Abstract

Purpose: Combination estrogen + progestin therapy has been associated with increased breast cancer risk in postmenopausal women. Selective estrogen receptor modulators (SERM) are potential alternatives to progestins, although the endometrial safety of estrogen + SERM co-therapies is not known. The goal of this study was to evaluate the endometrial profile of low-dose estradiol and the SERM tamoxifen alone and in combination.

Experimental Design: Twenty-four postmenopausal female cynomolgus macaques were randomized by social group to receive placebo, low-dose micronized estradiol (E2; 0.25 mg/1,800 kcal), the SERM tamoxifen (Tam; 20 mg/1,800 kcal), or E2 + Tam for 4 months in a parallel-arm design.

Results: Tamoxifen alone resulted in overlapping but distinct effects compared with E2. Both E2 and Tam increased uterine weight and endometrial thickness, whereas only E2 increased endometrial proliferation. Morphologic effects were similar for Tam and E2 + Tam, which both induced stromal fibrosis and cystic change. Tamoxifen inhibited E2-induced proliferation and expression of genes related to cell cycle progression while exhibiting mixed agonist and antagonist effects on gene markers of estrogen receptor activity. The gene expression profile for E2 + Tam was distinct from either E2 or Tam alone but dominated by the Tam effect for estrogen-regulated genes. Tam also attenuated E2 effects on both vaginal maturation and cervical epithelial height.

Conclusions: These findings characterize a novel phenotype resulting from estrogen + SERM co-therapy. The predominance of Tam effects on endometrial proliferation, morphology, and transcriptional profiles suggests that endometrial risks for E2 + Tam may be similar to Tam alone.

Estrogen exposure is an important risk factor for endometrial cancer (1, 2). Many key risk factors for endometrial cancer risk relate to lifetime exposure to endogenous estrogens (2), and long-term unopposed estrogen therapy (ET) results in markedly increased risk of endometrial hyperplasia and cancer in postmenopausal women (1). Progestogens protect the endometrium from adverse estrogen-induced effects (1) and, for this reason, are given with estrogen in combined postmenopausal hormone therapy regimens. Progestogens lack similar protective effects against breast cancer, however. Results from the Women’s Health Initiative randomized clinical trials (3, 4) and other observational studies (5) indicate that long-term use of estrogen + progestin therapy (EPT) results in a modest but significant increase in invasive breast cancer incidence among postmenopausal women, above that seen with ET. This evidence has generated interest in progestin alternatives that selectively block estrogen actions in both the endometrium and breast but not in other tissues such as bone, urogenital tract, and brain.

The most promising candidates for this application are selective estrogen receptor modulators (SERM), which exhibit tissue-specific estrogen agonist and antagonist effects (6). Tamoxifen (Tam) is a first-generation SERM widely used in the treatment and prevention of estrogen-responsive breast cancer. Tam metabolites competitively bind estrogen receptors (ER) and inhibit the growth-promoting activity of endogenous estrogens in the breast (6). In clinical trials, Tam treatment decreases the incidence of ER-positive breast cancer by 30% to 60% over 5+ years in women at high risk for the disease (7, 8). However, Tam is also associated with adverse side effects related to estrogen deficiency, most notably menopausal symptoms and urogenital atrophy (9–11), which negatively affect quality of life in breast cancer survivors and many other postmenopausal women. These observations have contributed to the idea that the combination of low-dose estrogen and Tam may provide a unique safety and
Therapeutic profile, particularly for women at high risk of breast cancer (12–15). Data from observational studies of Tam-treated women provide support for this idea, suggesting that Tam may reduce breast cancer risk even when given alongside hormone therapy (16, 17).

Tamoxifen and certain other SERMs elicit diverse and poorly understood effects in the human endometrium. Tam-associated changes include increased endometrial thickening, stromal fibrosis, cystic change, and polyp formation (18, 19). In addition, Tam increases the incidence of endometrial carcinoma from ~1 to 2 cases per 1,000 women per year (7, 20, 21) and of uterine sarcoma from 0.04 to 0.17 cases per 1,000 women per year (22). Tam also induces certain markers of ER activity in the endometrium (23, 24), suggesting that estrogen agonist activity may contribute to Tam-associated cancer risk (25). Endometrial profiles of Tam and other SERMs given with ET are not well known, despite recent interest in these co-therapies (12–15, 26–31). The purpose of this study was to evaluate the endometrial phenotype of low-dose oral estradiol and Tam in the postmenopausal endometrium.

