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# Expression of Gonadotropin Receptor and Growth Responses to Key Reproductive Hormones in Normal and Malignant Human Ovarian Surface Epithelial Cells[1]

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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 17$\beta$-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, 5$\alpha$-dihydrotestosterone was found to be more effective than testosterone in stimulating cell growth, but in OCa cell lines, 5$\alpha$-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[3] varies widely in frequency among different geographic regions and ethnic groups, with high incidences observed in the Scandinavia, 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 OCa, 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 progestins 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, $E_2$, the major postmenopausal estrogen, $E_1$, 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

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[3] The abbreviations used are: OCa, ovarian cancer; OSE, ovarian surface epithelial; HOSE, human OSE; LH, luteinizing hormone; LH-R, LH receptor; FSH, follicle-stimulating hormone; FSH-R, FSH receptor; RT-PCR, reverse transcription-PCR; $E_1$, estrone; $E_2$, 17$\beta$-estradiol; T, testosterone; DHT, 5$\alpha$-dihydrotestosterone; P4, progesterone; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PKA, protein kinase A; AR, androgen receptor; ERT, estrogen replacement therapy.

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, OVC A432, 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 $\beta$-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 $\beta$-induced growth arrest (39).

These cell lines were cultured and maintained at 37°C in a 5% $CO_2$ 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 $\mu$g/ml streptomycin (Sigma Chemical Co.) under 5% $CO_2$. Normal and malignant cells grown in this medium after two or more passages exhibited uniform epithelium-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%), $E_2$ (Sigma Chemical Co.), $E_1$ (Sigma Chemical Co.), DHT (Sigma Chemical Co.), T (Sigma Chemical Co.), or P4 (Sigma Chemical Co.). To study the synergistic action of FSH and $E_2$ on cell growth, cells were cultured with a combination of $E_2$ and FSH. Steroids were solubilized in absolute ethanol. The exposure concentrations ranged from $10^{-11}$ to $10^{-6}$ 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 12 h. Cell proliferation was measured by a MTT cell proliferation kit (Roche Diagnostics, Indianapolis, IN). After the incubation period, 10 $\mu$l of the MTT labeling reagent (final concentration, 0.5 mg/ml) were added to each well, and plates were incubated for 4 h in a humidified atmosphere. Finally, 100 $\mu$l of solubilization solution were added to each well, and plates were incubated overnight at 37°C. Cell growth was measured based on the cellular conversion of a tetrazolium compound to a colored formazan product over a period of 18 h. At the end of the incubation period, the amount of formazan formed was measured as absorbance at 570 nm in a spectrophotometer to determine the cell number in each well. Assays were performed in triplicate to generate mean values for the control and for each treatment group. Cell number, as measured by the rate of formazan formation, in control wells with untreated cells was arbitrarily assigned a value of 1. Relative cell growth was expressed as the fold increase over control untreated cultures. Data points in all figures are group mean values ± SDs from three separate experiments.

**Treatment of Normal and Malignant HOSE Cells with Hormones in the Absence and Presence of Hormone Receptor Antagonists.** Primary cultures of normal HOSE cells (HOSE 693, HOSE 770, HOSE 783, and HOSE 785), immortalized normal HOSE cell lines (HOSE 642, HOSE 301, HOSE 306, and HOSE 12-12), and OCa cell lines (OVCA 420, OVCA 429, OVCA 432, and OVCA 433) were seeded at $2 \times 10^5$ cells/T-25 flask (Falcon; Becton Dickinson Labware, Bedford, MA; 25-$cm^2$ culture area), allowed to attach during a 24-h period, and exposed to $10^{-8}$ M of either FSH, LH, $E_2$, T, or P4 in the presence or absence of the respective receptor or signaling antagonist. This dose was selected based on the results obtained from the cell proliferation assay, which demonstrated that for all hormones tested, this dose was at the midpoint of the dose-response curve. Two doses of receptor antagonist were used to block the action of the hormone. For FSH and LH, concentrations of $10^{-5}$ and $10^{-4}$ M of the PKA-selective inhibitor H89 (*N*-[2-(*p*-bromocinnamli)ethyl]-5-isoquinolinessulfonamide; 2HCL; Calibiochem) were added 30 min before treatment with $10^{-8}$ M of the gonadotropins. H89 has been shown to be a specific inhibitor of PKA ($K_i$ value, 0.048 $\mu$M) and to effectively block FSH and LH action at $10^{-4}$ M (41). For sex steroids, receptor-specific antagonists were used. A low ($10^{-5}$ M) and a high ($10^{-4}$ M) concentration of ICI 182,780 (a generous gift from Zeneca Pharmaceuticals, Macclesfield, United Kingdom), a pure estrogen receptor antagonist (42), 4-hydroxy flutamide (Schering, Kenilworth, NJ), an AR antagonist (43), or RU 38486 (Sigma Chemical Co.), a specific P4 receptor antagonist (44), were used to inhibit the action of $E_2$, T, or P4, respectively. The dosages of an antihormone used were based on literature reports of effective receptor antagonistic effect. The cell cultures were treated daily with hormones and hormone antagonists for a period of 5 days. After the treatment period, cell growth was determined by direct cell count on multiple aliquots of the cultures. Each experiment was carried out twice. The results are the means of two independent experiments.

