Tissue-Specific Pathways for Estrogen Regulation of Ovarian Cancer Growth and Metastasis

Monique A. Spillman1,2, Nicole G. Manning2, Wendy W. Dye2, Carol A. Sartorius2, Miriam D. Post3, Joshua Chuck Harrell2, Britta M. Jacobsen2, and Kathryn B. Horwitz2,3

Abstract

Menopausal estrogen (E2) replacement therapy increases the risk of estrogen receptor (ER)-positive epithelial ovarian cancers (EOC). Whether E2 is tumorigenic or promotes expansion of undiagnosed preexisting disease is unknown. To determine E2 effects on tumor promotion, we developed an intraperitoneal mouse xenograft model using ZsGreen fluorescent ERα 2008 and ERα PEO4 human EOC cells. Tumor growth was quantified by in vivo fluorescent imaging. In ERα tumors, E2 significantly increased size, induced progesterone receptors, and promoted lymph node metastasis, confirming that ERs are functional and foster aggressiveness. Laser-captured human EOC cells from ERα and ERα xenografted tumors were profiled for expression of E2-regulated genes. Three classes of E2-regulated EOC genes were defined, but <10% were shared with E2-regulated breast cancer genes. Because breast cancer selective ER modulators (SERM) are therapeutically ineffective in EOC, we suggest that our EOC-specific E2-regulated genes can assist pharmacologic discovery of ovarian-targeted SERM. Cancer Res; 70(21); 8927–36. ©2010 AACR.

Introduction

Estrogen replacement therapy (ERT) was hailed as the preserver of femininity, and before 1980, it represented >67% of postmenopausal hormone prescriptions (1). However, because unopposed ERT increased uterine cancer risk, hysterectomy became a prerequisite. This procedure removed the uterus, leaving the ovaries intact and susceptible to neoplastic transformation. Epithelial ovarian cancer (EOC) is a menopausal disease whose relative risk (RR) rises in the sixth and seventh decades of life from basal levels (RR, 1.0) in untreated women to RR of 1.22 to 1.72 in women prescribed ERT more than 5 years (2–6). Although epidemiologic data postulate a link between extended ERT and EOC, causality has not been proven. Two alternative hypotheses exist: that ERT directly causes neoplastic transformation of ovarian surface epithelium (OSE), or that ERT promotes proliferation and metastasis of preexisting, occult disease. Evidence for the first hypothesis is found in the facts that estrogen (E2) levels in ovarian tissues exceed circulating levels by 100-fold, and E2 has direct genotoxic effects in breast cancers (7). The second hypothesis postulates that prolonged ERT promotes proliferation and expansion of preexisting malignancy. In fact, 38% to 62% of EOCs are estrogen receptor-α (ER) positive (8, 9), making them good targets for E2 stimulation. Ovarian tumors from older women (≥65 years), although more likely to be ERα, are less likely to express progesterone receptors (PR; ref. 10). This paucity of PR may suggest that ER signaling becomes deficient with aging.

Here, we test aspects of the second hypothesis that ERT promotes expansion of preexisting malignancy. Using highly fluorescent ERα and ERα human ovarian cancer cell lines engrafted in the peritoneum of nude mice, we developed a site-specific, rigorously controlled, quantitative xenograft model of ERT-stimulated ovarian cancer. We show that ERT substantially increases proliferation and risk of distant lymph node (LN) metastasis only in ERα tumors, possibly through E2 regulation of cellular motility genes. Additionally, we identify E2-regulated genes specific to ERα ovarian cancers, and not shared with ERα breast cancers. We propose that these genes can be used for pharmacologic discovery and interventions with E2 antagonists that uniquely target ERα ovarian cancers.

Materials and Methods

Cell culture

PEO4 cells (11) were a gift from Dr. Anne Bowcock (Washington University, St. Louis, MO), and 2008 cells (12) were obtained from Southwestern Medical School (Dallas, TX). Cell lines were authenticated by polymorphic short tandem repeat repeat analysis at the University of Colorado DNA Sequencing and Analysis Core before xenografting (July 2008 and May 2010). Cells were routinely cultured at 37°C and 5% CO2 in RPMI 1640 with 10% fetal bovine serum. Cells were
tagged with ZsGreen by retroviral transduction (13) and routinely tested for absence of Mycoplasma.

