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Guest Editors: Donghai Dai, Andrew P. Bradford, and Eric R. Prossnitz

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Editorial Board

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Editorial

Endometrial Cancer: Molecular and Cellular Basis of Tumor Development, Novel Biomarkers and Therapeutic Agents, and Innovative Research Approaches

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Carcinoma of the endometrium is the most common cancer of the female reproductive tract with over 40,000 new cases diagnosed per year and over 7,000 deaths per year in the United States alone. Although a majority of endometrial tumors present with well-differentiated low grade endometrioid histology (Type 1), expressing high levels of estrogen and progesterone receptors (ER/PR) as well as epidermal growth factor receptor (EGFR), about one-quarter present as more advanced and aggressive tumors (Type 2), that are unlikely to be ER/PR-positive and have a much poorer prognosis. Although histology, genetic aberrations, and epidemiological profiles overlap between the two tumor types, they appear to represent distinct carcinogenic processes with distinct molecular characteristics. Whereas type 1 tumors are typically preceded by endometrial hyperplasia and are associated with a loss of PTEN expression as well as abnormalities in β-catenin, Kras, and DNA mismatch repair genes, type 2 tumors represent a heterogeneous group of tumors including high-grade (undifferentiated) endometrioid carcinomas, uterine papillary serous carcinomas, clear cell carcinomas, and carcinosarcomas. Whereas, uterine papillary serous carcinomas are typically associated with p53 mutations and often Her-2/neu mutations, with PTEN mutations being rare, carcinosarcomas, characterized by both malignant epithelial and mesenchymal components, are associated with many of the epidemiological risk factors linked to endometrioid carcinomas including obesity and tamoxifen therapy, which suggests that dysregulated estrogen signaling may have a role in its pathogenesis and may represent a therapeutic target. In this special issue on endometrial cancer, papers address not only molecular and cellular aspects of endometrial cancer formation but also novel studies of biomarkers as well as potential new therapeutic agents and approaches, which together could have great impact on the diagnosis and treatment of women with endometrial cancer.

The first paper of this issue examines the expression of Placenta-specific protein 1 (PLAC1), a small, secreted protein normally expressed only in trophoblast cells in the mammalian placenta. E. I. Devor and K. K. Leslie demonstrate that PLAC1 is ubiquitously expressed in tumors originating from the uterine epithelium and that expression is higher in more advanced and aggressive endometrial serous adenocarcinomas and carcinosarcomas. In the second paper, A. M. Thorne and colleagues demonstrate that expression of active, myristoylated PKCα confers ligand-independent activation of estrogen receptor-dependent promoters and enhances responsiveness to estrogen, suggesting that PKCα signaling, possibly via PI3K/Akt, may be a critical component of the hyperestrogenic environment with activation of ER that may underlie the development of estrogen-dependent endometrial hyperplasia and malignancy.

In the third paper, H. E. Dinkelspiel and colleagues discuss both risk factors for and protective factors against the development of endometrial cancer as well as primary and

alternative management options for this disease. In the fourth paper, K. K. Leslie and colleagues highlight new information linking the expression of the estrogen receptors (both α and β) to outcome, discussing the value of employing ER as a biomarker for positive outcome and hormonal treatment. The fifth paper in this issue examines the feasibility of RNA and DNA extraction from fresh Pipelle and archival endometrial tissues for use in gene expression and SNP assays, revealing that fresh frozen Pipelle samples, which are minimally invasive, yield excellent quantity and quality of RNA for gene expression arrays.

In the sixth paper of this special issue, S. Nair and colleagues demonstrate that adipocytes have potent proliferative paracrine effects on endometrial cells, which are in part mediated by TNF, since the proliferative effects of adipocyte-conditioned medium could be reversed by anti-TNF antibodies. In the seventh paper, W. K. Petrie and colleagues examine the mechanisms of estrogen signaling in ER-negative endometrial cancer cells. They demonstrate that estrogen, as well as SERMs and SERDs, such as tamoxifen, fulvestrant and raloxifene, continues to activate multiple signaling pathways in the absence of ER through the G protein-coupled estrogen receptor GPR30/GPER and that estrogen-stimulated ER-negative endometrial tumor growth is blocked by a selective GPER antagonist. Finally, in the last paper, X. Meng and colleagues test whether synthetic lethality can be achieved in endometrial cancer cells expressing mutant p53 by combining paclitaxel with agents to overcome G2/M arrest thereby inducing mitotic catastrophe. They reveal that synthetic lethality could be generated by combining paclitaxel with BIBF1120 (an investigational VEGFR, PDGFR, and FGFR multityrosine kinase inhibitor with established antiangiogenic activity), which together abrogated the G2/M checkpoint in p53-null endometrial cancer cells via modulation of G2/M checkpoint regulators followed by induction of mitotic cell death. Conversely, in endometrial cancer cells expressing an oncogenic gain-of-function p53 mutation, synthetic lethality was induced by combining paclitaxel with BIBF1120 and a histone deacetylase inhibitor, which served to destabilize mutant p53.

Basic and preclinical studies based on an improved understanding of the molecular and cellular aspects of tumor development and progression as described in this special issue will continue to serve as important approaches for the development of new biomarkers for diagnosis and prognosis as well as the innovative design of novel clinical trials utilizing molecularly targeted therapeutics.

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Research Article

Strategies for Molecularly Enhanced Chemotherapy to Achieve Synthetic Lethality in Endometrial Tumors with Mutant p53

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Serous uterine endometrial carcinomas are aggressive type II cancers with poor outcomes for which new treatment strategies are urgently needed, in particular, strategies that augment sensitivity to established chemotherapy regimens. The tumor suppressor gene *TP53* is dysregulated in more than 90% of serous tumors, altering master regulators of the G2/M cell cycle checkpoint in unique and predictable ways and desensitizing cells to chemotherapy. We hypothesized that synthetic lethality can be achieved in endometrial cancer cells with mutant p53 by combining paclitaxel with agents to overcome G2/M arrest and induce mitotic catastrophe. The combination of BIBF1120, an investigational VEGFR, PDGFR, and FGFR multityrosine kinase inhibitor with established anti-angiogenic activity, with paclitaxel abrogated the G2/M checkpoint in p53-null endometrial cancer cells via modulation of G2/M checkpoint regulators followed by induction of mitotic cell death. In endometrial cancer cells harboring an oncogenic gain-of-function p53 mutation, synthetic lethality was created by combining paclitaxel with BIBF1120 and a histone deacetylase inhibitor, which serves to destabilize mutant p53. These cells were also sensitive to an inhibitor of the G2/M kinase Wee1 in combination with paclitaxel. These findings reveal that, in addition to antiangiogenic activity, the angiokinase inhibitor BIBF1120 can be used to restore sensitivity to paclitaxel and induce mitotic cell death in endometrial cancer cells with non-functional p53. These preclinical data serve as a critical platform for the creative design of future clinical trials utilizing molecularly enhanced chemotherapy to achieve synthetic lethality based on the mutational landscape.

1. Introduction

While outcomes have substantially improved for many types of cancer, endometrial cancer incidence and deaths are on the rise, with the five-year survival rate being worse today than three decades ago [1]. Inadequate sensitivity to chemotherapy is a primary cause of therapeutic failure. In addition, the promise of targeted therapy with molecular inhibitors in combination with chemotherapy, now in phase III trials for the treatment of other forms of cancer, is in its infancy in this disease. Though recent studies of single agents such as the vascular endothelial growth factor (VEGF) inhibitor bevacizumab have yielded the first molecular therapies deemed "clinically active" in endometrial cancer [2], these studies were performed in patients with advanced or recurrent disease that had progressed after chemotherapy, and response rates were modest. Thus, it is clear that molecular therapies cannot be used alone or restricted to patients with advanced/recurrent disease who have failed chemotherapy. In order to improve patient outcomes, there is a critical need to identify strategies to restore chemosensitivity and increase efficacy of these agents. Towards that goal, bevacizumab was the first antiangiogenic agent to significantly prolong progression-free survival (PFS) when combined with carboplatin and paclitaxel as compared to chemotherapy alone for the treatment of epithelial ovarian cancer [3, 4]. Other investigational antiangiogenic agents, such as the triple angiokinase inhibitor BIBF1120 (also nintedanib; targets

receptors for VEGF, fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF)), have been studied in phase III maintenance therapy trials. These trials evaluated PFS as the primary endpoint (i.e., the AGO-OVAR12/LUME-Ovar1 trial) [4, 5]. Use of these angiokinase inhibitors may be superior to traditional tyrosine kinase inhibitors because they will target not only the signaling pathways that tumors rely on for survival, but also the tumor vasculature. However, we still must understand which tumors will respond and which will not.

One strategy is to identify Achilles' heels provided by distinct mutations within each tumor. Endometrial adenocarcinomas are broadly divided into two types based on histologic features: type I endometrioid adenocarcinomas and type II serous adenocarcinomas. Endometrioid endometrial cancer, a typically lower histological grade disease, is associated with gene mutations in *PTEN* (50%–83%), PI3Kinase (*PIK3CA, R1* and *R2* 40%–80%), *KRAS* (20%), and *FGFR2* (12%) and microsatellite instability (20%), while *TP53* mutations appear to be the key driver in serous lesions (~90% of nonendometrioid lesions) [6–9]. It is critically important to note that varying types of p53 mutant proteins exist, with different implications for chemosensitivity. Some mutations are relatively inconsequential from the perspective of p53 function, and proteins of this type retain wild-type activity. Other mutations are loss-of-function (LOF) in which single amino acid changes completely inactivate or destabilize the protein. Finally, an interesting category is the gain-of-function (GOF) p53 mutations that convert p53 from a tumor suppressor to an oncogene. Substantial clinical and preclinical data from a wide range of cancers indicate that GOF p53 mutations predict a poor response to treatment [10–12], though limited data are available for tumors of the endometrium.

In response to DNA damaging agents such as carboplatin and doxorubicin, cells activate a checkpoint signaling pathway downstream of ATM/ATR using effectors Chk1/Chk2 and a more recently identified branch through p38/MK2 in order to arrest cell cycle progression and repair DNA [13–15]. Chk1, Chk2, p53, and MK2 maintain the checkpoint by inhibiting the CDC25 phosphatases (activators of Cyclin B/Cdc2 in mitosis) [16–18]. The ability of cells to activate cell cycle checkpoints prevents progression into vulnerable phases of the cell cycle, M for paclitaxel and S for carboplatin and doxorubicin, leading to chemoresistance. The newly identified branch of cell cycle control via p38/MK2 is particularly relevant in endometrial cancer and is activated by the most common mutations driving this disease.

In cells with LOF p53, p38/MK2 and downstream components ultimately controlling the critical phosphatase CDC25C and Cyclin B/Cdc2 are activated as an alternative means to maintain the checkpoint [15]. We now understand that this pathway can be coopted by oncogenic alterations including p53 GOF (oncogenic) mutants and activated Ras mutants [19, 20]. Constitutive activation of p38 and downstream MK2 lead to an inhibitory phosphorylation event on the phosphatase CDC25C, inhibition of Cdc2, G2/M checkpoint maintenance, and chemoresistance. Polo-like kinase 1 (PLK1), which is upregulated in many cancers, plays a pivotal role in all phases of mitosis [21]. PLK1 is downregulated at the transcriptional level by p53 as part of the G2/M checkpoint [22–26]. Thus, p53 null cells are unable to downregulate PLK1 in response to chemotherapy, leading to chemoresistance [25, 26]. Indeed, PLK1 colocalizes with p38 and MK2 at the spindle during mitosis and is phosphorylated by MK2, linking their activities and suggesting pathway crosstalk [27].

We recently made the important discovery that endometrial cancer cells with inactivated p53 rely on the p38 pathway to maintain the G2/M checkpoint [28]. As such, these p53-null tumors are exquisitely sensitive to the combination of epidermal growth factor receptor (EGFR) inhibitor gefitinib with paclitaxel, which abrogates the G2/M checkpoint. Specifically, treatment of p53-null endometrial cancer cells with gefitinib lowered the IC50 of paclitaxel by 10-fold, with a combination index of 0.25 indicative of profound synergy. Since endometrial cancer cells express multiple angiogenic tyrosine kinase receptors, the objective in this study was to determine whether anti-angiogenic agents can be used to achieve synthetic lethality in combination with paclitaxel in p53 mutant endometrial cancer cells.

2. Materials and Methods

2.1. Reagents. All antibodies were purchased from Cell Signaling. Gefitinib (ZD1839, Iressa, AstraZeneca) and paclitaxel were suspended in dimethyl sulfoxide (DMSO). BIBF1120 (nintedanib), LBH589, and MK-1775 (Selleck Chemicals) were suspended in DMSO.

2.2. Cell Lines and Culture Conditions. Hec50co endometrial cancer cells, a subline of Hec50 cells, were kindly provided by Dr. Erlio Gurpide (New York University). Paclitaxel-resistant Hec50 cells, Hec50A and Hec50E, were obtained from parental Hec50co cells grown as xenograft tumors in mice as previously described [28]. KLE cells were purchased from ATCC. Cells were cultured in DMEM (Sigma-Aldrich) with 10% fetal bovine serum (Gemini Bio-Products) and 2 mM L-glutamine (Invitrogen).

2.3. Expression of p53 in Hec50 Cells. To generate p53 R175H GOF mutant, a vector containing wild-type p53 cDNA (Clontech) was subjected to site-directed mutagenesis (Stratagene) per manufacturer's instructions. Hec50 cells were transfected with constructs containing either WT or R175H p53 using Lipofectamine 2000 as per manufacturer's instructions (Invitrogen). Individual cell clones were selected for resistance to G418, expanded, and screened for p53 expression by Western blotting.

2.4. Western Blot Analysis. As previously described [28], cells were plated in 100 mm dishes and were allowed to grow for 24 h prior to treatment. After treatment for 24 h, cells were harvested, lysed with extraction buffer (1% Triton X-100, 10 mM Tris-HCl pH 7.4, 5 mM EDTA, 50 mM NaCl, 50 mM NaF, 20 μg/mL aprotinin, 1 mM PMSF, and 2 mM Na_3VO_4), and subjected to three freeze/thaw cycles as previously described [28]. Equal amounts of protein

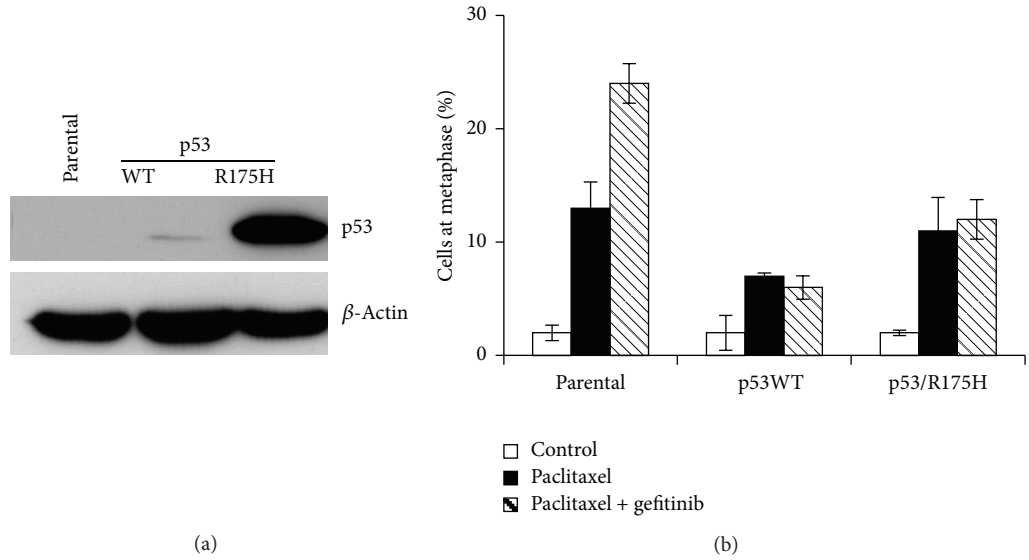

Figure 1: Synthetic lethality to paclitaxel + RTK inhibitor in endometrial cancer cells requires loss of functional p53. Hec50 endometrial cancer cells which are p53-null were transfected with either WT p53 or GOF p53 mutant R175H, one of the most commonly observed p53 GOF mutations in cancer. (a) Expression of p53 in Hec50 cells by Western blotting. (b) Percentage of mitotic cells after treatment with paclitaxel (10 nM) and/or EGFR inhibitor gefitinib (10 μM) for 24 h.

(determined by the method of Bradford, BioRad) were subjected to SDS-PAGE followed by transfer to nitrocellulose membranes (BioScience). Membranes were probed with primary antibodies against β-actin, p53, phospho-cdc2 Tyr15, CDC25C, phospho-Wee1 Ser642, phospho-Myt1 Ser83, phospho-stathmin Ser38, total stathmin, and phospho-histone H3 Ser10 followed by incubation with corresponding horseradish peroxidase-conjugated secondary antibody. The signal was visualized by chemiluminescence using ECL Western blotting detection reagents (Pierce).

2.5. Cell Cycle Analysis by Flow Cytometry. Cells were plated in 100 mm dishes with an equal number of cells in each dish and treated for 24 h. Cells were fixed in 70% ethanol. After washing with PBS, cells were incubated in Krishan's solution (3.8 mM sodium citrate, 0.014 mM propidium iodide, 1% NP-40, and 2.0 mg/mL RNase A) for 30 minutes at 37°C and analyzed by FacScan Flow Cytometer (Becton, Dickinson and Company) as previously described [28]. The data were subjected to further analysis by CellQuest software version 3.3, which generated DNA histograms indicating the fractions of the cell population in the sub-G1, G0-G1, S, or G2/M phase of the cell cycle. Experiments were performed in triplicate.

2.6. Quantitation of Percentage of Mitotic Cells. Cells were plated in 100 mm dishes with an equal number of cells and treated for 24 h. For metaphase spreads, cells were fixed with methanol : acetic acid (3 : 1). For visualization, cells were pipetted onto glass slides and stained with 4′,6-diamidino-2-phenylindole (DAPI). Mitotic spreads were viewed and imaged using fluorescence microscopy. The percentage of cells in mitosis was manually counted. A total of 300 cells for each treatment group were analyzed from three independent experiments.

2.7. Cell Viability Assays. Beginning 24 h after plating equal numbers of cells, cells were treated for 72 h followed by assessment of cell viability using the Wst-1 assay per manufacturer's instructions (Clontech). Data were quantitated relative to values obtained for control cells, which were set at 100% viability.

3. Results

3.1. BIBF1120 Increases Sensitivity to Paclitaxel in p53-Null Parental and Paclitaxel-Resistant Hec50 Cells by Producing a High Percentage of Mitotic Cells. Building on our recent study in which we achieved synthetic lethality by combining paclitaxel with the EGFR inhibitor gefitinib in p53-null endometrial tumors [25], we first sought to verify that the loss of p53 function is required for this effect. The p53-null poorly differentiated aggressive Hec50 endometrial cancer cells were transfected with either wild type (WT) p53 or R175H p53 GOF mutant. As anticipated [29], levels of WT p53 were very low in the absence of DNA damage, whereas the R175H p53 GOF mutant was very stable (Figure 1(a)). Expression of WT or GOF p53 prevented induction of mitotic arrest with paclitaxel and gefitinib as evidenced by significantly fewer cells in mitosis as compared to parental p53-null cells treated with this regimen (Figure 1(b)). These data validate the requirement for nonfunctional p53 to achieve synthetic lethality.

The use of gefitinib and paclitaxel represents the first-generation approach for synthetic lethality in p53-null endometrial tumors. Given that any strategy that inhibits activation of p38 should theoretically induce synthetic lethality when combined with paclitaxel in p53-null cells, we next explored the use of triple angiokinase inhibitor BIBF1120, which not only inhibits VEGFR, PDGFR, and FGFR2, but

also has antiangiogenic activity in the vascular endothelium. First, we established endometrial cancer cell lines that are highly resistant to paclitaxel. Parental Hec50 cells were grown as xenograft tumors in nude mice and treated with the PLK1 inhibitor BI2536 [28]. Tumors which did not respond to BI2536 were excised and cultured; these cell lines are referred to as Hec50A and Hec50E [28]. As compared to the parental Hec50 cells, Hec50A and Hec50E were extremely resistant to paclitaxel (Figure 2). However, synthetic lethality could be achieved in both the parental and the paclitaxel-resistant cells by the addition of 1 μM angiokinase inhibitor, BIBF1120, which targets VEGFR, PDGFR, and FGFR2 (Figure 2).

We next performed flow cytometry analysis to examine the effect of paclitaxel, BIBF1120, and the combination on cell cycle distribution. The percentage of cells in G2/M at baseline was similar among parental and paclitaxel-resistant Hec50 cells (Figure 3). Similarly, treatment with BIBF1120 alone had no effect on the percentage of cells in G2/M (Figure 3). We previously established that the IC50 of paclitaxel is 14 nM in parental Hec50 [28]. When the parental cells were treated with paclitaxel at 14 nM, there was a substantial increase in cells in G2/M (17% for control and 46% for paclitaxel, Figure 3(a)). Consistent with resistance to paclitaxel, the percentage of Hec50A and Hec50E cells in G2/M was unchanged with paclitaxel treatment as compared to control (Figures 3(b) and 3(c)). By contrast, the combination of paclitaxel and BIBF1120 produced a profound increase in the accumulation of cells in G2/M. We next examined the percentage of cells in mitosis. In the parental Hec50 cells and the paclitaxel-resistant cells, the combination of BIBF1120 and paclitaxel resulted in arrest in M phase (Figure 4), though the effect was dampened in Hec50E cells. This may be due to different mechanisms underlying resistance to paclitaxel in these clones, which may also impact sensitivity to the combination treatment at a particular dose. Consistent with this notion, viability studies in Figure 2 demonstrate that Hec50E cells require a slightly higher concentration of paclitaxel to reach an IC50 in combination with BIBF1120. Taken together, these data indicate that the combination of BIBF1120 and paclitaxel results in mitotic arrest and synergistic cell death.

3.2. Effect of Paclitaxel and BIBF1120 Combination Treatment on G2/M Cell Cycle Regulators. We next examined expression and activation of critical regulators of the G2/M checkpoint. The combination of BIBF1120 and paclitaxel resulted in activation of Cdc2 as evidenced by decreased phosphorylation at Tyr15 (Figure 5). The active form of CDC25C, a phosphatase that activates Cdc2 by dephosphorylating Tyr15, was significantly increased in cells treated with the combination of paclitaxel and BIBF1120 as demonstrated by a slower-migrating band compared to control or either drug alone (Figure 5). We also examined activation of other kinases, Wee1 and Myt1, that phosphorylate Cdc2 at Tyr15 to maintain Cdc2 in an inactive state. Wee1 phosphorylation at Ser642 is indicative of activation, whereas Myt1 phosphorylation at Ser83 reflects an inactive kinase. The combination treatment resulted in a decrease in phosphorylation of Wee1 at Ser642 and an increase in phosphorylation of Myt1 at Ser83, suggesting that both kinases are inactivated by BIBF1120 and paclitaxel. Consistent with the activation of Cdc2, treatment with paclitaxel and BIBF1120 promoted phosphorylation of stathmin-1 (STMN1), a microtubule destabilizer that is inactivated when phosphorylated. Finally, paclitaxel and BIBF1120 combination treatment resulted in a significant increase in phosphorylation of the histone H3 at Ser10, an established marker for mitosis. These data provide compelling evidence that the mechanism by which BIBF1120 induces synthetic lethality to paclitaxel is through abrogation of the G2/M checkpoint.

3.3. Strategies to Induce Synthetic Lethality in Cells with p53 GOF Mutation. Our preliminary data indicate that 15%–20% of serous tumors harbor p53 GOF mutations (Leslie, unpublished observations), which can lead to hyperactivation of the p38 pathway and resistance to gefitinib and paclitaxel combination therapy [28]. We first examined whether KLE endometrial cancer cells that contain a mutant p53 are also resistant to the combination of BIBF1120 and paclitaxel. As shown in Figure 6(a), addition of BIBF1120 to paclitaxel had no appreciable impact on cell viability. It has been reported that p53 mutants associate with the heat shock protein 90 (Hsp90) machinery, which serves to stabilize the mutated p53 protein [29, 30]. The interaction between the heat shock protein and its client proteins can be disrupted by acetylation of Hsp90 [31]. Therefore, we hypothesize that treatment with the HDAC inhibitor (HDACi) LBH589 has the potential to cause dissociation of the GOF p53-Hsp90 complex, leading to mutant p53 degradation. Consistent with this hypothesis, KLE cells were sensitized to BIBF1120+paclitaxel by treating with HDACi LBH589 (Figure 6(a)).

We also examined whether pathway inhibition downstream of constitutive p38 activation might circumvent the effect of the p53 GOF mutation and thereby sensitize cells to paclitaxel. We chose an inhibitor of Wee1, MK-1775. Treatment of KLE cells with Wee1 inhibitor MK-1775 in combination with paclitaxel significantly decreased cell viability as compared to paclitaxel alone, though it should be noted that we could not achieve complete cell killing with this strategy (Figure 6(b)).

4. Discussion

While the vast majority of endometrial cancer cases will be diagnosed at an early stage, those with advanced disease remain at high risk for relapse and ultimately death from their disease. For these women a priority must be the evaluation of new agents. Our objective in this study was to identify strategies to achieve synthetic lethality based on the p53 mutational status. For endometrial cancer cells with loss of functional p53 mutation or p53-null mutation, antiangiogenesis inhibitor BIBF1120 substantially increased paclitaxel sensitivity, including in cells that have high baseline resistance to paclitaxel. This cell death was achieved through induction of mitotic catastrophe as evidenced by abrogation of the G2/M checkpoint and a high percentage of cells in M phase (Figure 7(a)). For cells with p53 GOF mutation, we identified two strategies to induce synthetic lethality to paclitaxel (Figure 7(b)). The first utilized an HDACi in

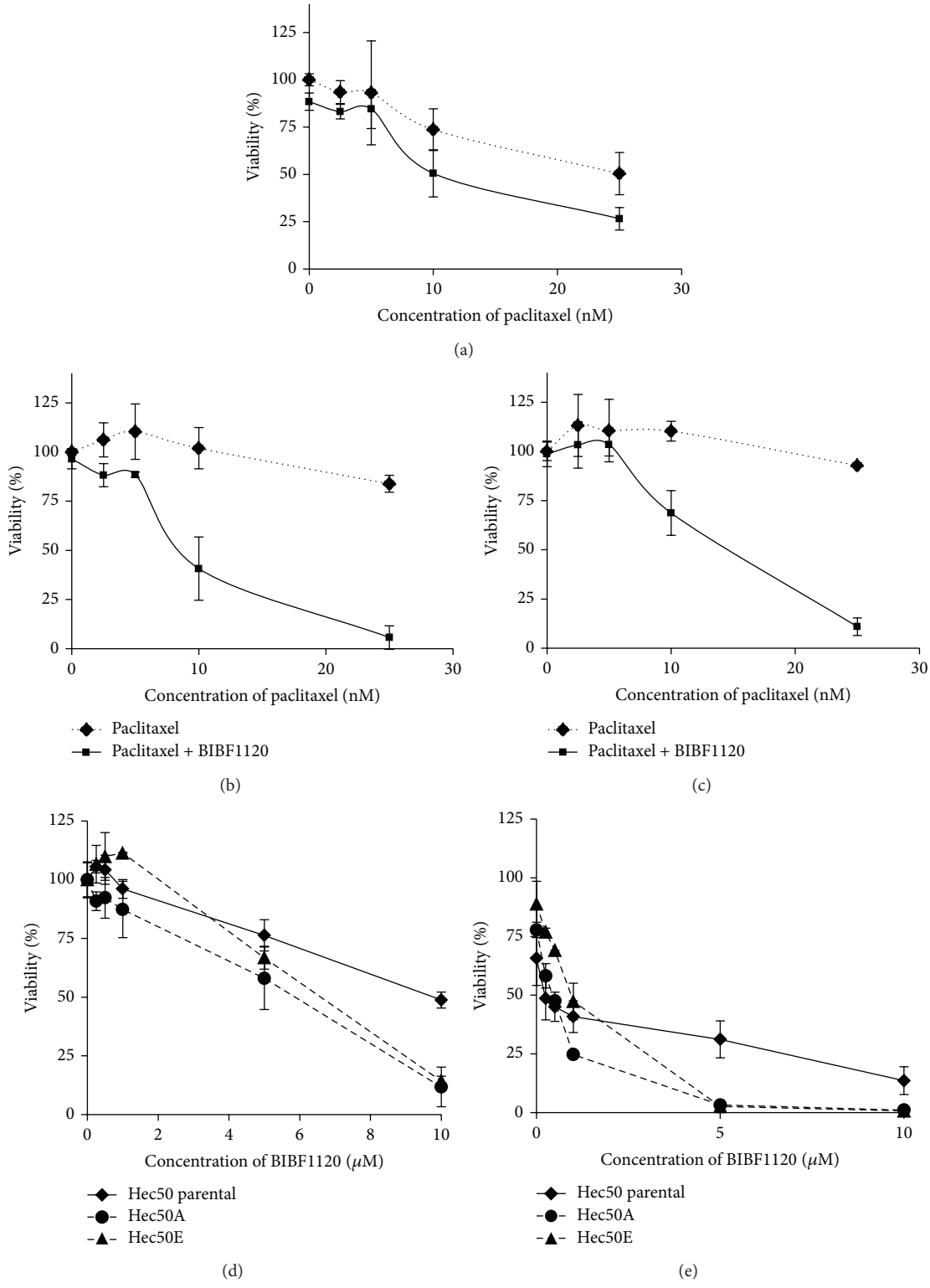

Figure 2: Angiokinase inhibitor BIBF1120 increases sensitivity to paclitaxel in parental and paclitaxel-resistant Hec50 cells. (a–c) Parental Hec50 (a) or paclitaxel-resistant Hec50A (b) or Hec50E (c) endometrial cancer cells, which are p53-null, were treated with increasing concentrations of paclitaxel in the absence or presence of 1 μM BIBF1120 for 72 h, followed by assessment of cell viability using the Wst-1 assay. (d, e) Parental Hec50 or paclitaxel-resistant Hec50A or Hec50E endometrial cancer cells were treated with increasing concentrations of BIBF1120 in the absence (d) or presence (e) of 10 nM paclitaxel for 72 h, followed by assessment of cell viability using the Wst-1 assay.

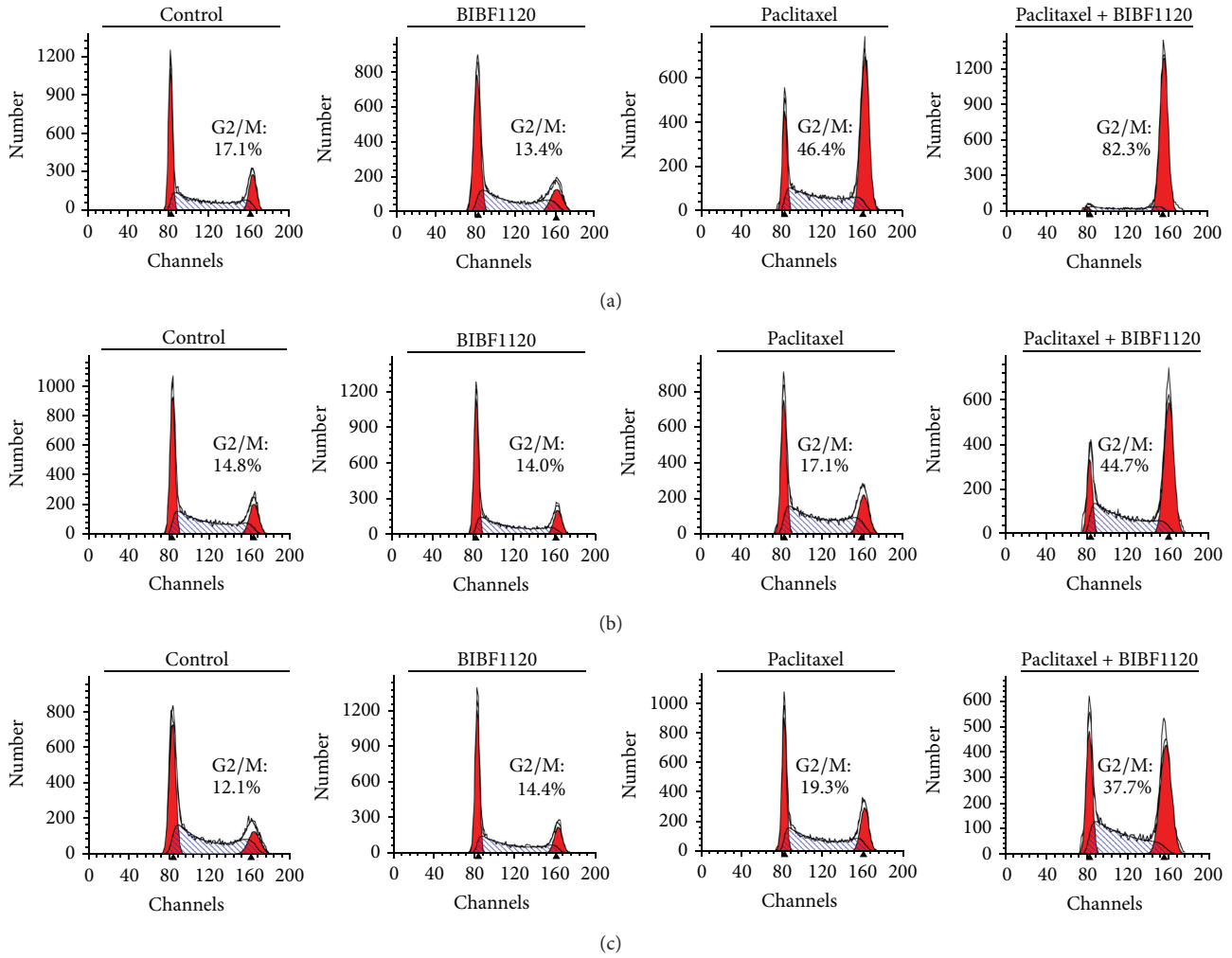

Figure 3: Combination of BIBF1120 and paclitaxel produces a profound increase in the percentage of cells in G2/M. Cell cycle profiles of parental (a) and paclitaxel-resistant Hec50A (b) and Hec50E (c) cells after treatment with 1 μM BIBF1120, 14 nM paclitaxel, or the combination for 24 h. The percentage of cells in G2/M is indicated in each plot.

combination with BIBF1120, which presumably destabilizes mutant p53. The second strategy inhibited the G2/M checkpoint controller Wee1. These data serve as a critical platform for future clinical trials in serous endometrial tumors to determine whether p53 status can be used to guide choice of therapy.

In normal and cancerous cells, WT p53 is normally expressed at very low levels. Levels rise precipitously in response to DNA damage, and WT p53 is then downregulated by MDM2. By contrast, expression of p53 GOF mutant protein is high in the absence of stress [32]. Most p53 GOF mutants fail to associate with MDM2 and instead acquire binding to new targets and protein interacting partners, such as p63 and p73 [32]. One reason for the high expression of R175H p53, as demonstrated in Figure 1, is its association with heat shock proteins, which increases its half-life [29, 30]. Another phenotype of gain of oncogenic function p53 R175H is inactivation of the Mre11/ATM-dependent DNA damage response, leading to chromosomal translocation and defects in the G2/M checkpoint [19, 33, 34]. Thus, p53 GOF mutants have acquired several key advantages that allow cells to continue to divide in the setting of stress, thereby contributing to drug resistance.

