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# Progesterone and Calcitriol Attenuate Inflammatory Cytokines CXCL1 and CXCL2 in Ovarian and Endometrial Cancer Cells

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## ABSTRACT

Cytokines/chemokines are key players in cancer-related inflammation. Increasing evidence suggests that chemokines produced by tumor cells are the mediators of metastasis. Thus, agents that can downregulate chemokines expression have potential against cancer metastasis. We have previously shown inhibition of ovarian and endometrial cancer cell growth with progesterone and calcitriol. In the present study, we evaluated the effect of these two agents on the expression of inflammatory genes. Using a RT-PCR array of inflammatory cytokines/chemokines and their receptors, we found a marked attenuation of CXCL1 and CXCL2 (GRO-α and -β) in cancer cells by both treatments. Knockdown of NFκB resulted in a reduced expression of CXCL1 and CXCL2 and the inhibitory effect of progesterone and calcitriol on the expression of chemokines was abrogated in NFκB-silenced cancer cells. Silencing of IκBα increased the expression of CXCL1 and CXCL2 in cancer cells, which can be attributed to the increased activation of NFκB-p65, caused by the lack of its inhibitor. Progesterone and calcitriol-induced inhibition was abolished in IκBα-knockdown cells. Our results demonstrate that suppression of IκBα phosphorylation by progesterone and calcitriol contributes to the reduced expression of CXCL1 and CXCL2. Downregulation of CXCL1 and CXCL2 was associated with a marked inhibition of metastasis-promoting genes. Overall, our results indicate that progesterone and calcitriol inhibit IκBα phosphorylation, NFκB activation, and the expression of NFκB regulated metastasis promoting genes. These results provide attractive data for the possible use of progesterone and calcitriol in the management of endometrial and ovarian tumors. J. Cell. Biochem. 113: 3143–3152, 2012. 

**KEY WORDS:** NFκB; METASTASIS; PROLIFERATION; IκBα; INVASION

Tumor-promoting inflammation has been recognized as one of the hallmarks of cancer [Hanahan and Weinberg, 2011]. NFκB is a powerful proinflammatory modulator that is constitutively activated in cancer [Pikarsky et al., 2004] and links inflammation and carcinogenesis [Darnell, 2002; Karin, 2006]. NFκB controls the expression of more than 400 genes involved in a plethora of functions, such as inflammation, growth, apoptosis, invasion, and metastasis [Aggarwal, 2004]. It is normally quiescent in cells and only becomes activated in response to stress signals, such as proinflammatory cytokines. NFκB consists of a family of transcription factors including p65 (RelA), p105/p50, p100/p52, RelB, and c-Rel. The classic form of NFκB is the p65/p50 heterodimer that contains the transcriptional activation domain and is sequestered in the cytoplasm as an inactive complex by IκB [Adli et al., 2010]. The activation of transcription factors is the key event in the progression of human cancer and can be a target for cancer therapy [Darnell, 2002; Karin, 2006]. Upon induction of the pathway, the inhibitor IκBα becomes phosphorylated by an IκB kinase (IKK), which leads to ubiquitination of IκB. The inhibitor is thus targeted to the ubiquitin-proteasome for degradation, and the NFκB heterodimer p65/p50 is free to translocate to the nucleus and begin altering gene expression [Karin et al., 2002; Hayden and Ghosh, 2008]. Some specific downstream NFκB targets include survival genes (Bcl-xl and XIAP), adhesion molecules (ICAM-1, VCAM-1, and ELAM-1), metastasis promoting gene (MMP9), angiogenesis factors (VEGF and TNFα), proliferation genes (cyclin D1 and C-myc), and a host of other genes

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known for their association with proliferation, inflammation, and immortalization of cells [Karin et al., 2002; Perkins and Gilmore, 2006; Van Waes, 2007].

Clinical, epidemiologic, and basic science evidence suggests that the progestin and calcitriol may be highly effective ovarian and endometrial cancer preventive agents. Calcitriol directly modulates basal and cytokine-induced NFκB activity in many cells [Yu et al., 1995; Stio et al., 2007]. It inhibits NFκB signaling in breast and prostate cells and thereby decreases levels of the angiogenic and proinflammatory cytokine IL-8 [Bao et al., 2006]. In colon cancer cells calcitriol inhibits NFκB activity by increasing IκBα and suppressing proinflammatory chemokine production, such as MCP-1 and IL-8 [Tse et al., 2007; Sun et al., 2008; Vanoirbeek et al., 2011].

Progesterone is a powerful anti-inflammatory and anti-proliferative hormone in the female reproductive tract [Dai et al., 2003; Yoshino et al., 2003]. In poorly differentiated endometrial cancer cells, Hec50c, progesterone was shown to inhibit NFκB transcriptional activity via modulation of A20 and ABIN-2 proteins [Davies et al., 2004]. Progesterone also inhibits the secretion of metalloproteinase, which is known to have inflammatory characteristics and is found in cancer [Di Nezza et al., 2003].

The molecular mechanism underlying the inhibitory effects of progesterone and calcitriol on inflammatory chemokines is not yet fully understood in ovarian and endometrial cancer. In this study, we investigated the mechanism by which progesterone and calcitriol inhibit the expression of inflammatory genes. Our results show that both inhibit the expression of CXCL1 and CXCL2 by inhibiting phosphorylated-IκBα and NFκB activation leading to suppression of NFκB-regulated proteins involved in tumor invasion and metastasis.