Materials and Methods

**Study design and treatments.** This study followed a parallel-arm design in which 24 ovariec-tomized female cynomolgus macaques (Macaca fascicularis) with a mean age of 14.7 ± 0.7 y were randomized to receive one of the following four treatments for 4 mo: (a) placebo (control; n = 6); (b) micronized 17β-estradiol (E2; Estrace, Mylan Pharmaceuticals) at a dose of 16.7 μg/kg body weight (0.25 mg/1,800 kcal; n = 6); (c) the SERM tamoxifen (Tam; Nolvadex, AstraZeneca Pharmaceuticals LP) at a dose of 1.3 mg/kg body weight (20 mg/1,800 kcal; n = 6); or (d) E2 + Tam (n = 6). Dose equivalents approximated a low ET dose of oral E2 in postmenopausal women (ref. 32; standard dose is 1.0 mg/d) and a standard maintenance dose of Tam following breast cancer diagnosis (7). In a previous study in this model, serum concentrations of 4-hydroxytamoxifen (one of the primary active metabolites of Tam) for the 20 mg/1,800 kcal dose were 5 ± 1 ng/mL, similar to those reported in women (33).

Hormone treatments were given in standard control diets with casein + lactalbumin as the protein source and macronutrient composition based on a typical North American human diet. Other than E2 and/or Tam treatments, group diets were the same in macronutrients, cholesterol, calcium, and phosphorus. Animals were fed 60 kcal/kg body weight (+10% extra to account for waste) twice daily. Daily E2 and Tam doses were scaled to 1,800 kcal of diet (the estimated daily intake for a U.S. woman) to account for differences in metabolic rates between monkeys and human subjects. All animals were originally imported from the Institut Pertanian Bogor in Bogor, Indonesia, and housed in stable social groups of three to four animals each. All animals were considered multiparous based on historical data from the original breeding colony, in which >90% of the adult females have had 2+ live births, and on myometrial evidence of prior pregnancy (expansion of venous adventitia).

Macaques are anthropoid primates with a high overall genetic coding sequence identity to humans, including important genes related to cancer susceptibility (34). Prior work from our lab and others has shown similarities between macaque and human endometrial biology, including responses to exogenous estrogen and SERMs, sex steroid receptor expression, and the presence of hyperplastic lesions (35, 36).

All procedures involving these animals were conducted in compliance with State and Federal laws, standards of the U.S. Department of Health and Human Services, and guidelines established by the Wake Forest University Animal Care and Use Committee. The facilities and laboratory animal program of Wake Forest University are fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care.

**Serum estradiol concentrations.** To confirm dietary intake of treatments, serum E2 concentrations were measured in blood samples collected by femoral venipuncture. Estradiol concentrations were measured during each month of treatment by RIA using a commercially available kit and protocol from Diagnostic Systems Laboratories (E2, DSL-4800 ultra-sensitive). Assays were done at the Yerkes National Primate Research Center Endocrinology Laboratory. Calibration standards ranged from 5 to 750 pg/mL.

**Endometrial tissue collection and processing.** At the end of the treatment period, animals were sedated with ketamine and euthanized using sodium pentobarbital (100 mg/kg, i.v.), as recommended by the Panel on Euthanasia of the American Veterinary Medical Association. Euthanasia was done for data collection related to cardiovascular and brain end points, to be described elsewhere. Uteri were
collected and weighed. Samples of endometrium were divided into two portions; the first part was snap-frozen in liquid nitrogen and stored at −70°C for gene expression analyses, whereas the remaining part was fixed for histology and immunohistochemistry. Fixed tissues were placed in fresh 4% paraformaldehyde solution at 4°C, transferred to 70% ethanol 24 h later, and then sectioned transversely immediately proximal to the uterotubal junction. All fixed samples were paraffin embedded, sectioned at 5 μm, and stained with H&E using standard histologic procedures.

**Histomorphometry and histology.** Endometrial thickness, stromal collagen, glandular area, cervical epithelial cell height, vaginal epithelial thickness, and vaginal keratin thickness were quantified by histomorphometric methods similar to those described previously (37). Briefly, H&E slides were digitized using a Labophot 3 light microscope (Nikon Instruments) and Infinity 3 digital camera (Lumenera), and measurements were taken with Image Pro-Plus software (Media Cybernetics). For each measure, six microscopic fields were randomly selected and examined at 200× magnification. Endometrial collagen content was determined from slides stained with Masson’s trichrome (containing Weigert’s iron hematoxylin, Crocein Scarlet MOO, 5% aqueous phosphomolybdic acid, and aniline blue; Fisher Scientific and Sigma); pale blue–stained areas (representing collagen and/or ground substance) were digitally measured using selective color-based analysis on Image Pro-Plus and values were averaged for each animal. Endometrial edema was quantified in a similar manner by digitally selecting and measuring clear or white areas within endometrial stroma. Cystic space was measured by manually tracing luminal area of endometrial glands. Epithelial area was determined by digital quantification of red positive staining for the glandular epithelial marker cytokeratin 18 (CK18; see below). Endometrial sections stained with H&E were evaluated for evidence of complex hyperplasia, neoplasia, and other histologic lesions by two board-certified veterinary pathologists (C.E.W. and J.M.C.). Endometrial sections were divided into two portions; the first part was snap-frozen in liquid nitrogen and stored at −70°C for gene expression analyses, whereas the remaining part was fixed for histology and immunohistochemistry. Fixed tissues were placed in fresh 4% paraformaldehyde solution at 4°C, transferred to 70% ethanol 24 h later, and then sectioned transversely immediately proximal to the uterotubal junction. All fixed samples were paraffin embedded, sectioned at 5 μm, and stained with H&E using standard histologic procedures.

