0013-7227/07/$15.00/0
Printed in U.S.A.

Endocrinology 148(8):3595–3604

doi: 10.1210/en.2007-0030

Ovarian Epithelial Inclusion Cysts in Chronically Superovulated CD1 and Smad2 Dominant-Negative Mice

Joanna E. Burdette, Rachel M. Oliver,* Victoria Ulyanov,* Signe M. Kilen, Kelly E. Mayo, and Teresa K. Woodruff

Institute for Women's Health Research (J.E.B., V.U., T.K.W.), Feinberg School of Medicine, Northwestern University, Chicago, Illinois 60611; Robert H. Lurie Comprehensive Cancer Center of Northwestern University (T.K.W.), Chicago, Illinois 60611; Department of Biochemistry (R.M.O.), Beloit College, Beloit, Wisconsin 53511; and Department of Biochemistry, Molecular Biology, and Cell Biology (K.E.M.) and Center for Reproductive Science (S.M.K., K.E.M., T.K.W.), Northwestern University, Evanston, Illinois 60208

Chronic ovulation as a contributing factor for the development of epithelial ovarian cancer in women has long been an outstanding hypothesis. To test the incessant ovulation hypothesis, mice were superovulated using weekly ip injections of pregnant mare serum gonadotropin (5 IU/animal), followed 48 h later by human chorionic gonadotropin (5 IU/animal). Wild-type CD1 mice were used along with CD1 mice expressing a Smad2 dominant-negative (Smad2DN) transgene under the control of the Müllerian inhibiting substance promoter that targets expression to the ovary and enhances cyst formation. After chronic injections, ovaries were analyzed from animals 6 months of age for the total adjusted number of cysts, cyst area, cyst location, and key signaling pathways. All observed cysts were confirmed to be of epithelial origin. The number of cysts was not significantly different between superovulated and control mice in either the wild-type or Smad2DN groups. However, the combination of the Smad2DN transgene and superovulation resulted in an increase in cyst formation compared with normal littermates that were unstimulated. Rapid proliferation of the cells lining the cysts was detected using bromodeoxyuridine and phospho-histone 3 immunohistochemistry but was not different in the ovarian surface epithelium or in the cyst lining between groups. These data suggest that chronic superovulation in Smad2DN mice results in a higher incidence of cyst formation compared with unstimulated controls, but the epithelial lined cysts did not progress to cancer over the course of this study. (*Endocrinology* 148: 3595–3604, 2007)

THE OVARIAN SURFACE epithelium (OSE) is important in maintaining the health and structure of the ovary. The OSE is a single layer of flattened-to-cuboidal cells that surrounds and protects the ovary. The dynamic nature of the OSE morphology and its lack of tissue-specific markers make it almost inconspicuous (1). Nonetheless, the OSE is responsible for nutrient transport and postovulatory epithelial wound repair (2). Despite performing its important endocrine and reproductive functions, the OSE provides the progenitor cells for 90% of human ovarian cancers (3). Ovarian cancer ranks first among the cause of death from a gynecological malignancy and accounts for more than 3% of cancer-related deaths in women.

Ovulation is a vigorous process that requires disruption of cell-cell junctions within the OSE to provide an exit for the released oocyte, followed by rapid migration and wound repair. Before ovulation, release of LH by the pituitary stimulates the OSE adjacent to a preovulatory follicle to induce the secretion of plasminogen activator, and this triggers TNFα breakdown of the OSE layer (4). The OSE expresses receptors for FSH and LH (5–8), and elevated amounts of gonadotropins stimulate proliferation of both rat and mouse OSE before and after ovulation (9, 10). In addition, homeostatic proliferation occurs in the OSE of cycling rats (11, 12). Although a drastic rise in OSE proliferation is necessary for reestablishing normal surface continuity, it may also be a source of uncontrolled cell growth involved in cyst or tumor formation.

Although ovulation is a natural and essential phenomenon, disturbances in the healing process may lead to cyst development and cancer. The wounding and healing process is necessary for oocyte release, but frequent rupture and repair also increases the chance for spontaneously developing mutations to accumulate in cellular DNA (13–15). Indeed, enhanced cyst formation and epithelial proliferation was observed in incessantly ovulating mice linking the process of ovulation with cyst formation (16, 17). In addition, factors that increase ovulation increase the risk of developing ovarian cancers, whereas factors that reduce total lifetime ovulation reduce cancer risk (18–25). An increased incidence of cysts and ovarian tumors in the adult hen is associated with ovulation number, and this serves as an animal model of human epithelial ovarian cancer (15, 26).

Ovarian inclusion cysts, which may form from epithelial invaginations, serve as precursors for the establishment of ovarian cancer (27, 28). Because most ovarian cancers are diagnosed during the last stages of disease, alterations in the phenotypes and genetic expression of ovarian cysts are important to study as an immediate antecedent to disease pro-

First Published Online April 12, 2007

* R.M.O. and V.U. contributed equally to this work.

Abbreviations: BrdU, Bromodeoxyuridine; CK8, cytokeratin 8; DAB, diaminobenzidine; hCG, human chorionic gonadotropin; MIS, Müllerian-inhibiting substance; NLM, normal littermate; OSE, ovarian surface epithelium; PMSG, pregnant mare serum gonadotropin; Smad2DN, Smad2 dominant-negative; TBS, Tris-buffered saline; TX, transgenic.

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

gression (29–32). Alternative precursor lesions identified in prophylactically removed BRCA (breast-ovarian cancer susceptibility gene)-positive ovaries include the appearance of a papillary phenotype of the epithelium, multiple invaginations that may provide the mechanism of cyst formation, stromal abnormalities, and hyperplasia. The ovary also develops cyst-like structures in women who have polycystic ovarian syndrome; however, these cysts are derived from follicles, a categorically different source, and do accumulate in response to ovulation (33). Therefore, studying the development of ovarian epithelial cysts in response to ovulation may provide insight into the incremental changes that lead to ovarian epithelial cancers.

The TGFβ and activin signaling pathways are present in OSE and have a variety of signaling properties important in cell maintenance. Both TGFβ and activin stimulate the phosphorylation of intracellular signaling molecules known as Smad2 and Smad3 by binding to and activating their own unique type II and type I receptors. The role of activin and TGFβ in cancer biology has been studied *in vitro*. Interestingly, these molecules can be both growth stimulatory and growth inhibitory (34–46). Two mouse models with impaired activin or TGFβ signaling in the ovary, the MT-inhibin α overexpressing mouse and the MIS-Smad2DN mouse, develop inclusion cysts that express the epithelial marker cytokeratin 8 (CK8) (47). Because these animals do not develop full-blown cancer, they may be used to study the role of ovulation during the progression of ovarian cysts to cancer. Cysts from Smad2DN and MT-inhibin α mice resemble the human condition of endosalpingiosis (47); therefore, the effect that ovulation has on these benign lesions may also be studied.

The purpose of the present study was to investigate the impact of chronic superovulation in control CD1 mice and Smad2DN mice on the formation of ovarian inclusion cysts and the progression of transgenically induced cysts toward neoplastic transformation. The ovaries were then characterized to determine whether ovulation contributed to a higher incidence of cyst formation, changes in cyst area, and alterations in the regions of cyst formation. In addition, cyst proliferation rate, induction of key activin and TGFβ signaling pathways, and their responsiveness to hormones were examined. These experiments demonstrate the effect of ovulation on cyst location and area as well as the role of TGFβ and activin signaling in these cysts.

Materials and Methods

Animals

Female CD1 mice were obtained through in-house breeding lines. Mice were maintained in accordance with the policies of the Northwestern University Animal Care and Use Committee. Mice were housed and bred in a controlled barrier facility within the Northwestern University Center for Comparative Medicine. Temperature, humidity, and photoperiod (12-h light, 12-h dark cycle) were kept constant. Animals were allowed access to phytoestrogen-free breeding chow 2919 (Harlan Teklad, Indianapolis, IN) and water *ad libitum*. The genetic cassette used for creating the Smad2 dominant-negative (Smad2DN) transgenic (TX) mice on a CD1 genetic background consists of a mouse minimal Müllerian-inhibiting substance (MIS) promoter (−180 bp), an epitope tag (Flag), a C-terminal truncation of the human Smad2 gene (dominant-negative), and a human GH polyadenylation sequence as described previously (47). Smad2DN transgene blocks Smad2 and Smad3 as demonstrated previously (47, 48).

