

Expression and Action of Transforming Growth Factor Alpha in Normal Ovarian Surface Epithelium and Ovarian Cancer¹

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ABSTRACT

Greater than 95% of ovarian cancers originate in the epithelial cells on the surface of the ovary. The current study investigates the expression and action of transforming growth factor alpha (TGF α) in ovarian surface epithelium (OSE) and the underlying stroma in both normal and tumorigenic ovarian tissues. Normal bovine ovaries are used in the current study as a model system to investigate normal OSE functions. Transforming growth factor alpha and its receptor, the epidermal growth factor receptor (EGFR), were detected in the OSE from normal ovaries by immunocytochemistry (ICC). Ovarian stromal tissue also contained reduced but positive TGF α and EGFR immunostaining. To examine TGF α and EGFR gene expression, RNA was collected from normal bovine OSE and ovarian stromal cells. The TGF α and EGFR transcripts were detected in both fresh and cultured OSE and stromal cells by a sensitive quantitative reverse transcription polymerase chain reaction (QRT-PCR) assay. Transforming growth factor alpha gene expression was found to be high in freshly isolated OSE, but low in freshly isolated stroma. In contrast, EGFR expression was higher in the stroma compared to the OSE. Both the ICC and QRT-PCR indicate that normal OSE express high levels of TGF α in vivo and in vitro. In vitro, normal ovarian stromal cells develop the capacity to express high levels of EGFR. Human ovarian tumors from stage II, stage III, and stage IV ovarian cancer cases were found to express TGF α and EGFR protein in the epithelial cell component of the tumor by ICC analysis. The stromal cell component of human ovarian tumors contained little or no TGF α /EGFR immunostaining. Observations suggest that tumor progression may in part require autocrine stimulation of the epithelia. Transforming growth factor alpha was found to stimulate the growth of normal bovine OSE and stroma cells to the same level as epidermal growth factor. Two ovarian cancer cell lines, SKOV3 and OCC1, were also stimulated to proliferate in response to TGF α . Transforming growth factor alpha was also found to stimulate the expression of two growth factors previously shown to be produced by OSE. Transforming growth factor alpha stimulates both kit ligand/stem cell factor and keratinocyte growth factor production by OSE. The effect of hormones on TGF α and EGFR expression by the OSE was also examined. Human chorionic gonadotropin stimulated TGF α expression, but not FSH. Both hCG and FSH stimulated EGFR expression by OSE. Combined observations suggest a role of systemic hormones and a locally produced growth factor, TGF α , in OSE biology. Insight is also provided into how the OSE may develop abnormal growth char-

acteristics involved in the onset and progression of ovarian cancer.

growth factors, ovary

INTRODUCTION

Ovarian cancer is the leading cause of death due to gynecological malignancies and is the fifth most common cause of death from cancer among women. It is estimated that there will be approximately 20 000 deaths from ovarian cancer in the United States this year [1]. Greater than 95% of these ovarian cancers originate in the epithelial cells on the surface of the ovary [2, 3].

The ovary is covered by a single layer of epithelial cells, commonly referred to as the ovarian surface epithelium (OSE). The OSE is a modified mesothelium consisting of cuboidal cells separated from underlying ovarian stromal tissue by collagenous connective tissue [4]. During the normal ovarian cycle, the OSE undergoes periodic changes and has the capacity to remodel the ovarian cortex through synthetic and proteolytic functions [4, 5]. Similarly, the stroma produces growth factors and cytokines that may act on the OSE [6]. Hence the interaction between these two cell types appears to be important for the normal functioning of the ovary. The OSE has been suggested to be involved in the process of ovulation and is known to produce lysosomal enzymes that contribute to follicular rupture [7]. The hypothesis has developed that aberrations in the proliferative wound repair process following ovulation leads to neoplasia. Repetitious ovulations appear to contribute to the etiology of ovarian cancer through the altered cellular activity of the OSE at ovulation [8]. The cellular associations between OSE and stroma have been shown to influence the intermediate filaments in the OSE that may be compared with the early stages of neoplastic progression [9]. Tumor invasion often requires an association with host stromal tissue and most ovarian tumors have a stromal-like component [10, 11]. Therefore, stromal-epithelial cell interactions appear to have a critical role in the function and growth of normal and tumorigenic OSE.

Transforming growth factor alpha (TGF α) is a 50-amino acid polypeptide of 5.6 kDa [12] that shares only modest homology with epidermal growth factor (EGF) but effectively competes with labeled EGF for the EGF receptor (EGFR) [13]. Transforming growth factor alpha is expressed by normal and tumorigenic OSE [14, 15]. The TGF α associated with ovarian cancer may act as a marker in effusions from cancer patients [16, 17]. It has been shown that TGF α signaling through the EGFR may be involved in regulating CA125 and tissue plasminogen activator secretion from human ovarian carcinomas [18]. Transforming growth factor alpha is associated with rapidly growing fetal tissues [20], neoplastic cells, and normal cells from a number of postnatal adult tissues [21–24]. In bovine and porcine ovarian follicles TGF α is produced by mes-

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enchymal/stromal-derived theca cells of the follicle and act primarily on the epithelial granulosa cells [25, 26]. The current study investigated a potential similar interaction between OSE and ovarian stromal cells.

Epidermal growth factor receptor is present in normal OSE and in human ovarian carcinomas [29–31]. The expression of EGFR in ovarian carcinoma appears to be related to both histological differentiation and/or advanced clinical stage [28]. Studies on ovarian epithelial tumors indicate that immunohistochemical detection of EGFR in ovarian epithelial tumors may act as a prognostic indicator for the tumor [32]. In the follicle, the EGFR gene is expressed primarily by the granulosa layers and appears to mediate the paracrine effects of TGF α produced by the theca [25, 33]. Whether the OSE and underlying stroma have a similar cell-cell interaction is examined.

Studies involving OSE have been limited by the lack of an efficient experimental model. Normal OSE of the rat, rabbit, and human have been isolated and cultured [34, 35], but the size and availability of these tissues often limit the use of these models. Bovine ovaries present a useful model for OSE and ovarian stromal interactions. The cow is a mono-ovulator that ovulates regularly and has an ovarian cycle similar to that of the human. Bovine ovarian cancer has been reported [36], suggesting that bovine OSE has similar tumorigenic potential to human OSE. Therefore, the bovine ovary provides a useful model for examining the biology of normal OSE [6] and was used in the current study.

Previous studies have examined the expression of TGF α and EGFR gene by the OSE in human and other species with procedures such as *in situ* hybridization and immunocytochemistry [18, 26, 27, 29, 31–33, 37, 38] but have not studied its expression in the stromal cells. The current study was designed to examine the local production and action of TGF α in bovine ovarian surface epithelium and the underlying stroma. The hypothesis is tested that TGF α may have an important role in normal OSE biology, as well as in ovarian cancer. Information regarding the expression of the TGF α gene and action of TGF α provides insight into potential cell-cell interactions that regulate OSE and ovarian tumor progression.