**Immunohistochemistry.** Fixed endometrial sections were immunostained using commercially available primary monoclonal antibodies for the proliferation marker Ki67 (Ki67/MIB1, Dako), the apoptosis marker cleaved caspase-3 (Cell Signaling Technologies), the glandular epithelial marker CK18 (clone DC10, Lab Vision), and estrogen receptor-α (ESR1; NCL-ER-6F11, Novocastra). Antibodies were diluted 1:50 for Ki67 and cleaved caspase-3 and 1:100 for CK18 and ESR1 in 1× Automation Buffer (Biomedia) containing 0.5% casein (Sigma). Immunostaining procedures included antigen retrieval with citrate buffer (pH 6.0), biotinylated rabbit anti-mouse Fc antibody as a linking reagent, alkaline phosphatase–conjugated streptavidin as the label, and Vector Red as the chromogen (Vector Laboratories). Negative control slides were run for each immunostain using the same protocol as for study slides except with nonimmune serum (from the same species as primary antibody) in place of the primary antibody. Nuclear cell labeling for Ki67 and ESR1 was quantified by a computer-assisted counting technique using a grid filter to select cells for counting and our modified procedure of cell selection, described previously (38). For endometrial glands and stroma, 200 cells were counted in both superficial and basal compartments. Immunolabeling counts were conducted blinded to experimental treatment and analyzed as a percentage of the total number of cells examined. Cytoplasmic CK18 labeling was quantified digitally as percent positive area across six microscopic fields per slide and values were averaged for each individual.

**Gene microarray analyses.** Endometrial total RNA was extracted from frozen samples using Tri Reagent (Molecular Research Center), purified using RNaseasy Mini Kit (QIAGEN), and quantitated using a NanoDrop ND-1000 UV-Vis spectrophotometer (NanoDrop). Nucleic acid intactness and quality were confirmed using an Agilent 2100 Bioanalyzer (Agilent Technologies). Biotinylated cRNA samples were prepared according to the standard Enzo Bioarray protocol (Enzo Life Sciences) and hybridized using the standard Affymetrix protocol for eukaryotic samples. Biotinylated cRNA from each sample was hybridized to Affymetrix GeneChip Rhesus Macaque Genome Arrays, washed, and stained in an Affymetrix GeneChip Fluidics Station, and then scanned with an Affymetrix GeneChip Scanner 3000. Intensity data were extracted from scanned images and checked for quality using Affymetrix GeneChip Operating Software and Expression Console (MAS5 algorithm). Microarray assays were done at Cogenics, a Division of Clinical Data (Morrisville, NC). Microarray data are publicly available on the National Center for Biotechnology Information Gene Expression Omnibus database (accession no. GSE14518).

Microarray data analyses were done using the GeneSifter software program (Geospiza). Intensity data were RNA normalized, converted to a log 2 scale, screened for heterogeneity among samples and groups, and evaluated using supervised ANOVA and pairwise comparisons between treatments. Principal components analysis, pattern navigation, cluster analysis, heatmapping, and KEGG pathway analyses were done on filtered data subsets, as described in Results. Differences in gene numbers altered by each treatment were compared using either Fisher’s exact test or χ² test. Euclidean distances (representing the numerical difference between treatment vectors) were calculated as part of hierarchical clustering dendrograms using average linkage. Pathways related to cell proliferation were evaluated using z-scores generated in KEGG analyses; a z-score ≥2.0 was considered significant overrepresentation of genes in a particular pathway. All P values were corrected when possible for multiple comparisons using the Benjamini and Hochberg method (P adj; ref. 39), which derives a false discovery rate estimate from the raw P values (40). Representation of differentially expressed genes within specific functional categories was evaluated using Ingenuity Pathway Analysis software v6 (Ingenuity Systems). Significance of gene numbers represented within a given category was determined in Ingenuity Pathway Analysis using Fisher’s
exact test with Benjamini and Hochberg correction and expressed as $-\log_{10} (P \text{ value})$ for gene numbers within each treatment group.

