Original Article

TGF-β signaling proteins and CYP24A1 may serve as surrogate markers for progesterone calcitriol treatment in ovarian and endometrial cancers of different histological types

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Background: Strategies are needed to coordinately block drivers and induce suppressors of cancer to reduce incidence and improve outcomes for individuals with inherited or acquired risk. We previously reported the chemopreventive and therapeutic efficacy of the combination of progestin and calcitriol in transformed and malignant endometrioid endometrial cancer (EC) and in ovarian cancer models involving attenuated expression of TGF-β signaling proteins and progestin-mediated inhibition of calcitriol-induced CYP24A1 expression. This study aims to expand the applications for this combination to other subtypes of endometrial and ovarian cancers, including those with mutations in ARID1A or PIK3CA, DNA mismatch repair (MMR) deficiency or BRCA1 null status.

Methods: Ovarian and EC cell lines of different histotypes were cultured with either progesterone, calcitriol, or the combination of progesterone and calcitriol for 3 or 5 days. The end points for this *in vitro* investigation included assessments of cell growth by (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) assays and the expression of TGF-β ligands, receptors, SMAD proteins and CYP24A1 by western blotting.

Results: Treatment of ovarian clear cell carcinoma, endometrioid carcinoma, papillary serous adenocarcinoma, BRCA1 null, and DNA MMR deficient EC cell lines with progesterone alone or in combination with calcitriol inhibited cell growth and expression of TGF-β1, TGF-β2, TGF-Rβ1, TGF-βR2, pSMAD2/3 and CYP24A1. Expression of TGF-βR3, SMAD-4, progesterone receptor (PR) and vitamin-D receptor (VDR) was not altered in any cell line tested except, ES-2, where VDR expression was upregulated in response to treatment.

Conclusions: These results suggest that progesterone alone and progesterone-calcitriol combination have broad application in both chemopreventive and therapeutic settings that merit further development in a wide variety of ovarian and ECs, including those derived from germline or somatic mechanisms. Moreover, our data suggest that TGF-β signaling proteins and CYP24A1 may be effective surrogate markers indicative of treatment response.

Keywords: Cell proliferation; gynecological cancer, treatment markers, cancer subtypes, chemoprevention

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Introduction

Endometrial cancer (EC) is the fifth most common type of cancer in developed countries. In 2019, there will be approximately 320,000 new cases of EC worldwide, leading to 76,000 deaths (1). Uterine cancers are generally treated with surgery, radiation, hormonal therapy, and/or chemotherapy contingent on stage and cancer type. Surgery is employed to treat patients with early stage disease (stages I and II). Approximately 28% of early stage patients have high-risk disease and also receive radiation and/or chemotherapy in addition to surgery. Among the women with advanced-stage disease (stage III and IV), the majority receive surgery followed by radiation and/or chemotherapy. Despite the success of primary treatment, nearly all women with advanced disease experience a recurrence of cancer, which is often resistant to chemotherapy (2). The unpretentious performance of therapeutic tactics has offered the impetus for the prevention of EC, a viable alternative to chemotherapy.

A compelling body of clinical and epidemiologic evidence suggests that progestins and vitamin-D are highly effective cancer preventive agents. In premenopausal women, progestin containing oral contraceptives use grants a significant reduction in cancer risk (3-5). Moreover, progestin-potent oral contraceptives have increased cancer protective effects compared to oral contraceptives containing weak progestins (6,7). The active form of vitamin D3-calcitriol, produced in the epidermis or obtained from the diet is known for the prevention of a number of tumors, including ovarian and ECs (8,9). Levels of vitamin-D are maintained by a number of enzymes that are involved in the synthesis, activation and inactivation (10). The active 1,25-D3 is neutralized by CYP24A1. This enzyme is vital in determining the antitumor activity of vitamin-D3. It has been shown that high expression of CYP24A1 promotes carcinogenesis in a number of cancers, including breast, thyroid and prostate (11-13). We compared the expression of CYP24A1 in endometrial and ovarian cancer cells and in immortalized endometrial and ovarian epithelial cells, and found elevated CYP24A1 expression in cancer cells compared to normal cells. Furthermore, our data demonstrated a marked reduction of CYP24A1 expression in progesterone treated endometrial and ovarian cancer cells. These findings imply that CYP24A1 overexpression diminishes the antitumor effects of calcitriol in cancer cells and that progestins can be promising for sustaining calcitriol's anti-cancer activity (14,15).

In a recent study, we examined the effects of progesterone, calcitriol, and their combination of EC cells and identified their targets of action. Our results demonstrated that combination treatment of EC cells with both agents impeded cell proliferation through increased vitamin-D receptor (VDR) expression, caspase-3 activation, induction of cell-cycle arrest and downregulation of cyclins (16) TGF-β signaling pathway performs important roles in several biological processes, such as cell growth, differentiation, apoptosis and migration. The TGF-β pathway is dysregulated in tumors and associated with cancer initiation and progression (17-19). Bokhari *et al.* (20) reported a significant decrease in the expression of three TGFβ isoforms, TGF-β receptor and SMAD2/3 in progesterone treated EC cells. Additionally, progesterone effectively reduced basal and TGF β1-stimulated cancer cell viability and invasion, which was associated with increased E-cadherin and decreased vimentin expression. An inhibitor of TGFβRI blocked TGFβ1-induced effects on cell viability and invasion and attenuated antitumor effects of progesterone.

Previously, we have shown that the progesterone and calcitriol combination is highly effective in inhibiting the growth of serous ovarian and endometrial tumors (14-16,20) by attenuating the expression of TGF-β signaling proteins and downregulating the expression of vitamin-D inactivating enzyme, CYP24A1. There is a critical need for developing effective chemopreventive and therapeutic strategies for distinct types of endometrial and ovarian cancer, and to determine if a consistent set of biomarkers may exist for evaluating the effectiveness of treatments initially in preclinical models and ultimately in investigations in human subjects. The availability of a breadth of cancer cell lines from different subtypes provides opportunities for the design of cancer prevention/

treatment studies that can test the specificity or conversely, broad applicability of therapeutic interventions in the clinic (21). The first goal of this study was to determine if the progesterone and calcitriol combination have restricted or broad potential applications in chemoprevention and treatment in a variety of subtypes of endometrial and ovarian cancers, including those with mutations in ARID1A or PIK3CA, DNA mismatch repair (MMR) deficiency or BRCA1 null status. The second goal was to test the hypothesis that TGF-β signaling proteins and CYP24A1 may be used as generalizable surrogate biomarkers of progesterone-calcitriol response in clear cell, endometrioid, BRCA1 null and DNA MMR deficient gynecologic cancers.