**Mouse xenografts**

Studies were approved by the University of Colorado Anschutz Medical Campus Institutional Animal Care and Use Committee. Female athymic nude mice (5–6 weeks of age; Harlan Sprague-Dawley) were anesthetized with Avertin and injected i.p. with $10^7$ ZsGreen-tagged ovarian cancer cells. A silastic pellet containing 2 mg cellulose or 17β-estradiol (E2) was implanted subcutaneously (14). Mice were weighed and monitored weekly for fluorescent tumor burden using Illumatool Bright Light System LT-9500 (Lightools Research). At necropsy, uteri were weighed to document appropriate estrogenization and fluorescent tumors were resected. Tumor samples were frozen in optimal cutting temperature compound for gene expression profiling or fixed overnight in 4% paraformaldehyde at 4°C and paraffin embedded for pathology and immunohistochemical analyses.

**IVIS imaging**

Biweekly fluorescent quantitative tumor imaging was done with IVIS 200 Optical Imaging System (Xenogen, Caliper Life Sciences) in live mice under isoflurane anesthesia. Living Image 2.60.1 (Caliper Life Sciences) software was used for quantitative analysis at 0.5-, 1-, and 5-second time points. Fluorescent background subtraction was performed, and data were displayed in log scale photons, flat fielded, with cosmic ray correction. Each abdominal region of interest (ROI) quantitation was 3.05 cm by 4.12 cm; a separate thoracic background ROI was 2.23 cm by 1.79 cm. Statistical analysis used Student’s t test for the difference between control and E2-treated groups at each time point, and Fisher’s exact test for differences in categorical variables. Cell doubling times were calculated from exponential growth curves derived from the fluorescent imaging data. Graphs were created in GraphPad Prism 5.

**Histology and immunohistochemistry**

Histology and immunohistochemistry were conducted according to standard protocols (15). Antibodies used were as follows: ER (1:20, SP1 clone RM9101; LabVision/NeoMarkers), PR (1:500, PgR1294 clone M3568; Dako), and bromodeoxyuridine (BrdUrd; 1:50, 347580; Becton Dickinson). A Nikon Eclipse Ti microscope, Ds-Fi1 color camera, and NIS Elements AR 3.1 software were used for digital photography. Human ovarian cancer tissue arrays were from BioChain Institute and stained for ER and PR.

**Proliferation**

After 10 to 12 weeks of tumor growth, 200 μL BrdUrd (Sigma) at 10 mg/mL in PBS was injected i.p. 3 hours before sacrifice. BrdUrd incorporation into DNA was assessed by immunohistochemistry. The proliferative fraction was calculated by dividing the number of BrdUrd-positive nuclei by the number of total nuclei per high-power field. A minimum of three high-power fields/tumor and a minimum of three independent tumors were counted for each cell line and condition.

**Gene expression profiling**

Intraperitoneal tumors from 2008 and PEO4 cells were frozen at necropsy from quadruplicate −E2- and +E2-treated mice. Human tumor cells were laser dissected from surrounding mouse tissues using an Autopix 100e (Molecular Devices). RNA was isolated (PicoPure RNA Isolation kit, Molecular Devices), amplified (WT-Ovation FFPE RNA Amplification System V2, NuGEN), and biotin labeled (FL-Ovation cDNA Biotin Module V2, NuGEN). RNA integrity was confirmed (Bioanalyzer 2100, Agilent). One −E2 PEO4 replicate was degraded and excluded from analyses. Samples were hybridized to U133 Plus 2.0 (Affymetrix) whole human genome expression arrays in the University of Colorado Cancer Center Microarray Core. Gene expression analysis used Partek software. An ANOVA analysis was done with hormone and cell type as fixed factors and an interaction between hormone and cell type included. Genes absent across all cell types were filtered out, and genes with a significant interaction ($P < 0.05$) between hormone and cell type were filtered in. Statistically significant genes were sorted by highest-fold expression change between PEO4 −E2 versus +E2 treatments, and with no significant change in 2008 −E2 versus +E2 treatment. Supervised clustering analysis was performed using standard correlation as similarity measure and an average linkage clustering algorithm (GeneSpring GX7.3.1). Overlapping gene lists were generated using GeneSpring (GX7.3.1). E2-regulated breast cancer gene lists were obtained from published data (16–18). Microarray data have been submitted to Gene Expression Omnibus (accession number GSE22600; http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE22600).