MATERIALS AND METHODS

Tissues

Bovine ovaries were obtained from young nonpregnant postpubertal cycling heifers less than 10 min after slaughter and transported on ice. Ovaries were selected that were in the follicular phase of the estrous cycle as previously described [6]. Human adult ovarian tissues were obtained from the ovarian cancer bank directed by Dr. Bethan Powell associated with a National Cancer Institute Ovarian Cancer Program at the University of California, San Francisco. The normal human tissues were collected from salpingo-oophorectomy specimens removed for benign diseases from women of child-bearing age. Human cancer tissues were surgically collected from women with stage II, stage III, and stage IV ovarian cancer. When required, ovaries were fixed in 10% buffered formalin, embedded in paraffin, and cut into 5- μ m sections. Sections were stored at 4°C until immunocytochemistry was performed.

Ovarian Surface Epithelium and Stromal Cell Isolation and Cell Culture

Ovarian surface epithelium cells and ovarian stromal cells were freshly isolated for RNA isolation or cultured.

The OSE cells were scraped from the surface of the ovary with a rubber policeman as previously described [6, 39]. Sheets of epithelial cells were suspended in Hanks buffered salt solution and then pelleted and washed prior to suspension for plating. After the removal of OSE cells, the ovarian surface stromal cells were microdissected from areas of the ovary devoid of follicles. A section of surface stromal cells 1–2 mm wide by 5–8 mm long and 1 mm deep was collected. The tissue piece was minced and digested with 1 mg/ml collagenase and 1 mg/ml hyaluronidase for 2 h at 37°C or 18 h at 4°C. Cells were plated with an initial density of approximately 10⁶ cells/2 cm² and were maintained at 37°C in 5% CO₂ atmosphere in Hams F-12 (Gibco Labs., Grand Island, NY) supplemented with 10% calf serum. Medium was changed every 48–72 h. Once the cells had grown to confluence, the cells were trypsinized and split into appropriate plates. For RNA isolation from cultured cells, OSE and stromal cells were plated in 100-mm large culture plates (Nunc, Rochester, NY) and maintained in Hams F-12 supplemented with 10% calf serum.

To study the effect of growth factor and gonadotropin treatment on TGF α and EGFR gene expression, OSE or stroma cells were plated in six-well plates Hams F-12 supplemented with 10% calf serum. When cells achieved confluency, cells were washed in Hams F-12 and maintained in Hams F-12 in the absence of calf serum. Cells were treated with no growth factor (control), TGF α (50 ng/ml), transforming growth factor β (TGF β) (10 ng/ml), basic fibroblast growth factor (bFGF) (40 ng/ml), EGF (50 ng/ml), FSH (50 ng/ml), or hCG (26.8 ng/ml). These doses have previously been shown to be optimal in all stimulations [6, 25]. Treated cells were cultured for 48 h and harvested for total RNA.

The purity of OSE isolated by this procedure is greater than 98% by keratin staining with no detectable stromal contamination [6]. The purity of the ovarian stromal cell preparation is also greater than 98% with no detectable OSE contamination [6]. The human ovarian cancer cell line, SKOV3 was obtained from the American Type Culture Collection (Rockville, MD) and the human ovarian cancer cell line OCC1 was kindly provided by Dr. Gordon Mills (M.D. Anderson Cancer Center, Houston, TX).

Growth Assays

For growth assays, OSE cells were plated in 24-well plates in Dulbeccos minimum essential medium (DMEM; Gibco) supplemented with 10% calf serum. When cells achieved 50–70% confluency, cells were washed in DMEM containing 0.1% calf serum to synchronize the cell population for growth assays. After 48 h, cells were treated with no growth factor (control), 50 ng/ml TGF α , 50 ng/ml EGF, or 10% bovine calf serum (Gibco). Cells were treated for 20 h. After treatment, 0.5 ml DMEM containing 2.5 μ Ci [³H]thymidine was added to each well, and the cells were incubated for 4 h at 37°C and then sonicated. The quantity of [³H]thymidine incorporated into DNA was determined, as previously described [6]. Data were normalized to total DNA per well.

DNA Assays

DNA was measured fluorometrically with ethidium bromide as previously described [6]. An aliquot of the sonicated cell suspension was added to an equal volume of ethidium bromide solution (0.25 mM ethidium bromide 100 U/ml heparin in ethidium bromide buffer (EBB: 20 mM

sodium chloride, 5 mM ethylene diamine tetracetate, 10 mM Tris, pH 7.8; Sigma Chemical Co., St. Louis, MO) was diluted 1:2 with EBB, and was allowed to incubate at room temperature for 30 min. Fluorescent emission at 585 nm with 350 nm excitation was then monitored. A standard curve with calf thymus DNA was used to quantify amounts of DNA in the culture wells. This assay has a sensitivity of approximately 0.1 μ g DNA.

Immunocytochemistry

Tissues were fixed in 10% formalin and embedded in paraffin according to standard procedures. Immunocytochemistry was performed as previously described [40]. Briefly, 5- μ m sections were deparaffinized and rehydrated, quenched in 3% hydrogen peroxide in methanol, and blocked in serum (normal goat serum; Vector Laboratories, Burlingame, CA) for 2 h at room temperature. Slides were incubated with polyclonal rabbit anti-human TGF- α or EGFR antibody (Santa Cruz Biotechnologies, Santa Cruz, CA) at 1:200 dilution overnight at 4°C. Secondary antibody (biotinylated goat anti-rabbit IgG; Vector) was detected by using the Vectastain kit (Vector) and diaminobenzidine (Vector). Controls were performed with each analysis and involved both the absence of primary antibody and the use of nonimmune antibody and serum at the same dilution as the primary antibody.

RNA Preparation

Total RNA was prepared from freshly isolated or cultured cells using Trizol reagent (Gibco). Trizol was added directly to freshly isolated cells or to the culture plate to prevent RNA degradation. RNA was stored at -70°C until use.