**RNA Isolation and Semiquantitative RT-PCR.** Untreated normal and malignant HOSE cells were harvested from cell cultures when they reached approximately 70–80% confluence. Total cellular RNA was isolated using Tri reagent (Sigma Chemical Co.) according to protocols provided by the manufacturer. Multiple cautionary steps were routinely taken to ensure RNA quality and linearity of the semiquantitation method. The quality of each cellular RNA sample was checked carefully and controlled by the following steps: (*a*) measurement of absorbance at 260 and 280 nm; (*b*) running of a denaturing RNA gel capable of detecting possible RNA degradation, as judged by the integrity and intensity of the 18S and the 28S rRNA signals; and (*c*) conducting semiquantitative RT-PCR amplification of the 18S rRNA at low cycle numbers to ensure RNA quality and linearity of transcript quantification.

To investigate the relative expression levels of FSH-R and LH-R mRNA, semiquantitative RT-PCRs were performed. The oligonucleotide primers used to amplify human FSH-R and LH-R cDNA were previously published sequences (45, 46). The forward primer sequence for FSH-R amplification was 5′-GAGAGCAAGGTGACAGAGATTCC-3′ (nucleotides 97–120), and the reverse primer sequence was 5′-CCTTTGGAGAGAATGAATCTT-3 (nucleotides 417–439). For human LH-R amplification, the sense primer was 5′-CTTGGATATTTCCCACACAAA-3′ (nucleotides 676–698), and the antisense primer was 5′-TGGCATGGTTATAGTACTGGC-3′ (nucleotides 1270–1290). For amplification of human 18 S rRNA, the sense primer was 5′-TGAGGCCATGATTAAGAGGG-3′, and the antisense primer was 5′-CGCTGAGCCAGTCAGTGTAG-3′. The amplimers from cDNA of FSH-R, LH-R, and 18S ribosomal mRNA were 343, 615, and 623 bp, respectively.

An equal amount of total RNA (1–3 $\mu$g) from the cellular total RNA sample was reverse-transcribed into cDNA using the GeneAmp RNA PCR kit (Perkin-Elmer, Foster City, CA). Aliquots (1–2 $\mu$l of 50 $\mu$l) 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 for FSH-R, LH-R, and 18S ribosomal mRNA was in the logarithmic phase, different amounts of total RNA were reverse transcribed, and aliquots were amplified using a different number of cycles. A linear relationship was observed between the amount of RNA and PCR products when 3 $\mu$g of total RNA were used in the reverse transcription reaction and when 35, 30, and 18 PCR amplification cycles were performed for FSH-R, LH-R, and 18S rRNA, respectively. PCR for 18S rRNA was used as a control to rule out the possibility of RNA degradation and to control the variation in mRNA concentration in the RT reaction. The PCR program was 1 min at 94°C, 1 min at 60°C (annealing temperature), and 1 min at 72°C. mRNA-specific modifications included an annealing temperature of 58°C for amplification of FSH-R cDNA and an annealing temperature of 55°C for amplification of LH-R. The PCR products were fractionated on a 2% agarose gel and visualized by ethidium bromide staining. The fluorescence images were visualized under UV transillumination, captured on 665 negative film (Polaroid Co., Cambridge, MA), and converted into digitized signals with an image scanner, and the intensities of each band, which were derived from the area under each peak, were quantified by ImageQuant (Molecular Dynamics, Sunnyvale, CA). Signal intensities of FSH-R and LH-R amplimers were normalized to those of 18S rRNA products. Message levels were expressed as the ratio of the signal intensity of the PCR product of the receptor message to that of the 18S rRNA to produce arbitrary units of relative abundance. The reproducibility of the quantitative measurements was evaluated by three independent cDNA synthesis and PCR runs from each preparation of RNA. The means of the replicated measurements were calculated and are shown in the figures.