## MATERIALS AND METHODS

### TREATMENT OF OVARIAN AND ENDOMETRIAL CANCER CELLS

Two ovarian (OVCA 420 and OVCA 429) and two endometrial (Ishikawa and HEC-1B) cancer cell lines were cultured as described previously [Syed and Ho, 2003; Saydmohammed et al., 2010]. Normal epithelial endometrial (EM-E6/E7/TERT) cells established and characterized by Kyo et al. [2003] and normal ovarian surface epithelial cells (HOSE 642) were used as controls. Briefly, the endometrial cancer cell lines Ishikawa and HEC-1B obtained from the American Type Culture Collection (Manassas, VA) were grown in DMEM:F12 (Invitrogen, Carlsbad, CA) and Eagle MEM (Invitrogen), respectively. The EM-E6/E7/TERT cells were grown in DMEM. Normal human ovarian surface epithelial cell line (HOSE 642) and ovarian cancer cell lines were grown in a 1:1 mixture of medium 199/MCDB 105 (Sigma, St Louis, MO), supplemented with 10% (v/v) fetal bovine serum (FBS, Invitrogen), 100 U/ml penicillin, and 100 U/ml streptomycin at 37°C in a humidified atmosphere containing 5% $CO_2$. The cells were treated with progesterone (25 μM P4, 99.9% pure; Sigma) or calcitriol (100 nM, Sigma) for 72 h 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 [Nguyen and Syed, 2011; Nguyen et al., 2011]. For a set of experiment ovarian and endometrial cancer cells transfected with siRNA targeting NFkB or scrambled siRNA and then treated with LPS (10 μg/ml) for 72 h. Protein extracts were prepared for the analysis of CXCL1 and CXCL2.

### REVERSE TRANSCRIPTION-PCR INFLAMMATION ARRAY

Effect of progesterone or calcitriol on inflammatory cytokines was studied by using the inflammatory cytokines and receptors PCR array (PAHS-011) from SABiosciences (Frederick, MD). Total RNA was isolated from control, progesterone, or calcitriol-treated endometrial cancer cells using TRI-reagent (Sigma). After DNase treatment, RNA was further cleaned using the Qiagen RNeasy Mini kit (Qiagen). cDNA was synthesized by $RT^2$ First Strand kit (SABiosciences) per the company's instructions. Quantitative reverse transcriptase PCR (RT-PCR) was conducted using the 7500RT-PCR System (AB Applied Biosciences) following the array manufacturer's instructions. Relative gene expression was determined using the $\Delta\Delta C_t$ method as described earlier [Nguyen et al., 2011].

### SILENCING OF NFκB, IκBα, CXCL1, AND CXCL2 IN OVARIAN AND ENDOMETRIAL CANCER CELLS

To establish that NFκB is a mediator of progesterone and calcitriol-induced growth inhibition, ovarian (OVCA 420 and OVCA 429) and endometrial (Ishikawa and HEC-1B) cancer cells were seeded in six-well plates and transfected the following day with the annealed double-stranded siRNAs targeting NFκB (accession number EF0403800) [r(GAUCAAUGGCUACACAGGA) d(TT) and r(UCCUGUGUAGCCAUUGAUC) d(TT)] or IκBα, (accession number AF080157) [r(GAAAAGGCACUGACCAUGG) d(TT) and r(CCAUGGUCAGUGCCUUUU d(TT)] from Thermo Scientific (Lafayette, CO) using the Lipofectamine 2000 reagent (Invitrogen). Transfected cells were treated with or without progesterone (25 μM) or calcitriol (100 nM) for 72 h. Cell extracts were used to assess expression of CXCL1, CXCL2, and NFκB. To better understand the role of CXCL1 and CXCL2 in invasion, cells were transfected with siRNAs directed against CXCL1 (accession number NM001511) and CXCL2 (accession number NM002089, 5 nmol/L; Santa Cruz Biotechnology, Inc.) or scrambled siRNA; 72 h later cell extracts were prepared to determine the expression of metastasis-related genes. The overall transfection efficiency for ovarian and endometrial cells assessed by luciferase assay was 72–77%. In a separate experiment transfected cells were used to assess their invasiveness using the Matrigel invasion assay.

### WESTERN BLOTTING

Ovarian and endometrial cancer cell extracts from progesterone or calcitriol-treated and non-treated control cells as well as extracts from NFκB and IκBα-silenced and control siRNA-transfected cells treated with or without progesterone or calcitriol were analyzed using antibodies against CXCL1 and CXC chemokines and their receptors (CXCR; Abcam Inc.), CXCL2 (Thermo Scientific), MMP2 (Abcam Inc.), MMP9 (Cell Signaling), COX2 (Cell Signaling), β-actin antibody (Sigma–Aldrich), NFκB-p65, IκBα, or CXCL4 (Santa Cruz), and p-IκBα (Millipore). 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 for visualization of the protein bands as recommended by the manufacturer (Pierce).

### INVASION ASSAY

The invasion assay was done using 24-well Biocoat Matrigel invasion chambers (BD Biosciences, San Jose, CA). The transfected cells and their respective control cells were suspended in medium at a concentration of $1 \times 10^5$ cells/ml, and 0.5 ml of each was added to the invasion chambers in triplicate. Medium (0.75 ml) supplemented with 10% FBS was added to each well of the plate to act as a chemoattractant and the plates were placed in an incubator. After 18 h of plating, non-invading 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 min in 100% methanol and stained with 1% toluidine blue in 1% sodium borate for 2 min. Cells that invaded through the insert were counted in five random fields per slide. All slides were coded to avoid biased counting.

### TRANS AM NFκB ACTIVATION ASSAY

Activation of p65 NFκB was examined using TransAM p65 NFκB chemiluminescence Transcription Factor Assay Kit from Active Motif (Carlsbad, CA) as per the instructions of the manufacturer. Briefly, 30 μl of complete binding buffer were added into each well coated with immobilized oligonucleotide containing the NFκB consensus site (5′-GGGACTTTCC-3′). Nuclear proteins (20 μg), prepared from cells incubated with or without progesterone or calcitriol for 72 h, were added to the wells in 80 μl complete lysis buffer and incubated for 1 h at room temperature. The wells were washed and incubated with anti-p65 NFκB antibody (1:1,000 dilution) for 1 h and washed thrice again followed by incubation with horseradish peroxidase-conjugated second antibody (1:10,000; 50 μl) for 1 h. The wells were washed four times followed by the addition of the chemiluminescent working solution. The resulting chemiluminescence, proportional to the levels of nuclear p65 NFκB, bound to the consensus sequence, was measured using a microplate reader (ELX800; Winooski, VT) at 450 nm. The experiment was performed in triplicate.

### STATISTICAL ANALYSES

Statistical analysis was carried out using ANOVA, followed by Tukey's post hoc test. Values are presented as the mean ± SD and are considered significant at a *P*-value of less than 0.05.