A goal of combinatorial therapy is to create synthetic lethality, where regimens are not simply additive, but synergistic. Synthetic lethality is the term for a historical genetic observation that in the presence of certain single gene mutations, blocking or mutating a second gene leads to cell death though neither mutation alone has a phenotype [35]. With respect to cancer therapy, synthetic lethality means capitalizing on the presence of a mutation in a driver protein to design novel treatments. To create therapeutic synthetic lethality, one must first know the driver mutation, understand the compensatory survival pathway which has been activated as a result of the mutation, and have an agent which can block this critical pathway. Mutations in p53, which are common in serous endometrial cancer, represent a platform upon which to design combinatorial regimens with the potential to result

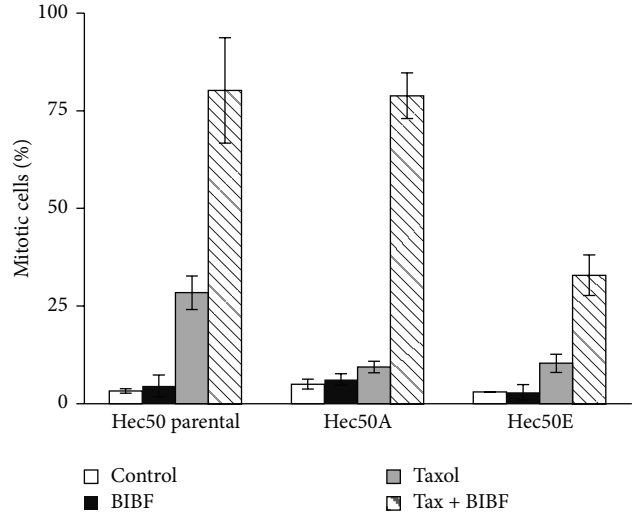

Figure 4: BIBF1120 and paclitaxel increase the percentage of mitotic parental and paclitaxel-resistant Hec50co cells. Parental Hec50 (a) or paclitaxel-resistant Hec50A (b) or Hec50E (c) endometrial cancer cells were treated with 14 nM paclitaxel for 24 h with or without 1 μM BIBF1120, followed by assessment of the percentage of mitotic cells.

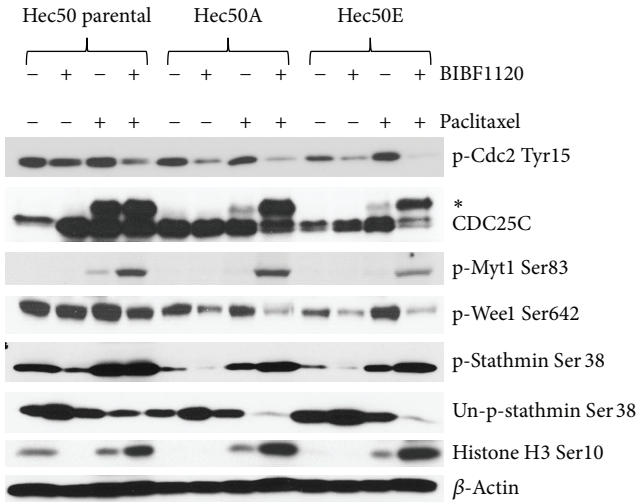

Figure 5: BIBF1120+paclitaxel modifies G2/M checkpoint controllers to induce transition into M phase. The effect of 1 μM BIBF1120, 14 nM paclitaxel, and combination treatment (24 h) on the posttranslational modification of cell cycle regulators was examined by Western blotting. *denotes slower-migrating CDC25C, indicative of activating phosphorylation.

in tumor cell synthetic lethality. Our data provide compelling evidence that the triple angiokinase inhibitor BIBF1120 combined with paclitaxel results in synthetic lethality in p53-null tumors that are resistant to either agent alone.

The rationale for evaluating the combination of BIBF1120 plus paclitaxel for p53-null tumors is two-fold. BIBF1120 has shown promising activity in combination with chemotherapy in ovarian cancer and is currently in phase III testing in combination with a backbone of carboplatin and paclitaxel. Therefore, issues related to dosing and safety of combining this agent with chemotherapy have been addressed. More importantly, our data demonstrate that BIBF1120 exhibits significant synergy with paclitaxel in endometrial cancer cells with loss of function mutations in p53. Such cells must activate alternative pathways to maintain critical cell cycle checkpoints [15]. One of these is the p38/MK2/CDC25C/CyclinB/Cdc2 signaling cascade, which allows cells to repair DNA at G2 prior to entering M [15]. Blocking this signaling pathway completely abrogates the checkpoint in cells which lack p53 (Figure 7(a)).

To move towards better therapies, we must first achieve a new understanding of cancer biology in the hopes to identify subpopulations of patients most likely to benefit from treatment. Work from The Cancer Genome Atlas (TCGA) project has significantly improved our understanding of the genomic heterogeneity of endometrial cancers, beyond the clinicopathologic characterization commonly used of type I versus type II tumors [9]. TCGA data indicate that up to

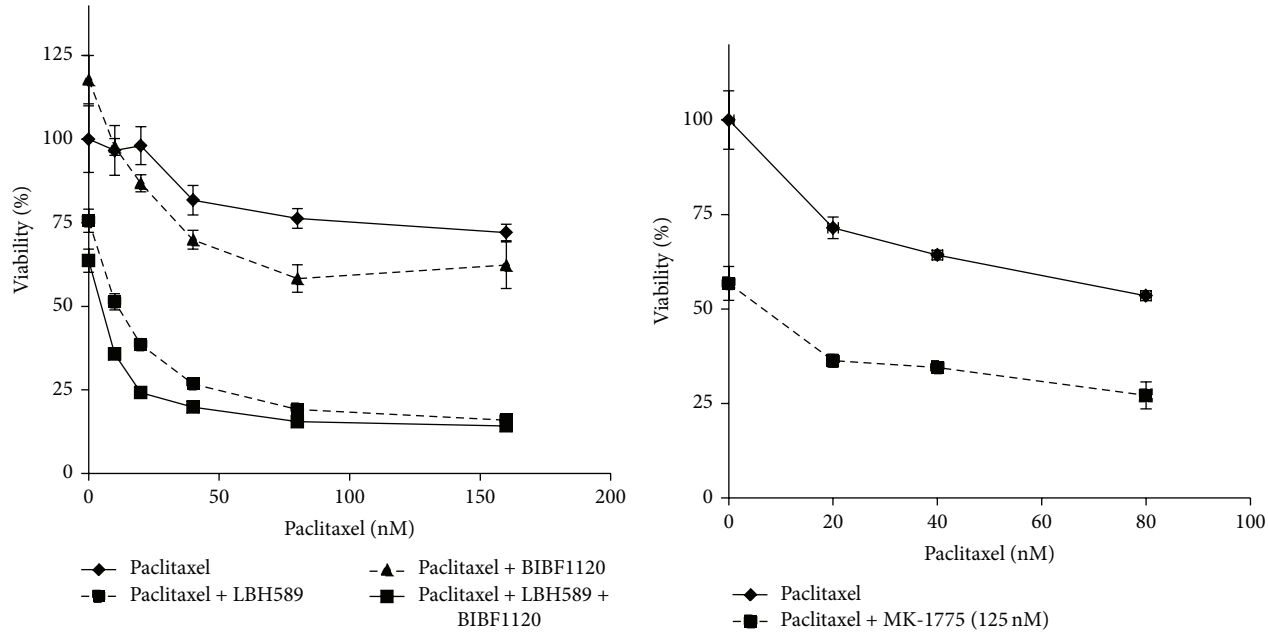

Figure 6: Strategies to achieve synthetic lethality in cells with p53 GOF mutation. (a) KLE endometrial cancer cells, which contain R175H p53 GOF mutation, were treated with increasing concentrations of paclitaxel in the presence of 10 nM LBH589 −/+ BIBF1120 (1 μM) for 72 h, followed by assessment of cell viability by Wst-1 assay. (b) KLE cells were treated with increasing concentrations of paclitaxel −/+ Wee1 inhibitor MK-1775 (125 nM) for 72 h, followed by assessment of cell viability.

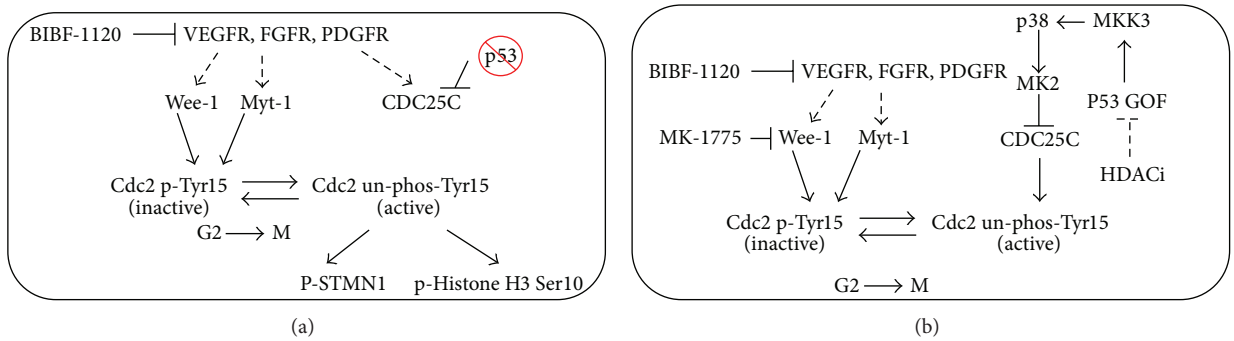

Figure 7: Proposed synergistic mechanisms for induction of mitotic cell death in endometrial cancer cells with mutant p53. (a) In the absence of functional p53, cells rely on the p38 pathway to maintain the G2/M checkpoint via inactivating phosphorylation of Cdc2 at Tyr15. Inhibition of RTK signaling with BIBF1120 results in premature entry into mitosis, where cells are sensitive to paclitaxel and thus undergo mitotic arrest and cell death. (b) In cells with p53 GOF mutation, the p38 pathway is hyperactivated through increased MKK3 transcription by p53 GOF. Synthetic lethality can be created by combining paclitaxel with BIBF1120 and an HDACi, which presumably disrupts the association of mutant p53 with Hsp90 and leads to its degradation. Alternatively, inhibition of Wee1, downstream of hyperactivated p38, is sufficient to restore sensitivity to paclitaxel.

25% of high-grade endometrioid tumors showed frequent mutations in *TP53* and extensive copy number alterations, both of which are key molecular characteristics in serous tumors. This pattern was not seen in grades 1-2 endometrioid tumors, suggesting that grade 3 endometrioid tumors were indeed more closely related to serous cancers [9]. In addition, these genomic similarities were shared between other tumors, including high-grade serous ovarian carcinoma and basal-like breast cancers insofar as these cancers share a high frequency of mutations in *TP53* (between 84 and 96 percent) and a low frequency in *PTEN*, with only 1 to 2 percent mutated. The fact that a high proportion of advanced endometrioid tumors fall into the same cluster as serous tumors suggests that these tumors should be treated similarly as serous tumors. In particular, loss of p53 in these tumors would suggest that the BIBF1120+paclitaxel regimen will induce synthetic lethality as in our studies.

In summary, our data provide clear evidence that abrogation of the G2/M checkpoint in cells with mutant p53, but not cells with normal p53, is a powerful strategy to

induce synthetic lethality to paclitaxel. Future advances in the treatment of endometrial cancer must take into account genomic heterogeneity, and our data suggest a way forward by using enriched trial designs.

Conflict of Interest

The authors declare no competing interests.

Acknowledgment

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Research Article

G Protein-Coupled Estrogen Receptor-Selective Ligands Modulate Endometrial Tumor Growth

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Endometrial carcinoma is the most common cancer of the female reproductive tract. GPER/GPR30 is a 7-transmembrane spanning G protein-coupled receptor that has been identified as the third estrogen receptor, in addition to ERα and ERβ. High GPER expression is predictive of poor survival in endometrial and ovarian cancer, but despite this, the estrogen-mediated signaling pathways and specific estrogen receptors involved in endometrial cancer remain unclear. Here, employing ERα-negative Hec50 endometrial cancer cells, we demonstrate that GPER mediates estrogen-stimulated activation of ERK and PI3K via matrix metalloproteinase activation and subsequent transactivation of the EGFR and that ER-targeted therapeutic agents (4-hydroxytamoxifen, ICI182,780/fulvestrant, and Raloxifene), the phytoestrogen genistein, and the "ERα-selective" agonist propylpyrazole triol also function as GPER agonists. Furthermore, xenograft tumors of Hec50 cells yield enhanced growth with G-1 and estrogen, the latter being inhibited by GPER-selective pharmacologic antagonism with G36. These results have important implications with respect to the use of putatively ER-selective ligands and particularly for the widespread long-term use of "ER-targeted" therapeutics. Moreover, our findings shed light on the potential mechanisms of SERM/SERD side effects reported in many clinical studies. Finally, our results provide the first demonstration that pharmacological inhibition of GPER activity *in vivo* prevents estrogen-mediated tumor growth.

1. Introduction

Carcinoma of the endometrium is the most common cancer of the female reproductive tract with over 40,000 new diagnoses and over 7,000 deaths per year in the United States. Although the majority (~75%) of endometrial tumors are of endometrioid histology (designated type I tumors), expressing high levels of estrogen receptor (ER), progesterone receptor (PR), and epidermal growth factor receptor (EGFR), about 25% of tumors are of advanced stage (designated type II tumors), are unlikely to be ER^+/PR^+, and have a poorer prognosis [1]. Although overlap exists with respect to histology, genetic aberations, and epidemiological profiles, the two tumor types appear to represent discrete carcinogenic processes with distinct molecular characteristics. Type I tumors consist of well-differentiated tumors preceded by endometrial hyperplasia and are associated with a loss of PTEN expression as well as abnormalities in β-catenin, Kras,

and DNA mismatch repair genes. Type 2 tumors are a heterogeneous group of tumors including high-grade (undifferentiated) endometrioid carcinomas, uterine papillary serous carcinomas, clear cell carcinomas, and carcinosarcomas, with different mutational profiles. Over 90% of uterine papillary serous carcinomas are associated with p53 mutations, 45–60% have Her-2/neu mutations, and PTEN mutations are rare [2, 3]. Carcinosarcomas, which are characterized by malignant epithelial and mesenchymal components, are associated with many of the epidemiological risk factors linked to endometrioid carcinomas including obesity and exposure to tamoxifen therapy, suggesting that dysregulated estrogen signaling has a role in their pathogenesis and may represent a therapeutic target. Moreover, recent mutational profiling studies indicate that whereas some carcinosarcomas share mutations with type 1 tumors (PTEN and ARID1A), others share mutations with uterine papillary serous carcinomas (notably p53 and PPP2R1A) [4].

The lack of estrogen receptor α (ERα) expression in most type II tumors has led to the assumption that these tumors must be "estrogen-independent" and that treatment with antiestrogens (selective estrogen receptor modulators (SERMs) such as tamoxifen and Raloxifene, or pure antagonists/selective estrogen receptor modulators (SERDs) such as ICI182,780/fulvestrant) commonly used in breast cancer treatment, would be ineffectual, a conclusion largely substantiated in a number of clinical trials [5, 6]. In fact, prolonged treatment of breast cancer with SERMs such as tamoxifen leads to an increased incidence of endometrial cancer [7], particularly those of high-risk histologic types [8], resulting in significantly poorer overall survival [9]. The effects of tamoxifen in the uterus have been ascribed to altered expression of nuclear coregulatory proteins in the endometrium compared to the breast, resulting in moderate agonist activity of tamoxifen in the uterus, compared to its antagonistic effects in the breast [10–13]. However, recent results have suggested that a heretofore-underappreciated estrogen receptor, the G protein-coupled estrogen receptor (GPER, formerly GPR30), may play an important role in both the increased incidence of endometrial cancer in women treated with tamoxifen [14] as well as representing an alternate mechanism through which endometrial cancers, particularly type II tumors, can maintain responsiveness to estrogen [15].

GPER is a member of the 7-transmembrane spanning G protein-coupled receptor (GPCR) superfamily, structurally unrelated to the nuclear receptor family members ERα and ERβ [16, 17]. Activation of GPER by estrogen has been demonstrated in many cancer cell lines [18, 19], including endometrial cancer cells [15, 20–27]. GPER is also activated by antiestrogens including tamoxifen (i.e., 4-hydroxytamoxifen) [28] and ICI182,780 (fulvestrant) [29], leading to the suggestion that GPER plays a role in hormone-resistance in breast cancer [30, 31] as well as in the increased incidence of endometrial cancer in women taking tamoxifen for breast cancer [14, 32]. Furthermore, GPER (over)expression has been associated with many cancers and in particular poor prognosis in a number of cancers, including breast [33], ovarian [34], lung [35], pancreatic [36], and endometrial [37] although observations to the contrary have also been reported [38, 39].

Because of the lack of specificity of estrogen and antiestrogens for the three known estrogen receptors (ERα, ERβ, and GPER), we have developed both a GPER-selective agonist (G-1, [40]) and antagonists (G15 [41] and G36 [42]) that display virtually no activity towards the classical estrogen receptors. In the current study, we examine the expression and function of GPER in the ERα^-/ERβ^- endometrial cancer cell line Hec50 [43, 44], which is representative of type II endometrial cancers. We demonstrate that, in Hec50 cells, GPER is localized predominantly in intracellular membranes and mediates PI3K and ERK activation in response to both estrogen and G-1 as well as the antiestrogens tamoxifen, Raloxifene, and ICI182,780 and the "ERα-selective" agonist propylpyrazole triol (PPT). We also demonstrate that Hec50 cells and primary patient endometrial adenocarcinomas maintain expression of GPER when grown as xenograft tumors. Finally, we demonstrate both estrogen and G-1 stimulate Hec50 xenograft tumor growth *in vivo* and that the GPER antagonist G36 greatly reduces growth of estrogen-stimulated Hec50 tumors. Overall, these results suggest that GPER may play a critical role in endometrial carcinogenesis, providing a novel target for prognosis and treatment.

2. Materials and Methods

Reagents. 17β-estradiol, 17α-estradiol, 4-hydroxytamoxifen, Raloxifene, genistein, LY294002, bovine serum albumin (BSA), normal goat serum, insulin, transferrin, hydrocortisone, fetuin, pancreatin, and trypsin were from Sigma (St. Louis, MO, USA). AG1478 and GM6001 were from Calbiochem (Billerica, MA, USA). DPN, PPT, and ICI182,780 were from Tocris Chemicals (Ellisville, MO, USA). G-1, G15, and G36 were synthesized as previously described [40–42]. Goat anti-rabbit Alexa-488, goat anti-rabbit Alexa-568, and donkey anti-mouse Alexa-568-conjugated secondary antibodies were from Invitrogen (Carlsbad, CA, USA). Rabbit anti-GPER C-terminal antiserum (cross-reactive to both human and murine sequences) was produced as previously described and used at a dilution of 1 : 10,000 [28]. Rabbit anti-GPER human N-terminal antiserum was produced against the peptide sequence MDVTSQARGVGLEMYPGTAQPAAC (with an added carboxy-terminal cysteine for conjugation to KLH) by New England Peptide, Inc. (Gardner, MA, USA) and used at a dilution of 1 : 5000. Polyclonal antibodies against total ERK and pERK were from Cell Signaling (Danvers, MA, USA); monoclonal anti-actin and anti-β-catenin antibodies were from Millipore (Burlington, MA, USA). Goat anti-rabbit HRP and donkey anti-mouse HRP were from GE-Amersham (Piscataway, NJ, USA). Dulbecco's MEM, RPMI 1640, and phenol red-free DMEM/F12 media, penicillin/streptomycin/glutamine, and fetal bovine serum were obtained from Fisher (Pittsburgh, PA, USA).

Cell Culture and Transfection. Human endometrial carcinoma Ishikawa H cells and Hec50 (specifically Hec50co [44]) cells (kindly provided by K. K. Leslie) were cultured in DMEM medium with FBS (10%) and 100 U/mL penicillin, 100 μg/mL

streptomycin, and 2 mm L-glutamine. Cells were grown as a monolayer at 37°C, in a humidified atmosphere of 5% CO_2 and 95% air. PH-RFP plasmid DNA was transfected with Lipofectamine2000 according to manufacturer's directions but using 1/4 the recommended amount of DNA. For "co-transfection" of PH-RFP plasmid DNA and siRNA on coverslips, Lipofectamine2000 was used to transfect siRNA on day 1 according to manufacturer's directions; on day 2, PH-RFP plasmid with additional siRNA as on day 1 was retransfected. For GPER knockdown, siRNA targeting GPER (ON-TARGET plus SMARTpool L-005563-00) was obtained from Dharmacon/Thermo-Fisher (Lafayette, CO, USA). The nontargeting siRNA ON-TARGETplus siControl Non-Targeting siRNA (D-001810-02) was used as a control. Cells transfected with siRNA were used in PH assays and stained for GPER expression 48 hours following the second siRNA transfection. For microscopy experiments, cells were seeded onto 12 mm glass coverslips and allowed to adhere for at least 24 h prior to antibody staining or 12 h prior to transfection.

Primary mouse uterine epithelial cells were harvested and cultured as described [45]. Briefly, uteri were removed from C57Bl6 mice between 21 and 35 days of age and slit longitudinally, followed by incubation in 2.5% pancreatin and 0.5% trypsin for 1 h at 4°C, followed by 1 h at RT. Digested uteri were then briefly vortexed, releasing epithelial sheets and fragments, which were transferred to a fresh tube containing 2% trypsin inhibitor (Invitrogen) in Hanks Balanced Salt Solution (HBSS). After two washes with HBSS, cells were seeded directly onto acid-washed, poly-L-lysine-coated glass coverslips and cultured in serum-free DMEM/F12 medium supplemented with 5 μg/mL insulin, 10 μg/mL transferrin, 10^{-7} M hydrocortisone, 2 mg/mL BSA, 1 mg/mL fetuin, and antibiotics.

Immunofluorescence Staining. Cells were seeded on 12 mm glass coverslips and fixed with 4% PFA (Electron Microscopy Sciences, Hatfield, PA, USA) in PBS for 15 min at 37°C. Coverslips were washed three times with PBS and blocked for 1 h with 3% BSA in PBS. Where indicated, permeabilization was accomplished with 0.05% Triton X-100 in the blocking buffer. Primary antibody was diluted in 3% normal goat serum and coverslips were incubated for 4 h at room temperature. Coverslips were washed three times with PBS and incubated with secondary antibody diluted in 3% normal goat serum. Coverslips were washed three times with PBS and mounted with Vectashield containing DAPI (Vector Labs, Burlingame, CA, USA). Confocal fluorescence images were collected on a Zeiss LSM 510 confocal microscope. Typical cell lengths ranged from 20 to 35 μm.

Western Blotting. Cells were harvested directly for receptor expression or starved in phenol red-free RPMI 1640 for 24 h prior to treatment. Cells were washed once with ice-cold PBS and lysed using NP-40 buffer. Twenty μg protein was loaded per lane and electrophoresed on 4–20% SDS-PAGE gels (Thermo Scientific, Waltham, MA, USA), transferred to PVDF membrane (Millipore), and blocked with 3% BSA in TBST (50 mm Tris, 150 mm NaCl, and 0.1% Tween-20) before overnight incubation with primary antibodies at 4°C. Blots were incubated with HRP-conjugated secondary antibodies, developed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific), exposed to film, scanned, and quantified using Image J software (NIH).

PI3K Activation. The pleckstrin homology (PH) domain (responsible for PIP3 binding) of Akt [46] fused to monomeric red fluorescent protein 1 (mRFP1) [47] generated the PH-RFP construct, which was used to localize sites of increased cellular PIP3 accumulation [28]. Hec50 cells were plated on coverslips, transfected with PH-RFP, followed by a 24 h recovery in complete DMEM, and serum starved in phenol red-free DMEM/F12 for 24 h before stimulation with ligands as indicated. The cells were fixed with 2% PFA in PBS, washed, mounted in Vectashield containing DAPI (Vector Labs), and imaged by confocal microscopy using a Zeiss LSM510 confocal fluorescence microscope. Images are representative of 75–85% of the transfected cells observed.

Xenograft Tumors. Ishikawa H cell and Hec50 cell xenograft tumors were produced by injecting ~ 3×10^6 cells (in 100 μL DMEM) subcutaneously into 6–8-week-old female athymic, Crl:Nu/Nu-nuBR "athymic nude" mice [48]. Subcutaneous tumors were recovered for histology and immunohistochemistry typically ~6 weeks after injection, when the tumors reached ~10 mm in diameter. For treatment models, Hec50 cells were injected into ovariectomized athymic nude mice 10 days after ovariectomy. Individual 60-day-release sham, estrogen (1.5 mg), G-1 (2.25 mg (equimolar with estrogen)), and G36 (11 mg pellets, (5-fold molar excess versus estrogen and G-1)), custom made by Innovative Research of America (Sarasota, FL), were introduced subcutaneously near the scapula with a trochar on the same day as Hec50 cell inoculation. When tumors became palpable (3–5 mm in diameter), tumor size was measured ~3 times per week by digital caliper, and upon sacrifice tumors were dissected and weighed. All protocols were approved by the Institutional Animal Care and Use Committee of the University of New Mexico Health Sciences Center.

For xenografts of fresh human tumor samples, tumors were obtained from the Department of Pathology at the University of New Mexico immediately after their arrival at the Surgical Pathology Gross Room. Fat and necrotic tissues were trimmed and remaining tumor tissues were rinsed with cell culture medium (DMEM). Tumors were minced into a fine homogenate and mixed with medium. Typically, 10 mg of tumor tissue was mixed with 100 uL medium for subcutaneous injection into a 6–8-week old athymic Crl:Nu/Nu-nuBR female mouse to create the first-generation xenografts, which were used for analysis as reported previously [49]. The collection of human endometrial tumors was approved by Human Research Protections Office at the University of New Mexico.

Histological Staining of Tumors. Five-micron sections from paraffin-embedded tumor tissues were prepared for immunohistochemistry (IHC) as previously described using the carboxy-terminus-targeted antibody against GPER [37].

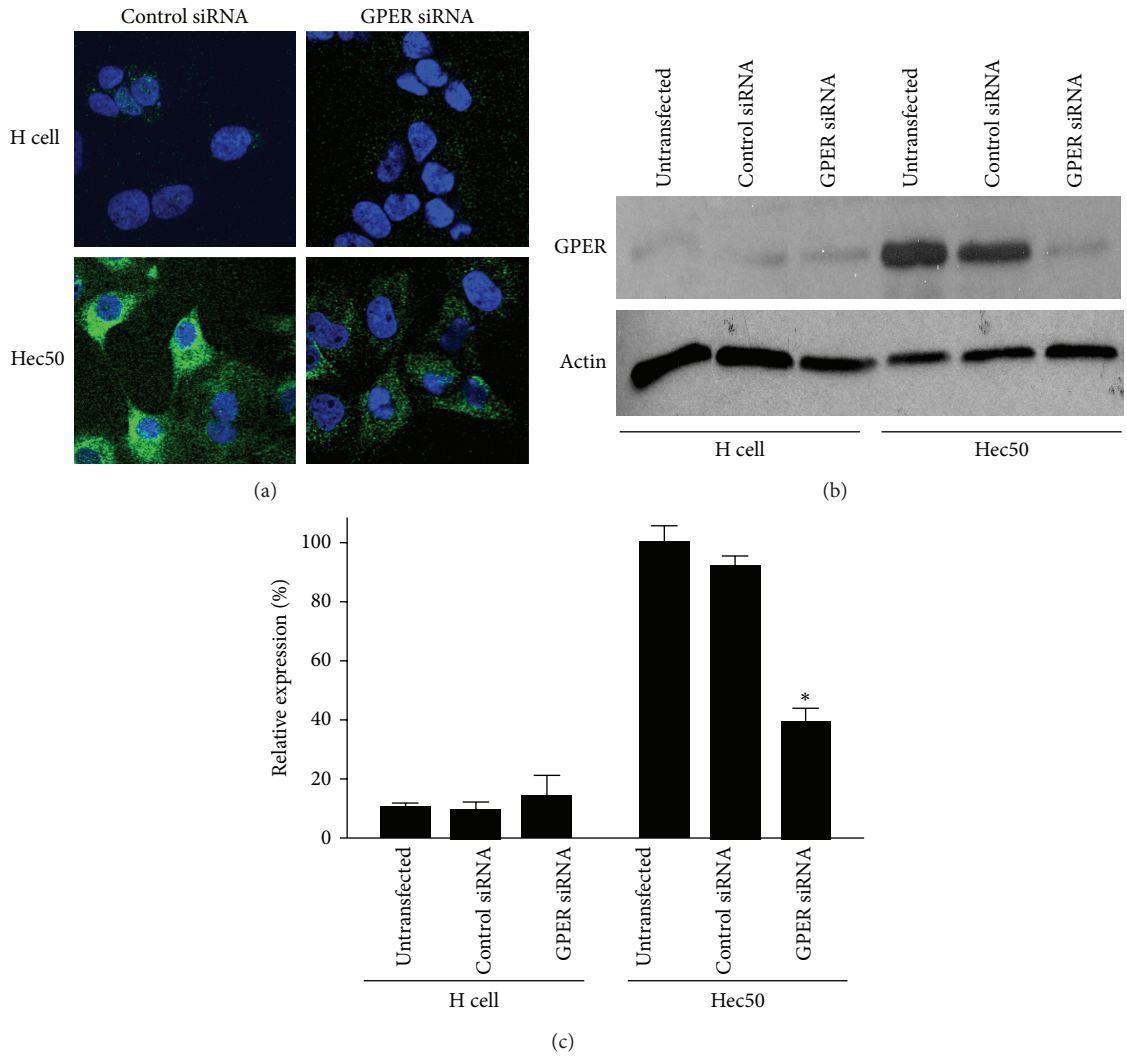

Figure 1: GPER expression in type I and type II endometrial cancer cells. (a) Representative immunofluorescence images of Ishikawa H cells (type I) and Hec50 cells (type II) transfected with either control siRNA or siRNA targeting GPER. GPER is shown in green; nuclei are stained blue with DAPI. (b) Western blot of GPER and actin in untransfected, control siRNA- and GPER-targeted siRNA-transfected H cells and Hec50 cells. (c) Western blot quantitation of GPER expression relative to actin and normalized to untransfected Hec50 cells. Data represent mean ± s.e.m. from three experiments. $^{*}P < 0.05$ compared to control siRNA.

In brief, sections were deparaffinized in CitriSolv clearing agent (Fisher, Pittsburgh, PA, USA) followed by rehydration in increasing H_2O : ethanol solutions. Antigen retrieval was accomplished by microwaving slides in 0.01 M sodium citrate buffer (pH 6.0) for 25 min, followed by incubation of cooled slides in fresh 2% H_2O_2 for 10 min. Permeabilization and blocking were performed by incubating the slides for 30 min in 200 μL of 0.1% Triton X-100 in PBS with 3% bovine serum albumin in a humid chamber. Slides were incubated with the affinity-purified GPER carboxy-terminal antibody diluted to a final protein concentration of 2 μg/mL in 3% normal goat serum for 1 h. Following multiple washes, bound antibody was detected using the immunoperoxidase system by incubating with goat anti-rabbit IgG conjugated to horseradish peroxidase (diluted 1 : 250 in 3% normal goat serum) for 45 min. Peroxidase was detected with the enzyme substrate 3′,3-diaminobenzidine tetrahydrochloride (DAB; Sigma, St. Louis, MO, USA).

3. Results

3.1. GPER Is Expressed Intracellularly in Hec50 Type II Endometrial Cancer Cells. Although the physiological and biological effects of estrogen have traditionally been described as being mediated by the nuclear estrogen receptors ERα and ERβ, recent evidence suggests an increasing role for the 7-transmembrane estrogen receptor GPER [16]. We have previously observed that, in many cell types, staining for GPER reveals a predominantly intracellular pattern associated with the endoplasmic reticulum and Golgi apparatus. Intracellular localization, including the nucleus [50, 51], has

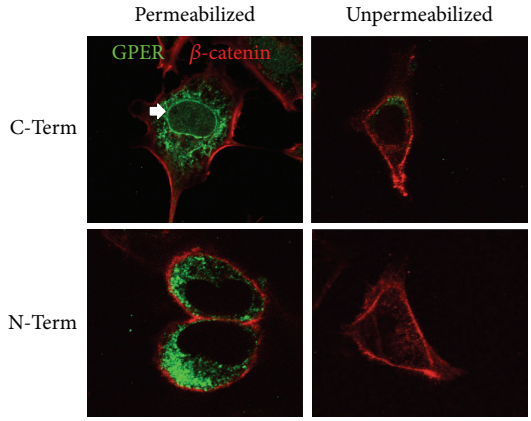

FIGURE 2: Subcellular localization of GPER expression in primary mouse uterine epithelial cells. Cells were stained with either an antibody targeted against the carboxy terminus (C-Term) or amino terminus (N-Term) of GPER under either permeabilizing or nonpermeabilizing conditions. As the amino terminus of GPCRs would be extracellular, any plasma membrane-localized receptor should be stained by the amino terminus-targeted antibody under nonpermeabilizing conditions. GPER is stained green; β-catenin is stained red as a plasma membrane marker. Arrow indicates nuclear membrane.

been reported by many [52–55] but not in other studies [56–58]. Recent results suggest that GPER undergoes constitutive internalization, which would suggest that at steady state a preponderance of GPER would be detected as intracellular [59, 60]. To address this further in endometrial epithelial cancer cells, we examined expression in type II Hec50 cells and compared it to type I Ishikawa H cells. Immunofluorescence staining and Western blotting (both with an anti-GPER carboxy terminus-targeted antibody) revealed that Ishikawa H cells express very low levels of GPER (~10%) compared to Hec 50 cells (Figures 1(a) and 1(b)). Treatment of both cell types with siRNA resulted in a significant reduction of GPER expression in Hec50 cells with no significant reduction in H cells (Figure 1). Immunofluorescence staining also revealed a pattern of GPER staining throughout the cytoplasm, consistent with localization to intracellular membranes.

To assess the localization of GPER in greater detail in primary cells, we cultured freshly isolated mouse uterine epithelial cells on glass coverslips and stained for both the carboxy-terminus as well as the amino-terminus of GPER in combination with β-catenin as a cell surface marker under both permeabilizing and nonpermeabilizing conditions (Figure 2). Staining for GPER with both antibodies under permeabilizing conditions revealed an intracellular localization with no significant overlap with β-catenin. Interestingly, in many cells (e.g., upper left panel), there was also staining of the nuclear membrane, which is continuous with the endoplasmic reticulum. Since GPCRs are oriented in the plasma membrane with their amino terminus to the cell exterior and their carboxy terminus to the cell cytoplasm, we expected that if GPERs were expressed in the plasma membrane, staining with an antibody targeting the amino terminus should be able to detect any receptor in the plasma membrane in the absence of permeabilization. However, staining with the amino terminus-targeted antibody in the absence of permeabilization revealed no significant staining, in contrast to the staining observed following permeabilization, indicating that little GPER is expressed on the cell surface compared to the intracellular pool.

