Identification of a Novel Estrogen-Regulated Gene, EIG121, Induced by Hormone Replacement Therapy and Differentially Expressed in Type I and Type II Endometrial Cancer

Lei Deng, Russell R. Broaddus, Adrienne McCampbell, Gregory L. Shipley, David S. Loose, George M. Stancel, James H. Pickar, and Peter J.A. Davies

Abstract

Purpose: The identification of genes and pathways that are affected by estrogenization may shed light on the mechanisms of estrogen action. Here, we describe the expression pattern of a novel estrogen-induced gene, EIG121, in distinct types of endometrial cancer.

Experimental Design: EIG121 was identified by cDNA microarray analysis of endometrial RNA from women receiving either placebo or estrogen replacement therapy. The expression level of EIG121 was then measured by real-time quantitative reverse transcription-PCR in benign, hyperplastic, and malignant endometrial samples. A polyclonal antibody was used to detect EIG121 protein by immunohistochemistry.

Results: In postmenopausal endometrium, estrogen replacement therapy with Premarin and synthetic estrogen sulfate conjugates induced the expression of EIG121-2- and 3-fold, respectively. In premenopausal endometrium, the expression of EIG121 was higher in the estrogen-dominated proliferative phase than the secretory phase. In endometrial complex, hyperplasia, and endometrioid adenocarcinoma, neoplastic proliferations associated with estrogen excess, the expression of EIG121 was significantly suppressed (on average 3.8-fold in hyperplasias and 21-fold in grade 1 tumors). Although the level of EIG121 mRNA in grade 3 endometrioid carcinoma was still 3.5-fold of that in benign endometrium, EIG121 expression tended to decline with increasing tumor grade and disease stage. Immunohistochemistry showed faint staining of normal endometrial epithelium, but intense staining of endometrioid tumors. In sharp contrast, EIG121 expression was significantly suppressed in both uterine papillary serous carcinoma and uterine malignant mixed mullerian tumor, two tumors not associated with estrogen exposure, to <5% of the level in benign endometrium.

Conclusions: Our results suggest that EIG121 is a good endometrial biomarker associated with a hyperestrogenic state and estrogen-related type I endometrial adenocarcinoma.
for the previous two to three decades for hormone replacement therapy, is associated with an increased risk for endometrial hyperplasia and endometrial cancer, especially type I carcinomas (11–13). In addition, an increased risk for endometrial cancer is associated with obesity, polycystic ovary disease,nulliparity, estrogen-producing tumors, and anovulation, all clinical conditions associated with relative estrogen excess (14). Although the positive association of endometrial hyperplasia and cancer with prolonged excessive estrogen stimulation has been well established, the underlying mechanisms are largely unknown. It is generally believed that estrogens are not genotoxic, although some estrogens may produce oxidative species under certain circumstances (15). The widely accepted hypothesis is that estrogens drive cell proliferation and thus allow for the accumulation of random genetic errors. Most functions of estrogens are mediated by estrogen receptors (ER), which belong to the nuclear receptor family of transcription factors. ERs bind to estrogen-responsive elements within enhancer regions of target genes to regulate transcription in a ligand-dependent manner. Because the effects of estrogen on the endometrium are mediated by an array of downstream genes, the identification of genes whose expression is affected by estrogentization may shed light on the mechanisms of estrogen-driven carcinogenesis in the endometrium. To this end, we have used cDNA microarray to screen for potential estrogen-regulated genes in endometrial biopsies from postmenopausal women undergoing estrogen replacement therapy. A novel estrogen-induced gene identified by this approach was termed EIG121. Here, we report its expression in normal endometrium, endometrial hyperplasia, and type I and type II endometrial cancer.

Materials and Methods

Human endometrial frozen tissues and RNA preparation. For the purpose of microarray screening and real-time quantitative PCR verification, two different subsets of normal endometrial biopsies were randomly selected from a large group of healthy postmenopausal women (n = 210) participating in an estrogen replacement therapy clinical trial (16). These 210 women were randomly divided into three groups receiving one of the following three treatments: (a) placebo, (b) conjugated estrogens (2.1, w/w) of estrone sulfate and equilin sulfate (EES; Wyeth Research, Philadelphia, PA) in the amount found in a 0.625 mg/d Premarin tablet, (c) Premarin (Wyeth Research), 0.625 mg/d, for 3 months under conditions approved by the Human Ethics Committee of Escola Paulista de Medicina Universidade Federal de São Paulo, Brazil. Endometrial biopsies were obtained at the end of the 3-month treatment. For the initial microarray screen, 10 endometrial RNA samples from each treatment group were pooled. For real-time quantitative PCR analysis, the second subset of endometrial biopsies (n = 10 each from the placebo, synthetic estrogens, or Premarin group) were analyzed individually.