## RESULTS

### PROGESTERONE AND CALCITRIOL ALTER THE EXPRESSION OF INFLAMMATORY GENES IN HUMAN CANCER CELLS

We have previously shown the anti-tumorigenic effects of progesterone and calcitriol on ovarian and endometrial cancer cells [Nguyen and Syed, 2011]. In the current study, we evaluated if anti-tumorigenic effects are mediated by modulation of inflammatory genes. RNA extracted from control, progesterone, or calcitriol-treated endometrial cancer cells were subjected to an inflammatory $RT^2$ Profiler RT-PCR array. The changes in mRNA levels of inflammation-related genes in progesterone or calcitriol-treated cancer cells are shown in Table I. A threefold change in mRNA expression was considered as significant when compared with the control cells. It is evident from the results that progesterone or calcitriol treatment led to decreased expression of proinflammaory genes and increased expression of anti-inflammatory genes (Table I). Since recent evidence links CXCR with the pathogenesis of metastasis in human cancer cells, we focused on the expression and regulation of CXCL1, CXCL2 in ovarian and endometrial cancer cells.

TABLE I. Fold Changes in Pro- and Anti-Inflammatory Genes in Progesterone and Calcitriol-Treated Endometrial Cancer Cells

| | Ishikawa (fold change) | | HEC-1B (fold change) | |
|---|---|---|---|---|
| Genes | Progesterone | Calcitriol | Progesterone | Calcitriol |
| Downregulated | | | | |
| IL-1β | 4.56 ± 0.87 | 3.38 ± 0.62 | 3.89 ± 0.34 | 3.12 ± 0.44 |
| TNF-α | 4.80 ± 0.62 | 4.23 ± 0.53 | 4.56 ± 0.22 | 3.78 ± 0.37 |
| CXCL1 | 5.21 ± 0.43 | 4.65 ± 0.56 | 4.78 ± 0.67 | 4.39 ± 0.63 |
| CXCL2 | 4.79 ± 0.19 | 4.33 ± 0.32 | 3.88 ± 0.46 | 3.73 ± 0.30 |
| Upregulated | | | | |
| IL-4 | 3.66 ± 0.29 | 4.32 ± 0.35 | 4.90 ± 0.26 | 3.87 ± 0.43 |
| IL-8 | 4.29 ± 0.20 | 4.03 ± 0.42 | 4.08 ± 0.24 | 4.54 ± 0.28 |
| IL-10 | 3.77 ± 0.31 | 3.98 ± 0.27 | 4.64 ± 0.36 | 3.34 ± 0.26 |
| IFNA2 | 4.22 ± 0.42 | 3.67 ± 0.57 | 4.76 ± 0.42 | 3.33 ± 0.19 |
| LTB | 4.35 ± 0.28 | 4.21 ± 0.49 | 4.28 ± 0.20 | 3.97 ± 0.25 |

To confirm the expression at the protein level, we assessed CXCL1, CXCL2, and their receptor CXCR2 expression levels by Western blotting in endometrial and ovarian cancer cells. Expression of CXCL1 and CXCL2 was higher in cancer cells compared to normal cells (Fig. 1A). Treatment of cells with progesterone or calcitriol markedly reduced the expression of CXCL1 and CXCL2 in cancer cells and confirmed the expression pattern shown by RT-PCR (Fig. 1B). Since both the chemokines under study here have the same receptor, we examined CXCR2 expression in progesterone and calcitriol-treated cells. CXCR2 was equally expressed in normal and malignant cells and progesterone and calcitriol treatment had no effect on its expression (Fig. 1A,B).

### PROGESTERONE AND CALCITRIOL DOWNREGULATE INFLAMMATORY CYTOKINES BY ATTENUATING NFκB IN CANCER CELLS

CXCL1 and CXCL2 promoters have respectively 7 and 13 perfectly conserved NFκB elements. These elements are within 1,000 nucleotides upstream and 500 nucleotides downstream of the transcription start site, indicating that these two cytokines are regulated by NFκB [Bachmeier et al., 2008]. We next sought to determine whether progesterone and calcitriol directly modify the binding of NFκB-p65 complex to the DNA. NFκB activity in nuclear lysates of ovarian and endometrial cancer cells treated with progesterone or calcitriol for 72 h was measured using TransAM NFκB kit (Active Motif). OVCA 420, OVCA 429, Ishikawa and HEC-1B cell lines had more NFκB binding activity than nuclear extracts from HOSE 642 and EM-E6/E7/TERT cells. Progesterone and calcitriol treatment markedly inhibited the binding of NFκB-p65 in ovarian (OVCA 420 and OVCA 429) and endometrial (Ishikawa and HEC-1B) cancer cells. In contrast, LPS-treated cancer cells used as positive controls showed further enhancement of NFκB activation

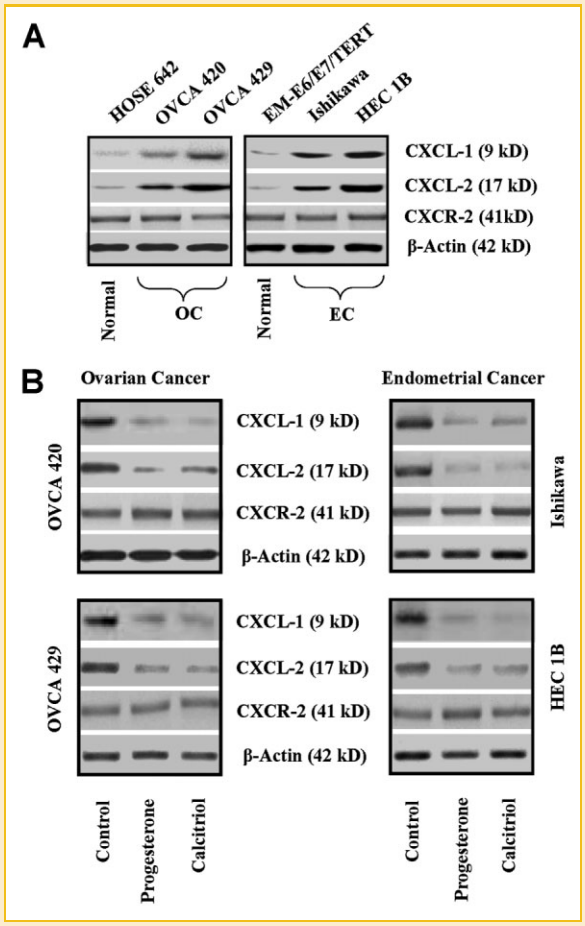


Fig. 1. Attenuation of CXCL1 and CXCL2 expression by progesterone and calcitriol in ovarian and endometrial cancer cells. A: Basal expression of CXCL1, CXCL2, and CXCR2 in normal, ovarian cancer (OC), and endometrial cancer (EC) cells. B: OVCA 420, OVCA 429, Ishikawa, and HEC-1B cells were treated with vehicle (control), progesterone (25 μM), or calcitriol (100 nM) for 3 days. Whole-cell extracts were resolved by SDS–PAGE and blotted with the indicated antibodies. Equal amount of protein was confirmed by using β-actin as a loading control.

compared to non-treated ovarian and endometrial cancer cells (Fig. 2A).

