

ESTROGEN STIMULATION OF OVARIAN SURFACE EPITHELIAL CELL PROLIFERATION

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SUMMARY

Ovarian cancer is the leading cause of gynecological cancer mortality, and 85–90% of this malignancy originates from the ovarian surface epithelium (OSE). The etiology of ovarian epithelial cancer is unknown, but a role for estrogens has been suspected. However, the effect of estrogens on OSE cell proliferation remains to be determined. Using the rabbit model, our studies have demonstrated that 17 β -estradiol stimulates OSE cell proliferation and the formation of a papillary ovarian surface morphology similar to that seen in human ovarian serous neoplasms of low malignant potential. Immunohistochemical staining of ovarian tissue sections with an antibody to the estrogen receptor α demonstrates its expression in both OSE cells and stromal interstitial cells. In primary ovarian cell cultures, the proliferative response of the epithelial cells to 17 β -estradiol depends on the expression of the estrogen receptor α in the epithelial cells. However, when the epithelial cells are grown together with ovarian stromal cells, their proliferative response to this hormone is greatly enhanced, suggesting the involvement of stromal–epithelial interactions. These studies suggest a role for estrogens and the estrogen receptor α in OSE growth.

Key words: ovarian cancer; estrogen receptor; ovarian surface epithelium; proliferation; stromal–epithelial interactions.

INTRODUCTION

Ovarian cancer is the leading cause of gynecological cancer mortality, and 85–90% of ovarian malignancy is thought to originate from the ovarian mesothelium, which is also termed ovarian surface epithelium (OSE) (Nicosia and Nicosia, 1988). OSE cells are mesodermal derivatives, a feature that they share with most reproductive tract epithelia (Nicosia, 1983). This embryological relationship may explain why both nonneoplastic and neoplastic OSE cells undergo Müllerian-oriented tubal or serous, endocervical, and endometrial metaplasias. Among the different ovarian epithelial tumors, serous ovarian cancer is the most common subtype. One of the histologic hallmarks of this neoplasm is the formation of OSE-lined fibrovascular structures, so-called papillae (Scully, 1979; Russell, 1994). The size and extension of these structures have been correlated with the malignant potential of serous neoplasms. Papillae are usually fewer, broader, and less proliferative in serous cystadenomas, but become much more complex and covered by stratified ovarian OSE cells in low malignant potential and frankly malignant serous tumors (Granberg et al., 1989; Ghossain et al., 1991). It has also been observed that pseudoneoplastic OSE papillae are associated with endocrine dysfunctions, such as polycystic ovaries and luteinized unruptured follicles (Motta et al., 1980), suggesting that their genesis may be stimulated by hormones.

Estrogens are the major female hormones with mitogenic and morphogenic activities on a variety of tissues including uterus, va-

gina, and mammary gland. A causative effect of estrogens on the proliferation and neoplastic transformation of OSE cells has long been suspected. Such a possibility has been based on high concentrations of estrogens in the follicular fluid that bathes the ovarian surface (OS) at the time of ovulation and on the reproduction-related risk factors that epithelial ovarian cancers share with other known estrogen-sensitive cancers such as those of breast and uterus. However, a controversy exists about the pathogenetic role of estrogens in ovarian tumorigenesis. To understand such a role, it is essential to determine the effect of estrogens on the putative precursor cell of epithelial ovarian cancer—the OSE cell.

The effect of estrogens is mediated through the estrogen receptor (ER) that belongs to the steroid/thyroid receptor superfamily, a group of ligand-regulated transcription factors (Evans, 1988; Tsai and O'Malley, 1994). Two ERs have been cloned thus far: the well-studied receptor, now designated as ER α , and the newly cloned ER β (Kuiper et al., 1996). They both have a structure typical of members of the steroid/thyroid receptor superfamily, which contains a conserved deoxyribonucleic acid (DNA)-binding domain or C region in the middle of the molecule and a conserved hormone-binding domain at the E region close to the C-terminus. Two transcriptional activation functions (AFs), AF2 and AF1, are located, respectively, at the conserved E region and the N-terminal A/B region; the latter is quite divergent between the two ERs as well as between ERs and other steroid receptors. In response to either estrogen binding or/and activation by growth signals, ERs act in a tissue- and gene-specific manner to stimulate or repress the expression of specific estrogen response element (ERE)-containing genes through the two AFs (Tora et al., 1989; Tzukerman et al., 1994). They also regulate the expression of genes whose regulatory sequence contains

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no typical EREs, presumably through protein–protein interaction with other transcription factors such as components of AP1 (Paech et al., 1997). It has been shown that ER β is expressed mainly in granulosa cells in the ovary (Saunders et al., 1997). However, ER α has been reported to be expressed in normal human OSE cells as well as in many ovarian epithelial tumors (Hamilton et al., 1981; Adams and Auersperg, 1983; Isola et al., 1990).

Our laboratory has utilized a rabbit model to investigate the effect of hormones and growth factors on OSE cell proliferation. Our studies indicate that 17 β -estradiol markedly stimulates OSE growth and papillogenesis in vivo and leads to the genesis of an ovarian surface morphology remarkably similar to that observed in human ovarian serous neoplasms of low malignant potential. Short-term ovarian cell cultures further demonstrate that the estrogen response depends on the OSE ER α expression and that the response is greatly enhanced by ovarian stromal (OS) cells. Treatment of rabbits with estrogen for 6 mo induced bilateral ovarian papillomas. Taken together, these studies suggest that 17 β -estradiol acts as a mitogen for OSE cells and stromal–epithelial interactions, mediated through ER α , may play an important role in the proliferative response of OSE cells to this hormone.

MATERIALS AND METHODS

Animals. Female New Zealand White rabbits, 4- to 5-mo-old, were purchased from Cummings (Dade City, FL). All studies were conducted in accordance with the principles and procedures outlined in the NIH Guidelines for Care and Use of Experimental Animals. Animals were fed with rabbit Purina chow and water ad libitum and individually kept for a minimum of 3 wk before tissue harvest or hormonal treatment to rule out pseudopregnancy.

In vivo hormonal treatment. Rabbits were implanted subcutaneously with time-release pellets (Innovative Research of America, Toledo, OH) containing either human follicle stimulating hormone (25 μ g/d), human luteinizing hormone (25 μ g/d), prolactin (250 μ g/d), 17 β -estradiol (10 μ g/d), progesterone (50 μ g/d), testosterone (50 μ g/d), or vehicle (ethanol for steroids and deionized water for peptides). After a 28-d treatment, animals were euthanized by sodium pentobarbital overdose (50 mg/lb body weight). One ovary from each rabbit was removed to determine hormone-induced cell proliferation while the contralateral ovary was used to quantify the number of papillae. To study the effect of long-term estrogen treatment on OSE cells, rabbits were treated with estrogen pellets for 6 mo and pellets were replaced with fresh ones every 4 wk.