3.2. PI3K Activation by Estrogen in Hec50 Endometrial Cancer Cells Is Mediated by GPER. As Hec50 cells lack ERα expression [44], we next asked whether, in the absence of ERα, estrogen could still mediate rapid signaling. To address this, we utilized a method we have previously employed [28, 41, 42], monitoring the activation of PI3K through the translocation of a fluorescent reporter of PIP3 localization, namely, a red fluorescent reporter (mRFP1 [47]) protein fused to the PH (PIP3-binding) domain of Akt [46]. In previous studies, we have observed that in serum-starved unstimulated cells (e.g., COS7 cells), the PH-RFP reporter is fairly uniformly distributed throughout the cytoplasm and nucleus [28]. However, when expressed in certain cancer cell lines (e.g., SKBr3 breast cancer cells), the reporter exhibits an enhanced plasma membrane localization [28], even under serum-starved conditions, that is likely due to constitutive activation of signaling pathways (e.g., EGFR activation or Her2 overexpression) that lead to activation of PI3K in the absence of exogenous stimuli.

To determine whether estrogen mediates rapid activation of PI3K in Hec50 cells, we transfected cells with PH-RFP and subsequently treated serum-starved cells with estrogen (17β-estradiol). The unstimulated cells yielded a plasma membrane localization similar to that previously observed in SKBr3 breast cancer cells, suggesting constitutive activation of PI3K at the plasma membrane (Figure 3(a)). However, upon estrogen stimulation for 15 min, the reporter translocated to the nucleus, suggesting activation of PI3K in the nucleus, as has been suggested by studies characterizing a nuclear pool of PI3K [61, 62]. Importantly, the inactive stereoisomer of estrogen (17α-estradiol) did not demonstrate PI3K activation, even at 1000x the concentration of 17β-estradiol, demonstrating the stereoselectivity of the receptor involved for the physiologically active isomer of estrogen. To test whether the activity of PI3K was required for the translocation of the PH-RFP reporter, we pretreated cells with the PI3K inhibitor LY294002, followed by estrogen stimulation, which yielded a uniform distribution of the PH-RFP reporter throughout the cell. This indicated not only that the nuclear localization of the PH-RFP reporter required PI3K activity, but that the membrane localization in unstimulated cells was also due to PI3K activity (as LY294002 treatment in the absence of estrogen yielded an identical distribution, data not shown). As rapid estrogen signaling has been demonstrated to involve/require EGFR activation through the generation of HB-EGF [29], we also pretreated cells with the EGFR kinase inhibitor AG1478 and the metalloproteinase inhibitor GM6001 (to block HB-EGF production). Both inhibitors blocked estrogen-mediated nuclear accumulation of the PH-RFP reporter, indicating a requirement for both HB-EGF

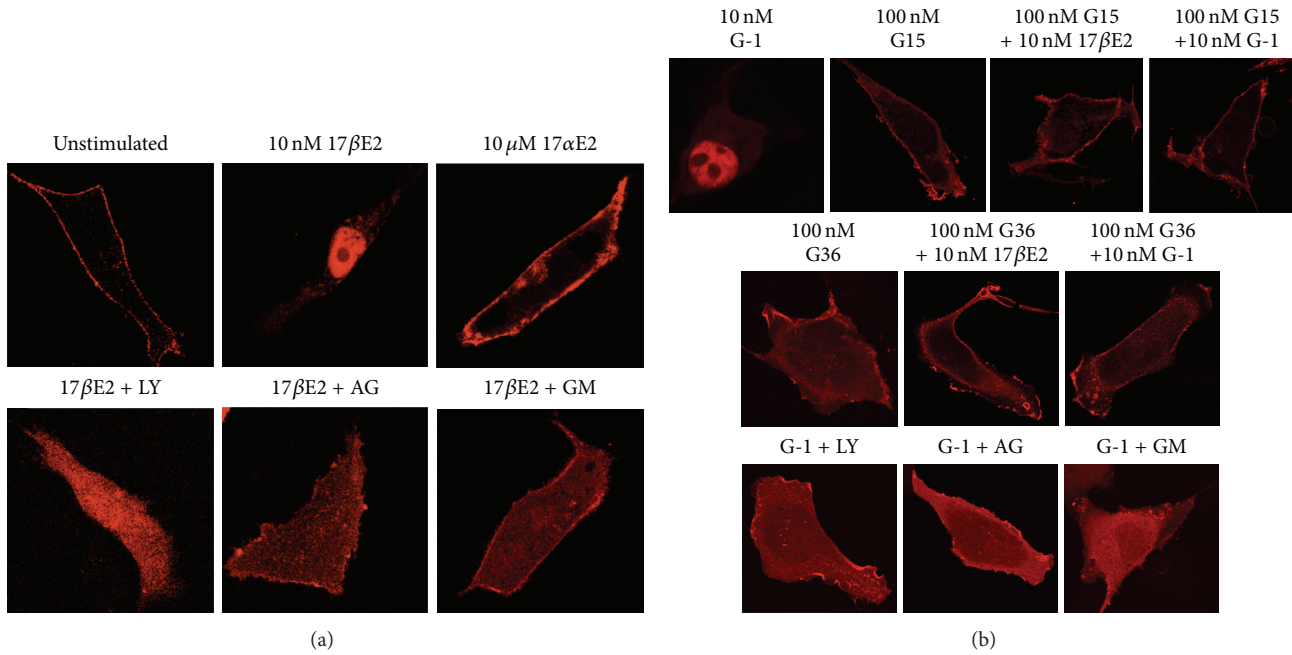

Figure 3: Activation of PI3K by estrogen in Hec50 cells is mediated by GPER. Hec50 cells were transfected with a marker of PIP3 production, the PH domain of Akt fused to monomeric red fluorescent protein (mRFP) yielding the marker PH-RFP. (a) PH-RFP-transfected Hec50 cells were stimulated with the following ligands: 10 nM estrogen (17βE2), 10 μM 17α-estrogen (17αE2), or 10 nM estrogen in the presence of the PI3K inhibitor LY294001 (10 μM, 20 min pre-incubation; 17βE2 + LY), the EGFR inhibitor AG1478 (25 μM, 60 min pre-incubation; 17βE2 + AG) or the metalloproteinase inhibitor GM6001 (10 μM, 30 min pre-incubation; 17βE2 + GM). Unstimulated designates vehicle only. (b) PH-RFP-transfected Hec50 cells were stimulated for 15 min with the GPER-selective agonist G-1 or estrogen at the indicated concentrations in the absence or presence of the GPER-selective antagonists G15 and G36 (cells were pretreated 15 min with G15 or G36 prior to stimulation with E2 or G-1) or in the presence of 10 nM G-1 and the PI3K, EGFR or metalloproteinase inhibitors as in (a).

and EGFR in estrogen-mediated PI3K activation in Hec50 endometrial cancer cells.

As Hec50 cells lack expression of the classical estrogen receptor ERα but express GPER, we next examined whether the activation of PI3K by estrogen might be mediated by GPER. Using the GPER-selective agonist G-1, we observed that, like estrogen, the PH-RFP reporter translocated to the nucleus, suggesting estrogen might be mediating its effects via GPER (Figure 3(b)). In support of this, the GPER-selective antagonists G15 and G36 not only prevented G-1-mediated activation of PI3K but also blocked estrogen-mediated PI3K activation (Figure 3(b)). G15 and G36 alone had no effect. As observed for estrogen-mediated activation of GPER, PI3K activation in response to G-1 also requires both EGFR kinase and metalloproteinase activity, as AG1478 and GM6001 also blocked nuclear translocation of PH-RFP following G-1 stimulation.

To further demonstrate the requirement for GPER in PI3K activation by estrogen and G-1 beyond pharmacological inhibition, we employed siRNA to knockdown expression of GPER (Figure 4). In mock-transfected (no siRNA) and control siRNA-transfected Hec50 cells, both estrogen and G-1 stimulated nuclear localization of the PH-RFP reporter. However, in cells transfected with GPER-targeted siRNA, neither estrogen nor G-1 stimulation resulted in nuclear translocation of the PH-RFP reporter (Figure 4(a)). Knockdown of GPER protein was confirmed by immunofluorescence staining of mock, control, and GPER siRNA-transfected cells (Figure 4(b)). The use of both a pharmacological approach (G15 and G36) and siRNA to prevent activation of PI3K by estrogen, as well as the ability of G-1 to activate PI3K, strongly indicates that GPER is the receptor mediating responsiveness to estrogen in Hec50 cells.

3.3. Multiple Estrogen Mimetics Activate PI3K and ERK via GPER. To examine the effects of a number of therapeutic antiestrogens and other ligands on PI3K activation in ERα^{-}/β^{-} Hec50 cells, we evaluated PH-RFP localization in cells treated with 4-hydroxytamoxifen, ICI182,780, the benzothiophene-based and recently FDA-approved SERM Raloxifene, the phytoestrogen genistein, and the widely used ERα- and ERβ-selective agonists propylpyrazoletriol (PPT) and diarylpropionitrile (DPN) (Figure 5(a)). There is evidence that ICI182,780 [29] and tamoxifen [15, 26, 28] can act through GPER to stimulate rapid cellular signaling. Specifically, we have demonstrated tamoxifen-mediated stimulation of PI3K in GPER-transfected COS7 cells and GPER^{+} SKBr3 breast cancer cells (both of which do not express ERα or for that matter ERβ) [28]. PPT and DPN have been used extensively as "selective" agonists of ERα and ERβ, respectively [63, 64]. Whereas PPT displays ~400-fold

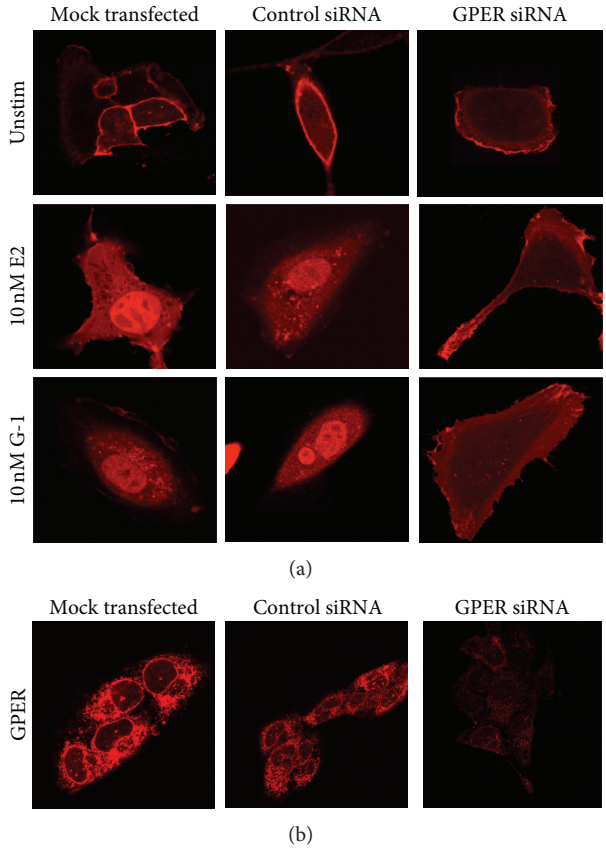

Figure 4: GPER mediates PI3K activation in Hec50 cells. (a) Hec50 cells were transfected with no siRNA (mock transfected), control siRNA or siRNA targeting GPER (GPER siRNA), and the PH-RFP reporter. Transfected Hec50 cells were stimulated with vehicle, estrogen (10 nM E2), or G-1 (10 nM). (b) Hec50 cells transfected with no siRNA (mock transfected) and control siRNA or siRNA targeting GPER (GPER siRNA) were stained for GPER (with carboxy-terminal antibody) to demonstrate the specific knockdown of GPER in the GPER siRNA-treated cells.

binding selectivity for ERα over ERβ, DPN exhibits only ~70-fold selectivity for ERβ over ERα [65–68]. Of these, all compounds (at 100 nM), with the exception of DPN (even at 10 μM), stimulated the nuclear translocation of the PH-RFP reporter to the nucleus (Figure 5(a)), as observed with estrogen and G-1 (Figures 3 and 4). To confirm the activity of DPN, we cotransfected Hec50 cells with ERβ-GFP and PH-RFP. In cells expressing ERβ, DPN was indeed able to stimulate PH-mRFP translocation at a concentration of 100 nM, demonstrating the ability of Hec50 cells to respond to DPN via ERβ and furthermore demonstrating that, without the exogenous expression of ERβ, Hec50 cells do not express sufficient ERβ (if any) to respond to DPN.

We and others have previously demonstrated that GPER is capable of activating ERK in addition to PI3K in multiple cancer and other cell lines, including other endometrial cancer cell lines [22, 27, 42, 69]. To further examine and quantify the ligand specificity of GPER, we determined pERK levels in Hec50 cells stimulated with the ligands above (Figures 5(b) and 5(c)). Although the only known estrogen receptor expressed in Hec50 cells is GPER, we confirmed the contribution of GPER to ligand-induced ERK activation using the GPER-selective antagonist G15. As for PI3K activation, estrogen, G-1, 4-hydroxytamoxifen, ICI182,780, and Raloxifene stimulated pERK ~5–8-fold. Activation of ERK by each of these ligands was completely inhibited by G15, indicating an essential role for GPER in the response to each ligand. The phytoestrogen genistein also acted as an agonist of GPER-mediated ERK activation in Hec50 cells and as observed with PI3K activation, PPT (100 nM), but not DPN (at concentrations up to 10 μM), was able to induce ERK activation in a GPER-dependent (i.e., G15-sensitive) manner. Finally, to ensure that G15 did not inhibit ERK activation downstream of the transactivated EGFR, we directly stimulated Hec50 cells with EGF. EGF was ~30% more potent than the next most potent ligand, estrogen, but unlike the other agonists of ERK activation via GPER, EGF stimulation was unaffected by G15, demonstrating that the inhibitory action of G15 on GPER activation is upstream of EGFR.

3.4. Expression of GPER in Xenografts of Endometrial Cancer Cell Lines and Human Endometrial Cancers. Hec50 cells are poorly differentiated endometrial cancer cells that were originally isolated from a metastatic lesion in a patient with advanced endometrial cancer who ultimately succumbed to the cancer [70, 71]. The cells do not form glands in tissue culture or in xenografts and do not express either ERα or PR [43]. They do however exhibit the capacity to subdifferentiate into a papillary serous phenotype when injected intraperitoneally in mice [72]. Thus, Hec50 cells are an excellent model of type II endometrial tumors [71]. In contrast, Ishikawa H cells were derived from a patient with stage 2 moderately differentiated endometrial adenocarcinoma who was treated with surgery and chemotherapy and survived without recurrence. These cells produce mucous, contain vacuoles, express both ERα and PR, and are thus an excellent model of type I endometrial cancer [71].

As demonstrated in Figure 1 by immunofluorescence and Western blotting, Hec50 cells express substantially (≥10-fold) more GPER than do H cells. In xenografts, H cells form endometrioid tumors whereas Hec50 can differentiate into a serous subtype [44]. To assess whether GPER expression patterns are maintained in xenografts, we performed immunohistochemistry for GPER on xenograft tumors of both H cells and Hec50 cells from nude mice (Figure 6). Tumors derived from H cells exhibited well-differentiated gland formation (Figures 6(a) and 6(b)), whereas, in tumors derived from Hec50 cells, cells were poorly or undifferentiated, nuclei were pleomorphic, and mitotic activity was abundant (Figures 6(d) and 6(e)). In addition, although GPER was detected to varying extents in the tumors from H cells (Figures 6(a) and 6(b)), it was expressed at far greater levels in tumors from Hec50 cells (Figures 6(d) and 6(e)), consistent with expression observed in cultured monolayers (Figure 1) [28].

We have previously reported that, in endometrial cancer, high GPER expression is prognostic of poor survival [37].

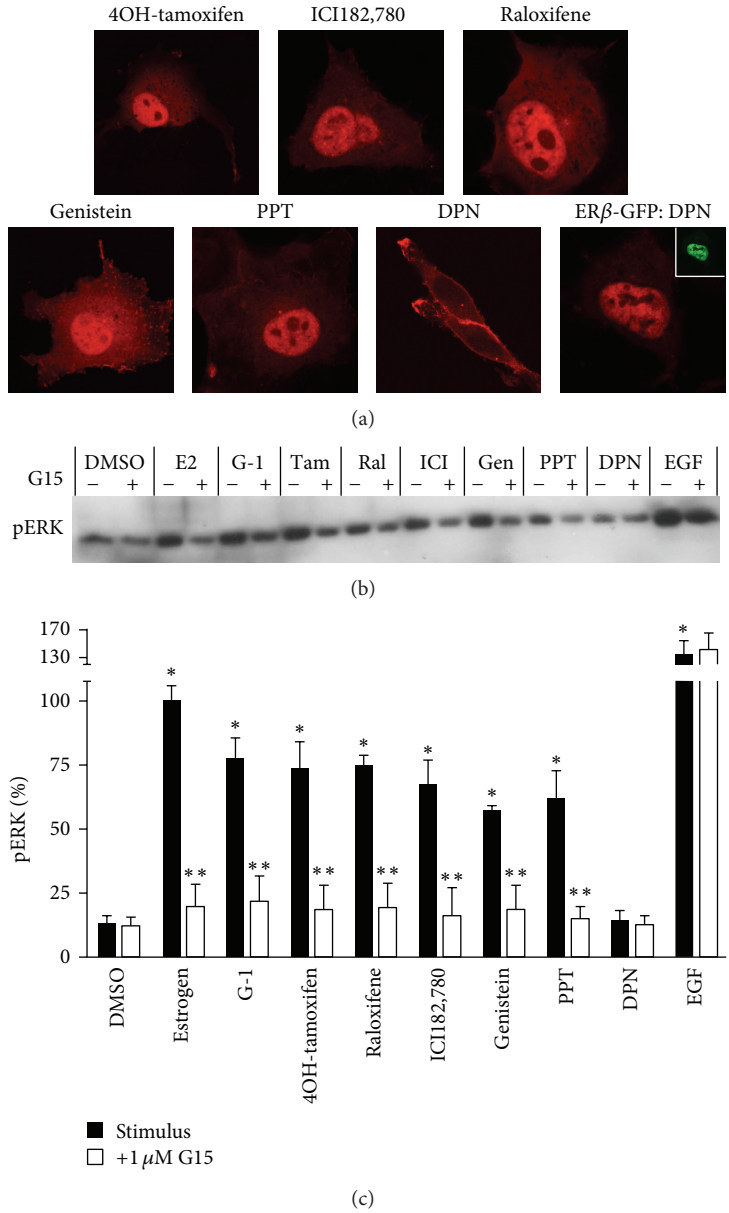

Figure 5: GPER-mediated activation of PI3K and ERK in Hec50 cells by SERMs, a SERD, and an ERα-selective agonist. (a) PH-RFP-transfected Hec50 cells were stimulated for 15 minutes with the following ligands: 4-hydroxytamoxifen (100 nM, 4OH-tamoxifen), ICI182,780 (100 nM), Raloxifene (100 nM), genistein (100 nM), PPT (100 nM), or DPN (10 μM). Hec50 cells were also cotransfected with ERβ-GFP (shown in inset) and PH-RFP and stimulated with 100 nM DPN to verify the activity of DPN as an ERβ agonist in Hec50 cells (ERβ-GFP:DPN). (b) Hec50 cells were stimulated for 15 min with vehicle (0.05% DMSO), estrogen (10 nM, E2), G-1 (10 nM), 4-hydroxytamoxifen (100 nM, Tam), Raloxifene (100 nM, Ral), ICI182,780 (100 nM, ICI), genistein (100 nM, Gen), PPT (100 nM), DPN (10 μM), or EGF (1 nM) either in the presence or absence of 1 μM G15 (10 min pretreatment, with 0.05% DMSO in samples without G15). (c) Band intensities of pERK were normalized to total ERK and plotted with estrogen as 100%. Data represent mean ± s.e.m. from three experiments. $^{*}P < 0.05$ versus DMSO; $^{**}P < 0.05$ versus paired stimulus without G15.

Moreover, in carcinosarcoma subtypes, advanced stage disease was more frequently associated with high levels of GPER and ERβ expression [73]. To assess whether GPER expression levels and patterns are maintained in xenografts of primary patient tumors, xenografts from tumors preoperatively characterized as type I and type II tumors were generated and immunostained for GPER using xenograft tissues and parallel paraffin-embedded tissue from the original patient tumor (Figures 6(f)–6(l)). Case studies of our illustrated cases are relevant. Patient 1 was originally diagnosed with superficially invasive (24%) grade 1, ERα^{+}/PR^{+}, endometrioid adenocarcinoma (FIGO stage IB) and presented two years later at our institution with a 20 × 21 × 10 cm mass involving the omentum, anterior abdominal wall, and bowel, which was

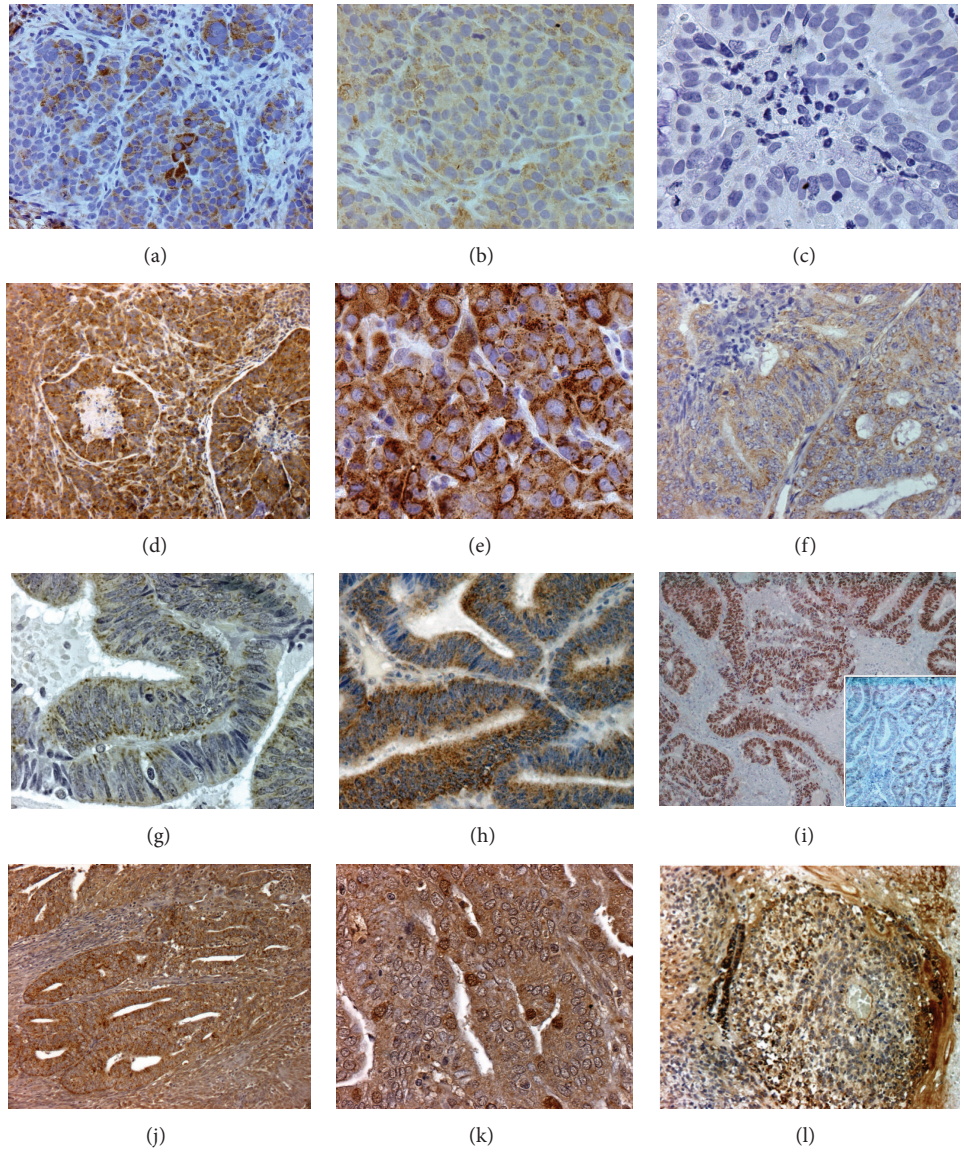

Figure 6: GPER expression in xenografts of Ishikawa H cells and Hec50 endometrial cancer cells as well as representative type I and type II human primary tumors. (a)–(e) Immunohistochemical staining of GPER in xenograft tumors of Ishikawa H cells ((a), 20x; (b), 40x) demonstrating the areas most strongly positive for GPER, and representative xenograft tumors of Hec50 cells ((d), 20x; (e), 40x; (c), negative control (irrelevant primary antibody), 40x). ((f)–(h)) GPER staining of a recurrent adenocarcinoma with endometrioid features ((f), mouse xenograft of patient tumor, 40x; (g), patient tumor, 40x; (h), patient tumor, 20x, demonstrating focal positivity; (i), ERα and PR (inset) staining of the same patient tumor). ((j)–(l)) GPER staining from a patient with Stage IA carcinosarcoma ((j), mouse xenograft of patient tumor, 20x, illustrating diffuse positive staining in both epithelial and stromal fractions; (k), patient tumor, 40x; (l), patient tumor, 20x, demonstrating similarly strong GPER staining in both the epithelial and stromal compartments).

resected. The patient refused postoperative chemotherapy and was instead treated with tamoxifen 40 mg daily and medroxyprogesterone acetate 200 mg daily, cycle day 16–30, on an IRB-approved institutional trial. With 6 months of hormonal therapy, the patient has remained radiographically free of disease (duration of followup: 4.5 years). Immunostaining of the recurrent tumor (the primary was unavailable for comparison) was scored as PR (3+, 10% of viable epithelial cells), ERα (3+, 100% of viable cells), and GPER (3+, less than 10% of viable cells, Figures 6(f)–6(i)).

Patient 2 was diagnosed with grade 3 adenocarcinoma based upon endometrial biopsy but at surgery was found to have carcinosarcoma, FIGO stage IA, with high-grade sarcomatous features. Following weekly cisplatin chemotherapy and radiation therapy this patient has remained disease-free 4 years after therapy. As carcinosarcoma tumors are considered biphasic (i.e., defined by having malignant epithelial and stromal compartments) [37, 74], we evaluated both epithelial and stromal cell staining for GPER expression and observed that both components were strongly positive for GPER.

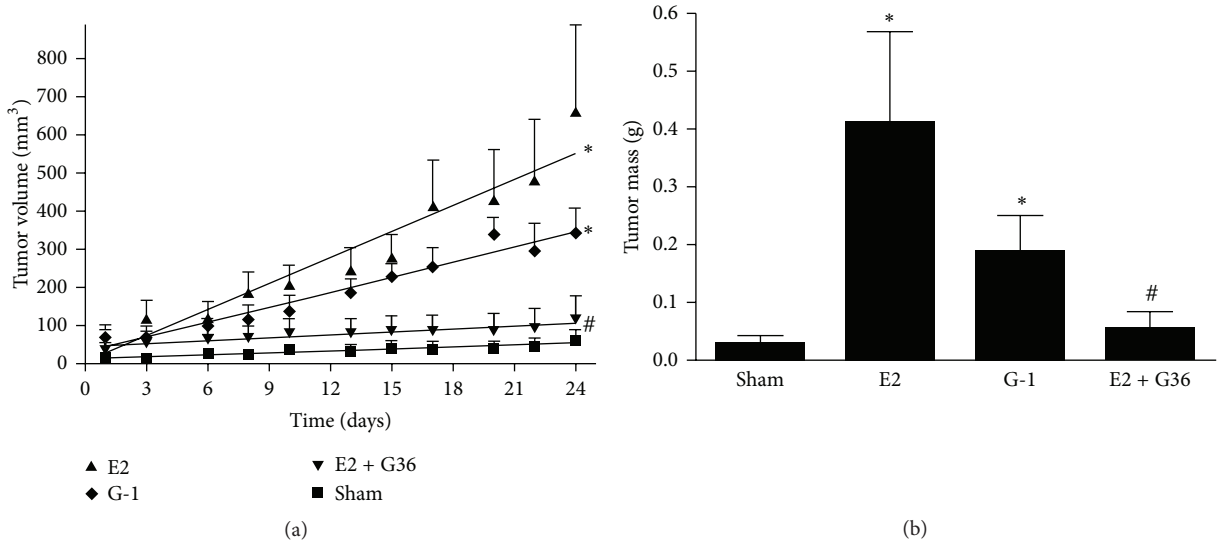

Figure 7: Inhibition of Hec50 tumor growth by GPER antagonist. Hec50 cell tumors were initiated in ovariectomized athymic mice and treated with a slow release pellet containing either no added compound (sham), estrogen (E2), G-1, or with two pellets (one of estrogen and one of G36, E2 + G36). (a) Tumor volume was measured with calipers over a 24-day period. (b) Upon sacrifice, tumors were dissected and tumor mass was determined. Data represent mean ± s.e.m. from 6–8 mice. $^{*}P < 0.05$ versus sham; $^{\#}P < 0.05$ versus E2 alone. Note that the tumor size for E2 + G36 was not significantly different from the sham.

These results demonstrate not only that Hec50 cells maintain high levels of GPER expression as xenografted tumors, but also that Hec50 cells mimic the levels of GPER expression observed in high grade endometrial tumors, as detected in paraffin-embedded patient samples and xenografted patient tumors, suggesting that primary xenografts of endometrial cancers (even complex tumors with biphasic characteristics) may represent an excellent model to test the therapeutic efficacy of GPER-targeted therapies.

Interestingly, within our endometrial cancer repository, we identified six patients who received tamoxifen-based therapy for recurrent endometrial cancer, and of these only one patient (case 1, above, with low GPER expression) experienced a complete response; in contrast, all nonresponders displayed increased GPER expression (defined as expression above the mean [37]) by immunohistochemistry of paraffin-embedded tumor samples (Fisher's $P = 0.03$), suggesting that a lack of or low GPER expression may be a predictor of tamoxifen responsiveness in endometrial cancer.

3.5. GPER Antagonist Inhibits Endometrial Tumor Growth. Type I endometrial cancer can result from excess and/or unopposed estrogen use and typically progresses from hyperplasia to atypical hyperplasia and finally to carcinoma. Type I tumors commonly express ER and PR [75] and are generally responsive to hormone treatment with therapeutic efficacy positively correlating with the level of receptor expression [2, 75, 76]. However, type II endometrial cancers are believed to develop through molecular pathways involving p53 mutations, which more closely resemble high-grade serous ovarian tumors in molecular alterations and morphology and commonly do not express either ER or PR [2, 44], suggesting that estrogen (or its inhibition) should have no effect on the growth of such tumors. To examine the estrogen dependence of a type II endometrial cancer *in vivo*, we generated Hec50 cell xenografts in mice. To determine the effect of estrogen, the tumors were initiated in ovariectomized mice, and estrogen was restored using slow release pellets (Figure 7). Surprisingly, we found very little tumor growth in ovariectomized mice (compared to our experience in ovary intact mice, data not shown). However, upon supplementation with estrogen, tumor growth was increased about 10-fold (compared to ovariectomized/sham-treated mice). To determine whether GPER was responsible for this estrogen-mediated enhancement of tumor growth, we also treated ovariectomized mice with slow release pellets containing G-1. The tumors in these mice were about 5-fold larger than those in sham-treated mice, suggesting that GPER was indeed capable of stimulating tumor growth *in vivo*. Finally, to further test whether the estrogen-stimulated tumor growth was mediated by GPER, we cotreated estrogen-supplemented mice with our recently identified GPER-selective antagonist G36 [42]. Estrogen-stimulated tumor growth was reduced by G36 to that of the sham treatment (i.e., estrogen deprived state), demonstrating a critical role for GPER in the estrogen-mediated response of ER^{-} type II endometrial cancer growth.

4. Discussion

Endometrial cancers, like most cancers, consist of multiple, distinguishable tumor types. At a minimum, endometrial cancers have been separated into type I and type II endometrial cancers, based on morphology and molecular phenotypes/genotypes [2]. Recent genomic studies from The Cancer Genome Atlas are shedding more light on the extent and range of mutations associated with this cancer, defining

4 categories of endometrial cancer [77]. Whereas survival is high among women with type I endometrial cancers, the opposite is true for type II cancers, which express high levels of GPER [37]. We have therefore focused our investigations on a model of type II endometrial cancer in this study, particularly due to the fact that these cancers are typically described as estrogen unresponsive due to their lack of ER expression. In this work, we have demonstrated that Hec50 cells, typical of type II endometrial cancer cells that do not express the classical ERα, do express GPER, which makes them responsive to estrogen in terms of rapid cellular signaling (PI3K and ERK). Furthermore, as chronic tamoxifen use (i.e., for breast cancer) results in an increased incidence of endometrial cancer, we demonstrated that, in endometrial cancer cells, GPER mediates cellular signaling in response to two SERMs (tamoxifen and Raloxifene) as well as a SERD (ICI182,780), revealing a possible additional mechanism for the increased risk of endometrial cancer with tamoxifen use. We also demonstrated that the ERα-selective (i.e., selective versus ERβ) agonist PPT activates GPER, raising questions about the conclusions drawn from its use in defining exclusive ERα function in a multitude of biological systems. Finally, we established that GPER is highly expressed in type II tumors, that Hec50 xenograft tumors display a strong dependence on estrogen *in vivo*, and that this occurs through GPER, the inhibition of which blocks estrogen-stimulated tumor growth.

Although GPCRs are traditionally thought of as cell surface receptors, mediating transmembrane signaling of membrane-impermeable ligands (e.g., ionic small molecules, peptides, and proteins), we originally described GPER localization as being predominantly intracellular [28]. Those and our continuing results have localized GPER primarily to intracellular membrane compartments (endoplasmic reticulum and Golgi membranes), even using immunohistochemistry of tumor and other tissues [34, 37, 53, 54]. In Hec50 cells, we again observed a strong intracellular localization. To examine whether this localization was also evident in primary cells (as opposed to cancer cell lines), we isolated and stained primary murine uterine epithelial cells. As with Hec50 cells, the staining pattern of GPER appeared intracellular but with a more punctate morphology. Consistent with an intracellular localization, no staining was observed using an antibody targeting the amino terminus of GPER under nonpermeabilizing conditions. In a fraction of cells, staining of the nuclear membrane was evident. As the endoplasmic reticulum is continuous with the nuclear membrane, such a pattern would not be unexpected. However, since nuclear membrane staining is not present in all cells and cell types, GPER localization to the nuclear and other membranes may be actively regulated. In fact, others have reported plasma membrane localization to varying extents in diverse cell types [56–58]. Whether the localization and trafficking are dynamically regulated within a cell, or simply different in distinct cell types, remains to be determined. The lack of extensive plasma membrane localization under steady-state conditions suggests either that GPER traffics poorly to the plasma membrane (being retained in the endoplasmic reticulum and Golgi apparatus) or that, if trafficked to the plasma membrane, it is rapidly internalized. Evidence for both of these mechanisms has been recently presented with Filardo et al. revealing constitutive internalization of GPER to a trans-Golgi compartment [78] and with Lenhart et al. suggesting a role for receptor activity-modifying protein 3 (RAMP3) in the trafficking of GPER to the cell surface [79]. With the identification of an increasing number of GPCRs being expressed intracellularly [80, 81], particularly those GPCRs for membrane-permeable ligands (such as lipids and steroids) [82], as well as the recognized activities of internalized GPCRs [83], the cellular mechanisms and functional consequences of regulating GPER localization remain to be elucidated. With the recent characterization of differential subcellular activation of calcium stores by GPR55 depending on its site of activation in cardiomyocytes (plasmalemmal versus intracellular) [82], the subcellular site of GPER expression/activation may similarly play an important role in regulating its downstream signaling activity, particularly given the broad scope of GPER expression and function throughout the body [16, 69, 84–87].