To understand the role of NFκB-p65 in modulating the expression of CXCL1 and CXCL2 in cancer cells, we knocked down the expression of NFκB using siRNA in four cancer cell lines. A significant loss of NFκB-p65 protein expression was observed in cancer cell cultures transfected with siRNA directed against NFκB compared to control cells that were transfected with non-target-directed siRNA (Fig. 2B). A marked decline in the expression of CXCL1 and CXCL2 was seen in NFκB-silenced cells. Cells transfected with non-target-directed siRNA and treated with progesterone and calcitriol showed a marked attenuation of NFκB-p65, CXCL1, and CXCL2 expression. However, progesterone and calcitriol failed to affect expression of CXCL1 and CXCL2 in NFκB-p65 knockdown cancer cells. To establish that NFκB is essential for the stimulation of CXCL1 and CXCL2 expression, we treated control, scrambled siRNA transfected, and NFκB-silenced cells with LPS. A marked increase in CXCL1, CXCL2, and NFκB was observed in LPS-treated control and scrambled siRNA-transfected cells compared to non-LPS-treated control and scrambled siRNA-transfected cells. LPS had no effect on the expression of CXCL1, CXCL2, and NFκB in NFκB-silenced cells, suggesting that NFκB-p65 is essential for the stimulation for CXCL1 and CXCL2. These results clearly demonstrate that knocking down NFκB results in a diminished expression of the two inflammatory chemokines suggesting that progesterone and calcitriol inhibit inflammatory chemokines via modulation of NFκB-p65 (Fig. 2B).

### SILENCING OF IκBα ATTENUATES PROGESTERONE AND CALCITRIOL INDUCED DOWNREGULATION OF CXCL1 AND CXCL2 EXPRESSION IN CANCER CELLS

We sought to study the contribution of IκBα, NFκB's inhibitor, in progesterone and calcitriol-induced inhibition of CXCL 1 and CXCL2. OVCA 420, OVCA 429, Ishikawa and HEC-1B cells were transfected with siRNA directed to IκBα. Suppression of IκBα expression is shown in Figure 3A. CXCL1 and CXCL2 expression levels increased in IκBα siRNA-transfected cells compared to control siRNA-transfected cells. The rise in CXCL1 and CXCL2 expression can be ascribed to increased activation of NFκB, which is caused by the lack of its inhibitor, IκBα. Control siRNA-transfected cells exposed to progesterone and calcitriol showed a marked decrease in the expression of CXCL1 and CXCL2 compared to IκBα-silenced cells, suggesting that intact IκBα is required for progesterone and calcitriol to exhibit an inhibitory effect on chemokines (Fig. 3B).

### PROGESTERONE AND CALCITRIOL DOWNREGULATE CXCL1 AND CXCL2 EXPRESSION BY DECREASING PHOSPHORYLATED IκBα

The degradation of IκBα and subsequent release of NFκB (p65–p50) requires prior phosphorylation at Ser32 and Ser36 residues. Thus, to investigate whether the inhibitory effects of progesterone and calcitriol are mediated through alteration of phosphorylated IκBα, OVCA 420, OVCA 429, Ishikawa, and HEC-1B cells were treated with progesterone and calcitriol, and their protein extracts were assessed for phospho-IκBα expression. Results in Figure 4 show that untreated cells constitutively expressed Ser32-phosphorylated IκBα. Upon progesterone and calcitriol treatment, the phosphorylated IκBα content markedly decreased.

### SUPPRESSION OF CXCL1 AND CXCL2 BY siRNA OR BY PROGESTERONE AND CALCITRIOL INHIBITS TUMOR CELL INVASIVENESS

Invasiveness and metastatic potential are important characteristics of malignant cells. The effect of CXCL1 or CXCL2 suppression by specific siRNAs or by treatment of cells with progesterone or calcitriol was determined by measuring the ability of the transfected/treated cells to pass through a layer of the Matrigel using invasion chambers. Silencing of CXCL1 or CXCL2 in cancer cells is shown in Figure 5A. CXCL1 or CXCL2-silenced cells and cells exposed to progesterone or calcitriol for 3 days showed significant decreases (65–75% CXCL1or CXCL2-knockdown and 20–40% progesterone- and calcitriol-treated) in their invasiveness compared to control cells, suggesting that downregulation of CXCL1 or CXCL2 results in inhibition of ovarian and endometrial cancer cells