Quantitative Reverse Transcription Polymerase Chain Reaction Assays

Steady-state amounts of TGF α and cyclophilin (i.e., 1B15) mRNAs were analyzed using a specific quantitative reverse transcription-polymerase chain reaction (QRT-PCR) assay for each gene. These QRT-PCR assays have previously been described in detail [41]. The primers used in this quantitative analysis of TGF α , EGFR, kertinocyte growth factor (KGF), kit ligand/stem cell factor (KL), and 1B15 were: TGF α , 5'-TAA TGA CTG CCC AGA TTC CC-3' (5' primer, 20-mer) and 5'-GAT GAT GAG GAC AGC CAG GG-3' (3' primer, 20-mer) that generated a specific 218-base pair (bp) TGF α PCR product; EGFR, 5'-CTG CTG GGG AAG AGG AGA GGA GAA C-3' (5' primer, 25-mer) and 5'-GAG TGG TGG GCA GGT GTC TT (3' primer, 20-mer) that generated a specific 207-bp EGFR PCR product; KGF, 5'-ATA CTG ACA TGG ATC CTG CCA AGT TTG CTC TAC AGA TCA TGC TTC-3' (5' primer, 45-mer) and 5'-TCC AAC TGC CAC GGT CCT GAT-3' (3' primer, 21-mer) that generated a specific 306-bp KGF PCR product; KL, 5'-GGACCA GTT TTC GAA TAT TTC TGA AGG CTT GAG TAA TTA TTG-3' (5' primer, 42-mer) and 5'-AGG CCC CAA AAG CAA ACC CGA TCA CAA GAG-3' (3' primer, 30-mer) that generated a specific 452-bp KL PCR product; 1B15, 5'-ACA CGC CAT AAT GGC ACT GGT GGC AAG TCC ATC-3' (5' primer, 33-mer) and 5'-ATT TGC CAT GGA CAA GAT GCC AGG ACC TGT ATG-3' (3' primer, 33-mer) that generated a specific 105-bp product. Before reverse transcription, tubes containing total RNA and specific

3'-primers were heated to 65°C for 10 min to facilitate denaturing and cooled to room temperature to facilitate annealing. Total RNA (1 μ g) was reverse transcribed for 1 h at 37°C using the following conditions: 1 μ g total RNA, 1 μ M specific 3'-primers of interest (up to four different primers including 1B15), 0.1 mM dNTPs, 10 mM dithiothreitol, 40 U RNase inhibitor (Promega, Madison, WI), and 200 U M-MLV reverse transcriptase (Gibco BRL, Gaithersburg, MD) in 40 μ l RT buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl₂). After 1 h samples were heated to 95°C for 5 min to inactivate the reverse transcriptase enzyme.

Samples were immediately diluted 2.5-fold and carrier DNA (Bluescript plasmid; Stratagene) was added to a final concentration of 10 ng/ μ l. This concentration of Bluescript carrier DNA (10 ng/ μ l) was included in all subsequent dilutions of samples and standards. Immediately before amplification, each unknown sample was further diluted 1:10 in order to improve the fidelity of the PCR reaction. Plasmid DNAs (i.e., Bluescript) containing bovine TGF α , EGFR, KGF, KL, or 1B15 subclones were used to generate standard curves from 1 attogram/ μ l (10⁻¹⁵ g/ μ l) to 10 pg/ μ l (10 \times 10⁻⁹ g/ μ l) each containing 10 ng/ μ l Bluescript carrier DNA. Identical 10- μ l aliquots of each sample and standard were pipetted in duplicate into a 96-well reaction plate (Marsh Biomedical Products, Rochester, NY) and sealed with adhesive film (Marsh Biomedical Products) for PCR amplification. By this design it was possible to assay simultaneously 5 known standard concentrations and 40 unknown samples for each gene. Amplification was performed in a Perkin Elmer 9600 equipped with a heated lid using the following conditions: 0.4 μ M each primer, 16 μ M dNTPs, and 1.25 U AmpliTaq polymerase in 50 μ l GeneAmp PCR buffer (containing 1.5 mM MgCl₂, Perkin Elmer). Each PCR amplification consisted of an initial denaturing reaction (5 min, 95°C); 26–34 cycles of denaturing (30 sec, 95°C), annealing (1 min, 60°C), and elongation (2 min, 72°C) reactions; and a final elongation reaction (10 min, 72°C). At least 0.25 μ Ci of ³²P-labeled dCTP (Redivue; Amersham Life Sciences, Arlington Heights, IL) was included in each sample during amplification for detection purposes.

Specific PCR products were quantitated by electrophoresing all samples on 4–5% polyacrylamide gels, simultaneously exposing the gels to a phosphor screen for 8–24 h, followed by quantitating the specific bands on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Each gene was assayed in separate PCR reactions from the same RT samples. Equivalent steady-state amounts of mRNA for each gene were determined by comparing each sample to the appropriate standard curve. All data were normalized for 1B15.

Optimal cycle number for amplification was determined for each assay in order to achieve maximum sensitivity while maintaining linearity (i.e., logarithmic phase of PCR reactions). Transforming growth factor alpha quantitative PCR products were amplified for 34 cycles, EGFR PCR products were amplified for 28 cycles, KGF PCR products were amplified for 28 cycles, KL PCR products were amplified 31, and 1B15 PCR products were amplified for 26 cycles. The sensitivity of each quantitative PCR assay was below 1 fg, which corresponds to less than 125 fg target mRNA/ μ g total RNA.

Statistical Analysis

All data were analyzed by a JMP 3.1 statistical analysis program (SAS Institute Inc., Cary, NC). Effects of growth

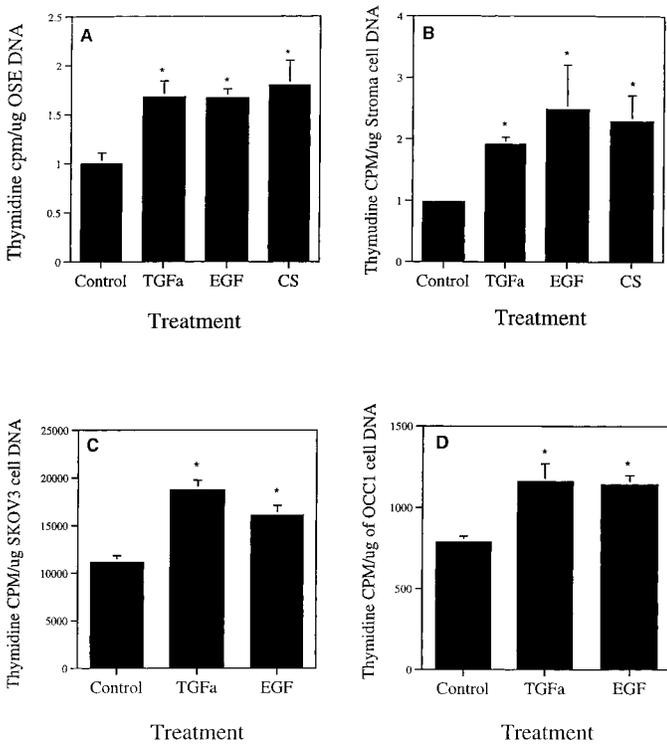


FIG. 1. Transforming growth factor alpha effects on DNA synthesis in bovine OSE, bovine stroma, and human cancer cells. Bovine OSE cells were isolated as described in *Materials and Methods*. Cells were deprived of serum when approximately 70% confluent. [3 H]Thymidine incorporation into DNA was determined after 20 h of treatment with 50 ng/ml TGF α , 50 ng/ml EGF, or 10% bovine calf serum (CS). Data are representative of three different experiments performed in replicate. An ANOVA was performed and bars with asterisks are statistically different from control ($P < 0.05$).

