Research Article

Progesterone Inhibits Endometrial Cancer Invasiveness by Inhibiting the TGFβ Pathway

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Abstract

Increased expression of TGFβ isoforms in human endometrial cancer correlates with decreased survival and poor prognosis. Progesterone has been shown to exert a chemoprotective effect against endometrial cancer, and previous animal models have suggested that these effects are accompanied by changes in TGFβ. The goal of this study was to characterize the effect of progesterone on TGFβ signaling pathway components and on TGFβ-induced protumorigenic activities in endometrial cancer cell lines. Progesterone significantly decreased expression of three TGFβ isoforms at 72 hours after treatment except for TGFβ2 in HEC-1B and TGFβ3 in Ishikawa cells. Progesterone treatment for 120 hours attenuated expression of the three isoforms in all cell lines. Progesterone exposure for 72 hours reduced expression of TGFβ receptors in HEC-1B cells and all but TGFβR1 in Ishikawa cells. Progesterone reduced TGFβ3 expression in RL-95 cells at 72 hours, but TGFβR1 and βR2 expression levels were not affected by progesterone at any time point. SMAD2/3 and pSMAD2/3 were substantially reduced at 72 hours in all cell lines. SMAD4 expression was reduced in RL-95 cells at 24 hours and in HEC-1B and Ishikawa cells at 72 hours following progesterone treatment. Furthermore, progesterone effectively inhibited basal and TGFβ1-induced cancer cell viability and invasion, which was accompanied by increased E-cadherin and decreased vimentin expression. An inhibitor of TGFβR1 blocked TGFβ1-induced effects on cell viability and invasion and attenuated antitumor effects of progesterone. These results suggest that downregulation of TGFβ signaling is a key mechanism underlying progesterone inhibition of endometrial cancer growth. Cancer Prev Res; 7(10); 1045–55. ©2014 AACR.

Introduction

The TGFβ family members are secreted cytokines that regulate a broad array of cellular responses, including proliferation, differentiation, migration, and apoptosis (1, 2). The role of TGFβ in tumor biology is complex. It has tumor-suppressing activity in the early stages of carcinogenesis, whereas in the later stages of carcinogenesis, it functions as a tumor promoter (2, 3). The growth-inhibitory function of TGFβ is selectively lost in advanced cancers due to mutational inactivation or dysregulated expression of various components of the TGFβ signaling pathway resulting in growth, invasion, and metastasis of cancer cells (4).

Human endometrial tumors contain the three TGFβ isoforms in both the epithelial and stromal counterparts of the tumors, and these proteins are responsible for autocrine as well as paracrine signaling in the microenvironment of these tumors. The different TGFβ isoforms can sometimes differentially activate signaling pathways in cancer cells, leading to isoform-specific effects on cellular phenotype (5, 6).

Overexpression of TGFβ1 has been reported in many (pancreas, breast, prostate, ovary, and endometrium) tumors and is associated with metastatic phenotypes and poor patient outcome (7, 8). TGFβ1 signals are largely known as tumor promoters of cellular responses such as proliferation, survival, migration, and invasion (4). Compared with TGFβ2 and TGFβ3, high levels of TGFβ1 are reported in endometrial cancer cell lines and these findings are corroborated with abundant TGFβ1 immunoreactivity in human endometrial tumors in vivo (8).

TGFβ2 is expressed in a variety of cancer cell lines, including glioma, prostate, breast, lung and kidney, and endometrium (9). In endometrial cancer of the three isoforms, TGFβ2 is the least expressed. Increase of TGFβ2 protein levels was observed in advanced-stage endometrial tumors (9). TGFβ2 promotes the survival of tumor cells by
activating NF-κB. Suppression of TGFβ2 expression by siRNA attenuates cancer cells growth, indicating that the TGFβ2–NF-κB pathway is important for viability of tumor cells (3).

High levels of TGFβ3 are detected in the glands in endometrial carcinoma compared with TGFβ3 detected by immunostaining in the glandular epithelial cells of the normal proliferative and secretory endometrium (6). TGFβ3 is shown to increase invasiveness of endometrial cancer (KLE and HEC-1A) cells through two signaling pathways: PI3K-dependent upregulation of X-linked inhibitor of apoptosis protein and through protein kinase C–dependent induction of matrix metalloproteinase-9 (MMP-9) expression (6). Upon progression toward a more invasive phenotype (from stage I to stage III endometrial adenocarcinoma), TGFβ3 immunoreactivity gradually extends from the epithelial compartment (in normal tissues) to the stroma (in adenocarcinoma), consistent with a role for TGFβ3 in endometrial cancer cell invasiveness (6, 8).