Whereas the classical estrogen receptors (ERα and ERβ) are traditionally thought to mediate primarily genomic responses [88], GPER has become recognized as an estrogen receptor that mediates rapid cellular signaling [89]. Nevertheless, there is also substantial evidence that ERα can mediate rapid signaling [90] and that GPER mediates transcriptional regulation [91]. Whereas ERα regulates transcription in part through direct binding to estrogen response elements in DNA [88], GPER presumably regulates transcription indirectly through kinase cascades [91]. While the transcriptional mechanisms of each receptor are distinct, there exist many possibilities for overlap and interactions of signaling activities (both synergistic and inhibitory) that remain largely undefined. The end results of GPER activation are also likely to be different in cells that also express ERα (or ERβ for that matter) compared to cells that express only GPER. For example, the role of GPER in ERα^+ breast cancer cells that are "addicted" to ER-mediated signaling/gene expression for growth, and thus sensitive to antiestrogen/hormone therapy, may be very different compared to ER$^-$/triple negative cells. The same may be true of endometrial cancers with ERα^+ type I tumors behaving very differently compared to ER$^-$ type II tumors, particularly with respect to estrogen signaling. Furthermore, the effects of additional growth promoting factors/pathways may obscure or minimize the effects of estrogen through GPER, or ERs for that matter.

In this study, we demonstrated that an endometrial cell line representative of ER$^-$ type II tumors maintains the ability to signal in response to estrogen via GPER. In Hec50 cells, estrogen signaling via GPER results in a metalloproteinase/EGFR-dependent activation of downstream kinase pathways, including PI3K and ERK, both important players in cancer cell survival and growth. Interestingly, the plasma membrane localization of PIP3 under unstimulated conditions suggests a level of constitutive PI3K activity in Hec50 cells. The ability of an EGFR inhibitor to reduce this further suggests that one or more members of the erbB family are involved through EGFR activation [92]. It is important to note that the effect of estrogen and G-1 might

not be easily detectable by measuring total cellular pAkt levels, for example, by Western blot, due to the constitutive activation of PI3K. Translocation of the PH-RFP reporter however allows selective detection of GPER-mediated PI3K activation in the absence of global changes in the total cellular level of PI3K activity. Such location/compartment-specific signaling activity may play an important role in the overall effect of any activated pathway.

The pharmacopeia of estrogen receptor ligands has been developed in the absence of consideration for their interaction with GPER. For example, the development of SERMs and SERDs was largely based on the development of compounds with high binding affinity to ERα and the effects of these compounds on transcriptional regulation (through estrogen response elements) and overall tissue-specific activities (breast cancer cell growth versus uterine effects such as imbibition). Furthermore, the search for ERα versus ERβ (and *vice versa*) selectivity [68, 93, 94] has also been carried without consideration of GPER function. In many cases, ligands or therapeutic agents were developed long before GPER was even identified, tamoxifen dating back to the 1970s. In many studies, these agents exhibit unexpected activities, often stimulating rapid cellular or physiological responses similar to estrogen without the expected inhibitory effects [95–100]. Although the disparate activities of SERMs have been attributed to tissue differences in the expression of ER coregulators [101], the agonistic effects of SERDs are perhaps less easily explained by such mechanisms. With previous reports of tamoxifen [28] and ICI182,780 [29] agonism through GPER, and now with our current demonstration of the activity of Raloxifene on GPER, the mechanisms of action of these compounds are greatly complicated. For example, in addition to ERα, what role does GPER play in hormone resistance in breast cancer [30, 31, 102] and the increased incidence of endometrial pathology and cancer in women taking tamoxifen [32]? The agonism of these compounds on GPER may well play an important role.

With the identification of ERβ in 1996 [103], it soon became clear that selective ligands would be a powerful tool in the characterization of the functions of the individual estrogen receptors as well as being therapeutically promising. Because the ligand binding pockets of ERα and ERβ are almost identical, achieving this goal has been challenging [104]. Today, the most widely used "selective" agonists for ERα and ERβ are propylpyrazoletriol (PPT) and diarylpropionitrile (DPN), respectively [63, 64]. PPT displays ~400-fold binding selectivity for ERα over ERβ, whereas DPN exhibits only ~70-fold selectivity for ERβ over ERα [65–68]. In our current study, we found that PPT (at 100 nM, with a weak response at 10 nM, unpublished observation) also acts as an agonist for GPER. The three estrogen receptor selective compounds PPT, DPN, and G-1 have recently been used to evaluate the estrogen receptor involved in a number of physiological or cellular responses [105–107]. Prior to the identification of G-1, only PPT and DPN could be used to "distinguish" between ERα and ERβ. A growing number of reports are concluding, for example, that both ERα and GPER mediate responses based on the activity of both PPT and G-1 [108, 109]; however, based on our current results, these responses *could* be mediated solely by GPER, if other measures of receptor involvement are not also employed. Clearly, PPT is a potent agonist of ERα, with enhanced selectivity versus GPER, but, without additional approaches (siRNA or selective antagonists such as G15 and G36), conclusions must be drawn with care and it is possible that many of the interpretations in the related literature (particularly using high PPT concentrations, e.g., ≥10 nM) should be reevaluated with these considerations in mind.

GPER stimulation has been demonstrated to increase cell proliferation in a broad array of cell lines, including some endometrial cancer cell lines, suggesting a potential importance in one or more aspects of carcinogenesis [15, 20, 24, 26, 27]. In fact, in a very recent study, knocking down GPER expression in Hec1A endometrial cancer cells (as well as Ishikawa H cells) resulted in a reduction of tumor growth in athymic mice [20]. This result is consistent with the many studies that suggest GPER expression correlates with poor survival or indicators of poor outcome in endometrial, ovarian, and other cancers [33–37]. In particular, GPER expression represents a mechanism by which ostensibly estrogen-unresponsive tumors (often stated as such based on the lack of ERα expression alone) can maintain estrogen responsiveness. The significance of this estrogen responsiveness will clearly depend on the type of cancer and the other mechanisms/mutations involved in a specific cancer. Although it has been amply demonstrated that, in breast cancer, antiestrogens targeting ERα are highly effective, the role of estrogen in gynecological cancers is thought to be of less importance due to the lack of clear efficacy of antiestrogens [110]. Nevertheless, the endometrium, like the breast, is highly estrogen responsive in terms of proliferation and elevated tumor estrogen levels have been reported not only in ERα^+ type I but also in ERα^- type II endometrial tumors [111]. Thus, if GPER expression and function play an important role, particularly in ERα^- type II endometrial cancers, then treatment with SERMs and SERDs, functioning as GPER agonists, would be highly contraindicated.

To examine the role of GPER in endometrial tumor growth, we sought to establish and investigate xenograft tumors in mice. We first examined whether the highly disparate levels of GPER expression in H cells and Hec50 cells observed in tissue culture were maintained as xenografts. Overall, xenograft tumors of each cell type displayed the expected histological properties with respect to tumor morphology and GPER expression with Hec50 cell tumors expressing substantially higher levels compared to H cell tumors. Importantly, primary xenografts displayed similar morphologies and GPER expression levels and patterns to those assessed directly from the patient samples. This suggests that the xenograft model may be a useful adjunct in which to test the therapeutic efficacy of GPER-selective compounds (particularly GPER-selective antagonists). Of particular relevance, in the clinical setting, we found that low expression of GPER was associated with the only observed response to tamoxifen; and since this patient was strongly ER$^+$/PR$^+$, we surmise this effect may have been mediated by classical steroid receptor pathways [112].

To determine if estrogen itself and GPER in particular contributes to tumor growth of ERα^- Hec50 cells, we established subcutaneous Hec50 tumors in ovariectomized athymic mice. Tumors remained small in untreated mice but were about 10-fold larger in mice treated with slow release estrogen pellets, indicating that the lack of ERα did not prevent responsiveness to estrogen. Interestingly, we were not able to demonstrate estrogen-stimulated growth in monolayer culture of Hec50 cells, suggesting estrogen-responsiveness may be a result of the tumor environment (unpublished results), highlighting the importance of tumorigenesis studies *in vivo*. To test whether GPER can mediate this estrogen responsiveness, we treated mice with slow release pellets containing G-1. These tumors were about half the size of the estrogen-treated mice, suggesting that GPER could mediate the effects of estrogen. The reduced tumor size of G-1-treated mice compared to estrogen-treated mice could be due to differential sensitivity to the two ligands (e.g., EC_{50} values) or differential distribution, metabolism, or excretion of the two compounds. Finally, to investigate more directly the role of GPER in the estrogen responsiveness, we treated mice with both estrogen and the GPER-selective antagonist G36. The tumors in these mice were restored to the size of the tumors in the sham-treated mice. These results indicate not only that the observed estrogen dependence of Hec50 cell tumors is due to GPER but also that pharmacological inhibition of GPER could represent an important new therapy for women, particularly those with aggressive type II endometrial cancer.

5. Conclusions

In this paper, we have demonstrated that GPER plays an important role in the estrogen-mediated signaling of a representative type II endometrial cancer cell line. In addition, we demonstrate for the first time that the SERM Raloxifene is an agonist for GPER, with potentially important clinical ramifications for its FDA-approved chronic use for osteoporosis. The ability of the widely used "ERα-selective" agonist PPT to activate GPER suggests that a large body of literature may have to be more carefully interpreted with respect to defining the roles of individual estrogen receptors. Finally, we have demonstrated that GPER-selective antagonists may represent important new therapeutic agents for endometrial and other cancers pathologically defined as estrogen unresponsive due to their lack of ERα expression.

Authors' Contribution

Whitney K. Petrie and Megan K. Dennis contributed equally.

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Research Article

Obesity and the Endometrium: Adipocyte-Secreted Proinflammatory TNFα Cytokine Enhances the Proliferation of Human Endometrial Glandular Cells

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Obesity, a state of chronic inflammation, is associated with poor fertility and low implantation rates and is a well-documented risk factor for endometrial cancer. Adipokines, such as tumor necrosis factor alpha, play an important role in initiation of endometrial cancer. The aim of this study is to evaluate *in vitro* effects of human adipocyte cells (SW872) on growth of endometrial glandular epithelial cells (EGE). *Methods.* We measured cell proliferation and expression of cell-growth proteins—proliferating cell nuclear antigen, cyclin D1, cyclin-dependent kinase-1, and apoptotic markers (BCL-2 and BAK) in human EGE cells cocultured with SW872 cells. EGE cells were also evaluated in SW872-conditioned media neutralized with anti-TNFα antibody. *Results.* A significant increase in EGE cell proliferation was observed in both SW872-conditioned media and in coculture ($P < 0.05$). We observed an upregulation of proliferation markers PCNA, cyclin D1, CDK-1, and BCL-2 and decrease in BAK ($P < 0.05$). Neutralization of SW872-conditioned media using anti-TNFα antibodies reversed EGE cell proliferation as indicated by BCL-2 expression. *Conclusions.* Adipocytes have potent proliferative paracrine effect on EGE cells which may be, in part, mediated via TNFα. Further understanding of the role of obesity in endometrial carcinogenesis should lead to better preventative and therapeutic strategies.

1. Introduction

Endometrial cancer is the most commonly diagnosed gynecologic cancer in American women [1]. National Cancer Institute estimates for 2013 indicate 48,560 new cases and 8,000 deaths with endometrial cancer expected in US alone [2]. Endometrial cancer-related mortality is about 14% in Black women compared to White women [3]. Altered sex hormones levels as well as dysregulated levels of cytokines and growth factors are involved in the biology of endometrial cancer [4]. One-third of cancers, including colon, breast, esophagus, and endometrium, are believed to be associated with increase in body weight and low physical activity [5]. Numerous studies have shown high body mass index and obesity to be great risk factors for endometrial cancer [6–8].

Adipokines are specific biological factors secreted from the adipose tissue, including several proinflammatory markers such as tumor necrosis factor α (TNF-α) [9], transforming growth factor-β (TGF-β) [10], interleukin-6 [11], and monocyte chemoattractant protein-1 [12]. Obesity, a state of low grade chronic systemic inflammation [13], changes the adipokine profiles causing an activation of inflammatory signaling pathways leading to morbid outcomes like tumorigenesis [14, 15]. An increased adipocyte expression and elevated serum level of TNFα have been reported in obese rodents and women [15–18]. TNFα is a major player in regulation of cell growth, differentiation, inflammation, and metastasis [19]. Inflammatory cytokines are reported to increase cell proliferation and angiogenesis, the hallmarks of tumorigenesis [20]. Although the mechanism involved in the

initiation of tumorigenesis via this pathway is not clear, the production of proinflammatory marker TNFα locally and/or systemically is believed to play an important role [21, 22].

Presence of higher levels of adiposity-related inflammatory cytokines as well as factors such as IL-6, TNFα, and C-reactive protein, in addition to lower levels of adiponectin has been implicated as possible contributing factors for initiation and progression of endometrial cancer in women [23]. Interestingly, TNFα is also synthesized and secreted from the human endometrial cells [24] and has been associated with physiological and pathological changes in the endometrium-like remodeling, implantation, and cancer [25, 26]. Elevated levels of TNFα along with its receptors have been strongly associated with a higher risk of endometrial cancer [27]. In the present study, we investigated the interaction of adipokine-secreting human adipocytes (SW872) with human endometrial glandular epithelial cells (EGE) to better understand the possible biological interplay of obesity and endometrial cancer. The central rationale of this study is that dysregulated adipokine levels secondary to obesity may contribute to the development of endometrial cancer. In this work, we used the *in vitro* cell culture model to investigate cell growth in EGE cells using SW872-conditioned media, and further cocultured the SW872 and EGE cells for additional verification.

2. Methods

2.1. Cell Culture. All cell culture experiments including coculture system were performed using human endometrial glandular epithelial cells (EGE, immortalized, nonmalignant human endometrial glandular cells, a generous gift from Dr. Satoru Kyo, Department of Obstetrics & Gynecology, Kanazawa University, Kanazawa, Japan) and human liposarcoma cells (SW872 cells obtained from American Type Culture Collection, Manassas, VA, USA). EGE cells were maintained in DMEM/F12 (1 : 1) supplemented with 10% fetal bovine serum and Insulin Transferrin Selenium (BD biosciences, Bedford, MA, USA) in 5% CO_2 at 37°C. SW872 cells were maintained in DMEM/F12 with 10% FBS and 1% Penicillin and Streptomycin (Invitrogen, Carlsbad, CA, USA). All coculture experiments were done in 24-well polycarbonate transwell plates with 0.4 μm pore size (Corning, Lowell, MA, USA) as described previously [28].

2.2. Colorimetric Assay. EGE cells were cocultured with SW872 cells as well as cultured in SW872-conditioned media and the cell proliferation measured using CyQuant assay, a method based on DNA quantification, as per manufacturer's instructions (Invitrogen, Grand Island, NY, USA). To prepare the conditioned media, SW872 cells were grown to 80% confluence and the media were collected, centrifuged and filtered to remove cell debris. It was diluted 2-fold, 4-fold, and 10-fold using unconditioned media before adding to the EGE cells.

Briefly, at day 2, day 4, and day 6 of coculture or conditioned media treatment, the culture plates were gently inverted to aspirate the medium from the wells and then washed carefully with PBS. The plates were immediately frozen at −70°C for one hour. The plates were then thawed at room temperature, and 200 μl of CyQuant GR dye/cell lysis buffer was added to each well and mixed gently. The plates were incubated for 5 minutes at room temperature in dark. The sample fluorescence was measured using a fluorescence microplate reader with filters set at 480 nm excitation and 520 nm emission.

2.3. Western Blot. After coculture, the EGE cells were harvested and lysed with a lysis buffer (CelLytic-M, Sigma, St. Louis, MO, USA) containing a protease inhibitor cocktail (Roche Applied Science, Tokyo, Japan). Protein concentration was determined by bicinchoninic acid (BCA) protein assay reagent (Thermo Scientific, Inc., Rockford, IL, USA). The samples were diluted with 4x SDS loading buffer containing β-mercaptoethanol. Equal amounts of protein (10 μg) were separated on SDS-polyacrylamide gel electrophoresis and electrotransferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore Corporation, Bedford, MA, USA). Proteins were detected by immunoblotting followed by ECL chemiluminescence detection (Amersham Biosciences, Piscataway, NJ, USA). Chemiluminescence signals were detected by a luminoimage analyzer SRX-101A (Konica Minolta, Ramsey, NJ, USA). Membranes were immunoblotted with the primary antibody against PCNA (1 : 500), BCL-2 (1 : 500), cyclin D1 (1 : 500), BAK 1 (1 : 500), and CDK-1 (1 : 500) purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Sigma (St. Louis, MO, USA). After washing, membranes were incubated with horseradish-peroxidase- (HRP-) conjugated secondary antibody (1 : 5000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Western blot with anti-β-actin antibody (1 : 5,000) was used as loading control. The intensity of each protein band was determined using a scanning densitometer (Alpha Innotech Imager, Santa Clara, CA, USA) and later normalized against the values obtained from β-actin. Western blot was also done to determine the expression of BCL-2 in EGE cells after culturing in anti-TNFα neutralized conditioned media. For the antibody neutralization experiments, SW872 cells were grown to 80% confluence. The cells were then starved by replacing their medium with FBS-free media and incubation continued for 48 hours. The FBS-free media were then collected, filtered, diluted to 1%, and treated with 1 ng/mL of anti-TNFα antibody purchased from R&D systems (Minneapolis, MN, USA) for 1 hour at 37°C.

2.4. Statistical Analysis. All data are presented as means ± standard error (SE) of all values obtained from three to four replicate wells repeated at least three times. Differences between groups were analyzed using Student's t-test. $P \leq 0.05$ was considered statistically significant.

3. Results

3.1. Enhanced Proliferation of Human Endometrial Glandular Epithelial Cells in Adipocyte-Conditioned Media. To determine the effect of SW872-conditioned media on EGE cells,

SW872 cells were grown to 80% confluence in T200 flasks. The media were collected, diluted to varying concentrations, and added to EGE cells grown to 30% confluence in a 96-well tissue culture plate. Cell proliferation in human endometrial glandular epithelial cells was measured on day 6 using CyQuant cell proliferation kit. Cell proliferation in EGE cells growing with adipocyte-conditioned media at 2-fold, 4-fold, and 10-fold dilutions showed a 14.5%, 22.3%, and 26.65% increase in cell growth, respectively ($P < 0.05$, Figure 1), compared to untreated control.

3.2. Enhanced Proliferation of Human Endometrial Glandular Epithelial Cells When Cocultured with Human Adipocyte Cells. To confirm potential humoral interaction between EGE cells and SW872 cells, cell proliferation was observed in a transwell coculture system (as described in Section 2) without direct cell to cell contact. The control group of EGE cells without SW872 cells coculture was compared with the treatment group of EGE cells and SW872 cells in the transwell coculture system till day 6. An increase in the number of EGE cells was observed with time when cocultured with adipocytes compared to the control. SW872 adipocytes cocultured endometrial cells increased by about 20% on day 6 compared to control cells ($P < 0.05$, Figure 2).

3.3. Adipocyte Coculture Modulates Expression of Protein Markers in Human Endometrial Cells. Western immunoblot assay showed changes in the expression of various protein markers: cell proliferation (PCNA), anti-apoptosis (BCL-2), cell cycle division (Cyclin D1), cell regulation (CDK-1), and apoptosis marker (BAX). PCNA expression in EGE cells cocultured with SW872 cells showed a significant twofold increase ($P < 0.05$) (Figure 3(a)). Similar significant increases in expression of anti-apoptotic protein marker BCL-2, cyclin D1, and CDK-1 were also recorded (Figures 3(b)–3(d)). The expression of BAK, an apoptotic protein, showed significant induction in control EGE cells compared to cocultured EGE cells (Figure 3(e)). Changes in expression of both cell proliferatory and apoptotic proteins in control versus cocultured EGE cells indicate a positive influence of SW872 cells on the EGE cells to induce proliferatory and inhibit apoptosis markers.

3.4. Adipocytes Influence Human Endometrial Cells via Proinflammatory Cytokine TNF-α. Several adipokines have been described in the literature as humoral factors mediating effects of adipocytes (and hence obesity) on various tissues [9, 15, 29, 30] of which TNFα is the most widely studied. We consequently evaluated the role of TNFα in mediating the proliferatory effects of SW872 adipocytes on EGE cells. Human endometrial glandular epithelial cells were treated with varying concentrations of TNFα (0.01, 0.1, and 0.5 μg/mL) for 72 hours and cell proliferation determined using CyQuant assay (Figure 4). We observed that concentrations above 0.1 μg showed a statistically significant increase in cell proliferation compared to control untreated EGE cells ($P < 0.05$). This result suggests that TNFα induces dose-dependent glandular endometrial cell proliferation. On the other hand elimination of TNFα in the SW872-conditioned media using anti-TNFα neutralizing antibody reversed the proliferatory effects in the EGE cells ($P < 0.05$, Figure 5). BCL-2, the antiapoptotic protein, showed reduced expression in cocultured antibody neutralized EGE cells ($P < 0.05$, Figure 6) suggesting the induction of apoptosis.

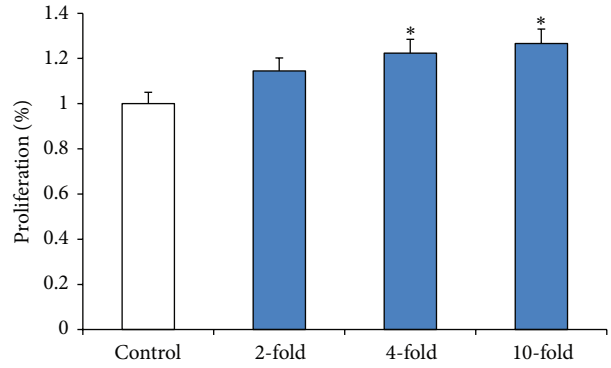

Figure 1: Effect of SW872-conditioned media on proliferation of EGE cells. EGE cells were cultured in 96-well cell culture plate and treated with conditioned media which were diluted from 2- to 10-fold concentrations. Cell proliferation in EGE treated with and without dilutions of SW872-conditioned media was assessed using CyQuant assay as described in Methods. Results are expressed as means ± SE from 3 separate experiments. *Significantly different from the control ($P < 0.05$).

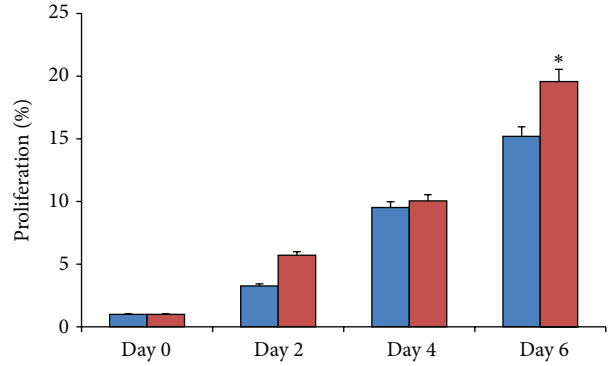

Figure 2: EGE cell proliferation with and without SW872 coculture. EGE cells were cocultured with SW872 cells for days 2, 4, and 6. Comparison in cell proliferation is made with EGE cells grown without SW872 coculture. Proliferation in EGE cells was measured using CyQuant assay. Results are expressed as means ± SE from 3 separate experiments. *Significantly different from the control ($P < 0.05$).

4. Discussion

In this study, we demonstrate that the effects of SW872 cells on EGE cell proliferation are mediated, at least partially, via paracrine effect of TNFα. Obesity, an established risk factor for initiation and progression of endometrial cancer, is characterized by a chronic state of inflammation and dysregulated adipokine levels and an activation of inflammatory signaling pathways resulting in pathogenic outcomes including cancers

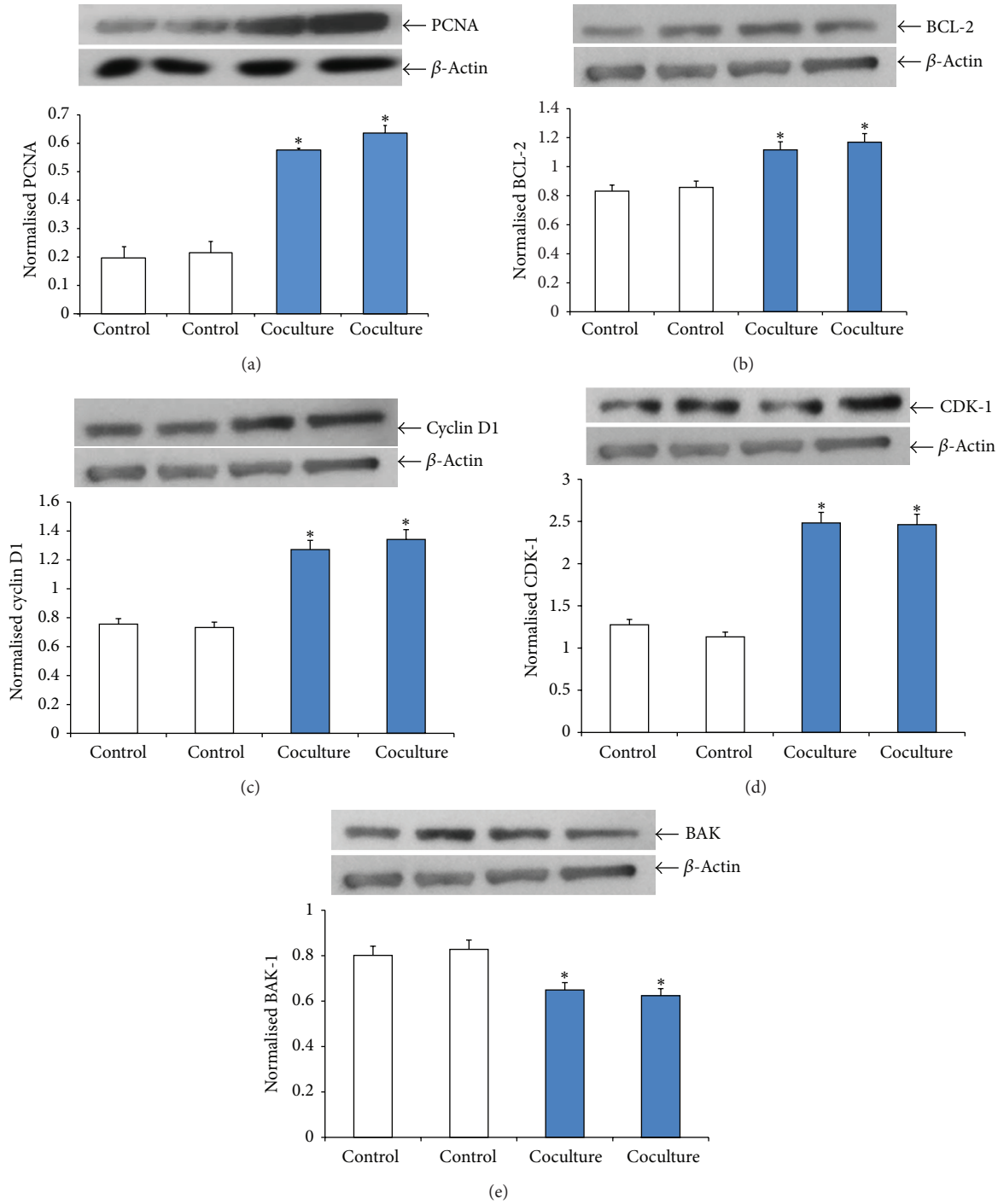

Figure 3: Western blot analysis of PCNA, BCL-2, cyclin D1, CDK-1, and BAK in EGE cells with and without coculture. EGE and SW872 cells were in coculture for 6 days. Lysates prepared from control and cocultured cells were analyzed by Western blotting with (a) anti-PCNA, (b) anti-BCL-2, (c) anticyclin D1, (d) anti-CDK-1, and (e) anti-BAK antibodies. The intensity of each protein signal was quantified and normalized with corresponding β-actin. Results shown represent three separate experiments with comparable results. $^{*}P < 0.05$ compared with control.

[31–34]. We evaluated the growth stimulatory effect of SW872 cells on EGE cells using a coculture system as an *in vitro* model for cell-cell interaction and demonstrated an increased proliferation of EGE cells.

Proinflammatory cytokine tumor necrosis factor alpha (TNFα) is a multifunctional cytokine shown to activate apoptosis, proliferation, differentiation, and survival responses in several cell types [35]. We also demonstrated that TNFα contributes to the EGE cell proliferation in a concentration-dependent manner. EGE cells cocultured with SW872 cells in the transwell system also showed an upregulation of cell proliferatory markers like PCNA, cyclin D-1, CDK-1, and

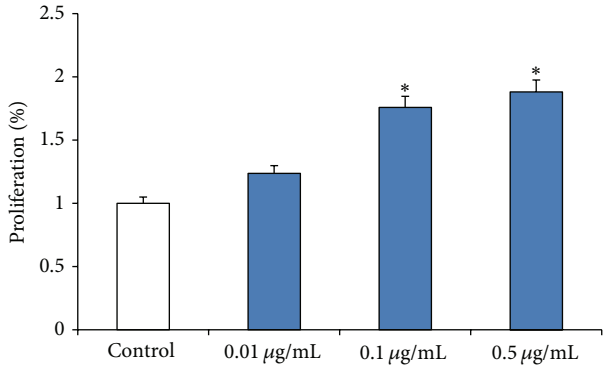

Figure 4: Effect of different concentrations of TNFα on EGE cell proliferation. Cell proliferation in EGE cell treated with different concentrations of TNFα was assessed using CyQuant assay as described in Methods. Results are expressed as means ± SE from 3 separate experiments. *Significantly different from the control ($P < 0.05$).

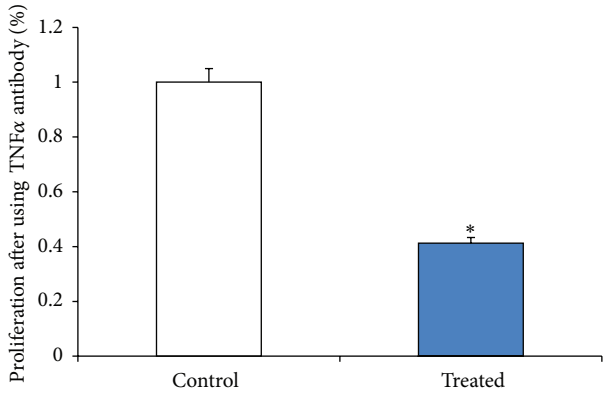

Figure 5: Effects of anti-TNFα neutralizing antibodies on EGE cell proliferation. Conditioned media collected from SW872 cells grown to more than 80 percent confluence were centrifuged, filtered, and diluted to 1%. These conditioned media were treated with 1 ng/mL of anti-TNFα antibody for an hour at 37°C. The neutralized conditioned media were then added to EGE cells and cell proliferation measured using CyQuant assay. Results shown represent three separate experiments with comparable results. *$P < 0.05$ (mean ± SE; $n = 3$).

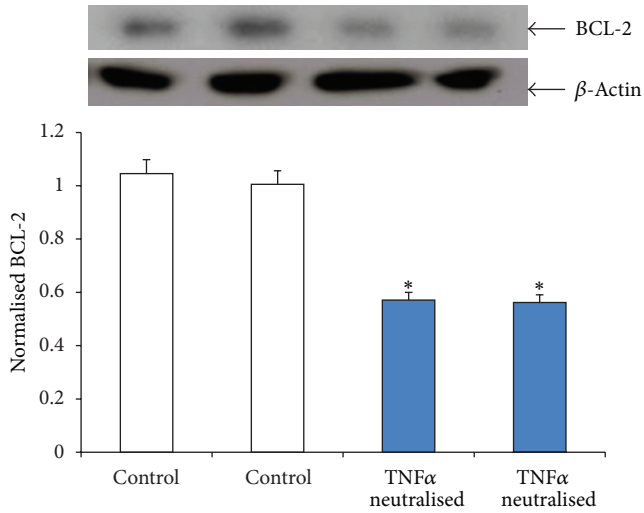

Figure 6: Effects of anti-TNFα neutralized conditioned media on the expression of anti-apoptotic BCL-2 in EGE cells. Conditioned media collected from SW872 cells grown to more than 80 percent confluence were centrifuged, filtered, diluted to 1%, and neutralized for an hour with 1 ng/mL of anti-TNFα antibody for an hour at 37°C. Lysates prepared from control and treated EGE cells were analyzed by Western blotting with anti-BCL-2 antibody. The intensity of each protein signal was quantified and normalized with corresponding β-actin. *$P < 0.05$ compared with control (mean ± SE; $n = 3$).

decrease in apoptosis marker BAK. Since these two chambers are physically separate and can communicate only via a membrane (pore size is 0.4 μm), this finding confirms the progrowth stimulatory role of soluble factors secreted from SW872 cells on EGE cell proliferation.

Endometrial cells have been shown to exhibit both receptors of TNFα and respond to TNFα [24]. In this context we found that neutralization of SW872 cells-conditioned media with anti-TNFα antibody reduced proliferation in EGE cells. The expression of anti-apoptosis gene, BCL-2, was downregulated in the EGE cells after treatment with anti-TNFα antibody, confirming the role of TNFα in an antiapoptotic activation of endometrial cells. Similar anti-apoptotic effect of TNFα via BCL-2 and nuclear factor kappa β (NFκB) pathway have been documented in activated hepatic stellate cells [36]. This indicates that TNFα secreted by the SW872 cells increases endometrial cell proliferation, and therefore it is plausible to speculate that TNFα secreted from adipose tissue may play an active role in endometrial tumorigenesis *in vivo*. Adipose tissue is reported to secrete TNFα both locally and into the circulation [37, 38]. TNFα has multiple functions which regulate complex intracellular signaling pathways like NFκB, Akt, p38 MAPK, and others which are well known to transduce TNFα signals [39–42]. Our findings suggest a major role of TNFα in EGE cell proliferation probably mediated by regulation of downstream proteins involved in inflammatory pathway. Another potential procancerous effect of TNFα is via modulating estrogen effects on endometrium. We have recently reported the effects of TNFα on estrogen homeostasis and metabolism in endometrial cells including hydroxylation of estrogen to the carcinogenic 4-hydroxy catechol-estrogen which acts as DNA adducts that introduce random DNA mutation and increase DNA instability eventually resulting in cancer initiation and progression [43]. Further studies are needed to identify and characterize adipokines with similar effects on endometrial cells and investigate their mechanism of action.

5. Conclusions

Our findings strongly suggest that adipose cells secrete active humoral factors which contribute to endometrial cell proliferation, and a prominent candidate in this panel is the proinflammatory cytokine TNFα. Our results are determined from studies conducted in an *in vitro* coculture system which has several limitations like low cell number, finite adipokine

volume secreted, and presence of other cytokines/cofactors with possible inhibitory/stimulatory effect. Additional work needs to be carried out to delineate the exact role of this cytokine in endometrial tumorigenesis.

Conflict of Interests

The author(s) declared no potential conflict of interests with respect to the research, authorship, and/or publication of this paper.

