Research Article

Progesterone Enhances Calcitriol Antitumor Activity by Upregulating Vitamin D Receptor Expression and Promoting Apoptosis in Endometrial Cancer Cells

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Abstract

Human studies suggest that progesterone and calcitriol may prove beneficial in preventing or inhibiting oncogenesis, but the underlying mechanism is not fully understood. The current study investigates the effects of progesterone, calcitriol, and their combination on immortalized human endometrial epithelial cells and endometrial cancer cells and identifies their targets of action. Combination treatment with both agents enhanced vitamin D receptor expression and inhibited cell proliferation through caspase-3 activation and induction of G0–G1 cell-cycle arrest with associated downregulation of cyclins D1 and D3 and p27 induction. We used mass spectrometry–based proteomics to measure protein abundance differences between calcitriol-, progesterone-, or combination-exposed endometrial cells. A total of 117 proteins showed differential expression among these three treatments. Four proteins were then selected for validation studies: histone H1.4 (HIST1H1E), histidine triad nucleotide-binding protein 2 (HINT2), IFN-induced, double-stranded RNA-activated protein kinase (EIF2AK2), and Bcl-2–associated X protein (BAX). Abundance levels of selected candidates were low in endometrial cancer cell lines versus the immortalized endometrial epithelial cell line. All four proteins displayed elevated expression in cancer cells upon exposure to calcitriol, progesterone, or the combination. Further BAX analysis through gain- or loss-of-function experiments revealed that upregulation of BAX decreased cell proliferation by changing the BAX:BCL-2 ratio. Knockdown of BAX attenuated progesterone- and calcitriol-induced cell growth inhibition. Our results showed that progesterone and calcitriol upregulate the expression of BAX along with other apoptosis-related proteins, which induce inhibition of endometrial cancer cell growth by apoptosis and cell-cycle arrest.


Introduction

Endometrial cancer is the most common gynecologic malignancy in the United States. In 2012, 47,130 new cases are anticipated, resulting in 8,010 deaths (1). Endometrial cancer is usually treated with surgical removal of the uterus and adjuvant therapy is administered in selected cases based on surgico-pathologic factors predictive of recurrence risk. For early-stage disease, outcomes are quite favorable with 5-year survival rates more than 90% (2). However, the number of women presenting with endometrial cancer in an advanced-stage or a high histologic grade, which is indicative of a poor prognosis, is increasing and mortality rates are rising (3, 4). Discovery of novel molecular targets for the diagnosis, prognosis, and treatment of endometrial cancer is imperative to improve the management and outcome of this disease.

A strong body of clinical and epidemiologic evidence suggests that progestins are highly effective endometrial cancer preventive agents. Routine use of progestins lowers endometrial cancer risk and the protective effect increases with increasing progestin potency. In premenopausal women, use of progestin containing oral contraceptives confers a significant reduction in endometrial cancer risk (5–7). In addition, progestin-potent oral contraceptives have enhanced endometrial cancer protective effects compared with oral contraceptives containing weak progestins (8, 9). In menopausal women, the addition of a
progestin to estrogen replacement therapy decreases the risk of precancerous endometrial hyperplasias, suggesting chemopreventive effects of progestins on the endometrium (10, 11). Currently, progestins are often used as salvage therapy in patients with recurrent endometrial cancer and in young patients to preserve childbearing capacity (12). Progesterone mediates its inhibitory effects on the endometrium and endometrial cancer via the progesterone receptor (PR), an intracellular steroid receptor with A and B isoforms. An increase in the response rate to progestin therapy and improved survival outcomes have been reported in tumors with a higher percentage of PR (12, 13). Progesterone potency can be enhanced by either increasing the dosage of progestin or by selecting a pharmacologically potent progestin. However, both of these approaches are likely to be associated with an increase in side effects that would be undesirable for long-term chemoprevention in women. An alternative strategy would be to combine a shorter course or lower dose of progestin with a second preventive agent that has an excellent safety profile and enhances the potency of the progestin.

There is a growing body of epidemiologic and laboratory evidence in support of vitamin D for the prevention of a number of malignancies including endometrial cancer (14, 15). Vitamin D3, produced in the epidermis or obtained from the diet, is metabolized to the dihydroxylated form [calcitriol, 1,25-(OH)2D3] to be biologically active. Calcitriol is well known for its antiproliferative roles through multiple mechanisms including the induction of cell-cycle arrest, apoptosis, and differentiation in a variety of cancer types, including prostate, breast cancer, colon, skin, and leukemic cells (16–18). The inhibitory role of calcitriol in cellular growth and proliferation potentially provides protection from various types of cancers, as indicated by several epidemiologic studies (19, 20). However, as calcitriol has been found to be efficacious in several preclinical and clinical studies, dose-limiting calcemic effects have proved a major obstacle for the use of this compound as a chemotherapeutic or chemopreventive agent (14, 15). One approach to limit the toxicity is to use lower concentrations of calcitriol in combination with other agents that enhance its anticancer activity.