Analysis of hormone-stimulated OSE cell proliferation. To quantify the growth effects of steroid and peptide hormones, one ovary from each animal ($n = 3$ ovaries per group) was incubated in 10 ml of antibiotics-rich M199 medium containing 50 μ Ci of 3 H-methylthymidine (0.5 μ Ci/ml, NEN, Boston, MA) under 5% CO₂/95% air with shaking at 100 rpm. After 1 h, OSE cells were isolated by a stepwise procedure involving successive collagenase digestion, mechanical dissection, 1 g sedimentation, and trypsinization (Giacomini et al., 1995). Isolated OSE cells were counted, sonicated for 15 min, and treated overnight at 4° C with 2% bovine serum albumin, and 0.4 N perchloric acid (PCA). After a wash with PCA, solubilization in Protosol® (NEN, Boston, MA) and resuspension in Econofluor® (NEN), the acid-precipitable 3 H-methylthymidine incorporated into the DNA was measured in a scintillation counter. OSE cell number and 3 H-methylthymidine incorporation were expressed as mean cell number $\times 10^{-6}$ per ovary and mean CPM $\times 10^{-3}$ per ovary, \pm standard error of mean (SEM), respectively.

Analysis of hormonal effect on rabbit ovarian surface morphology. To determine the morphogenetic effect of the hormones, the contralateral ovary of each animal was bisected along a perihilar sagittal plane, fixed in buffered 2.5% glutaraldehyde, and processed for scanning electronic microscopy. The number of OSE papillae was quantified at $\times 90$ magnification in six to eight 1.6-mm² areas of each bisected ovarian half using an eight-square grid directly superimposed on the screen monitor. Squares were chosen using a random number generator and the number of the OSE papillae was expressed as mean $\times 10^{-1}$ per ovary \pm SEM. To show the estrogen-induced papillomas, dissected whole ovaries were directly photographed.

In vitro analysis of the proliferative response to 17 β -estradiol of OSE cells cultured alone or with ovarian stromal cells. To assay the response of OSE cells to 17 β -estradiol in vitro, OSE cells were isolated from rabbits as described (Giacomini et al., 1995). The isolated OSE cells are over 98% pure as judged by general morphology and cytokeratin immunoreactivity. Cells were expanded in vitro in M199 medium containing 15% fetal bovine serum (FBS). After 1 wk, cells were digested with trypsin and plated at about 8 \times 10⁴ cells/well density in six-well plates precoated with 1 ml of HL-1 medium (Ventre Laboratories, Portland, ME) containing 8 μ g/ml fibronectin. OSE cells were then exposed to 17 β -estradiol, ICI 182,780 and ethanol in serum-free HL-1 medium. Cells were fed every 2 d with fresh HL-1 medium, and such a treatment continued for 6 d before dissociation by trypsin and cell-counting in a hemocytometer.

To assess the influence of OS cells on the proliferative response of OSE cells to 17 β -estradiol, OSE cells were cocultured with equal number of OS cells isolated as described (Giacomini et al., 1995). OS cell isolates are composed mainly of interstitial cells and fibroblasts. OS cells and cocultures were treated similarly to OSE cells cultured alone (see above). After culture and determination of cell number, representative cell aliquots were spun onto glass slides, fixed immediately in 95% ethanol, and stained with an anti-ER α antibody (see below) to determine the ER expression or with an anti-cytokeratin antibody (see below) to distinguish OSE cells (cytokeratin-positive) from OS cells (cytokeratin-negative). The percentage of OSE and OS cells in the mixed cultures was determined by counting cytokeratin-positive and -negative cells in 10 randomly chosen microscopic areas at $\times 10$ magnification.

Each experiment was done at least twice and duplicate samples were analyzed in parallel for each data point.

Cytokeratin and ER α immunohistochemistry. The preparation of tissue sections and cytocentrifuge slides of cultured cells and the immunostaining technique for low molecular weight cytokeratin (CAM 5.2) have been previously described (Giacomini et al., 1995). To detect ER α expression in cultured cells, OSE cells were spun onto glass slides, fixed with 95% ethanol, and stained with mouse anti-human ER α antibody, 1D5 (DAKO Corporation, Carpinteria, CA) at 1:100 dilution. To detect ER α expression with 1D5 in ovarian paraffin sections, antigen retrieval was first achieved by heating the deparaffinized sections in a microwave oven set at 1100 W four times at 5 min each in 10 mM sodium citrate, pH 6.0. The signal was detected by an immunoperoxidase method (Giacomini et al., 1995).

Cell transfection and ER transcriptional assay. Isolated OSE cells with or without trypsin digestion were grown for 1 wk in M199 medium supplemented with FBS and antibiotics (penicillin and streptomycin). Twenty-four hours before transfection, cells were plated in six-well plates in M199 medium at a density of 2 \times 10⁵ cells/well. Subsequently, cells were washed with Hank's balanced salt solution (HBSS), and 2 ml of HL-1 serum-free medium or phenol red-free Dulbecco modified Eagle medium (DMEM) supplemented with 5% charcoal-stripped FBS was added to each well. Cells were then transfected with 0.5 μ g/well of a β -galactosidase expression vector, Plemgal (Lee et al., 2000), 0.5 μ g /well of an estrogen reporter, ERE e/b Luc, and with or without 0.1 μ g/well of a mammalian expression vector for human ER α , PlemER (Smith et al., 1997). Cells were transfected using Lipofectamine Plus from GIBCO/BRL (Gaithersburg, MD) following their procedures. After incubation with the DNA complex for 2 h, cells were washed three times with HBSS and 2 ml of HL-1 serum-free medium, or phenol red-free DMEM medium containing 5% charcoal-stripped FBS, were added to the cells. Cells were then treated for another 24 h with either ethanol (vehicle control) or 10⁻⁸ M 17 β -estradiol before being harvested.

Luciferase and β -galactosidase activities were determined using the Dual-Light kit from Tropix Inc. (Bedford, MA), following the company's protocols. After the activity was determined using a microtiter luminometer from Dynex (Chantilly, VA), luciferase activity was normalized to the β -galactosidase activity. Duplicate samples were analyzed for each data point.