**Results**

**Fluorescent ovarian cancer intraperitoneal xenograft mouse model**

Two different human serous ovarian carcinoma cell lines were engrafted in immunodeficient mice: 2008 cells (12), which are ER− PR−, served as controls for PEO4 cells (11), which are ER+ PR+. Both cell lines were stably transduced with a ZsGreen expressing retrovirus for bright permanent green fluorescence. Female athymic nude mice, ovariectomized 4 to 6 weeks after birth to remove endogenous E2 and estrous cycling, were injected i.p. with $10^7$ cells, and tumors were allowed to grow for 60 to 70 days. Figure 1A shows fluorescent imaging at necropsy of ER− 2008 ovarian tumor small-bowel implants. Figure 1B shows ER+ PEO4 disease, which exhibits the hallmarks of advanced ovarian cancer—peritoneal studding (arrow), bowel implants, and omental implants (asterisk). At necropsy, intraperitoneal disease was visible in 67% (42 of 62) of mice bearing ER− 2008 tumors and in 69% (50 of 72) of mice bearing ER+ PEO4 tumors. Concordance between the two indicates that the ovariectomized mouse peritoneum provides a hospitable environment for tumor growth. Both 2008 and PEO4 tumors show similar histology, morphologically consistent with high-grade EOC. The ER− 2008 implants (Fig. 1C) have irregular nuclei, prominent nucleoli, loose cell association, and...
moderate mitotic activity. The ER− PEO4 nodules (Fig. 1D) show cohesive cells with distinct cell membranes, prominent nuclei, large nucleoli, and moderate mitotic activity.

**Intraperitoneal ovarian tumor burden is significantly increased by ERT**

E2 falls from 128 pg/mL physiologic levels in the normal cycling mouse to 31 pg/mL in ovariectomized mice. E2 supplementation with 17β-estradiol–releasing pellets implanted subcutaneously restores blood levels to 139 pg/mL (14). We confirmed restoration of physiologic E2 by measuring uterine weights. Mean control uterine weights were 0.06 g in E2-deprived ovariectomized mice and 0.18 g in E2-supplemented mice, a statistically significant increase ($P = 0.002$).

After establishing the physiologic effectiveness of E2 supplementation, we assessed E2 effects on tumor volumes. Qualitative fluorescent examination of intraperitoneal ER− 2008 tumors at necropsy failed to reveal a significant E2 effect on disease volume (Supplementary Fig. S1A, B, E, and F). In contrast, prolonged E2 significantly increased the intraperitoneal disease volume in ER+ PEO4 implants (Supplementary Fig. S1C, D, G, and H). Differences in tumor volume in response to E2 were confirmed by quantifying tumor fluorescence by photon flux IVIS imaging (Fig. 2). Figure 2A shows serial quantitation of ER− 2008 implants. Fluorescent images were obtained 22, 49, and 63 days after i.p. injection of tumor cells in five control (left) and five E2-treated (right) mice. Data plotted as mean and SEM for nine control and nine E2-treated mice (Fig. 2B) clearly show that the points overlap ($P = 0.83, 0.69$ for 49, and 63 days, respectively), indicating that ER− 2008 ovarian tumor implants are not E2 responsive. In contrast, quantitation of ER+ PEO4 tumor volume showed a significant increase with E2. Figure 2C illustrates 10 ovariectomized mice bearing PEO4 implants 22, 49, and 63 days after i.p. injection without (left) or with (right) E2 supplementation. At 22 days, the control and ERT points overlap ($P = 0.46$), but by 49 days, disease volume in the ERT mice was significantly increased ($P = 0.033$) and rose further at 63 days ($P = 0.013$). Exponential growth curves plotted from the fluorescent data show that 2008 control cells have a doubling time of 38.74 days, which is similar to the 35.31 day doubling time for 2008 E2 cells (not shown). On the other hand, PEO4 control cells have a doubling time of 93.25 days, which is accelerated to 18.08 days for PEO4 E2 cells (not shown).