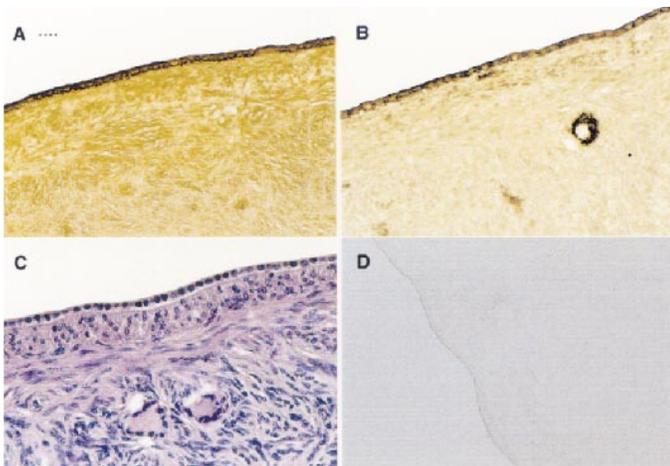


FIG. 2. Transforming growth factor alpha and EGFR protein expression in normal bovine ovaries. Bovine ovaries were immediately fixed in Carnoy's solution followed by paraffin embedding. Sections were cut at 5 μ m thickness and stored on glass slides at 4°C. Immunocytochemistry was performed using a TGF α and EGFR antibody as described in *Materials and Methods*. Sections were visualized and photographed at approximately $\times 400$ magnification. Similar results were obtained in three separate experiments using four different bovine ovaries. **A**) Bovine ovary stained with TGF α antibody. **B**) Bovine ovary stained with EGFR antibody. **C**) Bovine ovary stained with hematoxylin and eosin. **D**) Bovine ovary stained with nonimmune IgG.

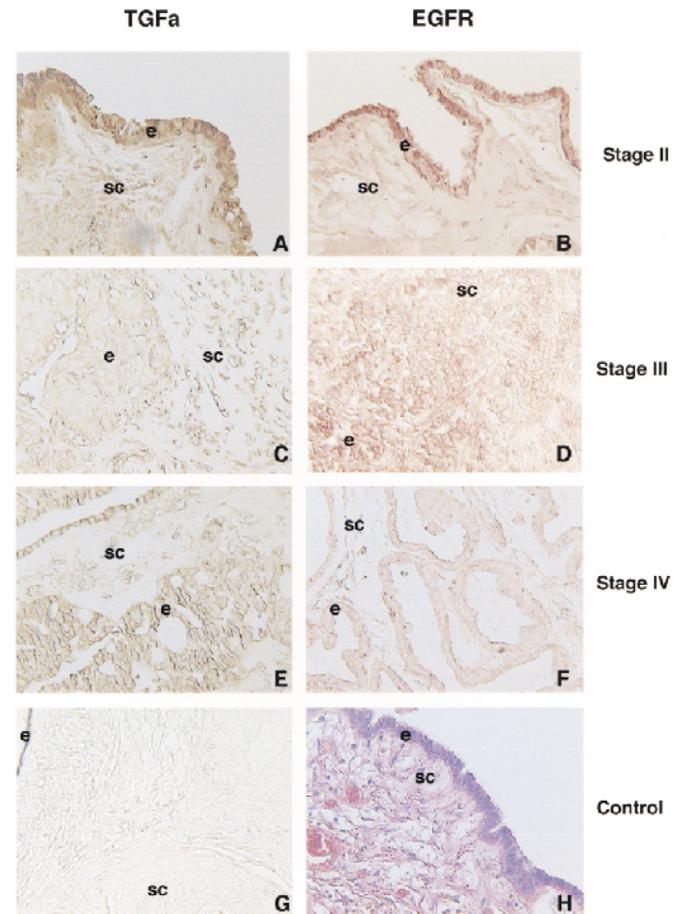


FIG. 3. Transforming growth factor alpha and EGFR protein expression in human tumor ovarian tissue. Human ovarian tumors were surgically removed and immediately fixed in 4% paraformaldehyde followed by paraffin embedding. Ovaries were obtained from patients diagnosed with stage II, stage III, and stage IV ovarian cancer. Sections were cut at 5 μ m thickness and stored on glass slides at 4°C. Immunocytochemistry was performed using a TGF α and EGFR antibody as described in *Materials and Methods*. Sections were visualized and photographed at approximately $\times 400$ magnification. Similar results were obtained in three separate experiments using four different ovarian tumors. **A, B**) Stage II ovarian cancer. **C, D**) Stage III ovarian cancers. **E, F**) Stage IV ovarian cancers. **G**) Control section stained with nonimmune IgG. **H**) Control section stained with hematoxylin and eosin. e, Epithelia; sc, stroma.

factors on [3 H]thymidine incorporation into DNA and OSE and stroma cell mRNA were analyzed by a one-way ANOVA. Observed significance probabilities of 0.05 ($P > F$) or less were considered evidence that an ANOVA model fits the data. Significant differences between treated cells and control (untreated) cells were determined using the Dunnett's test that guards against the high alpha-size (type I) error rate across the hypothesis. All data were obtained from a minimum of three different experiments performed in replicate, and the reproducibility of the data is discussed in each figure legend.

RESULTS

Growth Assay

The ability of TGF α to influence the growth of bovine OSE and stroma cells was investigated. Transforming growth factor alpha and EGF stimulated DNA synthesis in bovine OSE (Fig. 1A) and stroma (Fig. 1B) in a similar manner. Therefore, TGF α can act as a growth factor for

OSE. Similar growth experiments were performed using two human ovarian cancer cell lines, SKOV3 and OCC1 [42]. Transforming growth factor alpha stimulated DNA synthesis in both SKOV3 and OCC1 cells (Fig. 1, C and D). Epidermal growth factor was used as a positive control. Observations demonstrate that TGF α can stimulate the growth of human ovarian cancer cell lines, as well as normal bovine OSE and stromal cells.

Immunocytochemistry

Transforming growth factor alpha protein was examined in normal bovine ovaries by immunocytochemistry. Transforming growth factor alpha and EGFR protein were detected and prominent in the OSE (Fig. 2, A and B). While TGF α staining was intense in the OSE, light staining could also be detected in the stromal cells that border the epithelial cells. Staining for EGFR was present in the OSE and to a lesser extent in the stroma cells. No detectable staining was seen in control slides (Fig. 2D). Observations suggest that TGF α protein is present at high levels in normal bovine OSE and at lower levels in the adjacent stromal cells.

The presence of TGF α protein was examined in tumor tissue from different stages of ovarian cancer. Tissues were examined from stage II, stage III, and stage IV ovarian tumors. Transforming growth factor alpha was detected in the epithelial cell component of the tumor at all stages of disease examined (Fig. 3). In the stage II ovarian tumor the human OSE clearly had high levels of TGF α staining (Fig. 3A). Light staining could also be detected in the stromal cells that border the epithelial cells, but the most intense staining was in the epithelium. Control slides were analyzed using nonimmune IgG and showed no staining (Fig. 3). The EGFR staining at all stages was primarily present in the epithelial cells (Fig. 3). These results support the hypothesis that expression of TGF α may be important during different stages of ovarian cancer in humans.