TGFβ ligands bind to TGFβ receptor type 2 (TGFβR2), a constitutively active transmembrane serine/threonine kinase, and forms an oligomeric complex with TGFβ receptor type 1 (TGFβR1). TGFβR1 is phosphorylated by the TGFβR2. The activated TGFβR1 initiates intracellular signaling through phosphorylation of specific receptor-regulated (R)-SMAD proteins (SMAD2 and SMAD3). Activated (R)-SMADs form a complex with SMAD4, and the complex translocates to the nucleus, where it binds to DNA in a sequence-specific manner, and regulates gene transcription (3, 4).

Immunohistochemical analysis has shown higher expression of TGFβR1, TGFβR2, and TGFβR3 in endometrial tumors compared with normal tissues (10, 11). Deregulation of TGFβR3 expression not only results in loss of antiproliferative activity of TGFβ signaling at early stages of endometrial cancer development but also leads to acquisition of cell motility. High TGFβ3 expression plays a role in mediating cell proliferation and migration in endometrial tumors (9). TGFβR3 binds all three TGFβ isoforms. The function of TGFβR3 is to present ligands to TGFβR2 and TGFβR1, which, upon ligand binding, transphosphorylate to activate the type I TGFβR1 (12, 13).

An important step in the metastatic process is epithelial to mesenchymal transition (EMT), in which adherent and nonmotile epithelial cells acquire motility and invasiveness (14). Increased expression of TGFβ induces EMT by enhancing the expression of zinc-finger transcriptional factors Snail and Slug and downregulating expression of epithelial marker E-cadherin, which is critical in mediating epithelial cell integrity and cell–cell adhesion (15). Subsequently, mesenchymal markers N-cadherin, vimentin, and MMP proteins are elevated and thus cell motility and invasive phenotypes (14, 16). Cancer cells overexpressing active TGFβ have increased metastatic ability (17, 18), and targeting of TGFβ signaling prevents metastasis in several neoplastic tumors, including breast, prostate, and colorectal cancers (19–21).

Progestins are highly effective endometrial cancer–preventive agents. Extensive epidemiologic investigations demonstrate that long-term progestins use is associated with significant endometrial cancer risk reduction (22–23). Progesterone has also been used as a primary treatment for endometrial carcinoma in premenopausal women, and response rates in these women can be as high as 60%, indicating that progesterone is a potent inhibitor of endometrial carcinogenesis (24, 25). Recent studies in primates have demonstrated a potential mechanism underlying the chemopreventive effect of progestins [levonorgestrel and medroxyprogesterone acetate (MPA)] against ovarian and endometrial cancer. Progestin exposure was associated with increased apoptosis and a concomitant marked decrease in the expression of TGFβ1 in the ovarian and endometrial glandular epithelium, and a concomitant increase in the expression of TGFβ2 and 3 in the ovarian epithelium and endometrial stroma, respectively (26, 27). This suggests that the antitumorigenic effects of levonorgestrel and MPA may be at least, in part, mediated by altering the expression of TGFβ signaling components.

Despite the importance of TGFβ in regulating several endometrial cellular activities (4, 28), the effect of progesterone on the expression and regulation of TGFβ signaling components in human endometrial cancer has not been well characterized. In the present study, we examined the effect of progesterone on the TGFβ/SMAD signaling pathway in endometrial cancer cells and showed that progesterone inhibits TGFβ-induced R-SMAD expression, nuclear translocation, and subsequently attenuates tumor cell growth and invasiveness by decreasing vimentin and increasing E-cadherin.