RESULTS

Proliferative and morphogenetic response of OSE cells to hormones in vivo. To understand the hormonal regulation of OSE cells, rabbits were treated with various hormones, and the growth as well as the morphogenetic response of OSE cells to such hormones was analyzed as described in "Materials and Methods."

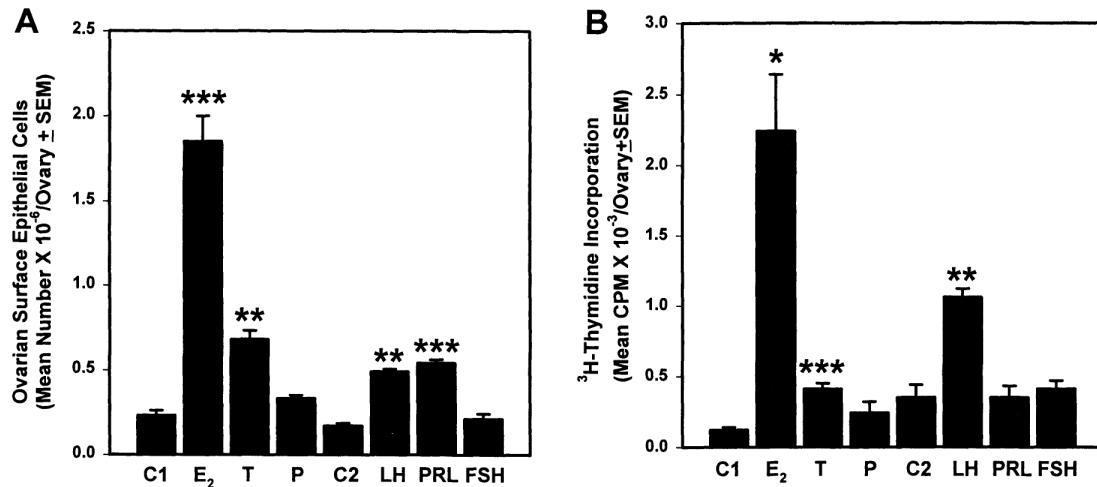


FIG. 1. Growth stimulation of OSE cells by steroid and peptide hormones. Groups of three animals each were treated with various hormones as described in "Materials and Methods." OSE cell growth (A) and ^{3}H -thymidine incorporation (B) were determined using one ovary from each animal. Abbreviations: FSH: human follicle stimulating hormone; LH: human luteinizing hormone; PRL: prolactin; E₂: 17 β -estradiol; P: progesterone; T: testosterone; C1: ethanol control for steroids; C2: deionized water control for peptides. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

As shown in Fig. 1, among all the hormones tested in our studies, 17 β -estradiol was the predominant hormone tested that induced a dramatic increase in cell proliferation based on cell number (about eightfold, Fig. 1A) ($P < 0.001$) and thymidine incorporation (about 20-fold, Fig. 1B) ($P < 0.05$). In the same experiment, testosterone ($P < 0.05$) and luteinizing hormone ($P < 0.01$) induced minor increases compared to corresponding controls. Prolactin induced a minor increase ($P < 0.001$) in cell number, but no measurable increment in DNA synthesis.

Consistent with the pattern of OSE cell proliferation, 17 β -estradiol was the predominant hormone tested that stimulated the formation of papillae (Fig. 2). The stimulation is about 16-fold comparing to ethanol controls ($P < 0.001$). Testosterone was the only other hormone that induced a smaller increase (about fivefold) in the formation of papillae ($P < 0.05$). Overall, this *in vivo* analysis suggests that 17 β -estradiol is the major hormone that stimulates OSE cell proliferation and papillogenesis *in vivo*.

Surface morphology of rabbit ovaries after 17 β -estradiol stimulation. To visualize the morphogenetic changes associated with estrogen treatment, ovaries treated for 28 d with vehicle (Fig. 3A) or 17 β -estradiol (Fig. 3B and C) were fixed and processed for scanning electron microscopy as described in "Materials and Methods."

As shown in Fig. 3, papillae in vehicle-treated ovaries are sparse and simple, while 17 β -estradiol-treated ovaries are densely and uniformly covered with papillae, assuming a configuration similar to that seen in human papillary serous neoplasms of low malignancy (Nicosia and Nicosia, 1988). The estrogen-stimulated papillae are complex and branching, and covered by cells whose surface is rich in blebs, ruffles, microvilli, and occasional single cilia (data not shown).

To investigate the pathological consequence of long-term estrogen stimulation of OSE cell proliferation, female rabbits were continuously treated for 6 mo with estrogen pellets as described in "Materials and Methods." Bilateral papillomas were observed in approximately 30% of the rabbits (Fig. 4, panel A). Sections of the

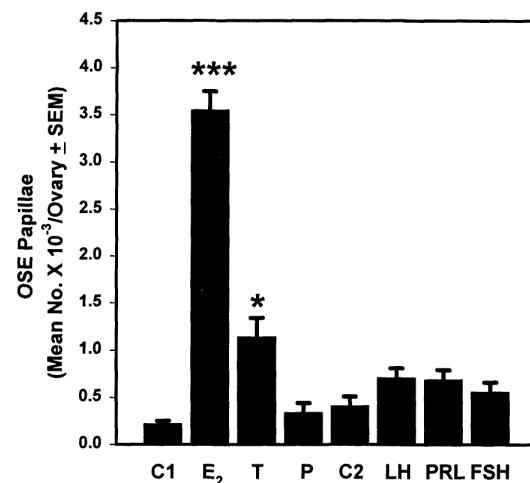


FIG. 2. Stimulation of OSE papillae formation by steroid and peptide hormones. The contralateral ovary of each animal, treated as described in Fig. 1, was used to determine the number of the OSE papillae by scanning electron microscopy. Abbreviations are used as in legend of Fig. 1. * $P < 0.05$; *** $P < 0.001$.

ovaries through the tumors detected multiple papillary processes with secondary and tertiary branchings (Fig. 4B).