Quantitative RT-PCR

Transforming growth factor alpha and EGFR gene expression in normal OSE and ovarian surface stromal cells was examined using a sensitive QRT-PCR assay [41]. Steady-state levels of TGF α and EGFR mRNA gene expression were determined and normalized for the constitutively expressed gene cyclophilin, termed 1B15. Normalization for 1B15 gene expression corrected for changes in cell number, amount of RNA, the integrity of initial mRNA, and for small differences in the efficiency of reverse transcription reaction between samples.

Transforming growth factor alpha gene expression was observed in both freshly isolated and cultured OSE and ovarian surface stromal cells (Fig. 4A). A representative singlet experiment was presented from three different experiments. The amount of TGF α expression varied between individual samples, but in general TGF α gene expression was much greater in OSE cells than in stromal cells. While freshly isolated and cultured stromal cells had low but detectable TGF α gene expression, OSE had a greater level of TGF α gene expression (Fig. 4A). The level of TGF α gene expression increased in cultured OSE relative to freshly isolated cells. Epidermal growth factor receptor gene expression was observed in both fresh and cultured OSE at similar levels (Fig. 4B). Epidermal growth factor receptor gene expression was present in freshly isolated stromal cells and the level of expression increased in cultured stromal cells.

The ability of OSE/stroma cells to express the TGF α

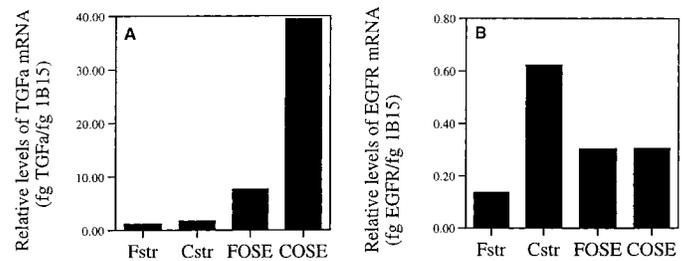


FIG. 4. Quantitation of TGF α and EGFR mRNA expression in OSE and ovarian stromal cells. Bovine OSE cells and ovarian stromal cells were isolated and grown to confluence as described in *Materials and Methods*. Total RNA was extracted from freshly isolated cells or cultured cells. Steady-state TGF α and EGFR mRNA levels were determined in duplicate using QRT-PCR as described in *Materials and Methods*. Amounts of TGF α and EGFR mRNA were determined and normalized to levels of cyclophilin (1B15) mRNA (fg TGF α or EGFR mRNA/fg 1B15 mRNA). A representative singlet experiment of three different experiments is presented. **A**) Transforming growth factor alpha in freshly isolated OSE (FOSE) and stroma cells (Fstr) and cultured OSE (COSE) and stroma cells (Cstr). **B**) Epidermal growth factor receptor in freshly isolated and cultured OSE (FOSE and COSE) and stroma cells (Fstr and Cstr).

gene and respond to TGF α was further investigated by examining the factors that regulate the expression of the TGF α and EGFR genes. The effect of TGF α and gonadotropins on OSE TGF α gene expression was investigated. Gene expression for TGF α was determined using QRT-PCR as described in *Materials and Methods*. Transforming growth factor alpha, EGF, and hCG stimulated TGF α gene expression while FSH and TGF β had no effect on TGF α gene expression in OSE cells (Fig. 5). Epidermal growth factor receptor gene expression was stimulated by EGF, FSH, and hCG, but not by TGF α or TGF β (Fig. 5). Therefore, TGF α can regulate in an autocrine manner TGF α expression by the OSE. Interestingly, the gonadotropins can also regulate TGF α and EGFR gene expression by OSE.

Although TGF α can influence growth of OSE in a direct manner (Fig. 1), potential indirect effects mediated through other growth factors were investigated. Previously, OSE have been shown to express KGF and KL genes, and both KGF and KL can act as autocrine factors to stimulate OSE growth. Transforming growth factor alpha was found to stimulate the expression of both KGF and KL genes by OSE cells (Fig. 6). Basic FGF was used as a negative control and had no effect on KGF or KL gene expression. Transforming growth factor beta was also found to stimulate both KGF and KL gene expression, similar to TGF α (Fig. 6). These results demonstrate that the locally produced TGF α can stimulate expression of other growth factor genes. The interactions between these growth factors appear to act as a positive feedback loop for OSE.

DISCUSSION

Previously within the ovarian follicle the stromal-derived theca cells have been shown to produce TGF α that acts in a paracrine manner to stimulate granulosa cell growth [25]. The current study investigated whether a similar stromal-epithelial interaction occurs between OSE and ovarian stromal cells. Observations indicate that the principle site of TGF α production is the OSE that can act in an autocrine manner to influence OSE growth. Therefore, the interactions between OSE and stroma are distinct from the stromal-epithelial interactions in the follicle. Unlike the granulosa cells in the follicle, the OSE does not represent a true epithelial cell type. The OSE cells are modified mesenthe-

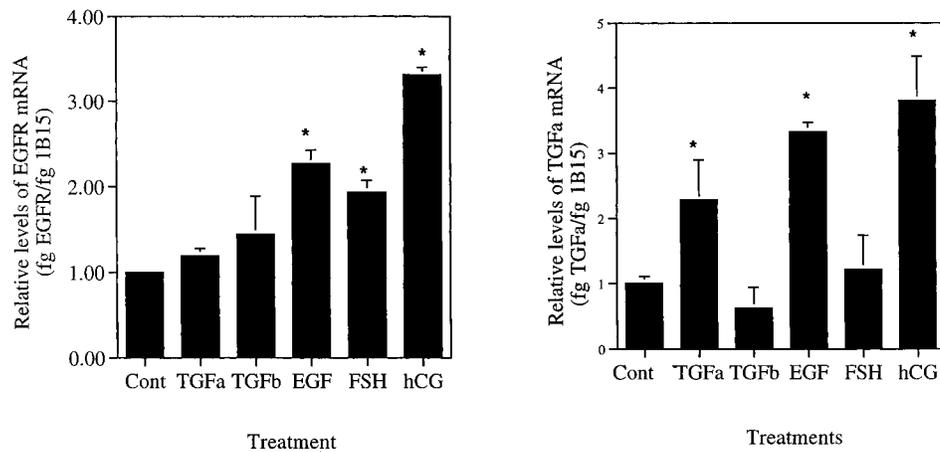


FIG. 5. Regulation of amounts of TGF α and EGFR mRNA in OSE cells. Bovine OSE cells were grown to confluence as described in *Materials and Methods*. Cells were deprived of serum and treated with 50 ng/ml TGF α , 50 ng/ml EGF, 10 ng/ml TGF β , 500 fg/ml FSH, or 26.8 ng/ml hCG for 48 h. Steady-state levels of mRNA were determined in replicate using QRT-PCR as described in *Materials and Methods*. Levels of TGF α and EGFR mRNA were determined and normalized to levels of cyclophilin (1B15) mRNA (fg TGF α or EGFR mRNA/fg 1B15 mRNA). Data are presented as the mean \pm SEM from three different experiments performed in duplicate wells. An ANOVA was performed and bars with asterisks are statistically different from control ($P < 0.05$).