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Research Article

Feasibility of RNA and DNA Extraction from Fresh Pipelle and Archival Endometrial Tissues for Use in Gene Expression and SNP Arrays

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Identifying molecular markers of endometrial hyperplasia (neoplasia) progression is critical to cancer prevention. To assess RNA and DNA quantity and quality from routinely collected endometrial samples and evaluate the performance of RNA- and DNA-based arrays across endometrial tissue types, we collected fresh frozen (FF) Pipelle, FF curettage, and formalin-fixed paraffin-embedded (FFPE) hysterectomy specimens (benign indications) from eight women. Additionally, neoplastic and uninvolved tissues from 24 FFPE archival hysterectomy specimens with endometrial hyperplasias and carcinomas were assessed. RNA was extracted from 15 of 16 FF and 51 of 51 FFPE samples, with yields >1.2 μg for 13/15 (87%) FF and 50/51 (98%) FFPE samples. Extracted RNA was of high quality; all samples performed successfully on the Illumina whole-genome cDNA-mediated annealing, selection, extension, and ligation (WG-DASL) array and performance did not vary by tissue type. While DNA quantity from FFPE samples was excellent, quality was not sufficient for successful performance on the Affymetrix SNP Array 6.0. In conclusion, FF Pipelle samples, which are minimally invasive, yielded excellent quantity and quality of RNA for gene expression arrays (similar to FF curettage) and should be considered for use in genomic studies. FFPE-derived DNA should be evaluated on new rapidly evolving sequencing platforms.

1. Introduction

Though endometrial carcinoma is the most common gynecologic malignant neoplasm [1], diagnostic capabilities and management of endometrial precancer (intraepithelial neoplasia) lag far behind those of cervical carcinoma [2]. A neoplastic continuum from simple, to complex, to atypical hyperplasia, to endometrial carcinoma is suggested from longitudinal epidemiologic studies [3–5]. Identification of molecular alterations present in various stages of endometrial neoplasia will provide the basis for early detection and therapeutics [6]. Present diagnostic capabilities utilizing histologic evaluation for endometrial hyperplasia/neoplasia alone are limited by poor diagnostic reproducibility [7] and relatively low prognostic value. Risk of progression to carcinoma among women with a diagnosis of endometrial hyperplasia with atypia is not well understood, though exposure to

progestin therapy has been reported to be associated with an approximately 60% decreased risk of progression [4, 8]. Future studies that attempt to elucidate molecular biomarkers of endometrial hyperplasia progression risk will require the development of two methodologies: the ability to perform array studies from extremely small fresh endometrial samples, as well as high-fidelity large-scale affordable array interrogation of archived FFPE samples. Potential challenges exist with both.

The growing field of genomic technologies has made large array-based studies of somatic mutations possible; whole exome sequencing and mutation detection have led to identification of potential driver cancer genes in endometrial cancer [9, 10]. Tissue archives of longitudinal endometrial neoplasia (intraepithelial neoplasm) specimens represent a potentially valuable resource for genomic studies, but they are typically comprised of formalin-fixed paraffin-embedded (FFPE) samples [4, 5]. High-quality nucleic acids necessary for gene expression profiling are most readily obtained from fresh or fresh frozen samples, as the purity and quantity of extracted RNA and DNA from archival tissues can be highly variable [11]. However, the whole-genome cDNA-mediated annealing, selection, extension, and ligation (WG-DASL) Assay (Illumina, San Diego, CA, USA) [12] has been implemented using partially degraded RNA extracted from FFPE tissues for breast carcinoma [13–15] and ovarian carcinoma [16], with varying degrees of success. It is essential to evaluate the quantity and quality of RNA and DNA obtained from endometrial FFPE samples and their performance on high-throughput arrays to determine the feasibility of using these readily available tissue archives to further our understanding of longitudinal progression of endometrial neoplasms and improve diagnostic accuracy and thereby, therapeutic choices.

Women diagnosed with endometrial neoplasia present with abnormal or heavy bleeding patterns and are usually first evaluated with office endometrial biopsies [17, 18], often sampled at multiple time points. Samples are obtained blindly using a disposable suction device placed into the uterus, which results in random sampling that is not oriented to uterine site. The most common device is a Pipelle. The small diameter (3 mm) and flexible plastic suction curette improve tolerability of this office procedure. Volumes of tissue samples obtained are usually 1-2 cubic centimeters (cc) or less than 1 cc in atrophic samples, and can be greater than 3 cc in proliferative samples, for example, endometrial hyperplasia/neoplasia. Moving forward, in the clinical scenario of a woman presenting with abnormal bleeding, we envision holding a small amount of fresh office endometrial biopsy specimen for biomarker analysis while retaining the bulk of the specimen for FFPE clinical diagnostic analysis.

To address whether the quantity and quality of RNA obtained from office endometrial samples and archived FFPE endometrial tissues were suitable for downstream array analyses, we compared the quality and quantity of RNA isolated from three endometrial specimen types from the same individual (a) Pipelle suction (blind, random, small volume, single pass only); (b) curettage (nonblind, oriented fundus to cervix from bivalve hysterectomy specimen); and (c) FFPE surgical hysterectomy specimens, as well as paired endometrial hyperplasias and carcinomas and uninvolved myometrium from archived FFPE specimens. We also examined DNA yield and DNA performance on the Affymetrix SNP 6.0 platform in a subset of samples.

2. Materials and Methods

2.1. Study Participants and Biological Samples. This study was approved by the University of Washington Human Subjects Division.

2.2. Fresh Tissue Collection. To assess whether quantity, quality, and performance of RNA and DNA obtained from different types of FF and FFPE endometrial samples from the same person varied by sample type, we enrolled eight women undergoing total abdominal hysterectomy for benign conditions in July and August of 2009. Indications for hysterectomy (not mutually exclusive) were irregular heavy bleeding ($n = 6$); uterine prolapse ($n = 1$), and fibroids ($n = 4$). For each patient, three types of samples were collected: (1) fresh Pipelle (CooperSurgical Inc.) biopsy; (2) fresh curettage biopsy; and (3) FFPE hysterectomy surgical sections. Immediately following removal of the uterus, a single pass with a Pipelle curette was performed and tissues were placed into tissue culture media (minimum essential media (MEM) with 5% fetal calf serum, 5 mM HEPES buffer, and 10% DMSO) on ice for storage at −30°C. No RNAlater sample was obtained as we had no data to guide whether the endometrial sample obtained with one pass would be of sufficient volume for both diagnostic and research purposes; our primary focus was on the performance of the MEM-preserved specimens. The uterus was then bivalved and the endometrium was sharply curetted in two passes from the fundus to the junction of the uterus and the cervix. One curettage sample was placed into MEM, and the second sample was placed into RNAlater (Qiagen, Valencia, CA, USA), a medium suitable for long-term storage of tissues for both RNA and DNA extractions. Both curettage samples were placed on ice at −30°C until frozen for storage at −70°C. The uterus was then sectioned and processed into FFPE hysterectomy samples for histopathology. Each method of tissue harvesting potentially samples different cell types. Pipelle biopsies sample the endometrial lining, while the curettage biopsies are comprised of predominantly endometrial tissue but may contain myometrial cells. The FFPE hysterectomy specimens contain both endometrial and myometrial cell types.

We were able to obtain sufficient tissue for analysis from 7 of 8 FF Pipelle (MEM), 8 of 8 FF curettage (MEM), 5 of 8 FF curettage (RNAlater) samples, and all 8 hysterectomy samples (FFPE) (Table 1(a)).

2.3. Archival Tissue Collection. To assess quantity and quality of DNA and RNA from older archived clinical samples and performance on the WG-DASL and SNP 6.0 platforms, we selected 24 FFPE hysterectomy specimens: 6 complex hyperplasias; 6 atypical hyperplasias; and 12 carcinomas from the University of Washington Pathology Department. The

Table 1: (a) Benign hysterectomy specimens: RNA and DNA yield for FF pipelle samples preserved in MEM, FF curettage samples preserved either in MEM or in RNAlater, and FFPE hysterectomy samples. (b) Archived FFPE Specimens: RNA and DNA yield and year of collection for normal and involved tissue samples, for complex hyperplasia and atypical hyperplasia. (c) Archived FFPE Specimens: RNA and DNA yield and year of collection for endometrial carcinomas and normal myometrium.

(a)

Study ID	Specimen collection date	Age	Menopause status	Pipelle, MEM			Curettage, MEM			Curettage, RNAlater	Hysterectomy, FFPE	
				DNA yield (μg)	Weight of RNA tissue (mg)	RNA yield (μg)	DNA yield (μg)	Weight of RNA tissue (mg)	RNA yield (μg)	DNA yield (μg)	Weight of RNA tissue (mg)	RNA yield (μg)
1	2009	46	Pre	24	31	62	26.6	26	50.1	35	20	13.4
2	2009	50	Peri	n/a[*]	29	23	8.5	29	32.8	28.1	21	16.8
3	2009	70	Post	101.9	n/a[*]	n/a[*]	23.4	n/a[§]	0[†]	X[‡]	21	7.8
4	2009	45	Pre	20.8	19	25.4	40.3	30	11.8	21	24	18.2
5	2009	51	Pre	37.7	31	20.9	17.5	n/a[§]	0.5[†]	X[‡]	25	2.7
6	2009	52	Peri	69	32	27.9	35.8	18	25.1	X[‡]	21	3.2
7	2009	47	Peri	25.3	27	44	48.7	20	34	49	21	4.4
8	2009	48	Pre	46.3	23	44.6	17.2	26	42.2	137.5	37	26
			Average yield	*46.4*	*27.4*	*35.4*	*27.3*	*24.8*	*32.7*	*54.1*	*23.8*	*11.6*

[*]Not enough tissue remaining for nucleic acid extraction.

[†]Not enough yield to use tissue on downstream applications; <1.2 μg. These are not included in the average yield.

[‡]No tissue available.

[§]Too little tissue to weigh accurately.

(b)

				Complex hyperplasia, $n = 6$			
Study ID	Specimen collection date	Age	Menopause status	Involved		Uninvolved	
				Weight of RNA tissue (mg)	RNA yield (μg)	Weight of RNA tissue (mg)	RNA yield (μg)
9	2000	65	Post	16	12.6	11	24.9
10	2001	53	Post	23	16.1	28	18.3
11	2003	38	Pre	27	20.9	27	0.2[†]
12	2005	38	Pre	36	1.6	32	14
13	2008	55	Post	24	48.5	25	19.1
14	2008	62	Post	25	3.1	17	5.7
			Average yield	*25.2*	*17.1*	*23.3*	*16.4*

(b) Continued.

Study ID	Specimen collection sate	Age	Menopause status	Atypical hyperplasia, $n = 6$			
				Involved		Uninvolved	
				Weight of RNA tissue (mg)	RNA yield (μg)	Weight of RNA tissue (mg)	RNA yield (μg)
15	2001	32	Pre	21	10.8	28	18.6
16	2003	54	Pre	29	8.2	27	18.2
17	2003	69	Post	24	17.8	26	12.1
18	2005	43	Peri	27	9.9	26	9.7
19	2005	54	Pre/Peri	25	8	25	6.9
20	2008	72	Post	29	11.8	26	21.4
			Average yield	*25.8*	*11.1*	*26.3*	*14.5*

(c)

Study ID	Specimen Collection Date	Age	Menopause status	Endometrial Carcinoma, $n = 12$					
				Involved			Uninvolved		
				DNA yield (μg)	Weight of RNA tissue (mg)	RNA yield (μg)	DNA yield (μg)	Weight of RNA tissue (mg)	RNA yield (ng/ul)
21	1999	70	Post	14.7	16	30	40.2	23	43.1
22	1999	84	Post	89.7	32	36.4	18.3	24	6.8
23	1999	80	Post	8.7	26	20.9	36.8	28	13.7
24	2004	77	Post	12.5	25	29.6	51.3	29	6.1
25	2004	70	Post	33.2	30	14.7	57.3	34	11
26	2004	55	Post	83.04	21	27.4	X[‡]	X[‡]	X[‡]
27	2007	81	Post	40.9	23	19.4	X[‡]	X[‡]	X[‡]
28	2007	59	Post	17.38	35	27.1	39.4	28	6.4
29	2007	63	Post	9.6	31	11.1	X[‡]	X[‡]	X[‡]
30	2009	56	Peri	81.5	24	32.1	35.2	24	10.1
31	2009	44	Pre	49.2	32	18.1	X[‡]	X[‡]	X[‡]
32	2009	52	Post	44.6	32	39.7	X[‡]	X[‡]	X[‡]
			Average yield	*40.4*	*27.3*	*25.5*	*39.8*	*27.1*	*13.9*

[*]Not enough tissue remaining for nucleic acid extraction.

[†]Not enough yield to use tissue on downstream applications; <1.2 μg. These are not included in the average yield.

[‡]No tissue available.

[§]Too little tissue to weigh accurately.

samples were collected between 1999 and 2009 and stored for an average of 4.4 years (range 1 to 11 years) before tissue cores were collected for RNA and DNA extraction.

All FFPE surgical histopathology slides were reviewed by a pathologist to identify and mark blocks with representative and adequate areas of the various tissue types for RNA and DNA extraction. For the specimens with hyperplasia, uninvolved endometrium was marked for sampling 7–10 millimeters away from the involved hyperplastic endometrium. For the specimens with carcinoma, uninvolved myometrium was identified for sampling from separate blocks without carcinoma. Samples were obtained from the paraffin blocks by coring the marked area(s) of the identified tissue with a warm 18-gauge needle. Approximately 15 cores were collected per sample and the total core weight was recorded; up to 37 mg of paraffin-embedded tissue (range, 11–37 mg). Cores collected from each tissue type were placed into separate microcentrifuge tubes, deparaffinized, and extracted using the Qiagen Blood and Tissue DNeasy spin columns according to the manufacturer's protocol.

In total, we obtained FFPE tissue for 51 samples, including the benign hysterectomy samples described above ($n = 8$; Table 1(a)), as well as complex hyperplasia ($n = 6$ involved, $n = 6$ uninvolved); atypical hyperplasia ($n = 6$ involved, $n = 6$ uninvolved; Table 1(b)); and endometrial carcinoma ($n = 12$ involved, $n = 7$ uninvolved myometrium; Table 1(c)). We were unable to obtain uninvolved myometrial samples from 5 of the 12 endometrial carcinoma samples because there were no tissue blocks without carcinoma for these individuals.

2.4. RNA Extraction, Expression Array, and RT-PCR Validation of Expression Array. RNA extraction was performed on samples with sufficient tissue. For the FF samples, the specimens were split for RNA and DNA extraction, and for FFPE tissues, the weight of the cores for RNA extraction was recorded (Table 1(a)). Extractions were performed using the RecoverAll Total Nucleic Acid Isolation Kit (cat no. AM1975, Applied Biosystems/Ambion, Austin, TX, USA). RNA was quantitated using the NanoDrop ND-1000 spectrophotometer. Quality was assessed by measuring the Ct threshold using QuantiTect SYBR-Green RT-PCR mix using the protocol with the WG-DASL kit (Illumina, San Diego, CA, USA) on a quantitative real-time PCR (qRT-PCR) system (Applied Biosystems/Life Technologies 7900HT, Carlsbad, CA) in the Fred Hutchinson Cancer Research Center (FHCRC) Genomics Core.

The amount of input RNA recommended by Illumina for the WG-DASL (Illumina, San Diego, CA, USA) is 10–100 ng for RNA derived from FF samples and 50–200 ng for RNA derived from FFPE samples. Therefore, samples were excluded if they yielded less than 40 ng of RNA. Thirteen FF and 49 FFPE samples were assayed. To test reproducibility of the assay, we randomly selected 8 FF samples (4 Pipelle; 4 curettage) and 26 FFPE samples (4 benign; 3 complex, both involved and uninvolved; 3 atypical involved and uninvolved; and 5 carcinoma, both involved and uninvolved) to run in replicate for a total of 21 FF and 75 FFPE samples. The samples were processed and run on the WG-DASL arrays by the FHCRC Genomics Shared Resource according to the manufacturer's protocol. We examined whether the quality and quantity of the RNA varied by tissue type and fixation method, and for the FFPE samples, whether RNA quality varied by year of sample processing. Various performance metrics were examined, including the average number of probes detected (out of the 24,526 transcripts assayed), the average number of genes detected (out of the 18,391 genes assayed), the average signal p95, which is the 95th percentile of the probe intensities on the array, and the signal-to-noise ratio, which compares the strength of the signal to the background signal. Using Genome Studio (Illumina, San Diego, CA, USA), a detection P value was calculated for each transcript, which represents the probability of observing a given transcript if in fact the signal is not above the noise, with the background defined using negative control probes. We examined two levels of confidence: $P < 0.01$ and $P < 0.05$. While a larger number of probes are defined as detected for $P < 0.05$, they might not be as reproducible as the probe set defined by $P < 0.01$. Probe concordance is defined as the percentage of the number of probes with matching detected calls (at $P < 0.01$ or $P < 0.05$) in two replicate samples, over the total number of probes detected in either of the two samples.

To validate the relative expression values obtained by WG-DASL, expression levels of six genes were examined by qRT-PCR in a subset of RNA samples ($n = 44$). Five genes (*PTEN, CD79B, CD82, S100A4,* and *FOLR1*) were selected because they have been reported to be associated with endometrial hyperplasia or endometrial carcinoma (http://www.proteinatlas.org/) [6, 19], and one (*KCNMA1*) was selected because we observed a wide range of expression levels (as assayed using WG-DASL) across the tissues examined. The 44 samples included 13 FF (7 MEM Pipelle and 6 MEM curettage) and 31 FFPE (8 benign hysterectomy, involved tissue from 6 complex hyperplasias, 5 atypical hyperplasias, and 12 endometrial cancers). All 44 samples were run in duplicate, resulting in 88 samples for a total of 528 data points. Averaged ratios of expression obtained by WG-DASL and qRT-PCR were compared for the six genes. We determined the fold difference in expression of a given gene between two sets of tissues by dividing the level of expression in one tissue by the level in the other. We then compared the fold differences between the tissue types for measures obtained by DASL versus those obtained by qRT-PCR. The two different fold changes for a given gene were plotted as X and Y coordinates on a correlation graph and an R^2 value was calculated to determine how well the two sets of measures were correlated across all 6 genes.

2.5. DNA Extraction and Genotyping. For FF samples, DNA extraction was performed on 20 Pipelle and curettage samples stored either in MEM (7 Pipelle and 8 curettage samples; one Pipelle did not have sufficient tissue for extraction) or RNAlater (5 of 8 curettage samples had sufficient tissue; no Pipelle samples were stored in RNAlater as the entire Pipelle samples for research were placed in MEM to maximize quantity). FF samples were minced with scalpel blades and sheared with NST buffer (146 mM NaCl, 10 mM Tris Base (pH 7.5), 1 mM $CaCl_2$, 0.5 mM $MgSO_4$, 0.05% Bovine serum

albumin (BSA), 21 mM $MgCl_2$, and 0.2% Igepal) with a 1 cc syringe as needed prior to extraction using Puregene DNA Isolation Kit (Gentra Systems, Inc., Minneapolis, MN, USA). DNA was quantitated with PicoGreen (Quant-iT dsDNA Assay, Invitrogen, Carlsbad CA, USA).

For FFPE samples, we performed DNA extraction on 12 endometrial cancers with paired uninvolved myometrium ($n = 7$; 5 cancers did not have corresponding blocks of uninvolved tissue). The samples were deparaffinized and extracted using the Blood and Tissue DNeasy spin columns according to the manufacturer's protocol (Qiagen, Valencia, CA, USA). DNA quality was assessed by measuring its size and concentration using a microfluidics-based platform offering qualitative and semiquantitative analysis (Agilent 2100 Bioanalyzer) with the DNA 7500 LabChip assay (Agilent Technologies, Santa Clara, CA, USA) in the Fred Hutchinson Cancer Research Center (FHCRC) Genomics Core.

To evaluate the performance of DNA derived from RNAlater and FFPE, we planned to genotype the DNA samples from the 5 FF curettage samples preserved in RNAlater and the 7 paired FFPE carcinomas and myometrium from uninvolved tissue blocks on the Genome-Wide Human SNP Array 6.0 (Affymetrix, Santa Clara, CA, USA) according to the standard Affymetrix protocol by HudsonAlpha Institute for Biotechnology, Huntsville, AL, USA. According to that protocol, DNA was digested with Nsp I and Sty I restriction enzymes and ligated to adaptors to amplify DNA prior to genotyping. However, the DNA from FFPE samples was fragmented and failed to amplify. Therefore, these samples were not genotyped. DNA from the RNAlater samples was resuspended at 50 ng/μL prior to analysis on arrays.

3. Results

3.1. RNA Quantity and Quality and WG-DASL Array Performance and Reproducibility. We extracted RNA from 15 of 16 FF MEM-preserved biopsy samples (7 Pipelle, 8 curettage) and 51 of 51 FFPE tissues. The extraction was considered successful if the sample yielded ≥1.2 μg of RNA and had a 260/280 ratio of 1.80 to 2.20. Extraction was successful for 13/15 (87%) FF and 50/51 (98%) FFPE samples. All three failures were attributed to low yields <1.2 μg (2 curettage samples and one uninvolved FFPE sample from complex hyperplasia). One postmenopausal individual (patient ID no. 3) was found to have an atrophic endometrium. Not surprisingly, there was insufficient tissue from both Pipelle and curettage (MEM and RNALater) for analysis (Table 1(a)). The yields from the FF tissues were higher than those from the FFPE tissues, but the extracted RNA was very pure for all of the sample types, as evidenced by the 260/280 ratios being close to 2.0, which is the 260/280 ratio for pure RNA (Table 2).

A prequalification qRT-PCR assay was performed to test the quality of the RNA for optimal performance prior to running the WG-DASL array. A Ct threshold above 29 cycles was used as a cutoff to indicate poor quality samples. While the FF samples performed slightly better as evidenced by lower numbers of Ct cycles than did the FFPE samples, all 63 extracted samples passed the prequalification assay (Table 2).

The overall performance of the RNA samples on the WG-DASL array was excellent and was only slightly better for the FF samples than the FFPE samples with respect to the 95th intensity percentile (p95) and signal-to-noise ratio. The average number of probes detected ($P < 0.05$) was similar for both FF and FFPE tissues, with 75% (18421/24526) detected for FF, 73% (17959/24526) detected for FFPE, and 69% of genes detected for both groups (12780/18401 for FF and 12296/18401 for FFPE). The average number of probes detected at $P < 0.05$ and $P < 0.01$, respectively, ranged from 17,197 (70.1%) and 15,640 (63.8%) for benign hysterectomy FFPE tissue to 18,640 (76.0%) and 17,064 (69.6%) for FF Pipelle tissue. The proportion of genes detected was lowest for both benign hysterectomy FFPE tissue and FFPE carcinoma at 64.8%, and highest for Pipelle, at 70.2% (Table 2). The Pipelle and curettage samples were highly comparable with an average expression level correlation $R^2 = 0.939$. The average expression level correlations for FF Pipelle and FF curettage compared to their matched FFPE hysterectomy samples were similar (0.690 and 0.692, resp.).

To assess the reproducibility of the WG-DASL array, we assessed the probe correlation for sample replicates. Expression levels were highly correlated for both the FF and FFPE replicate samples ($R^2 = 0.991$ and $R^2 = 0.985$, resp.). Probe detection concordance rates of the replicates were calculated at P values of <0.05 and <0.01, and all samples had high concordance with an average of 96% gene overlap at both P values (Table 2). The lowest concordance rates were observed in the carcinomas at 94%. There was no difference in the quality of FFPE RNA samples, probe concordance across replicates, RNA quality (260/280 ratio), or quantity (RNA yield), by year of sample collection (data not shown).

3.2. Validation of WG-DASL Array with qRT-PCR. Expression of six genes was assayed using qRT-PCR on the same RNA samples used for the WG-DASL arrays. Correlations between fold differences in expression assayed using WG-DASL and qRT-PCR were evaluated for each gene between tissue types (FF Pipelle and curettage, FFPE atypical and complex hyperplasia, FFPE cancer, and uninvolved myometrium). Agreement between the two methods was good for Pipelle and curettage ($R^2 = 0.90$) and cancer and myometrium ($R^2 = 0.82$) but was poor for atypical and complex hyperplasia samples ($R^2 = 0.02$). To investigate whether the atypical or complex hyperplasia RNA samples had deteriorated over time, we examined the fold change in expression of the panel of genes as determined by WG-DASL and qRT-PCR in the atypical and complex hyperplasia samples compared to FFPE hysterectomy from the same women. The gene expression changes between atypical hyperplasia and FFPE hysterectomy assayed using the WG-DASL and qRT-PCR were well correlated ($R^2 = 0.82$), but this was not the case for complex hyperplasia compared to FFPE hysterectomy ($R^2 = 0.18$). We did not find evidence of suboptimal qRT-PCR due to technical issues (e.g., well position in the PCR block), suggesting the discrepancy was due to sample degradation specific to the complex hyperplasia samples in the time between the WG-DASL and qRT-PCR analyses (36 weeks).

Table 2: RNA quality metrics and assay performance.

	Benign			Complex hyperplasia		Hyperplasia with atypia		Cancer	
	FF Pipelle	FF Curettage	FFPE	Normal	Involved	Normal	Involved	Normal	Involved
RNA Extraction									
Number successfully extracted (over number attempted)*	7/7	6/8	8/8	5/6	6/6	6/6	6/6	7/7	12/12
Mean RNA yield† (μg) (range)	35.4 (20.9–62.0)	32.7 (25.1–50.4)	11.6 (2.7–26)	16.4 (5.7–24.9)	17.1 (1.6–48.5)	14.5 (6.9–21.4)	11.1 (8.0–17.8)	13.9 (6.1–43.1)	25.5 (11.1–39.7)
Mean 260/280 ratio† (range)	2.05 (2.01–2.08)	2.06 (2.05–2.09)	2.00 (1.9–2.0)	2.02 (1.93–2.05)	2.05 (1.91–2.1)	2.02 (1.97–2.05)	1.98 (1.8–2.03)	2.04 (1.93–2.09)	2.00 (1.9–2.1)
DASL Pre-qualification‡									
Mean Ct threshold (range)	15.7 (15.0–16.4)	15.7 (15–18.7)	21.0 (18.0–25.4)	20.9 (19.6–22.5)	19.5 (18.2–20.7)	20.5 (19.3–21.3)	19.9 (20.0–21.7)	21.5 (19–24.7)	21.0 (18.5–27.6)
DASL Expression Array									
Array pass rate§	7/7	6/6	8/8	5/5	5/5	6/6	6/6	7/7	12/12
Average number of probes detected (of 24,526, $P < 0.05$)	18,640 (76.0%)	18,165 (74.1%)	17,197 (70.1%)	18,455 (75.2%)	18,244 (74.4%)	18,239 (74.4%)	18,446 (75.2%)	18,177 (74.1%)	17,648 (72%)
Average number of probes detected (of 24,526, $P < 0.01$)	17,064 (69.6%)	16,637 (67.8%)	15,640 (63.8%)	16,912 (69.0%)	16,846 (68.7%)	16,647 (67.9%)	16,823 (68.6%)	16,442 (67.0%)	15,874 (64.7%)
Average number of genes detected (of 18,401, $P < 0.01$)¶	12,917 (70.2%)	12,630 (68.6%)	11,931 (64.8%)	12,434 (67.6%)	12,580 (68.4%)	12,642 (68.7%)	12,645 (68.7%)	12,348 (67.1%)	11,930 (64.8%)
Average Signal p95¶	7,436	8,495	6,630	6,906	6,944	7,202	6,921	6,703	6,725
Signal-to-noise ratio¶	191	188	184	179	184	176	179	178	192
Replicate Concordance									
Number of replicates run on array	3	4	4	3	3	3	3	5	5
Correlation of expression levels (r^2)	0.99	0.99	0.99	0.99	0.99	0.98	0.99	0.99	0.98
Probe concordance, $P < 0.05$	0.97	0.98	0.96	0.96	0.96	0.95	0.96	0.96	0.94
Probe concordance, $P < 0.01$	0.98	0.98	0.97	0.96	0.97	0.96	0.96	0.96	0.94

*Extractions were defined as successful if the yield was >1.2 μg and the 260/280 ratio was between 1.80 and 2.20, as pure RNA has a 260/280 ratio of 2.0.

†Excluding the three samples that had yields <1.2 μg.

‡A Ct threshold of 29 or below qualifies RNA for the DASL array.

§All replicates also passed.

¶In our high-throughput facility, the average number of genes detected is 10,000, the average p95 signal is 5,000, and the average signal-to-noise ratio is 40.

3.3. DNA Quantity, Quality, and Genotyping Success. DNA extraction was successfully performed on all 20 FF samples and all 19 FFPE samples (12 carcinomas and 7 uninvolved myometrium) with excellent yields from all sample types. The average DNA yield was considerably higher for MEM Pipelle (46.4 μg) than MEM curettage (27.3 μg; Table 1(a)), though the DNA yield from RNAlater curettage was the highest (54.1 μg). The DNA yield from FFPE samples was more than adequate for genomic studies (40.2 μg). Genotyping was planned only for the 5 RNAlater-stored curettage samples and 14 of the FFPE samples (7 carcinomas and their paired uninvolved myometrium samples). However, because DNA fragmentation of the FFPE samples was detected, these samples were not genotyped. Of the five FF RNAlater-stored curettage samples, one failed due to low input DNA. The remaining four samples performed well, with call rates of 98.2% to 99.3% (mean, 98.8%).

4. Discussion

This pilot study assessed the feasibility of using fresh and FFPE uninvolved, hyperplastic, and endometrial carcinoma specimens collected during routine clinical care for high-throughput, array-based, and quantitative methodologies. We demonstrated the ability to extract high-quantity and-quality RNA from both FF and FFPE specimens for all tissue types and showed generally successful performance on the WG-DASL platform. We also demonstrated that DNA derived from fresh samples stored in RNAlater can be used successfully for genotyping on the Affymetrix SNP 6.0 platform. The use of RNAlater has implications for simplifying clinical tissue collection, since samples can remain at room temperature during surgery rather than stored on ice as is necessary for samples placed in MEM. The SNP 6.0 array platform was not successful for DNA isolated from FFPE carcinomas and myometrium, despite there being a substantial volume of DNA. It is possible that our FFPE-derived DNA sample preparation protocol was not optimal; others have had success with the SNP 6.0 platform on DNA derived from FFPE tissue after implementing adjusted preparation protocols to improve hybridization performance and a modified data analysis procedure [20]. The protocol we used to collect DNA from FFPE tissues yielded far more DNA than is necessary for most genomic applications; future studies could take a smaller number of cores for DNA extraction to preserve the tissue for additional assays. The techniques validated in this pilot study have immediate potential to be used in samples from our existing longitudinal, retrospective cohort [4, 8] and to ultimately be translated to future clinical applications with specimens collected during outpatient gynecologic visits for abnormal bleeding in women at risk for endometrial neoplasia.

Of particular interest is the assessment of the quality and quantity of RNA extracted from Pipelle specimens, since this method of endometrial sampling is minimally invasive and can be performed repeatedly over time. We demonstrated that single pass Pipelle tissue specimens (disordered blinded specimens) are equivalent to curettage specimens with regard to DNA and RNA yield and RNA performance on the WG-DASL array. A limitation of our study was a lack of direct comparison of WG-DASL results between FF Pipelle and FFPE Pipelle samples. To our knowledge, RNA and DNA quality and quantity from Pipelle samples have not been evaluated previously. Because we needed to assure sufficient endometrium for clinical diagnostic procedures, and the amount of tissue needed to extract high-quality RNA and DNA was unknown, we conservatively chose to use the remaining sample for FF analysis. Given that the optimal Pipelle endometrial sampling process typically includes multiple (rather than single) uterine passes, and that we observed high RNA and DNA yields from the single pass Pipelle specimens, we propose that tissue collected in each pass could be split to use for diagnostic histopathologic evaluation and for research purposes.

We observed moderate success with RNA expression assayed using the WG-DASL method. While some prior studies have reported concordance between FF and FFPE samples [13], others have described poor correlations at the gene or probe level [15, 16], noting that combinations of genes (particularly those already identified as members of a predictive signature, that is, for ovarian cancer subtypes based on The Cancer Genome Atlas data) performed reasonably well. Given that there are no gene expression signatures to date that identify endometrial hyperplasia subgroups that are likely to respond to progestin, or are likely to progress, it is of critical importance to have complete and reliable gene expression data. Thus, while the WG-DASL approach appears to work reasonably well for validation, it is not as effective for discovery and is therefore not the ideal platform to use to identify such subgroups.

Sequencing of both RNA and DNA derived from FFPE tissues is feasible, but performance of these methods will need to be assessed within specific endometrial sample types [21, 22]. Development of novel analytic methods for quality control of RNA expression data generated from FFPE tissues, such as the quality control pipeline described by Waldron et al. [23], are particularly important as data from multiple study sites over longer time periods are combined. Additionally, as new methods are developed, such as a novel method to simultaneously extract DNA, RNA, and microRNA from a single FFPE sample without splitting it [24], performance of the methods will need to be evaluated for endometrial samples.

5. Conclusions

Our study demonstrates that the Pipelle specimen, which is a common preferred method of clinical endometrial sampling in women with abnormal bleeding, can be used effectively for RNA expression studies and also provides a high yield of DNA. We propose that in the future, while most of the Pipelle would be reserved for FFPE diagnostic assays, a portion of the sample could be retained for fresh tissue analysis. Until endometrial biomarker studies become a routine aspect of care, our study demonstrates that instituting research practices that collect fresh specimens as well as retaining the majority of the specimen for FFPE should not affect current diagnostic capabilities and health outcomes. RNA expression

data from FF Pipelle samples can be used to generate prognostic signatures, which can then be validated in archived FFPE tissues. Interrogation of longitudinal endometrial FFPE tissue banks to better understand alterations that occur in the progression of endometrial neoplasms is certainly feasible.

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Review Article

The Estrogen Receptor Joins Other Cancer Biomarkers as a Predictor of Outcome

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Endometrial cancer, the most common gynecologic malignancy in the United States, is on the rise, and survival is worse today than 40 years ago. In order to improve the outcomes, better biomarkers that direct the choice of therapy are urgently needed. In this review, we explore the estrogen receptor as the most studied biomarker and the best predictor for response for endometrial cancer reported to date.

1. Endometrial Cancer as a Hormonally Regulated Disease

Endometrial cancer is the most common gynecologic malignancy in the United States, with an estimated 47,130 cases and over 8,500 deaths expected in 2013. The disease is on the rise and, unlike cancers arising in most other sites, five-year survival is worse today than in 1975 (87% in 1975–77; 83% in 2003–08) [1]. Biomarkers that can be used to guide treatment selection are urgently needed in order to address this alarming trend of decreasing survival. The purpose of this paper is to review the most consistently studied marker for response to therapy on clinical trials in endometrial cancer, the estrogen receptor (ER), and to highlight new information linking its expression to the outcomes.

Estrogen binds to at least three major classes of receptors, ER-α, ER-β, and GPR30 (Figure 1). ER-α predominates in the endometrium and is the best studied of the three. 17 β-OH-estradiol is the most active ligand and, upon binding to ER-α, causes the transactivation of numerous growth-promoting genes, including growth factors such as epidermal growth factor (EGF) and its receptor (EGFR), insulin-like growth factor-1 (IGF-1), and growth-enhancing protooncogenes such as c-fos and c-myc [2–13]. Most relevant for this discussion are growth factors such as EGF, vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and IGF. These ligands, in turn, activate cognate growth factor receptors, leading to multiple signaling cascades which drive cellular proliferation.