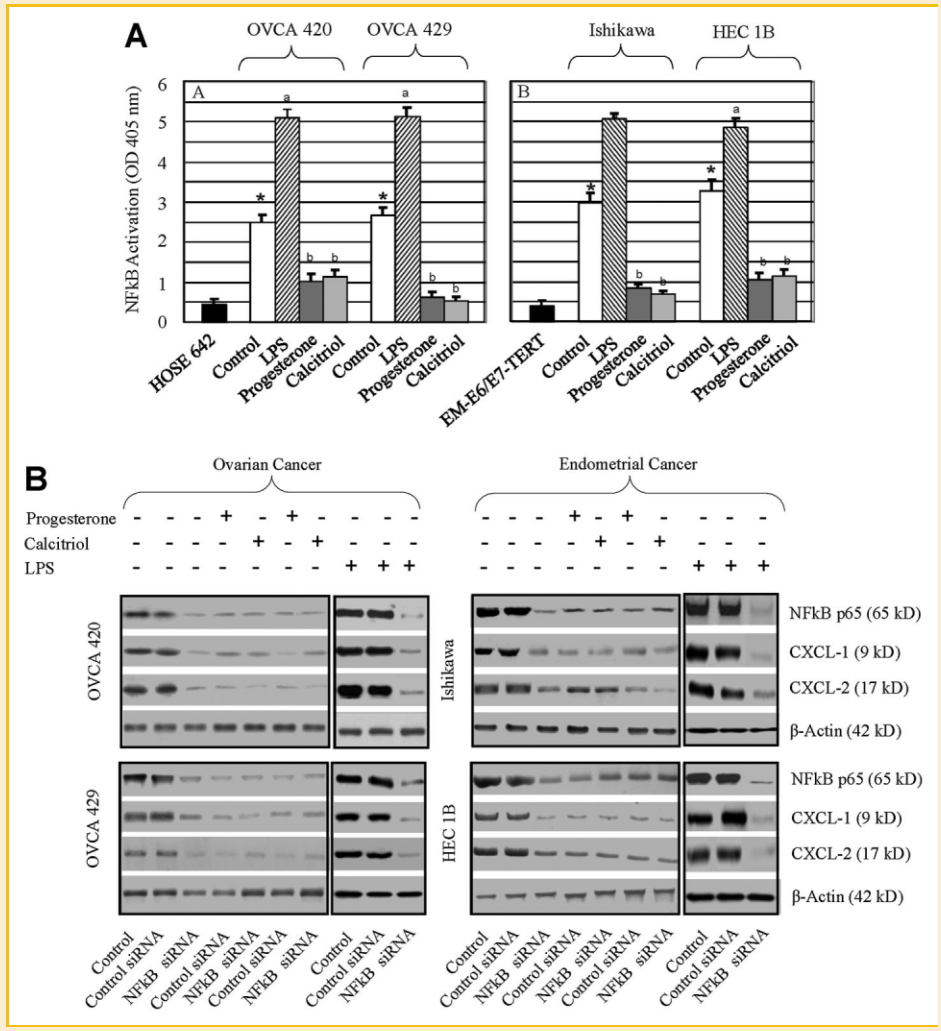


Fig. 2. CXCL1 and CXCL2 expression is regulated via the NFκB in ovarian and endometrial cancer cells. A: Progesterone or calcitriol inhibit activation of NFκB-p65 as measured in a DNA-binding assay. Cancer cells were treated with progesterone, calcitriol, or LPS for 72 h and processed for the preparation of nuclear protein extracts. The presence of activated NFκB-p65 in the nuclear extracts was examined with the TransAM NFκB-p65 Chemi Transcription Factor Assay kit. Normal ovarian surface epithelial cells (HOSE 642) and normal endometrial cells (EM-E6/E7-TERT) were used as controls. B: Western blots of cellular extracts from ovarian and endometrial cancer cell lines transfected with siRNA directed against NFκB or with non-targeted siRNA revealed downregulation of CXCL1 and CXCL2 in NFκB knockdown cells compared to non-targeted siRNA-tranfected non-treated controls. Cells transfected with non-targeted siRNA and treated with progesterone or calcitriol for 72 h showed significantly diminished expression of the two chemokines and NFκB expression compared to non-treated non-targeted siRNA-transfected cells. Progesterone or calcitriol showed no effect on the expression of NFκB, CXCL1, and CXCL2 in NFκB knockdown cells. Control and scrambled siRNA-transfected cells exposed to LPS (10 μg/ml) for 72 h showed a marked increase in the expression of CXCL1 and CXCL2 compared to NFκB knockdown cells. β-Actin was used as a loading control.

invasiveness (Fig. 5B). To further substantiate whether down-regulation of the two cytokines can affect the invasive potential of cells, we analyzed the expression of a series of metastasis-related genes in CXCL1- and CXCL2-silenced or progesterone or calcitriol-treated cancer cells. Marked attenuation of the metastasis-related genes CXCR4, COX2, MMP9, and MMP2 was seen in ovarian and endometrial cancer cells (Fig. 6A,B).

## DISCUSSION

Accumulating evidence supports that inflammation contributes to the development and progression of many cancers [Allavena et al., 2008; Mantovani et al., 2008]. Ovarian and endometrial cancers are emerging as an inflammation-driven condition that involves activation of complex cytokine and chemokine networks [Ness et al., 2000; Fleming et al., 2006; Furuya et al., 2007]. Recent research, including results from our laboratory, suggests that calcitriol and progesterone exhibit anti-tumorigenic actions that may contribute to their beneficial effects in several cancers [Ho, 2003; Maxwell et al., 2006; Rodriguez et al., 2008; Nguyen et al., 2011]. However, the mechanisms by which progesterone and calcitriol exert an inhibitory effect on inflammation are not known in ovarian and endometrial cancers.

The goal of this study was to elucidate the role of anti-tumorigenic agents, progesterone and calcitriol in the regulation of