**Prolonged ERT promotes LN metastases**

Proliferation of intraperitoneal tumors is a hallmark of advanced-stage EOC. Additionally, 10% of patients exhibit extraperitoneal LN metastases, an ominous finding (19). Figure 3 shows our ability to model ovarian tumor cell translocation from the abdominal cavity to extraperitoneal LN. Figure 3A shows in situ liver, heart, and lungs of a mouse bearing ER− PEO4 tumors. Paired bilateral LNs are clearly fluorescent (arrows), indicating the presence of tumor cells. Figure 3B shows histologic confirmation of PEO4 tumor cells (arrow) infiltrating under the nodal capsule and pushing against lymphoid tissues. Histologically, PEO4 cells metastatic to LN resemble PEO4 cells in the peritoneum (compare Figs. 1C and 3B). LN metastases were present in 6% (2 of 33).
of PEO4 and in 0% (0 of 26) of 2008 tumor-bearing, ovariec-
omized mice without E2 supplementation. This rose to 26% (10 of 39) of ER+ PEO4 mice and to 9% (3 of 33) of ER-
− 2008 mice with E2 supplementation. In this model, ERT is associ-
ated with a significant (P = 0.03) increase in metastases in
ER+ PEO4 mice.

In ER+ tumors, prolonged ERT induces PRs
Thirty-six percent of EOCs express both ER+ and PR+ (8).
The quantitative increase in PEO4 tumor burden in E2-
supplemented mice (Fig. 2D) suggested that ERs were pre-
sent and functional. Figure 4 is an immunohistochemical
analysis of ER and PR in 2008 and PEO4 tumor implants in
the absence of E2, and of PEO4 intraperitoneal implants and
LN metastases in the presence of E2. In contrast to 2008
tumors that lack ER (Fig. 4A), PEO4 implants from ovariec-
tomized mice without E2 supplementation express high ER
levels (Fig. 4B). PRs are absent in both types of tumors
(Fig. 4E and F). With E2, high levels of ER were maintained
in the PEO4 intraperitoneal implants and LN metastases
(Fig. 4C and D). Additionally, E2 treatment induced PR,
indicating that the ERs were functional (Fig. 4G and H).

We noted that ER and especially PR expression was
heterogeneous, with some cells strongly positive and others
having lower or negative receptor levels. We asked whether
similar heterogeneity existed in a human ovarian serous

Figure 2. ERT significantly increases ER+ intraperitoneal disease volume. A, 10 ovariectomized nude mice received 10⁷ ER− ZsGreen-tagged 2008
cells i.p. (−E2 or +E2 supplementation). The five −E2 mice are on the left; the five +E2 mice are on the right. IVIS imaging heat maps were obtained in
live mice at 22, 49, and 63 d after i.p. injection. Disease burden ranges from lowest (blue) to highest (red). B, quantitation of fluorescent photon flux in nine
mice/cohort show the mean and SEM. Statistical comparison of the means was done by a two-tailed t test. ERT does not change the volume of ER−
disease. C, 10 ovariectomized nude mice received 10⁷ ZsGreen-tagged PEO4 cells, −E2 (left) or +E2 (right), and disease was quantified by IVIS imaging
at 22, 49, and 63 d. D, data for 10 mice/cohort were graphed. ERT significantly stimulated disease at 49 and 63 d (red star). (One mouse showed no
visible intraperitoneal disease at 22 and 49 d, died from infection, and is absent from the Panel A day 63 +E2 cohort.)
adenocarcinoma array by ER and PR immunohistochemistry (Supplementary Fig. S2). Overall, 17 of 66 (27%) of tumors were ER+/PR+, whereas another 10 of 66 (15%) were ER+/PR−. They showed heterogeneity of receptor expression analogous to the xenografts.