Methods

Cell culture and treatment

Human ovarian and endometrial cell lines, ES-2, TOV-21G, TOV-112D, HEC-1A, OV-90, and UWB1.289 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). HEC-59 cells were purchased from AddexBio (San Diego, CA, USA). These human-derived cell lines were authenticated by DNA short-tandem repeat analysis by ATCC and Sigma. All 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. TOV-21G, TOV-112D and OV-90 were cultured in a 1:1 mixture of MCDB (Sigma, St. Louis, MO, USA): medium 199 with 15% fetal bovine serum (FBS). ES-2 and HEC-1A were grown in McCoy's 5a Medium (ATCC, Manassas, VA, USA) with 10% FBS. The cell line UWB1.289 was cultured in a 1:1 mixture of Mammary Epithelial Basal medium (MEBM, Lonza, Walkersville, MD, USA) and Roswell Park Memorial Institute (RPMI) medium 1640 (Theromo Fisher Scientific, Waltham, MA, USA) supplemented with bovine pituitary extract (BPE), hydrocortisone, human recombinant epidermal growth factor (hEGF), insulin, penicillin and streptomycin and 3% FBS. HEC-59 cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) from Thermo Fisher Scientific (Waltham, MA, USA). All media were supplemented with penicillin and streptomycin to a final concentration of 1%. The cells were cultured at 37 ℃ in a humidified atmosphere containing 5% CO_2. Forty-eight hours later, the media were replaced with the same media but containing charcoal-stripped FBS. The cells were treated with progesterone (10–80 μmol/L), calcitriol (10–80 nmol/L) or a combination for 72 or 120 hours. To avoid the toxicity associated with higher doses of progesterone in most experiments, we used the lower dose of progesterone (20 μmol/L) that has a potent inhibitory effect on the growth of cancer cells. Because high doses of calcitriol are associated with hypercalcemia *in vivo*, we therefore used a concentration of calcitriol that would not induce hypercalcemia *in vivo* (22).

Cell viability assay

Cell viability of cancer cell lines treated with progesterone, calcitriol or the combination was evaluated using the CellTiter 96 AQueous One Solution cell viability as previously reported (14,16,20). Briefly, CellTiter 96 AQueous One Solution reagent 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 (Winooski, VT, USA). Relative cell viability was expressed as % change of treated cells over vehicle-treated cells. The half maximal inhibitory concentration (IC50) values were calculated based on the four-parameter non-linear regression method by Graphpad Prism 4.0 software (San Diego, CA, USA). The combination of progesterone and calcitriol was characterized by a combination index (CI) as described by Chou *et al.* (23) and calculated with CompuSyn (ComboSyn, Inc., Paramus, NJ, USA). CI values were interpreted as follows: CI <1, synergism; CI =1, additive; CI >1, antagonism.

Western blot analysis

Cancer cell extracts from cells treated with progesterone or calcitriol and the combination of the two were analyzed using antibodies against TGFβ1, TGFβ2, TGFβ3, TGFβRI, TGFβRII, TGFβRIII, pSMAD2/3, SMAD2/3, SMAD4, progesterone receptor (PR) and VDR from Santa Cruz Biotechnology (Dallas, TX, USA), CYP24A1 from Abgent (San Diego, CA, USA), and β-actin from Sigma-Aldrich (St. Louis, MO, USA). Equal amounts of protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the intracellular amount of β-actin was analyzed as a loading control. The enhanced chemiluminescence system was used to visualize the protein bands as recommended by the manufacturer (Thermo Fisher Scientific, Waltham, MA, USA). Bands were quantified by densitometry using ImageJ software (version1.51j8, NIH, Bethesda, MD, USA) and protein

band intensities were normalized to β-actin. The bars represent the means ± standard error of the mean (SEM) of normalized levels of three independent experiments.

Statistical analysis

Each experiment was conducted independently at least three times. Cell proliferation experiments were performed in quadruplicates and values were presented as the means ± SEM. Statistically significant differences between control and treatment groups were identified using two-way analysis of variances (ANOVAs) followed by Tukey *post-hoc* tests. A P value of less than 0.05 was considered statistically significant.

Results

Progesterone and calcitriol inhibit proliferation of clear cell ovarian cancer by inhibiting TGF-β signaling pathway proteins

We examined the dose response effects of progesterone and calcitriol alone or in combination on the growth of ovarian clear cell cancer lines ES-2 and TOV-21G. Cells were treated with progesterone (10, 20, 40 or 80 μmol/L), calcitriol (10, 20, 40 or 80 nmol/L), or the combination for 72 or 120 hours. At the end of the treatment time, cell viability was assessed by (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) assay. Although progesterone and progesterone-calcitriol inhibited cell proliferation in all cell lines at both treatment times, only results of cells incubated with hormones for 120 hours are presented. In both cell lines, progesterone reduced cell viability in a dose-dependent manner. The IC50 values of progesterone, calcitriol and the combination of two for ES-2 were 24.86±2.62, 30.25±2.59, 18.64±1.78 and for TOV-21G were 41.64±1.59, 47.66.25±3.41, 25.84±1.96, respectively. Combined progesterone and calcitriol treatment showed a pronounced synergistic inhibitory effect (CI <0.62 for ES-2 and 0.57 for TOV-21G) on cell numbers compared to either single agent in both cell lines tested (*Figure 1A*). Endometrial tumors have been reported to express high levels of three TGF-β isoforms and TGF-β receptors *in vivo* (24,25). In addition, it has been suggested that TGF-β plays a major role in the initiation of endometrial carcinoma invasion (26). To determine whether progesterone regulates the expression of TGF-β and their receptors, ES-2 and TOV-21G cell lines were exposed to progesterone, calcitriol or their combination for 120 hours. Exposure of cells to progesterone and calcitriol-progesterone combination significantly decreased the expression of TGF-β1, TGF-βR1 and TGF-βR2 in ES-2 and TOV-21G cells. Expression of TGF-β2, TGF-β3, and TGF-βR3 was not affected by progesterone or calcitriol-progesterone combination. While the expression of total SMAD2/3 and pSMAD2/3 was attenuated with both progesterone and calcitriol-progesterone combination in both clear cell cancer cell lines, SMAD4 was not affected with any treatment (*Figure 1B,C*).

Effect of progesterone and calcitriol on the expression of PR, VDR and CYP24A1 in clear cell ovarian cancer

Both isoforms of PR were expressed in clear cell cancer cell lines but no marked change was observed in the expression of PR isoforms following progesterone, calcitriol, or combination treatments (*Figure 1B*). We also analyzed the effect of progesterone, calcitriol, or both on VDR protein expression in ovarian clear cells. As shown in *Figure 1B*, VDR expression was enhanced in ES-2 cells treated with progesterone and calcitriol-progesterone combination and treatment failed to alter VDR expression in TOV-21G cells. CYP24A1 protein levels were examined by Western blotting after 120 h of exposure to progesterone and calcitriol either alone or in combination. Progesterone and calcitriol-progesterone inhibited CYP24A1 levels in both clear cell lines. Calcitriol alone showed no effect on CYP24A1 expression (*Figure 1B,C*).