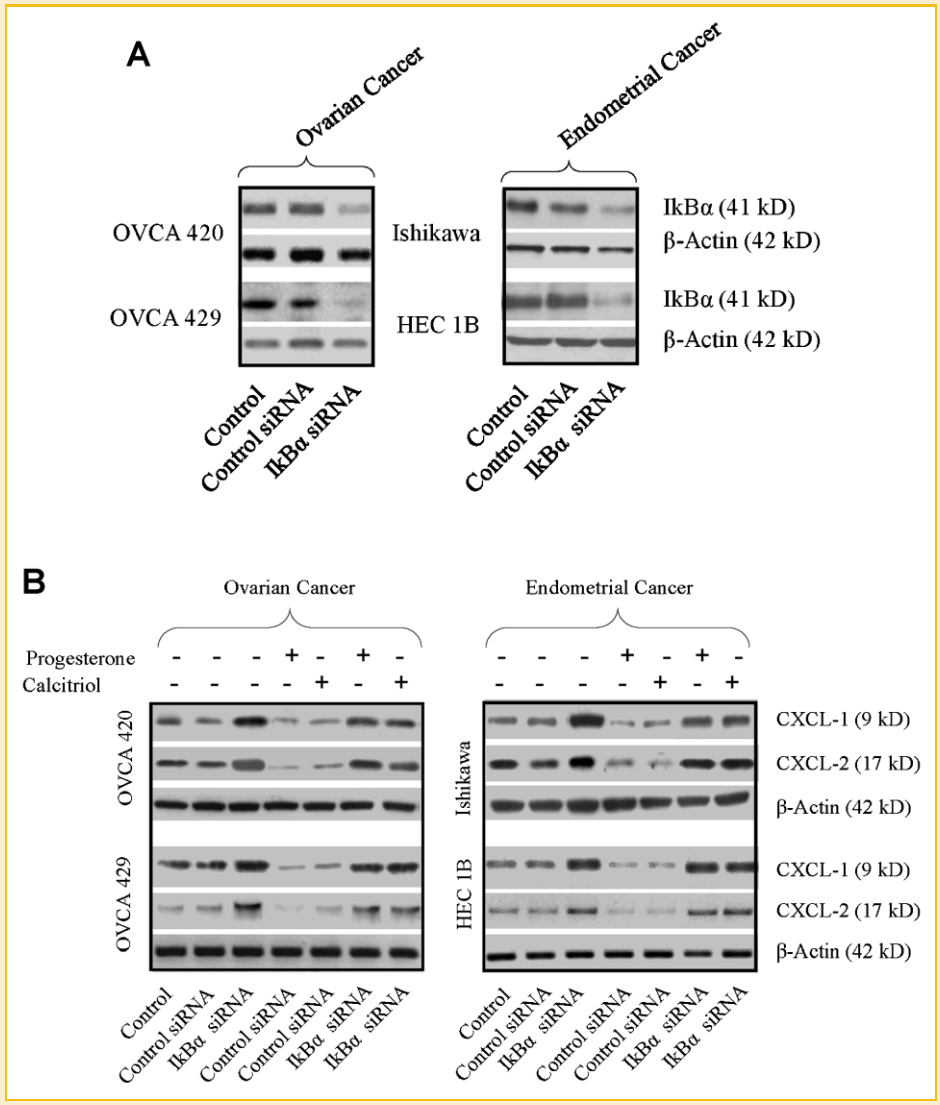


Fig. 3. Silencing of IκBα increases expression of CXCL1 and CXCL2 in cancer cells. A: Cellular extracts from ovarian and endometrial cancer cell lines transfected with siRNA directed against IκBα showed silencing of IκBα. B: Western blots of cellular extracts from ovarian and endometrial cancer cell lines transfected with IκBα siRNA or with non-targeted siRNA showed upregulation of CXCL1 and CXCL2 in IκBα knockdown cells compared to scrambled siRNA-transfected non-treated controls. Cells transfected with non-targeted siRNA and treated with progesterone or calcitriol for 72 h showed significantly diminished expression of the two cytokines expression compared to non-treated scrambled siRNA-transfected cells. Progesterone or calcitriol showed no effect on the expression of CXCL1 and CXCL2 in IκBα knockdown cells. β-Actin was used as a loading control.

inflammatory chemokines in ovarian and endometrial cancers. Our results demonstrate that (1) progesterone and calcitriol decreased the expression of CXCL1 and CXCL2 in cancer cells; (2) ovarian and endometrial cancer cells showed a higher level of NFκB transcriptional factor than normal ovarian and endometrial cells; (3) progesterone and calcitriol suppressed NFκB activity in cancer cells; (4) progesterone- and calcitriol-induced inhibition of CXCL1 and CXCL2 expression was abolished by silencing NFκB; (5) knockdown of Iκβα increased the expression of CXCL1 and CXCL2 in cancer cells; and (6) silencing of CXCL1 and CXCL2 expression was associated with reduced cell invasiveness and a marked decrease in the expression of metastasis-related proteins. To our knowledge, these findings are the first to report the inhibition of chemokines in ovarian and endometrial cancer cells by progesterone and calcitriol.

There are several reports that document the increased expression of chemokines, such as CXCL1, CXCL2, and CXCL12 in ovarian, endometrial, and breast cancer [Furuya et al., 2007; Son et al., 2007; Mantovani et al., 2008]. CXCL1 and CXCL2 are associated with tumors and are known to promote tumor growth, metastasis, as well as angiogenesis in squamous cell carcinomas [Loukinova et al., 2001] and in breast cancer [Youngs et al., 1997]. Our results demonstrate for the first time that progesterone and calcitriol implement their anti-carcinogenic effects by downregulating the

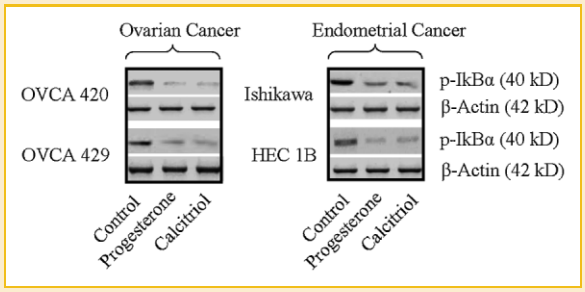


Fig. 4. Progesterone and calcitriol inhibit phosphorylated IκBα. OVCA 420, OVCA 429, Ishikawa, and HEC-1B cells were treated with progesterone (25 μM) or calcitriol (100 nM) for 72 h. Extracts were prepared for assessing the level of phosphorylated IκBα by Western blotting. β-Actin was used as a loading control.

expression of the two chemotactic cytokines in ovarian and endometrial cancer cells.

Inflammatory mediators such as cytokines and chemokines enhance tumorigenesis through activation of NFκB pathway [Allavena et al., 2008, 2011; Mantovani et al., 2008; Mantovani, 2010]. The NFκB pathway plays a pervasive role in the pathogenesis of cancer and aberrant NFκB signaling has also been identified in tumors of epithelial origin including breast, colon, and lung carcinomas [Mantovani, 2010; Wallace et al., 2010; Allavena et al., 2011].