Experimental design for chronic ovulation

Smad2DN and their normal littermate (NLM) counterparts were genotyped at d 18 and subsequently placed into one of four groups: NLM ovulation suppressed and nonsuperovulated, NLM superovulated, Smad2DN ovulation suppressed and nonsuperovulated, and Smad2DN superovulated. Each experimental group contained at least 10 animals. Superovulated animals were injected with pregnant mare serum gonadotropin (PMSG) (5 IU/mouse) (Sigma, St. Louis, MO), followed by human chorionic gonadotropin (hCG) (5 IU/mouse) (Sigma) 48 h later once a week starting at age 6 wk and continuing until 6 months of age. Control animals were group housed to suppress ovulation, and superovulated animals were singly housed to improve the chance of continuous ovulation (49–54). At 6 months of age, the animals were killed, and the serum, ovaries, and uteri of all mice were collected. Organs were fixed in 4% paraformaldehyde for 8–12 h, dehydrated with ethanol, paraffin embedded, and serial sectioned at 4 μm. Images were obtained using ×10, ×20, or ×40 objectives on a Nikon (Tokyo, Japan) Eclipse E600 microscope with a Spot camera (Diagnostic Instruments, Sterling Heights, MI). Every 10th slide, each slide containing five sections, from cumulative serial sections was analyzed for cyst and invagination number. Two separate investigators, blinded to the conditions, independently counted the total number of cysts and the total number of invaginations and recorded cyst location. Counts for each investigator were averaged, and then counts for the treatment groups were averaged. Images were acquired for each cyst, and the total cyst area at the widest point was calculated using Spot Advanced software.

Experimental design of proliferation study in immature mice

In this study, immature mice, with no previous ovulations, were injected with either PBS saline control or a combination of 5 IU of PMSG and hCG to induce superovulation. Once an injection of bromodeoxyuridine (BrdU) was given to an animal, cumulative labeling was achieved by placing BrdU into the drinking water of the animals until the time they were killed. The animals were injected with BrdU to label either background proliferation or that induced from PMSG and hCG. Total basal proliferation was assessed by injecting animals with PBS and BrdU at 0900 h on d 25 of life and continuing to label all dividing cells until the time of death on d 27 for a total of 60 h. Abridged basal proliferation was quantified in animals labeled with BrdU at 0900 h on d 27 of life until death for a total of 12 h. Total periovulatory proliferation was defined as the mitosis of OSE occurring from 0900 h on d 25 until 2100 h on d 27 for a total of 60 h in animals injected with PMSG and hCG. Proliferation measured in PMSG- and hCG-injected animals from 0900 h on d 27 until death is defined as periovulatory proliferation and depicts cell division for 12 h from the time of the hCG injection until the animals were killed. These labels apply to both NLM and Smad2DN animals. Images were obtained around the perimeter of at least one section per animal using a ×20 objective, and the ovarian surface was reconstructed using Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA). After reconstruction and printing of the image, two separate investigators, blinded to the conditions, independently counted the total number of cells and the total number of positively stained cells. Counts for each investigator were averaged, and then counts for the treatment groups were averaged.

Immunohistochemistry

All reagents were obtained from Vector Laboratories (Burlingame, CA) unless otherwise indicated. Slides were deparaffinized using xylenes and rehydrated with subsequent ethanol dilutions. Antigen retrieval was performed using 1 mM sodium citrate by microwaving 2 min on high and 7 min on low, followed by cooling in solution for 20 min. Slides were washed in Tris-buffered saline (TBS) with Tween [20 mM Tris, 500 mM NaCl, and 0.1% Tween 20 (pH 7.4)]. Tissues were blocked for 15 min in 3% hydrogen peroxide (Fisher Scientific, Pittsburgh, PA), followed by avidin and biotin according to the instructions of the manufacturer. Slides were incubated in 10% serum of the secondary antibody host in 3% BSA in TBS for 1 h at room temperature. After blocking, slides were incubated overnight at 4 C in primary antibody in 3% BSA-TBS-10% serum. Slides were rinsed three times for 5 min in TBS-Tween 20

and then incubated at room temperature for 1 h in secondary antibody in 3% BSA-TBS. After washing slides in TBS-Tween 20, avidin-biotin complex reagent was added and incubated for 30 min at room temperature. Slides were then washed in TBS, and antigen-antibody-horseradish peroxidase complex was visualized using diaminobenzidine (DAB) reagent for 3 min. For the phospho-Smad2 and Smad3 antibodies, the method of enzyme detection was the tyramide signal amplification fluorescein kit (PerkinElmer, Wellesley, MA) used with biotinylated antirabbit secondary at 1:400 dilution (Vector Laboratories). Control slides received serum block instead of primary antibody.

Antibodies

The primary antibodies used were raised against BrdU (BrdU antibody, sheep; 1:50 dilution; Abcam, Cambridge, MA), CK8 (CK8 TROMA-1 antibody, rat; 1:50; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), phospho-Smad2 (3101, rabbit; 1:50; Cell Signaling Technology, Boston, MA), Smad3 (rabbit, 1:200; Invitrogen, Carlsbad, CA), phospho-histone 3 (9764, rabbit; 1:100; Cell Signaling Technology), estrogen receptor α (sc-543, rabbit, 1:100; Santa Cruz Biotechnologies, Santa Cruz, CA), progesterone receptor (sc-538, rabbit; 1:100; Santa Cruz Biotechnologies), and MIS (sc-6886, goat; 1:50; Santa Cruz Biotechnologies), and they were incubated overnight at 4 C with ovary sections. The following secondary antibodies were incubated with their respective sections for 1 h at room temperature: biotinylated antisheep (1:200), biotinylated antigoat (1:200), biotinylated antirabbit (1:200), and biotinylated antirat (1:200) antibodies.

Statistical analysis

All numerical data were analyzed using ANOVA, followed by secondary paired *t* tests using the Bonferroni's correction factor. For data representing a percentage, a χ^2 analysis was performed using the Pearson's correction factor and then grouped using a correspondence analysis. Significance of the data were determined as $P < 0.05$.

Results

Chronic superovulation of Smad2DN mice increased cyst number compared with CD1 animals

To study the impact of repeated ovulatory events, CD1 mice and Smad2DN TX mice were subjected to chronic superovulation. The four groups analyzed are as follows: NLM, NLM superovulated, TX, and TX superovulated. Superovulation was induced by injecting animals beginning at 6 wk of life with 5 IU/animal PMSG, followed 48 h later with an injection of hCG (5 IU/mouse) once per week. Cysts were systematically evaluated in superovulated and control ovaries to examine whether the process of superovulating mice until 6 months of life would induce cystic lesions (Fig. 1A). Ovarian epithelial cysts were confirmed by immunostaining the adjacent slide with CK8 antibody. Epithelial cysts were defined as those expressing CK8 (Fig. 1B). The impact of superovulation increased the average amount of cysts formed in NLM animals compared with control mice, but the difference did not reach significance.