**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. 1, *A* 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. 1*B*). Conversely, relative LH-R mRNA expression levels in normal HOSE cell cultures/lines were higher than those observed in OCa cell lines (Fig. 1*D*). 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 $10^{-6}$ M 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. 2*A*. 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. 2*A*). 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

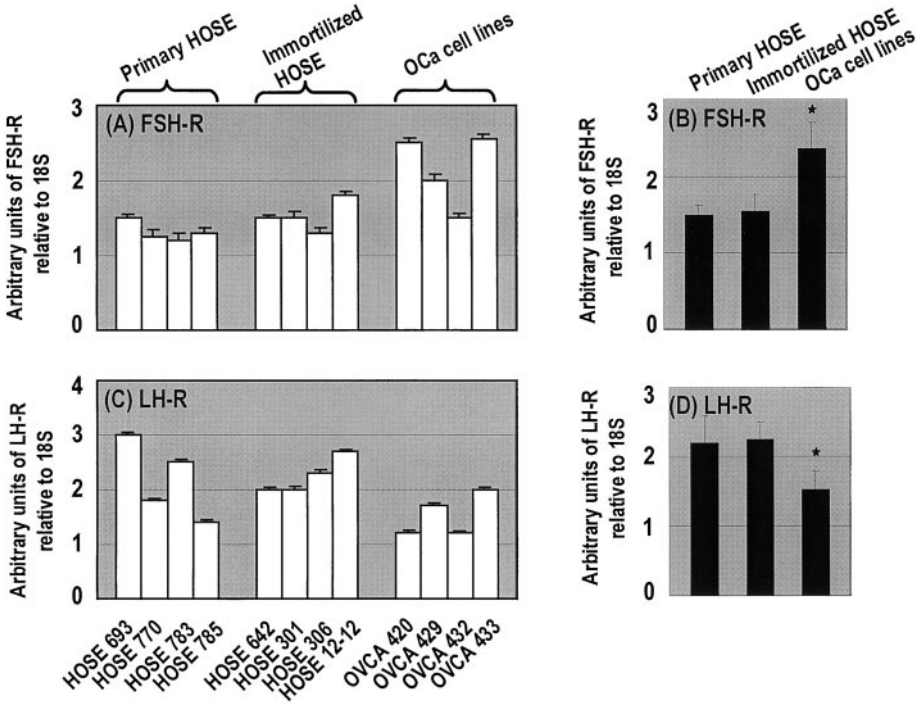


Fig. 1. Detection of mRNA for human FSH-R and human LH-R transcripts in total RNA samples from normal primary, normal immortalized, and malignant HOSE cell lines. Four normal primary (HOSE 693, HOSE 770, HOSE 783, and HOSE 785), normal immortalized (HOSE 642, HOSE 301, HOSE 306, and HOSE 12-12), and malignant OSE cell lines (OVCA 420, OVCA 429, OVCA 432, and OVCA 433) were used to isolate total RNA. Semiquantitative RT-PCRs were performed as described in "Material and Methods." *Open columns* represent the relative abundance of (*A*) FSH-R mRNA and (*C*) LH-R mRNA in individual primary HOSE cell cultures, immortalized HOSE cell lines, and OCa cell lines. Three independent RT-PCRs were performed with total RNA of primary HOSE cell cultures, immortalized HOSE cell lines, and OCa cell lines. Relative mRNA levels were expressed as arbitrary units derived from signal intensities of ethidium bromide-stained PCR products of the target cDNA normalized to those of 18S rRNA cDNA. The *open columns* and *error bars* represent the mean relative mRNA abundance ± SD ($n = 3$). The *black columns* represent group means of FSH-R (*B*) and LH-R (*D*) mRNA levels of all four primary HOSE cultures, four immortalized HOSE cell lines, and four OCa cell lines in each group. The data are shown as the means ± SD. *, statistically significant difference between mean transcript levels in OCa cell lines and those observed in primary HOSE cell cultures at $P < 0.05$.