Progesterone and progesterone-calcitriol combination attenuated proliferation of ovarian endometrioid carcinoma by suppressing TGF-β signaling

The TOV-112D cells were cultured with various doses of progesterone (10–80 μM) and proliferation was inhibited in a dose-dependent manner with an IC50 of 25.48±2.54. Exposure of cells to calcitriol alone for 120 hours resulted in inhibition of cell growth and an IC50 of 34.18±3.39. Concurrent treatment of cells with progesterone and calcitriol produced a greater reduction in growth compared to single treatment with an IC50 of 18.22±1.98 and CI <0.74 (*Figure 2A*). Expression of TGF-β1, TGF-β2, TGF-βR1, TGF-βR2, total SMAD2/3 and pSMAD2/3 was attenuated in TOV-112D cells with progesterone alone and progesterone-calcitriol combination. There was no change in expression of TGF-β3, TGF-βR3 and SMAD4 (*Figure 2B,C*).

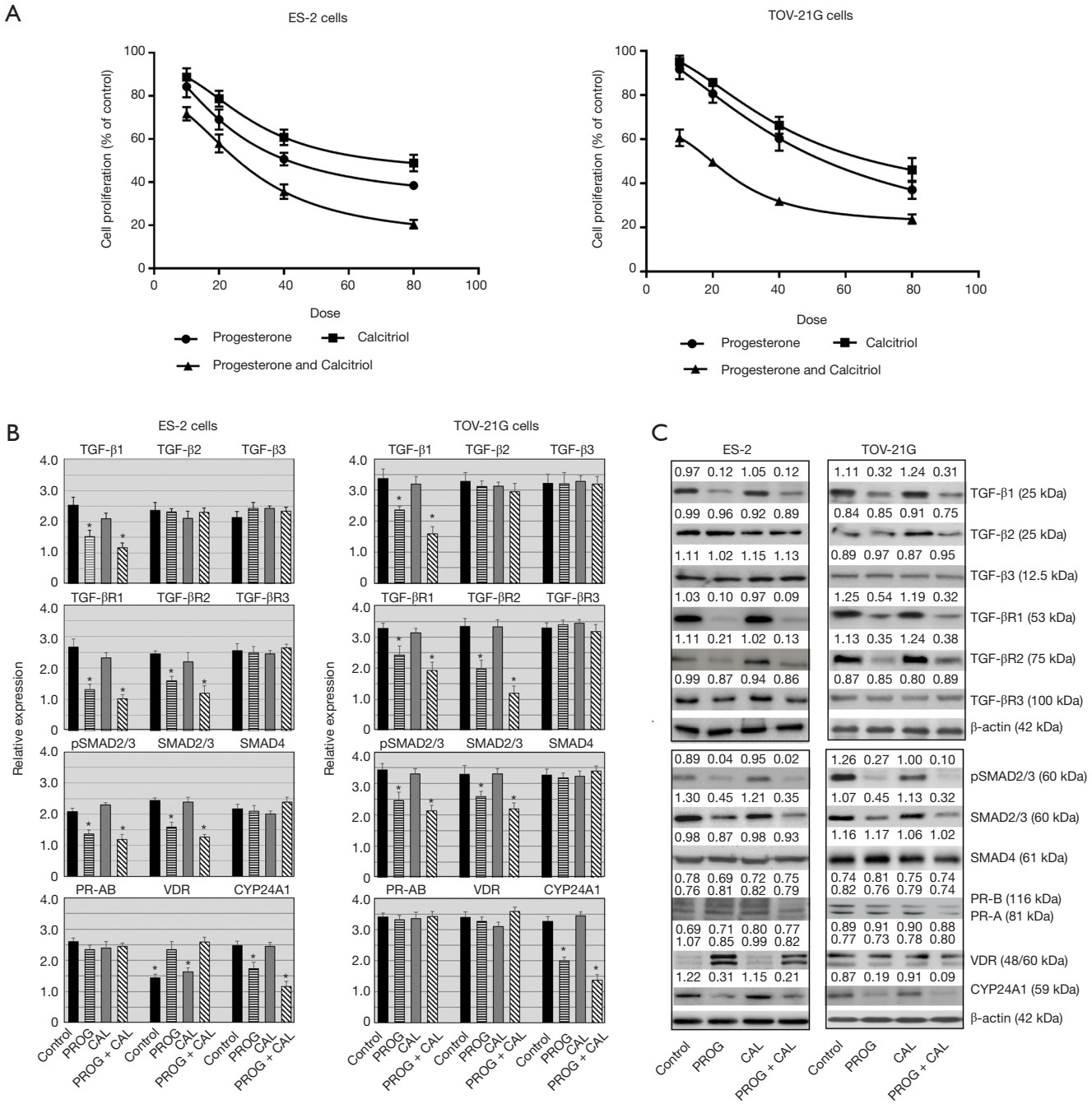

Figure 1 Progesterone and calcitriol-progesterone combination inhibited the growth of clear cell cancer by attenuating the expression of TGF-β signaling proteins and CYP24A1. (A) Clear cell ovarian cancer lines ES-2 and TOV-21G were treated with various doses of progesterone, calcitriol or their combination for 120 h. Cell viability was measured by MTS assay. Results are expressed as percentage of untreated controls; (B) the expression of TGF-β ligands, TGF-β receptors, SMADs, PR, VDR and CYP24A1 in clear cells is shown as bars after normalization to β-actin. Data are expressed as means ± SEM of three independent experiments; (C) representative images of western blots showing expression of various proteins in progesterone, calcitriol and progesterone-calcitriol combination treated cells. β-actin was used as a loading control. *, P<0.05 (statistically significant) between the control and treatment groups. MTS, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium); PR, progesterone receptor; VDR, vitamin-D receptor; SEM, standard error of the mean.

