# Estrous Cycle Modulates Ovarian Carcinoma Growth

Guillermo N. Armaiz-Pena,[1,3] Lingegowda S. Mangala,[1] Whitney A. Spannuth,[1] Yvonne G. Lin,[1] Nicholas B. Jennings,[1] Alpa M. Nick,[1] Robert R. Langley,[2] Rosemarie Schmandt,[1] Susan K. Lutgendorf,[4] Steven W. Cole,[5] and Anil K. Sood[1,2]

**Abstract**

**Purpose:** The effects of reproductive hormones on ovarian cancer growth are not well understood. Here, we examined the effects of estrous cycle variation and specific reproductive hormones on ovarian cancer growth.

**Experimental Design:** We investigated the role of reproductive hormones in ovarian cancer growth using both *in vivo* and *in vitro* models of tumor growth.

**Results:** *In vivo* experiments using the HeyA8 and SKOV3ip1 ovarian cancer models showed that tumor cell inoculation during proestrus significantly increased tumor burden (251-273%) compared with injection during the estrus phase. Treatment of ovariectomized mice with 17β-estradiol resulted in a 404% to 483% increase in tumor growth compared with controls. Progestins had no significant effect, but did block estrogen-stimulated tumor growth. Tumors collected from mice sacrificed during proestrus showed increased levels of vascular endothelial growth factor (VEGF) and microvessel density compared with mice injected during estrus. HeyA8, SKOV3ip1, and mouse endothelial (MOEC) cells expressed estrogen receptor α and β and progesterone receptor at the protein and mRNA levels, whereas 2774 ovarian cancer cells were estrogen receptor – negative. *In vitro* assays showed that 17β-estradiol significantly increased ovarian cancer cell adhesion to collagen in estrogen receptor – positive, but not in estrogen receptor – negative cells. Additionally, 17β-estradiol increased the migratory potential of MOEC cells, which was abrogated by the mitogen-activated protein kinase (MAPK) inhibitor, PD 09859. Treatment with 17β-estradiol activated MAPK in MOEC cells, but not in HeyA8 or SKOV3ip1 cells.

**Conclusion:** Our data suggest that estrogen may promote *in vivo* ovarian cancer growth, both directly and indirectly, by making the tumor microenvironment more conducive for cancer growth.

There is growing evidence that the menstrual cycle, through variation of reproductive hormone levels, can affect tumor growth and metastasis (1–4). Although this observation is best characterized in breast cancer patients, it likely also impacts the behavior of tumors in women with gynecologic tumors, such as ovarian cancer. Premenopausal breast cancer patients who undergo surgery during the luteal phase of the menstrual cycle have higher disease-free intervals and better overall survival rates than those who had surgery during other phases of the cycle (5, 6). However, the mechanisms underlying this observation are not clearly understood. It is known that in the follicular phase, higher levels of estrogen can alter some aspects of immune response, reduce phagocytic activity, and increase growth factor levels, potentially increasing the growth and spread of cancer cells (7). Obese patients, who produce more peripheral aromatase, have higher estrogen levels when compared with thin patients and this fact has been suggested to contribute to a higher risk of developing premenopausal breast cancer (8, 9). Furthermore, higher circulating levels of estrogen are known to increase angiogenesis by promoting the expression of vascular endothelial growth factor (VEGF; refs. 3, 10–13). Conversely, in the luteal phase, the increase in circulating progesterone may decrease the proliferation of normal and tumor tissue and increase intercellular cohesion resulting in reduced tumor growth and decreased metastatic potential (14, 15). Progesterone has also been found to reduce the number of estrogen receptors expressed on myoepithelial breast cells, which can potentially result in apoptosis (16). Both clinical and preclinical studies suggest that estrogen receptor–negative cancers still depend on reproductive hormones for

**Authors' Affiliations:** Departments of [1]Gynecologic Oncology and [2]Cancer Biology, The University of Texas M. D. Anderson Cancer Center, [3]Program in Cancer Biology, The University of Texas Graduate School of Biomedical Sciences, Houston, Texas; [4]Department of Psychology, University of Iowa, Iowa City, Iowa; and [5]Division of Hematology-Oncology, Department of Medicine, University of California Los Angeles School of Medicine, Los Angeles, California

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**Requests for reprints:** Anil K. Sood, Departments of Gynecologic Oncology and Cancer Biology, The University of Texas, M. D. Anderson Cancer Center, 1155 Herman Pressler, Unit 1352, Houston, TX 77030. Phone: 713-745-5266; Fax: 713-792-7586; E-mail: asood@mdanderson.org.



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## Translational Relevance

The effects of reproductive hormones on ovarian cancer growth are not well understood. In this study, we examined the role of estrous cycle variation and specific reproductive hormones on ovarian cancer growth by utilizing *in vitro* and *in vivo* models. Our data suggest that estrogen promotes *in vivo* ovarian cancer growth by making the tumor microenvironment more conducive for cancer growth. These findings offer new insights into the mechanisms underlying the effects of reproductive hormones on ovarian carcinoma and therein identify potential avenues for improvement in targeted therapies.

their formation and progression by influencing tumor-associated endothelial cells (17–20).