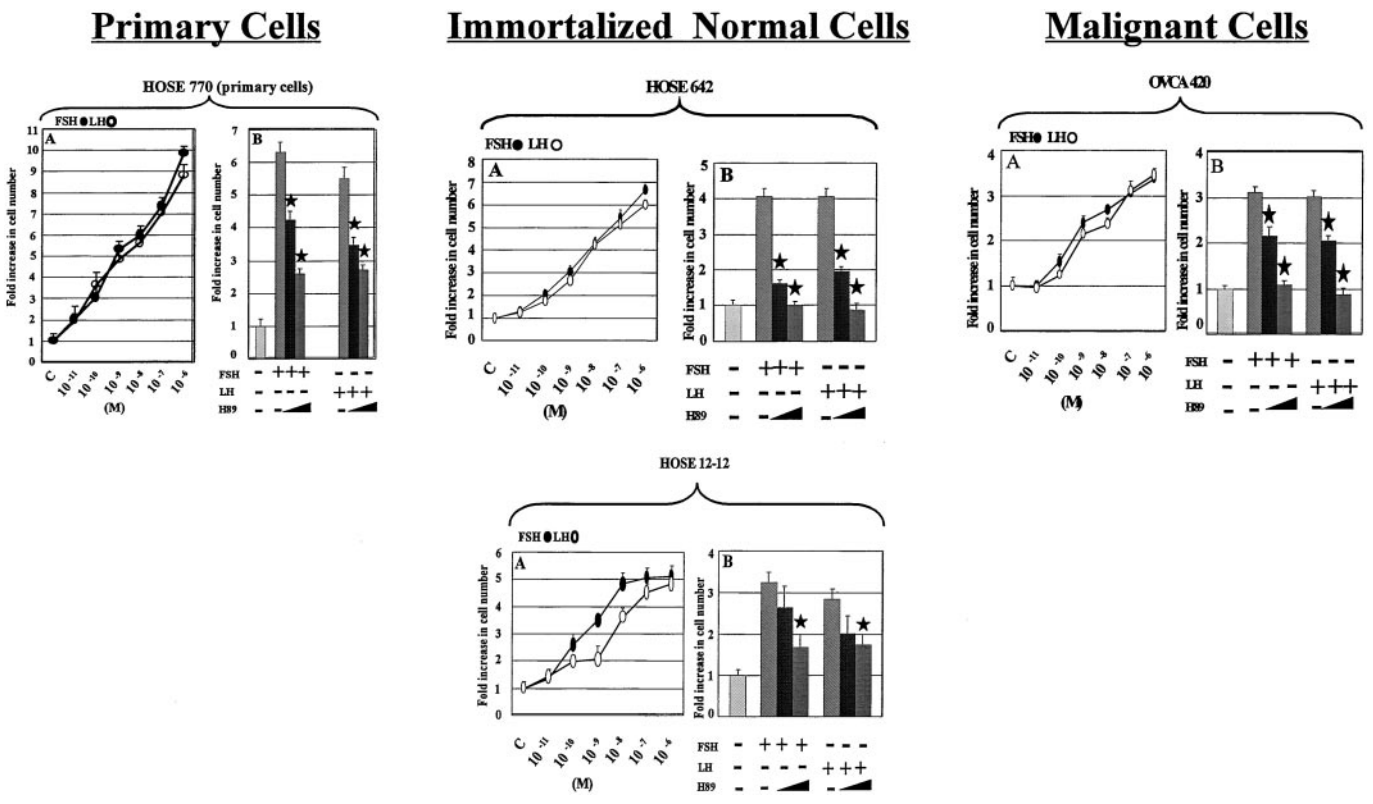


Fig. 2. Effect of FSH and LH on cell proliferation in normal primary HOSE, immortalized HOSE, and OCa cells. Representative cell lines from each group and any cell line that showed divergence from the other cell lines in a group are shown under the representative cell line. Primarily HOSE (HOSE 693, 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 ($10^{-11}$ to $10^{-6}$ 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, $2 \times 10^5$ cells/T-25 flask were cultured alone (▨) or cotreated with $10^{-8}$ M of either FSH or LH (▧) and two doses of the PKA inhibitor H89 ($10^{-5}$ 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 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 cyclic AMP, followed by activation of PKA and subsequent biological responses. To ascertain whether the observed gonadotropin-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 $10^{-4}$ 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. 2*B*). 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. 3*A*), immortalized normal HOSE cell lines (HOSE 642, HOSE 301, HOSE 306, and HOSE 12-12; HOSE 642, representative cell line shown in Fig. 3*A*), and OCa cell lines (OVCA 420, OVCA 429, OVCA 432, and OVCA 433; OVCA 420, representative cell line shown in Fig. 3*A*), 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. 3*A*). 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 $10^{-8}$ M $E_2$ 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. 3*B*). ICI 182,780 is recognized as a pure antiestrogen, and it has been shown to inhibit the action of both estrogen receptor-$\alpha$ and estrogen receptor-$\beta$ (44).

**Differential Responsiveness of Normal and Malignant HOSE Cells to DHT- and T-induced Cell Growth Enhancement.** Testosterone 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