A general approach to cancer chemotherapy entails simultaneous administration of 2 or more chemotherapeutic drugs to patients, in anticipation that the drug combination will be more effective than any single agent. Several studies have reported that calcitriol works synergistically with chemotherapeutic drugs (21, 22). Combining progestosterone with low doses of calcitriol might be a beneficial chemopreventive or therapeutic strategy in endometrial cancer. Therefore, in the present study, we evaluated the effects of combinations of calcitriol and progesterone on endometrial cancer cells. The data show that combinations achieve significantly greater inhibition of tumor cell growth and enhanced anticancer activity than individual agents. Characterizing the underlying mechanisms of progesterone’s synergism with calcitriol will provide an important rationale for chemoprevention or treatment trials using this combination.

Materials and Methods

Cell lines and culture conditions

The immortalized human endometrial epithelial cell line, EM-E6/E7-TERT, established and characterized by Dr. Satoru Kyo, (Kanazawa University, School of Medicine, Kanazawa, Japan) was kindly provided to us in 2008. This line was not authenticated by us once received in our laboratory. Human endometrial cancer cell lines HEC-1B and RL-95 were obtained from the American Type Culture Collection (ATCC) and Ishikawa cell line was obtained from Sigma. These human-derived cell lines were authenticated by DNA short-tandem repeat analysis by ATCC and Sigma. All 4 cell lines were initially expanded and cryopreserved within 1 month of receipt. Cells were typically used for 3 months, at which time a fresh vial of cryopreserved cells was used. The cells were routinely tested for Mycoplasma.

Immortalized epithelial endometrial (EM-E6/E7-TERT) cells were established and characterized by Kyo and colleagues (23). Primary endometrial epithelial cells were transformed by the combination of human papillomavirus-16 E6/E7 expression and telomerase activation by the introduction of human telomerase reverse transcriptase (hTERT). The immortalized cells contained no chromosomal abnormalities, retained responsiveness to sex-steroid hormones, exhibited glandular structure on 3-dimensional culture, and lacked transformed phenotypes on soft agar or in nude mice. The cells were grown in Dulbecco’s modified Eagle medium (DMEM). Ishikawa cells were derived from a well-differentiated adenocarcinoma of the human endometrial epithelium from a 39-year-old woman, and express functional steroid receptors for estrogen, progesterone, and androgen. Cells were grown in DMEM:F12 supplemented with insulin (Invitrogen). HEC-1-B cells derived from a moderately differentiated adenocarcinoma of endometrial epithelium from a 71-year-old patient were cultured in Eagle minimum essential medium (Invitrogen). RL95-2 was derived from a grade 2 moderately differentiated adenosquamous carcinoma of the endometrium. The cells are characteristically epithelioid with well-defined junctional complexes, tonofilaments, filopodia-like extensions, and surface microvilli. Nuclei are large, irregular, and invaginated frequently with multiple, prominent, and lamellar nucleoli. The RL95-2 cells were grown in DMEM:F12 medium supplemented with insulin (0.005 mg/mL) and FBS as described earlier (24, 25). The cells were treated with progesterone (20 μmol/L P4, 99.9% pure; Sigma), calcitriol (100 nmol/L; Sigma), or both for 24, 72, or 120 hours and collected for protein extraction. The time of treatment and doses of progesterone and calcitriol were based on our previous studies showing inhibition of cell growth and apoptosis of cancer cells (24). For a set of experiments, endometrial cancer cells were transfected with siRNA-targeting Bcl-2–associated X protein (BAX) or scrambled siRNA and then treated with progesterone, calcitriol, or...
both for 120 hours. Protein extracts were prepared for BAX analysis.

**Cell growth and death assays**
Endometrial cells were plated at 5,000 cells/cm² and fed after 24 hours with fresh medium and treated with progesterone, calcitriol, or both. After 120 hours of these treatments, total cells were collected by brief trypsinization, and washed with PBS. Total cell number was determined by counting each sample in triplicate using a hemocytometer under an inverted microscope. Cell viability was determined using the Trypan blue exclusion method. Each treatment had 3 independent plates. The data shown in this study are the mean of 3 independent experiments.