ER-α is induced in estrogen-driven tumors, typically grade 1 and 2 lesions, and is associated with surrounding endometrial hyperplasia. The etiology of such tumors is clearly linked to overexposure to estrogen in the absence of progesterone, the principal differentiating hormone which downregulates ER expression and counters its actions on multiple levels. Such tumors are on the rise in obese postmenopausal women, where adipose tissue produces estrone which is readily converted to estradiol in the endometrium, and are also a concern in younger women who do not ovulate due to PCOS. Such tumors have been classically referred to as type I lesions [14].

Type I endometrial cancer is of endometrioid morphology, is preceded by endometrial hyperplasia, and comprises approximately 80% of sporadic tumors. On a molecular level, type I cancers have been linked to mutations or downregulation of *PTEN*, among other targets, leading to constitutive activation of Akt and mTOR [15–18]. New data from the Cancer Genome Atlas (TCGA) also categorize endometrial tumors into multiplatform subtypes based on mRNA expression, somatic copy number alterations, microsatellite instability (MSI), and somatic nucleotide substitutions [19]. These data confirm that high ER-α expression (ESR1) is a

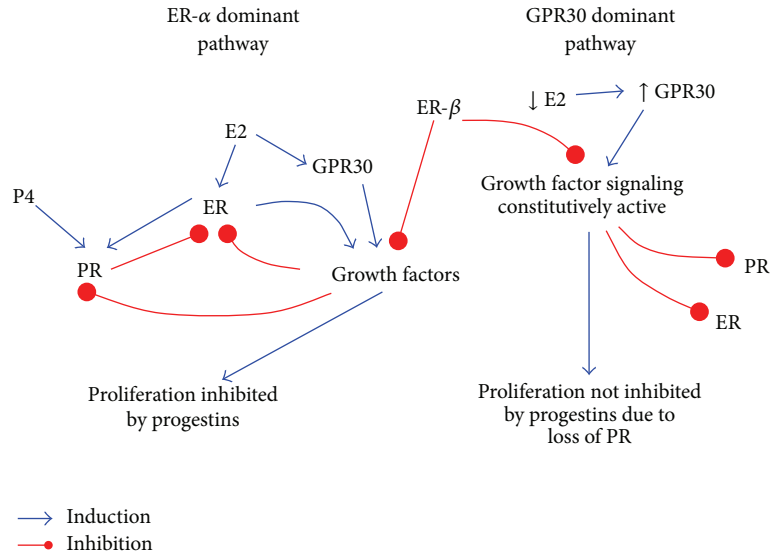

Figure 1: Hormone receptors in endometrial tumors. In ER-α dependent tumors (left side), estrogen induces growth factors and PR through ER-α. This creates a positive feedback loop between ER-α and growth factor signaling. However, progesterone (P4), when bound to PR, downregulates ER and PR. In addition, MAPK activation downstream of growth factor signaling results in phosphorylation of ER and PR and the ligand-dependent loss of PR and ER proteins by ubiquitination-mediated proteasomal degradation. ER-α and PR levels are increased again at the level of transcription by estrogen stimulation. Hence, the growth of these tumors is dependent upon estrogen and is limited by progesterone, suggesting that the patient will respond to progestin hormonal therapy. High expression of ER-β, if present, can inhibit the function of ER-α. For GPR30 dependent tumors (right panel), we hypothesize that proliferation is driven by the constitutive activation of one or more components of a growth factor pathway. Growth does not depend upon the presence of estrogen and is not limited by progesterone. Also, the classical steroid hormone receptors are downregulated as a result of constitutive phosphorylation via MAPK. This is predicted because the phosphorylation of the receptors leads to its targeting the proteasome for degradation. By virtue of the constitutive activation of a growth factor pathway, such tumors grow independently of classical hormonal signaling.

characteristic of lower grade tumors which are also associated with mutations in *PTEN* (the "copy-number low" cluster). Moreover, RNA-seq and reverse phase protein array (RPPA) data demonstrate that ER-α mRNA and protein expression and phosphorylation on Ser118, indicating its activity, strongly correlate with the PTEN-null, MSI hyper-mutated, and copy-number low cluster which is also associated with E-cadherin expression and activation of polycystic kidney disease 1 (PDK1) and Akt [20]. Exome sequencing has also revealed a high prevalence of mutations in *ARIDIA* [20, 21], which likely contributes to PI3K activation in the "copy-number low" cluster.

In comparison, type II tumors comprise a heterogeneous, poorly differentiated group of tumors of high grade endometrioid, serous papillary, or clear cell morphology that primarily occurs in older postmenopausal women. Type II cancers are well known to harbor mutations in *TP53* and demonstrate higher expression of *ErbB2* [18, 22–24]. These tumors are often locally advanced and/or metastatic, and they carry a very poor prognosis [25]. For such lesions, survival is often less than six months despite aggressive chemotherapy and radiation. The TCGA confirms the general categorization of type II lesions to include serous, serous-like, and a subset of endometrioid tumors, mostly of high grade, which make up approximately 25% of all type II tumors when segregated based upon genomic data. Again, the strong correlation of *TP53* mutations, resulting in aberrant protein expression, is noted in this "high copy-number cluster" TCGA subtype. CHK2 phosphorylation on T68 and the high expression of cell cycle regulators, Cyclin E, Cyclin D, and CDK1, are characteristics of these tumors [19]. In addition, genes involved in chromatin remodeling and ubiquitin ligase complexes are frequently mutated in serous tumors [26, 27]. RPPA and RNA-seq data demonstrate that PTEN expression is present, and ER-α expression is generally low [20]. However, it is possible that other estrogen receptors are present in type II tumors, and further analysis of the TCGA and other datasets should shed light on this question, as discussed below.

ER-β expression, though lower than ER-α in most endometrial cancers, may be induced in some tumors, in particular endometrial tumors of a higher grade [28]. Reports suggest that it may inhibit the function of ER-α and/or that it may be a marker for poor outcome [29, 30]. However, these data are complicated by the presence of several ER-β splice variants that are differentially associated with tumor grade [28].

A novel intracellular seven-transmembrane G protein-coupled estrogen receptor (GPR30) appears to function alongside the traditional estrogen receptor to regulate physiological responsiveness to estrogen and is now considered a new estrogen receptor [31]. GPR30 has also been linked to poor clinical outcomes in endometrial cancer patients [32]. GPR30 signals through EGFR to control PI3K and MAPK activity [33, 34]. In turn, these phosphorylate ER and

progesterone receptor (PR), resulting in their degradation in the proteasome. In addition to estrogen, classic ER antagonists such as tamoxifen activate multiple cellular signaling pathways via GPR30 [31, 34, 35]. Partial or biologically weak estrogens can also activate ER-β to a greater degree than ER-α, indicating that these receptors may be functional despite low levels of estradiol. In conclusion, the three different receptors for estrogen appear to segregate between a classic versus an alternative, or GPR30-driven, pathway, as shown in Figure 1.

2. ER-α and PR Markers for Sensitivity to Hormonal Therapy

The expression of ER and PR is linked because transcription of the PR gene is induced by estrogen and inhibited by progestins [36, 37]. Data from the Gynecologic Oncology Group (GOG) Core Laboratory for Receptors have shown that ER-α on a pretreatment biopsy predicts response to hormonal therapy in GOG study 119, tamoxifen and intermittent medroxyprogesterone acetate for advanced endometrial cancer [38]. These data were more recently confirmed in a preliminary analysis of GOG study 248, where hormonal therapy with tamoxifen and intermittent progestin with an mTOR inhibitor was compared to the mTOR inhibitor alone. A general theme from these studies is that ER-α expression correlates with PR expression, but interestingly, the correlation differs somewhat by PR isoform. ER was most strongly associated with PRA expression compared to PRB. The implications of this finding with respect to endometrial carcinogenesis and progression are substantial, given the different functions of the PR isoforms, and should be validated in future clinical trials. Indeed, the importance of identifying PRA compared to PRB has been assessed. Our laboratory published on the expression of the isoforms in well-differentiated compared to poorly differentiated endometrial cancer cell lines and showed that loss of PRB is associated with loss of differentiation [25].

The requirement of PR for endometrial function, secretion, and immunomodulation, as well as limiting the proliferative effects of estrogen, has been well documented [39–43]. The use of PR and ER as markers of response to therapy is generally supported by the literature [38], yet unfortunately in our view, the receptors are not routinely assessed in endometrial cancer specimens. Perhaps the introduction of more effective hormonal regimens, whereby PR expression is enhanced and maintained by epigenetic modulation, will provide new opportunities to treat patients with endometrial cancer [44, 45]. With improved hormonal regimens on the horizon, the assessment of tumors for receptor expression will become even more imperative.

3. Molecular Inhibitors and ER

In addition to hormonal therapy, targeted treatments are also used for advanced endometrial cancer [46–50]. Somewhat surprisingly, ER-α has been the most consistent and robust marker for overall survival (OS) in patients on these trials. The GOG has studied a number of agents in the 229 queue, including gefitinib (229C), lapatinib (229D), bevacizumab (229E), and brivanib (229I). One explanation for the finding that ER-α positively correlates with OS is that such tumors are better differentiated and less aggressive. However, even after controlling for stage and grade, ER-α remains a predictive marker. An alternative explanation is that each of these inhibitors blocks an estrogen-induced growth factor pathway (EGF, Her-2, VEGF, and FGFR). Tumors with high ER-α expression, which have developed in the setting of estrogen excess, are reliant on estrogen-driven pathways for survival and are the most responsive to treatment when such pathways are blocked. This hypothesis should be further evaluated in future studies of molecular inhibitors which impact growth factors downstream of ER-α.

4. Conclusion

Despite the predictability of ER-α, its expression has not been clinically evaluated in the routine care of patients with endometrial cancer. We propose that ER-α should be recognized as a biomarker for positive outcome in endometrial cancer and its presence assessed on patient specimens. Immunohistochemistry (IHC) is the most appropriate methodology to measure ER-α from clinical samples due to feasibility (general lack of access to fresh frozen tissue) and the long track-record of IHC as a reliable measure of ER-α expression. Singh et al. (GOG119) serves as the basis for determining ER-α expression in primary tumor tissue by IHC [38].

ER-α is predictive of positive outcomes in endometrial cancer, both OS in general and to therapy. It is a marker for a hormone responsive tumor, and such cases should be considered for hormonal therapy. In addition, tumors with high ER-α expression are also dependent upon downstream growth factor signaling and may respond better to molecular inhibitors of the EGF, VEGF, and FGF pathways.

While other cancer biomarkers such as PSA and CEA are negative markers which indicate the presence of cancer or its recurrence, ER-α is a positive marker for better clinical outcomes in women with endometrial malignancy. The usefulness of a positive biomarker may not be intuitively obvious. However, we propose that positive biomarkers can be helpful in directing therapy to agents with a higher potential of improving outcomes, that is, hormonal treatment and specific targeted agents, allowing ER-α negative tumors to be treated by other means such as adjuvant chemotherapy. While the impact of the other forms of estrogen receptors (GPR30 and ER-β) on outcomes deserves further study, it is clear that ER-α is a confirmed biomarker. ER-α positive tumors are more likely to be cured with hysterectomy alone. Such cases may require no additional treatment, a hypothesis which should be tested prospectively in future studies. Limiting excessive therapy may provide substantial benefit to patients and is an important goal which may be positively impacted by the use of ER-α as a biomarker.

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgments

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Research Article

The Oncoplacental Gene Placenta-Specific Protein 1 Is Highly Expressed in Endometrial Tumors and Cell Lines

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Placenta-specific protein 1 (PLAC1) is a small secreted protein expressed exclusively in trophoblast cells in the mammalian placenta. PLAC1 is expressed early in gestation and is maintained throughout. It is thought to function in trophoblast invasion of the uterine epithelium and, subsequently, to anchor the placenta to the epithelium. In recent years, evidence has accumulated that PLAC1 is also expressed in a variety of human solid tumors, notably in breast cancers. We demonstrate for the first time that PLAC1 is ubiquitously expressed in tumors originating in uterine epithelium. Further, we find that PLAC1 expression is significantly higher in the more advanced, more aggressive endometrial serous adenocarcinomas and carcinosarcomas relative to endometrioid adenocarcinomas by more than 6-fold and 16-fold, respectively. We also show that PLAC1 is simultaneously transcribed from two promoters but that, in all cases, the more distal P1 promoter dominates the more proximal P2 promoter. While the function of the two PLAC1 promoters and their regulation are as yet unknown, overall expression data suggest that PLAC1 may serve as a biomarker for endometrial cancer as well as a potential prognostic indicator.

1. Introduction

Placenta-specific protein 1 (PLAC1), encoded on human chromosome Xq26, is a small (212 amino acid) secreted protein whose normal expression is almost exclusively limited to placental trophoblast cells [1, 2]. Comparative genomics reveals that PLAC1 evolved after the divergence of placental mammals (Eutheria) from marsupials (Metatheria) as there are homologs throughout the former but no evidence in either of the other mammalian subclasses Metatheria and Prototheria (egg laying mammals) [3]. Among all Eutheria so far studied, PLAC1 shows a well conserved signal peptide (residues 1–23), a very highly conserved transmembrane domain (TMD) (residues 20–50), and a highly conserved region in the extracellular domain homologous to the N-terminal subdomain of the zona pellucida ZP3 glycoprotein (residues 58–118) [4, 5]. Normal expression of the PLAC1 protein is limited to the apical villous surface of syncytiotrophoblasts suggesting that it is involved in anchoring the placenta to the endometrium and maintaining that contact throughout gestation [2]. Moreover, the ZP3-like extracellular domain suggests that strong protein binding interactions are likely [6], and evidence that PLAC1 and F-actin colocalize further supports this view [2].

In addition to the highly specific expression in normal placental development and maintenance, several studies have detected strong PLAC1 expression in a number of human solid tumors prompting the classification of PLAC1 as an oncoplacental protein [7], a class of protein in which PLAC1 remains the sole member. Among the tumors where PLAC1 expression has been detected are nonsmall cell lung cancers [8], breast cancers [5], hepatocellular and colorectal cancers [9, 10], and gastric cancers [11]. In addition, PLAC1 expression has been demonstrated in nearly one hundred cancer cell lines representing fourteen different cancers [5, 8, 9]. However, to date, PLAC 1 expression has not been reported in endometrial cancers. Here, we demonstrate ubiquitous PLAC1 expression in a panel of endometrial tumors as well as in endometrial cancer cell lines. Moreover, we show

Table 1: Characteristics of the patients and cancer cell lines used in this study.

(a) Endometrial cancer patient panel

ID no.	Age	Tumor type	Stage	Grade
BE226	45	Benign endometrium		
BE227	46	Benign endometrium		
BE243	32	Benign endometrium		
BE253	43	Benign endometrium		
EA45	71	Endometrioid adenocarcinoma	2	IB
EA54	84	Endometrioid adenocarcinoma	2	IB
EA68	49	Endometrioid adenocarcinoma	2	IIB
EA69	58	Endometrioid adenocarcinoma	1	IB
EA70	62	Endometrioid adenocarcinoma	1	IB
EA74	74	Endometrioid adenocarcinoma	2	IA
EA81	44	Endometrioid adenocarcinoma	1	IA
EA83	85	Endometrioid adenocarcinoma	1	IA
EA115	55	Endometrioid adenocarcinoma	2	IB
SA48	60	Serous adenocarcinoma	3	IVB
SA72	83	Serous adenocarcinoma	3	IIIC
SA79	87	Serous adenocarcinoma	3	IIIC
SA93	85	Serous adenocarcinoma	3	IIIC
SA169	81	Serous adenocarcinoma	3	II
SA178	71	Serous adenocarcinoma	3	IC
SA208	70	Serous adenocarcinoma	3	IA
SA289	83	Serous adenocarcinoma	3	IIIC2
CS5	63	Carcinosarcoma	3	IIIC
CS21	77	Carcinosarcoma	3	IC
CS32	61	Carcinosarcoma	3	IIIC
CS114	47	Carcinosarcoma	3	IIIC
CS335	78	Carcinosarcoma	3	IA
CS352	54	Carcinosarcoma	3	IA
CS355	60	Carcinosarcoma	3	IIIA

(b) Endometrial cancer cell lines

Cell line	Age	Tumor type	Source	Reference
Ishikawa H	39	Endometrioid adenocarcinoma	Gift	[12]
ECC-1	68	Endometrioid adenocarcinoma*	ATCC	[13]
KLE	64	Endometrioid adenocarcinoma	ATCC	[14]
RL95-2	65	Adenosquamous carcinoma	ATCC	[15]
Hec50co	na	Endometrioid adenocarcinoma#	In-house	[16]
AN3CA	55	Endometrioid adenocarcinoma mets§	ATCC	[17]
SK-UT-1b	75	Leiomyosarcoma	ATCC	[18]

*tumor from luminal epithelium.
#will produce serous tumors in mouse explants.
§associated with a primary diagnosis of acanthosis nigricans.

that PLAC1 expression is significantly greater in the higher stage, more aggressive uterine serous adenocarcinomas and carcinosarcomas.

2. Materials and Methods

2.1. Study Subjects, Tissue Collection, and RNA Preparation. The endometrial tissue panel used in this study is composed of four benign endometrium tissues, nine endometrioid adenocarcinomas, eight serous adenocarcinomas, and seven endometrial carcinosarcomas (Table 1(a)). All tissues were obtained under informed consent, and with IRB approvals, from patients undergoing surgery at the University of Iowa Hospitals and Clinics. Endometrial cancer cell lines used were Ishikawa-H, ECC-1, KLE, RL95-2, KLE, Hec50co, An3CA, and SK-UT-1b (Table 1(b)). All cell lines were grown under optimum conditions, and cells were harvested for RNA preparation at 80% to 90% confluence.

Table 2: Primer sequences used for conventional and quantitative PCR assays.

Amplicon	Size	Sequence	T_m
Exon 6 coding region	812 bp	Forward: 5′-TCCTGTTTCCTGTGGTTCATT-3′	62.0°C
		Reverse: 5′-TCATGAAGTTGCTATAGGTTTCTCT-3′	62.0°C
Exon 5-6*	232 bp	Forward: 5′-CACCAGTGAGCACAAAGCCACATT-3′	60.3°C
		Reverse: 5′-CCATGAACCAGTCTATGGAG-3′	52.3°C
Exon 4-5-6#	361 bp	Forward: 5′-GTGACTCTCCTATGAAGGTAAAGG-3′	54.4°C
		Reverse: 5′-CCATGAACCAGTCTATGGAG-3′	52.3°C
Exons 1-5-6§	283 bp	Forward: 5′-AAACTTACACGAGGAGTCTGTC-3′	57.2°C
		Reverse: 5′-CTGTGACCATGAACCAGTCTAT-3′	54.2°C
18S rRNA	104 bp	Forward: 5′-AACTTTCGATGGTAGTCGCCG-3′	57.3°C
		Reverse: 5′-CCTTGGATGTGGTAGCCGTTT-3′	57.6°C

*qPCR assay for total PLAC1 mRNA.
#qPCR assay for Promoter 2 transcribed PLAC1 mRNA.
§qPCR assay for Promoter 1 transcribed PLAC1 mRNA.

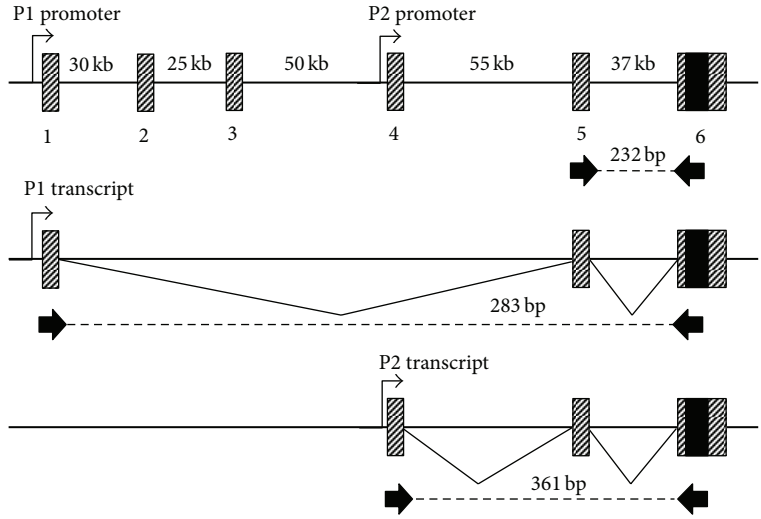

Figure 1: Genomic organization of the human PLAC1 gene on chromosome Xq26. The five 5′ UTR exons and the 5′ UTR and 3′ UTR components of Exon 6 are cross-hatched. The protein coding region in Exon 6 is the solid box. The P1 and P2 promoters are indicated relative to Exons 1 and 4 along with the composition of the P1 and P2 transcripts. Locations of the RT-PCR/qPCR primers in Table 2 are indicated along with their amplicon sizes.

Total cellular RNAs were purified from flash frozen tumor tissue samples and harvested cultured cells using the miRvana RNA isolation kit according to manufacturer's instructions (Ambion, Life Technologies). RNA yield and quality was determined using a NanoDrop M-1000 spectrophotometer and an Agilent 2100 Bioanalyzer. Acceptable RNA quality were assigned to RNAs having RIN ≥ 7.00, and those RNAs were standardized to 100 ng/μL for subsequent expression assays.

2.2. PCR and qPCR Primer Design. PLAC1 genomic organization is presented in Figure 1. The gene is composed of six exons, of which five constitute a series of alternately spliced 5′ UTRs [19]. The 3′ end of the 5′ UTR, the entire coding region, and the 3′ UTR are all contained within the 898 bp long Exon 6. Two 5′ UTR variants dominate PLAC1 expression (Figure 1). One transcript, containing Exons 1-5-6, is transcribed from a promoter termed P1 lying 5′ of Exon 1, while the second transcript, containing Exons 4-5-6, is transcribed from a promoter termed P2 lying in Intron 3 [19, 20]. Using genome sequence information from Ensembl as well as from Chen et al. [19], we designed PCR primers for use in both conventional and quantitative PCR. Primer sequences are shown in Table 2. All PCR primers were purchased from IDT (Integrated DNA Technologies). Primer secondary structure and dimer formation were evaluated using PrimerQuest (IDT). In some instances, PrimerQuest-designed sequences partially overlap previously published sequences [8, 19]. Primer specificity was further evaluated via BLAST.

Prior to qPCR, all primer pairs were validated by conventional PCR and direct sequencing of amplicons.

2.3. Reverse Transcription PCR. Reverse transcription was performed on 250 ng aliquots of total RNA from all cell lines and tissue samples using SuperScript III RT (Invitrogen).

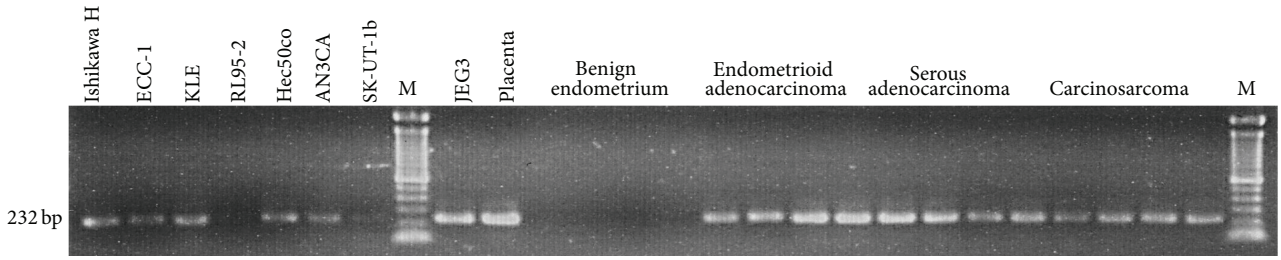

Figure 2: RT-PCR amplification of PLAC1 message in seven endometrial cancer cell lines and representative endometrial tumors ($n = 4$ each). Primers used for this assay are the Exon 5-6 pair (Table 2). JEG3 is a choriocarcinoma cell line known to express PLAC1 [8]. Placental tissue is from a normal 38-week delivery. Molecular weight markers (M) are Invitrogen Trackit 100 bp ladder.

PLAC1 expression was determined via amplification of cDNA with the Exon 5-6 PCR primers (Table 2). PCR conditions were conventional three-step amplification for 35 cycles at an annealing temperature of 60.0°C.

RT-PCR amplicons were run out on a 1.5% agarose gel in 1X TBE. The expected 232 bp amplicon was confirmed with a 100 bp ladder (Invitrogen Trackit).

2.4. Quantitative PCR. Quantitative PLAC1 expression was assessed via SYBR Green qPCR assay. Reverse transcription was performed on 350 ng aliquots of total RNA from endometrial cancer cell lines and both benign and cancerous endometrial tissue samples using SuperScript III RT (Invitrogen). Resulting cDNAs were equally aliquoted into four reactions for qPCR of total PLAC1 message (Exon 5-6 primers), P1 transcribed message (Exon 1-5-6 primers), P2 transcribed message (Exon 4-5-6 primers), and 18S rRNA endogenous control (Table 2). All primer pairs were validated for specificity by conventional gel electrophoresis and dissociation curve.

SYBR Green qPCR amplifications were carried out in triplicate in Power SYBR Green mix (Applied Biosystems, Life Technologies) in a 384-well format on an Applied Biosystems Model 7900 Genetic Analyzer. Cycle thresholds were normalized against 18s rRNA. Fold change was determined via the standard ΔΔCt method [21, 22] and statistical significance assessed by a conventional t-test with unequal variances [23].

3. Results

3.1. PLAC1 Expression in Endometrial Tumors and Cell Lines. The presence of PLAC1 mRNA transcripts in both endometrial cancer cell lines and endometrial tumors is seen in Figure 2. PCR amplification was carried out using the Exon 5-6 primer pair that produces a 232 bp amplicon from cDNA (Table 2). Consistent with the known PLAC1 expression pattern, none of the four benign endometrium samples produced PLAC1 amplicons though all four did produce 18S rRNA amplicons. PLAC1 transcript was detected to varying degrees among the seven endometrial cancer cell lines. RNAs from the choriocarcinoma cell line JEG3, known to express PLAC1 [8], and from a 38-week human placenta were included as positive controls. The three major endometrial cancer tumor types, endometrioid adenocarcinoma, serous adenocarcinoma, and carcinosarcoma, are represented by four samples each. These were selected from the full panel of nine endometrioid adenocarcinomas, eight serous adenocarcinomas, and seven carcinosarcomas, all of which produced both PLAC1 and 18S rRNA amplicons.

3.2. PLAC1 Quantitative PCR. The SYBR Green qPCR assay of all seven endometrial cancer cell lines and all twenty-four endometrial tumors, using the Exon 5-6 primers, was consistent with the conventional PCR results. Among the endometrial cancer cell lines Ishikawa H and Hec50co cells displayed the highest, nearly equal PLAC1 expression with Hec50co cells being 1.26-fold higher relative to Ishikawa H. KLE cells presented PLAC1 expression of −3.26-fold relative to Ishikawa H. Both ECC-1 and AN3CA cells were more than 80-fold lower than Ishikawa H, but neither RL95-2 nor SK-UT-1b cells presented any appreciable PLAC1 expression relative to the others.

Endometrial tumors showed far less volatility in PLAC1 expression than did the cell lines. However, the higher stage, more aggressive serous adenocarcinomas and carcinosarcomas did display significantly higher PLAC1 expression than the lower stage, less aggressive endometrioid tumors (Figure 3). Serous adenocarcinomas display a 6.6-fold higher PLAC1 expression ($P < 0.01$) relative to endometrioid adenocarcinomas, and carcinosarcomas display a 16.5-fold higher PLAC1 expression ($P < 0.07$) relative to endometrioid adenocarcinomas. When tumor stage is considered, Stage 3 tumors display 10.41-fold higher PLAC1 expression than do Stage 1 and 2 tumors ($P < 0.01$). However, this result is essentially redundant as there were no Stage 3 endometrioid tumors in our sample nor were there any Stage 1 or Stage 2 serous adenocarcinomas or carcinosarcomas (see Table 1). The importance of stage versus tumor type cannot be determined until PLAC1 expression is determined in higher stage endometrioid adenocarcinomas as well as lower stage serous adenocarcinomas and carcinosarcomas.

3.3. Promoter-Specific qPCR. P1 and P2 transcript-specific SYBR Green assays showed that P1 transcribed message is significantly more abundant than P2 transcribed message in all twenty-four tumors. In the endometrioid adenocarcinomas P1 transcript abundance was 22.1-fold higher than P2

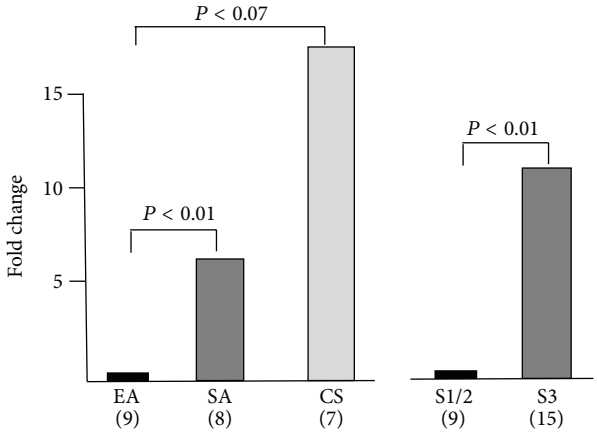

Figure 3: Relative PLAC1 expression among endometrial tumor types (left) and between tumor stages (right). Fold changes, assessed by ΔΔCt, between endometrioid adenocarcinoma (EA) and serous adenocarcinoma (SA) and between endometrioid adenocarcinoma (EA) and carcinosarcoma (CS) are 6.58 and 16.47, respectively. PLAC1 fold change between Stage 1 and 2 tumors (S1/2) and Stage 3 (S3) tumors is 10.41. Statistical significance is assessed by a two-tailed t-test with unequal variances using normalized expression values (ΔCt). Sample sizes are shown.

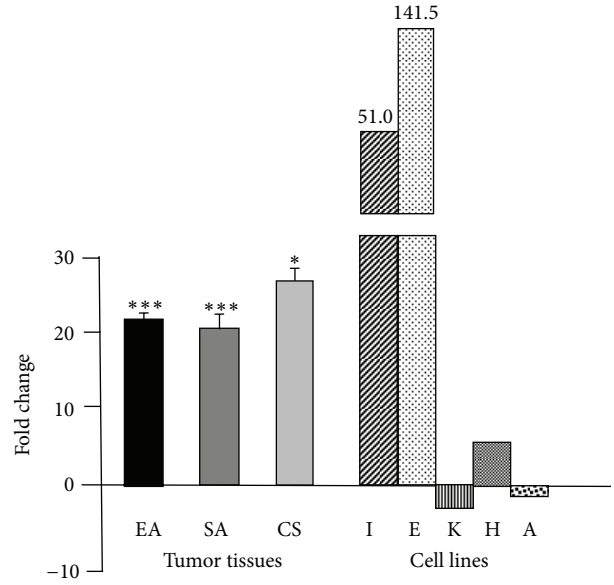

Figure 4: Relative expression of P1 transcripts and P2 transcripts in both endometrial tumors and cultured cell lines. Fold change of P1 transcript versus P2 transcript in each tumor type, assessed by ΔΔCt, was calculated for each individual sample and then averaged. Statistical significance is assessed by a two-tailed t-test with unequal variances using normalized expression values (ΔCt). EA is endometrioid adenocarcinoma, SA is serous adenocarcinoma, and CS is carcinosarcoma. Cell lines are Ishikawa H (I), ECC-1 (E), KLE (K), Hec50co (H), and AN3CA (A). $^{*}P < 0.05$, $^{***}P < 0.001$.

transcript abundance ($P < 0.001$), in the serous adenocarcinomas P1 transcript abundance was 20.3-fold higher than P2 transcript abundance ($P < 0.001$), and in carcinosarcomas P1 transcript abundance was 28.8-fold higher than P2 transcript abundance ($P < 0.05$) (Figure 4). There was a considerable range of P1 transcript abundance from a low of 2.1-fold in one of the carcinosarcomas to a high of 430.2-fold in a serous adenocarcinoma. Though no individual tumor showed an overabundance of P2 transcript, two of the five cell lines, KLE and AN3CA, did (Figure 4). Such variation in relative transcript abundance is consistent with other cultured cell line data [19], but, as this is the first time similar data have been collected from individual tumors of any kind, it is unknown if other tumors display similar variation.

4. Discussion

We have shown both through conventional RT-PCR and SYBR Green qPCR assays that the gene encoding the oncoplacental protein PLAC1 is expressed in endometrial cancer cell lines and in all three of the most common endometrial tumors. These results add to the growing list of human solid tumors in which PLAC1 expression has been demonstrated [5, 8–10]. It is important to note that PLAC1 expression is seen in all twenty-four endometrial tumors irrespective of tumor type. Ubiquitous PLAC1 expression has not previously been reported. The closest any cancer has come is 29 of 32 breast cancers (90.6%) [5], while other tumors such as nonsmall cell lung cancer (5 of 8, 62.5%) [8], hepatocellular cancer (32 of 69, 46.4%) [9], and colorectal cancer (22 of 42, 52.4%) [10] present PLAC1 expression much less often. The reason for such nearly universal PLAC1 expression in breast and uterine tumors may be that these cancers are far more hormone sensitive than are the others so far reported, and there is evidence from breast cancers that one of the PLAC1 promoters, P2, is estrogen responsive. In a paired set of ERα-positive and ERα-negative breast tumors ($n = 20$ each), there was a statistically significant increase in P2 transcript in the ERα-positive group [20]. Detailed examination of the P2 promoter in the ERα-positive MCF-7 breast cancer cell line demonstrated that ERα activates the P2 promoter via a pathway independent of estrogen response elements [20]. The relationship between ERα and PLAC1 promoters in tumors has not been explored. However, it is well established that presence or absence of ERα in endometrial cancers, often in relation to progesterone receptor and Erβ, is related to treatment response and survival [24]. Thus, this dimension must also be investigated.

In general, the reason for the presence or absence of PLAC1 expression at all in a tumor is unknown. Several excellent studies of PLAC1 expression in placentae have shown that the protein is exclusively expressed at the apical surface of trophoblasts [2, 4, 25] and that expression begins very early in gestation and remains throughout [26]. Mature PLAC1 protein localizes in the cell membrane with the entire post-TMD, including the highly conserved, potentially reactive ZP3 domain, in the intracellular milieu between placenta and uterine epithelium. This has led to speculation that PLAC1 likely serves to assist in trophoblast invasion of the endometrium and subsequent anchoring of the placenta. Indirect support for this idea comes from experiments in

MCF-7 and BT-549 breast cancer cells showing that PLAC1 knockdown significantly reduces cell motility, proliferation, and invasiveness [5]. It seems reasonable to assume that it is these very properties, derived from the gene's normal function, which lead cancer cells to coopt the PLAC1 gene.