Ovaries collected from Smad2DN TX mice were similarly evaluated for an increase in cyst formation from chronic superovulation. The average cyst number for TX animals was higher than NLMs as reported previously (Fig. 1A). Superovulation did not induce a statistically significant increase in cyst number in Smad2DN mice. However, when the total number of cysts was compared between superovulated Smad2DN mice and control NLM mice, there was a significant increase. These data suggest that the combination of

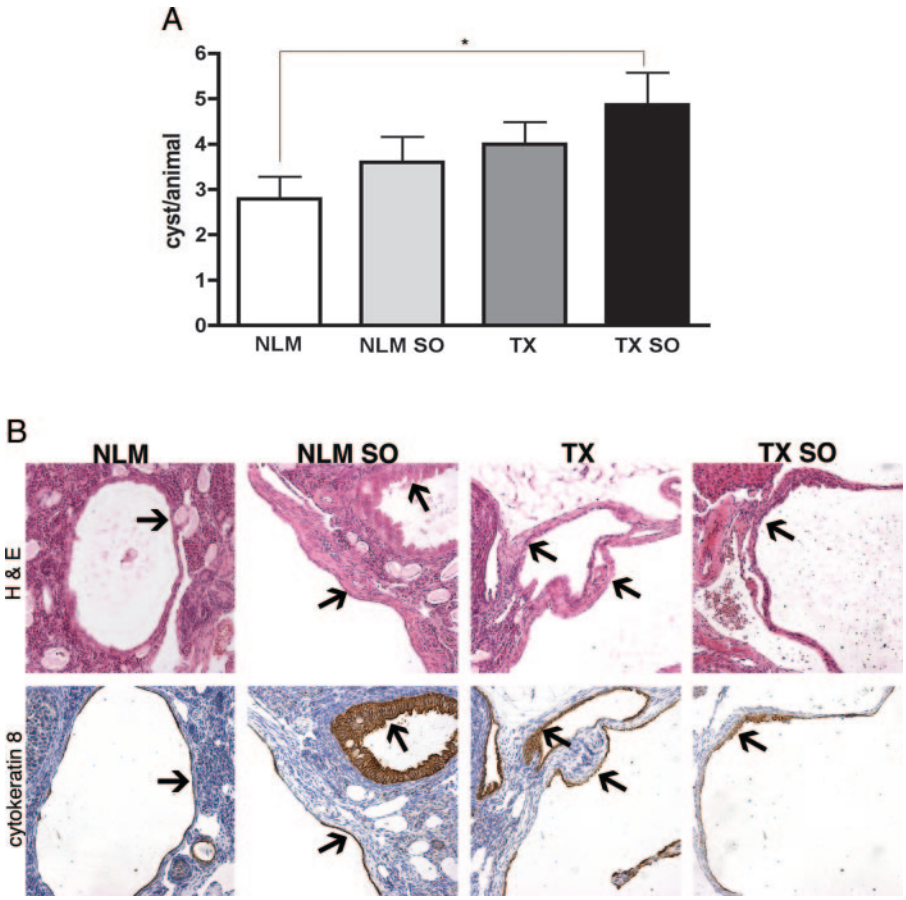

FIG. 1. Epithelial cyst adjusted number in wild-type and Smad2DN mouse ovaries after chronic superovulation. A, The data shown represent the average number of cysts per animal as measured in both ovaries on every 10th slide through serial sections and the SE from the mean. *, $P < 0.05$. B, Immunohistochemical analysis of cyst cell type and origin. Sections adjacent to hematoxylin and eosin-stained tissue in which cysts were located were stained with the epithelial marker CK8. Cysts from each treatment group acquired with ×200 magnification after staining. *Black arrows* indicate cyst lining. NLM SO, NLM superovulated; TX SO, transgenic superovulated.

chronic superovulation and Smad2DN TX expression results in an increase in cyst number compared with control.

Invaginations typically occur in human ovaries with age as a result of the loss of total ovarian size from repeated follicular extrusion. Therefore, the amount of surface invaginations were counted in each ovary from all treatment groups and averaged for the individual animals. The number of invaginations formed did not differ significantly between the groups (data not shown).

Superovulation and cyst location in Smad2DN mouse ovaries

The formation of cysts has been reported previously to be location dependent after chronic ovulation (16). To confirm and extend these previous observations, the location of each cyst was determined as either located in the hilus (this did not include cysts that were on the oviduct side but not physically located within the hilus), perpendicular to the hilus at either edge, opposite the hilus, or in the middle of the ovary (Fig. 2, A and B). For each treatment group, the percentage of the cysts in each location are reported in Fig. 2A. An average increase in the percentage of cysts in the middle of the ovary was seen with superovulation in combination with TX Smad2DN expression. These data indicate that the two-hit combination of having a Smad-deficient signaling pathway in the ovary combined with chronic superovulation tends to result in cysts accumulating in the middle of the ovary.

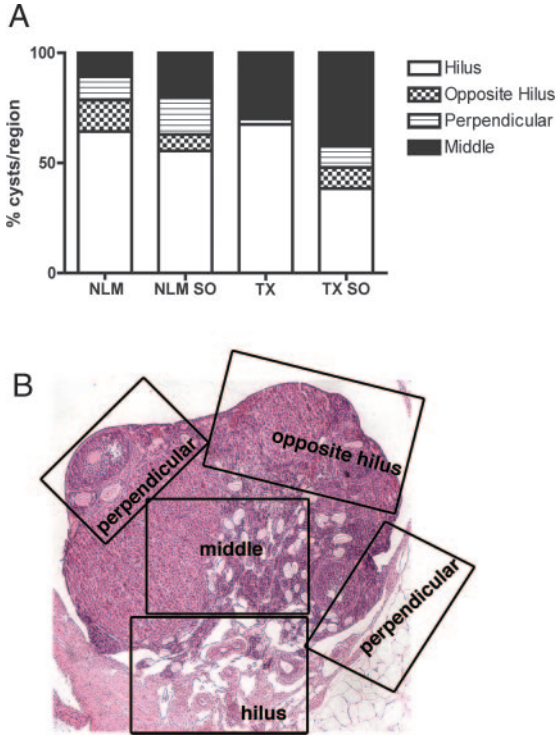

FIG. 2. Epithelial ovarian cyst location in mouse ovaries. A, Data represent the percentage of total cysts in each discrete location of the ovary. B, Cysts were classified into one of four anatomical locations: hilus, perpendicular to the hilus, opposite from the hilus, or in the middle of the ovary section analyzed. NLM SO, NLM superovulated; TX SO, transgenic superovulated.

Superovulation and cyst area

Cyst area varied greatly between genotypes and treatment groups. To quantify these changes, ovarian cysts that were confirmed using CK8 stain were measured. The average cyst area for each treatment group is reported in Fig. 3. The average area of unstimulated TX mice cysts was significantly larger than superovulated NLM cysts using a paired Student's t test ($P < 0.05$). The average cyst area decreased in NLM and TX mice subjected to chronic superovulation. The reduction in the average cyst area with superovulation suggests that it drives the formation of new cysts that begin as small structures.

Proliferation attributable to Smad inactivation does not result in excess cyst formation

Activin and TGFβ have both been reported to reduce human OSE cellular proliferation in cells grown in culture (34–46). Because activin and TGFβ both signal through Smad2, overexpression of a Smad2DN TX might eliminate the antiproliferative effects of activin and TGFβ and thereby induce proliferation responsible for cyst formation (47). To directly test this hypothesis, experiments were designed to investigate whether an increase in proliferation of the OSE resulted from a single superovulation event (Fig. 4A). Animals injected with BrdU to label all cells that had synthesized DNA during the time of superovulation revealed that Smad2DN animals have the same increase in proliferation in response to PMSG and hCG as do CD1 mice (Fig. 4B). Therefore, the increase in cyst rate in superovulated TX animals is likely not attributable to an increase in ovarian surface epithelial proliferation from gonadotropins.