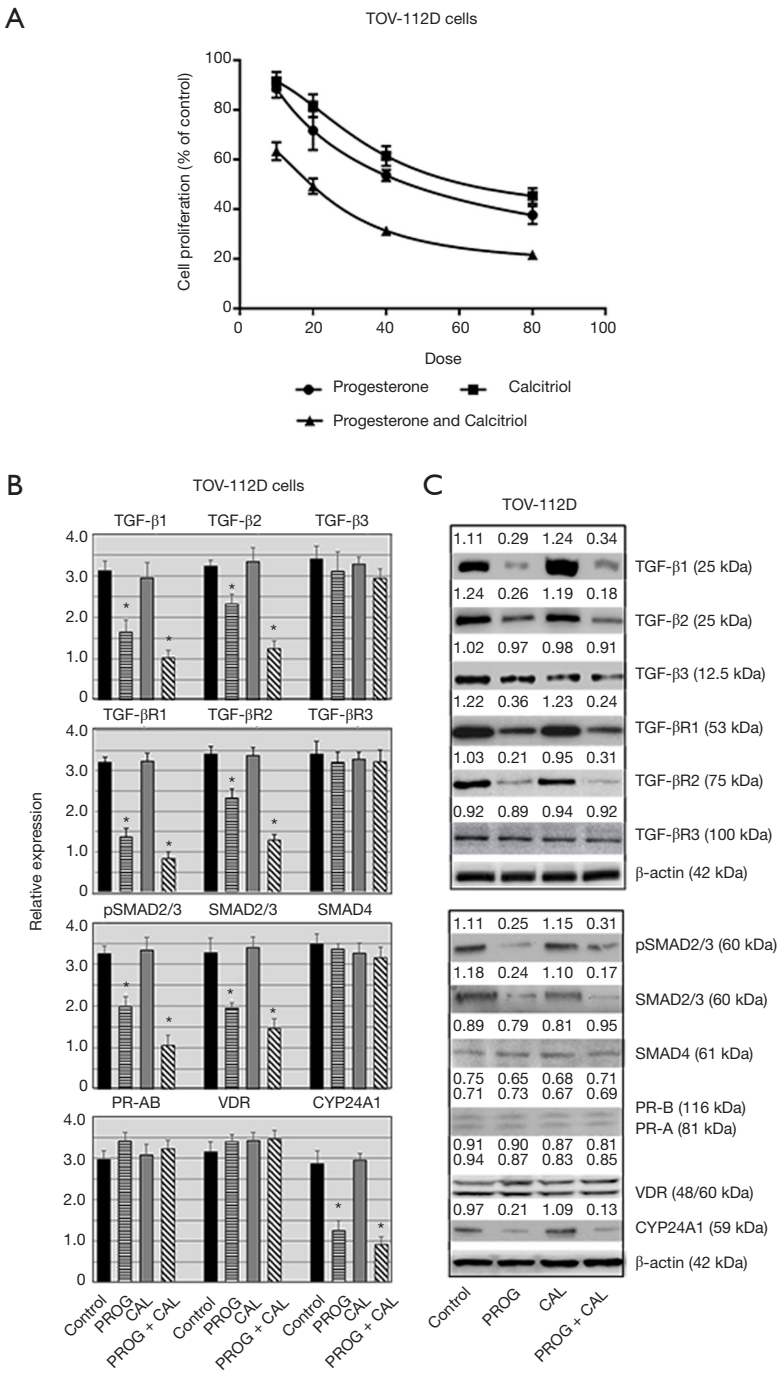

Figure 2 Progesterone and calcitriol-progesterone combination inhibited the growth of ovarian endometrioid cells by attenuating the expression of TGF-β signaling proteins and CYP24A1. (A) Ovarian endometrioid cells were treated with progesterone, calcitriol or their combination for 120 h. Cell viability was measured by MTS assay. Results are expressed as percentage of untreated controls; (B) the expression of TGF-β ligands, TGF-β receptors, SMADs, PR, VDR and CYP24A1 in ovarian endometrioid cells is shown as bars after normalization to β-actin. Data are expressed as means ± SEM of three independent experiments; (C) representative images of western blots showing expression of various proteins in progesterone, calcitriol and progesterone-calcitriol combination treated cells. β-actin was used as a loading control. *, P<0.05 (statistically significant) between the control and treatment groups. MTS, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium); PR, progesterone receptor; VDR, vitamin-D receptor; SEM, standard error of the mean.

Effect of progesterone and calcitriol on the expression of PR, VDR and CYP24A1 in of ovarian endometrioid carcinoma

The effect of progesterone-calcitriol treatment was analyzed on the ovarian endometrioid carcinoma cell line, TOV-112D. The expression of PR and VDR was not affected by progesterone and calcitriol treatment. Expression of CYP24A1 in TOV-112D was markedly inhibited by progesterone alone and in combination with calcitriol (*Figure 2B*,*C*).

Progesterone and progesterone-calcitriol combination attenuated proliferation of ovarian papillary serous adenocarcinoma by suppressing TGF-β signaling

To investigate the cytotoxic effect of progesterone-calcitriol, OV-90 cells were exposed to different concentrations of progesterone or calcitriol ranging from 0 to 80 μM for 120 h. The MTS assay was used to determine the cell viability (*Figure 3A*). A dose-dependent attenuation of cell viability was noticed with progesterone and calcitriol. The IC50 values for progesterone and calcitriol were 30.42±2.11 and 46.35±4.50 respectively. The combination treatment synergistically enhanced growth inhibitory effects (IC50 21.30±3.16 and CI <0.45). Exposure of OV-90 cells to progesterone, calcitriol alone or calcitriol-progesterone combination exhibited reduced expression of TGF-β1, TGF-β2, TGF-βR1, TGF-βR2, pSMAD2/3 and total SMAD2/3. Expression of TGF-β3, TGF-βR3 and SMAD4 was not affected by any treatment (*Figure 3B*,*C*).

Effect of progesterone and calcitriol on the expression of PR, VDR and CYP24A1 in ovarian papillary serous adenocarcinoma

Treatment of the OV-90 cell line with progesterone alone or with progesterone-calcitriol combination inhibited expression of CYP24A1. There was no effect on the expression of PR-B and VDR. However, expression of PR-A was suppressed by progesterone and calcitriol-progesterone combination (*Figure 3B*,*C*).

Progesterone and calcitriol attenuated proliferation of DNA MMR deficient EC cell lines by suppressing TGF-β signaling

The effect of various doses of progesterone, calcitriol and their combination on the growth of two DNA MMR deficient cell (HEC-1A and HEC-59) lines was investigated. Both cell lines demonstrated a dose-dependent reduction (IC50 27.86±4.47 and 32.39±2.47 for HEC1A and HEC59, respectively) in cell viability after 120 hours of culture with progesterone. Simultaneous exposure of cells to progesterone and calcitriol resulted in further reduction (HEC-1A, IC50 22.43±2.57, CI <0.54; HEC-59, IC50 27±3.12, CI <0.48) of cell growth compared to progesterone alone treatment (*Figure 4A*). The expression of TGF-β ligands, TGF-β receptors and downstream signaling proteins were evaluated in HEC-1A and HEC-59 cell lines. In HEC-1A, a marked decrease of TGF-β1, TGF-βR1, total SMAD2/3, and pSMAD2/3 expression was seen following progesterone and progesterone-calcitriol combination. HEC-59 cell lines showed a similar response as HEC-1A, with the addition of TGF-β2 and TGF-βR2 suppression with progesterone-calcitriol treatment. Neither cell line showed changes in TGF-β3, TGF-βR3, or SMAD4 after treatment with progesterone and calcitriol (*Figure 4B*,*C*).