Endometrial samples from premenopausal cycling women were obtained from hysterectomy surgical specimens submitted to the Department of Pathology, University of Texas M.D. Anderson Cancer Center (Houston, TX). The endometrial samples were classified as proliferative (n = 15) or secretory (n = 9) after microscopic examination of H&E-stained slides. Frozen tissues from 118 endometrial cancers (95 endometrioid adenocarcinoma, 12 uterine papillary serous carcinomas [UPSC], and 11 malignant mixed mullerian tumors [MMMT]) were obtained at the time of hysterectomy. Diagnoses were confirmed following light microscopic examination of H&E-stained slides by a gynecologic pathologist.

RNA preparation from the frozen endometrial tissues was carried out as previously described (16). For the initial microarray screen, pools of total RNA were passed over Oligotex mRNA isolation columns (Qiagen, Valencia, CA) twice to isolate polyadenylated RNA. RNA isolation from formalin-fixed, paraffin-embedded tissues. Formalin-fixed, paraffin-embedded human endometrial biopsies of proliferative endometrium (n = 9), secretory endometrium (n = 4), endometrial complex hyperplasia with atypia (n = 14), and grade 1 endometrioid adenocarcinoma (n = 17) were obtained from the files of the Department of Pathology, the University of Texas M.D. Anderson Cancer Center. H&E-stained slides from each block were carefully examined by a pathologist to confirm the tissue histology and the absence of contaminating tissues such as normal cervix or myometrium. Hyperplasia cases with contaminating normal endometrium were not used. Five 10-μm sections were cut from the paraffin-embedded blocks using a T35 microtome. Tissue sections were deparaffinized using two xylene washes. DNA was then extracted using the MasterPure Reagent Kit (Epicenter, Madison WI). Proteinase K digestion was done for 4 hours at 65°C in a SDS-containing lysis solution followed by isopropanol precipitation and two ethanol washes. DNA was digested by incubation with RNase-free DNase I with RNase Inhibitor for 15 minutes at 37°C. DNase I was heat-inactivated at 75°C for 10 minutes.

cDNA microarray screen. The mRNA samples were converted to Cy3- or Cy5-labeled cDNA and subsequently hybridized to the Human V 43K cDNA microarray by Incyte Genomics (St. Louis, MO). The data were analyzed using the Incyte GEM Tools 2.0 software. Defective cDNA spots (signal/noise ratio <2.5, irregular geometry, or <10% spot area compared with average) were eliminated from the data set. The identity of spotted DNA on the chips was confirmed by PCR and the spots with failed PCR or multiple bands with PCR were also excluded.

Reverse transcription and real-time quantitative PCR. Reverse transcription and quantitative PCR were done as previously described (16). The sequences of primers and probes used in this study are listed in Table 1.

Immunohistochemistry. A polyclonal antibody was raised against the COOH-terminal peptide (CEDDLIFTSKKLFGK) of EIG121 protein by Sigma Genosys (Houston, TX). The antibody was purified by affinity purification using peptide-conjugated CNBr-activated sepharose 4B (Amersham Biosciences, Piscataway, NJ). Antibody concentration was determined using the bicinchoninic acid assay kit from Pierce Laboratories (Rockford, IL). After initial deparaffinization, endogenous peroxidase activity was blocked by using 0.3% H2O2. Sections were then microwaved in 10 mmol/L citrate buffer (pH 6.0) to unmask the epitopes. Slides were incubated in primary antibody (1:100) in PBS containing 10% normal goat serum overnight at 4°C and with biotin-labeled secondary antibody for 30 minutes at room temperature, and finally with streptavidin-peroxidase for 30 minutes. Tissues were then stained for 5 minutes with 0.05% 3,3-diaminobenzidine tetrahydrochloride that had been freshly prepared in 0.05 mol/L Tris buffer (pH 7.6) containing 0.024% H2O2, counterstained with hematoxylin, dehydrated, and mounted.