Paralleling clinical findings, there is some preclinical evidence that the timing of the estrous cycle can affect cancer growth and metastasis (1–4). For example, in preclinical breast cancer models, a 96% surgical cure rate is noted with resection during the estrus phase, when progesterone is the dominant hormone. Conversely, surgical cure rates drop to 33% when the tumor was resected during proestrus, when estrogen levels peak (2, 3). Additionally, in a preclinical melanoma model, a third of the mice injected during metestrus developed ovarian metastases, whereas no such metastases were found in mice injected during proestrus (4). These results suggest a possible role for circulating reproductive hormones in modulating the growth and metastatic spread of cancer cells.

Based on the paucity of information regarding the effects of reproductive hormones in ovarian cancer growth and progression, we examined the *in vivo* effects of variations in the estrous cycle on ovarian cancer growth and progression. In addition, we tested the effects of pharmacologic exogenous estrogen and progestin therapy in tumor-bearing ovariectomized animals.

## Materials and Methods

***Reagents.*** Unless otherwise indicated, chemicals were purchased from Sigma-Aldrich.

***Cell lines and culture.*** HeyA8, SKOV3ip1, and 2774 epithelial ovarian cancer cell lines were maintained as previously described (21, 22). The cells were cultured in phenol red-free RPMI-1640 supplemented with 15% charcoal-stripped fetal bovine serum and 0.1% gentamicin sulfate (Gemini Bioproducts) at 37°C in 5% $CO_2$/95% air. The derivation and characterization of murine ovarian endothelial cells (MOEC; a kind gift of Dr. Robert Langley, Department of Cancer Biology, M. D. Anderson Cancer Center, Houston, TX) have been described previously (23). MOEC cells were cultured in phenol red free DMEM supplemented with 10% charcoal stripped fetal bovine serum and 0.1% gentamicin sulfate at 33°C in 5% $CO_2$/95% air. *In vitro* studies were done with 70% to 80% confluent cultures.

***Characterization of the estrous cycle.*** Female athymic mice (NCr-*nu*) were purchased from the National Cancer Institute-Frederick Cancer Research and Development Center. The mice were housed 5 per cage on a 12-h day/night light cycle and maintained under specific pathogen-free conditions in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with the current regulations and standards of the U.S. Department of Agriculture, the U.S. Department of Health and Human Services, and the NIH. All studies were approved and supervised by the University of Texas M. D. Anderson Cancer Center Institutional Animal Care and Use Committee. All mice used in these experiments were 8-10 wk old. To avoid periods of anestrous and to synchronize the estrous cycle, female mice were exposed to bedding containing male urine prior to the start of the experiments (24). To determine the estrous cycle phase, vaginal sampling took place daily 3 to 4 h after light onset for at least one full cycle before the experiments started, and daily sampling continued throughout the experiment. Samples were obtained by vaginal lavage, the washout deposited on a glass slide, mixed with a drop of Gill's No. 3 hematoxylin, and coverslipped. Slides were examined using light microscopy and estrous phase determined based on standard vaginal cytology (Fig. 1A).

***Orthotopic* in vivo *model of ovarian cancer.*** Animals determined to be in either proestrus or estrus were injected i.p. with either SKOV3ip1 ($1 \times 10^6$ cells per 0.2 mL HBSS; Life Technologies Invitrogen) or HeyA8 cells ($2.5 \times 10^5$ cells per 0.2 mL HBSS). For *in vivo* experiments, cells were collected by trypsinization and centrifugation at 1,100 rpm for 7 min at 4°C. Cells were then washed twice in PBS before being reconstituted in HBSS. Only single-cell suspensions with >95% viability, as determined by trypan blue exclusion, were used for the *in vivo* injections. Mice ($n$ = 10 per group) were sacrificed on day 28 (HeyA8) or day 35 (SKOV3ip1). Total body weight, tumor incidence and mass, and the number of tumor nodules were recorded. Tumors were either fixed in formalin and embedded in paraffin or snap-frozen in optimal cutting temperature compound (Sakura Finetek) in liquid nitrogen.

***Hormone replacement therapy.*** Anesthetized nude female mice underwent bilateral ovariectomy. The animals were then allowed to recover for 2 wk to remove residual estrogen and progesterone from the body and to allow inflammation to subside. For the first study, slow-release pumps (Alzet Osmotic Pumps) containing water-soluble 17β-estradiol (28 μg/d), water-soluble progesterone (167 μg/d), β-cyclodextrin (carrier molecule for water-soluble hormones), or a combination of 17β-estradiol and progesterone were introduced s.c. 3 d before SKOV3ip1 cells were injected i.p. into the mice. The mice were sacrificed 31 d after tumor cell inoculation. To validate our findings a similar experiment was done in the HeyA8 model. In this experiment, the mice were treated with water-soluble 17β-estradiol, daily megestrol acetate (250μg/d), β-cyclodextrin, or a combination of 17β-estradiol and megestrol acetate. The mice were sacrificed 28 d after tumor cell inoculation.