*Expression of the ER α in rabbit OSE and OS cells *in vivo*.* Due to the observed effect of 17 β -estradiol on OSE cell proliferation and morphogenesis, the ER status of ovarian tissue was determined immunohistochemically using an ER α -specific antibody shown to react with the rabbit ER (Bodker et al., 1993). As indicated in Fig. 5, the ER is expressed in the nuclei of both rabbit OSE cells (A) and OS cells (C). Ovarian sections stained with a nonspecific mouse IgG (B and D) were not immunoreactive, indicating that the signals detected by the ER antibody is specific to the ER. Figure 5 also

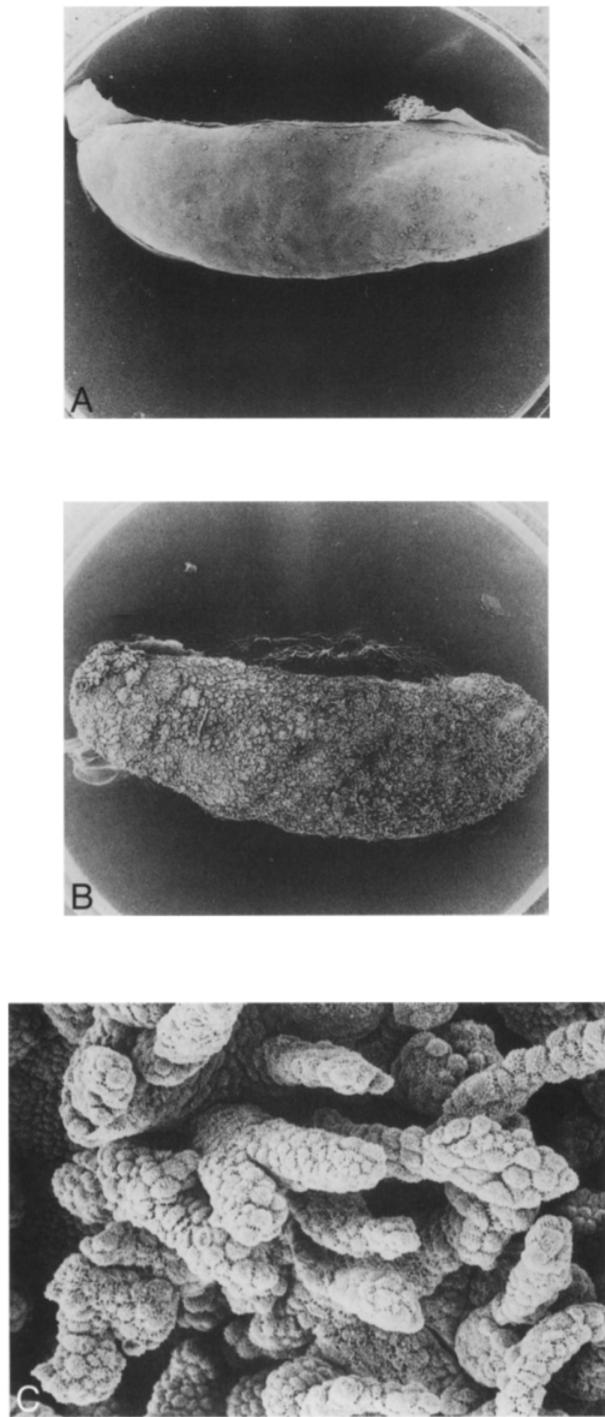


FIG. 3. Rabbits ovarian surface morphology before and after estradiol treatment. (A) An ovary treated with vehicle alone for 28 d showing sparse papillae on its surface, (B) an ovary after 17 β -estradiol treatment showing a surface densely covered with papillae, and (C) a 17 β -estradiol-treated ovary showing prominent and branching papillae. Magnification: A, B, $\times 10$; C, $\times 500$.

shows that the ER α -positive cells in rabbit stroma are interstitial cells (C), the major component of the rabbit ovarian stroma, while fibroblasts underlying the OSE cells (A) contain little ER α .

The growth stimulation of rabbit OSE cells by 17 β -estradiol *in vivo* could be due to either the direct effect of the hormone on OSE cells or, alternatively, its indirect effect on other ER-positive cells. To determine whether 17 β -estradiol directly acts on rabbit OSE cells, the OSE cells were isolated and their response to 17 β -estradiol was analyzed *in vitro*.

Lack of proliferative response to estradiol of OSE cells losing transcriptionally active ER α . OSE cells represent less than 1% of total ovarian cells. In order to obtain an adequate number of cells for *in vitro* study, primary OSE cells, isolated as described (Giacomini et al., 1995), were expanded in M199 medium containing 15% fetal calf serum and antibiotics before their response to 17 β -estradiol was examined.

Expanded OSE cells were treated with either 10 $^{-7}$ M 17 β -estradiol or ethanol as control. Cells treated with 10 $^{-6}$ M ICI 182,780 were also evaluated due to the concern that the ER may be activated by buffer components such as phenol red (Berthois et al., 1986). ICI 182,780 has been shown to be a potent and specific ER antagonist. Therefore, the total ER-mediated response in our assay is expressed as the difference between the proliferation of cells treated with 17 β -estradiol, and cells treated with ICI 182,780.

As shown in Fig. 6, panel A, no significant difference in cell numbers was observed between OSE cells treated with vehicle, 10 $^{-7}$ M 17 β -estradiol or 10 $^{-6}$ M ICI 182,780 for 6 d. This suggests that OSE cells after tryptic digestion and *in vitro* expansion no longer respond to estrogen.

Consistent with the loss of estrogen response, OSE cells cultured for 1 wk after tryptic digestion have a much lower level of ER α expression (Fig. 6B [3]) as compared to primary OSE cells (Fig. 6B [1]). Based on measurements with an image analysis cytometer (CAS 200, Cell Analysis System Inc., Elmhurst, IL), the nuclear ER α level in OSE cells, after 1-wk culturing following trypsin digestion, was about 30% of the ER α level in primary OSE cells. Controls exposed to a nonspecific mouse IgG were not immunoreactive (Fig. 6B [2] and [4]), indicating that the signal detected with the anti-ER α antibody is specific to the receptor. This decrease in level of OSE ER α expression during *in vitro* culturing appears to be unique to rabbit since human ER α expression is retained during *in vitro* culturing, although the immunohistochemical signal changes from distinctly nuclear observed *in situ* to a diffused pattern after culturing (data not shown).