Next the proliferation rate of cells lining the ovarian cyst from chronically superovulated animals was compared between the treatment groups and with the ovarian surface. Two methods were used to compare proliferation. First, chronically ovulated animals were injected with BrdU 24 h before death, and an immunostain directed against BrdU was used to mark cells that had divided at any time during the 24 h after the injections. Second, an antibody directed against phosphorylated-histone 3 was used to mark cells dividing at the time of death. Using both methods, there was no differ-

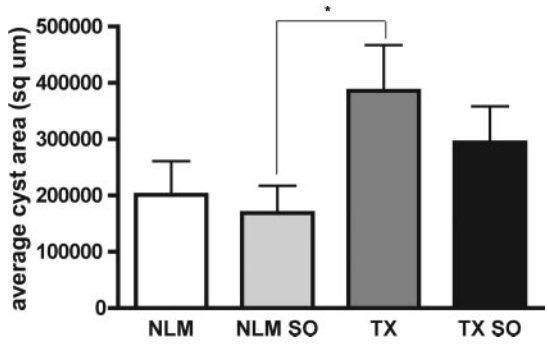

FIG. 3. Superovulation and cyst area. Cysts were followed through serial sections, and cyst area was calculated at the widest measured diameter. The data represent the average total cyst area at its widest point from all cysts observed in each group and the SE from the mean. *, $P < 0.05$. NLM SO, NLM superovulated; TX SO, transgenic superovulated.

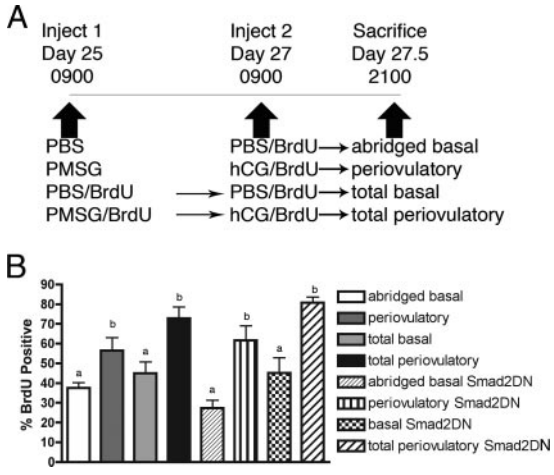

FIG. 4. BrdU incorporation into the OSE over the total surface area of the ovary after gonadotropin stimulation with PMSG and hCG from one superovulation cycle. A, Injection schedule for gonadotropin stimulation is depicted. B, BrdU-positive cells were divided by the total number of OSE in one histological section taken from ovaries, counted, and averaged between seven ovaries. The shown data represent the least square mean (percentage) of total proliferation and the SE from the mean. $P < 0.05$ significant differences between groups labeled with a *vs.* b.

ence in the amount of proliferating cells in the ovarian cysts of TX mice compared with NLM regardless of superovulation (Fig. 5). Ovarian cysts in aged superovulated animals have been reported to have a higher incidence of proliferating cells in the cyst lining than in the ovarian surface layer (55). No difference was detected in the number of proliferating cells in the ovarian cysts compared with the OSE (data not shown). These data again demonstrate that, although cystic structures are highly proliferative, the overall rate of proliferation was not different.

Ovarian cysts in Smad2DN mice regain Smad2 phosphorylation

Our laboratory previously reported that the OSE cells of Smad2DN TX mice have significantly less Smad2 phosphorylation compared with their NLMs attributable to the expression of the transgene under MIS control in these cells (47). Because both the cysts and the surface contain the epithelial cell marker CK8 (47), the ovarian cysts may be derived from OSE cells that also lack Smad phosphorylation, thus explaining their high incidence in TX mice compared with NLMs. These data would suggest that eliminating activin or TGFβ signaling through Smad2 is permissive for cyst formation. To investigate these mechanisms of cyst formation, the amount of Smad2 phosphorylation was compared between cysts and the ovarian surface in both normal and TX animals. Sixty percent of unstimulated TX animals had OSEs that did not display Smad2 phosphorylation as reported previously, whereas only 30% of NLMs had undetectable phosphorylation. In all cases, the ovarian cysts themselves were found to have more Smad2 phosphorylation compared with the ovarian surface of the same ovary analyzed (Fig. 6, *top* and *middle*). These data suggest that, once epithelial ovarian cysts form in both normal and TX animals, a phosphorylated Smad2 pathway is observed.

Because the Smad2DN transgene was expressed under the MIS promoter, a reduction of Smad2 phosphorylation might be expected to correlate with expression of MIS (47). In fact, the ovarian surface of the TX and normal animals was found previously to express MIS, thus explaining why Smad2 phosphorylation is reduced in the TX animals (47). To correlate this reacquisition of Smad2 signaling with MIS expression, ovarian cysts were stained for MIS. In the NLMs cysts, MIS expression was retained, whereas in the Smad2DN animals, MIS expression was lost. The loss of MIS expression suggests

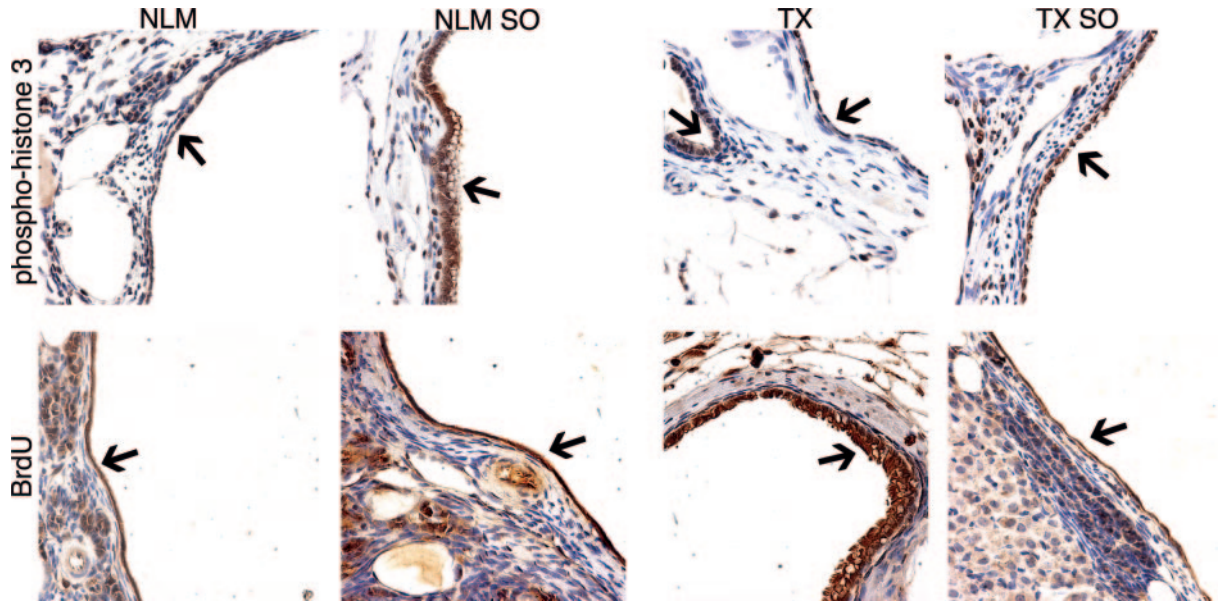

FIG. 5. Ovarian inclusion cysts undergo rapid proliferation. *Top* shows phospho-histone 3-positive cells lining the cysts undergoing proliferation. *Bottom* shows BrdU incorporation into the epithelial lined cysts. Sections were stained with DAB and counterstained with hematoxylin. *Black arrows* indicate cyst lining. NLM SO, NLM superovulated; TX SO, transgenic superovulated.