In addition to ubiquitous PLAC1 expression in the tumor panel, of the seven endometrial cancer cell lines examined here, only the five cell lines derived from endometrioid adenocarcinomas, Ishikawa H, ECC-1, KLE, Hec50co, and AN3CA, evidenced appreciable PLAC1 expression. Of the two cell lines that failed to express PLAC1 mRNA one, RL95-2, is from a Grade 2 adenosquamous tumor [15], and the other, SK-UT-1b, is from a Grade 3 leiomyosarcoma [18]. It is presently unknown whether or not the origin and type of endometrial cancer triggers PLAC1 expression, but it may not be coincidental that the five endometrial cancer cell lines that do express PLAC1 all originated in uterine epithelium as did, by definition, all seventeen adenocarcinomas in the tumor panel. Further, uterine carcinosarcomas have been shown to originate as monoclonal tumors in uterine epithelium that subsequently differentiate into carcinomatous/epithelial and sarcomatous/mesenchymal components [27]. Whether this warrants a conclusion that tumors arising in uterine epithelium either preferentially or exclusively activate PLAC1 expression must await further study of a range of uterine tumors.

Another question that must receive further attention is the mechanism of transcription of PLAC1 message both in tumors and placenta. As shown in Figure 1, PLAC1 genomic structure is composed of six exons wherein the $3'$-most 58 bp of the $5'$ UTR, the entire 639 bp coding region, and the 201 bp $3'$ UTR are entirely contained within the sixth exon. The other five exons form several alternately spliced $5'$ UTRs [19]. Among these, only two, one composed of Exons 1-5-6 and another composed of Exons 4-5-6, are expressed, but they are expressed simultaneously through different promoters. Exon 4-5-6 mRNA, the P2 transcript, is transcribed from a canonical SP1 site and from an unusual isoform of CCAAT/enhancer-binding protein β (C/EBP β-2), both of which are located just upstream from Exon 4 and are estrogen responsive [20]. A second promoter, termed P1, is just upstream from Exon 1 [19]. Moreover, mRNA transcribed from both promoters is present in total PLAC1 message [19]. P2-driven transcription accounts for the majority of PLAC1 message in human placenta, but P1-driven transcription accounts for the majority of PLAC1 message in several cancer cell lines. We see from our own promoter-specific PCR and qPCR amplifications that the same holds true for the endometrial cancer cell lines and tumors. Our own data show that P1 accounts for significantly more PLAC1 message in all of the endometrial tumors, but there is a mixture of P1-driven and P2-driven messages in the endometrial cancer cell lines derived from adenocarcinomas. The importance of this dual promoter transcription is not clear. To date, our data are the only data from primary tumors tissues to assess the relative contribution of the two promoters to total PLAC1 message even in a preliminary way.

5. Conclusion

Placenta specific protein 1 (PLAC1), which normally is exclusively expressed in placental trophoblasts, is an important element in the establishment and maintenance of the placenta. Several studies have shown that the PLAC1 gene is turned on in a variety of human solid tumors and cancer cell lines. We have shown here that PLAC1 expression appears to be ubiquitous in cancers originating in uterine epithelium. We also demonstrate that total PLAC1 message originates in two promoters simultaneously though the specific mechanism and reason for this remain unknown at the present time. Our study suffers from two weaknesses. First, our sample sizes are small. Second, while we succeeded in purifying high quality RNA from the tissues, we did not obtain protein lysates with which to examine PLAC1 expression at that level. We believe, however, that further detailed study of PLAC1 in endometrial cancers is warranted and will ultimately lead to elucidation of the role of PLAC1 in uterine carcinogenesis, the mechanism determining PLAC1 transcription initiation, the role of estrogen in PLAC1 transcription, and development of PLAC1 as a biomarker of endometrial carcinogenesis and prognosis.

Acknowledgments

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Review Article

Contemporary Clinical Management of Endometrial Cancer

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Although the contemporary management of endometrial cancer is straightforward in many ways, novel data has emerged over the past decade that has altered the clinical standards of care while generating new controversies that will require further investigation. Fortunately most cases are diagnosed at early stages, but high-risk histologies and poorly differentiated tumors have high metastatic potential with a significantly worse prognosis. Initial management typically requires surgery, but the role and extent of lymphadenectomy are debated especially with well-differentiated tumors. With the changes in surgical staging, prognosis correlates more closely with stage, and the importance of cytology has been questioned and is under evaluation. The roles of radiation in intermediate-risk patients and chemotherapy in high-risk patients are emerging. The therapeutic index of brachytherapy needs to be considered, and the best sequencing of combined modalities needs to balance efficacy and toxicities. Additionally novel targeted therapies show promise, and further studies are needed to determine the appropriate use of these new agents. Management of endometrial cancer will continue to evolve as clinical trials continue to answer unsolved clinical questions.

1. Epidemiology of Endometrial Cancer

Endometrial cancer is the most common gynecologic malignancy in the United States and the fourth most common cancer in women, comprising 6% of female cancers. Only breast, lung, and colon cancers have higher incidence rates. The American Cancer Society estimated that there were 47,130 new cases of endometrial cancer and 8,010 deaths from endometrial cancer in 2012 [1]. Based on 2004–2008 Surveillance Epidemiology and End Results (SEER) data on endometrial cancer, the age-adjusted incidence rate is 23.9 per 100,000 women per year, and the age-adjusted death rate is 4.2 per 100,000 per year [2]. In the United States, the lifetime risk of developing endometrial cancer is 3%. Excluding women who have had a hysterectomy, 6% of women are diagnosed with endometrial cancer in their lifetime [3, 4]. Rising life expectancy and increasing rates and severity of obesity have contributed to the increasing incidence of endometrial cancer [5]. The National Health and Nutrition Examination Survey (NHANES) in 2009-2010 reported that 36% of adult females in the United States are obese [6].

While the absolute number of estimated new cases of endometrial cancer each year is similar between developed and developing countries, it occurs in a higher percentage of the population in developed countries. The developing world accounts for nearly 80% of the world's population but only about half of endometrial cancer cases [7]. Specifically, the International Agency for Research on Cancer through the GLOBOCAN series estimated 287,000 new cases of endometrial cancer and 74,000 deaths from endometrial cancer worldwide in 2008 [8]. There is a similar absolute distribution between developed and developing countries: GLOBOCAN estimated 142,000 new cases in developed countries and 145,000 new cases in developing countries, with 32,000 deaths in developed countries, in contrast to 41,000 deaths in developing countries [9]. The incidence rates of endometrial cancer are higher in Northern European and industrialized countries than in developing countries [3].

The incidence and 5-year survival rates of endometrial cancer also vary by race. The incidence of endometrial cancer in Caucasian women has remained stable, while the incidence in African American women has increased 2% per year. The death rate from endometrial cancer has remained both

Table 1: Endometrial cancer stage distribution and five-year survival.

Stage	Stage distribution*	Five-year survival
Local disease	68%	96%
Regional disease	20%	67%
Distant disease	8%	16%

*Based on SEER 2001–2007 data. Total not 100% since stage of disease is sometimes unknown.

Table 2: Type I and II endometrial cancers.

	Type I endometrial cancers	Type II endometrial cancers
Hormonal impact	Estrogen dependent	Estrogen independent
Histology	Endometrioid adenocarcinomas	Clear-cell, serous, uterine carcinosarcomas
Patient population	Younger, obese, perimenopausal	Older, thin, postmenopausal
Distribution	85%	15%
Prognosis	Better differentiated	More aggressive, proportionally higher mortality
Genetic mutations	Kras, PTEN, MLH1	p53, erbB2

stable and disparate in both Caucasian and African American women. The relative 5-year survival in Caucasians is 84% in contrast to 60% in African Americans including all stages [2]. Overall, the 1-year survival rate is 92%, and the 5-year survival rate is 82%. Most endometrial cancers are diagnosed at early stage and have over 95% five-year survival rates (Table 1) [2].

Endometrial cancer is a diagnosis of older women, with a median age at diagnosis of 61 years. Over half of endometrial cancers are diagnosed in women who are 50 to 69 years old, and 32% of endometrial cancers are diagnosed between ages 55 and 64 [2].

Most endometrial cancers are adenocarcinomas and separated into type I and type II endometrial cancers based on clinical, pathologic, and molecular characteristics (Table 2) [3]. Grade 3 endometrioid adenocarcinomas have a propensity to behave as aggressively as type II tumors, which leads to controversy about how to classify them [9].

2. Risk Factors for Endometrial Cancer

2.1. Lifestyle and Behavioral Factors. Women exposed to unopposed estrogen are at risk for developing endometrial cancer. Increasing BMI significantly increases the risk for developing endometrial cancer (RR 1.59–2.89) with a higher relative risk for endometrial cancer-related death of 2.53 for obese women (BMI 30–34.9 kg/m^2) and of 6.25 for morbidly obese women (BMI > 40 kg/m^2) [10]. Multiple mechanisms explain the elevated endometrial cancer risk in obese women. Obesity increases the conversion of androstenedione to estrone by aromatase in adipose tissue. Obesity also leads to insulin resistance and decreased serum hormone binding globulin with a resulting increase in unbound biologically active estrogen and an increased inflammatory response [10, 11]. Occupations that are sedentary independently increase the risk of endometrial cancer by 28% [12]. A high-fat diet and diabetes (RR 3) are additional risk factors for endometrial cancer.

2.2. Reproductive and Menstrual History. Risk factors for endometrial cancer related to the reproductive and menstrual cycle include early menarche (before 12 years) (RR 1.5–2), late menopause (after 55 years) (RR 2-3), more lifetime menstrual cycles, nulliparity (RR 3), and infertility [13]. Similarly, pregnancy decreases the time that a woman menstruates, and duration of full term pregnancies creates a 22% per year cancer risk reduction [13]. The impact of duration of menstruation on endometrial cancer risk is likely multifactorial. Incessant menstruation from early menarche to late menopause in combination with nulliparity may lead to repetitive turnover of the cells of the endometrial lining, increasing the probability for sporadic DNA replication errors and consequent mutations in *PTEN* and *p53* [3]. Over 40% of type I endometrial cancers have a loss of *PTEN* and an activation of the PI3K/AKT/mTOR pathway [10, 14].

2.3. Genetic Conditions. Hereditary nonpolyposis colorectal cancer (HNPCC) is an autosomal dominant disorder, diagnosed by the Amsterdam criteria and resulting primarily from mutations in *MLH1* or *MSH2*. The lifetime risk of endometrial cancer is 40–60% in women with HNPCC. Cowden Syndrome, an autosomal dominant disorder characterized by multiple noncancerous hamartomas, is primarily caused by mutations in the *PTEN* gene. Five to 10% of women with Cowden Syndrome develop endometrial cancer [15].

2.4. Cancer and Precancer. Fifteen to 20% of granulosa-theca cell ovarian tumors and 30% of endometrioid ovarian cancers are associated with endometrial cancer. Other risk factors include a 10-fold increased risk with a family history of endometrial cancer at age younger than 50 years, personal history of breast or ovarian cancer, prior pelvic radiation, and endometrial hyperplasia [5, 10]. One percent of women with simple hyperplasia without atypia, 3% of women with complex hyperplasia without atypia, 8% of women with simple atypical hyperplasia, and 30–40% of women with complex atypical hyperplasia develop endometrial cancer.

2.5. Polycystic Ovarian Syndrome. Women with polycystic ovarian syndrome (PCOS) experience chronic anovulation with unopposed estrogen, leading to a 4-fold increased risk of developing endometrial cancer when compared to the general population, with an over two-fold increased risk when adjusted for BMI [10].

2.6. Use of Estrogen-Only Hormone Therapy. Women who take estrogen-only hormone therapy are at increased risk for developing endometrial cancer; progestins counter the effects of estrogen on the endometrial lining. The increased risk of

endometrial cancer using estrogen-only hormone therapy is most pronounced in nonobese women [16].

2.7. Impact of Medications and Environment. The rate of endometrial cancer in women who take tamoxifen is 2-3 per 1000 women per year, and raloxifene is 1.25 per 1000 women per year. Talcum powder use has been shown to be associated with endometrial cancer. This may be due to increased inflammation with lower levels of antiMUC1 antibodies, activation of cytokines and macrophages, increased release of reactive oxygen species, increased cell turnover, and increased risk for DNA damage [10, 17].

3. Protective Factors against the Development of Endometrial Cancer

Oral contraceptives, physical activity, multiparity, and nonhormonal intrauterine device (IUD) use protect against endometrial cancer [10, 13].

3.1. Oral Contraceptives. Oral contraceptives decrease the risk of endometrial cancer by up to 50%. The duration of oral contraceptive use impacts the risk reduction, and that risk reduction is maintained for 10 years following discontinuation of oral contraceptives [10].

3.2. Physical Activity. Physical activity reduces endometrial cancer risk by 33–39%, an effect that is more pronounced in obese women [10, 12, 18]. Although physical activity reduces the risk for endometrial cancer, the Centers for Disease Control report that 49% of the US population does not engage in the recommended level of physical activity [19]. Increased insulin sensitivity, decreased body fat, and decreased circulating estrogen levels are possible explanations for the mechanism of the risk-reducing effects of physical activity on endometrial cancer risk.

3.3. Possible Protective Factors, Associations, and Areas for Future Study. Studies are inconclusive on the impact of hormonal IUDs, bariatric surgery, metformin, breastfeeding, and tubal sterilization on endometrial cancer risk. The levonorgestrel IUD has been shown to reverse complex atypical hyperplasia in multiple studies and may exert a protective effect against developing endometrial cancer, but more studies are needed to determine the impact of hormonal IUDs on endometrial cancer risk [10]. Metformin inhibits aromatase and therefore has the potential to exert a protective effect against endometrial cancer [10]. The impact of breastfeeding and duration of lactation on endometrial cancer risk is debated. Studies show a decreased risk of endometrial cancer with breastfeeding that is directly proportional to the duration of lactation; this risk reduction decreases with time, and lactation has no effect on endometrial cancer risk after age 50 [13, 20]. There is also debate about whether or not bariatric surgery and tubal sterilization decrease endometrial cancer risk [3, 21, 22]. Similarly, tobacco use is associated with a decreased risk of endometrial cancer. This may be because of the antiestrogenic effect of smoking, decreased BMI, or earlier menopause; the effect is most pronounced in current smokers and postmenopausal women [23].

Table 3: 2009 FIGO endometrial cancer staging.

Stage	
I	Tumor confined to the corpus uteri.
IA	No or less than half myometrial invasion.
IB	Invasion equal to or more than half of the myometrium.
II	Tumor invades cervical stroma but does not extend beyond the uterus.
III	Local and/or regional spread of the tumor.
IIIA	Tumor invades the serosa of the corpus uteri and/or adnexae.
IIIB	Vaginal and/or parametrial involvement.
IIIC	Metastases to pelvic and/or para-aortic lymph nodes.
IIIC1	Positive pelvic nodes.
IIIC2	Positive para-aortic lymph nodes with or without positive pelvic lymph nodes.
IV	Tumor invades bladder and/or bowel mucosa, and/or distant metastases.
IVA	Tumor invasion of bladder and/or bowel mucosa.
IVB	Distant metastases, including intra-abdominal metastases and/or inguinal lymph nodes.

3.4. Pathologic Associations with a Decreased Endometrial Cancer Risk. A history of bone fractures is associated with a lower risk of endometrial cancer likely because of the prolonged hypoestrogenic state that frequently leads to fractures. Systemic lupus erythematosus is also associated with a decreased risk for endometrial cancer (OR 0.71), possibly because these women tend to start menopause at a younger age [3, 24].

4. Endometrial Cancer Staging Revisions

In 2009, the International Federation of Gynecologists and Obstetricians (FIGO) revised the staging for endometrial cancer for the first time since the initial surgical staging in 1988. The 2009 FIGO staging for endometrial cancer now has separate staging systems for the 97% of epithelial carcinomas and the 3% of uterine sarcomas (Table 3). The notable changes are the combination of stages IA and IB into IA, encompassing superficial disease and disease with <50% myoinvasion, the elimination of stage IIA or cervical glandular involvement, the removal of peritoneal cytology, and the subdivision of stages IIIC into IIIC1 with positive pelvic nodes and IIIC2 with positive para-aortic nodes. Overall, the revisions in the 2009 staging appear to correlate more precisely with prognosis than the 1988 staging system. Formerly, survival had been better for IIA than IC, and currently all stage I cancers have improved survival over stage II cancers. Stages IIIC1 and IIIC2 also differ in prognosis. Whether or not peritoneal cytology is an independent prognostic factor is debated, but currently cytology has been removed from the staging system yet is still reported. There

is also debate about the optimal extent of staging, specifically with regard to which patients need a lymphadenectomy and how aggressive the lymphadenectomy should be [25–27]. Sentinel lymph node biopsy in early-stage endometrial cancer may avoid the morbidity of a more extensive lymph node dissection while providing prognostic significance that could influence treatment decisions [27, 28].

5. Endometrial Cancer Presentations and Screening

Most women with endometrial cancer present with abnormal uterine bleeding or postmenopausal bleeding and are therefore diagnosed at an early stage [5]. Other common presenting symptoms include pain with urination, dyspareunia, pelvic pain, vaginal discharge, and weight loss [1]. Over 95% of women diagnosed with endometrial cancer present with symptoms. The less than 5% of women diagnosed without symptoms are diagnosed through workup of abnormal Pap smear, abnormal finding on imaging, or as an incidental finding on pathology at time of hysterectomy. Although postmenopausal bleeding is the most common presenting symptom, only 10% of women with postmenopausal bleeding have endometrial cancer. Pipelle endometrial biopsy (EMB) is the preferred method for evaluation of abnormal uterine bleeding because of its high sensitivity, low cost, and low morbidity in comparison to other sampling devices. The false negative rate of Pipelle EMB increases when less than 50% of the endometrial cavity is affected by disease [29]. Postmenopausal women with an endometrial stripe by transvaginal ultrasound (TVUS) less than 4 or 5 mm are at low risk for endometrial cancer [30]. Saline infusion sonohysterography has a higher sensitivity and specificity for detection of endometrial polyps but with potential increased patient discomfort, lack of tissue diagnosis, and higher costs, making it an alternative but not preferred method for evaluation of abnormal uterine bleeding [31]. Women who continue to be symptomatic should have a fractional dilation and curettage (D and C) with or without hysteroscopy [5]. Both saline infusion sonohysterography and hysteroscopy have theoretical risks of dissemination of tumor cells.

Routine screening for endometrial cancer is not recommended in the general population. In women with HNPCC Syndrome, the American Cancer Society recommends annual screening with endometrial biopsy and/or transvaginal ultrasound starting at age 35 [1], and the National Comprehensive Cancer Network (NCCN) recommends that all women with HNPCC undergo yearly EMBs until hysterectomy and bilateral salpingo-oophorectomy after completion of childbearing [5]. Asymptomatic women who are taking tamoxifen should not be routinely screened. Women on tamoxifen should be evaluated if they develop vaginal bleeding with an EMB or D and C [32]. Patients who are undergoing endometrial ablation should have an EMB prior to ablation. No routine screening is recommended in women with Cowden Syndrome.

6. Endometrial Cancer Treatment

Treatment of endometrial cancer is on one level very straightforward and yet on another level evolving and fraught with controversies. The mainstay of treatment for endometrial cancer is surgery including total hysterectomy, peritoneal cytology, and bilateral salpingo-oophorectomy followed by intraoperative staging as indicated. Adjuvant therapy is based upon final stage, patient characteristics, and peritoneal cytology status.

6.1. Role of Lymphadenectomy in Endometrial Cancer. The role of pelvic and para-aortic lymphadenectomy in endometrial cancer is controversial. Experts debate whether lymphadenectomy is simply diagnostic or also therapeutic and whether or not there is benefit to node dissection for all or only a selected group of patients. The Gynecologic Oncology Group surgicopathology study (GOG 33) identified multiple prognostic factors that impact the likelihood of nodal disease and overall survival (OS). Endometrial cancer is now categorized into low, intermediate and high-risk disease based on tumor size, grade, extent of myometrial invasion, cervical stromal involvement, lymphovascular space invasion (LVSI), and increased age [33–36].

Multiple randomized controlled trials have shown no OS benefit from lymphadenectomy in early-stage, low-risk disease. Panici et al. in a randomized trial of over 500 patients with stage I endometrial cancer reported no difference in disease-free survival (80% vs. 82%) or OS (90% vs. 86%) between the lymphadenectomy and no lymphadenectomy groups [37]. Although the lymphadenectomy group in comparison to the no lymphadenectomy group had a higher rate of upstaging, they also had a higher complication rate ($P = 0.001$). Similarly A Study in the Treatment of Endometrial Cancer (ASTEC) trial from the United Kingdom examined 1400 women, with endometrial cancer confined to the uterus on preoperative assessment, and demonstrated no OS benefit from pelvic lymphadenectomy in early-stage endometrial cancer with a hazard ratio for OS of 1.04 and recurrence-free survival of 1.25 in favor of no lymphadenectomy in comparison to lymphadenectomy [38]. Both of these trials were performed in low-risk populations; they were underpowered and have been criticized for their study design. The second randomization to adjuvant radiation in the ASTEC trial has led many to conclude that by attempting to assess the impacts of both lymphadenectomy and radiation on survival, the authors were not able to assess either condition [39]. Additionally critics of the ASTEC trial note the baseline differences in the lymphadenectomy and no lymphadenectomy arms as well as the inadequacy of lymph node dissection as weaknesses of the trial [39]. In contrast to the ASTEC and Italian trial findings, Chan et al. in a Surveillance, Epidemiology, and End Results (SEER) study of 12,333 patients recognized improved 5-year disease-specific survival with lymphadenectomy in stage IB grade 3 and higher patients when patients were matched by stage and those with and without lymphadenectomy were compared ($P < 0.001$) [40].

Table 4: Mayo criteria for omission of lymphadenectomy in surgical management of endometrial cancer.

Omit lymphadenectomy if no disease beyond the uterine corpus AND
(1) Endometrioid grade 1 or 2, myometrial invasion ≤50%, and tumor diameter ≤2 cm OR
(2) Endometrioid and no myometrial invasion independent of grade and tumor diameter

One approach to this controversy of the value of lymphadenectomy has been the development of algorithms for patient selection such as the Mayo Clinic criteria (Table 4) [41]. Although an analysis showed increased cost and morbidity without survival benefit with lymphadenectomy in low-risk patients as defined by the Mayo criteria, these criteria depend on intraoperative frozen pathology, and frozen and final pathology discrepancies vary by institution, limiting the generalizability of these data [42].

Although these data demonstrate a lack of therapeutic benefit in early-stage endometrial cancer, Bristow et al. in retrospective study of 40 patients with stage IIIC endometrial cancer showed a statistically significant disease-specific survival benefit of 37.5 months versus 8.8 months ($P = 0.006$) from debulking macroscopic adenopathy with node-positive, advanced disease [43]. In addition to this therapeutic benefit, lymph node dissection identifies patients who do not need adjuvant therapy or who can receive less aggressive adjuvant therapy. Proponents of routine lymph node dissection debate the extent of lymphadenectomy as well as the criteria for determining if a lymphadenectomy is adequate. Fotopoulou et al. concluded that lymphadenectomy should be extended superior to the inferior mesenteric artery (IMA) to the level of the renal veins after finding that, in intermediate and high-risk node-positive patients, 76% of patients will have positive para-aortic nodes, with LVSI and incomplete tumor resection being the largest predictors of positive nodal status [44]. Mariani et al. reported in a prospective assessment of lymph node metastases that 16% of patients had isolated positive para-aortic lymph node, and, of patients with para-aortic lymph node involvement, 77% had positive nodes above the IMA [41].

Those in opposition to routine lymphadenectomy have concerns about the short- and long-term complications especially from lymphocyst and lymphedema. Rates of lymphedema and lymphocyst range from 1.2 to 3.1% [36, 45–47]. Given the potential morbidity of lymphadenectomy, a prospective study of 115 patients examined the efficacy of sentinel lymph node mapping using isosulfan or methylene blue dye and technetium-99 in endometrial cancer. An overall 85% detection rate was found with sentinel lymph node mapping when followed by confirmatory regional lymph node dissection. Rate of successful mapping improved from 77% to 94% after an individual completed 30 cases [48]. Sentinel lymph node mapping requires further validation prior to routine use.

6.2. Mode of Primary Surgical Treatment and Alternative Primary Management of Endometrial Cancer. Although traditionally surgical management of endometrial cancer was performed via laparotomy, current management of endometrial cancer incorporates minimally invasive approaches when feasible, which offer the least morbid, optimal treatment option for these women who often have significant comorbidities. The GOG LAP-2 randomized over 2600 patients to laparoscopy versus laparotomy. The laparoscopic group had fewer postoperative complications (14% vs. 21%, $P < 0.0001$) and shorter hospital stays over 2 days (52% vs. 94%, $P < 0.0001$) but longer operating times (204 minutes vs. 130 minutes, $P < 0.001$). A secondary survival analysis demonstrated similar recurrence risk (11% vs. 10% at 3 years), which did not meet the protocol-specified definition of noninferiority, and OS (90% at 5 years in both groups) [49, 50].

Similar to LAP-2, other studies have demonstrated benefits to minimally invasive techniques, including robotic-assisted surgical management of endometrial cancer, making it an acceptable alternative to laparoscopy [51]. Importantly, a study of 2,464 women undergoing minimally invasive hysterectomy for endometrial cancer found no difference in morbidity but increased cost with robotic hysterectomy compared to laparoscopic hysterectomy [52].

Total vaginal hysterectomy is also a reasonable approach to management of early endometrial cancer but is limited in exploration of the abdominal cavity, lymph node dissection, peritoneal washings, and further staging as indicated.

With the overall favorable prognosis for early-stage, low-grade, and type I histology endometrial cancer, fertility preservation is a temporizing treatment option for women who understand and accept the risks. In a SEER database study of over 3200 premenopausal women with stage I endometrial cancer, ovarian preservation was not associated with increased cancer-related or overall survival difference [53]. In a prospective, multi-institution study in Japan, women desiring fertility less than 40 years of age with presumed stage IA endometrial cancer or atypical endometrial hyperplasia were treated with primary medroxyprogesterone acetate and low-dose aspirin. Complete response rates were 55% and 82% for endometrial cancer and atypical hyperplasia, respectively, with 47% recurrence rate at 3 years [54].

In presumed stage I-II patients who are medically inoperable, primary radiation therapy offers a feasible alternative to primary surgery with a 16% recurrence rate and a 3.4 times higher likelihood of death from a cause other than cancer [55].

6.3. Adjuvant Treatment for Endometrial Cancer. Optimal adjuvant treatment for endometrial cancer is controversial. Four randomized controlled trials of early-stage endometrial cancer patients, the Norwegian trial, Post Operative Radiation Therapy in Endometrial Carcinoma (PORTEC-1), GOG-99, and ASTEC/EN 5, showed improved locoregional control but no OS benefit with radiation therapy (Table 5) [56–59]. Additionally gastrointestinal (GI) toxicity was higher

Table 5: Randomized trials of adjuvant radiation therapy in early-stage endometrial cancer.

Trial	Norwegian trial	PORTEC-1	GOG-99	ASTEC/EN 5
Authors	Aalders et al.	Creutzberg et al.	Keys et al.	ASTEC/EN 5 Study Group et al.
Endometrial cancer stage	I	I, grade 1, 2, 3	IB, IC, II	I, IIA
Number of patients	540	715	392	905
Adjuvant treatment	Vaginal brachytherapy (VBT) versus VBT and pelvic radiation therapy (RT)	Observation versus RT	Observation versus RT	Observation versus RT
Vaginal and pelvic recurrence	7% versus 2% ($P < 0.01$)	14% versus 4% ($P < 0.001$)	12% versus 3%	6% versus 3%
Overall survival	89% versus 91%	85% versus 81% ($P = 0.31$)	86% versus 92% ($P = 0.557$)	84% versus 84%
Extent of staging	Staging not mandated	Staging not mandated	Staging mandated	50% staged

Table 6: High-intermediate risk endometrial cancer patients.

Age ≥70 with 1 risk factor
Age ≥50 with 2 risk factors
Age ≥18 with 3 risk factors
Risk factors: grade 2/3 tumors, LVSI, outer 1/3 myometrium

in the external beam radiation therapy group, and adjuvant radiotherapy had a negative impact on quality of life [60].

In contrast to the four randomized controlled trials showing no survival benefit with adjuvant radiation therapy in early endometrial cancer, in an analysis of over 21,000 women from the Surveillance, Epidemiology, and End Results (SEER) database, stage IC grades 1 and 3 showed improved overall and relative survival from adjuvant radiation with hazards ratios of 0.44 ($P < 0.001$) and 0.72 ($P = 0.009$), respectively [61]. Despite randomized controlled trials showing no survival benefit with adjuvant radiation in early endometrial cancer, these data elucidate the importance of identifying an early-stage, high-risk population that might benefit from adjuvant therapy. GOG-99 identified this high-intermediate risk group of patients based on clinical and pathologic features (Table 6) [58]. Adjuvant radiation therapy in this high-intermediate risk group decreased recurrence risk. Additionally, the high-intermediate risk subgroup treated with adjuvant radiation therapy appeared to have a survival advantage (RH 0.73, 90% CI 0.43–1.26). Unfortunately, because of the predominance of low-risk patients and the fact that so many patients died of causes other than endometrial cancer in both arms, the study was underpowered both for overall survival and subgroup analysis of high versus low intermediate risk. As a result, adjuvant radiation therapy may be considered in high-intermediate risk but not low-risk patients. NCCN presents guidelines for management of early-stage and advanced local endometrial cancer (Tables 7 and 8) [62]. Note that current investigation, as well as these guidelines, has advocated consideration of chemotherapy for high-risk, local disease.

The long-term morbidity of whole pelvic radiotherapy and the frequency vaginal cuff recurrences prompted study of vaginal brachytherapy in comparison to pelvic radiotherapy. PORTEC-2 demonstrated similar locoregional recurrence and OS but decreased GI toxicity (13% vs. 54%) with adjuvant vaginal brachytherapy in comparison to external beam radiation therapy [63]. Based on these findings and the low morbidity of vaginal brachytherapy, vaginal brachytherapy is frequently used for adjuvant treatment in intermediate-risk patients.

Chemotherapy has emerged as an important component of adjuvant treatment due to the recognition that many patients with high-risk disease will have a component of the recurrence outside the pelvis. Multiple trials compare chemotherapy with radiation with combination treatment (Table 9) [64–68]. GOG 122 demonstrated the value of chemotherapy in stage III-IV patients with improved survival with doxorubicin and cisplatin in comparison to whole abdominal radiotherapy, PFS HR = 0.71($P < 0.01$); OS HR = 0.68 ($P < 0.01$) [69]. Adding paclitaxel to this chemotherapeutic regimen did not improve survival and demonstrated greater toxicity [68]. Carboplatin and paclitaxel have demonstrated efficacy in adjuvant treatment for advanced stage disease with minimal toxicity in retrospective analysis [70]. In stages III and IV patients with extrauterine disease, chemotherapy is the treatment of choice. Although no prospective trials have examined the optimal sequencing of chemotherapy and radiation, a multicenter retrospective cohort of advanced stage endometrial cancer showed overall and progression free-survival benefit with the sandwich technique of chemotherapy followed by radiation followed by chemotherapy [71].

Although the role of adjuvant chemotherapy for advanced stage endometrial cancer is standard, the role of adjuvant chemotherapy in early endometrial cancer is controversial. Randomized trials in Europe examined adjuvant treatment in surgically treated women with stages I, II, and III endometrial cancer who had no residual tumor. The addition of sequential chemotherapy to adjuvant radiotherapy led to a reduced risk of both relapse and death and improved cancer-specific survival ($P = 0.01$) versus adjuvant radiotherapy alone

Table 7: NCCN guidelines for adjuvant treatment of early-stage endometrial cancer.

Stage	Adverse risk factors[a]	Grade		
		1	2	3
IA	Not present	Observe	Observe or brachytherapy	Observe or brachytherapy
	Present	Observe or brachytherapy	Observe or brachytherapy ± WPRT (category 2B)	Observe or brachytherapy ± WPRT
IB	Not present	Observe or brachytherapy	Observe or brachytherapy	Observe or brachytherapy ± WPRT
	Present	Observe or brachytherapy ± WPRT	Observe or brachytherapy ± WPRT	WPRT ± brachytherapy ± chemotherapy (category 2B) or observe (category 2B)

[a]Risk factors: age >60; lymphovascular space invasion (LVSI); Tumor size >2 cm; lower uterine (cervical/glandular) involvement.
WPRT: whole pelvic radiation therapy.

Table 8: Adjuvant treatment of advanced local endometrial cancer.

Stage	Grade		
	1	2	3
II	Brachytherapy ± WPRT	WPRT + brachytherapy	WPRT ± brachytherapy ± chemotherapy (category 2B)
IIIA	Chemotherapy ± WPRT or tumor-directed RT ± chemotherapy or WPRT ± brachytherapy	Chemotherapy ± WPRT or tumor-directed RT ± chemotherapy or WPRT ± brachytherapy	Chemotherapy ± WPRT or tumor-directed RT ± chemotherapy or WPRT ± brachytherapy

WPRT: whole pelvic radiation therapy; RT: radiation therapy.

Table 9: Adjuvant treatment in endometrial cancer trials: radiation versus chemotherapy versus combination.

Study	*N*	Stage	Drug regimen	5-year PFS (%)	5-year OS (%)
Randall GOG 122	386	III/IV	AP versus WAI	50 38	55 42
Maggi	340	I-III	CAP versus PRT	63 63	66 60
Susumu JGOG	385	I-III	CAP versus PRT	82 84	85 87
Hogberg	372	I-III	Various versus PRT	79 72	88 78
Homesely GOG 184	552	III/IV	PRT + AP PRT + TAP	62 (3 year) 63 (3 year)	NS

AP: doxorubicin-cisplatin;
WAI: Whole-abdominal irradiation;
CAP: cyclophosphamide-doxorubicin-cisplatin;
PRT: Pelvic radiation therapy;
TAP: paclitaxel-doxorubicin-cisplatin.

Table 10: GOG trials of hormone therapy in endometrial cancer.

GOG study and dosing	RR (%)	PFS (months)	OS (months)	DOR (months)
153 MA 80 mg BID × 3 weeks alternating with T 20 mg BID × 3 weeks	27%	2.7 months	14.0 months	28 months
121 high dose MA 800 mg daily	24%	2.5 months	7.6 months	8.9 months
81 MA high dose 1000 mg daily versus low dose 200 mg daily	15% high dose 25% low dose	2.5 months 3.2 months	7.0 months 11.1 months	NR
119 T 200 mg BID + MPA 100 mg BID intermittently weekly	33%	3.0 months	12.8 months	NR
81F T 20 mg BID	10%	1.9 months	8.8 months	NR

MA: megastrol acetate; T: tamoxifen; MPA: medroxyprogesterone acetate; RR: response rate; PFS: progression free-survival (median); OS: overall survival (median); DOR: duration of response (median); NR: not reported.

Table 11: Selected treatment controversies in endometrial cancer.

Question/controversy	Comment
Role of lymphadenectomy in low risk	ASTEC trial did not support but results not universally accepted
Prognostic value of peritoneal cytology	Removed as a staging variable recently but still to be collected and reported
Role of radiation in intermediate risk	Local control improved but no overall survival benefit
Best adjuvant for high-risk disease	Recent data supports carboplatin/paclitaxel combination
Should targeted therapies be utilized	Further clinical and basic science research required; mTOR inhibtors promising

(HR = 0.74) [67]. Given these encouraging data on the role of adjuvant chemotherapy in high-risk endometrial cancer, two trials, examining the role of chemotherapy in high-risk, uterine-confined endometrial cancer, are currently ongoing. The PORTEC-3 trial is comparing chemoradiation with radiotherapy alone in high-risk stages I, II, and III endometrial cancer, and GOG-249 is comparing whole pelvic radiation with vaginal brachytherapy and carboplatin and paclitaxel in high-intermediate risk endometrial cancer.