Materials and Methods

Cell culture and treatment

Human endometrial cancer cell lines HEC-1B and RL-95 were obtained from the ATCC and the Ishikawa cell line was obtained from Sigma. The cell lines were authenticated by DNA short-tandem repeat analysis by the ATCC and Sigma. All three 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. Ishikawa cells were grown in DMEM:F12 supplemented with insulin (Invitrogen), HEC-1B cells were cultured in Eagle minimum essential medium (Invitrogen), and RL-95 cells were grown in DMEM:F12 medium supplemented with insulin (0.005 mg/mL). All three media were supplemented with 10% (v/v) FBS (Invitrogen), 100 U/mL penicillin, and 100 U/mL streptomycin. The cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂. Forty-eight hours later, the media were replaced with same media but containing charcoal-stripped FBS. The cells were treated with progesterone (20 μmol/L, 99.9% pure; Sigma) for 24, 72, and 120 hours and collected for protein extraction. The time of treatment and dose of progesterone was based on our previous studies showing inhibition of cell growth and apoptosis of cancer.
cells (29). For a set of experiments, cells were treated with progesterone (20 μmol/L) or TGFβ1 (10 ng/mL) in the presence or absence of TGFβR1 blocker (SD-208, 10μmol/L; Santa Cruz Biotechnology) for 72 hours and the effects on cell viability and invasion were assessed. Progesterone solution was in absolute ethanol, TGFβ1 solution was in aqueous saline, and TGFβR1 blocker was in DMSO. The final concentrations of ethanol and DMSO in the media were 0.1%. Untreated control cultures were exposed to equal concentrations of ethanol, aqueous saline, or DMSO vehicle alone.

Measurement of TGFβ1 concentration in the conditioned media
To study the effect of progesterone on the secretion of TGFβ in culture, endometrial cells were treated for 72 hours with progesterone or TGFβ1 in the presence or absence of progesterone receptor antagonist Milifepristone (0.1 mmol/L). After culture, cells were removed by centrifugation and the cell-free media were collected and stored at −80°C until use. After thawing at room temperature, the media were assayed for immunoreactive latent TGFβ by enzyme-linked immunosorbent assay (BioLegend) according to the manufacturer’s specifications with absorbance at 450 nm in a plate reader (ELX800). The immunoassay kit used in this experiment offered a dynamic range starting in the low ng/mL and covered three logarithms. Experiments were repeated at least three times with different media to minimize the intra- and interassay variation.

Cell viability assay
Cell viability of endometrial cancer cells treated with progesterone, TGFβ1, or the combination in the presence or absence of TGFβR1 blocker was evaluated 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 an ELX800 microtiter Reader. Relative cell viability was expressed as percent change of treated cells over vehicle-treated cells.

Cell invasion assay
Endometrial cancer cells treated with progesterone, TGFβ1, or the combination in the presence or absence of TGFβR1 blocker for 72 hours were detached by trypsin and resuspended in serum-free medium. Medium containing 10% FBS medium was applied to the lower chambers of BD BioCoat Matrigel Invasion Chambers (BD Biosciences) as chemoattractant, and then, cells were seeded on the upper chambers at a density of 2.5 × 10⁴ cells per well in 100 μL of serum-free medium without progesterone or TGFβ1. The chambers were incubated for 16 to 18 hours at 37°C. At the end of incubation, noninvading cells were removed from the upper surface of the membrane by scrubbing. The cells on the lower surface of the membrane were fixed for 2 minutes in 100% methanol and stained with 1% toluidine blue in 1% sodium borate for 2 minutes. Excess stain was removed by rinsing the inserts with water. Each membrane was removed from the insert and placed on a microscope slide. The cover slip was placed on the slide and cells were counted in five random fields per slide. All slides were coded to avoid biased counting. The assay was run in triplicates.

Western blot analysis
Cell lysates were prepared in RIPA buffer [50 mmol/l Tris-HCl (pH 8.0), 150 mmol/l NaCl, 0.5% deoxycholate, 1.0% NP40, and 0.1% SDS] supplemented with a protease inhibitor mixture solution (Roche Molecular Biochemicals). After sonication, cell debris was pelleted by centrifugation, and protein concentration was determined with the BCA Protein Assay Reagent (Pierce). Equivalent amounts of proteins were resolved by 10% SDS–PAGE and transferred to PVDF membranes. The blots were then incubated with primary antibodies against TGFβ1, TGFβ2, TGFβ3, TGFβR1, TGFβR2, TGFβR3, pSMAD2/3, SMAD2/3, SMAD4 (Santa Cruz Biotechnology), E-cadherin, vimentin (Cell Signaling Technology, Inc.), and β-actin (Sigma-Aldrich). The Enhanced Chemiluminescence Detection System (Pierce), followed by autoradiography, was used for protein visualization. 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.