**Cell-cycle analysis**
Cells were plated onto tissue culture flasks and the medium was replaced the next day with medium containing progesterone, calcitriol, combined treatment, or vehicle. Cell-cycle status was measured after 24 hours of exposure to treatments. Cells (1 × 10⁶) were collected and centrifuged at 250 × g for 5 minutes. Supernatant was removed and cells were fixed by slowly adding 1.0 mL of a chilled ethanol (−20°C) while vortexing the tube at low speed. Cells were kept at −20°C until DNA staining. On the day of DNA staining, samples were centrifuged at 250 × g for 5 minutes. Then supernatant was removed. DNA staining buffer (1 mL containing 100 μg propidium iodide; Sigma) was added to cell pellet and briefly vortexed. Cells were kept in the dark for 15 minutes at room temperature. The distribution of cells between phases of the cell cycle was determined using a BD LSR II Flow Cytometer (Becton Dickinson, LSR 2) and ModFit LT software (Verity Software House). Data are means ± SD of 3 independent experiments.

**Liquid chromatography/tandem mass spectrometry**
To identify differentially expressed proteins in endometrial cancer cells exposed to progesterone, calcitriol, or both, 40 μg of each total cell lysate was resolved by one-dimensional SDS-PAGE. Twenty equivalently sized gel slices were excised and digested using trypsin. Tryptic digests were resuspended in mobile phase A and each subjected to 5 recursive nanoflow liquid chromatography/tandem mass spectrometry (LC/MS-MS) analyses (LTQ-Orbitrap Velos; Thermo Fisher Scientific) as previously described (26). Tandem mass spectrometry (MS-MS) spectra were searched against the UniProt human protein database using Mascot (Matrix Science) with a decoy database to result in a false peptide discovery rate of less than 1%. Differences in protein abundance between the samples were derived by spectral counting (SC), and peptides whose sequence mapped to multiple protein isoforms were grouped as per the principle of parsimony.

**Silencing of BAX in endometrial cancer cells**
To establish that BAX is a mediator of progesterone and calcitriol-induced growth inhibition, endometrial (Ishikawa, HEC-1B, and RL95-2) cancer cells were seeded in 6-well plates and transfected the following day with BAX siRNA (SC29212; Santa Cruz Biotechnology) or scrambled siRNA (SC37007; Santa Cruz Biotechnology) using the Lipofectamine 2000 reagent (Invitrogen). Transfected cells were treated with or without progesterone (20 μmol/L), calcitriol (100 nmol/L), or both for 5 days. Cell extracts were used to assess expression of BAX. The overall transfection efficiency for endometrial cells assessed by luciferase assay was 76% to 83%.

**Cell viability assay**
Cell viability was evaluated after transfection of endometrial cancer cells with BAX siRNA or scrambled siRNA by using the CellTiter 96 AQueous One Solution cell viability assay (Promega) according to the instructions of the manufacturer. CellTiter 96 AQueous One Solution reagent (20 μL) was added into each well of the 96-well assay plate containing the samples in 100 μL of culture medium. Absorbance was measured at 490 nm using a microtiter plate reader. Relative cell viability was expressed as percentage change of transfected cells over scrambled siRNA-transfected cells.

**Western blot analysis**
Endometrial cancer cell extracts from progesterone- or calcitriol-treated and nontreated control cells as well as extracts from BAX-silenced and control siRNA-transfected cells treated with or without progesterone or calcitriol were analyzed using antibodies against BAX, caspase-3, cyclin D1, cyclin D3, p27 (Cell Signaling Technology), BCL-2 (BioLegend), PR (PgR 1294; Dako Corporation), VDR, histone H1.4 (HIST1H1E), histidine triad nucleotide-binding protein 2 (HINT2), IFN-induced, double-stranded RNA-activated protein kinase (EIF2AK2; Santa Cruz Biotechnology), and β-actin antibody (Sigma-Aldrich). Equal amounts of protein were subjected to SDS-PAGE. The enhanced chemiluminescence system was used to visualize the protein bands as recommended by the manufacturer (Pierce). Protein bands were quantified using densitometry software (Bio-Rad), and normalized using actin as a loading control. To calculate the relative intensity of each band, individual bands were divided by the corresponding loading control intensity.

**Statistical analysis**
Data are presented as the mean of triplicate determinants with SEM. Experiments carried out in triplicate were repeated at least 3 times. Statistically significant differences were determined between control and treatment groups using two-way ANOVA followed by Tukey post hoc test. A P value of less than 0.05 was considered statistically significant. The Pearson correlation coefficient test was used to calculate the correlation between BAX expression and cell growth. Western blot analysis data were quantified using densitometry.

A representative of 3 immunoblots is presented in the figures along with average relative density of the bands normalized to β-actin. Statistically significant differences were determined between control and treatment groups by
quantification of 3 immunoblots and \( P \) values are provided in the results section.