**Quantitative gene expression.** Expressions of genes associated with proliferation (MKI67, Ki67 antigen), matrix remodeling (OVOS2, ovostatin 2), and ER activity [ESR1, estrogen receptor-α; TFF1, trefoil factor 1, also known as pS2; STC2, stanniocalcin 2; IGFBP2, insulin-like binding protein 2; PGR, progesterone receptor; and CXCL12, chemokine (C-X-C motif) ligand 12, also known as SDF1] were measured in endometrial samples using quantitative real-time reverse transcriptase PCR (qRT-PCR). Macaque-specific qRT-PCR primer-probe sets for internal control genes [glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin (ACTB)] were generated through the Applied Biosystems (ABI) TaqMan Assay-by-Design service. Sources for target primer-probe sets are given in Supplementary Table S1. All probes spanned an exon-exon junction to eliminate genomic DNA contamination. qRT-PCR reactions (20 μL volume) were done on an ABI Prism 7000 Sequence Detection System using standard TaqMan reagents and thermocycling protocol (41). Relative expression was determined using the $\Delta\Delta C_t$ method described in ABI User Bulletin #2 (available online). The Ct values for the control genes GAPDH and ACTB were averaged for use in internal calibration, whereas reference premenopausal breast tissue RNA was run in parallel for plate-to-plate calibration. Calculations were done using ABI Relative Quantification SDS Software v1.1.

**Statistical analysis.** Data were analyzed using the SAS statistical package (version 8, SAS Institute). All data were evaluated for normal distribution and homogeneity of variances among groups. A general linear model was used to determine mean values and calculate group differences for body weight, age, serum E2, uterine weight, endometrial morphometric measures, Ki67 immunolabeling, and qRT-PCR expression data. Immunolabeling data for ESR1 and cleaved caspase-3 were evaluated using a nonparametric Kruskal-Wallis test followed by two-sided Wilcoxon rank sum pairwise analysis. Gene expression data were log transformed to improve distribution, and data were then retransformed to original scale and reported as fold-change of control with 90% confidence interval. All other data are reported as mean ± SE. One animal randomized to the Tam group was excluded from all analyses based on repeated baseline serum E2 values >30 pg/mL, indicating ectopic

Fig. 1. Tamoxifen increases uterine weight and endometrial thickness while antagonizing E2-induced proliferation. A, uterine weight and endometrial thickness were higher in all treatment groups. B and C, endometrial proliferation, determined by immunolabeling and relative gene expression for the Ki67 marker, was greater only in the E2 group. The addition of Tam antagonized E2-induced proliferation (B). Images of Ki67 labeling (C) were taken at 200× magnification. Gene expression values were measured by qRT-PCR, corrected for internal control gene expression, and expressed relative to control group values. Vertical bars indicate 90% confidence intervals (gene expression) or SEs (other measures). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, compared with the respective control group values; #, $P < 0.05$, compared with the E2 group.
and/or remnant ovarian tissue. Final group sizes were thus \( n \) = 6 for Con, E2, and E2 + Tam and \( n \) = 5 for Tam for all end points. All pairwise \( P \) values were adjusted for the number of pairwise tests using a Bonferroni correction. A two-tailed significance level of 0.05 was chosen for all comparisons.

**Results**

**Treatment group characteristics.** No treatment group differences were noted in age or body weight at baseline (\( P > 0.1 \) for both; Supplementary Table S2). During 4 months of treatment, no significant differences in body weight or body weight changes among groups were noted. Serum E2 was higher in the E2 and E2 + Tam groups compared with control in each month of treatment (\( P < 0.01 \) for all), whereas no significant differences were observed between the E2 and E2 +Tam groups.

**Tamoxifen and estradiol effects on endometrial thickness and proliferation.** Uterine weight and endometrial thickness were at least 2-fold higher in the E2, Tam, and E2 + Tam groups compared with placebo (\( P < 0.01 \) for all; Fig. 1A). Endometrial thickness was marginally higher in the E2 + Tam group compared with the E2 alone group (\( P = 0.06 \)). In contrast, endometrial proliferation, indicated by MKI67 gene expression and Ki67 immunolabeling within glandular and stromal compartments, was greater only in the E2 alone group (\( P < 0.05 \) for all; Fig. 1B and C). The addition of Tam significantly abolished E2-induced stromal and epithelial Ki67 expression (\( P < 0.05 \) for E2 + Tam compared with E2; Fig. 1B). No significant treatment effects were seen on endometrial apoptosis, measured by the expression of the marker cleaved caspase-3 (data not shown). No neoplastic or complex/atypical hyperplastic lesions were noted on histology.