Similar to the low level of ER α expression, 17 β -estradiol-induced transcriptional activity of the endogenous ER α in these cells is undetectable (Fig. 6C). As a control, the transcriptional activity of transfected ER α was induced by 17 β -estradiol and blocked by ICI 182,780 under the same assay conditions, indicating that the transcriptional machinery is intact in expanded OSE cells. Interestingly, transfected ER α exhibits some basal transcriptional activity in the absence of 17 β -estradiol, and such activity was blocked by ICI 182,780 (Fig. 6C). The basal activity was observed in both phenol red-free DMEM containing 5% charcoal-stripped FBS and HL-1 synthetic medium which is free of serum but contains phenol red. This suggests that the basal activity is probably due to endogenous estrogens of OSE cells rather than medium components. Our experience is that OSE cells do not grow well in DMEM complemented with 5% charcoal-stripped FBS, an environment widely

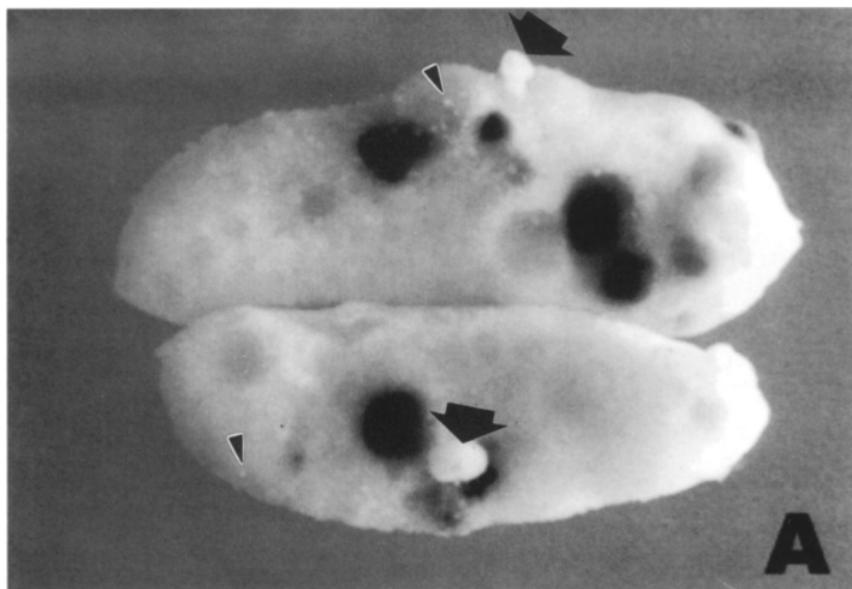
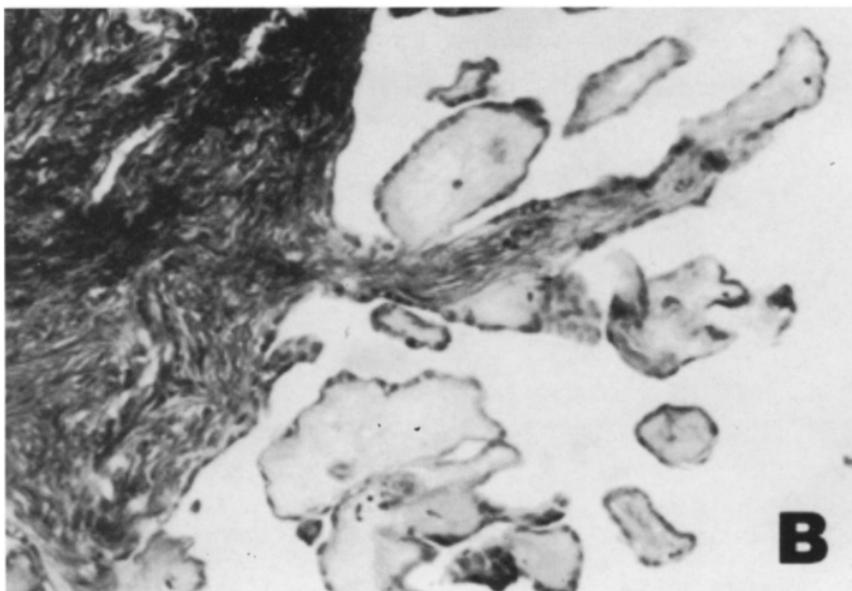


FIG. 4. Induction of papillomas by continuous estrogen treatment in rabbits. Rabbits ($n = 3$) were treated with estrogen pellets for 6 mo as described in "Materials and Methods." (A) Ovaries from one of the estrogen-treated rabbits. Note bilateral papillomas (large arrows) and smaller, dot-like papillary projections (small arrows) in the ovarian surface, and (B) histology of a papilloma showing multiple and branching papillary process (Masson's trichrome). Magnification: A, $\times 4$; B, $\times 60$.



used to measure the proliferative response of ER-positive cancer cells to estrogens. Therefore, the HL-1 medium was used in our studies due to its ability to maintain OSE cells successfully under serum-free conditions (Giacomini et al., 1995).

These data strengthen the interpretation that the decreased expression of transcriptionally active ER α in OSE cells is the reason

for the diminished response of OSE cells to 17β -estradiol after short-term culture.

Estrogen responsiveness of OSE cells retaining transcriptionally active ER α . OSE tissue fragments after collagenase digestion were directly plated in M199 containing 15% of FBS without trypsin dissociation. After expansion for 7 d in culture, the ER expression