lial cells derived from the same progenitor cells that give rise to the stroma cells. Hence, OSE stain positively for both epithelial cell markers (e.g., cytokeratin) and mesenchymal cell markers (e.g., vimentin). In culture, these cells produce epithelial (e.g., laminin and collagen type IV) and mesenchymal (e.g., collagen types I and III) components of extracellular matrix [43]. A variety of environmental cues cause OSE cells to change from an epithelial to mesenchymal morphology [34]. Therefore, OSE may be relatively immature and uncommitted cells with a dual epithelial-mesenchymal phenotype [34]. The uncommitted differentiated state of this cell may be a factor in its susceptibility to become transformed and develop tumors. The current study establishes that TGF α and its receptor, EGFR, gene expression is observed in normal and tumorigenic OSE. Observations suggest that TGF α acts as an autocrine factor to induce cell proliferation in both normal and tumorigenic OSE.

The current study demonstrates that the TGF α protein is primarily present in the OSE, although less abundant

amounts are detected in the stroma. The EGFR is also present in the OSE and at reduced levels in the stromal cells. This finding supports and extends the view that TGF α could have both an autocrine/paracrine role in OSE-stroma interactions. Growth assays with OSE and stroma cells indicate that TGF α is capable of stimulating both these cell types to proliferate. Growth assays with human cancer cell lines also indicate that TGF α is a potent stimulatory agent for these cells. Immunocytochemical analysis of TGF α in tumor tissue from several stages of ovarian cancer indicates that TGF α is expressed primarily by the epithelial component of the tumor at all stages. In the early stage II ovarian tumor the human OSE also clearly expresses high levels of TGF α . Transforming growth factor alpha gene expression is negligible in the tumor stroma. Epidermal growth factor receptor is also present primarily in the epithelial compartment of the tumors. The presence of TGF α and EGFR in the epithelial compartment of the tumor suggests that TGF α also may stimulate tumorigenic OSE growth in an autocrine manner.

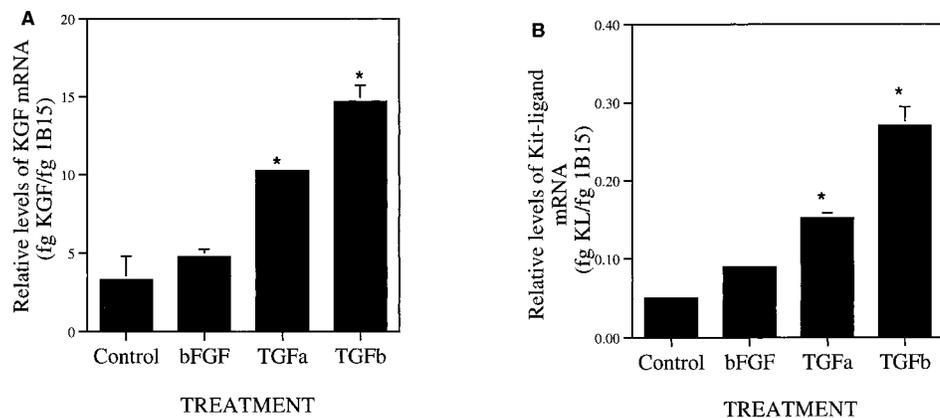


FIG. 6. Regulation of amounts of KGF and KL mRNA in OSE cells. Bovine OSE cells were grown to confluence as described in *Materials and Methods*. Cells were deprived of serum and treated with 50 ng/ml TGF-alpha, 10 ng/ml TGF β , or 40 ng/ml bFGF for 48 h. Steady-state levels of KGF and KL mRNA were determined in replicate using QRT-PCR as described in *Materials and Methods*. Levels of KGF or KL mRNA were determined and normalized to levels of cyclophilin (1B15) mRNA (fg KGF or KL mRNA/fg 1B15 mRNA). Data are presented as the mean \pm SEM from three different experiments performed in duplicate. An ANOVA was performed and bars with asterisks are statistically different from controls ($P < 0.05$). **A**) Keratinocyte growth factor expression by OSE cells. **B**) Kit ligand expression by OSE cells.

The relative amounts of TGF α and EGFR mRNA in freshly isolated OSE and stroma cells indicate that both are present in OSE and at reduced levels in stroma cells that support the immunocytochemical results. The ability of growth factors to regulate TGF α and EGFR expression was examined. Transforming growth factor alpha gene expression was stimulated by TGF α and EGF. Epidermal growth factor receptor gene expression was stimulated by EGF, but not TGF α . Although TGF α acts at the same receptor as EGF (i.e., EGFR), the mechanism for this difference in TGF α and EGF actions remains to be determined. The inability of TGF α to stimulate EGFR gene expression could be a factor of the bioactivity of the factors used. Interestingly, TGF α was able to stimulate its own gene expression, suggesting a positive feedback loop in the autocrine growth regulation of OSE by TGF α . It is likely that such an autocrine growth control will be a factor in the onset and progression of ovarian cancer.

Ovarian cancer primarily affects women in the peri- or postmenopausal period. The role of the changing hormones during this time may be a factor in the progression of the disease. The current study considered the potential role of these hormones in regulating the expression and function of specific growth factor genes. Results from the QRT-PCR experiments indicate that LH stimulates the expression of TGF α by OSE cells. Both LH and FSH stimulates EGFR gene expression by OSE. As menopause is characterized by increased serum concentrations of FSH and LH, it is possible that TGF α and EGFR gene expression are upregulated in the OSE during menopause and may be a factor in the onset and/or progression of the disease. Additionally, the low circulating concentrations of estradiol during menopause may contribute to overexpression of the TGF α gene indirectly, by removing the steroid feedback inhibition on gonadotropins. Thus, changing systemic and local hormone concentrations may affect local growth factor production and eventually OSE biology.

The current study confirms previous observations that normal OSE express the EGFR and a tumorigenic OSE at several tumor stages also express the EGFR [27]. Transforming growth factor alpha has been associated with ovarian cancer [44, 45] and may act as an autocrine growth factor to induce cell proliferation in both normal and tumorigenic OSE [46, 47]. The data presented on normal bovine ovaries and human tumor tissue extend the previous observations with normal human and rodent ovaries with immunocytochemistry and in situ hybridization [27–33, 46, 47]. Several additional growth factors have also been shown to influence OSE. Basic FGF and its receptor are present in human ovarian epithelial cancer tissue [48, 49]. Several ovarian cancer cell lines also proliferate in response to bFGF. Transforming growth factor beta is a multifunctional protein that has a major role in inhibiting the actions of growth stimulators such as EGF/TGF α and bFGF. Transforming growth factor beta has been shown to be produced by OSE [50], and TGF β can inhibit the growth of normal OSE cells and some tumorigenic OSE cells [43, 51, 52].