***Immunoprecipitation.*** All Western blot analyses for estrogen receptor β expression were completed after immunoprecipitation with anti–estrogen receptor β (Calbiochem). Briefly, 500 μg of protein in a total volume of 650 μL of modified radio immunoprecipitation assay lysis buffer with protease inhibitor were allowed to incubate with 7 μL of anti–estrogen receptor β antibody overnight in 4°C. Subsequently, 50 μL of purified 50% protein G agarose slurry (Upstate Cell Signaling Solutions) were added to the samples and rotated for 2 h in 4°C. This was followed by three sequential washings with modified radio immunoprecipitation assay lysis buffer. Finally, an appropriate amount of 3× Laemmli's sample buffer was added and boiled for 5 min. Anti–estrogen receptor β antibody was used at a 1:1,000 dilution, and visualized using antimouse horseradish peroxidase at 1:1,000 dilution (Amersham Biosciences UK Limited).

***Western blot analysis.*** Cell lysates were prepared by washing cells with PBS and incubating them for 10 min at 4°C in modified radio immunoprecipitation assay lysis buffer. Cells were scraped from plates, centrifuged at 13,000 rpm for 20 min at 4°C, and the supernatant was stored at -80°C. Protein concentrations were determined using a bicinchoninic acid protein assay reagent kit (Pierce), and 50 μg of whole cell lysate were subjected to 10% SDS-PAGE separation. Samples were transferred to a nitrocellulose membrane by wet electrophoresis (Bio-Rad Laboratories), blocked

with 5% nonfat milk in TBS Tween 20 for 1 h at room temperature, and incubated with 1 μg/mL of anti–estrogen receptor α, anti-phosphorylated mitogen-activated protein kinase (MAPK) or anti-MAPK overnight at 4°C. Primary antibody was detected with antirabbit IgG linked to horseradish peroxidase (Amersham Biosciences), and developed with an enhanced chemiluminescence detection kit (Pierce).

***Reverse transcriptase PCR.*** Total RNA was isolated from MOEC and ovarian cancer cells using Qiagen RNAeasy kit (Qiagen Inc.). Next, cDNA was synthesized from 5 μg of total RNA using the Superscript First-Strand Kit (Invitrogen) as per the manufacturer's instructions. The cDNA was subjected to PCR using specific primers (Table 1) along with *β-actin* as a housekeeping gene. PCR was done with 1 μg of reverse-transcribed RNA and 100 ng/μL of sense and antisense primers in a total volume of 20 μL. Each cycle consisted of 45 s at 94°C for denaturing the sample, 1 min of annealing at varying temperatures (Table 1), and 45 s of elongation at 72°C (35 cycles). Amplified PCR products were analyzed by electrophoresis on 1% agarose gel with Tris-borate-EDTA buffer and visualized under UV light after staining with ethidium bromide.

***Adhesion assay.*** HeyA8 and SKOV3ip1 cells ($5 \times 10^4$) were plated on a 96-well plate precoated with either collagen 1 (20 μg/mL) or 2% denatured bovine serum albumin. At the time of seeding, cells were exposed to 100 nmol/L of 17β-estradiol and incubated for 60 min at 37°C. Nonadherent cells were removed by washing wells with PBS. Adherent cells were then fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. Adherent cells were quantified by counting the number of attached cells per 40× high-power fields over 5 randomly selected fields.

***Migration assay.*** Viable MOEC cells (cell viability determined by trypan blue exclusion) were resuspended in serum-free medium ($1 \times 10^5$ cells per mL), and 1 mL was added to the upper wells of a membrane invasion culture system as previously described (25). A 0.1% gelatin-coated membrane separated the upper wells from the lower wells, which were filled with serum-free media that lacked (control) or contained 17β-estradiol, PD 09859, or a combination. The membrane culture system was incubated for 6 h at 37°C. Cells that had migrated into the bottom wells were removed and collected in 0.1% EDTA, loaded onto a 3.0-μm polycarbonate filter (Osmonics) using a Minifold I Dot-Blot System (Schleicher & Schuell), and fixed, stained,