Effect of progesterone and calcitriol on the expression of PR, VDR and CYP24A1 in DNA MMR deficient EC cells

In both DNA MMR deficient cell lines, the expression of PR and VDR was not altered after treatment with progesterone and progesterone-calcitriol combination. CYP24A1 was markedly downregulated with progesterone and calcitriol-progesterone combination in HEC-1A and HEC-59 cell lines (*Figure 4B*,*C*).

Progesterone and calcitriol attenuated proliferation of ovarian cancer BRCA1 null cell ovarian cancer line by suppressing TGF-β signaling

The ovarian cancer cell line UWB1.289 was treated with various doses of progesterone, calcitriol or a combination of progesterone and calcitriol. Progesterone attenuated proliferation of cells in a dose-dependent manner and the IC50 was 27.93±1.85. Cells exposed to the combination of calcitriol and progesterone showed greater decrease (IC50 20.46±2.68 and CI <0.68) in cell number than either of the treatments alone (*Figure 5A*). The expression of TGF-β signaling components were analyzed in progesterone, calcitriol and progesterone-calcitriol treated ovarian cancer BRCA1 null ovarian cancer cells (*Figure 5B*). Progesterone and progesterone-calcitriol combination downregulated the

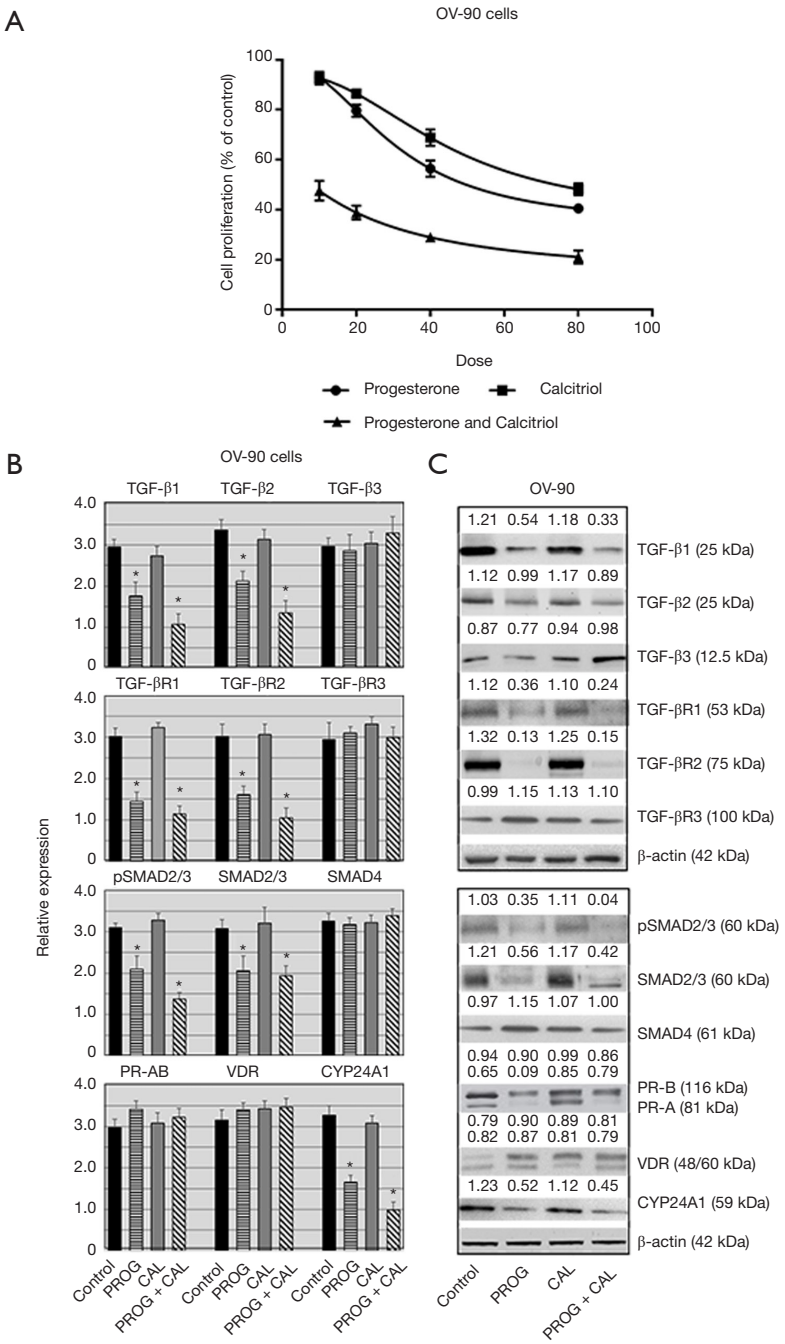

Figure 3 Progesterone and calcitriol-progesterone combination inhibited the growth of ovarian papillary serous adenocarcinoma cells by attenuating the expression of TGF-β signaling proteins and CYP24A1. (A) Ovarian papillary serous adenocarcinoma cells were treated with progesterone, calcitriol or their combination for 120 h. Cell viability was measured by MTS assay. Results are expressed as percentage of untreated controls; (B) effect of progesterone, calcitriol and progesterone-calcitriol combination on the expression of TGF-β ligands, TGF-β receptors, SMADs, PR, VDR and CYP24A1 in ovarian endometrioid cells is shown as bars after normalization to β-actin. Data are expressed as means ± SEM of three independent experiments; (C) representative images of Western blots showing expression of various proteins in progesterone, calcitriol and progesterone-calcitriol combination treated cells. β-actin was used as a loading control. *, P<0.05 (statistically significant) between the control and treatment groups. MTS, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium); PR, progesterone receptor; VDR, vitamin-D receptor; SEM, standard error of the mean.