The relationship of progesterone and NFκB is complex. Progesterone has been shown to suppress constitutive NFκB activation in cancer cells by many different mechanisms. Davies et al. [2004] suggested that progesterone induces the transcription of a zinc finger protein A20 which is a cellular inhibitor of NFκB and a binding inhibitor of NFκB activation-2 (ABIN-2) genes resulting in the inactivation of free NFκB in the cytoplasm. Other studies revealed repression of NFκB activity in cancer cells and associated the repression with increased transcription of the Ikβα promoter [McKay and Cidlowski, 1999; Deroo and Archer, 2002]. Still another report showed that NFκB(p65 RelA) and progesterone receptor (PR) may negatively influence each other by direct interaction [Kalkhoven et al., 1996; Kobayashi et al., 2010]. Using TansAm NFκB kit that detects NFκB only when NKκB is activated and bound to its target DNA, a significant increase in NFkB-p65 activation was observed in ovarian and endometrial cancer cells compared to their normal control cells. We found that progesterone and calcitriol suppressed constitutive NFκB activation in all cell lines suggesting a direct action of the two agents on NFκB. Our results are in agreement with studies reporting inhibition of NFκB signaling by calcitriol in prostate and breast cancer cells [Bao et al., 2006; Tse et al., 2010].

Knockdown of NFκB resulted in a reduced expression of the two proinflammatory chemokines in ovarian and endometrial cancer cells. Progesterone and calcitriol had no effect on inflammatory cytokines in NFκB-silenced cells compared to control siRNA-transfected cells where marked inhibition of cytokines expression was seen, suggesting that progesterone and calcitriol need NFκB to illustrate their effect on proinflammatory chemokines.

We performed silencing experiments in order to delineate whether IκBα, which acts upstream of NFκB, influences chemokine expression. Using siRNA oligo directed against IκBα, a marked increase in the expression of CXCL1 and CXCL2 was observed, which can be attributed to the increased activation of NFκB-p65 caused by the lack of its inhibitor. Progesterone and calcitriol failed to inhibit expression of CXCL1 and CXCL2 in IκBα-silenced cells, implying that progesterone and calcitriol mediate their effect by modulating IκBα.

The phosphorylation of IκBα occurs through the activation of Iκβ kinase (IKK) which results in dissociation of IκBα from NFκB. The activated NFκB is then translocated into the nucleus where it binds to specific sequences of DNA and initiates gene transcription. The translocation of NFκB to the nucleus is preceded by the proteolytic degradation of IκBα [Ghosh and Karin, 2005]. We found that progesterone and calcitriol suppressed phosphorylation of IκBα attenuated NFκB and eventually downregulated the expression of CXCL1 and CXCL2 in cancer cells. Whether progesterone and calcitriol suppress phosphorylation of IκBα by suppressing IKK activation that resulted in inhibition of IκBα phosphorylation and abrogation of IκBα degradation remains to be seen.

Since NFκB binding sites have been identified in the promoter region of many metastasis-related genes, including COX2 and MMP [Himelstein et al., 1998; Chen et al., 2005], we studied the effect of CXCL1 and CXCL2 knockdown/downregulation on cell invasiveness and several NFκB regulated proteins, such as COX2, MMP9, MMP2, and CXCL4. Downregulation of CXCL1 and CXCL2 was associated with a decreased number of cells that migrated through the Matrigel membrane, implicating a decrease in the invasive potential of ovarian and endometrial cancer cells. The discrepancy between the 65% and 75% decrease in cell invasiveness with CXCL1 or CXCL2 knockdown and 20–40% observed with progesterone or calcitriol treatment can be explained by the fact that hormone-induced downregulation of CXCL1 and CXCL2 required more time to achieve levels comparable to levels of CXCL1 and CXCL2 in siRNA-transfected cells. By comparing the expression levels of two chemokines in CXCL1 and CXCL2 silenced cells and scrambled siRNA-transfected cells treated with calcitriol and progesterone for 3 days, it was evident that the levels of CXCL1 and CXCL2 were significantly decreased in CXCL1 and CXCL2 silenced cells compared to scrambled siRNA-transfected hormone-treated cells. Thus, inhibition of cell invasion is inversely dependent on the levels of CXCL1 and CXCL2 expression.

Results also revealed a marked decrease in the expression of NFκB regulated metastasis suppressor proteins in CXCL1 and CXCL2-silenced cells or cells exposed to progesterone and calcitriol. Our results are in concurrence with studies demonstrating marked decrease in metastatic potential of CXCL1 and CXCL2-silenced MDA-MB-231 and MCF-7 cells that was associated with reduced expression of several prometastatic genes [Li and Sidell, 2005; Minn et al., 2005; Bachmeier et al., 2008].

In conclusion, we provide evidence that progesterone and calcitriol inhibit ovarian and endometrial cancer migration and