Statistical analysis. Statistical differences between groups were calculated using the Kruskal-Wallis test. Correlation between any two transcripts was evaluated by the Pearson correlation analysis and confirmed by Spearman’s and Kendall’s tests. Differences were considered significant if P < 0.05.

Results

Identification of a novel estrogen-induced gene, EIG121, by cDNA microarray. We used cDNA microarray to screen for potentially novel genes induced or suppressed by estrogen replacement therapy. Among the ~10,000 expressed sequence tags with unknown identities on the cDNA chip, one clone with an ID of 1808121 was induced 3-fold by estrogen replacement
therapy (Fig. 1). We termed this clone estrogen induced gene 121 (EIG121) and studied it in more detail.

Searching the GenBank database, we found that this expressed sequence tag fragment is identical to the 3' untranslated region of an unknown gene KIAA1324 (NM_020775), which encodes the large, 1,013 amino acid protein maba1 of unknown function. By BLAST search against the human genome database, EIG121 was mapped to the chromosome 1p13.3 region. The EIG121 genomic sequence spans 89.3 kb on chromosome 1 and contains 22 exons and a large intron 1 of >47 kb. By BLAST search, we also found that EIG121 is highly conserved among species during evolution. The rodent EIG121 mRNAs share >90% identity with the human EIG121 mRNA, and the mammalian EIG121 genes are very similar to a few genes with unknown identities in lower organisms including Caenorhabditis elegans, fish, Xenopus, and chicken (data not shown).

**Estrogen regulation of EIG121 expression.** To confirm the data from the initial microarray analysis that estrogen induces endometrial EIG121 expression, a real-time quantitative reverse transcription-PCR assay specific for the 3'-untranslated region of EIG121 transcript was designed. The EIG121 mRNA levels in a different set of 30 endometrial RNA samples (not overlapping with the samples used in the initial microarray screen) obtained from postmenopausal women receiving placebo, Premarin or EES treatments were measured. As shown in Fig. 2A, both Premarin and EES significantly induced EIG121 expression, but EES seemed to induce EIG121 expression more efficiently (3-fold; \( P < 0.01 \)) than Premarin (1.4-fold; \( P < 0.05 \)).

During the proliferative phase of the human menstrual cycle, peripheral blood estrogen levels are relatively high, while progesterone levels remain relatively low. Therefore, the proliferative phase is generally regarded as an estrogen-dominated phase. To determine whether endogenous estrogen has a role in regulating the expression of EIG121, we measured EIG121 mRNA by real-time PCR in endometrial samples obtained from premenopausal women undergoing hysterectomy for nonmalignant uterine diseases. As shown in Fig. 2B, EIG121 mRNA was significantly higher in the proliferative phase than in the secretory phase. The levels of EIG121 expression in placebo-treated postmenopausal endometrium were lower than in the endometrium of premenopausal women at secretory phase (\( P = 0.019 \)), suggesting that endogenous estrogen may be necessary for maintaining endometrial EIG121 expression.

**Overexpression of EIG121 in endometrial hyperplasia and type I endometrial adenocarcinoma.** We next determined whether EIG121 is overexpressed in endometrial complex hyperplasia and type I endometrial carcinoma (endometrioid adenocarcinoma),