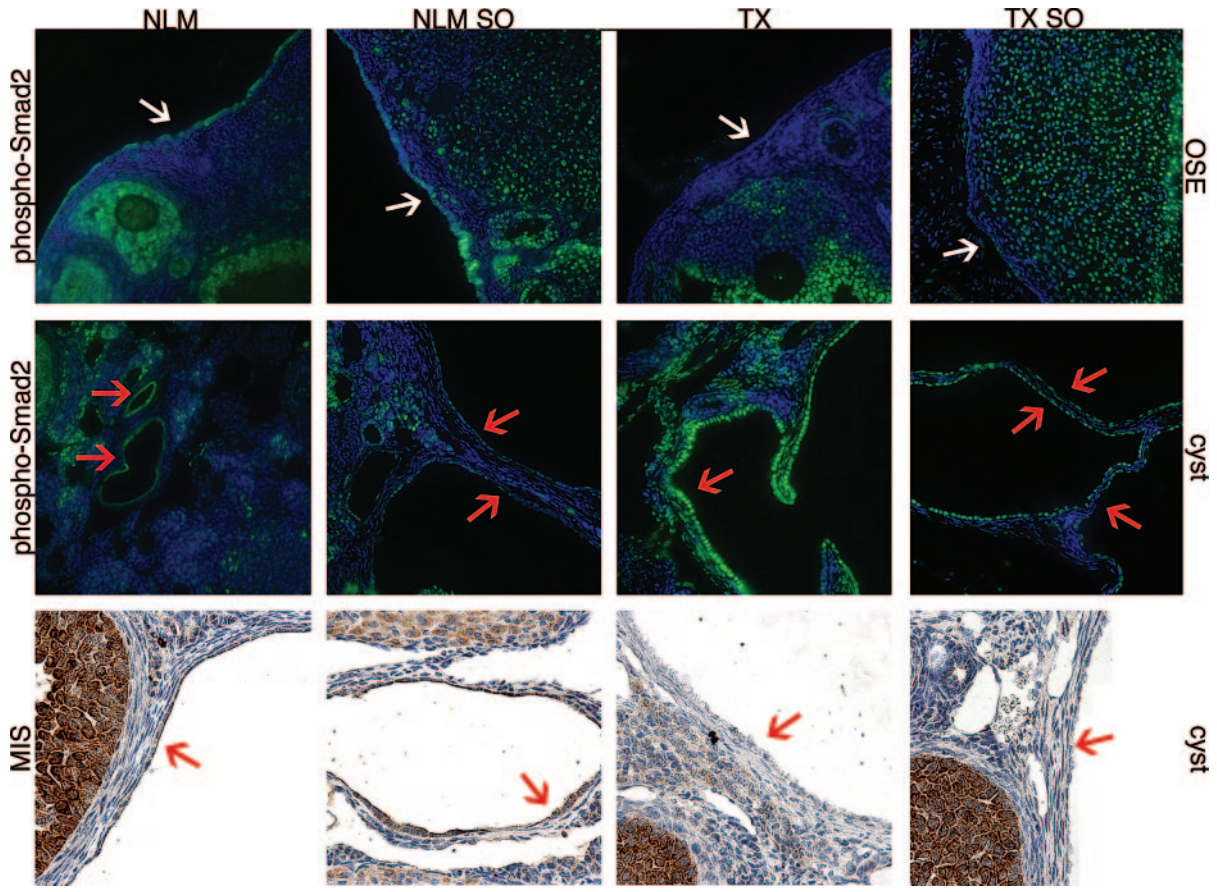

FIG. 6. Smad2 phosphorylation of OSE compared with the lining of epithelial cysts. Images in the *top* depict the phosphorylation of Smad2 in the OSE from ovaries acquired in each treatment group. Ovarian surface is labeled with a *white arrow*. Images in the *middle* depict phosphorylation of Smad2 in cysts determined to be of epithelial origin. Cysts lining are labeled with a *red arrow*. The *bottom* represents MIS immunostained with DAB and counterstained with hematoxylin, demonstrating the loss of gene activation from the endogenous MIS promoter in cysts of TX mice. *Red arrows* point toward cyst lining. NLM SO, NLM superovulated; TX SO, transgenic superovulated.

that the cysts of Smad2DN mice lose MIS promoter expression and thereby regain Smad2 phosphorylation (Fig. 6, *bottom*). The cyst may originally be dependent on the transgene during formation, and, as the cyst progresses and persists, expression may be lost. Alternatively, the cyst may produce additional endogenous Smad2, allowing for a high level of phosphorylation after cyst progression.

Ovarian cysts are hormonally responsive

Epithelial lined inclusions cysts of the ovary are exposed to different stimuli from those cells lining the outside of the ovary that are separated from the stroma by the tunica albuginea. Because the epithelial lined cysts in this study were found inside the ovary and progressively further inside the middle of the ovary after TX alteration and superovulation, the hormone receptor status was investigated to determine whether the cells lining these cysts were hormone responsive. Receptor expression in the cyst could identify a potential source of signaling alteration and growth characteristics different from normal surface epithelia. Using an antibody directed against estrogen receptor α, estrogen receptors were detected in all of the cysts analyzed regardless of genotype or superovulation (Fig. 7, *top*). The expression of the receptors was found in large and small cysts regardless of location. Therefore, cystic lesions may be responsive to estrogens that are generated by the growing follicles stimulated from PMSG injections and natural ovulation events.

To confirm whether the cysts in this study expressed progesterone receptor, an immunostain was performed using an antibody detecting both forms of progesterone receptors. This antibody does not distinguish between the two isoforms. Progesterone receptors were detected in all of the cysts analyzed, and their expression did not differ between groups (Fig. 7, *bottom*). However, the expression of progesterone receptors in the cyst was found to be constant, whereas OSE expression was only detected in approximately 50% of the ovaries. The OSE expression did not change based on genotype or treatment but appeared random. Therefore, ovarian lined inclusion cysts in both NLM and TX animals were found to express estrogen and progesterone receptors.

Discussion

Epidemiological data collected from humans indicates that the total number of ovulatory events is a risk factor for the development of ovarian cancer. Because ovarian cancers are usually diagnosed in the late stages, generating animal models to study the initiation of precancerous events is of critical importance (56). The incidence of ovarian cysts in the

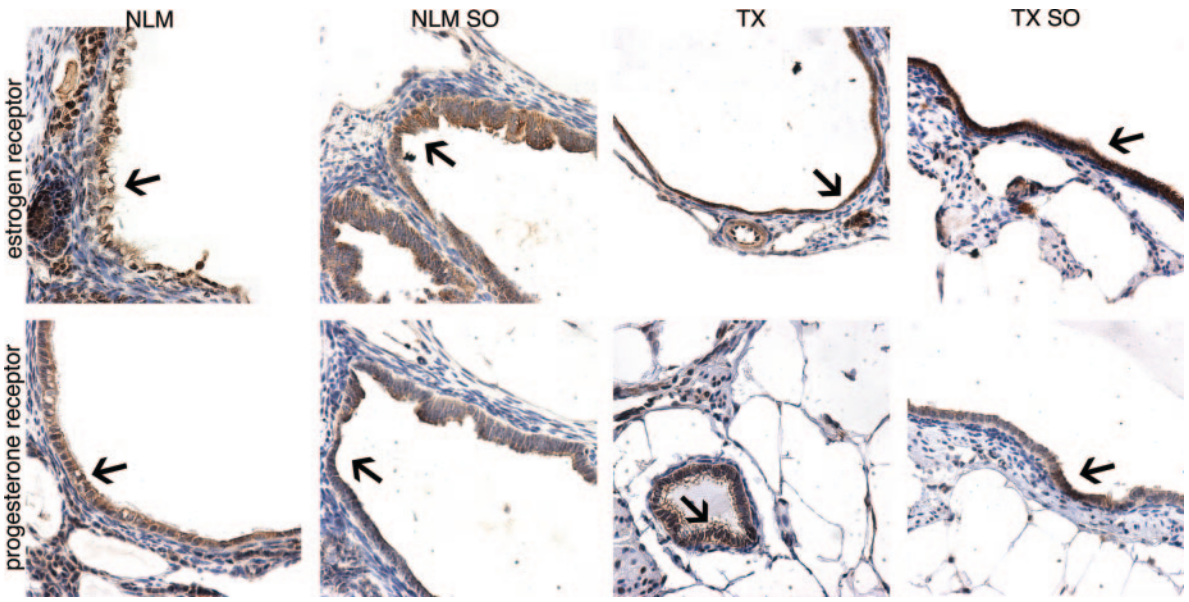

FIG. 7. Ovarian inclusion cysts express hormone receptors. *Top* shows the estrogen receptor α expression in epithelium lining inclusion cysts found in each treatment group. *Bottom* depicts progesterone receptor expression in epithelium lining of inclusion cysts from each treatment group. *Black arrows* point toward inclusion cyst lining. NLM SO, NLM superovulated; TX SO, transgenic superovulated.