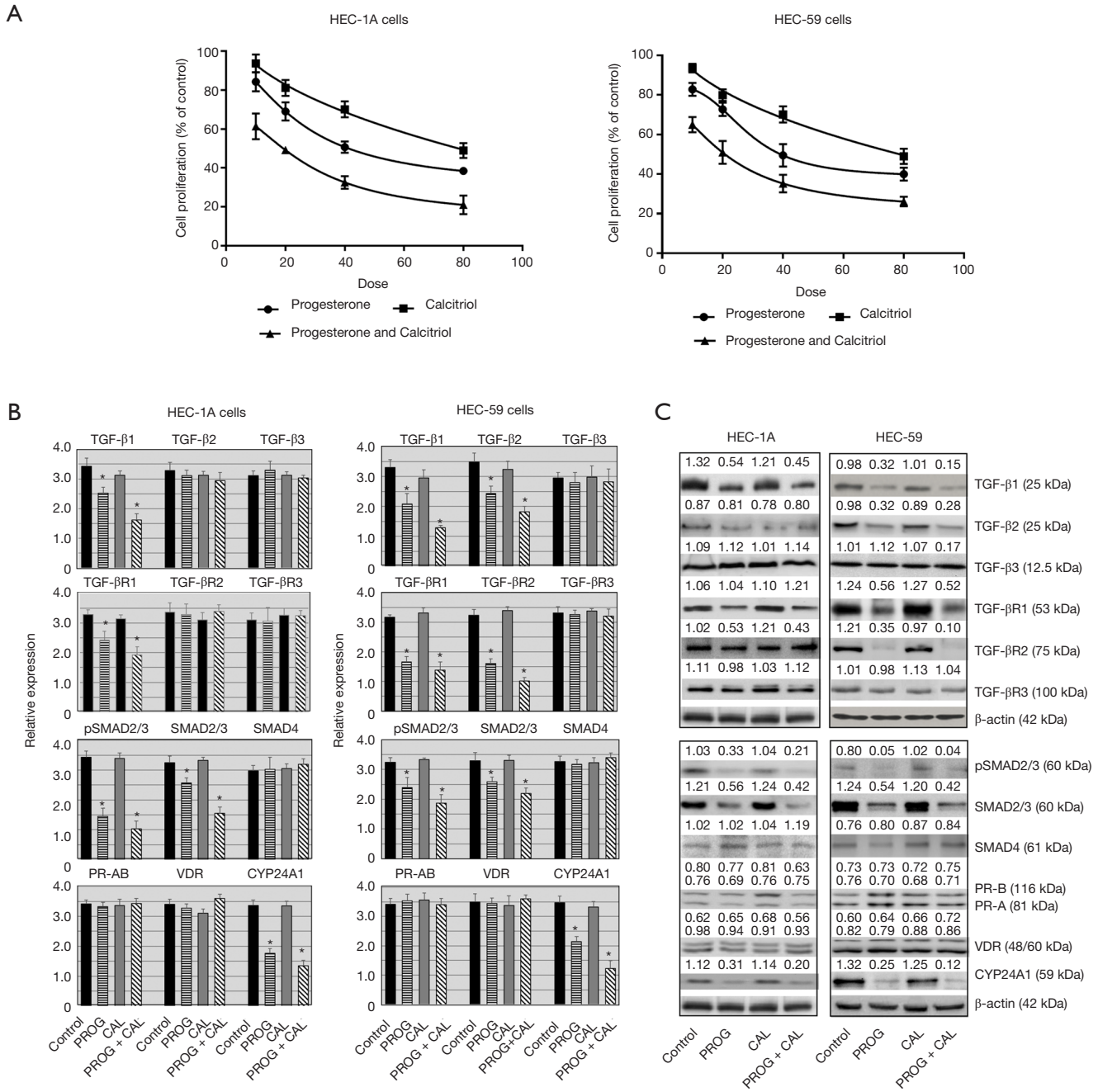

Figure 4 Progesterone and calcitriol-progesterone combination inhibited the growth of EC DNA MMR deficient cell lines by attenuating the expression of TGF-β signaling proteins and CYP24A1. (A) EC DNA MMR deficient cell lines were treated with progesterone, calcitriol, or their combination for 120 h. Cell viability was measured by MTS assay. Results are expressed as percentage of untreated controls; (B) effect of progesterone, calcitriol and progesterone-calcitriol combination on the expression of TGF-β ligands, TGF-β receptors, SMADs, PR, VDR and CYP24A1 in ovarian endometrioid cells is shown as bars after normalization to β-actin. Data are expressed as means ± SEM of three independent experiments; (C) representative images of western blots showing expression of various proteins in progesterone, calcitriol and progesterone-calcitriol combination treated cells. β-actin was used as a loading control. EC, endometrial cancer. MMR, mismatch repair. *, P<0.05 (statistically significant) between the control and treatment groups. MTS, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium); PR, progesterone receptor; VDR, vitamin-D receptor; SEM, standard error of the mean.