### Table 1. Sequences for real-time quantitative PCR assays

<table>
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<tr>
<th>Transcripts</th>
<th>Sequences</th>
<th>Accession no.</th>
</tr>
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<tr>
<td>18S rRNA</td>
<td>(535+) GAGGGAGCCTGAGAAACGG</td>
<td>M10098</td>
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<tr>
<td></td>
<td>(602–) GTGCGGATGGGAATTTGC</td>
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<tr>
<td></td>
<td>(555+) FAM-TACACACATCAAGGAGAGCGAGG</td>
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<tr>
<td>Cyclophilin</td>
<td>52(+) AGCGGAGCCTTGG</td>
<td>NM_021130</td>
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<tr>
<td></td>
<td>117(–) TTCCTGCTCTTTGGAACCT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>69(+) FAM-CGCTCTCTCTTTGAGGTTGTTCA</td>
<td></td>
</tr>
<tr>
<td>EIG121</td>
<td>3074(+) CTTGCAATAGCCCTT TGCAA</td>
<td>NM_020775</td>
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<tr>
<td></td>
<td>3136(–) CAGTGGGCTTGTCAGGATG</td>
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<td></td>
<td>3096(+1) FAM-CTGCGGGCAGTTTGGT</td>
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<tr>
<td>ER(\alpha)</td>
<td>1394(+) TACTGCAACACTGCGACAGG</td>
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<td>ER(\beta)</td>
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![Fig. 1. Identification of a novel estrogen-regulated gene (EIG121) by cDNA microarray screen. Pooled endometrial RNA from postmenopausal women either receiving placebo (\( n = 10 \)) or EES (\( n = 10 \)) for 3 months was subjected to microarray analysis using Incyte Human V cDNA arrays. Scanned images of the spot on the array corresponding to the EIG121 clone hybridized to Cy3 or Cy5 fluorescent probes made from placebo-treated or EES-treated groups, respectively.](https://www.aacrjournals.org/doi/abs/10.1158/1078-0432.CCR-05-0860)
two conditions known to be associated with excessive estrogen exposure (11–14, 17). Because of the lack of frozen tissues from complex hyperplasia cases, we modified the RNA extraction and EIG121 quantitative PCR protocols to allow for the use of formalin-fixed, paraffin-embedded tissues. As shown in Fig. 3A, the expression of EIG121 transcripts showed a progressive increase in complex hyperplasia and grade 1 endometrioid carcinoma. Compared with the benign endometrium, EIG121 mRNA increased 3.8-fold in hyperplasia and >21-fold in the grade 1 tumors. Immunohistochemistry using a polyclonal antibody raised against a peptide in the COOH terminus of EIG121 protein showed strong staining in the cytoplasm and cell membrane of the normal endometrial surface and glandular epithelial cells, especially in the apical aspect, with minimal staining in the stroma (Fig. 3B). Compared with normal endometrium, both the number of EIG121-positive cells and the intensity of EIG121 staining in individual cells were significantly increased in type I endometrial carcinomas (Fig. 3B).

Type I endometrial carcinoma is associated with exposure to unopposed estrogens and can evolve from complex hyperplasia. Using a larger set of frozen tissues of type I tumors, we then sought to determine whether tumor differentiation (grade) influences the expression of EIG121. As shown in Fig. 4A, the level of EIG121 expression was significantly elevated (P < 0.01) in type I endometrial carcinoma overall, to >4.6-fold of its level in benign endometrium. Although the level of EIG121 expression was still significantly higher in the grade 3 tumors, EIG121 level seemed to decline stepwise with increasing tumor grade (Fig. 4A). EIG121 expression also declined stepwise with tumor stage, with the levels in stage III tumors significantly lower than in stage I tumors (Fig. 4B).

Down-regulation of EIG121 expression in type II endometrial cancer. UPSC and MMMT are type II endometrial carcinomas that are associated with advanced clinical stage and a poor prognosis (18, 19). In contrast to the endometrioid tumors, UPSC and MMMT are typically not associated with estrogen exposure (2, 5). As shown in Fig. 5, compared with benign endometrium, EIG121 mRNA was significantly decreased in both UPSC and MMMT. The level of EIG121 mRNA in UPSC and MMMT was only <5% of that of the benign endometrium.

Correlation of EIG121 expression with the expression of estrogen receptor and progesterone receptor. As shown in Fig. 6, statistical analysis revealed that the level of EIG121 transcripts in the endometrial tumors was highly correlated with the expression of transcripts for ERα (r = 0.641; P < 0.01) and progesterone receptor (PR; r = 0.529; P < 0.01), but there was no correlation between the levels of EIG121 and ERβ (r = −0.005; P = 0.96).

Discussion

In this study, we have identified a novel estrogen-induced gene, EIG121, which is induced in normal postmenopausal endometrium by estrogen replacement therapy. In premenopausal women, endogenous estrogen also induces endometrial...
expression of EIG121, as expression is higher in the estrogen-dominant proliferative phase compared with the secretory phase. In endometrial cancer, EIG121 is differentially expressed; it is up-regulated in type I (estrogen-related, good prognosis) endometrial cancer, but suppressed below benign endometrial levels in type II (not estrogen-related, poor prognosis) endometrial cancer.