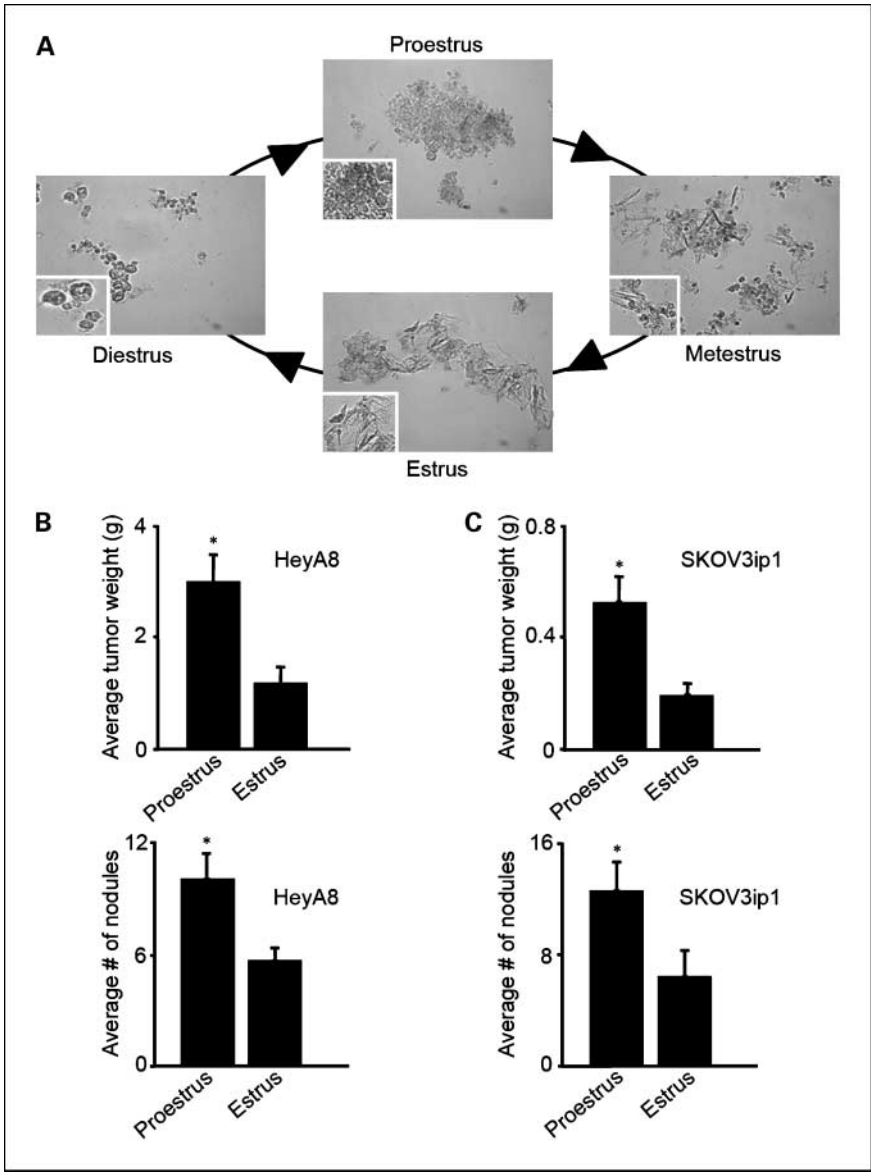


**Fig. 1.** Inoculation of tumor cells into the peritoneal cavity of mice during proestrus results in higher tumor weights and nodule formation. *A,* characterization of the estrous cycle in nude mice by vaginal cytology. *In vivo* effects of the estrous cycle on tumor growth was assessed on mice injected i.p. with either HeyA8 (*B*) or SKOV3ip1 (*C*) cells during proestrus or estrus. Three to four weeks later, the animals were sacrificed and a necropsy was done. Mice injected during proestrus showed increased tumor burden (251-273%; $P < 0.05$) and increased tumor nodule formation (178-196%; $P < 0.05$).

**Table 1.** Primer sequences and PCR strategy used in this study

| Gene | Primer sequences* | Annealing temperature (°C) |
|---|---|---|
| Human ER α | F, 5′-AGGCTGCGGCGTTCGGC-3′<br>R, 5′-AGCCATACTTCCCTTGTCAT-3′ | 53 |
| Murine ER α | F, 5′-CGTGTGCAATGACTATGCCTC-3′<br>R, 5′-TTTCATCATGCCCACTTCGTAA-3′ | 65 |
| Human ER β | F, 5-TTCCCAGCAATGTCACTAACT-3′<br>R, 5-CTCTTTGAACCTGGACCAGTA-3′ | 54 |
| Murine ER β | F, 5-CTGTGATGAACTACAGTGTTCCC-3′<br>R, 5-GCAGTGGGTGGCTAAAGGA-3′ | 62 |
| Human PR | F, 5-GATTCAGAAGCCAGCCAGAG-3′<br>R, 5-AGTTGCCTCTCGCCTAGTTG-3′ | 55 |
| Murine PR | F, 5-GAGCTGCATTCTACTCGCTG-3′<br>R, 5-CGCGGATATAGCTTGATCTCATC-3′ | 62 |

Abbreviations: ER, estrogen receptor; PR, progesterone receptor.
*F, Forward primer, R, reverse primer.

and counted by light microscopy, as previously described (26). The cells in 10 randomly chosen fields (×400 final magnification) were counted.

***Immunohistochemistry.*** CD31 immunohistochemistry was done on freshly cut frozen tissue. Briefly, slides were fixed in cold acetone and then incubated with anti-CD31 [platelet/endothelial cell adhesion molecule 1 (PECAM-1)] antibody (Pharmingen). Microvessel density was quantified by counting the number of microvessels per 200× high power fields over 10 randomly selected 0.159-$mm^2$ fields. A microvessel was defined as a discrete CD31-positive cluster or single cell adjacent to a lumen. VEGF immunohistochemistry analyses were done as previously described (27). Briefly, after deparaffinization and rehydration, antigen retrieval was carried out using pepsin in a 37°C humidified incubator. Endogenous peroxidases and nonspecific epitopes were blocked with 3% $H_2O_2$/methanol and 5% normal horse serum with 1% normal goat serum, respectively. Slides were then incubated with the appropriate primary antibody at 1:100 in blocking solution overnight at 4°C. After incubating with the appropriate secondary antibody conjugated to horseradish peroxidase, detection was achieved with 3,3′-diaminobenzidine substrate and counterstained with Gill's No. 3 hematoxylin.