**Tamoxifen and estradiol effects on endometrial gene expression profiles.** Gene microarrays were used to further investigate treatment effects on endometrial proliferation. Global expression profiles showed greater numbers of genes altered by E2 compared with Tam but greater percent overlap between Tam and E2 + Tam. For example, among significantly altered (named) genes with fold-change >2 (ANOVA \( P \_adj < 0.05 \)), E2 (\( n = 634 \)) had 38% overlap with Tam (\( n = 364 \)) and 45% overlap with E2 + Tam (\( n = 498 \)), whereas Tam had 67% overlap with E2 and 84% overlap with E2 + Tam (Fig. 2A). Supervised hierarchical clustering also indicated that Tam and E2 + Tam were the most closely associated groups with a Euclidean distance of \( \sim 15 \) for genes significantly altered at fold-change >2 (Fig. 2A). The number of genes significantly altered only in the E2 group was greater than that for the Tam and E2 + Tam groups at all fold-change values <5 (\( P < 0.001 \) by \( \chi^2 \) test). This divergence of E2 from Tam and E2 + Tam was evident qualitatively from
principal components analysis vectors and heatmaps for both overall altered genes and altered genes specifically related to cell proliferation and cell cycle (based on ontology classification; Fig. 2B and C). A similar pattern was seen when altered genes were sorted by functional category, which showed significant overrepresentation of genes (−log P > 1.2) related to cancer, cell cycle, and cell proliferation functions in all groups, with the greatest representation in the E2 group (Fig. 2D).

Complementary pathway analyses evaluated the representation of altered genes at fold-change >2 in nine preselected KEGG pathways related to cell proliferation. Cell cycle was the only one of these pathways to have a significant z-score (Table 1). Fourteen of the 18 cell cycle genes identified were upregulated in the E2 group, in 13 of these 14 genes (the exception being cyclin D1), E2 had a greater fold-change effect than Tam and E2 + Tam (Table 2). Three of the four downregulated genes were cyclin-dependent kinase inhibitors involved in negative regulation of cell cycle progression. Expanding the filter to genes significantly altered at fold-change >1.2 provided cell cycle z-scores of 5.27 (33 genes represented out of 89 on array) on KEGG analysis and 7.75 on ontology analysis (308 genes represented out of 686 on array). Of the KEGG genes, 37 of 53 were upregulated in the E2 group, and 34 of these 37 genes had the greatest magnitude of change in the E2 group (P < 0.0001 compared with Tam and E2 + Tam by Fisher’s exact test), indicating partial or full antagonism of Tam on E2 effects related to cell cycle gene expression. A complete list of all significantly altered genes related to cell cycle (KEGG and ontology) and proliferation (ontology) is provided in Supplementary Table S3A to C.

### Table 1. Representation of significantly altered genes in preselected pathways related to cell proliferation

<table>
<thead>
<tr>
<th>KEGG pathway</th>
<th>List</th>
<th>Array</th>
<th>z-score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell cycle</td>
<td>18</td>
<td>89</td>
<td>3.90</td>
</tr>
<tr>
<td>mTOR signaling pathway</td>
<td>5</td>
<td>35</td>
<td>1.17</td>
</tr>
<tr>
<td>TGF-β signaling pathway</td>
<td>5</td>
<td>46</td>
<td>0.52</td>
</tr>
<tr>
<td>Jak-STAT signaling pathway</td>
<td>10</td>
<td>103</td>
<td>0.36</td>
</tr>
<tr>
<td>ErbB signaling pathway</td>
<td>6</td>
<td>68</td>
<td>0.03</td>
</tr>
<tr>
<td>Insulin signaling pathway</td>
<td>9</td>
<td>105</td>
<td>−0.06</td>
</tr>
<tr>
<td>Wnt signaling pathway</td>
<td>9</td>
<td>106</td>
<td>−0.09</td>
</tr>
<tr>
<td>VEGF signaling pathway</td>
<td>4</td>
<td>49</td>
<td>−0.14</td>
</tr>
<tr>
<td>Notch signaling pathway</td>
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<td>−0.59</td>
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<tr>
<td>MAPK signaling pathway</td>
<td>9</td>
<td>156</td>
<td>−1.35</td>
</tr>
</tbody>
</table>

NOTE: Microarray gene expression data were screened using a threshold fold-change >2.0 (in at least one group), Benjamini and Hochberg-adjusted ANOVA P value < 0.05, and quality setting >2. The resulting gene set (n = 2,065) was then subjected to KEGG pathway analysis in the Genesifter software program.

### Table 2. Treatment effects on relative expression (fold-change versus control) of individual genes involved in cell cycle regulation on microarray analysis