**E2 promotes proliferation of ER+ ovarian tumors**

Having shown that ERs are functional in PEO4 tumors, we next asked whether the known mitogenic effects of E2 in endometrial and breast cancers were similarly demonstrable in ovarian cancers (20). To that end, tumor-bearing mice were injected with BrdUrd before necropsy, and its incorporation into S-phase DNA was quantified by immunohistochemistry (Fig. 5). Proliferating cells were present at basal states in both 2008 and PEO4 tumors of ovariectomized mice without E2 supplementation (Fig. 5A). With E2, the number of positive nuclei increased in the PEO4 implants but not in the 2008 implants (Fig. 5A). Quantitation of the BrdUrd incorporation (Fig. 5B) shows that without E2, more cells are undergoing mitosis in 2008 cells (10.7%) than in PEO4 cells (5.5%), suggesting that, on average, 2008 cells are more aggressive. However, E2 significantly (P = 0.0001) increased the proliferative fraction of ER+ PEO4 tumor implants (23.9%) while having nonsignificant (P = 0.188) effects on ER−2008 tumors (13.6%). This suggests that E2 increases the aggressiveness of ER+ disease. The BrdUrd proliferation data mirror the growth doubling times calculated from the fluorescent data in Fig. 2A and C. PEO4 control cells had...
the slowest doubling time, which was significantly accelerated by E2. The intermediate doubling times of 2008 cells were unaffected by E2.

**E2-regulated genes of ER<sup>−</sup> ovarian cancer differ from those of ER<sup>+</sup> breast cancer**

To define transcriptional pathways regulated by ERT in ovarian cancers, ER<sup>−</sup> PEO4 and ER<sup>+</sup> 2008 tumor implants from ovariectomized control and E2-treated mice were gene expression profiled. To insure that only human cells were analyzed, quadruplicate sets of human cells were first laser captured from tumors to separate them from surrounding mouse cells. RNA was obtained from human tumor cells of the four experimental conditions, which were hybridized to human microarrays and gene expression profiled (Materials and Methods). Fig. 6 shows the supervised hierarchical cluster analysis of genes from untreated (−) and E2-treated (+) PEO4 and 2008 tumors that are relatively upregulated (red) or downregulated (blue) compared with unchanged genes. Each column represents an individual tumor, and each row a unique gene probe set. To be considered significantly regulated, each gene had to pass strict statistical ANOVA criteria for cell line and hormone interaction (P < 0.05) as well as >1.5-fold expression changes.

The heat map indicates that E2-responsive genes fall into one of three categories arbitrarily defined as class A, B, and C. In Fig. 6A, genes from ER<sup>−</sup> PEO4 +E2 tumors cluster to the far left, whereas genes from the −E2 control tumors cluster to their right. The two tumor types originate from different gene branches (top). In contrast, the ER<sup>+</sup> 2008 samples cluster randomly with regard to control or +E2 status. Class A genes (Fig. 6A, top) are ones whose expression is low (blue) and independent of E2 in ER<sup>−</sup> 2008 tumor implants, similarly low in −E2 ER<sup>−</sup> PEO4 implants, but upregulated (red) by +E2 in ER<sup>−</sup> PEO4 implants. Class A genes were expected to include classic E2-inducible transcripts. In fact, PR transcripts called PGR, well known to be E2 regulated (21), fell into this category and were significantly induced by E2 in ER<sup>−</sup> PEO4 tumors. E2 induction of PR transcript expression validates the statistical analysis and confirms the PR protein immunohistochemical data (Fig. 4G). A second known E2-regulated transcript of interest, called gene regulated by estrogen in breast cancer 1 (GREB1), was also identified (22). Supplementary Table S1 lists the class A genes.