For isolated vaginal cuff recurrence, radiation is the treatment of choice [72]. Chemotherapy or progestational agents are treatment options for recurrent endometrial cancer that is not localized. Surgery is an option for patients who have already had radiation treatment. Hormonal therapy with progestins or tamoxifen has also demonstrated efficacy in recurrence. Multiple GOG trials have examined hormone therapy in endometrial cancer (Table 10) [73]. Additionally many novel biologic therapies are under investigation; temsirolimus, an mTOR inhibitor, has activity in advanced stage and recurrent endometrial cancer [74].

7. Conclusions

In conclusion, although endometrial cancer management is in many ways straightforward, many controversies still require further debate and investigation (Table 11). Given that patients frequently present with symptoms and providers are able to assess disease with EMB and D and C, most patients are diagnosed with early-stage disease and have a favorable prognosis. As a result, many general practitioners perform hysterectomies for complex atypical hyperplasia despite its 25–40% rate of concurrent endometrial cancer and generalists' lack of training in lymph node dissection. One of the primary benefits of lymphadenectomy is identification of patients who can either avoid or receive less aggressive adjuvant therapy. The role and extent of lymphadenectomy especially in low-risk patients are debated. The Mayo criteria are one approach to providing guidance for determination of low-risk patients in whom to omit lymphadenectomy, although these criteria rely on intraoperative frozen pathology, which may be discrepant from final pathology. The change in surgical staging correlates more closely with prognosis and removes peritoneal cytology from the staging system. The importance of cytology is unknown, and currently data are being prospectively collected for evaluation. Adjuvant treatment of endometrial cancer is changing with debates about the roles of radiation in intermediate-risk patients and chemotherapy in high-risk patients. Additionally targeted therapies such as mTOR inhibitors show promise in endometrial cancer. Management of endometrial cancer will continue to evolve as studies begin to answer these controversial questions.

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There are no financial disclosures for any of the authors.

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Research Article

Protein Kinase Cα Modulates Estrogen-Receptor-Dependent Transcription and Proliferation in Endometrial Cancer Cells

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Endometrial cancer is the most common invasive gynecologic malignancy in developed countries. The most prevalent endometrioid tumors are linked to excessive estrogen exposure and hyperplasia. However, molecular mechanisms and signaling pathways underlying their etiology and pathophysiology remain poorly understood. We have shown that protein kinase Cα (PKCα) is aberrantly expressed in endometrioid tumors and is an important mediator of endometrial cancer cell survival, proliferation, and invasion. In this study, we demonstrate that expression of active, myristoylated PKCα conferred ligand-independent activation of estrogen-receptor- (ER-) dependent promoters and enhanced responses to estrogen. Conversely, knockdown of PKCα reduced ER-dependent gene expression and inhibited estrogen-induced proliferation of endometrial cancer cells. The ability of PKCα to potentiate estrogen activation of ER-dependent transcription was attenuated by inhibitors of phosphoinositide 3-kinase (PI3K) and Akt. Evidence suggests that PKCα and estrogen signal transduction pathways functionally interact, to modulate ER-dependent growth and transcription. Thus, PKCα signaling, via PI3K/Akt, may be a critical element of the hyperestrogenic environment and activation of ER that is thought to underlie the development of estrogen-dependent endometrial hyperplasia and malignancy. PKCα-dependent pathways may provide much needed prognostic markers of aggressive disease and novel therapeutic targets in ER positive tumors.

1. Introduction

Endometrial cancer is the most common invasive gynecological malignancy in the United States, accounting for 45,000 new cancer cases and over 7,500 deaths annually [1]. However, molecular mechanisms underlying its etiology and pathophysiology are poorly understood. Endometrial carcinomas are derived from glandular epithelium and typically divided into two subtypes based on clinical, histological, and molecular characteristics [2, 3]. Type I tumors, comprising 80% of cases, are generally well or moderately differentiated with endometrioid morphology and are associated with chronic unopposed estrogen exposure and hyperplasia. By contrast, type II tumors are more heterogeneous, poorly differentiated and may be estrogen independent, arising in a background of atrophic endometrium [2, 4]. The prevalence of advanced stage, high-grade tumors, of both types, with recurrent metastatic disease is increasing [5, 6]. Such cancers typically have a poorer prognosis and are refractory to current therapeutic regimens [7].

Endometrioid tumors retain expression of estrogen (ER) and progesterone (PR) receptors [8], and estrogen is a critical regulator of endometrial proliferation [9, 10]. Indeed, the majority of endometrial cancers are thought to arise due to unopposed estrogen action leading to hyperplasia and malignant transformation [2, 11]. However, our understanding of the molecular mechanisms underlying the pathophysiology of endometrial cancer lags far behind that of other hormone-dependent malignancies such as breast, prostate and ovarian cancer [2, 8, 12, 13].

The protein kinase C (PKC) family has been implicated in the regulation of numerous signal transduction pathways, modulating cell growth, differentiation, and survival [14–16]. In endometrial cancer cells and primary endometrial epithelium, expression of PKCα is increased in response to treatment with estrogen and tamoxifen and may underlie the proliferative actions of these agents in the endometrium [17, 18]. We have previously shown that PKCα is aberrantly expressed in human endometrial tumors [19, 20] and is a critical regulator of endometrial cancer cell survival, proliferation, transformation, invasion, and response to chemotherapy [21, 22]. In addition, we demonstrated that knockdown of PKCα inhibits growth of estrogen-dependent endometrial cancers in an *in vivo* model [20].

In this study, we present evidence that, in type I endometrial cancer cells, PKCα induces hormone-independent activation of ER, potentiates estrogen transcriptional responses, and regulates estrogen-dependent proliferation and gene expression. Thus, PKCα signaling may be a critical element of the supraphysiologic activation of ER thought to underlie the development of endometrial hyperplasia and malignancy.

2. Materials and Methods

2.1. Cell Lines. Ishikawa and HEC-50 endometrial carcinoma cells were a generous gift from Dr. Leslie (University of Iowa). Ishikawa cells expressing luciferase (luc) or PKCα shRNAs have been described [21]. Unless stated otherwise, all cell lines were maintained in 5% CO_2, phenol red free DMEM, supplemented with charcoal stripped 10% fetal bovine serum, 10 units/mL penicillin, 10 μg/mL streptomycin, and 200 μM L-glutamine. Prior to estrogen treatment (100 nM Estradiol, Sigma Aldrich, St. Louis, MO, USA), cells were transferred to phenol red free DMEM containing 1x SR-1 serum replacement (Sigma Aldrich, St. Louis, MO, USA). Cell lines used were authenticated by analysis of DNA microsatellite short tandem repeats (STRS), as described previously [23].

2.2. Cell Proliferation. Cell number and viability were determined from subconfluent cultures using a Vi-Cell Coulter Counter (Beckman-Coulter, Inc., Fullerton, CA, USA) as described in [20].

2.3. Luciferase Reporter Assays. The ERE-luc and pS2-luc promoter reporter constructs have been described in [24–26]. Myristoylated PKCα vector [27] was obtained from Addgene (Cambridge, MA). Cells (2.0×10^5) were transiently transfected with 0.5 μg ERE-Luc or pS2-luc reporter plasmids using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) as per the manufacturers protocol. 0.5 μg pCMVβ, encoding β-galactosidase under control of the CMV constitutive promoter, was included as a control for transfection efficiency and cell number. Total DNA was kept constant by addition of empty vectors. Promoter activity was determined by Luciferase and β-galactosidase assays, as described in [28].

2.4. RNA Isolation and Quantitative RTPCR. RNA was isolated from 10^6 cells using a Qiagen RNeasy kit (Qiagen, Germantown, MD, USA) according to the manufacturer's directions and quantitated using a NanoDrop ND1000 spectrophotometer. Aliquots were evaluated by chromatography using an Agilent RNA 6000 Nano LabChip kit (Agilent Technologies, Santa Clara, CA, USA) on an Agilent Bioanalyzer 2100 system. cDNAs were prepared using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) as per the manufacturers instructions. The samples were amplified by real-time PCR using iQ SYBR green supermix (Bio-Rad, Hercules, CA, USA) on a Bio-Rad CFX96 C1000 Thermal Cycler using the following conditions: 10 minutes at 95°C and 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Negative control RNA samples were not reverse transcribed or did not lack PCR template. Results were analyzed with qbase^{PLUS} software (Bio-Rad Hercules, CA, USA), and changes in expression, relative to β-actin and rpl13a controls, were estimated using the ΔCT method [29]. Primer pair sequences (forward and reverse, 5′ to 3′) were as follows: β-Actin: AGCCTCGCCTTTGCCGA and GCGCGGCGATATCATCATC; RPL13A: TACCAGAAAGTTTGCTTACGTGGG and TGCCTGTTTCCGTAACCTCAAG; PRKCA: GCTTCCAGTGCCAAGTTTGC and GCACCCGGACAAGAAAAAGTAA; LTF: ATGGTGGTTTCATATACGAGGCA and GCCACGGCATAATAGTGAGTT; c-FOS: AAAAGGAGAATCCGAAGGGAAA and GTCTGTCTCCGCTTGGAGTGTAT; pS2 (TFF1): AGGCCCAGACAGAGACGTGTAC and CGTCGAAACAGCAGCCCTTA. Primers were designed using Primer3 software (http://primer3.wi.mit.edu) and obtained from Eurofins MWG Operon (Huntsville, AL, USA) or Integrated DNA Technologies (Coralville, IA, USA).

2.5. Statistical Analysis. Data were expressed as mean ± standard deviation or standard error of the mean and analyzed using Student's t-test. P values <0.05 were considered significantly different.

3. Results

To investigate the functional role of PKCα signal transduction in the regulation of ER-dependent transcription, Ishikawa endometrial cancer cells were transiently transfected with a myristoylated PKCα construct (myrPKCα) that is targeted to membranes and thereby rendered constitutively active [21, 27]. As shown in Figure 1, expression of myrPKCα, in the absence of estrogen, resulted in a dose-dependent activation of transcription from a promoter containing 3 copies of a canonical estrogen response element (ERE) fused to luciferase [30]. Treatment of Ishikawa cells with estradiol (E2) increased the activity of the ERE promoter approximately 30-fold (Figure 2(a)). In the presence of activated myrPKCα, E2-stimulated ERE promoter activity was further increased over 170-fold. Thus, PKCα induced hormone-independent activity of an ERE and potentiated the effect of estrogen. Similar results were obtained using the pS2 (TFF1) promoter, an endogenous E2 regulated gene [31] (Figure 2(b)). myrPKCα expression induced a marked increase in basal pS2 promoter

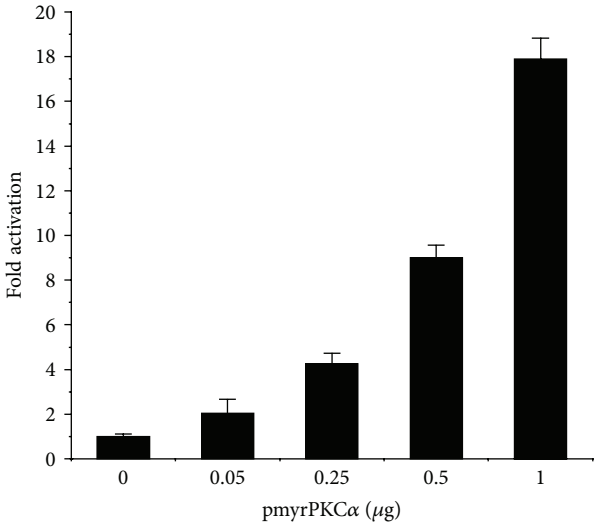

FIGURE 1: PKCα activates an estrogen responsive promoter. Ishikawa cells were transiently transfected with 0.5 μg pEREluc, 0.3 μg pCMVβ, and the indicated amounts of pmyrPKCα or vector control (pCDNA3). Luciferase activity was normalized to β-galactosidase and promoter activity expressed as fold increase over control. Data are mean ± s.d. (n = 3).

activity and enhanced the stimulatory effect of E2. Treatment with E2 had no effect on the level of myrPKCα expression in Ishikawa cells (not shown).

In HEC-50 endometrial cancer cells, which lack estrogen receptor (ER) [32], activity of the ERE and pS2 promoters was minimal (Figure 3). Expression of active PKCα or treatment with E2 (in the presence or absence of myrPKCα) had no effect on pS2 or ERE promoter activity, indicating that the effects of PKCα and E2 are dependent on ER expression (Figure 3). Accordingly, transfection of HEC-50 cells with pHEGO encoding ERα reconstituted ERE and pS2 transcriptional responses to both E2 and myrPKCα (Figure 4). Expression of ERα in HEC-50 cells also restored the enhancement of E2-stimulated promoter activity by PKCα. (Figure 4). Together, these results (Figures 1–4) indicate that PKCα signaling induces ligand-independent activation of ER-dependent transcription and thereby potentiates responses to E2.

Activation of the phosphoinositide 3-kinase (PI3K)/Akt pathway is one of the most critical steps in endometrial carcinogenesis [11] and has been shown to mediate ligand-independent activation of ER [33, 34]. Moreover, we have previously implicated PKCα in the regulation of Akt in endometrial cancer cells [22]. To investigate the role of PI3K/Akt signaling in PKCα regulation of transcription, we treated Ishikawa cells with pharmacological inhibitors of PI3K (LY 29004) or Akt (Akt-I-1/2) [35, 36] and examined their effects on the ERE promoter (Figure 5). Treatment of Ishikawa cells with LY29004 or Akt-I-1/2 significantly inhibited the ability of myrPKCα to enhance E2 activation of the ERE promoter (Figure 5(a)). Similar results were obtained in HEC-50 cells transfected with ERα (Figure 5(b)). LY29004 and Akt-I-1/2 treatment resulted in the expected decrease in phosphorylation of Akt and GSK3, respectively, and did not impact expression of myrPKCα (not shown). Thus, the effects of PKCα on E2- and ER-dependent transcription are mediated, in part, by the PI3K/Akt pathway.

To confirm the results, using the ERE and pS2 promoter constructs, we examined expression of a panel of estrogen-dependent genes implicated in endometrial neoplastic transformation [33, 34]. Levels of pS2 (TFF1), lactotransferrin (Ltf), and c-fos mRNA were determined by real-time reverse transcription PCR, in Ishikawa cells stably expressing shRNA to knockdown PKCα. Control cells were transduced with shRNA targeting luciferase [20]. As shown in Figure 6, knockdown of PKCα in Ishikawa cells significantly reduced expression of the estrogen-dependent genes pS2, ltf, and c-fos. PKCα shRNA expressing cells also exhibited the expected decrease in PKCα mRNA levels (Figure 6).

Estrogen is a critical regulator of type I endometrial cancer growth and stimulates proliferation of Ishikawa cells [9, 37–39]. We therefore determined the effect of PKCα knockdown on estrogen-dependent proliferation. E2 treatment stimulated proliferation of Ishikawa cells expressing a control shRNA targeting luciferase, reflected by an increase in the number of viable cells (Figure 7). Knockdown of PKCα significantly reduced the E2-dependent increase in cell number at 72 h and essentially abrogated the E2 proliferative response at 144 h. Cell viability (89%–96%) was not significantly different between cell lines and was not affected by E2 treatment.

Together, these results indicate that PKCα is a critical regulator of ER-dependent gene expression and modulates both E2-stimulated transcription and cell proliferation in ER positive endometrial cancer cells.

4. Discussion

Estrogen, acting through ER, is a major contributor to endometrial proliferation. Indeed, hormone-dependent, type I endometrial cancers are thought to arise due to excess estrogen stimulation, unopposed by progesterone, promoting mitogenesis, atypical hyperplasia, and the transition to malignant adenocarcinoma [4, 8, 11]. In this study, we have shown that activation of PKCα is a critical element of such an estrogenic environment, resulting in estrogen-independent activation of ER-dependent transcription and potentiating the effects of estrogen on both gene expression and endometrial cancer cell proliferation. The primary effect of PKCα is to stimulate basal, unliganded ER transactivation, thereby amplifying estrogen-stimulated promoter activity and enhancing levels of genes linked to endometrial hyperplasia and malignancy.

To confirm the observed interaction of PKCα and ER signaling on estrogen responsive promoters, we examined levels of a subset of estrogen responsive genes (lactotransferrin, pS2/TFF1, and c-fos) implicated in proliferation of normal and transformed endometrial cells and linked to the development of endometrial carcinoma [11, 33, 34, 40, 41]. Knockdown of PKCα in endometrial cancer cells reduced expression of these genes (Figure 6) consistent with their regulation by both ER and PKCα. Accordingly, treatment of breast

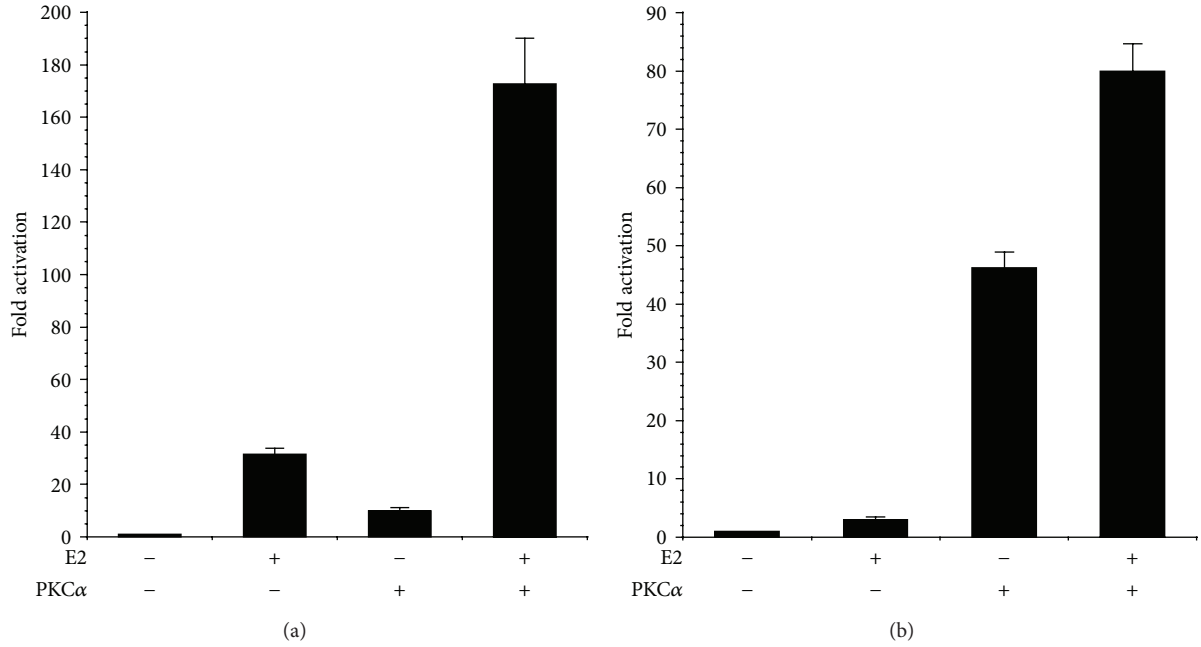

Figure 2: PKCα enhances ER-dependent promoter activity. Ishikawa cells were transiently transfected with (a) 0.5 μg pEREluc or (b) 0.5 μg pPS2luc and 0.3 μg pCMVβ in the presence or absence of 0.5 μg pmyrPKCα or vector control (pCDNA3). Cells were treated with ±100 nM estradiol (E2), as indicated. Luciferase activity was normalized to β-galactosidase and promoter activity expressed as fold increase over control. Data are mean ± s.e.m of 6 experiments conducted in triplicate.

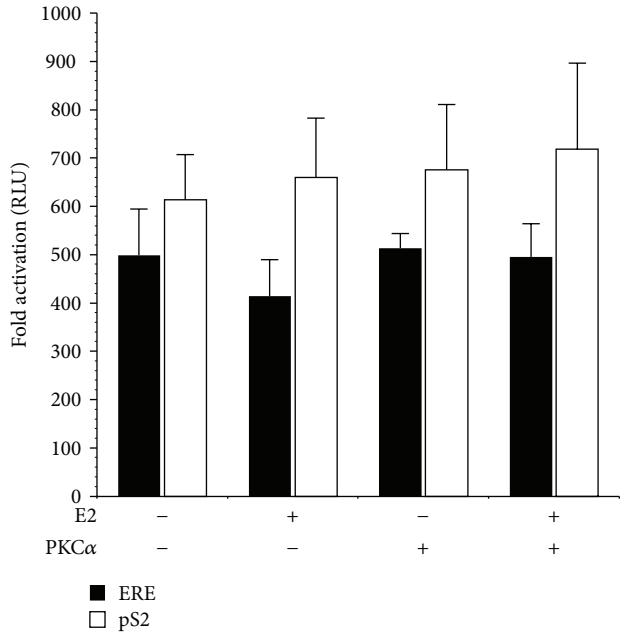

Figure 3: Estrogen and PKCα responses are ER dependent. HEC-50 cells, lacking ER, were transiently transfected with 0.5 μg pEREluc or 0.5 μg pPS2luc and 0.3 μg pCMVβ in the presence or absence of 0.5 μg pmyrPKCα or vector control (pCDNA3). Cells were treated with ±100 nM estradiol (E2), as indicated. Luciferase activity was normalized to β-galactosidase and promoter activity expressed as Relative Light Units (RLU). Data are mean ± s.e.m of 4 experiments conducted in triplicate.

and endometrial cancer cells with phorbol esters, to activate PKC, has been shown to induce expression of pS2 and c-fos and augment their increased levels observed in response to estrogen treatment [41–43].

Cyclin D1 is also an important mediator of estrogen-dependent endometrial cell proliferation and is over expressed in endometrioid tumors [9, 37]. Consistent with interaction of E2 and PKCα mitogenic signaling pathways, we previously demonstrated that PKCα activates the cyclin D1 promoter in endometrial cancer cells [20]. In addition, expression of the cyclin-dependent kinase (CDK) inhibitor p21 is decreased in endometrial cancers, correlating with poorer prognosis [44, 45]. Estrogen-induced Ishikawa cell proliferation paralleled a decline in p21 protein expression [9], whilst progesterone mediated growth inhibition was linked to elevated p21 levels [46]. Expression of p21 was also upregulated in response to knockdown of PKCα [20], suggesting that the CDK inhibitor is a target of both PKCα and estrogen signaling pathways, regulating endometrial cancer cell proliferation.

The PI3K/Akt pathway is commonly dysregulated in type I endometrial cancers. More than 80% of endometrioid carcinomas exhibit loss of the tumor suppressor PTEN and/or activating mutations in PI3K [47–49]. PTEN heterozygous mice develop endometrial hyperplasia and adenocarcinoma, characteristic of human endometrioid tumors [11, 33, 34]. Endometrial tumorigenesis in this model is associated with upregulation of estrogen-stimulated gene expression and ligand-independent activation of ER [34], mediated by Akt [33]. Consistent with these results, we have shown that PKCα

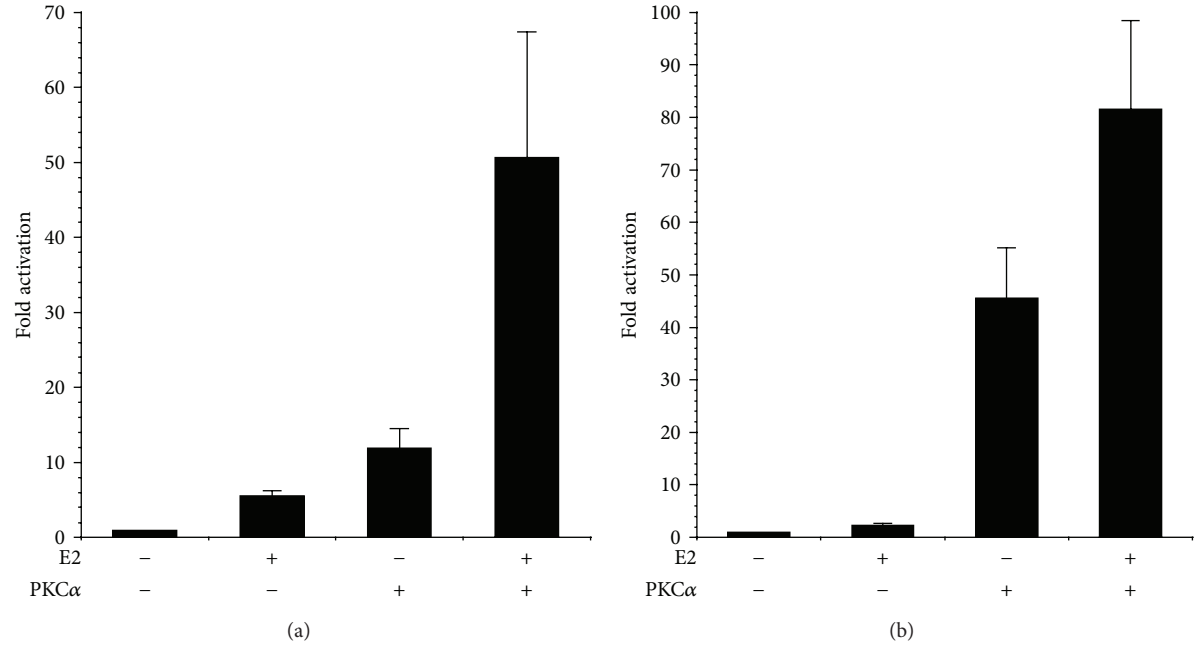

Figure 4: Reconstitution of PKCα regulated, ER-dependent transcription in HEC-50 cells. Cells were transiently transfected with 0.5 μg pHEGO (ERα) and (a) 0.5 μg pEREluc or (b) 0.5 μg pPS2luc and 0.3 μg pCMVβ in the presence or absence of 0.5 μg pmyrPKCα or vector control (pCDNA3). Cells were treated with ±100 nM estradiol (E2), as indicated. Promoter activity was determined as in Figure 2. Data are mean ± s.e.m of 6 experiments conducted in triplicate.

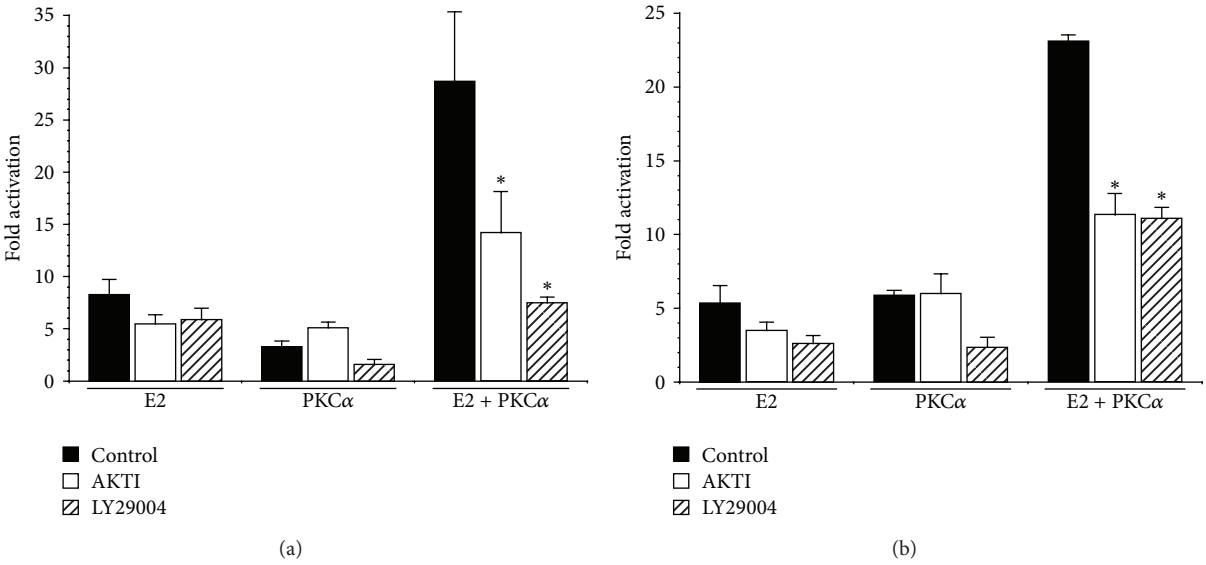

Figure 5: PKCα effects on ER-dependent transcription are mediated by the PI3-kinase/Akt pathway. (a) Ishikawa cells were transiently transfected with 0.5 μg pEREluc and 0.3 μg pCMVβ, in the presence or absence of 0.5 μg pmyrPKCα. (b) HEC-50 cells were transiently transfected with ERα (0.5 μg pHEGO), 0.5 μg pEREluc, and 0.3 μg pCMVβ± 0.5 μg pmyrPKCα or pCDNA3. Cells were treated with ±100 nM estradiol (E2) in the presence or absence of the Akt and PI3K inhibitors, Akt-I-1/2 (1 μM) and LY29004 (10 μM), respectively. Promoter activity was determined as in Figure 2 and expressed as fold increase over the appropriate inhibitor or diluent control. Results are mean ± s.d. ($n = 6$). $^*P < 0.05$.

is required to maintain Akt activity in endometrial cancer cells [20] and that amplification of estrogen/ER mediated transcription by PKCα is dependent upon the PI3K/Akt pathway (Figure 5).

Phosphorylation of ER has been implicated in regulation of its transcriptional activity and DNA binding [50, 51]. Phosphorylation of serine 167, by Akt, induces activation of ER [33], and phosphorylation of serines 104, 106, and 118

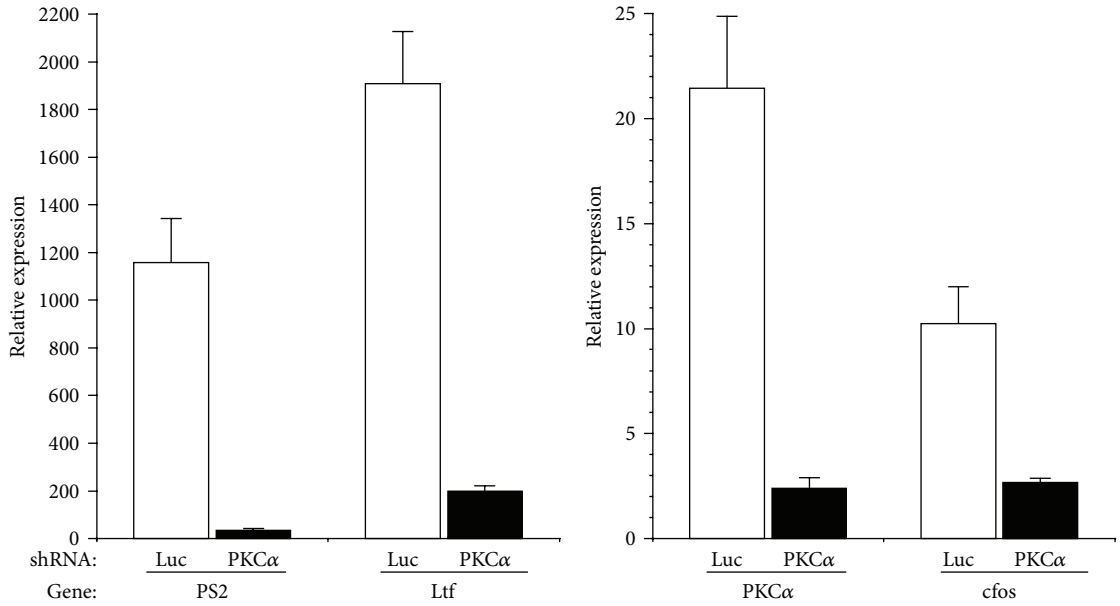

Figure 6: Knockdown of PKCα reduces ER-dependent gene expression. Ishikawa cells were stably transduced with shRNAs targeting PKCα or luciferase (luc). RNA was isolated and analyzed by real time reverse transcription PCR, using primers specific for the indicated gene, as described in Section 2. ΔCt values were calculated relative to a control gene (rp13) and relative levels expressed as $2^{\Delta ct}$. Data are mean ± s.e.m ($n = 6$).

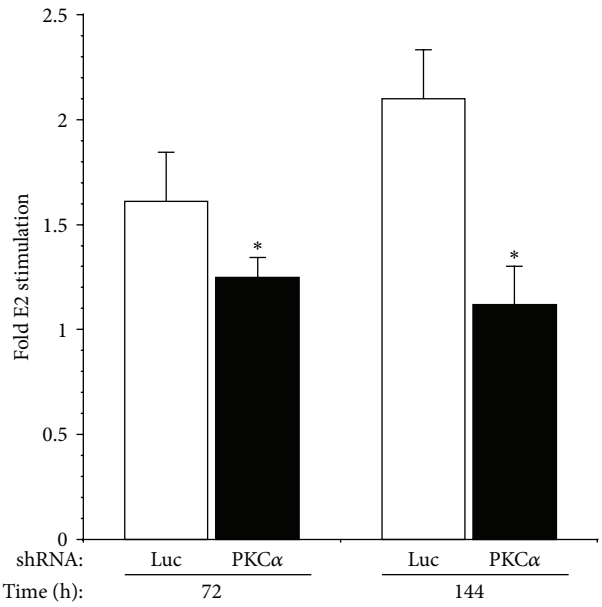

Figure 7: Knockdown of PKCα inhibits estrogen-stimulated growth of Ishikawa cells. Cells were stably transfected with shRNAs targeting PKCα or luciferase. Control (luc) or PKCα knockdown cell lines were treated with ±100 nM estrogen (E2) and harvested at the indicated time points. Cell number and viability were determined using a Beckmann Coulter Vi-CELL analyzer. Results are expressed as the fold increase in cell number induced by estrogen treatment. Data are mean ± s.e.m ($n = 6$). $^{*}P \leq 0.05$.

modulates ER interaction with co activators [52]. PKCα-dependent ER phosphorylation and its functional role in endometrial cancer cells remain to be established; however, these latter sites match the consensus substrate sequence for PKC and, since PKCα regulates Akt activity [20], suggest that the effects of PKCα may be mediated by direct or indirect phosphorylation of ER.

5. Conclusions

In summary, we have shown that activation of PKCα induces estrogen-independent activation of ER-dependent gene expression and potentiates the effects of estrogen on transcription. Evidence also implicates PKCα in the regulation of estrogen-dependent endometrial cancer cell proliferation. Thus, PKCα-dependent signal transduction is a critical component of the environment of excessive estrogen and supraphysiologic activation of ER, which is thought to underlie the development of endometrial hyperplasia and endometrioid adenocarcinoma. Furthermore, estrogen exposure may increase PKCα expression and/or activity in endometrial cancer cells [17, 18, 53], providing a potential positive feedback loop to amplify estrogen and ER-dependent responses.

The incidence of endometrial cancer continues to rise, and, despite advances in hormonal and chemotherapy, overall survival has not significantly improved [54–56]. Thus, there is an evident need to develop novel, molecular targeted therapies. PKCα is a critical element in the estrogen, PI3K/Akt, and growth factor/ERK-dependent signal transduction pathways regulating the growth of type I tumors [20–22]. Hence, inhibition of PKCα-dependent signaling would enable the simultaneous targeting of multiple estrogen-dependent and -independent pathways implicated in the development and progression of endometrial carcinogenesis. PKCα specific

inhibitors [57–59] may provide novel avenues, for primary or adjunct therapeutic intervention, to target tumors resistant to current regimens.

Conflict of Interests

The authors have no conflict of interests.

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