***Statistical analyses.*** For *in vivo* experiments, differences in continuous variables (mean body weight, tumor weight, and microvessel density) were analyzed using Student's *t*-test for comparing two groups and by ANOVA for multiple group comparisons with $P < 0.05$ considered statistically significant. For values that were not normally distributed, the Mann-Whitney rank sum test was used. The Statistical Package for the Social Sciences (SPSS, Inc.) was used for all statistical analyses.

## Results

***Effect of estrous cycle timing on ovarian cancer growth.*** Prior to examining the effects of estrous cycle variation on cancer growth, we first characterized the duration of estrous cycle in nude mice. Daily vaginal smears were obtained and analyzed by cytology (Fig. 1A). The diestrus phase was characterized by few cells and occasional epithelial cells, whereas proestrus was defined by the presence of >75% of nucleated epithelial cells. Metestrus was characterized by the presence of approximately equal proportions of nucleated epithelial cells and anucleated cornified epithelial cells. Estrus was defined as >75% of anucleated cornified epithelial cells.

To account for variability in the estrous cycle, we sought to synchronize the cycle prior to assessing effects on tumor growth. Therefore, mice were maintained for two weeks in cages pretreated with male urine. After this period, the estrous cycle was synchronized, as determined by daily vaginal cytology (data not shown). After this pretreatment, mice were injected i.p. with either SKOV3ip1 or HeyA8 ovarian cancer cells ($n = 10$ mice/group) during either proestrus or estrus. Three to four weeks after tumor cell injection, the animals were sacrificed and a necropsy was done. In the HeyA8 tumor model, mice injected during proestrus showed a 251% increase in tumor weight accompanied by a 178% increase in tumor nodules compared with the group injected in estrus ($P = 0.01$; Fig. 1B). Similar results were obtained in the SKOV3ip1 tumor model where tumor weight was increased by 273% and tumor nodules by 196% when mice were injected with tumor cells in proestrus compared with estrus (Fig. 1C).

***Effects of 17β-estradiol on ovarian cancer growth.*** Based on the increased tumor growth observed with tumor cell injection in proestrus, we next asked whether the increased tumor growth was related to higher estrogen levels. To answer this question, we administered estrogen or progesterone to tumor-bearing mice following bilateral ovariectomy. Treatment with 17β-estradiol alone resulted in a 404% increase in tumor weight in the SKOV3ip1 model compared with treatment with the β-cyclodextrin control ($P < 0.01$; Fig. 2A). Although progesterone alone did not have any effect on tumor growth, the combination of progesterone with 17β-estradiol restored tumor burden levels to near control levels (Fig. 2A). Furthermore, we did not see any significant differences in tumor nodule counts (data not shown) in this model after hormone treatment. To confirm our findings a second experiment was done utilizing the HeyA8 model. In this experiment, 17β-estradiol treatment resulted in a 483% increase in tumor weight compared with controls ($P < 0.01$; Fig. 2B). Consistent with the SKOV3ip1 model, the combination of megestrol acetate with 17β-estradiol restored tumor burden to near control levels (Fig. 2B). Similar effects were noted on the number of tumor nodules (data not shown).

***Proestrus promotes angiogenesis.*** To explore possible mechanisms that might explain the increase in tumor growth resulting from inoculation of tumor cells during proestrus, we examined whether angiogenesis was affected. Microvessel density was calculated following CD31 staining of tumors from each group. Mice injected in proestrus showed a 191% increase in microvessel density when compared with that of the estrus group ($P < 0.05$; Fig. 2C). Because it is known that VEGF is an important mediator of angiogenesis and that it is increased in proestrus, we examined its expression in tumor samples. VEGF levels were increased in tumors harvested from mice that were sacrificed in proestrus, compared with mice sacrificed in estrus (Fig. 2D).

***Hormone receptor expression in tumor and endothelial cells.*** To examine whether the effects of estrogen on tumor growth might be direct (tumor cells) or indirect (microenvironment), we next analyzed the expression of estrogen receptor α, estrogen receptor β, and progesterone receptor mRNA in the HeyA8, SKOV3ip1, 2774, and MOEC cells using reverse transcription-PCR. All cell lines expressed estrogen receptor α, estrogen receptor β, and progesterone receptor (Fig. 3A), whereas 2774 cells did not express either estrogen receptor

(data not shown). Furthermore, Western blot analysis showed that all HeyA8, SKOV3ip1, and MOEC cells expressed estrogen receptor α, estrogen receptor β, and progesterone receptor at the protein level (Fig. 3B).