Class B genes (Fig. 6A, bottom) are constitutively high (red) in ER<sup>−</sup> 2008 tumor implants independent of E2 treatment. In ER<sup>−</sup> PEO4 tumors, their expression is low in the absence of E2 (blue), but they are E2 inducible (red). Class B genes have lost regulation by E2 in ER<sup>−</sup> ovarian cancer cells—their upregulation having been hijacked by other signaling pathways—while retaining E2 regulation in ER<sup>+</sup> ovarian cancer cells. Several class B genes previously defined as having roles in ovarian cancer metastasis and stem cell signatures included CD44 (23). Palladin (24) and fascin 1 (25) are cellular motility genes whose expression was not previously suspected as being E2 regulated. Supplementary Table S2 lists class B genes.

Class C genes (Fig. 6B) are constitutively high (red) in E2-deprived ER<sup>−</sup> PEO4 cells but downregulated by E2 (blue). Sixteen class C genes were identified with known E2 regulation, including KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 3 (KDEL.R3), an interesting gene linked to chemotherapy response in ovarian cancers (26). ROPN1 is a cancer-associated testis antigen, which is more highly expressed in ER<sup>−</sup> than in ER<sup>+</sup> breast cancers.
Supplementary Table S3 lists class C genes. Quantitative PCR confirmed the E2-regulated expression of a subset of class A, B, and C genes (Supplementary Fig. S3). Six genes were selected for quantitative PCR, and each one was statistically differentially expressed in each class for a 100% validation rate.

Our laboratory has previously published E2-regulated genes in T47D human breast cancer cells using a similar E2-supplemented nude mouse xenograft model (17). We compared our E2-regulated ovarian cancer genes to the previously defined breast cancer genes and found that only three class A genes (NEDD9, FBXL7, and CA12) and three class C genes (B3GNT3, GLDC, and PPAP2B) were shared. Next, we compared the class A and B E2-regulated ovarian cancer genes with other previously published E2-regulated breast cancer genes (16–18). Surprisingly, only 6.8% (10 of 146) of class A and 9.9% (15 of 152) of class B ovarian cancer genes overlapped with E2-regulated breast cancer genes (16–18). Shared class A and B genes are listed in Supplementary Table S4.

Discussion

Epidemiologic meta-analyses assessing menopausal ERT show a combined RR of 1.22 of developing EOC after 5 years of E2 use (3). The role of E2 in the development and promotion of EOC is unclear. We hypothesized that prolonged ERT promotes growth and metastasis of preexisting ER+ EOC cells through activation of specific gene expression programs. To test this, we developed a mouse model of preexisting disease that allows quantitative assessment of ovarian tumor burden in response to E2 exposure. Our model uses ZsGreen fluorescence, allowing external serial visualization of tumor implants and quantitation by emission fluorescent photon flux, in living mice.

By using E2 supplementation in an ovariectomized mouse, the specific effects of estrogenization can be analyzed. This model produces serum E2 levels of 125 pg/mL (14), similar to the range obtained in women (28). We find that compared with ovariectomized mice, continuous ERT significantly increased fluorescence signal intensity in mice bearing ER+ PEO4 tumor implants (Fig. 2D). The increase was due to E2-induced cell proliferation as quantified from exponential growth curves (Fig. 2D) and by BrdUrd incorporation (Fig. 5B). As expected, ERT had no effect on the proliferative index of ER+2008 tumors. This confirmed our hypothesis that ERT-induced growth of ovarian cancer cells is limited to ER+ disease. Modeling the action of ERT in ovariectomized mice at the natural site of ovarian cancer implantation is critically important. Previously, Langdon and colleagues (29) implanted PEO4 cells for 4 weeks in the flank of intact cycling nude mice.