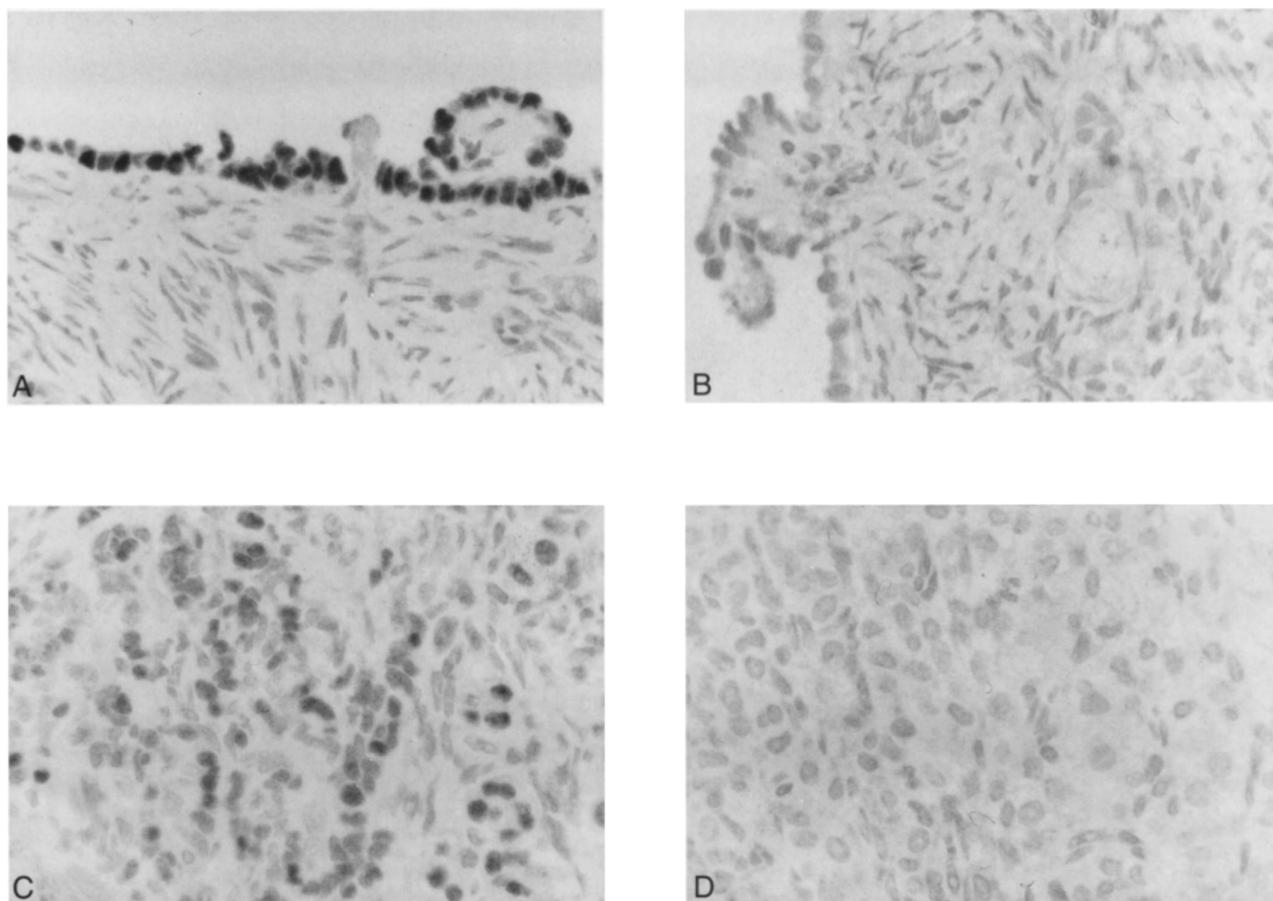


FIG. 5. Expression of the ER α in OSE and OS cells. (A) and (C) anti-ER α ; (B) and (D) mouse IgG controls; (A) and (B) OSE cells; (C) and (D) OS cells. Note distinct ER nuclear staining in OSE (A) and interstitial (C) cells. (Immunoperoxidase; Magnification: A-D, $\times 400$).

of OSE cells was examined by immunohistochemistry. Based on image analysis cytometry, these cells retained the majority (about 70%) of the ER α signal detected in native OSE cells. Consistent with higher ER α expression, the estrogen-induced transcriptional activity of endogenous ER was detectable in these cells (Fig. 7A).

Retaining the expression of a transcriptionally active ER α in expanded OSE cells would anticipate responsiveness to estrogens. Therefore, the effect of 17 β -estradiol was examined as described for the experiment shown in Fig. 6. In Figure 7, panel B, expanded OSE cells exhibited a measurable growth response to 17 β -estradiol ($P < 0.01$), while the antiestrogen was inhibitory ($P < 0.05$) relative to ethanol controls. Although growth stimulation by 17 β -estradiol was modest, the overall ER-mediated response, measured by the difference between estrogen- and antiestrogen-treated samples, was significant (2.5-fold) ($P < 0.01$).

The correlation between OSE ER α expression and response to 17 β -estradiol (Figs. 6 and 7) strongly suggests that the OSE ER α is required for estrogen-induced OSE cell growth. This is further supported by the inhibition of 17 β -estradiol-induced OSE cell proliferation by the specific ER antagonist ICI 182,780 when the two compounds are added together (Fig. 7A). The inhibition of OSE cell growth by ICI 182,780 to a degree lower than ethanol control in

both transcriptional (Fig. 7A) and proliferation assays (Fig. 7B) indicates the existence of basally active endogenous ER under these experimental conditions. The blocking effect of ICI 182,780 is apparently not due to a nonspecific toxic effect since the same level of this antiestrogen does not affect the growth of OSE cells lacking detectable levels of transcriptionally active ER α (Fig. 6).

Potentiation of 17 β -estradiol-stimulated OSE cell proliferation by ovarian stromal cells. The role of stroma in epithelial cells' response to estrogens has been suggested in other reproductive tissues. To examine the influence of stromal cell growth in the presence of estrogens, OSE and OS cells expanded without trypic digestion were plated either alone or as mixed cultures and their growth response to 17 β -estradiol examined as described in Fig. 6. To eliminate the potential influence of cell density on growth, equal total number of cells was plated in either separate or mixed cultures (i.e., the number of OSE and OS cells in the mixed cultures was half of that in single cell cultures).

Figure 8 shows that OSE cells cultured alone were growth-stimulated by 17 β -estradiol and inhibited by ICI. More interestingly, the response of OSE cells to 17 β -estradiol in mixed cultures was much greater than that observed for OSE cells cultured alone ($P < 0.05$). Based on cytokeratin immunoreactivity, the majority (about