Immunofluorescence
Cells were plated on a glass bottom culture dish (MatTek Corporation) at a density of 0.3 × 10⁶ cells per dish. Cells were grown to approximately 50% confluence in chamber slides and then treated with or without TGFβR1 blocker for 2 hours before treatment with either vehicle, progesterone, TGFβ1, or a combination of progesterone and TGFβ1 for a 24-hour period. After treatment, cells were washed twice with PBS and fixed with 4% parafomaldehyde for 15 minutes. Cells were then permeabilized with 100% methanol at −20°C, and care was taken to prevent desiccation of the samples. After complete removal of methanol, the samples were blocked using buffer prepared with normal goat serum for 60 minutes. SMAD2/3 antibody (Santa Cruz Biotechnology) diluted at 1:250 was applied overnight at 4°C. After complete removal of primary antibody, the diluted secondary antibody, Alexa Fluor 488 Goat Anti-mouse IgG (Invitrogen; Cat# A-11001), was applied. Samples were incubated for 2 hours at room temperature in the dark. Once excess secondary antibody was removed by rinsing with PBS, 4’6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI, 1 μg/mL; Sigma) was applied to cover slides for 30 seconds in the dark and then rinsed away with PBS. Coverslips were then mounted with Prolong Gold Antifade Reagent (Invitrogen; Cat# P36930). Slides were imaged on a Zeiss Pascal Confocal Microscope (Carl Zeiss Microscopy GmbH) immediately following preparation. Z stack slices obtained through confocal microscopy were then summed in ImageJ (NIH).
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 the Tukey post hoc test. A P value of less than 0.05 was considered statistically significant. Western blots 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 attenuates TGFβ ligands in endometrial cancer cells
Endometrial tumors have been reported to express high levels of three TGFβ isoforms and TGFβ receptors in vivo (6). In addition, it has been suggested that TGFβ1 plays a major role in the initiation of endometrial carcinoma invasion (21). To determine whether progesterone regulates the expression of TGFβs and their receptors, HEC-1B, Ishikawa, and RL-95 human endometrial carcinoma cell lines were exposed to progesterone for 24, 72, and 120 hours. Exposure of endometrial cells to progesterone for 120 hours decreased secretion of TGFβ1, -β2, and -β3 in all cell lines tested. All isoforms showed a marked decrease in expression following 72 hours of exposure except for TGFβ2 and TGFβ3 in HEC-1B and Ishikawa cells, respectively. In RL-95 cells, all three TGFβ isoforms showed a significant decrease with progesterone in 72 hours of culture. No alteration of TGFβ isoforms was seen in any cell line with progesterone in the first 24 hours of exposure (Fig. 1A).

Progesterone inhibits latent TGFβ concentration in the culture medium of cancer cells
To ascertain if progesterone regulates secretion of TGFβ, the amount of TGFβ secreted into the culture media was assayed by ELISA. As evident in Fig. 1B, cells exposed to TGFβ1 secreted high levels of latent TGFβ compared with vehicle-treated cells. TGFβ secretion by endometrial cancer cells was markedly inhibited by addition of 20 μmol/L progesterone for 72 hours compared with vehicle-treated cells. TGFβ1-induced secretion of latent TGFβ was attenuated by progesterone. No such inhibiting effect was observed in the culture media of cells treated with progesterone receptor antagonist (Mifepristone) and progesterone or TGFβ1 progesterone combination. Mifepristone-treated cells showed no effect on TGFβ. These results clearly suggest that cancer cells secrete TGFβ, and progesterone, at least in vitro, inhibits basal and TGFβ1-induced secretion. Down-regulation of TGFβ1 was blocked by the antagonist Mifepristone providing evidence for the specificity of progesterone action in cancer cells.

TGFβ receptors are downregulated by progesterone in endometrial cancer cells
TGFβ acts through a membrane-associated receptor complex. We evaluated the protein expression of TGFβR1, TGFβR2, and TGFβR3 in endometrial cultures exposed to progesterone for 24, 72, and 120 hours. In HEC-1B cells, expression of TGFβR1, TGFβR2, and TGFβR3 was significantly (P < 0.05) downregulated following progesterone exposure for 72 and 120 hours compared with vehicle-treated cells. Ishikawa cells showed a marked reduction of TGFβR1 expression at 120 hours of cell culture with progesterone, whereas TGFβR2 and TGFβR3 were inhibited at 72 and 24 hours, respectively. Progesterone inhibited TGFβR3 expression in RL-95 at 72 and 120 hours exposure and had no effect on TGFβR1 and TGFβR2 at any time point (Fig. 2).