Figure 6. Supervised hierarchical clustering of genes expressed in ovarian cancers identifies three ERT-regulated subgroups in ER+ disease. ER+ 2008 or ER+ PEO4 ovarian tumor cells were grown i.p. in ovariectomized nude mice supplemented without (−) or with (+) E2-releasing pellets for ~11 wk. Tumors were harvested and human cells were laser captured from frozen sections. RNA was hybridized to U133 Plus 2.0 whole human genome expression arrays. Statistical analyses were performed as described in Materials and Methods. Statistically significant genes were sorted by highest-fold expression change between PEO4−E2 versus +E2, and with no significant change in 2008−E2 versus +E2. Each column represents a separate tumor, and each row a transcript. Red (high) and blue (low) indicate relative transcript expression levels. A, genes regulated in ER+ PEO4 versus ER+ 2008 showing class A and B genes that are upregulated by E2 only in ER+ cells; they are constitutively low (class A) or high (class B) in ER− cells. B, class C genes are downregulated by E2 from high basal levels in ER+ cells; they are constitutively low in ER− cells.
mice before ERT supplementation. Paradoxically, growth was inhibited in comparison with controls. Significant experimental differences between our models include use of cycling mice by Langdon and colleagues (29), in which progesterational influences are retained (30). Our ovariectomized model allows independent future assessment of the role of progesterone alone or when combined with E2 as in EPRT hormone replacement therapies.

Two previously published reports of E2-regulated genes in ovarian cancer cell lines were in vitro and used short-term E2 stimulation. O’Donnell and colleagues (31) identified 5 E2-upregulated and 23 E2-downregulated genes in PEO1 ovarian cancer cells. We compared our class A genes with their upregulated genes, and our class C genes with their downregulated genes, and only two overlapped. One of these, Cyr61, whose protein product promotes cell adhesion, was E2 downregulated in the study by O’Donnell and colleagues, but E2-upregulated class B in ours. E2 upregulation of Cyr61 agrees with previous reports in MCF-7 breast cancer cells (32). The second study (33) used transformed human ovarian surface epithelial cells and ovarian cancer cell lines. The authors identified 155 E2-regulated genes in ovarian surface epithelium cells, and 315 in cell lines, of which 19 overlapped between the two models. There were no exact matches between those 19 and our E2-regulated genes.

Our study is the first to identify E2-regulated genes in ER− ovarian cancer xenografts using ER− tumors as negative controls. This comparison was essential to identification of class B genes, which are constitutively high in ER− tumors, but E2 regulated in ER+ tumors. Class B may represent genes critical to the transition from E2 sensitivity to E2 independence. Intriguingly, 15 of the class B genes were “cell motility” genes, including CD44 (34) and fascin 1 (35). None of the class B motility genes have been previously identified as E2 regulated.

The importance of E2 regulation of cell motility in ovarian cancer is further supported by our striking observation that mice treated with prolonged ERT preferentially developed distant LN metastases (Fig. 3). Although metastases occurred in both ER+ and ER− tumors, they were significantly increased by ERT only in ER+ tumors. ERT may also promote metastasis through direct action on the metastatic niche in the host compartment explaining its more subtle effects in ER− disease. Previously, E2 has been shown to regulate the host compartment of ovariectomized mice to promote lung metastasis of ER− lung and mammary carcinoma cell lines (36).

PR, the canonical E2-regulated gene in the breast, is also E2 regulated in PEO4 cells (37). E2 induction of PR served as an internal control for our models and was a highly expressed class A gene. E2 regulation of PR was confirmed by immunohistochemistry in PEO4 tumors (Fig. 4) and was found to be heterogeneous, analogous to human ovarian tumors (Supplementary Fig. S2). Areas of high PR expression are not exclusively restricted to areas of high ER expression, which may indicate that ERs are downregulated in some cells within tumors. Heterogeneity of ER and PR expression within ovarian tumors may explain conflicting results on the correlation between receptor expression and survival advantage (38–40). Maintenance of functional ER in younger ovarian cancer patients, as evidenced by coexpression of PR (10), gives support to our hypothesis that a subset of ovarian cancers is E2 regulated. Our gene expression profiles are most applicable to this subgroup of patients.

The presence of E2-upregulated genes in EOC makes anti-estrogen therapy an attractive premise. Although antiestrogens have been a mainstay of breast cancer treatment, in EOC they have been reserved for chemotherapy-refractory tumors, where they have a relatively low overall response rate. In a Gynecologic Oncology Group study of recurrent EOC (41), tamoxifen elicited an 18% response rate, correlated with ER expression. The pure antiestrogen fulvestrant stabilized 50% of disease, but few tumors regressed (42). A similar response rate was seen with the aromatase inhibitor letrozole, where 20% of patients had stable disease for at least 12 weeks on therapy (43). The response correlated with high expression of ER and PR, defining an “endocrine-sensitive” subgroup (43).