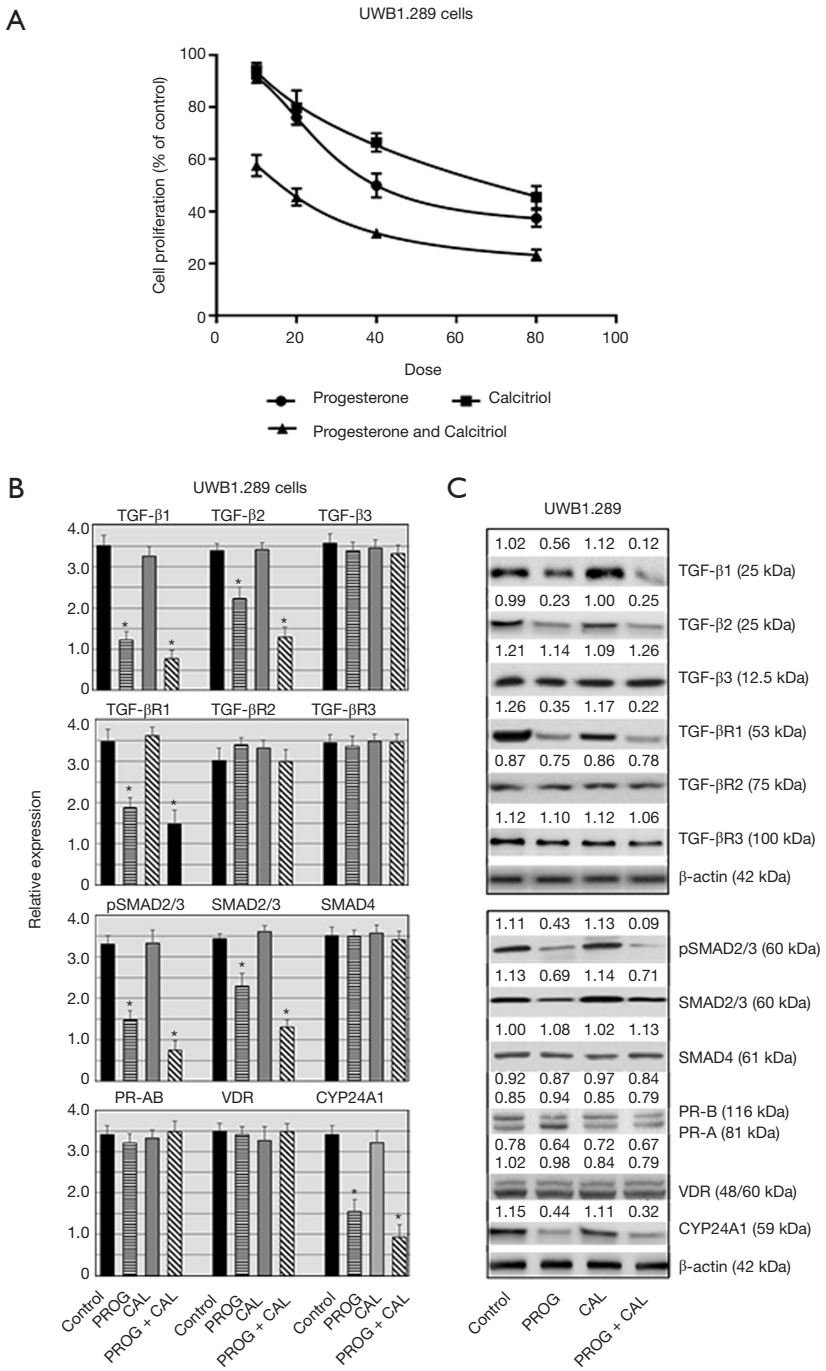

Figure 5 Progesterone and calcitriol-progesterone combination inhibited the growth of ovarian cancer BRCA1 null cells by attenuating the expression of TGF-β signaling proteins and CYP24A1. (A) Ovarian cancer BRCA1 null cells were treated with progesterone, calcitriol or their combination for 120 h. Cell viability was measured by MTS assay. Results are expressed as percentage of untreated controls; (B) effect of progesterone, calcitriol and progesterone-calcitriol combination on the expression of TGF-β ligands, TGF-β receptors, SMADs, PR, VDR and CYP24A1 in ovarian endometrioid cells is shown as bars after normalization to β-actin. Data are expressed as means ± SEM of three independent experiments; (C) representative images of western blots showing expression of various proteins in progesterone, calcitriol and progesterone-calcitriol combination treated cells. β-actin was used as a loading control. *, P<0.05 (statistically significant) between the control and treatment groups. MTS, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium); PR, progesterone receptor; VDR, vitamin-D receptor; SEM, standard error of the mean.

expression of TGF-β1, TGF-β2, TGF-βR1, pSMAD2/3 and total SMAD2/3 in cells. No change in TGF-β3, TGF-βR3, and SMAD4 was observed (*Figure 5B,C*).

Effect of progesterone and calcitriol on the expression of PR, VDR and CYP24A1 in BRCA1 null ovarian cancer cells

The progesterone-calcitriol combination failed to alter the expression of PR and VDR in BRCA1 null cells. However, the expression of vitamin-D induced gene, CYP24A1, was attenuated with progesterone-calcitriol combination and progesterone alone (*Figure 5B,C*).

Discussion

Previously, our group showed that the combination of a low dose of progesterone with a low dose of calcitriol markedly inhibited growth of serous ovarian and EC cells. The mechanism for growth inhibition was by enhanced expression of the VDR, activation of caspase-3, induction of cell cycle arrest and downregulation of cyclins. Furthermore, our results revealed downregulation of TGF-β signaling proteins and CYP24A1, an enzyme that breaks down the active form of vitamin-D (14-16,20).

Ovarian and ECs are not single diseases but a compendium of diseases with distinct histologic subtypes, molecular alteration(s) and ecosystems of tumor cells, stromal and immune cells compositions (27-29). Owing to the heterogeneous nature of both cancers, it is expected that they might respond to the treatment in different ways. Thus, it is important to test whether the progesterone-calcitriol combination that successfully suppressed the growth of serous ovarian and endometrioid EC would be effective in attenuating growth of different cancer subtypes and those with various mutations. Analyzing the mechanistic activity of the progesterone and calcitriol combination on different subtypes of endometrial and ovarian cancer has the potential to increase our understanding of these cancers and may translate into expanded applications of the progesterone-calcitriol combination in clinical practice in chemopreventive and therapeutic settings.

TGF-β is a superfamily of cytokines with pleiotropic functions. It regulates a number of biological processes such as cell proliferation, differentiation, migration, survival, apoptosis, angiogenesis, extracellular matrix production and immune response (30,31). There are three homologous isoforms of TGF-β ligands (TGF-β1, TGF-β2 and TGF-β3). The ligands exercise their effects by binding to the TGF-βR2, which recruits the TGF-βR1. SMAD2 and 3 are phosphorylated by type I receptor, which in turn form heteromeric complexes with SMAD4. The activated SMAD complexes accumulate in the nucleus and bind to a specific promoter region on target genes along with transcription factors and/or co-activators/repressors (30,31). Our published work demonstrated an intact TGF-β pathway in EC cells. Progesterone markedly inhibited basal and TGF-β1-induced proliferation and invasive potential of EC cells. Furthermore, TGF-βR1 blocker (SD-208) abrogated TGF-β1 induced growth as well as progesterone induced growth inhibition. These results suggest that progesterone exerts its growth inhibitor effects via the TGF-β1/SMAD signaling pathway (20). Phosphorylation and subsequent translocation of SMAD2 and SMAD3 to the nucleus are crucial steps in TGF-β signal transduction. Western blotting revealed that the total and phosphorylated SMAD2/3 levels were reduced by progesterone and the combination of progesterone-calcitriol in all cell lines tested. This indicates that reduction of pSMAD2/3 is due to the decreased total-SMAD2/3. These findings are in concert with our previous immunofluorescence staining study (20) showing that TGF-β1 treatment of the EC cells increased and caused translocation of SMAD2/3 from the cytoplasm to the nucleus and treatment with progesterone decreased expression in the cytoplasm and the nucleus of cells.

Local levels of vitamin-D_3 are maintained in the cells by a delicate balance between the activities of CYP27B1 and CYP24A1, which promote synthesis and deactivate vitamin-D_3 respectively. CYP24A1 is a vitamin-D_3 induced gene. Factors that modify the activity of CYP27B1 and CYP24A1 have the potential to impact vitamin D_3 signaling. A number of microarray studies have shown induction of CYP24A1 and TGF-β signaling proteins in response to vitamin-D3 in prostate, colon, and breast cancer cells (32-34). Towsend *et al.* (33) showed that co-treatment with vitamin-D_3 and TGF-β2 enhanced the accumulation of CYP24A1 further in the MCF-7 cells compared to cells treated with vitamin-D_3 alone. The relationship between the TGF-β pathway and CYP24A1 has been further substantiated in prostate cancer and stromal cells. Treatment of cancer/stromal cells with TGF-β increased expression of CYP24A1, which metabolizes vitamin-D_3 and thus reduces VDR activity. Knock down of a TGF-β-inducible nuclear receptor co-regulator also known as Hic-5, reduced basal VDR expression, vitamin-

D_3-induced CYP24A1 expression and TGF-β-enhanced CYP24A1 expression (34).