Previously, OSE were found to express KGF and KL, and both factors promote OSE growth [53, 54]. The possibility that TGF α may promote other growth factor gene expression by OSE was examined. It was found that TGF α is capable of stimulating the expression of two stimulatory growth factors, KGF and KL, by the OSE. Both KGF and KL can stimulate normal and tumorigenic OSE growth [53, 54]. The possibility that the actions of TGF α on OSE growth may be in part indirect through stimulating the ex-

pression of other growth factors remains to be examined. Basic FGF was used as a negative control in this experiment and did not influence KGF or KL gene expression. Transforming growth factor beta was used as a positive control and found to stimulate KGF and KL gene expression. This is in contrast to a previous observation that demonstrated that TGF β can inhibit the ability of cAMP to stimulate KL gene expression [55]. Whether differences in assay sensitivity or basal levels of gene expression is the reason for differences between this and the current study remains to be investigated.

Given the unique characteristics of the OSE, it is likely that the autocrine stimulation of growth, through factors such as TGF α , may be important for normal OSE biology. Abnormal regulation of OSE growth and growth factor gene expression is postulated to be involved in the development and progression of ovarian cancer. The current study helps establish the concept that locally produced growth factors such as TGF α are critical for normal and tumorigenic OSE growth and function.

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REFERENCES

1. ACS Cancer Facts and Figures. Atlanta: American Cancer Society; 1998.
2. Piver MS, Baker TR, Piedmonte M, Sandecki AM. Epidemiology and etiology of ovarian cancer. *Semin Oncol* 1991; 18:177–185.
3. Weiss NS, Homonchuk T, Young JL Jr. Incidence of the histologic types of ovarian cancer: the U.S. Third National Cancer Survey, 1969–1971. *Gynecol Oncol* 1977; 5:161–167.
4. Auersperg N, Maclaren IA, Kruk PA. Ovarian surface epithelium: autonomous production of connective tissue-type extracellular matrix. *Biol Reprod* 1991; 44:717–724.
5. Woessner JF Jr, Morioka N, Zhu C, Mukaida T, Butler T, LeMaire WJ. Connective tissue breakdown in ovulation. *Steroids* 1989; 54: 491–499.
6. Vigne JL, Halburnt LL, Skinner MK. Characterization of bovine ovarian surface epithelium and stromal cells: identification of secreted proteins. *Biol Reprod* 1994; 51:1213–1221.
7. Murdoch WJ. Ovarian surface epithelium, ovulation and carcinogenesis. *Biol Rev Camb Philos Soc* 1996; 71:529–543.
8. Godwin AK, Testa JR, Handel LM, Liu Z, Vanderveer LA, Tracey PA, Hamilton TC. Spontaneous transformation of rat ovarian surface epithelial cells: association with cytogenetic changes and implications of repeated ovulation in the etiology of ovarian cancer [see comments]. *J Natl Cancer Inst* 1992; 84:592–601.
9. Hornby AE, Pan J, Auersperg N. Intermediate filaments in rat ovarian surface epithelial cells: changes with neoplastic progression in culture. *Biochem Cell Biol* 1992; 70:16–25.
10. Kurman RK. *Bleustein's Pathology of the Female Genital Tract*, 3rd ed. New York: Springer-Verlag; 1987: 571–597.
11. Scully RE. Pathology of ovarian cancer precursors. *J Cell Biochem* 1995; 23(suppl):208–218.
12. de Larco JE, Todaro GJ. Growth factors from murine sarcoma virus-transformed cells. *Proc Natl Acad Sci USA* 1978; 75:4001–4005.
13. Massague J. Epidermal growth factor-like transforming growth factor. II. Interaction with epidermal growth factor receptors in human placenta membranes and A431 cells. *J Biol Chem* 1983; 258:13614–1320.
14. Jindal SK, Snoey DM, Lobb DK, Dorrington JH. Transforming growth factor alpha localization and role in surface epithelium of normal human ovaries and in ovarian carcinoma cells. *Gynecol Oncol* 1994; 53:17–23.
15. Jindal SK, Ishii E, Letarte M, Vera S, Teerds KJ, Dorrington JH. Regulation of transforming growth factor alpha gene expression in an ovarian surface epithelial cell line derived from a human carcinoma. *Biol Reprod* 1995; 52:1027–1037.