SMAD expression is inhibited by progesterone in endometrial cancer cells
In the canonical signaling pathway, SMAD phosphorylation is an indicator of functional TGFβ receptors. To confirm that progesterone inhibits TGFβ signaling transduction, protein levels of downstream SMADs were investigated. We examined levels of total and phosphorylated SMAD2/3 (p-SMAD2/3) in endometrial cancer cells exposed to progesterone for 24, 72, and 120 hours. Incubation with an antibody recognizing p-SMAD2 and 3 at Serine 423/425 showed that SMAD2/3 is phosphorylated in nontreated cancer cells. A significant reduction in SMAD2/3 and pSMAD2/3 was apparent 72 and 120 hours after exposure to progesterone in all cell lines except RL-95, where pSMAD2/3 showed significant inhibition at 24 hours of treatment. Total and pSMAD2/3 levels were not affected by progesterone in HEC-1B and Ishikawa cells during the first 24 hours of treatment (Fig. 3). These results imply that progesterone is a potent TGFβ antagonist in endometrial cancer cells. HEC-1B and Ishikawa cells exposed to progesterone for 72 and 120 hours showed pronounced decrease in SMAD4 expression. RL-95 cells exhibited a marked decrease in SMAD4 expression after 24 hours of progesterone treatment (Fig. 3).

Furthermore, we investigated the effect of progesterone on the TGFβ1-induced subcellular localization of SMAD2/3 in HEC-1B and Ishikawa endometrial cancer cells. In vehicle-treated cells, SMAD2/3 was expressed in the cytoplasm and in the nucleus. Progesterone and TGFβ1 treatment decreased cytoplasmic SMAD2/3 (Fig. 4A and C). To establish that high SMADs localization in cancer cells is activated via an autocrine pathway, endometrial cancer cells pretreated with the TGFβ receptor I blocker and then exposed to TGFβ1 or a combination of TGFβ1 and progesterone showed significantly attenuated nuclear localization of SMAD2/3 (Fig. 4B and D) compared with non-TGFβ receptor I blocker pretreated cells (Fig. 4A and C). These findings strongly suggest that nuclear localization of SMAD2/3, which is an indication of the activated TGFβ/SMAD signaling pathway, is attributed to TGFβ produced and secreted by endometrial cancer cells.
Figure 1. A, downregulation of TGFβ isoforms expression in endometrial cancer cells by progesterone. Endometrial cancer cells (HEC-1B, Ishikawa, and RL-95) treated with progesterone (PROG, 20 μmol/L) for 24, 72, and 120 hours were evaluated by Western blot analysis for expression of TGFβ1, TGFβ2, and TGFβ3. β-Actin was used as a loading control. Data, representative of three independent experiments. B, concentration of latent TGFβ present in the culture media of endometrial cancer cells treated for 72 hours with progesterone (20 μmol/L), TGFβ1 (10 ng/mL), or the combination in the absence or presence of progesterone receptor antagonist Mifepristone (0.1 mmol/L). The concentrations of TGFβ present in the conditioned media of cells were measured by ELISA, as described in Materials and Methods. Measurement was made at least three times per sample and presented as mean ± SD. *: P < 0.05 compared with control.

**Progesterone attenuates TGFβ-mediated cell viability and invasion**

TGFβ1 has a principal role at the initiation of endometrial carcinoma invasion through the promotion of the EMT that leads to the acquisition of an invasive phenotype. In addition, although TGFβ1 is a potent inhibitor of proliferation of most types of normal epithelia, it has a paradoxical proliferative effect in carcinomas (30). To explore the effect of progesterone and TGFβ1 on cell proliferation and invasion, we examined the effect of progesterone, TGFβ1, or the combination in the presence or absence of TGFβR1 blocker (72 hours) on cell viability and cellular invasiveness. As shown in Fig. 5A, TGFβ1 treatment of endometrial cancer cells induced a marked increase in cell viability compared with cells treated with vehicle. As expected, progesterone alone inhibited cell viability. Interestingly, progesterone markedly abrogated TGFβ1-induced cell viability in both cell lines shown. TGFβ1 failed to enhance cell viability in the presence of TGFβR1 blocker. Moreover, progesterone-induced inhibition of cell viability was attenuated by TGFβR1 blocker.