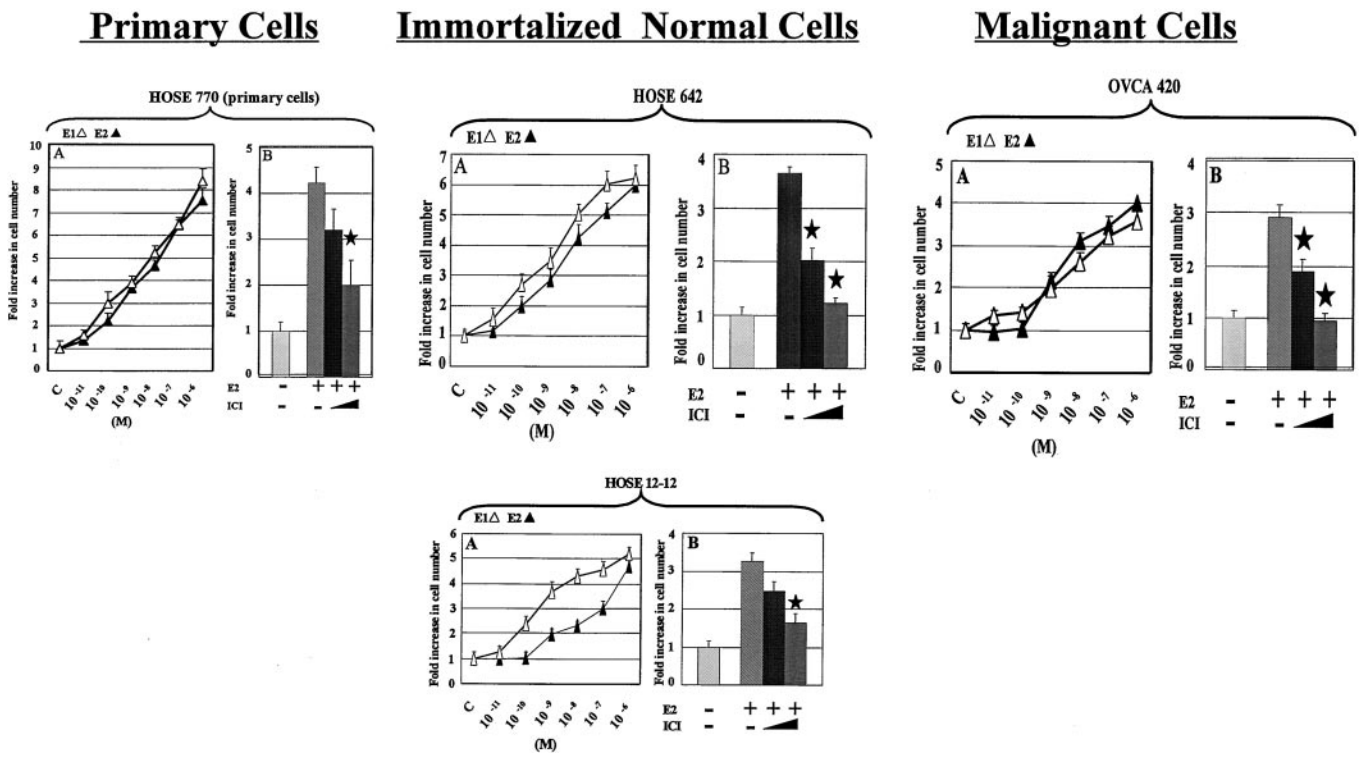


Fig. 3. Effect of $E_2$ and $E_1$ on cell proliferation in normal primary HOSE, immortalized HOSE, and malignant cells. Primarily HOSE (HOSE 693, HOSE 770, HOSE 783, and HOSE 785), immortalized HOSE (HOSE 642, HOSE 301, HOSE 306, and HOSE 12-12), and OCa (OVCA 420, OVCA 429, OVCA 432, and OVCA 433) cell lines were cultured as described in the legend of Fig. 2. After preincubation for 48 h in heat-inactivated charcoal-stripped FBS, the cells were treated with different concentrations ($10^{-11}$ to $10^{-6}$ M) of $E_2$ (▲) or $E_1$ (△) 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 $E_2$- and $E_1$-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 (OVCA 420) cells is shown. The immortalized HOSE cell line (HOSE 12-12) that showed divergence from the other cell lines in the group is shown under the representative cell line. *B*, to confirm the specificity of $E_2$, $2 \times 10^5$ cells/T-25 flask were cultured alone (▨) or cotreated with $10^{-8}$ M of $E_2$ (▧) and two doses of ICI 182,780 ($10^{-5}$ 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 $E_2$ and $E_1$ induced proliferation of cells in a dose-dependent manner. Cotreatment with ICI 182,780 abolished the response of normal HOSE cells to $E_2$. The data are shown as the mean of two experiments with triplicate samples and represent the mean ± SD. ∗, $P < 0.05$.

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. 4*A*. Primary cell cultures of normal HOSE cells (HOSE 770, Fig. 4*A*) and immortalized normal HOSE cell lines (HOSE 642, Fig. 4*A*) were more responsive to DHT than T, whereas the OCa cell lines (Fig. 4*A*) 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. 4*A*). The OCa cell line OVCA 433 failed to respond to both T and DHT stimulation (Fig. 4*A*). The androgen-induced cell growth enhancement was found to be dose dependent (Fig. 4*A*) and reversible by cotreatment of cells with the antiandrogen 4-hydroxy flutamide (Fig. 4*B*) 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 783, HOSE 785, and HOSE 770; HOSE 770 is shown as the representative cell line in Fig. 5*A*) except HOSE 693 (Fig. 5*A*) 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. 5*A*) and HOSE 301 (data not shown)], whereas the other two cell lines, HOSE 642 (shown in Fig. 5*A*) 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. 5*A*) 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. 5*A*). 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. 5*A*, 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 progestin 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. 5*B*). 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