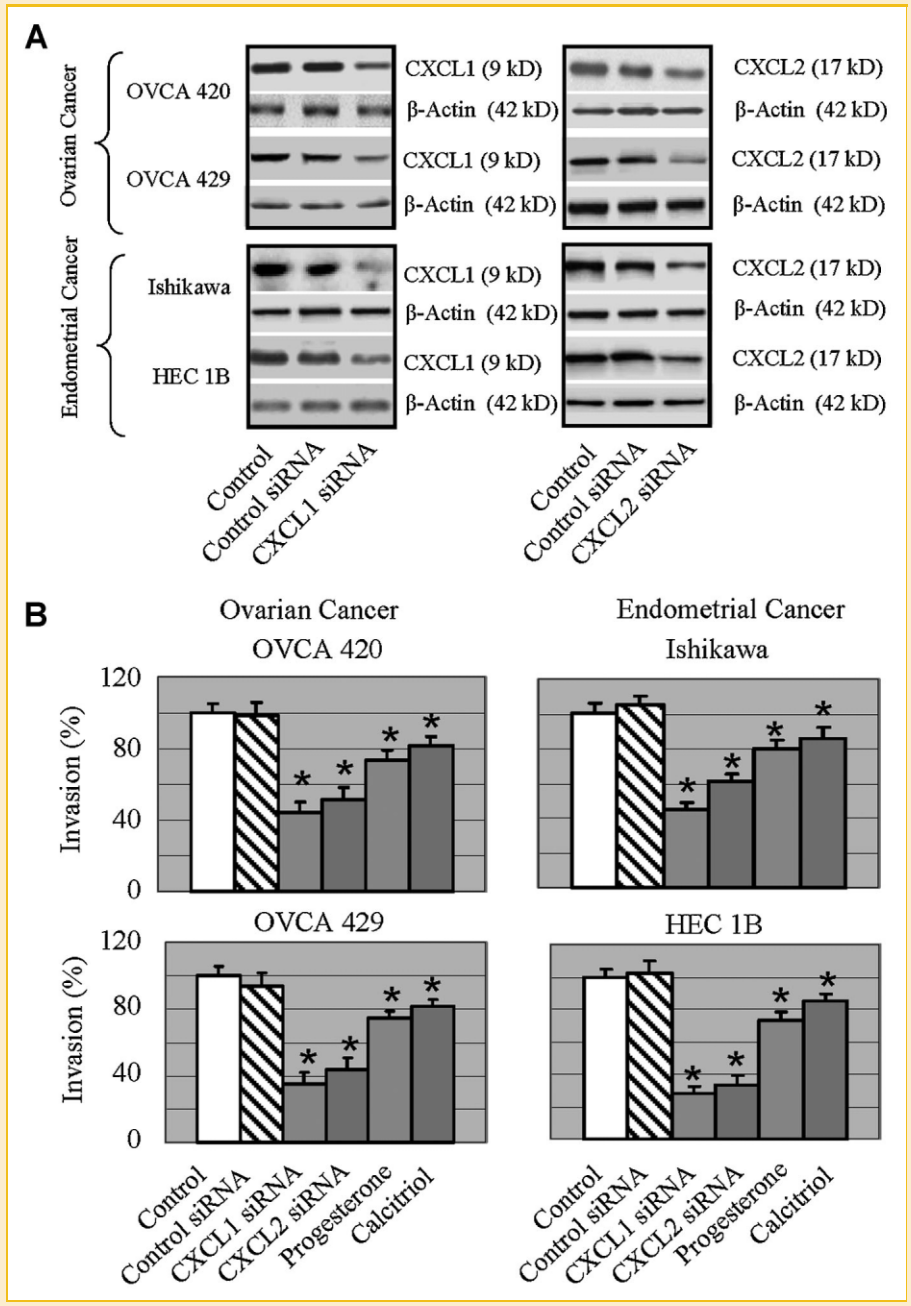


Fig. 5. The effect of CXCL1 and CXCL2 downregulation on ovarian and endometrial cancer cell invasion. A: Cellular extracts from ovarian and endometrial cancer cell lines transfected with siRNA directed against CXCL1 or CXCL2 showed silencing of CXCL1 or CXCL2. B: The cell lines (OVCA 420, OVCA 429, Ishikawa, and HEC-1B) transfected with CXCL1- or CXCL2-specific siRNA, scrambled siRNA or non-transfected cells treated with progesterone or calcitriol were plated on Matrigel to assess cell invasion. After 22 h, cells that migrated through the Matrigel were counted. Columns, mean of three independent experiments; bars ± SD. *Statistically significant changes in cell invasion, compared with those seen in control cells ($P < 0.05$).

invasive potential by inhibiting phosphorylated Iκβα and NFκB activation, which consequently decrease the expression of prometastatic chemokines, which in turn decreases the expression of metastasis-promoting genes. Our findings indicate that progesterone and calcitriol may have potential therapeutic benefits against ovarian and endometrial cancers because of their anti-inflammatory and anti-invasive properties.

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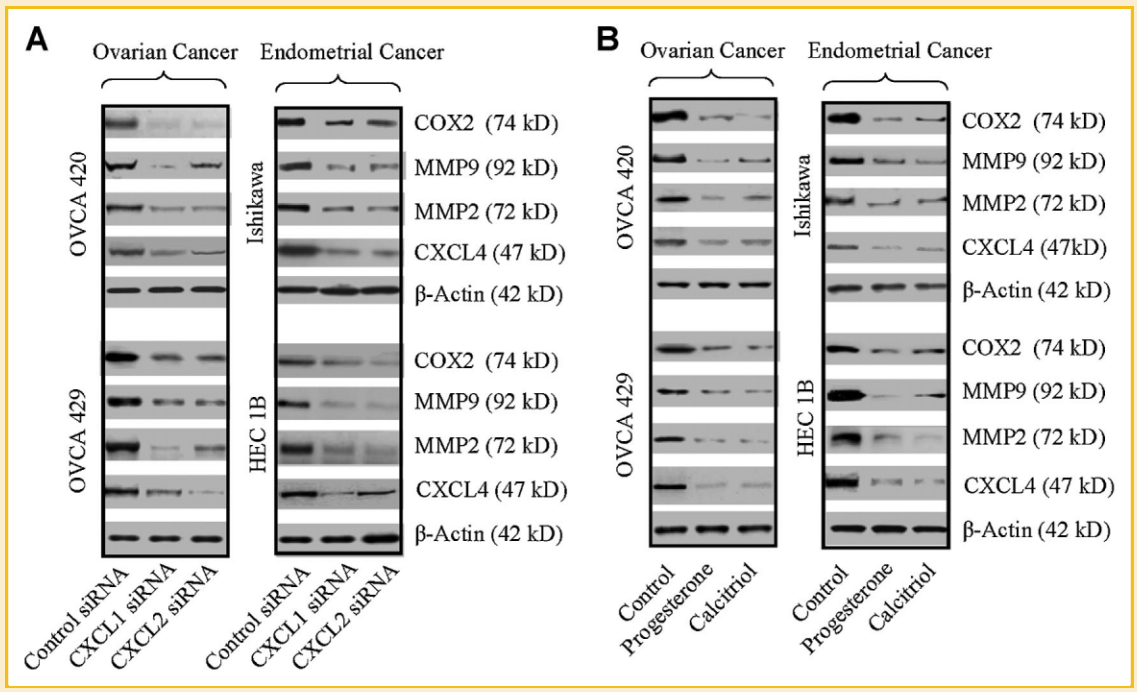


Fig. 6. The effect of CXCL1 and CXCL2 downregulation on the metastasis-related proteins in ovarian and endometrial cancer. A: Cells extracts from CXCL1 and CXCL2 silenced cells, or (B) from cells treated with progesterone or calcitriol for 72 h were analyzed by Western blotting for the expression of CXCR4, COX2, MMP9, and MMP2. β-Actin was used as a loading control.

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