Results

Progesterone receptors were not affected by progesterone or calcitriol treatment

The protein expression levels of PR-A and PR-B were studied in EM-E6/E7-TERT and 3 endometrial cancer (Ishikawa, HEC-1B, and RL95-2) cell lines by quantification of Western blot analyses (Fig. 1), using a mouse anti-human PR antibody that recognizes both human PR isoforms: PR-A (85–94 kDa) and PR-B (116–120 kDa). Both isoforms were expressed in endometrial cancer and in immortalized cell lines (Fig. 1A). However, expression of both isoforms was reduced in cancer cell lines compared with the immortalized cell line (\( P < 0.05 \)). We studied the effects of progesterone, calcitriol, or both on PR protein expression in the same normal and endometrial cancer cell lines. No marked change was noticed in the expression of PR isoforms in immortalized cells and in endometrial cancer cells following progesterone, calcitriol, or combination treatments (Fig. 1A).

Progesterone enhances vitamin D receptors in endometrial cancer cells

We analyzed the effect of progesterone, calcitriol, or both on VDR protein expression in normal and malignant endometrial cells. As shown in Fig. 1B, Western blotting of cell lysates for VDR revealed a doublet at approximately 60 and 48 kDa, which likely represents multiple phosphorylated forms of the receptor. Interestingly, quantification of VDR expression data in progesterone-treated cancer cell lines resulted in a marked (\( P < 0.05 \)) stimulation of VDR expression. Exposure to the combined treatment showed similar expression of VDR versus cells treated with only progesterone. Although calcitriol enhanced VDR expression, the enhancement was not as pronounced as that shown by progesterone-treated cells. In EM-E6/E7-TERT cells, calcitriol, but not progesterone, enhanced (\( P < 0.05 \)) VDR expression (Fig. 1B).

Progesterone and calcitriol inhibit cell proliferation and cause cell death

We examined the dose response effects of these agents alone or in combination on cell growth and death in EM-E6/E7-TERT and 3 endometrial cancer lines (Ishikawa, HEC-1B, and RL95-2). Cells were treated with progesterone (10, 20, or 40 \( \mu \)mol/L), calcitriol (50, 100, and 200 \( \mathrm{nmol/L} \)), or the combination of the 2 for 120 hours. This treatment time was selected on the basis of our previous studies, which indicated significant inhibition of cell growth after 5 days (25). At the end of treatment time, determination of total cell number as well as dead cells showed that both progesterone and calcitriol inhibit cell growth and cause cell death in a dose-dependent manner (Fig. 2A and B). Combined progesterone and calcitriol treatment showed a pronounced dose-dependent decrease in cell numbers compared with either single agent in all the cell lines tested. Using the

![Figure 1. Expression of PR and VDR in immortalized human endometrial (EM-E6/E7-TERT) and endometrial cancer (Ishikawa, HEC-1B, and RL95-2) cell lines. Whole-cell extracts of progesterone- (20 \( \mu \)mol/L), calcitriol- (100 \( \mathrm{nmol/L} \)), or combination-treated cell lines containing an equal amount of protein (100 \( \mu \)g) for PRs (A) and (20 \( \mu \)g) for VDR (B) were separated by electrophoresis, and immunoblots were probed with 3 mg/mL of mouse monoclonal anti-human PR antibody from Dako Corporation or VDR antibody at 4°C. The immunoblots shown here are representative of 3 independent experiments with similar results. The values above the bands represent relative density of the bands normalized to \( \beta \)-actin. The upper values represent quantification of PR (B) and lower represent PR (A). The quantitation of VDR includes both the upper and lower bands.](image-url)
Trypan blue dye exclusion method, we observed that the decrease in cell number by both the agents was accompanied by an increase in cell death. However, no treatment caused the percentage of cell death to increase beyond 20%, suggesting that the overall decrease in cell number was partially caused by the cell death–inducing effect of both the agents. In an attempt to identify the mechanism of cell death caused by each of the 3 treatments, we assessed the levels of cleaved caspase-3 in cells. Although treatment of cells with progesterone or calcitriol alone showed moderate elevation of cleaved caspase-3, their combination substantially increased \( (P < 0.05) \) caspase-3 levels (Fig. 2C). Next, we investigated the effect of progesterone and calcitriol on cell-cycle arrest in endometrial cancer cells to determine
whether the inhibitory effect on cell proliferation is accompanied by modulation of cell-cycle progression.