The main histological subtypes of ovarian and ECs are serous, endometrioid, clear cell, and mucinous adenocarcinoma. These subtypes reflect significant biological alterations in the behavior of tumors which demonstrate different phenotypes with distinct biological and genetic backgrounds (29,35). To develop treatment for cancer, it is crucial to test the efficacy of drugs on individual cancer subtypes to understand whether treatment is effective for a wide variety of cancers. In our previous studies, we prioritized evaluations of the effects of progesterone and calcitriol in the most common EC histologies, endometrioid adenocarcinoma and ovarian serous carcinoma, and discovered the mechanisms by which these two agents suppressed tumor growth. In this study, we extended our analysis to other ovarian and EC subtypes. The TGF-β signaling pathway is activated in ovarian and ECs as seen by high levels of TGF-β and pSMAD2/3 in different ovarian and endometrial cell lines (20,36). In the present study, the expression of TGF-β ligands, TGF-β receptors and SMADs were assessed in clear cell, endometrioid and papillary serous ovarian cancer cell lines exposed to progesterone, calcitriol and the combination of the two. All cancer subtypes showed high expression of TGF-β signaling proteins. These findings concur with earlier studies showing elevated expression of these proteins in ovarian clear cell, endometrioid and papillary serous cancers (37,38). Treatment of these subtypes of cancer with progesterone and combination of progesterone with calcitriol markedly inhibited the growth and the expression of TGF-β1, TGF-β2, TGF-βR1, TGF-βR2 and SMAD2/3. These results support the therapeutic use of progesterone-calcitriol combination as an effective strategy in suppressing growth of different histological subtypes of tumors by abrogating the expression of TGF-β signaling proteins.

Progesterone elicits action through PR and cancers with high PR expression have good prognosis compared to those with lower expression of PR (39). The cell lines used in the present study representing clear cell, endometrioid and papillary serous subtypes of ovarian tumors, express both forms of PR in varying levels depending on the cell line. A number of studies reported higher PR positivity in serous (58%) and endometrioid (76%) carcinoma compared to clear-cell (8%) carcinoma (40-42). Previously, we showed expression of PR-A and PR-B on serous ovarian and EC cells and demonstrated anti-proliferative actions of progesterone, primarily through the induction of apoptosis (14,15). We examined the effects of progesterone, calcitriol, or both on PR protein expression in cancer cell lines of different subtypes. No noticeable changes were observed in any cell line following progesterone, calcitriol, or combination treatments except OV-90, a papillary serous ovarian cancer cell line, where progesterone and combination suppressed PR-A expression. This observation indicates that reduced PR-A may have different functional outcomes, which remain to be investigated.

MMR deficient cells typically have many DNA mutations that lead to cancer. Alterations in the MMR pathway lead to high levels of microsatellite instability. Lately, it has been established that high levels of TGF-β induce genomic instability by impairing DNA repair. TGF-β has been shown to down-regulate the expression of MSH2 and ataxia telangiectasia mutated (ATM) (43-45), leading to an impaired DNA repair efficiency. The effect of progesterone and calcitriol on the HEC-1A cell line with the complete loss of MMR (hMSH6/hPMS2-defective), and HEC-59 with no hMSH2, was assessed. We found downregulation of TGF-β signaling proteins in both DNA MMR deficient EC cells and observed inhibition of growth with progesterone and calcitriol-progesterone combination treatments. These results suggest that calcitriol-progesterone may control growth of tumor cells by downregulating TGF-β signaling proteins.

Germline mutations of tumor suppressors BRCA1 and BRCA2 confers with an increased risk of developing breast and ovarian cancer (46-48). BRCAs participate in DNA damage repair via homologous recombination and impact genomic instability and malignant transformation (25,49). Treatment of UWB1.289 BRCA1 null cells with progesterone or calcitriol progesterone combination attenuated expression of TGF-β1, TGF-β2, TGF-βR1 and subsequently phosphorylated SMAD2/3. A link between BRCA1 and TGF-β1/SMAD pathway is well established. In breast cancer cells, interruption of endogenous BRCA1 in MCF-7 cell line changes their anti-proliferation responses, while maintenance of BRCA1 upholds TGF-β1 responsiveness through enhancing the stability of SMAD4 (50).

The CYP24A1 is upregulated in a number of cancers, including ovarian and endometrial, and impairs the activity of calcitriol (14,15). Here, we showed increased expression of CYP24A1 in endometrioid, clear cell, papillary serous carcinomas, endometrial tumors with DNA MMR deficiency and ovarian cancer with BRCA1/BRCA2 mutations. These findings indicate that cancer cells can evade the anti-tumorigenic effects of calcitriol by inducing the expression of CYP24A1. Moreover, progesterone inhibited the expression

of CYP24A1 in not only serous ovarian and ECs, but also attenuated CYP24A1 expression in all types of cancer tested in this study. Similarly, recent studies have shown sensitization of cancer cells by inhibition of CYP24A1 using pharmacological inhibitors or genetic knockdown approaches (51,52). VDRs were expressed in all the cells lines tested. These results concur with investigations showing no significant differences in the expression of VDR in serous, mucinous, clear cell, and endometrioid subtypes of ovarian cancer (53). Previously, we have shown that progesterone upregulates the expression of VDR in serous EC cells and thus, enhances the anticancer effects of calcitriol (16). However, we have not seen upregulation of VDR in response to progesterone in any cell line tested, except ES-2 cells.

We acknowledge that in the current study we have used only one serous cell line. Our goal was to validate our findings in serous cancers to show the synergistic effect of progesterone and calcitriol combination on cell proliferation. Only one serous cancer cell line was commercially available at the time. Further studies will be performed to confirm the results of this study after procurement of more than one serous cancer cell lines from non-commercial sources.

In summary, our data reveal that the progesterone-calcitriol combination not only inhibited growth of different subtypes of endometrial and ovarian cancers, but also those with mutations in ARID1A or PIK3CA, DNA MMR deficiency or BRCA1 null status. Furthermore, in all cell lines of different histotypes, progesterone-calcitriol combination attenuated the expression of TGF-β signaling proteins and CYP24A1.

Collectively, these results underscore the potential expanded applications of the progesterone and calcitriol combination for further development as a chemopreventive and therapeutic strategy for endometrial and ovarian cancer, and the important role that surrogate biomarkers of TGF-β signaling proteins and CYP24A1 may play in future investigations of activity of this combination in preclinical model systems and human subjects.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr.2019.07.36). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The institutional ethical approval and informed consent were waived.

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