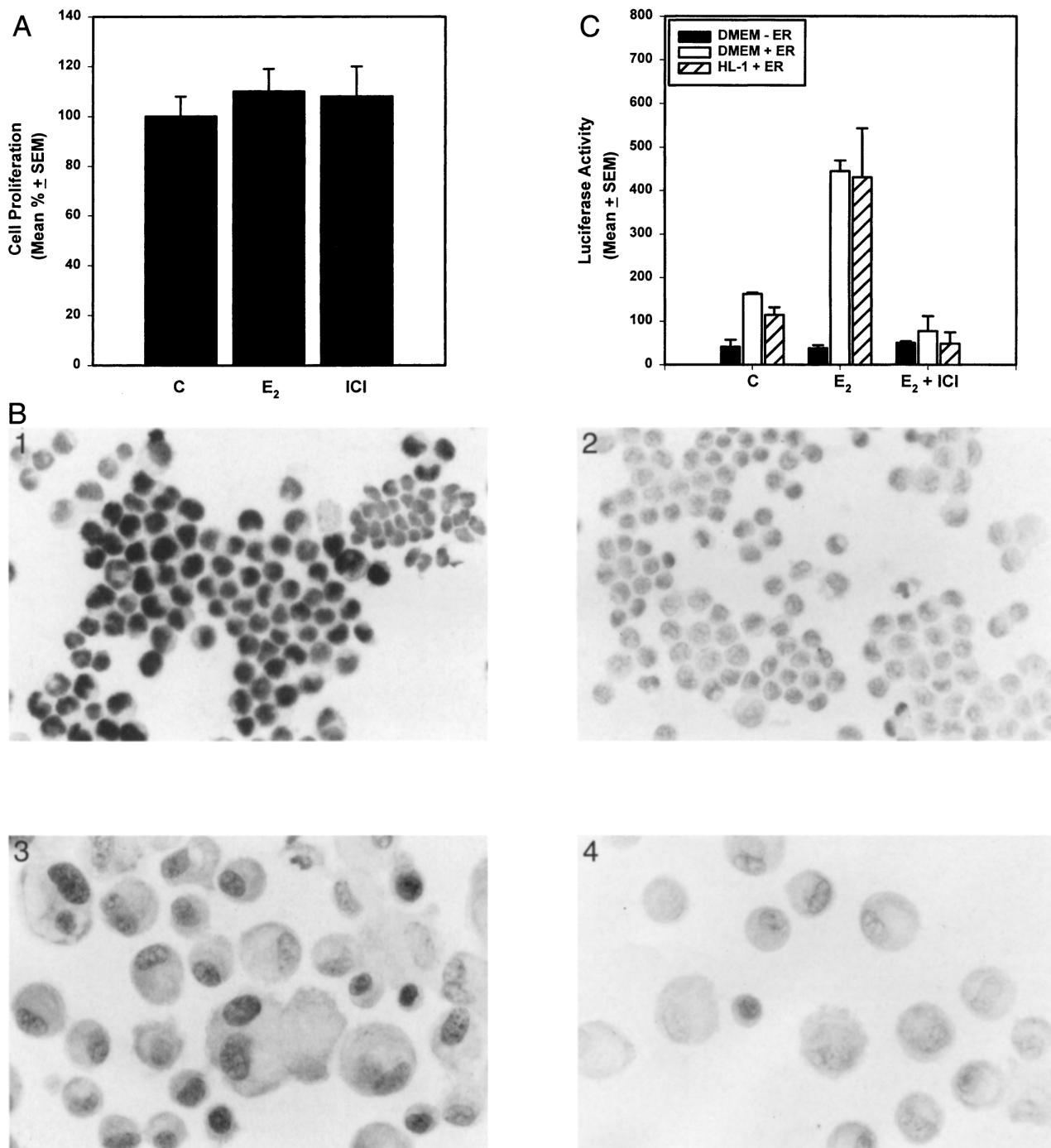


FIG. 6. Lack of growth response to 17β -estradiol of OSE cells after tryptic digestion and expansion in culture. (A) Cell proliferation assay. OSE cells expanded after tryptic digestion were treated either with ethanol (C), $10^{-7} M$ 17β -estradiol (E_2), or $10^{-6} M$ ICI 182,780 (ICI). (B) ER α expression in OSE cells immediately after isolation (6B [1] and [2]) or after being grown for 1 wk following tryptic digestion (6B [3] and [4]). 6B (1) and (3): 1D5; 6B (2) and 6B (4): IgG control. (C) Transcriptional assay for the endogenous (solid bars) and transfected (open and hatched bars) ER in DMEM medium containing 5% charcoal-stripped FBS (solid and open bars) or HL-1 serum-free medium (hatched bars). Expanded OSE cells were transfected with ERE l bLuc and Plemgal with (open and hatched bars) or without (solid bars) PlemER α . Data are normalized to β -galactosidase activity as described in "Materials and Methods." (Immunoperoxidase; Magnification: B [1]-[4], $\times 400$).

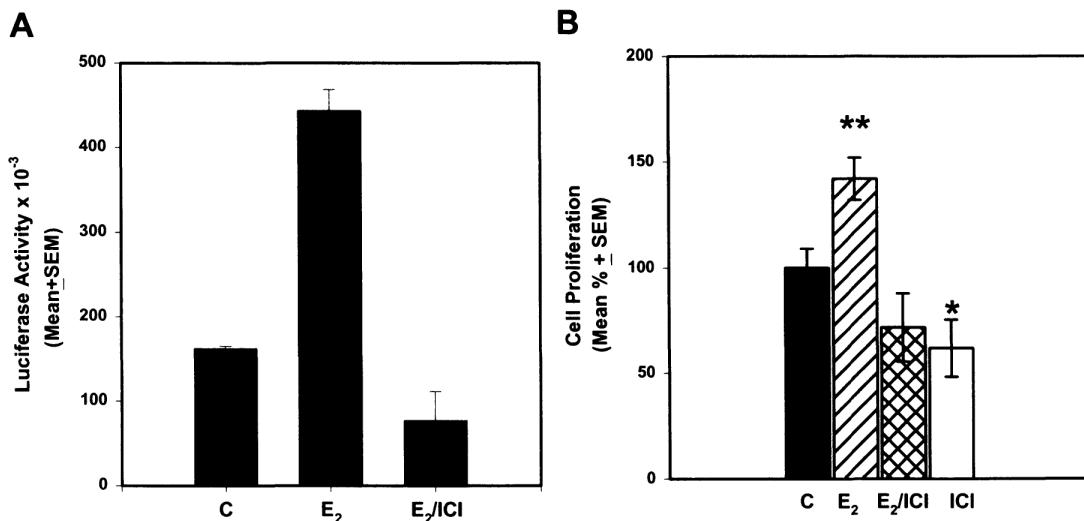


FIG. 7. Growth response to 17 β -estradiol of OSE cells expanded as cell organoids. (A) Transcriptional activity of endogenous ER in OSE cells expanded as cell organoids. OSE cells expanded as cell organoids were transfected with EREe1blue and Plemgal. Luciferase activity was normalized to β -galactosidase activity. (B) Cell proliferation assay. OSE cells expanded as cell organoids were treated with either ethanol (C), 10^{-7} M 17 β -estradiol (E_2), 10^{-6} M ICI 182,780 (ICI), or 10^{-7} M 17 β -estradiol and 10^{-6} M ICI 182,780. * $P < 0.05$; ** $P < 0.01$.