contralateral ovary removed from cancer patients suggests that these inclusions are the early stages of transformation in the ovary (31). In addition, as women age, ovaries acquire more ovarian surface invaginations, which may pinch off to form cysts (30). Although several mouse models have been generated that develop ovarian tumors reminiscent of the human condition, all of these models have genetic manipulation in oncogenes and tumor suppressors that result in tumorigenesis independent of ovulation (57–59). Therefore, this study addressed the role of ovulation in CD1 mice as well as transgenically altered animals with a Smad2DN protein expressed under the control of the MIS promoter that develop cysts but not cancer. Superovulated TX mice had significantly more cysts than unstimulated NLMs. In response to chronic superovulation, cysts tended to appear in the middle of the ovary as opposed to the hilar region. The cysts produced in all of the mice expressed estrogen and progesterone receptors. Although proliferation of the OSE did not differ between genotypes, all cysts were highly proliferative, indicating fast growth and expansion.

Superovulation of Smad2DN TX mice generated more cysts than in unstimulated NLM. Previous attempts to generate ovarian cancer in mouse models have required multiple genetic insults to produce similar human phenotypes such as papillary epithelium, ascites, and tumors (57–59). For example, generation of mice with Ras overexpression in combination with PTEN (phosphatase and tensin homolog) knockout produced far more tumors *in vivo* than PTEN or Ras genetic changes alone (60). Similarly, knockout of p53 produced mice with tumors, but the combination of eliminating p53 and retinoblastoma enhanced the formation of ovarian tumors (58). When ovarian surface cells were collected from both rats and mice and passaged in culture multiple times, the cells formed tumors when injected into immunodeficient mice, indicating that several genetic alterations were required for transformation (61, 62). In this study, chronic superovulation did not produce significantly more cysts, but the combination of Smad2 phosphorylation-deficient signaling and superovulation did generate significantly more cysts. Therefore, in mouse models, multiple insults to epithelial cells are required for generating cysts and ovarian cancer.

The most common form of ovarian cancer in humans is derived from epithelial cells. Whether the epithelial cells that generate human cancers are derived from the OSE, the Müllerian system, or the organs that they resemble such as fallopian tubes, endometrium, or cervix is still debated, but many scientists conclude that the OSEs represent a naive cell type capable of differentiating into many morphologically different epithelia (1). In Smad2DN mice, the cysts expressed the epithelial marker CK19, CK8, and lack the follicular marker inhibin α (47). In addition, other markers to distinguish the OSE from the rete ovarii were investigated and found to not differentiate these two epithelial cells in the mouse using existing antibodies directed against activin βC and calretinin (data not shown) (63, 64). The lack of specific markers makes absolute identification of the cyst origin difficult. Interestingly, chronic superovulation tended to generate ovaries with a higher percentage of cysts in the middle similar to previous reports (55). Inclusion cysts may garner growth advantages from stromal-derived growth factors that would otherwise be sequestered away from the OSE cells by the tunica albuginea. The inflammatory process of ovulation is thought to be a key part of transformation and may contribute more toward cyst formation in the middle of the ovary compared with the hilus. Alternatively, ovulation may increase the process of involution at the outer edge of the ovary, increasing the chance that cysts would form away from the hilus or the cysts may move after formation (13). However, in this study, the total number of invaginations did not differ significantly with ovulation and seemed more dependent on the overall age of the animal. Therefore, chronic

superovulation of both CD1 and Smad2DN mice generates epithelial lined inclusion cysts that do not differ in their expression of CK8, activin βC, calretinin, or inhibin α.

Chronic superovulation of mice generated a higher incidence of cyst formation but did not result in ovarian cancer formation within 6 months. Mice do not naturally develop ovarian cancer and therefore may have several aspects of ovarian biology that differ from humans, making the study of ovulation only indirectly applicable. Human ovarian cancers generally do not express E-cadherin until after transformation, which is unlike most other epithelial cancers (65). The mouse OSE normally expresses E-cadherin, and therefore acquisition of this protein differs significantly in the overall biology, possibly altering the ability of ovulation to generate cancers similar to human. Second, human epithelial ovarian cancer often forms metastasis in the peritoneal cavity and ascites, which may be fundamentally different in the mouse attributable to the presence of the bursa sac that forms a physical barrier around the ovary precluding immediate access to the peritoneal space. One hypothesis regarding human epithelial ovarian cancer involves the process of inflammation at ovulatory sites, yet mice ovulate multiple follicles at once and still fail to spontaneously develop epithelial ovarian cancer. In addition, the mouse OSE seems to proliferate readily without much apoptosis (9, 66). Interestingly, passage of the mouse OSE cells in culture permits a transformed phenotype once reinjected into nude mice, perhaps indicating that removal from specific signals *in vivo* permits the development of cancer-initiating events in the mouse (57). Although the present study demonstrates that ovulation plays a role in generating more, smaller ovarian inclusion cysts, the chronic superovulation in mice did not provide direct evidence associating cysts as precancerous lesions. To that end, the most commonly noted change in ovarian cysts associated with a precancerous lesion is CA-125, which is not produced in the mouse. Finally, the Smad2DN mouse develops cysts that specifically model the human condition of endosalpingiosis, and chronic superovulation apparently does not push these structures in the mouse toward tumor formation.

The incidence and size of ovarian inclusion cysts is significantly higher in TX animal models that lack a functional activin and TGFβ signal either attributable to inhibin overexpression or Smad2DN compared with wild-type mice (47, 67). In culture, activin and TGFβ have been shown to slow cellular proliferation and induce apoptosis, suggesting that a loss of the signal could encourage aberrant cell growth (36, 37). This study did not find a significant difference in the overall proliferation rate of OSE between immature CD1 mice and Smad2DN animals subjected to one superovulation event. In addition, the proliferation rate within the cysts themselves as determined by BrdU incorporation and phospho-histone 3 expression was too high to distinguish a difference between genotypes and treatments but did indicate that the cells lining these structures are rapidly dividing. Therefore, an increase in cyst formation from the Smad2DN transgene may arise from enhanced motility and invasiveness of epithelial cells into the stroma rather than proliferation. The cysts in both genotypes demonstrated a high level of Smad2 phosphorylation, and this was significantly higher in the cyst compared with the ovarian surface of TX mice. Therefore, an advantage may be incurred from acquiring Smad2 signaling based on exposure to stromal factors, or the cells lining the cysts may no longer be exposed to the proper transcription factors necessary to propagate expression from the MIS promoter. Finally, activin has been implicated in wound healing of the skin. Ovulation is similar to the wounding process in that the OSE must form a rupture site for the release of the oocytes, followed by rapid movement and proliferation to cover the ruptured site. Typically, activin-overexpressing mice have enhanced wound healing, and follistatin-overexpressing mice have a severe delay in wound healing (68–70). A delay in wound healing of the ovary from Smad2DN transgene expression may produce more cysts in response to chronic superovulation.

Estrogen and progesterone are potent mitogens in many tissues, and each plays a role in proper function of the ovary. Because ovarian cysts are adjacent to stromal- and follicular-derived growth factors, such as estrogen and progesterone, they may respond directly and proliferate. All cysts investigated appeared to express high levels of estrogen receptor α, and this expression pattern did not differ between cell types in the cysts, genotypes, or ovulation number. Progesterone receptors were more abundantly expressed in cysts compared with ovarian surface, and this is consistent with the cycling rat (11). Therefore, progesterone does not seem to be directly reducing cyst formation because the receptor is more highly expressed in cysts compared with the surface. The expression of hormone receptors might allow this model to be used to determine whether excess of estrogen as in hormone replacement therapy increases the incidence of cyst formation or whether antiestrogens and aromatase inhibitors block cyst formation. Also, the expression of hormone receptors may explain cyst persistence because superovulation generated more small cysts that must survive and grow to form the larger cysts seen from inactivation of Smad2.