Current selective ER modulators (SERM) such as tamoxifen and fulvestrant were designed to regulate genes in breast and bone. The lack of significant response to SERM in EOC may reflect poor tissue specificity of current agents, including differences in the tissue repertoire of ER coregulators in ovarian versus breast tumors, rather than bona fide attenuation of ERs signaling. Our ERT-regulated ovarian cancer genes represent the largest collection of potential therapeutic targets in the literature. SERM could be screened against these genes to identify compounds likely to have efficacy and specificity in EOC.

Our data would suggest that administration of ERT to women who have ovarian cancer should be viewed with caution, but there are no clinical trials to suggest that this worsens prognosis (44). One small prospective clinical trial of ERT administered to ovarian cancer patients suggested that ERT was associated with prolonged survival. However, the study failed to stratify the patients by tumor ER expression either at enrollment or at time of recurrence (45).

Interestingly, addition of a progesterin to estradiol (EPRT) for menopausal hormone replacement therapy seems to be protective against EOC, but deleterious in breast cancer (46). The differential tissue specificity of ERT versus EPRT in the breast and ovary is likely to be mediated by differences in gene expression patterns. We have compared the gene expression profile of our PEO4 EOC tumors with previously published E2-regulated gene data sets in breast cancer (16–18). Less than 10% of the ovarian cancer genes are shared with breast cancer genes. No single signaling pathway was predominant in the shared cohort, a finding that emphasizes the importance of tissue-specific targeting of pharmacologic therapy. Although we have attributed the paucity of overlap between our ovarian E2-regulated genes and E2-regulated breast cancer genes to tissue specificity, differences in cDNA arrays, data analysis, and time of E2 stimulation may have also contributed to the perceived discrepancy.

In summary, we have developed a site-specific, rigorously controlled, quantitative mouse model of E2-stimulated human ovarian tumors may explain conflicting results on the correlation between receptor expression and survival advantage (38–40). Maintenance of functional ER in younger ovarian cancer patients, as evidenced by coexpression of PR (10), gives support to our hypothesis that a subset of ovarian cancers is E2 regulated. Our gene expression profiles are most applicable to this subgroup of patients.
Estrogen Promotes Ovarian Cancer Growth and Metastasis

Estrogen Promotes Ovarian Cancer Growth and Metastasis

ovarian cancer growth. ERT substantially increased the risk of distant LN metastasis, possibly through E2 regulation of cellular motility genes. Additionally, we have identified E2-regulated genes specific to ovarian cancers. These can be used for pharmacologic discovery and interventions that specifically target ERα ovarian cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

7. Langdon S, Lawrie S, Hay F, et al. Characterization and properties of ovarian cancer motility genes. Additionally, we have identified E2-regulated genes specific to ovarian cancers. These can be used for pharmacologic discovery and interventions that specifically target ERα ovarian cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


Grant Support

Gynecologic Cancer Foundation/Susan G. Komen Career Development Award (M.A. Spillman), Ovarian Cancer Research Fund/Liz Tilberis Scholars (M.A. Spillman), NIH Women’s Reproductive Health Research Scholar grant 5K22HD001277-10 (M.A. Spillman), NIH National Cancer Institute CA000469 (K.B. Horwitz), Avon Foundation (K.B. Horwitz), Breast Cancer Research Foundation (K.B. Horwitz), and National Foundation for Cancer Research (K.B. Horwitz).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 04/13/2010; revised 08/19/2010; accepted 08/30/2010; published OnlineFirst 10/19/2010.
cultures derived from ovarian cancer and in borderline and carcino-
Tissue-Specific Pathways for Estrogen Regulation of Ovarian Cancer Growth and Metastasis


Cancer Res 2010;70:8927-8936. Published OnlineFirst October 19, 2010.