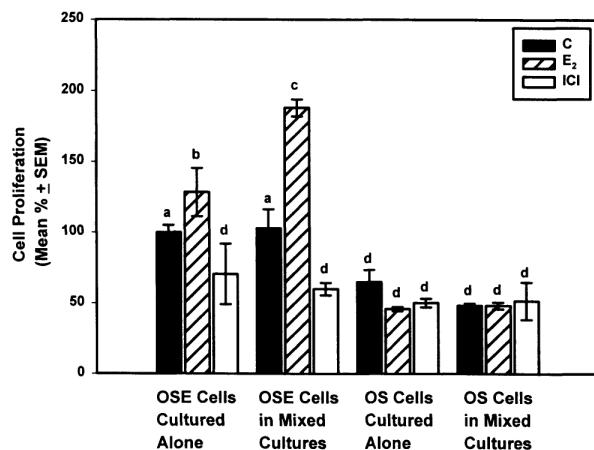


FIG. 8. Enhancement by OS cells of the growth response of OSE cells to 17 β -estradiol. OSE cells, OS cells, or combined OSE/OS cells were treated with either ethanol (C), 10^{-7} M 17 β -estradiol (E_2), or 10^{-6} M ICI 182,780 (ICI). Cell phenotype was identified by cytokeratin immunoreactivity as described in "Materials and Methods." The fraction of OSE and OS cells in cocultures was calculated based on the average number of cytokeratin-positive and -negative cells, respectively. Different letters above columns indicate significant differences among groups ($P < 0.05$).

70%) of the cells in the mixed cultures after 17 β -estradiol treatment were OSE cells, while the ratio between OSE cells and OS cells in the mixed cultures treated with ICI 182,780 remained close to the starting ratio of 1:1. This suggests that there is a preferential growth response of OSE cells to 17 β -estradiol in mixed cultures. No difference in cell numbers were detected among OS cells, alone or in mixed cultures, treated with ethanol, 17 β -estradiol, or ICI, suggesting that OS cells are growth-insensitive to 17 β -estradiol.

DISCUSSION

Our studies demonstrate that 17 β -estradiol stimulates OSE cell proliferation both in vivo and in vitro. Taken together, these data suggest that OSE cells are responsive to 17 β -estradiol. Rabbit OSE cells expanded as cell organoids retain transcriptionally detectable ER α as well as estrogen responsiveness and thus, provide a useful in vitro cellular model system for investigating the mechanism of estrogen action in the OSE.

Although human follicle stimulating hormone (FSH) is known to increase the endogenous estrogen level, the administration of FSH did not induce measurable growth response of OSE cells in vivo (Figs. 1 and 2). This may reflect the inability of FSH to sustain endogenous estrogen to a steady high level as the estrogen pellets would do. The ability of ICI 182,780 to block the proliferative effect of 17 β -estradiol indicates that the stimulatory effect of 17 β -estradiol is mediated through intracellular receptors. The expression of ER α in OSE cells as well as the correlation between the ER α expression and the proliferative response of OSE cells to 17 β -estradiol suggest that the effect of 17 β -estradiol may be mediated through the alpha subtype of the receptor. Although ER β is the major subtype of ERs expressed in the ovary, a direct role of ER β in OSE cell proliferation has yet to be established. ER β protein was shown to be expressed in granulosa cells but not in OSE cells in the rodent ovary (Saunders et al., 1997). However, ER β messenger ribonucleic acid (mRNA) was detected by reverse transcriptase-polymerase chain reaction in some human ovarian epithelial cancer cells (Bradenberger et al., 1998; Lau et al., 1999), and one report described the detection of ER β mRNA in human OSE cells by in situ hybridization (Pujol et al., 1998). The detection of ER β protein in OSE cells has not been reported and our investigation aiming at detecting specific nuclear ER β protein signal in rabbit OSE cells with several commercial anti-ER β antibodies has been unsuccessful.

ful, presumably due to the lack of reactivity of the available antibodies.

Although the growth response of OSE cells to 17 β -estradiol depends on the OSE ER, our studies also demonstrate that OS cells enhance the proliferative response of OSE cells to 17 β -estradiol. Immunostaining results show that the loss of ER α expression in expanded OSE cells, cultured alone or with OS cells, is similarly observed (data not shown). This finding eliminates the possibility that the better growth response of OSE cells to 17 β -estradiol in the presence of stromal cells is due to an effect of OS cells on OSE ER α expression. ERs can be activated by growth factors (Nelson et al., 1991; Ignar-Trowbridge et al., 1992) and it has been suggested that, in steroid-sensitive organs such as the prostate, the stromal activity mediating the epithelial growth response to androgens is indeed due to growth factors (Cunha, 1996). Growth factors stimulate the growth and morphogenesis of rabbit OSE cells in vitro (Pierro et al., 1996). Although it remains to be demonstrated, similar paracrine OS secretions may augment the growth response of OSE cells to estrogens by enhancing the transcriptional activity of ER α .

The finding that the OSE cells' proliferative response to 17 β -estradiol depends to a large extent on OSE ER expression is somewhat at variance with data in uterine tissues, where the growth response of epithelial cells to estrogens is mediated through the ER of stromal cells (Cooke et al., 1997). Vaginal epithelial ERs alone are not essential to epithelial cell growth, but, together with stromal ER, are required for estrogen-induced cornification and epithelial stratification (Buchanan et al., 1998). Similar to our studies, short-term cultures of normal breast epithelial cells depend on estrogens for enhanced cell growth (Malet et al., 1988) and microvilli formation (Chambon et al., 1984). Using an *in vivo* system, it has also been shown that regulation of ductal and lobulo-alveolar development in mammary gland by progesterone requires the expression of both epithelial and stromal progesterone receptors (Humphreys et al., 1997). These studies suggest that the effect of female sex steroids is mediated through distinct mechanisms in different systems and that the mechanism of estrogen action is divergent among known target tissues, although the involvement of stromal cells seems common.

Since cell proliferation is a hallmark of cancer, the demonstration of growth stimulation of putative precursor cells of ovarian epithelial cancer cells suggests a role for estrogens and their receptors in ovarian tumorigenesis. This notion is consistent with the finding that a significant percentage of ovarian epithelial neoplasms, both of low malignant potential and frankly malignant, are ER-positive (Abu-Jawdeh et al., 1996; Kommonss, 1996). It is also consistent with several studies showing estrogen stimulation of epithelial ovarian cancer cell lines (Langdon et al., 1988, 1990; Nash et al., 1989; Geisinger, 1990; Galtier-Dereure et al., 1992) and estrogen induction of tumor-like and frankly neoplastic OSE in rabbits and other experimental animals (Fig. 4) (Jabara, 1959). The papillary morphology of OS and the formation of papillomas induced by 17 β -estradiol suggest that the rabbit may be a relevant animal model, at least for studying the early proliferative events that contribute to the formation of serous ovarian neoplasms, the major type of epithelial ovarian tumors in humans.

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