It has been well-documented that endometrial cancers occurring in women receiving exogenous estrogen are usually grade 1 endometrioid tumors with minimal myometrial invasion. We have found that EIG121, a gene induced by exogenous and endogenous estrogens, is elevated in endometrial complex hyperplasia and is especially high in grade 1 endometrioid tumors, but not in type II tumors such as UPSC and MMMT. The pattern of EIG121 expression in normal endometrium, hyperplastic endometrium, and endometrioid tumors is similar to the expression patterns of two classic estrogen target genes, PR and pS2/TFF1 (a member of the trefoil factor family that is involved in mucosal maintenance and repair). The expression of both PR and pS2 is under estrogen control (20–22). Ghareb et al. (23) recently reported that the expression of PR gradually increases in non-atypical and atypical endometrial hyperplasia and reaches the highest level in grade 1 endometrioid adenocarcinoma, then decreases stepwise in grade 2 and grade 3 endometrioid tumors. Koshiyama et al. (24) has reported that the expression of pS2 increases progressively from normal endometrium to endometrial hyperplasia and well-differentiated carcinoma, but pS2 expression decreases stepwise in grade 2 and grade 3 endometrioid carcinomas and becomes undetectable in non-endometrioid tumors. In addition, we have also found that the expression of EIG121 is highly correlated with that of ERα and PR, but not with ERβ (Fig. 6). The levels of ERβ in the endometrium are extremely low compared with ERα (~100-fold less), suggesting that the regulation of EIG121 is predominantly via ERα in the uterus. Although the expression of EIG121 generally correlated well with the levels of ERα and PR, EIG121 expression was more dramatically altered in both type I and type II tumors than the expression of steroid hormone receptors. For example, EIG121 expression can be up-regulated several hundred-fold in some grade 1 tumors, whereas showing >95% reduction in type II tumors. These results indicate that EIG121 expression may also be modulated independently of hormones during tumor development and progression. We are currently exploring the possibility that EIG121 expression may be altered by DNA amplification or methylation.

Interestingly, we have noted that there was a difference in the activity of Premarin versus the conjugated estrogen preparation (EES) in inducing EIG121 expression (Fig. 2). Although we do not know the pharmacologic basis for this difference, it may be related to the fact that Premarin is a complex mixture of multiple steroids with overall estrogenic activity, whereas EES is known to be relatively pure in its estrogenic activity. Differences in Premarin’s pharmacologic activity when compared with the activity of estrone and equilin sulfates may represent subtle differences in pharmacokinetic or pharmacodynamic activity between the two preparations.
As previously discussed, traditional models of endometrial cancer pathogenesis have divided this malignancy into two relatively distinct clinical/pathologic types (2). However, it has recently been debated that there is overlap between the two types, particularly between grade 3 endometrioid adenocarcinoma and UPSC (25). For example, several studies have found that, when corrected for stage, survival for UPSC is about the same as that for endometrioid grade 3 (26–29).

However, one study found that the presence of even a small percentage (10%) of UPSC within a grade 3 endometrioid tumor caused significantly worse survival compared with patients with pure grade 3 endometrioid tumors (30). These reports suggest that compartmentalization of grade 3 endometrioid and UPSC into two discrete categories can be clinically and pathologically difficult. Interestingly, in our study, expression of EIG121 was clearly much lower in UPSC compared with grade 3 endometrioid tumors, and, in fact, significantly lower than levels detected in benign endometrium (Figs. 4 and 5). A recent gene expression profiling analysis of endometrial cancer also found that EIG121 (KIAA1324) showed the greatest difference in levels of gene expression between endometrioid carcinoma and UPSC (31). Therefore, despite the controversy surrounding the clinical behavior of UPSC and grade 3 endometrioid tumors, at the molecular level, EIG121 is an excellent discriminator between these two types of endometrial cancer. Because our quantitative PCR assay has successfully been applied to formalin-fixed, paraffin-embedded tissues (Fig. 3), EIG121 transcripts could potentially be quantified as a diagnostic adjunct in tumors that are difficult to pathologically classify at the level of light microscopy. Such clinical use of EIG121 would require a prospective clinical trial for evaluation.

The EIG121 genes show high sequence conservation among different species, and their genomic structure, exon/intron boundaries, exon sizes are nearly identical (data not shown). This strongly suggests that EIG121 fulfills some important physiologic function. Generation of EIG121 null mice and its overexpression in various biological systems will allow us to define the physiologic functions of EIG121 and to better dissect its role in endometrial cancer.

References


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