16. Hanauske AR, Arteaga CL, Clark GM, Buchok J, Marshall M, Hazarika P, Pardue RL, Von Hoff DD. Determination of transforming growth factor activity in effusions from cancer patients. *Cancer* 1988; 61:1832-1837.
17. Arteaga CL, Hanauske AR, Clark GM, Osborne CK, Hazarika P, Pardue RL, Tio F, Von Hoff DD. Immunoreactive alpha transforming growth factor activity in effusions from cancer patients as a marker of tumor burden and patient prognosis. *Cancer Res* 1988; 48:5023-508.
18. Kurachi H, Adachi H, Morishige K, Adachi K, Takeda T, Homma H, Yamamoto T, Miyake A. Transforming growth factor-alpha promotes tumor markers secretion from human ovarian cancers in vitro. *Cancer* 1996; 78:1049-1054.
19. Lee DC, Rochford R, Todaro GJ, Villarreal LP. Developmental expression of rat transforming growth factor-alpha mRNA. *Mol Cell Biol* 1985; 5:3644-3666.
20. Derynck R, Goeddel DV, Ullrich A, Gutterman JU, Williams RD, Bringman TS, Berger WH. Synthesis of messenger RNAs for transforming growth factors alpha and beta and the epidermal growth factor receptor by human tumors. *Cancer Res* 1987; 47:707-712.
21. Wilcox JN, Derynck R. Localization of cells synthesizing transforming growth factor-alpha mRNA in the mouse brain. *J Neurosci* 1988; 8:1901-1904.
22. Madtes DK, Malden LT, Raines EW, Ross R. Induction of transforming growth factor-alpha in activated human alveolar macrophages. *Cell* 1988; 53:285-293.
23. Kobrin MS, Samsouondar J, Kudlow JE. Alpha-transforming growth factor secreted by untransformed bovine anterior pituitary cells in culture. II. Identification using a sequence-specific monoclonal antibody. *J Biol Chem* 1986; 261:14414-14419.
24. Coffey RJ Jr, Derynck R, Wilcox JN, Bringman TS, Goustin AS, Moses HL, Pittelkow MR. Production and auto-induction of transforming growth factor-alpha in human keratinocytes. *Nature* 1987; 328:817-820.
25. Skinner MK, Coffey RJ. Regulation of ovarian cell growth through the local production of transforming growth factor-a by theca cells. *Endocrinology* 1988; 123:2632-2638.
26. Lobb DK, Kobrin MS, Kudlow JE, Dorrington JH. Transforming growth factor-alpha in the adult bovine ovary: identification in growing ovarian follicles. *Biol Reprod* 1989; 40:1087-1093.
27. Owens OJ, Leake RE. Epidermal growth factor receptor in normal ovaries and benign ovarian tumours. *Eur J Obstet Gynecol Reprod Biol* 1992; 47:229-233.
28. Wang DP, Konishi I, Koshiyama M, Nanbu Y, Iwai T, Nonogaki H, Mori T, Fujii S. Immunohistochemical localization of c-erbB-2 protein and epidermal growth factor receptor in normal surface epithelium, surface inclusion cysts, and common epithelial tumours of the ovary. *Virchows Arch A Pathol Anat Histopathol* 1992; 421:393-400.
29. Henzen-Logmans SC, Berns EM, Klijn JG, van der Burg ME, Foekens JA. Epidermal growth factor receptor in ovarian tumours: correlation of immunohistochemistry with ligand binding assay. *Br J Cancer* 1992; 66:1015-1021.
30. Henzen-Logmans SC, van der Burg ME, Foekens JA, Berns PM, Brussee R, Fieret JH, Klijn JG, Chadha S, Rodenburg CJ. Occurrence of epidermal growth factor receptors in benign and malignant ovarian tumors and normal ovarian tissues: an immunohistochemical study. *J Cancer Res Clin Oncol* 1992; 118:303-307.
31. Kohler M, Bauknecht T, Grimm M, Birmelin G, Kommos F, Wagner E. Epidermal growth factor receptor and transforming growth factor alpha expression in human ovarian carcinomas. *Eur J Cancer* 1992; 1432-1437.
32. Khalifa MA, Lacher DA, Lage JM, Mannel RS, Walker JL, Angros LH, Min KW. Immunohistochemical assessment of proliferation markers and altered gene expression in archival specimens of ovarian epithelial tumors. *Cancer Detect Prev* 1997; 21:532-539.
33. Tamura M, Sasano H, Suzuki T, Fukaya T, Funayama Y, Takayama K, Takaya R, Yajima A. Expression of epidermal growth factors and epidermal growth factor receptor in normal cycling human ovaries. *Hum Reprod* 1995; 10:1891-1896.
34. Siemens CH, Auersperg N. Serial propagation of human ovarian surface epithelium in tissue culture. *J Cell Physiol* 1988; 134:347-356.
35. Adams AT, Auersperg N. A cell line, ROSE 199, derived from normal rat ovarian surface epithelium. *Exp Cell Biol* 1985; 53:181-188.
36. Marchant J. Animal models for tumors of the ovary. In: Murphy ED, Beamer WG (eds.), *Biology of Ovarian Neoplasia*. Geneva, Switzerland: International Union Against Cancer (UICC) Tech. Report Series, vol. 50; 1980: 50-56.
37. Mason HD, Carr L, Leake R, Franks S. Production of transforming growth factor-alpha by normal and polycystic ovaries. *J Clin Endocrinol Metab* 1995; 80:2053-2056.
38. May JV, Stephenson LA, Turczynski CJ, Fong HW, Mau YH, Davis JS. Transforming growth factor beta expression in the porcine ovary: evidence that theca cells are the major secretory source during antral follicle development. *Biol Reprod* 1996; 54:485-496.
39. Kruk PA, Maines-Bandiera SL, Auersperg N. A simplified method to culture human ovarian surface epithelium. *Lab Invest* 1990; 63:132-136.
40. Doraiswamy V, Knutson DL, Grazul-Bilska AT, Redmer DA, Reynolds LP. Fibroblast growth factor receptor (FGFR)-1 and -2 in the ovine corpus luteum throughout the estrous cycle. *Growth Factors* 1998; 16:125-135.
41. Parrott JA, Skinner MK. Developmental and hormonal regulation of keratinocyte growth factor expression and action in the ovarian follicle. *Endocrinology* 1998; 139:228-235.
42. Wong WS, Wong YF, Ng YT, Huang PD, Chew EC, Ho TH, Chang MZ. Establishment and characterization of a new human cell line derived from ovarian clear cell carcinoma. *Gynecol Oncol* 1990; 38:37-45.
43. Auersperg N, Maines-Bandiera SL, Dyck HG, Kruk PA. Characterization of cultured human ovarian surface epithelial cells: phenotypic plasticity and premalignant changes. *Lab Invest* 1994; 71:510-518.
44. Yeh J, Yeh YC. Transforming growth factor-alpha and human cancer. *Biomed Pharmacother* 1989; 43:651-659.
45. Owens OJ, Leake RE. Growth factor content in normal and benign ovarian tumours. *Eur J Obstet Gynecol Reprod Biol* 1992; 47:223-228.
46. Bast RC Jr, Jacobs I, Berchuck A. Malignant transformation of ovarian epithelium [editorial; comment]. *J Natl Cancer Inst* 1992; 84:556-558.
47. Stromberg K, Collins 4th TJ, Gordon AW, Jackson CL, Johnson GR. Transforming growth factor-alpha acts as an autocrine growth factor in ovarian carcinoma cell lines [published erratum appears in *Cancer Res* 1992 Mar 1;52:1382]. *Cancer Res* 1992; 52:341-347.
48. Di Blasio AM, Carniti C, Vigano P, Vignali M. Basic fibroblast growth factor and ovarian cancer. *J Steroid Biochem Mol Biol* 1995; 53:375-379.
49. Di Blasio AM, Centinaio G, Carniti C, Somigliana E, Vigano P, Vignali M. Basic fibroblast growth factor messenger ribonucleic acid levels in eutopic and ectopic human endometrial stromal cells as assessed by competitive polymerase chain reaction amplification. *Mol Cell Endocrinol* 1995; 115:169-175.
50. Berchuck A, Rodriguez G, Olt G, Whitaker R, Boente MP, Arrick BA, Clarke-Pearson DL, Bast RC Jr. Regulation of growth of normal ovarian epithelial cells and ovarian cancer cell lines by transforming growth factor-beta. *Am J Obstet Gynecol* 1992; 166:676-684.
51. Auersperg N, Edelson MI, Mok SC, Johnson SW, Hamilton TC. The biology of ovarian cancer. *Semin Oncol* 1998; 25:281-304.
52. Grunt TW, Somay C, Oeller H, Dittrich E, Dittrich C. Comparative analysis of the effects of dimethyl sulfoxide and retinoic acid on the antigenic pattern of human ovarian adenocarcinoma cells. *J Cell Sci* 1992; 103:501-509.
53. Parrott JA, Skinner MK. Expression and action of keratinocyte growth factor (KGF) in normal ovarian surface epithelium and ovarian cancer. *Mol Cell Endocrinol* 2000; (in press).
54. Parrott JA, Skinner MK. Expression and action of kit ligand/stem cell factor in normal ovarian surface epithelium and ovarian cancer. *Biol Reprod* 2000; 6:1600-1609.
55. Ismail RS, Cada M, Vanderhyden BC. Transforming growth factor-beta regulates kit ligand expression in rat ovarian surface epithelial cells. *Oncogene* 1999; 19:4734-4741.