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>GenBank ID</th>
<th>E2</th>
<th>Tam</th>
<th>E2 + Tam</th>
</tr>
</thead>
<tbody>
<tr>
<td>BUB1 (+/-)</td>
<td>AF043294</td>
<td>4.3</td>
<td>1.4</td>
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</tr>
<tr>
<td>BUB1B (+/-)</td>
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<td>1.4</td>
<td>1.6</td>
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<td>3.9</td>
<td>1.4</td>
<td>1.5</td>
</tr>
<tr>
<td>CCNB2 (+)</td>
<td>NM_004701</td>
<td>5.1</td>
<td>1.5</td>
<td>1.8</td>
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<tr>
<td>CCND1 (+)</td>
<td>BC000076</td>
<td>1.8</td>
<td>2.2</td>
<td>2.7</td>
</tr>
<tr>
<td>CDC14B (+/-)</td>
<td>AK024868</td>
<td>0.5</td>
<td>0.6</td>
<td>0.5</td>
</tr>
<tr>
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<td>NM_001786</td>
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<td>1.1</td>
<td>1.1</td>
</tr>
<tr>
<td>CDC20 (+)</td>
<td>NM_001255</td>
<td>2.0</td>
<td>1.0</td>
<td>1.2</td>
</tr>
<tr>
<td>CDC6 (+)</td>
<td>NM_001254</td>
<td>3.8</td>
<td>1.2</td>
<td>1.3</td>
</tr>
<tr>
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<td>AW274756</td>
<td>2.6</td>
<td>2.1</td>
<td>2.4</td>
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<td>0.5</td>
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<td>0.5</td>
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<td>0.4</td>
<td>0.5</td>
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<td>1.8</td>
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<td>1.4</td>
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</tr>
<tr>
<td>MCM4 (+)</td>
<td>AI936556</td>
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<td>1.6</td>
<td>1.4</td>
</tr>
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</table>

NOTE: (+) and (−) indicate the respective effects on cell cycle progression.

To examine genes altered specifically in the E2 + Tam group, differentially expressed transcripts (ANOVA P adj < 0.05) were screened for a pattern of fold-change >2.0 in the E2 + Tam group but not in the E2 or Tam group. A total of 169 transcripts (with GenBank accession numbers) were identified (Supplementary Table S3D). Notable genes in this list included androgen receptor (AR; ↑ 2.0×), retinoic acid receptor–related orphan receptor B (RORB; ↓ 2.2×), V-erb-a erythroblastic leukemia viral oncogene homolog 4 (ERBB4; ↑ 2.6×), trefoil factor 3 (TFF3; ↑ 2.3×), tumor protein D52 (TPD52; ↑ 2.3×), myosin heavy chains 1 (MYH1; ↑ 3.0×) and 2 (MYH2; ↑ 7.1×), and breast carcinoma amplified sequence 1 (BCAS1; ↑ 2.2×).

**Divergent effects of estradiol and tamoxifen on endometrial morphology.** Endometrial morphometric measures were used to evaluate changes contributing to the increased uterine weight and endometrial thickness. In the Tam and E2 + Tam groups, these effects were due in part to greater endometrial fibrosis (P < 0.001 for both groups compared with control), which was evident on histology and confirmed using morphometry on sections stained with Masson’s trichrome for collagen (Fig. 3A and B). This change has been noted previously in the human endometrium in response to Tam and other SERMs and may contribute to formation of polyps (18, 19).

To explore gene expression changes related to the Tam effect on endometrial collagen, we evaluated pathways...
involved in matrix remodeling. Among significantly altered genes with fold-change >2 in the Tam and E2 + Tam groups (but not in E2 group) on microarray analysis, no significant ontology or KEGG pathways directly related to extracellular matrix or collagen remodeling were identified. Similarly, no effects specific to the Tam and E2 + Tam groups were observed for genes within related classes such as collagens, matrix metalloproteinases, fibrogenic cytokines, and tissue inhibitors of matrix metalloproteinases. However, markedly increased gene expression of the protease inhibitor ovostatin 2 (OVOS2) was noted in the Tam (24×) and E2 + Tam (12×) groups (P < 0.01 for both compared with control group; Fig. 3A). Whereas the exact role of ovostatin 2 is undetermined, the highly similar ovostatin 1 protein is a potent inhibitor of matrix metalloproteinases, including collagenase (42). An incidental but potentially related change noted on histology was distinctive thickening of glandular basement membranes in the Tam and E2 + Tam groups.

A second contributing factor to increased endometrial thickness in the Tam and E2 + Tam groups was cystic dilation of glands, indicated by greater glandular luminal area (P < 0.01 for both compared with control group; Fig. 3C). The addition of E2 to Tam had modest, if any, abrogating effects on fibrosis or cystic changes. In contrast to Tam, E2 effects on endometrial thickness were due largely to superficial stromal edema, which was marginally higher in the E2 group but not in the Tam or E2 + Tam group (ANOVA P = 0.06; Fig. 3C). Glandular epithelium measured by CK18 expression was higher in the E2, Tam, and E2 + Tam groups for deep, but not for superficial, endometrial glands but did not contribute substantially to overall endometrial thickness, occupying <3% of the sectional area (data not shown).