**Combination of progesterone and calcitriol induces G₁ cell-cycle arrest and modulates cell-cycle regulators in endometrial cancer cells**

Many apoptotic stimuli induce cell-cycle arrest before cell death, thereby affecting both cell cycle and apoptotic machinery. To determine whether inhibition of cell proliferation was associated with cell-cycle arrest, endometrial cells EM-E6/E7-TERT, Ishikawa, HEC-1B, and RL95-2 were treated for 24 hours with vehicle, progesterone, calcitriol, or combined treatment and analyzed by flow cytometry. The initial cell population in the G₀–G₁ phase was 56.39% ± 0.42%, 36.44% ± 0.35%, 49.69% ± 0.41%, and 47.14% ± 0.14% in EM-E6/E7-TERT, HEC-1B, Ishikawa, and RL95-2 cells, respectively. However, after 24 hours of incubation with the combined progesterone-calcitriol treatment, a significantly greater number of cells were arrested in the G₁ phase (62.86% ± 0.57%, 60.24% ± 0.4%, 59.97% ± 0.48%, and 58.97% ± 0.48% in EM-E6/E7-TERT, HEC-1B, Ishikawa, and RL95-2 cells, respectively). However, after 24 hours of incubation with the combined progesterone-calcitriol treatment, a significantly greater number of cells were arrested in the G₁ phase (62.86% ± 0.57%, 60.24% ± 0.4%, 59.97% ± 0.48%, and 58.97% ± 0.48% in EM-E6/E7-TERT, HEC-1B, Ishikawa, and RL95-2 cells, respectively). These results suggest that a combination treatment induces G₀–G₁ phase arrest in endometrial cancer cells. To identify cell-cycle regulatory molecules, we examined the expression of cyclins and cyclin-dependent kinases (CDK) inhibitors. Cells were treated with progesterone, calcitriol, or the combination for 5 days and cell lysates were prepared for Western blot analysis. Our results showed a marked decrease ($P < 0.05$) in cyclins D1 and D3 in endometrial cancer cell lines with concomitant elevation ($P < 0.05$) of p27 protein expression following treatment with progesterone, calcitriol, or the combination compared with untreated cells (Fig. 3E). Together, these results suggested that progesterone and calcitriol treatments in endometrial cancer cells strongly modulate cyclins and inhibit their normal regulation of cell-cycle progression.

**Identification of novel mediators of calcitriol and progesterone signaling in endometrial cancer cells**

We used a proteomic approach to identify additional mediators of progesterone and calcitriol signaling in endometrial cancer cells. Comparative analysis of protein expression in HEC-1B cells treated with progesterone, calcitriol, or the combination for 24 hours resulted in the identification of alterations in the abundance levels of 609, 387, and 519 proteins, respectively. Interestingly, between the 3 treatments, only 117 proteins were found in common that exhibited altered expression in HEC-1B relative to the untreated cells (Fig. 4A). The list of 117 proteins is provided in the Supplementary Table. The proteins that were differentially altered by these 3 treatments in the HEC-1B cells were categorized into different groups: oncogenes, tumor suppressors, binding proteins, membrane proteins, structural proteins, transport proteins, ribosomal proteins, receptors, and transcription factors, as well as proteins involved in apoptosis and cell-cycle regulation. Of the 117 proteins, 4 apoptosis-related proteins were selected for validation studies, HIST1H1E, HINT2, EIF2AK2, and BAX (Fig. 4A). The expression levels of the selected candidates were successfully verified by Western blot analysis to be at low levels in 3 endometrial cancer cell lines and their expression was significantly elevated ($P < 0.05$) upon exposure to progesterone, calcitriol, or the combination (Fig. 4B). We selected BAX for further analysis and investigated the time-dependent induction of BAX and BCL-2 in response to progesterone, calcitriol, or both. A time-dependent increase in BAX and concomitant decrease in BCL-2 expression was observed in all cell lines (Fig. 5). Quantification of the Western blot images and calculation of the BAX:BCL2 ratio clearly showed a marked ($P < 0.05$) increase of BAX:BCL2 following combined treatment versus either single treatment in cancer cells (Fig. 5). However, in immortalized cells no significant changes were noted in BAX:BCL2 between vehicle-treated cells versus single or combination treatment cells (Fig. 5).

**Knockdown of BAX enhances growth of cancer cells and restoration of BAX expression by progesterone and calcitriol attenuates proliferation**

To confirm that the progesterone and calcitriol-induced inhibition of endometrial cancer cell growth is mediated via BAX expression, we transfected cells with BAX-targeted siRNA oligonucleotides. Cells were harvested at 24 hours after transfection and BAX expression was analyzed by Western blot analysis (Fig. 6A). Although BAX-specific siRNA suppressed BAX synthesis almost completely, control (scrambled) siRNA had no effect. Next, we assessed the effect of BAX-specific siRNA on cell viability. Progesterone-, calcitriol-, and combination-induced growth inhibition was attenuated in BAX knockdown cells compared with scrambled siRNA-transfected cells (Fig. 6B). BAX expression was correlated with cell proliferation using Pearson correlation. Strong correlation (correlation coefficient $r = 0.81$; $P < 0.001$, HEC-1B, $r = 0.90$; $P < 0.001$, Ishikawa, $r = 0.89$; $P < 0.001$, RL-95) was observed between BAX expression and inhibition of cell growth.