***17β-estradiol increases tumor cell adhesion to collagen coated plates.*** Given the observed effects of the estrous phase on the number of nodules, we next asked whether estrogen might affect cell adhesion, thereby enhancing metastatic spread. Results showed that 100 nmol/L 17β-estradiol increased the collagen-adhesive potential of HeyA8 cells by 30% and SKOV3ip1 cells by 38% ($P < 0.01$) but not in estrogen receptor–negative 2774 cells (Fig. 4A). These effects could be detected with 17β-estradiol levels as low as 100 pmol/L (Fig. 4A).

***17β-estradiol increases the migratory potential of endothelial cells through activation of the MAPK pathway.*** It is known that estrogen can cause the activation of the MAPK pathway (28–30). Specifically, it has been shown in various endothelial cell lines that estrogen-induced activation of the MAPK pathway might play a role in endothelial cell migration (31, 32). We next examined whether estrogen treatment activates MAPK in ovarian cancer and endothelial cells. Phospho-MAPK levels were significantly increased by 17β-estradiol in MOEC cells, but did not change significantly at any time point tested in HeyA8 or SKOV3ip1 cells (Fig. 4B). Total MAPK levels remained unchanged in all cases. Based on growing information regarding the role of estrogen on endothelial cell migration, we assessed *in vitro* migration using the membrane invasion culture system assay. 17β-Estradiol increased the migratory ability of MOEC cells by 2.5-fold when compared with controls ($P < 0.02$; Fig. 4C). To evaluate the functional role of MAPK, we treated cells with 20 μmol/L PD 90859 (MAPK inhibitor) with or without 17β-estradiol. PD 90859 abrogated 17β-estradiol–mediated induction of endothelial cell migration (Fig. 4C).

## Discussion

The key finding of this study is that hormonal variations during the estrous cycle can affect ovarian cancer growth and progression. We found that inoculation of mice during proestrus results in enhanced tumor growth. This effect was also seen in tumor-bearing ovariectomized mice treated with exogenous estrogen. Additionally, we showed that progestins could abrogate estrogen-induced cancer growth. These observations are further supported by the inhibitory effects of progestins on *in vivo* and *in vitro* growth of some cancers, including ovarian cancer (33–36). Furthermore, mechanistic studies done in our laboratory reveal that estrogen promotes angiogenesis, endothelial cell migration, and tumor cell adhesion.

An association between the reproductive cycle and malignant progression has long been suspected (1–6). In the present study, we sought to identify direct and indirect processes and pathways that link the estrous cycle to malignant cell behavior in ovarian carcinomas. Both estrogen and progesterone serve as dominant reproductive hormones in the menstrual cycle and are therefore likely key mediators in this process. Proestrus is associated with higher levels of estrogen, whereas estrus is characterized by decreased estrogen (3). Consistent with estrogen induction of VEGF production by endothelial and ovarian cancer