The effects of progesterone and TGFβ1 on the invasiveness of HEC-1B and Ishikawa cell lines were determined by using a boyden chamber invasion assay. Treatment of endometrial cancer cells with TGFβ1 produced a significant increase in invasiveness as compared with vehicle-treated control. In addition, HEC-1B and Ishikawa cells treated with progesterone alone, or TGFβ1 and progesterone in combination, underwent significant decreases in invasiveness when compared with vehicle-treated cells and TGFβ1-treated cells, respectively (Fig. 5B). TGFβR1 blocker markedly reduced the antitumorigenic effect of progesterone on cell invasiveness. These results demonstrate that progesterone inhibits both basal and exogenous TGFβ1-induced promigratory and invasive activity of endometrial cancer cells by suppressing TGFβ signaling.

**Progesterone reduces expression of mesenchymal markers and inhibits EMT process**

The EMT process, which is a crucial step in tumor progression, can be induced by several cytokines and chemokines, including TGFβ (4). To examine if a progesterone-induced decrease in cell invasiveness is associated with the EMT process, we evaluated the expression of EMT marker proteins (vimentin and E-cadherin) in extracts of HEC-1B and Ishikawa cells treated with vehicle, progesterone, TGFβ1, or combination of progesterone and TGFβ1 for 72 hours. Vimentin was upregulated and E-cadherin was downregulated in vehicle-treated cells. The TGFβ1-exposed cells showed decreased expression of E-cadherin and enhanced expression of vimentin. Progesterone treatment caused a significant decrease in vimentin and an increase in E-cadherin expression in cancer cells. A similar expression pattern was observed in TGFβ1- and progesterone-exposed cells. These results suggest that progesterone inhibits the EMT process in endometrial cancer cells (Fig. 6).
TGFβ endometrial carcinoma cells
receptors, and SMADs. High levels of TGFβ
progesterone on TGFβ fill this data gap, we explored the antitumor effect of
TGFβ of information available about the effect of progesterone on
proliferation, and carcinogenesis (36, 37). There is a paucity
can result in loss of restraint of growth, initiation of cell
late stages (2, 4). Impairment of the TGFβ
tumor suppressor at early stages and a tumor promoter at
affected endometrial latent precursors, as well as endome-
therapy has been shown to reverse preexisting PTEN-inac-
30% or more (31, 32). In addition, high-dose progestin
markedly lowers endometrial cancer risk by as much as
Routine use of progestin-containing oral contraceptives
gestins are potent inhibitors of endometrial carcinogenesis.

Figure 2. Progesterone inhibited TGFβ receptor expression in endometrial
cancer cells. Endometrial cancer cells (HEC-1B, Ishikawa, and RL-95)
treated with progesterone (PROG, 20 μmol/L) for 24, 72, and 120 hours
were evaluated by Western blot analysis for expression of TGFβR1, 
TGFβR2, and TGFβR3. β-Actin was used as a loading control. Data,
representative of three independent experiments.

Discussion
A strong body of clinical evidence has shown that pro-
gestins are potent inhibitors of endometrial carcinogenesis. 
Routine use of progestin-containing oral contraceptives
markedly lowers endometrial cancer risk by as much as
30% or more (31, 32). In addition, high-dose progestin
treatment has been shown to reverse preexisting PTEN-inac-
tivated endometrial latent precursors, as well as endome-
trial hyperplasia and even-low grade endometrial cancer in
some women (33–35).

TGFβ can play multiple roles in carcinogenesis, acting as a
tumor suppressor at early stages and a tumor promoter at
late stages (2, 4). Impairment of the TGFβ–SMAD pathway
can result in loss of restraint of growth, initiation of cell
proliferation, and carcinogenesis (36, 37). There is a paucity
of information available about the effect of progesterone on
TGFβ/SMAD signaling in endometrial carcinoma cells. To
fill this data gap, we explored the antitumor effect of
progesterone on TGFβ signaling at three levels: ligands,
receptors, and SMADs. High levels of TGFβs are present in
endometrial carcinoma cells in vitro and in vivo (38–39).
TGFβ isoforms have been shown to act on endometrial cell
growth by autocrine or paracrine mechanisms and are
implicated in several processes of tumorigenesis and metas-
tasis (8). In the current study, progesterone inhibited
expression of TGFβ ligands in all of the tested endometrial
cancer cell lines. Our study provides clear evidence that
the TGFβ produced and secreted by endometrial cancer
is inhibited by progesterone, and the progesterone
receptor antagonist Mifepristone abolished progesterone-
induced inhibition of TGFβ secretion from endometrial
cancer cells.