**Estrogen agonist and antagonist effects of tamoxifen on endometrium.** Nuclear expression of ESR1 protein was detected within endometrial glands and stroma. No significant treatment effects were seen for ESR1 immunolabeling (Supplementary Fig. 1A) or gene expression (data not shown). Two major patterns of expression were seen for gene markers of ER activity. In the first pattern, E2-induced genes such as TFF1, STC2, and IGFBP2 were higher in all treatment groups (P < 0.01 for all compared with control; Supplementary Fig. 1B), consistent with an ER agonist
effect of Tam. Other ER-driven genes such as PGR and CXCL12 showed a similar pattern, although these changes were not significant on qRT-PCR (ANOVA P > 0.05; data not shown). In the second pattern, E2-induced genes were higher only in the E2 group, with full or partial antagonism of E2 by Tam (Table 3). As with MKI67 and many of the cell cycle targets (Table 2), E2-induced gene expression for E2 + Tam seemed to be dominated by Tam rather than by E2.

Antagonism of estradiol effects by tamoxifen in the genital tract. Treatment with E2 resulted in ∼3-fold greater vaginal epithelial thickness (Fig. 4A and B), vaginal keratin thickness (Fig. 4C), and cervical gland height (Fig. 4D; P < 0.01 for all compared with control). Tam had no effects on any of these measures when given alone, completely antagonized E2 effects on vaginal maturation, and partially antagonized E2 effects on cervical epithelial height (Fig. 4A-D).

Discussion

Estrogen + SERM co-therapy is an emerging alternative to traditional EPT, especially for postmenopausal women concerned about the promotional effects of progestins on breast cancer. The endometrial safety of estrogen + SERM combinations is not known, however. The primary goal of this study was to evaluate the endometrial profile of low-dose E2 and Tam alone and in combination. Our findings from this study indicate that Tam may also inhibit beneficial estrogen effects on urogenital atrophy. Collectively, these data indicate that Tam may override two of the primary indications for ET and EPT and is thus not a suitable SERM co-therapy for estrogen.

Other SERMs investigated as estrogen co-therapies include raloxifene and bazedoxifene. A small clinical trial reported that raloxifene at 60 mg/d given with oral E2 at 1 mg/d improved menopausal symptoms but increased endometrial thickness compared with baseline and treatment with raloxifene alone (28). Similar findings were noted in an earlier pilot study using transdermal estradiol (27). One additional study examining vaginal atrophy found no adverse attenuating effects of raloxifene on the efficacy of an estradiol-releasing vaginal ring in postmenopausal women (26). Preclinical data indicate that the third-generation SERM bazedoxifene may antagonize estrogen effects on both mammary gland and uterine measures while maintaining estrogen effects on vaginal maturation (29–31). These latter effects include dose-dependent attenuation of estrogen-induced proliferation of ER-positive MCF-7 breast cancer cells in culture (29) and uterine weight gain in mice (30, 31).

Endometrial safety is an important concern in the development and long-term use of SERMs. Preclinical and clinical evaluation of endometrial SERM effects is often based on markers of estrogen agonist activity. Previous studies have shown that Tam induces estrogen-responsive markers in the endometrium (23, 24) and increases endometrial thickness and cancer risk in postmenopausal women (7, 20, 21), supporting the idea that carcinogenic effects of Tam are due to ER agonist signaling. In this study, Tam induced a subset of ER-driven genes in the endometrium while antagonizing others, indicating that Tam is not simply acting as a weak ER agonist relative to E2 but instead exhibiting more complex mixed patterns of ER transactivation. Given the heterogeneity of tissue samples used for gene expression analyses, it is possible that Tam estrogen agonist/antagonist effects may even differ within specific compartments (e.g.,

Table 3. Treatment effects on relative expression (fold-change versus control) of selected estrogen-induced genes on microarray analysis

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>GenBank ID</th>
<th>E2</th>
<th>Tam</th>
<th>E2 + Tam</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tamoxifen agonist pattern</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>STC2</td>
<td>BC000658</td>
<td>2.9</td>
<td>2.1</td>
<td>2.4</td>
</tr>
<tr>
<td>IGFBP2</td>
<td>NM_000597</td>
<td>2.9</td>
<td>2.8</td>
<td>3.4</td>
</tr>
<tr>
<td>CDH1</td>
<td>L08599</td>
<td>2.2</td>
<td>2.5</td>
<td>2.0</td>
</tr>
<tr>
<td>EGFR</td>
<td>S75916</td>
<td>3.0</td>
<td>2.3</td>
<td>2.4</td>
</tr>
<tr>
<td>MUC1</td>
<td>A1610869</td>
<td>2.0</td>
<td>2.8</td>
<td>3.6</td>
</tr>
<tr>
<td>Tamoxifen antagonist pattern</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MKI67</td>
<td>AU132185</td>
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</tr>
<tr>
<td>PCNA</td>
<td>NM_002592</td>
<td>1.4</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>TOP2A</td>
<td>AL561834</td>
<td>5.3</td>
<td>1.5</td>
<td>1.8</td>
</tr>
<tr>
<td>PTEN</td>
<td>U96180</td>
<td>1.6</td>
<td>1.3</td>
<td>1.1</td>
</tr>
<tr>
<td>PTTG1</td>
<td>NM_004219</td>
<td>3.5</td>
<td>1.1</td>
<td>1.2</td>
</tr>
</tbody>
</table>

