displayed equal potency as the immortalized or transformed counterparts. The representative cell line from primary HOSE (HOSE 770), immortalized HOSE (HOSE 642, HOSE 301, HOSE 306, and HOSE 12-12), and OCa (OVCA 420, OVCA 429, OVCA 432, and OVCA 433) cell lines were cultured as described in the legend of Fig. 2. A, the cells were treated with different concentrations (10^{-11} to 10^{-8} M) of P4 (A) for 5 days. The cell growth was assessed by MTT assay as described in “Materials and Methods.” The absorbance of wells not exposed to hormones was arbitrarily set as 1, and P4-treated cell growth was expressed as the fold increase/decrease as compared with the control. The representative cell line from primary HOSE (HOSE 770), immortalized HOSE (HOSE 642), and malignant (OVCA 420) cells is shown. The primary cell line (HOSE 693), immortalized HOSE cell line (HOSE 306), and malignant cell line (OVCA 433) that showed divergence from the other cell lines in their respective groups are shown under the representative line. B, to confirm the specificity of P4, 2 x 10^5 cells/T-25 flask were cultured alone (□) or cotreated with 10^{-7} M P4 (□) and two doses of RU 38486 (10^{-5} M, □, 10^{-4} M, □) for 5 days. The control cells were treated with vehicle. After 5 days, the number of cells was counted. Treatment of cells with P4 inhibited proliferation of cells in a dose-dependent manner in all cell lines tested. Cotreatment with RU 38486 abolished the response of normal HOSE cells to P4. The data are shown as the mean of two experiments with triplicate samples and represent the mean ± SD. *P < 0.05.

Appreciable evidence implicates androgens in the pathogenesis of OCa. In premenopausal women, the circulating T levels are around 380 pg/ml or 10^{-9} M (52). Postmenopausal ovary is rich in androgen, as evidenced by T concentrations seen in ovarian veins. T (21) and DHT (37), at concentrations between 10^{-11} and 10^{-6} M, are well within the range capable of stimulating HOSE and OCa cell growth. According to the inclusion cysts theory, normal HOSE cells entrapped into inclusion cysts are predisposed to undergo neoplastic transformation, probably due to exposure to an androgen-rich stromal environment (2, 9, 10). In the present study, we observed an AR- and dose-dependent enhancement of cell growth in all normal and malignant HOSE cell cultures/lines. The cellular androgen, DHT, is apparently more potent than the circulating androgen, T, in stimulating normal HOSE cell growth. However, both androgens are equally effective in stimulating OCa cell growth. The differential cellular responses to T and DHT may be related to differential activities of 5α-reductase in these cell lines (65). Our finding that OVCA 433 fails to respond to both androgens could be explained by our previous report of a complete loss of AR mRNA expression in this OCa cell line (66). In addition, we have observed loss of AR expression in several other OCa cell lines (66). Hence, although androgens may play a significant role in the early genesis of OCa, such as when the OSE is entrapped in inclusion cysts, their contribution in OCa growth regulation may be significantly reduced during tumor progression in postmenopausal women with declining androgen levels (67) and in ovarian tumors with notable loss of AR expression (63).

Perhaps the most intriguing and novel finding of this study is the inverted U-shape dose-response curves observed for many, but not all, normal HOSE cell cultures in response to P4. P4 present at low doses (10^{-11} to 10^{-9} M) was proliferative, whereas P4 present at higher...
doses (10⁻⁸ to 10⁻⁶ m) was antiproliferative to most normal and malignant HOSE cells. In premenopausal women, serum P4 levels fluctuate in the range of 2–14 ng/ml or 6–47 × 10⁻⁹ m (52). The higher concentrations are only reached during the midluteal phase of the female cycle. Furthermore, a 10-fold increase in P4 is noted during pregnancy (68). Previous studies on the influence of P4 on OCA cell growth demonstrated a growth-inhibitory effect for the steroid (20, 36). Induction of apoptosis and p53 up-regulation were proposed as mechanisms mediating the P4-induced growth-inhibitory action on OCA cells (20). We recently obtained flow cytometry data to indicate that all HOSE and OVCA cell lines die via apoptosis after treatment with high doses of P4 (10⁻⁹ m).4 It is worthwhile to note that the proapoptotic effects of low-dose P4 on normal and malignant HOSE cell cultures/lines have not been reported previously. Taken together, the antiproliferative effects of P4 could explain the observed protective effect offered by pregnancy, sometimes referred to as the “pregnancy clearance effect.” According to this theory, pregnancy rids the OSE of early transformed cells. In this regard, our data would suggest that only high levels of P4, which are present during pregnancy, are effective in inducing massive cell death in the OSE and therefore offer a cancer prevention effect. Ironically, lower levels of P4, which are found during the luteal phase of the female cycle, are likely to be proproliferative to the OSE. Thus, whereas pregnancy may offer protection against ovarian carcinogenesis, continuous ovarian cycling may increase OCA risk.

In conclusion, we have observed coexpression of FSH-R and LH-R transcripts in all normal and malignant HOSE cell cultures/lines examined. Our data have identified FSH, LH, E1, E2, T, DHT, and P4, as potent antiproliferative factor for HOSE cells. Collectively, these results support the notion that elevated gonadotropin levels after menopause, rising estrogen and P4 levels during the female cycle, exposure of OSE to a high androgenic environment such as that seen in the inclusion cysts, and exposure to exogenous estrogens such as that seen during ERT are probable risk factors for OCAs. Conversely, high levels of P4 may offer protection against OCA development by ridding the OSE of early transformed cells, hence providing a mechanistic explanation for the phenomenon of pregnancy clearance effect. The putative protective effect of P4 also raises the issue of whether combined estrogen and progesterin replacement therapy is a safer alternative than ERT with respect to OCA development.

REFERENCES