In summary, chronic superovulation in the absence of additional genetic manipulation does not significantly increase cyst formation or ovarian cancer in CD1 mice. The process of superovulation did increase the average number of inclusion cysts, primarily in the less than 1000 μm^2 area class and in the middle of the ovary. Epithelial markers, follicular markers, and Smad signaling was consistent between these cysts, suggesting a common signaling defect in cyst formation between normal and TX animals. Inclusion cysts were highly proliferative and likely hormonally responsive. Smad2DN lesions that closely resemble the human condition endosalpingiosis did not advance to cancer in response to chronic superovulation. Therefore, chronic superovulation in combination with genetic changes in mice provides a link between ovulation and cyst formation but not between cyst formation and ovarian cancer.

Acknowledgments

We thank Dr. Alfred Rademaker for assistance in statistical analyses, Tyler Wellington for histological embedding, sectioning, and staining, and the Schweppe Foundation for support of R.M.O. during this project.

Received January 10, 2007. Accepted April 2, 2007.

Address all correspondence and requests for reprints to: Teresa K. Woodruff, Ph.D., Department of Obstetrics and Gynecology, Institute for Women's Health Research, Feinberg School of Medicine, Northwestern University, 2205 Tech Drive, Chicago, Illinois 60208. E-mail: tkw@northwestern.edu.

This work was supported by National Institutes of Health/National Institute of Child Health and Human Development Hormone Signals that Regulate Ovarian Differentiation Grant PO1 HD021921 and Oncogenesis and Developmental Biology Training Grant T32 CA80621.

Disclosure Statement: The authors have nothing to declare.

References

1. **Auersperg N, Wong AS, Choi KC, Kang SK, Leung PC** 2001 Ovarian surface epithelium: biology, endocrinology, and pathology. Endocr Rev 22:255–288
2. **Osterholzer HO, Johnson JH, Nicosia SV** 1985 An autoradiographic study of rabbit ovarian surface epithelium before and after ovulation. Biol Reprod 33:729–738
3. **Auersperg N, Edelson MI, Mok SC, Johnson SW, Hamilton TC** 1998 The biology of ovarian cancer. Semin Oncol 25:281–304
4. **Murdoch WJ, McDonnel AC** 2002 Roles of the ovarian surface epithelium in ovulation and carcinogenesis. Reproduction 123:743–750
5. **Syed V, Ulinski G, Mok SC, Yiu GK, Ho SM** 2001 Expression of gonadotropin receptor and growth responses to key reproductive hormones in normal and malignant human ovarian surface epithelial cells. Cancer Res 61:6768–6776
6. **Ivarsson K, Sundfeldt K, Brannstrom M, Hellberg P, Janson PO** 2001 Diverse effects of FSH and LH on proliferation of human ovarian surface epithelial cells. Hum Reprod 16:18–23
7. **Parrott JA, Doraiswamy V, Kim G, Mosher R, Skinner MK** 2001 Expression and actions of both the follicle stimulating hormone receptor and the luteinizing hormone receptor in normal ovarian surface epithelium and ovarian cancer. Mol Cell Endocrinol 172:213–222
8. **Choi JH, Choi KC, Auersperg N, Leung PC** 2004 Overexpression of follicle-stimulating hormone receptor activates oncogenic pathways in preneoplastic ovarian surface epithelial cells. J Clin Endocrinol Metab 89:5508–5516
9. **Burdette JE, Kurley SJ, Kilen SM, Mayo KE, Woodruff TK** 2006 Gonadotropin-induced superovulation drives ovarian surface epithelia proliferation in CD1 mice. Endocrinology 147:2338–2345
10. **Davies BR, Finnigan DS, Smith SK, Ponder BA** 1999 Administration of gonadotropins stimulates proliferation of normal mouse ovarian surface epithelium. Gynecol Endocrinol 13:75–81
11. **Gaytan M, Sanchez MA, Morales C, Bellido C, Millan Y, Martin de Las Mulas J, Sanchez-Criado JE, Gaytan F** 2005 Cyclic changes of the ovarian surface epithelium in the rat. Reproduction 129:311–321
12. **Stewart SL, Querec TD, Gruver BN, O'Hare B, Babb JS, Patriotis C** 2004 Gonadotropin and steroid hormones stimulate proliferation of the rat ovarian surface epithelium. J Cell Physiol 198:119–124
13. **Murdoch WJ** 2005 Carcinogenic potential of ovulatory genotoxicity. Biol Reprod 73:586–590
14. **Murdoch WJ, Townsend RS, McDonnel AC** 2001 Ovulation-induced DNA damage in ovarian surface epithelial cells of ewes: prospective regulatory mechanisms of repair/survival and apoptosis. Biol Reprod 65:1417–1424
15. **Murdoch WJ, Van Kirk EA, Alexander BM** 2005 DNA damages in ovarian surface epithelial cells of ovulatory hens. Exp Biol Med (Maywood) 230:429–433
16. **Tan OL, Hurst PR, Fleming JS** 2005 Location of inclusion cysts in mouse ovaries in relation to age, pregnancy, and total ovulation number: implications for ovarian cancer? J Pathol 205:483–490
17. **Celik C, Gezginc K, Aktan M, Acar A, Yaman ST, Gungor S, Akyurek C** 2004 Effects of ovulation induction on ovarian morphology: an animal study. Int J Gynecol Cancer 14:600–606
18. **Modan B, Ron E, Lerner-Geva L, Blumstein T, Menczer J, Rabinovici J, Oelsner G, Freedman L, Mashiach S, Lunenfeld B** 1998 Cancer incidence in a cohort of infertile women. Am J Epidemiol 147:1038–1042
19. **Fathalla MF** 1971 Incessant ovulation—a factor in ovarian neoplasia? Lancet 2:163
20. **Whittemore AS, Harris R, Itnyre J** 1992 Characteristics relating to ovarian cancer risk: collaborative analysis of 12 US case-control studies. IV. The pathogenesis of epithelial ovarian cancer. Collaborative Ovarian Cancer Group. Am J Epidemiol 136:1212–1220
21. **Risch HA, Marrett LD, Howe GR** 1994 Parity, contraception, infertility, and the risk of epithelial ovarian cancer. Am J Epidemiol 140:585–597
22. **Riman T, Persson I, Nilsson S** 1998 Hormonal aspects of epithelial ovarian cancer: review of epidemiological evidence. Clin Endocrinol (Oxf) 49:695–707
23. **Bjersing L, Cajander S** 1975 Ovulation and the role of the ovarian surface epithelium. Experientia 31:605–608
24. **Bosetti C, Negri E, Trichopoulos D, Franceschi S, Beral V, Tzonou A, Parazzini F, Greggi S, La Vecchia C** 2002 Long-term effects of oral contraceptives on ovarian cancer risk. Int J Cancer 102:262–265
25. **Brinton LA, Lamb EJ, Moghissi KS, Scoccia B, Althuis MD, Mabie JE, Westhoff CL** 2004 Ovarian cancer risk after the use of ovulation-stimulating drugs. Obstet Gynecol 103:1194–1203
26. **Fredrickson TN** 1987 Ovarian tumors of the hen. Environ Health Perspect 73:35–51
27. **Scully RE** 1995 Early de novo ovarian cancer and cancer developing in benign ovarian lesions. Int J Gynaecol Obstet 49 (Suppl):S9–S15
28. **Scully RE** 1995 Pathology of ovarian cancer precursors. J Cell Biochem (Suppl) 23:208–218
29. **Cvetkovic D** 2003 Early events in ovarian oncogenesis. Reprod Biol Endocrinol 1:68
30. **Feeley KM, Wells M** 2001 Precursor lesions of ovarian epithelial malignancy. Histopathology 38:87–95
31. **Schlosshauer PW, Cohen CJ, Penault-Llorca F, Miranda CR, Bignon YJ, Dauplat J, Deligdisch L** 2003 Prophylactic oophorectomy: a morphologic and immunohistochemical study. Cancer 98:2599–2606
32. **Roland IH, Yang WL, Yang DH, Daly MB, Ozols RF, Hamilton TC, Lynch HT, Godwin AK, Xu XX** 2003 Loss of surface and cyst epithelial basement membranes and preneoplastic morphologic changes in prophylactic oophorectomies. Cancer 98:2607–2623
33. **Schneider JG, Tompkins C, Blumenthal RS, Mora S** 2006 The metabolic syndrome in women. Cardiol Rev 14:286–291
34. **Rodriguez GC, Haisley C, Hurteau J, Moser TL, Whitaker R, Bast Jr RC, Stack MS** 2001 Regulation of invasion of epithelial ovarian cancer by transforming growth factor-β. Gynecol Oncol 80:245–253
35. **Berchuck A, Rodriguez G, Olt G, Whitaker R, Boente MP, Arrick BA, Clarke-Pearson DL, Bast Jr RC** 1992 Regulation of growth of normal ovarian epithelial cells and ovarian cancer cell lines by transforming growth factor-β. Am J Obstet Gynecol 166:676–684.
36. **Choi KC, Kang SK, Nathwani PS, Cheng KW, Auersperg N, Leung PC** 2001 Differential expression of activin/inhibin subunit and activin receptor mRNAs in normal and neoplastic ovarian surface epithelium (OSE). Mol Cell Endocrinol 174:99–110
37. **Choi KC, Kang SK, Tai CJ, Auersperg N, Leung PC** 2001 The regulation of apoptosis by activin and transforming growth factor-β in early neoplastic and tumorigenic ovarian surface epithelium. J Clin Endocrinol Metab 86:2125–2135
38. **Di Simone N, Crowley Jr WF, Wang QF, Sluss PM, Schneyer AL** 1996 Characterization of inhibin/activin subunit, follistatin, and activin type II receptors in human ovarian cancer cell lines: a potential role in autocrine growth regulation. Endocrinology 137:486–494
39. **Fukuda J, Ito I, Tanaka T, Leung PC** 1998 Cell survival effect of activin against heat shock stress on OVCAR3. Life Sci 63:2209–2220
40. **Havrilesky LJ, Hurteau JA, Whitaker RS, Elbendary A, Wu S, Rodriguez GC, Bast Jr RC, Berchuck A** 1995 Regulation of apoptosis in normal and malignant ovarian epithelial cells by transforming growth factor β. Cancer Res 55:944–948
41. **Hurteau J, Rodriguez GC, Whitaker RS, Shah S, Mills G, Bast RC, Berchuck A** 1994 Transforming growth factor-β inhibits proliferation of human ovarian cancer cells obtained from ascites. Cancer 74:93–99
42. **Lambert-Messerlian GM, DePasquale SE, Maybruck WM, Steinhoff MM, Gajewski WH** 1999 Secretion of activin A in recurrent epithelial ovarian carcinoma. Gynecol Oncol 74:93–97
43. **Nilsson EE, Skinner MK** 2002 Role of transforming growth factor β in ovarian surface epithelium biology and ovarian cancer. Reprod Biomed Online 5:254–258
44. **Steller MD, Shaw TJ, Vanderhyden BC, Ethier JF** 2005 Inhibin resistance is associated with aggressive tumorigenicity of ovarian cancer cells. Mol Cancer Res 3:50–61
45. **Welt CK, Lambert-Messerlian G, Zheng W, Crowley Jr WF, Schneyer AL** 1997 Presence of activin, inhibin, and follistatin in epithelial ovarian carcinoma. J Clin Endocrinol Metab 82:3720–3727
46. **Zheng W, Luo MP, Welt C, Lambert-Messerlian G, Sung CJ, Zhang Z, Ying SY, Schneyer AL, Lauchlan SC, Felix JC** 1998 Imbalanced expression of inhibin and activin subunits in primary epithelial ovarian cancer. Gynecol Oncol 69:23–31
47. **Bristol-Gould SK, Hutten CG, Sturgis C, Kilen SM, Mayo KE, Woodruff TK** 2005 The development of a mouse model of ovarian endosalpingiosis. Endocrinology 146:5228–5236
48. **Burdette JE, Jeruss JS, Kurley SJ, Lee EJ, Woodruff TK** 2005 Activin A mediates growth inhibition and cell cycle arrest through Smads in human breast cancer cells. Cancer Res 65:7968–7975
49. **Van Der Lee S, Boot LM** 1955 Spontaneous pseudopregnancy in mice. Acta Physiol Pharmacol Neerl 4:442–444
50. **Lamond DR** 1958 Infertility associated with extirpation of the olfactory bulbs in female albino mice. Aust J Exp Biol Med Sci 36:103–108
51. **Lamond DR** 1959 Effect of stimulation derived from other animals of the same species on oestrous cycles in mice. J Endocrinol 18:343–349
52. **Whitten WK** 1959 Occurrence of anoestrus in mice caged in groups. J Endocrinol 18:102–107
53. **Marsden HM, Bronson FH** 1964 Estrous synchrony in mice: alteration by exposure to male urine. Science 144:1469
54. **Vandenbergh JG, Whitsett JM, Lombardi JR** 1975 Partial isolation of a pheromone accelerating puberty in female mice. J Reprod Fertil 43:515–523
55. **Tan OL, Fleming JS** 2004 Proliferating cell nuclear antigen immunoreactivity