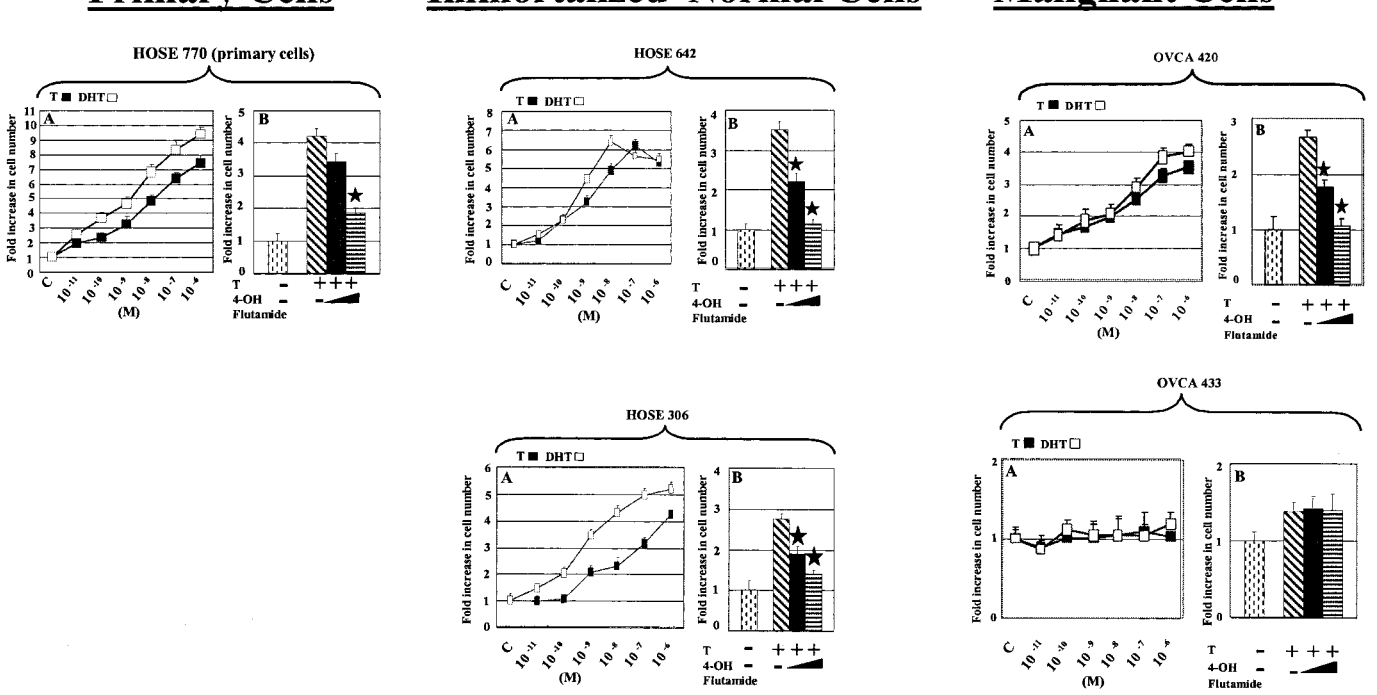


Fig. 4. Effect of T and DHT on cell proliferation in normal primary HOSE, immortalized HOSE, and malignant cells. Primarily HOSE (HOSE 693, HOSE 770, HOSE 783, and HOSE 785), immortalized HOSE (HOSE 642, HOSE 301, HOSE 306, and HOSE 12-12), and OCa (OVCA 420, OVCA 429, OVCA 432, and OVCA 433) cell lines 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 $10^{-6}$ 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, $2 \times 10^5$ cells/T-25 flask were cultured alone (▨) or cotreated with $10^{-8}$ M T (▧) and two doses of 4-hydroxy flutamide ($10^{-5}$ 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 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 ($E_1$ and $E_2$) 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, P4 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 OCa (7, 9, 16). The incidence of OCa peaks 10–20 years after menopause, when gonadotropin levels are elevated. Case studies have reported development of epithelial OCa 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 $10^{-10}$ 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 OCa and are in disagreement with the hypothesis that high levels of gonadotropins are protective against OCa development (54).