TGFβ mediates its effects from the surface of cells to
the nucleus through TGFβ receptors. Thus, TGFβRs are the
gateways of intracellular signaling (4). Therefore, drugs
blocking TGFβ receptors’ intracellular activity are urgently
needed to inhibit TGFβ-induced tumor progression. In

Figure 3. Downregulation of SMAD expression in endometrial cancer cells
by progesterone. Endometrial cancer cells (HEC-1B, Ishikawa, and RL-
95) treated with progesterone (PROG, 20 μmol/L) for 24, 72, and 120
hours were evaluated by Western blot analysis for expression of p-
SMAD2/3, SMAD2/3, and SMAD4. β-Actin was used as a loading control.
Data, representative of three independent experiments.
the present report, we show that one of the possible mechanisms by which progesterone inhibits cancer cell viability and invasiveness is through targeting TGFβRs as evident by a marked decrease in TGFβR1, TGFβR2, and TGFβR3 expression.

The TGFβR3 plays a vital role in presenting TGFβ to the signaling receptors, enhancing receptor–ligand interactions and promoting the cooperation between TGFβ type I and II receptors. Decreasing the expression of TGFβR3 in endometrial cancer cells lines by progesterone may lead to decreased endometrial responsiveness to TGFβ, which is a natural modulator of endometrial cell proliferation and ultimately inhibition of endometrial cancer cells invasion and metastasis.

SMAD proteins are major signaling molecules acting downstream of TGFβ receptors. In normal cells, RSMADs are predominantly concentrated in the cytoplasm. Upon binding of TGFβ to TGFβR2 and phosphorylation of TGFβR1, the activated receptor complex phosphorylates SMAD2/3, which forms a complex with SMAD4. This complex then translocates into the nucleus (4). We analyzed the localization of SMAD2/3 and its nuclear translocation in TGFβ1 and progesterone-treated endometrial cancer cells. SMAD2/3 was found in the cytoplasm of endometrial cancer cells. However, it was found at even higher levels in the nucleus, despite the absence of exogenous TGFβ. We analyzed the localization of SMAD2/3 and its nuclear translocation in TGFβ1 and progesterone-treated endometrial cancer cells. SMAD2/3 was found in the cytoplasm of endometrial cancer cells. However, it was found at even higher levels in the nucleus, despite the absence of exogenous TGFβ. This can be explained by the fact that endometrial cancer cells secrete TGFβs, which activate the signaling pathway by an autocrine mechanism. As a result, SMADs eventually translocate into the nucleus where they interact with other transcription factors to induce or repress
transcription of a number of target genes. Our findings show that TGFβ1 treatment of the cells caused translocation of SMAD2/3 from the cytoplasm to the nucleus. Progesterone reduced SMAD2/3 in the cytoplasm. This suggests disruption of SMAD signaling may be in part the mechanism by which progesterone affects tumor cell growth. To establish that SMAD activation in endometrial cancer cells is via an autocrine pathway, cells exposed to TGFβR1 blocker showed lack of SMAD2/3 nuclear localization compared with cells where TGFβR1 was not blocked. These findings substantiate that endometrial cancer cells secrete TGFβ, which activate the signaling pathway by an autocrine mechanism. The discrepancy between confocal results and time-dependent effect of progesterone on SMAD2/3 expression in endometrial cancer cells by Western blotting can be explained by the fact that confocal microscopy is more sensitive than Western blotting. Thus, small decrease of SMAD2/3 in the cytoplasm was not detected by Western blotting. However, when cells were exposed to progesterone for a longer period of time, a marked decrease in SMAD2/3 observed in total cellular extracts can be attributed to an overall decrease in TGFβ/SMAD signaling pathway components and could be implicated in the observed decrease in cell viability and invasion.