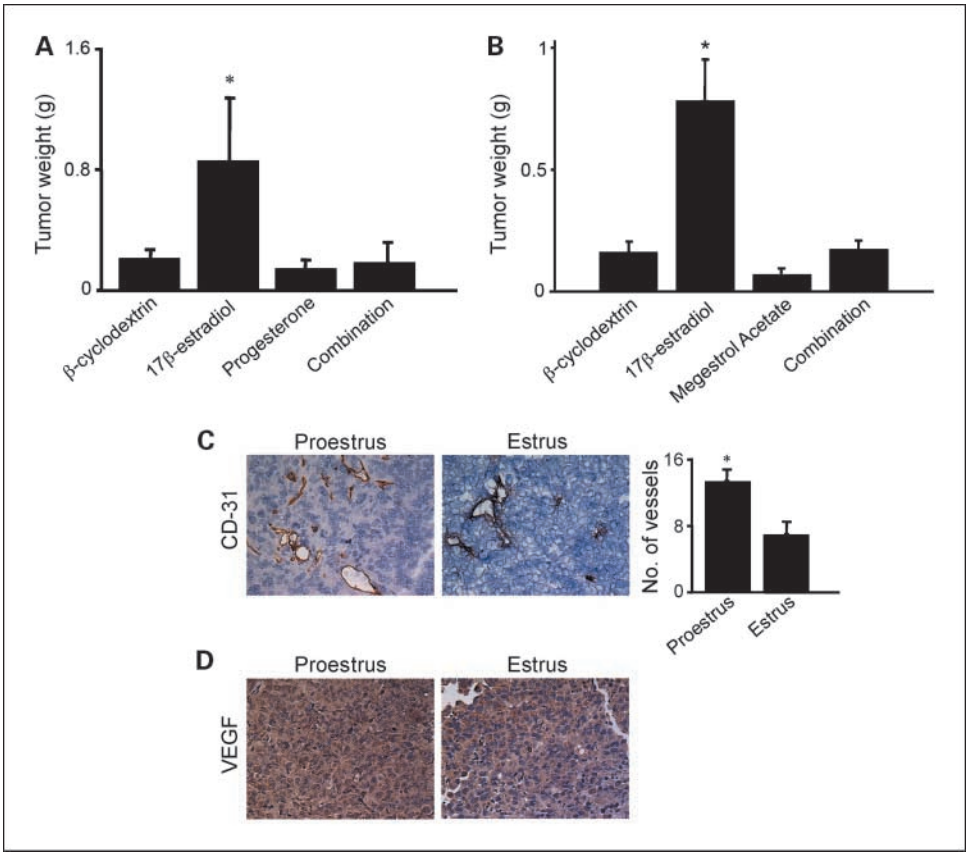


**Fig. 2.** Effects of reproductive hormones on ovarian cancer growth and angiogenesis. Following recovery from ovariectomy, the mice received either 17β-estradiol, progestin, or combination treatment. After 3 d of treatment, the mice were injected with either (*A*) SKOV3ip1 or (*B*) HeyA8 ovarian cancer cells. Four weeks later, the mice were sacrificed and the tumor burden was assessed. Effect of estrous cycle variation on vessel density (*C*) and VEGF (*D*) was assessed in tumors from mice inoculated during proestrus or estrus. All pictures were taken at original magnification, ×200.

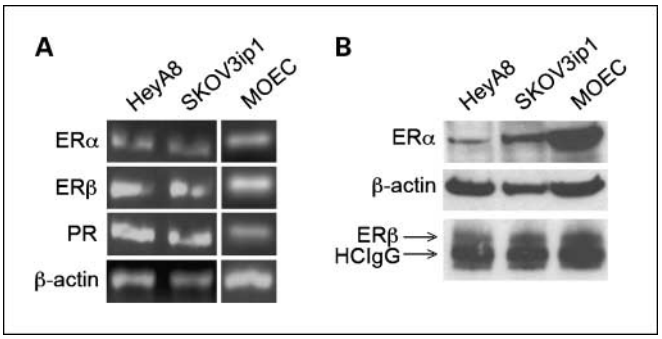


**Fig. 3.** Hormone receptor expression in tumor and endothelial cell. *A,* mRNA expression of estrogen receptor α, estrogen receptor β, and progesterone receptor in HeyA8, SKOV3ip1, and MOEC cells. *B,* Western blot analyses show that all three cell lines expressed all three receptors.

cells (10–13), we found that VEGF levels are increased at the tumor level during proestrus. In other models, estrogen-induced VEGF production was attributed to estrogen receptor α and estrogen receptor β activity at the transcriptional level (40). Specifically, it was found that estrogen receptor α and estrogen receptor β could bind to estrogen response elements near the transcriptional start site of the *VEGF* gene (40). Also reminiscent of previous observations in other tumor systems (41), we found that estrogen can increase the migratory potential of tumor-associated endothelial cells and this process is mediated by activation of the MAPK signaling pathway. These two mechanisms suggest that estrogen may increase the angiogenic potential of the tumor microenvironment by increasing VEGF secretion and increasing endothelial cell migration.

In our study, we found more tumor nodules in mice inoculated during the proestrus phase of the menstrual cycle, suggesting that estrogen promotes metastatic colonization by increasing the adhesive potential of cancer cells to extracellular matrix proteins. This finding is supported by other observations related to the role of estrogen in enhancing endothelial cell adhesion to extracellular matrix proteins (42). Although the effect of estrogen on ovarian cancer cell adhesion is not well known, it has been studied in other gynecologic diseases. In an orthotopic mouse model of endometriosis, pretreatment with estrogen resulted in a higher percentage of animals developing endometriotic-like nodules, whereas treatment with progesterone alone did not affect implantation rates (43).

MAPK has been suggested to play a major role in cell migration, proliferation, differentiation, and survival (44). In some experimental models, estrogen has been shown to activate MAPK (28–30). For example, various studies have shown that estrogen induced porcine aortic endothelial cells and human umbilical vein endothelial cells migration, and data suggest that this effect is mediated by MAPK (31, 32). Thus, we examined whether estrogen treatment resulted in