**Discussion**

Although both progesterone and calcitriol may have potential as therapeutic interventions in selected cancers, adverse side effects associated with high dosages may limit their clinical use, especially for calcitriol, which can cause harmful hypercalcemia. These limitations could be overcome if the 2 agents act additively or synergistically or via a different mechanism of action, thereby allowing the use of lower doses of each. On the basis of this rationale, in the present study, we tested the efficacy of progesterone in combination with calcitriol against endometrial cancer. The results show that progesterone and calcitriol produced dose-dependent growth inhibition in endometrial cancer and in immortalized endometrial cells, although growth
Inhibition of Cancer by Progesterone and Calcitriol

Figure 3. Progesterone and calcitriol induce cell-cycle arrest in endometrial cancer cells. A–D, cells were treated with progesterone (20 μmol/L), calcitriol (100 nmol/L), or a combination for 24 hours and stained with propidium iodide. DNA content was analyzed using flow cytometry. Results are shown as the percentage of cell population in G1, S, and G2-M phases of the cell cycle. E, Cell-cycle regulatory proteins expression in progesterone- and calcitriol-treated cells. The immunoblots shown are representative of 3 independent experiments with similar results. The values above the bands represent relative density of the bands normalized to β-actin.
inhibition was less pronounced with calcitriol treatment alone. Of note, cells exposed to a combination of these agents showed greater inhibition than did cells exposed to either agent alone, suggesting an additive effect on tumor cell growth. Our results agree with preclinical studies showing that calcitriol has additive effects when used in combination with progesterone.

Table 1. Effect of progesterone and calcitriol on cell-cycle arrest

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<thead>
<tr>
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<th>EM-E6/E7-TERT</th>
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<td></td>
<td>G0–G1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>S</td>
<td>G2–M</td>
<td>G0–G1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>S</td>
<td>G2–M</td>
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<tr>
<td>Control</td>
<td>56.39 ± 0.43</td>
<td>24.58 ± 0.56</td>
<td>19.03 ± 0.95</td>
<td>36.44 ± 0.35</td>
<td>45.28 ± 0.84</td>
<td>18.28 ± 0.58</td>
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<tr>
<td>PROG</td>
<td>59.19 ± 0.84</td>
<td>25.70 ± 0.73</td>
<td>15.11 ± 0.17</td>
<td>60.94 ± 0.45</td>
<td>30.54 ± 0.53</td>
<td>8.53 ± 0.16</td>
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<tr>
<td>Calcitriol</td>
<td>63.71 ± 0.37</td>
<td>21.34 ± 0.73</td>
<td>14.95 ± 0.44</td>
<td>38.01 ± 0.57</td>
<td>43.05 ± 0.26</td>
<td>18.94 ± 0.42</td>
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<tr>
<td>Prog + calcitriol</td>
<td>62.86 ± 0.57&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20.04 ± 0.46</td>
<td>17.11 ± 0.11</td>
<td>60.24 ± 0.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>30.38 ± 0.83</td>
<td>9.38 ± 0.48</td>
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Abbreviation: HSD, honest significant difference.

<sup>a</sup>Statistically significant difference from PROG, calculated by Tukey HSD (P < 0.05).

<sup>b</sup>Statistically significant difference from calcitriol calculated by Tukey HSD (P < 0.05).

<sup>c</sup>Statistically significant difference across the entire cell line, calculated by two-way ANOVA (P > 0.05).

Figure 4. Identification and validation of new progesterone and calcitriol targets in endometrial cancer cells. A, total number of proteins altered in endometrial cancer cells by progesterone, calcitriol, and combination treatment. Common proteins differentially regulated by all treatments are shown in the center and 4 proteins selected for validation are listed. B, verification of a subset of progesterone- and calcitriol-regulated proteins in endometrial cancer cell lines by Western blotting. Representative Western blot analyses of 3 independent experiments with similar results are shown. The values above the bands represent relative density of the bands normalized to β-actin.
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Combination therapy with chemotherapeutic agents including cisplatin and paclitaxel (27, 21). Such combination therapy potentially offers a therapeutic advantage over a standard dose of a single drug by enhancing the effectiveness of the therapy without increasing the dose, thus limiting dose-dependent toxic side effects.