Because the findings reported herein suggest a role of progesterone in regulation of the TGFβ/SMAD signaling pathway, it is conceivable that the response of cells to progesterone depends on progesterone receptors. In our recently published study (40), we have shown reduced expression of both isoforms (PR-A and PR-B) in cancer (Ishikawa, HEC-1B, and RL95-2) cell lines compared with an immortalized endometrial epithelial (EM-E6/E7-TERT) cell line, and no marked changes were noticed in the expression of progesterone receptor isoforms in immortalized cells and in endometrial cancer cells following progesterone treatment. Treatment of endometrial cancer cells with progesterone caused a dose-dependent decrease in cell viability, and progesterone receptor antagonist abrogated that effect (29), suggesting the presence of functional progesterone receptors on endometrial cancer cells. Levels of progesterone receptor expression vary in cancer cell lines, which would explain the differences in the time required for progesterone to downregulate TGFβ, TGFβR, and SMADs among cancer cell lines.

Our results showed reduced levels of TGFβ secreted into culture media of cells exposed to progesterone compared with vehicle-treated cells. Abrogation of progesterone-induced decrease of TGFβ into culture media of cells exposed to progesterone receptor antagonist is a testament of the fact that reduction of TGFβ by progesterone is mediated through progesterone receptors. Furthermore, treatment of endometrial cancer cells with progesterone inhibited TGFβ-induced SMAD2 translocation in the nucleus. This effect was not observed when cancer cells were cultured with the progesterone receptor antagonist.
Mifepristone (results not shown). These findings strongly suggest that alterations in TGFβ signaling pathway components are specifically mediated by progesterone.

Results presented herein demonstrate a decrease in basal and TGFβ1-induced viability, and invasive potential of endometrial cells exposed to progesterone. TGFβR1 blockade has shown effects on cellular responses, such as cell-cycle arrest and EMT of epithelial cells in vitro (41, 42). The fact that TGFβR1 blocker could completely block TGFβ1-induced viability and invasion and markedly attenuated progesterone-induced suppression of both phenotypes suggests that progesterone is exerting its effects via the TGFβ/SMAD signaling pathway. A significant decrease in cell viability and invasion seen with progesterone in TGFβR1 blocker–treated cells indicates that progesterone uses other pathways as well to inhibit malignant phenotypes. These results are in agreement with studies demonstrating that blocking TGFβ receptors inhibits TGFβ1-induced proliferation and reduces the invasive and metastatic potential of human glioma colon, breast, and pancreatic cancer cells (42–44).

In the present study, the inhibitory effect of progesterone treatment alone on cell viability and invasion is more potent than blocking TGFβR. In the previous study, we investigated the effects of progesterone on endometrial cancer cells and identified its targets of action using mass spectrometry–based proteomics. A total of 278 proteins, including 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), showed differential expression with progesterone (40). The dominant inhibitory effect of progesterone on cell viability and invasion can be attributed to upregulation of several tumor suppressor proteins suppressing cancer cell viability and invasive potential independent of the TGFβ signaling pathway.

Although many signaling pathways have been implicated in the regulation of cell migration and invasion, TGFβ is known to be a major modulator of tumor cell migration (4, 45) and invasion, partly due to its promotion of EMT (2). TGFβ has emerged as a promising target for treatment of cancer metastasis (4). The expression of TGFβ1 is increased in various tumors, including endometrial cancer, and correlates with poor patient prognosis (11, 45). TGFβ1 triggers EMT by direct phosphorylation of SMAD2/3, nuclear translocation of active SMAD complexes, and subsequent interaction with nuclear transcription factors and cofactors and activation of tumor-promoting genes. Hence, suppression of the activated TGFβ pathway by progesterone may be a new avenue for potential therapy in human endometrial tumors.

In summary, these findings suggest that progesterone markedly suppresses the TGFβ pathway in endometrial cancers. Progesterone suppresses expression of the TGFβ ligands, thus inhibiting autocrine TGFβ-mediated growth of tumor cells. Progesterone also suppresses TGFβ downstream signaling events via suppression of TGFβ receptors and SMADs. These inhibit the metastatic characteristics of endometrial cancer cells by increasing E-cadherin and decreasing vimentin expression. These data suggest that modification of TGFβ signaling may be a major mechanism underlying the anticancer effects of progesterone in the endometrium.

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

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Authors’ Contributions
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Grant Support
This study was funded by the United States Medical Acquisition Activity Award W81XWH-11-2-0131 (to C.A. Hamilton, G.L. Maxwell, G.C. Rodriguez, and V. Syed).

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Received February 11, 2014; revised July 11, 2014; accepted July 11, 2014; published OnlineFirst July 28, 2014.
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