NOTE: Microarray gene expression data were screened using a threshold fold-change >2.0 (in at least one group), Benjamini and Hochberg–adjusted ANOVA P value < 0.05, and quality setting >2. Known estrogen-induced genes shown were selected from the resulting gene set.
stroma and epithelium) of the same tissue. Of note, Tam has also been shown to induce certain estrogen-responsive markers in normal mammary gland (44) and breast cancer cells (45) despite having well-documented ER antagonist effects. This information suggests that individual estrogen response markers are not necessarily the best predictors of SERM effects or risk profile.

Increased proliferation is associated with the development of many cancers and provides a useful biomarker of potential cancer-promoting effects. In the uterus, epithelial cell proliferation serves as an important prognostic marker in human endometrial cancers (46) and may help predict risk associated with different hormone therapies (47). In this study, Tam decreased E2-induced Ki67 labeling and inhibited or partially inhibited the expression of numerous E2-induced genes related to proliferation. Many of the proliferation-related genes with this pattern directly involve cell cycle progression. These findings, in combination with phenotypic features dominated by Tam, suggest that E2 + Tam may be associated with cancer risk estimates more similar to that of Tam than of E2 alone.

Numerous studies have shown that standard doses of unopposed ET (for oral E2, 1 or 2 mg/d) increase endometrial hyperplasia and cancer risk in postmenopausal women (1, 2). The endometrial effects of newer low-dose ETs are less clear, however. A small clinical trial previously reported increased endometrial thickness following oral E2 doses of 0.5 and 1.0 mg/d but not 0.25 mg/d (32), whereas a separate trial using oral conjugated equine estrogens noted a dose-related increase in endometrial hyperplasia incidence from 3% for 0.3 mg/d to 27% for the standard 0.625 mg/d dose after 2 years (48). Although no neoplastic or complex hyperplastic lesions were observed in the current study, our results suggest that lower doses of oral E2 (≤0.5 mg/d) may still exert a clear stimulatory effect, increasing endometrial weight, thickness, glandular area, and proliferation. It is also worth noting that the peak serum E2 concentrations (40-80 pg/mL) were comparable to

![Fig. 4. Tamoxifen antagonizes E2 effects on lower reproductive tract measures. A, representative images of vaginal epithelium show increased overall epithelial maturation and keratinization (superficial laminar zone) following E2 and antagonism of this effect by Tam. Images were taken at 100× magnification; H&E stain. B to D, Tam fully antagonized E2 effects on overall epithelial thickness (B) and keratin thickness (C) and partially antagonized stimulatory E2 effects on cervical glandular height (D). Vertical lines indicate SEs. *, P < 0.05; **, P < 0.01; ***, P < 0.001, compared with the respective control group values. ##, P < 0.01, compared with the E2 group.](image-url)
the steady-state concentrations reported in postmenopausal women receiving E2 via vaginal ring at 150 μg/d (49) or transdermal patch at 50 μg/d (50). Serum E2 concentrations (and pharmacodynamics) vary widely for different E2 formulations and routes of administration, however, and it is unclear whether Tam or other SERMs may exert similar effects to those seen in the present study when given alongside lower doses of parenteral E2. Dose-dependent endometrial effects may also be seen with Tam (19), and additional studies are needed to determine whether lower Tam doses than those used here (<20 mg/d human equivalent) would similarly influence E2 effects.

The ideal postmenopausal hormone therapy would provide estrogen agonist effects in tissues such as bone and urogenital tract while minimizing the risk of breast and endometrial cancer. Estrogen + SERM combinations have been proposed recently as a potential way to achieve this profile. Data from this study provide an initial step in profiling the uterine effects of these therapies. Our results show that a SERM given at a standard dose may dominate the estrogen phenotype in the endometrium, at least for lower doses of oral E2. In the case of Tam, this profile may still be associated with adverse long-term effects, including cancer risk. Nevertheless, the dominance of the SERM signature suggests that other SERMs with more favorable profiles in the endometrium and elsewhere may be used as progestin alternatives in future postmenopausal therapies. Further studies directly comparing different SERM-estrogen combinations are needed to identify the safest SERM for this purpose.

Disclosure of Potential Conflicts of Interest

J.M. Cline, J.R. Kaplan, and C.E. Wood are unpaid co-investigators on an investigator-initiated preclinical study funded by Wyeth Pharmaceuticals (now part of Pfizer) on the effects of the SERM bazedoxifene.

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