Calcitriol has antiproliferative and antineoplastic activities, which include activation of apoptosis, induction of cell-cycle arrest, differentiation, inhibition of invasion and motility, and reduction of angiogenesis (28, 29). These activities are exerted through both genomic and nongenomic pathways. The classic genomic response is believed to be the most responsible for the vitamin D action, and it is mediated through the VDR. The VDR is highly expressed in a large number of tumor tissues including ovarian, endometrial, cervical, breast, and colorectal cancer (30–36). Herein, we showed for the first time that progesterone upregulated VDR expression in endometrial cancer but not in immortalized endometrial cells, suggesting that progesterone is able to improve calcitriol’s biologic effects in cancer cells. This is corroborated by our results showing a significant decrease in cell proliferation in cells treated with a combination of progesterone and calcitriol. Although progesterone-treated cells showed a dramatic increase in VDR expression, calcitriol-treated cells displayed a much more subtle increase. This suggests low basal VDR expression in the cell lines of interest, which may explain the moderate antiproliferative activity caused by calcitriol treatment in comparison with progesterone or combination treatment.
Because apoptotic stimuli often arrest growth before inducing cell death and as significant apoptosis was not evident in calcitriol-treated endometrial cells for the first 24 hours (results not shown), we opted to investigate the early effects of progesterone, calcitriol, and combined treatment on the cell cycle in the first 24 hours of exposure. Our results showed G0–G1 cell-cycle arrest following progesterone treatment. The combined treatment resulted in a greater number of cells undergoing cell-cycle arrest in the Ishikawa cell line. However, combined treatment failed to show an additive effect on the cell cycle in EM-E6/E7-TERT, RL-95, and HEC-1B cell lines. In some cell lines, the delayed effect of calcitriol and combined treatment can be attributed to the sensitivity of cell lines to treatment as 48-hour treatment (data not shown) significantly increased cell-cycle arrest in immortalized endometrial cell line. In EM-E6/E7-TERT cells, increased cell-cycle arrest in G0–G1 in response to calcitriol can be ascribed to increased VDR expression (Fig. 2). In nontumorigenic cells, this is not surprising because vitamin D3/VDR signaling is involved in maintenance of cellular homeostasis and modulation of growth by interactions with other transcriptional regulators and cell signaling systems, including TGF-β and β-catenin, to control cell growth and differentiation.

Cells progress through the various phases of the cell cycle via the interactions of different cyclins with their respective
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CDK subunits (37–39). Quiescent cells enter the cell cycle after mitogenic stimuli and upregulate cyclins D and E during the G1 phase of the cell cycle (38). Cyclins D1 and D3 are known to bind with and activate CDK4, which phosphorylates Rb (Rb) protein to release E2F transcription factors to transcribe genes needed for the G1 to S transition (40). Cyclin D1 is considered an oncogene and is over-expressed in many cancers including prostate, breast, esophagus, lung, head and neck, and colon (41–43). The Cip/Kip family of CDK inhibitors binds to and inhibits the activity of CDK-cyclin complexes that regulates G1–S and G2–M phase transitions (38, 40). The Cip/Kip family plays a critical role in the cellular response to DNA damage for cell-cycle arrest (44, 45) and is upregulated in response to antiproliferative signals for cell-cycle arrest (46, 47).

Endometrial cancer cells treated with a combination of progesterone and calcitriol showed a decrease in cyclins D1 and D3, and an increase in p27 expression. Others (48, 49) have shown an increase in the steady-state levels of p27 protein in prostate and endometrial cancer cells in response to calcitriol. Although cyclins are downregulated, we cannot conclude from our studies that cyclins are direct transcriptional targets of calcitriol and progesterone. Cyclin suppression may have occurred as a downstream consequence of the prior modulation of direct target genes.

Induction of apoptosis in cancer cells is another approach to limit their uncontrolled proliferation (50, 51). In this process, activation of caspases is the central event (50, 51). Once activated, the executioner caspases downstream of the caspase cascade act on the key molecules inside the cells to orchestrate cell death (51). In fact, many cancer preventive and chemotherapeutic agents have been shown to activate the caspases. Cleavage of caspase-3 is considered to be a marker for apoptotic death (51). Confirming these reports, our study shows treatment of endometrial cancer cells with progesterone, calcitriol, and the combination significantly enhanced levels of cleaved caspase-3. Together these observations suggest that progesterone and calcitriol inhibit cell growth through apoptosis.

Many proteins either sensitize cancer cells to apoptosis or are directly involved in apoptosis. In an effort to identify the molecular targets of progesterone and calcitriol in endometrial cancer cells, we used a proteomic approach. Our data showed 117 proteins differentially regulated by progesterone, calcitriol, and the combination. The 4 apoptosis-related proteins selected for validation were upregulated by all 3 treatments in endometrial cancer cells. To our knowledge, this is the first report showing BAX, HIST1H1E, HINT2, and EIF2AK2 as tumor suppressors of endometrial cancer and showed their regulation by progesterone and calcitriol. These proteins have not been reported to be regulated by progesterone or calcitriol in endometrial cancer.