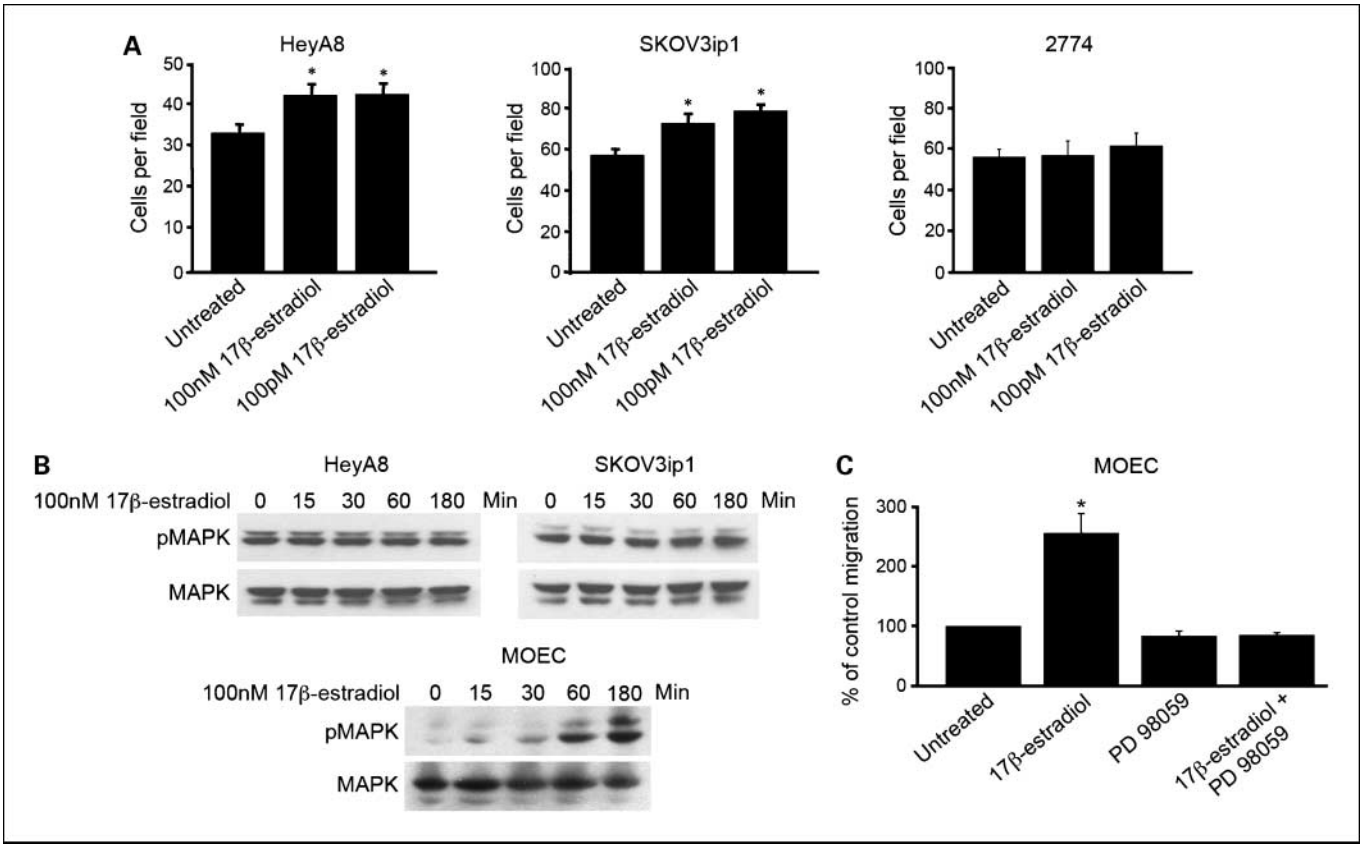


**Fig. 4.** Estrogen increases ovarian cancer cell adhesion, endothelial cell migration, and activates MAPK in endothelial cells. *A,* effect of 17β-estradiol on the adhesive potential of HeyA8, SKOV3ip1, and 2774 cells to collagen I was assessed. *B,* effect of 17β-estradiol on MAPK activation in HeyA8, SKOV3ip1, and MOEC cells. *C,* effect of 17β-estradiol, PD 09859, or in combination, on migration of MOEC cells was examined with a gelatin-coated membrane.

MAPK activation in ovarian cancer and endothelial cells. Incubation with estrogen at a dose known to reflect the physiologic conditions in the ovary was not effective in activating MAPK in either of the human ovarian cancer cells we tested (45). However, estrogen treatment rapidly increased the levels of activated MAPK in MOEC cells. These data suggest that estrogen impacts tumor biology by stimulating endothelial cells in the tumor microenvironment. Our finding is further supported by data that suggest that estrogen can promote tumor growth even in estrogen receptor–negative breast cancer models (17–20).

Progesterone is the dominant hormone during the estrus phase of the menstrual cycle. Fluctuations in progesterone levels could serve as another plausible reason for the differences in ovarian tumor growth observed in our study. Progesterone has been found to cause apoptosis of ovarian cancer cells *in vitro* and to reduce the tumorigenic capacity of cells inoculated into the peritoneal cavity of mice (46, 47). Pretreatment with progesterone has been shown to reduce the number of tumor implants and metastases, thus prolonging the animal life span (46). Additionally, long-term progesterone treatment of ovarian cancer cells was able to suppress the transformed phenotype as indicated by the acquisition of contact inhibition and loss of anchorage independence (48). Our observations of decreased tumor growth in the progestin-treated arms validate these findings in the literature and could serve as an explanation for the protective effect of progesterone in ovarian cancer.

Some studies have shown that estrogen can promote ovarian cancer cell proliferation, motility, and invasion, and reduce apoptosis (49–52). However, tamoxifen treatment is effective even in estrogen receptor–negative ovarian cancers, suggesting that other factors might be responsible for decreased tumor growth (53). In contrast, progesterone treatment inhibits cell growth and invasion and promotes apoptosis (46, 54–59). Our findings extend previous work in that the effects of reproductive hormones were found to be mediated via the tumor and endothelial cells.

In summary, we have shown that the timing of ovarian cancer cell inoculation within the estrous cycle modulates the growth of ovarian cancer cells. In addition, we have identified that the reproductive hormone mileu functions as a key predictor of ovarian tumor progression. These results show that cancer cell growth and angiogenesis are highly coordinated within the estrous cycle, raising the possibility that the effectiveness of therapeutic strategies might depend in part upon when in the reproductive cycle they are used.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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