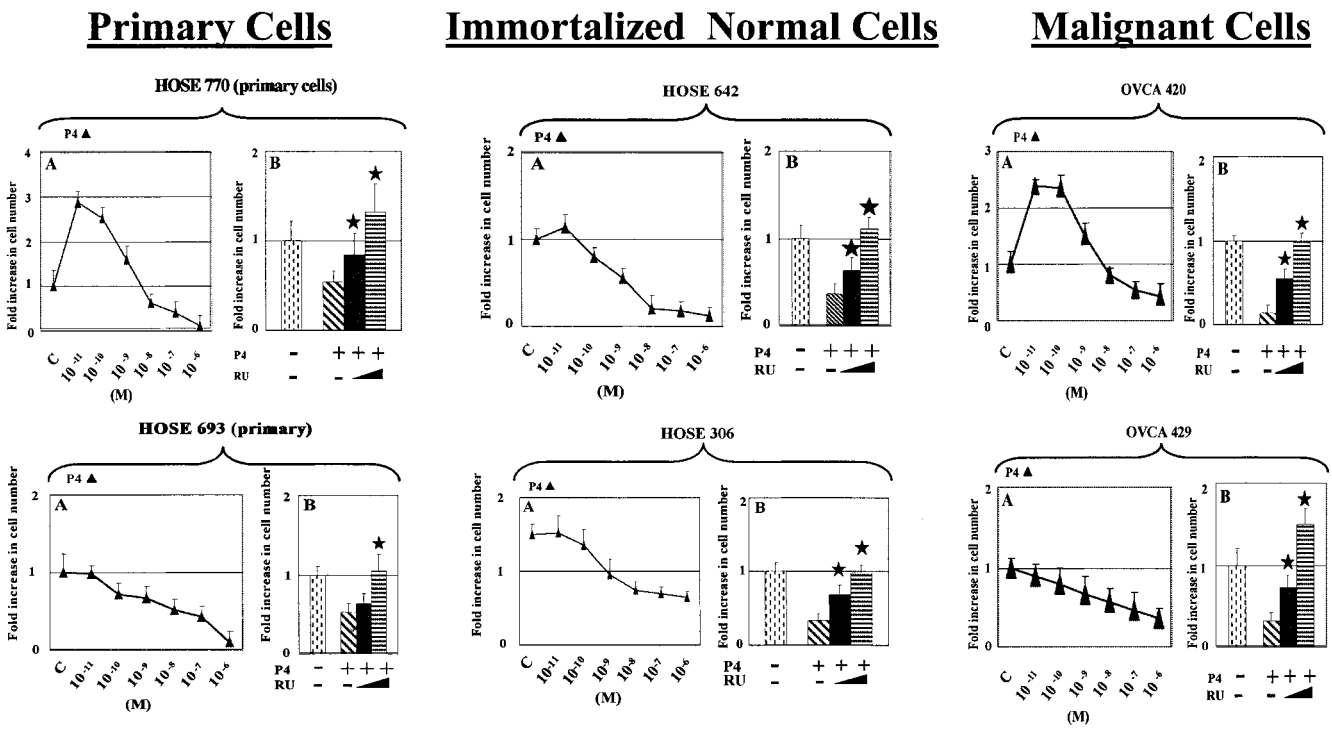


Fig. 5. Effect of P4 on cell growth in primary HOSE, immortalized normal HOSE, and malignant cell lines. Primarily HOSE (HOSE 693, HOSE 770, HOSE 783, and HOSE 785), immortalized HOSE (HOSE 642, HOSE 301, HOSE 306, and HOSE 12-12), and OCa (OVCA 420, OVCA 429, OVCA 432, and OVCA 433) cell lines were cultured as described in the legend of Fig. 2. *A*, the cells were treated with different concentrations ($10^{-11}$ to $10^{-6}$ M) of P4 (▲) 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, $2 \times 10^5$ cells/T-25 flask were cultured alone (▨) or cotreated with $10^{-8}$ 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. *, $P < 0.05$.

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 immortalized or transformed counterparts. In addition, the major postmenopausal estrogen, $E_1$ (62), displayed equal potency as the premenopausal ovarian-derived circulating estrogen, $E_2$, in stimulating normal and malignant HOSE cell growth. Because $E_1$ is a weak ligand for estrogen receptors (63), the popular view maintains that this estrogen exerts little estrogenic effect on target cells. Our data therefore provide a contrary perspective that suggests the effectiveness of postmenopausal estrogen in promoting OSE cell proliferation. In premenopausal women, circulating $E_2$ ranges from 10–20 pg/ml during the follicular and luteal phases and peaks at 200 pg/ml during ovulation (52). These circulating $E_2$ levels, at $3 \times 10^{-11}$ to $6 \times 10^{-10}$ M, are definitely effective in stimulating normal and malignant HOSE cell growth under our culture conditions. In perimenopausal women, $E_1$ sulfate, which serves as a stable circulating reservoir of estrogen, reaches levels as high as 100 pg/ml or $10^{-9}$ M. Ovaries of postmenopausal women do not secrete estrogens, but postmenopausal women have significant levels of $E_2$ (9 pg/ml or $3 \times 10^{-11}$ M) and $E_1$ (13.3–350 pg/ml or $4 \times 10^{-11}$ to $1 \times 10^{-9}$ M) in their circulation (64). These levels are still high enough to promote HOSE and OCa cell growth, based on the results of the current study.

Appreciable evidence implicates androgen 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 simulating 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 proproliferative, 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 $6-47 \times 10^{-9}$ 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 ($10^{-6}$ M).[4] It is worthwhile to note that the proproliferation 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" (19). 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, $E_1$, $E_2$, T, DHT, and low-dose P4 as positive growth regulators for HOSE cells. Conversely, P4 at high concentrations has been shown to be a 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 OCa. 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 progestin replacement therapy is a safer alternative than ERT with respect to OCa development.

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