BAX resides in the cytosol and translocates to mitochondria upon induction of apoptosis. BAX has been shown to induce cytochrome c release and caspase activation. Its expression is considerably diminished at the mRNA and protein level in cancerous tissues. Suppression of BAX gene expression promoted tumorigenesis, whereas its overexpression showed an inhibitory effect on cell growth (52).

In the present study, lower basal levels of BAX expression were observed in endometrial cancer cell lines than in immortalized endometrial cells. Progesterone and calcitriol were shown to upregulate BAX expression in cancer cells. Cell survival is maintained by a fine balance of the proapoptotic (e.g., Bad and Bax) and antiapoptotic proteins (e.g., BCL-2 and BCL-xL), which control the process of apoptosis through release of caspases (51). Therefore, the increase in the ratio of BAX to BCL-2 proteins observed following treatment of endometrial cancer cells with a combination of progesterone and calcitriol could be responsible for the observed apoptotic effect. Experimental alterations of BAX levels disrupt cellular processes. We were able to elevate BAX protein expression via progesterone and calcitriol treatment in endometrial cancer cells and subsequently relate their effects to cellular functions such as proliferation. The highlight of this study is that BAX knock-down attenuated progesterone- and calcitriol-induced growth inhibition. Interestingly, the upregulation of BAX by combined treatment of endometrial cancer cells resulted in a greater decrease in cell proliferation compared with either progesterone or calcitriol alone. This suggests that the upregulation of BAX by a combined treatment may have a cumulative effect on inhibition of cell growth.

Our results showed no significant effects of progesterone and calcitriol treatment on BAX-BCL2 expression in EM-E6/E7-TER cells. Normal cells maintain critical balance of proteins promoting or inhibiting cell proliferation. Knocking down of BAX perturbs the balance and exposure to the 2 agents triggers expression of proteins that may inhibit cell growth.

These results provide evidence that calcitriol and progesterone produce cell death in endometrial cells by apoptosis. All investigations into vitamin D and cell death to this point have pointed to an apoptotic mechanism. Although caspase cleavage and the action of other apoptosis-related molecules usually occurs within 24 hours or less, it has been shown that vitamin D acts much more slowly to effect apoptosis. For example, Zhang and colleagues (53, 54) have shown the strongest apoptotic response at 6 days following treatment using a vitamin D analog in several cell lines. Mathiasen and colleagues (55) also showed peak vitamin D–induced apoptosis at 6 to 7 days posttreatment. The latter group also concluded that vitamin D–induced apoptosis may not be caspase-dependent. Although we have not conducted caspase inhibition studies, it should be noted that cleaved caspase-3 increased primarily in response to progesterone treatment, particularly in combination with calcitriol. The cumulative evidence presented herein points strongly to an apoptotic pathway for cell death in these endometrial cancer cell lines in response to progesterone and calcitriol.

Induction of chromatin and condensation and cleavage of DNA are characteristic steps of apoptosis, leading to the formation of oligomers of nucleosomes. HIST1H1E is
essential for the generation of condensed structures and initiation of the subsequent internucleosomal cleavage of DNA, which is a hallmark of programmed cell death. During early apoptosis, induction of internucleosomal DNA cleavage and increase in HIST1H1E expression is a prerequisite for DNA accessibility and/or endonuclease. Similar to our findings in human leukemic cells, HIST1H1E is low and expression increases when cells are exposed to an apoptosis-inducing monoclonal anti-CD95 antibody (56).

HINT proteins are AMP–lysine hydrolases. HINT2 is a mammalian-specific, nuclear-encoded mitochondrial HINT hydrolase. We have shown low expression of HINT2 in endometrial cancer cells compared with control cells and expression of HINT2 was upregulated by progesterone and calcitriol treatment. These results are in agreement with a study suggesting that HINT2 overexpression sensitizes hepatocellular carcinoma to apoptosis (57).

EIF2AK2 is a member of the eukaryotic initiation-factor 2 α kinase family. EIF2AK2 has been shown to play a significant role in signaling pathways involved in other cellular processes such as cell proliferation, differentiation, metabolism, DNA repair, tumor suppression, and apoptosis (58). EIF2AK2 regulates TNF-β-induced p53-dependent apoptosis in U937 cells. In addition, it can interact directly with p53 and phosphorylate it (58). The identification of these new progesterone and calcitriol molecular targets in endometrial cancer cells offers unique opportunities for future development of novel strategies for endometrial cancer therapies.

In summary, we conclude that progesterone combined with calcitriol enhances expression of proapoptotic (BAX, HIST1H1E, HINT2, and EIF2AK2) proteins and causes apoptosis and cell-cycle arrest in endometrial cancer cells.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References
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Cancer Prevention Research

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