in the ovarian surface epithelium of mice of varying ages and total lifetime ovulation number following ovulation. Biol Reprod 71:1501–1507
56. **Vanderhyden BC, Shaw TJ, Ethier JF** 2003 Animal models of ovarian cancer. Reprod Biol Endocrinol 1:67
57. **Roby KF, Taylor CC, Sweetwood JP, Cheng Y, Pace JL, Tawfik O, Persons DL, Smith PG, Terranova PF** 2000 Development of a syngeneic mouse model for events related to ovarian cancer. Carcinogenesis 21:585–591
58. **Flesken-Nikitin A, Choi KC, Eng JP, Shmidt EN, Nikitin AY** 2003 Induction of carcinogenesis by concurrent inactivation of p53 and Rb1 in the mouse ovarian surface epithelium. Cancer Res 63:3459–3463
59. **Connolly DC, Bao R, Nikitin AY, Stephens KC, Poole TW, Hua X, Harris SS, Vanderhyden BC, Hamilton TC** 2003 Female mice chimeric for expression of the simian virus 40 TAg under control of the MISIIR promoter develop epithelial ovarian cancer. Cancer Res 63:1389–1397
60. **Dinulescu DM, Ince TA, Quade BJ, Shafer SA, Crowley D, Jacks T** 2005 Role of K-ras and Pten in the development of mouse models of endometriosis and endometrioid ovarian cancer. Nat Med 11:63–70
61. **Godwin AK, Testa JR, Handel LM, Liu Z, Vanderveer LA, Tracey PA, Hamilton TC** 1992 Spontaneous transformation of rat ovarian surface epithelial cells: association with cytogenetic changes and implications of repeated ovulation in the etiology of ovarian cancer. J Natl Cancer Inst 84:592–601
62. **Testa JR, Getts LA, Salazar H, Liu Z, Handel LM, Godwin AK, Hamilton TC** 1994 Spontaneous transformation of rat ovarian surface epithelial cells results in well to poorly differentiated tumors with a parallel range of cytogenetic complexity. Cancer Res 54:2778–2784
63. **Gold EJ, O'Bryan MK, Mellor SL, Cranfield M, Risbridger GP, Groome NP, Fleming JS** 2004 Cell-specific expression of βC-activin in the rat reproductive tract, adrenal and liver. Mol Cell Endocrinol 222:61–69
64. **Woolnough E, Russo L, Khan MS, Heatley MK** 2000 An immunohistochemical study of the rete ovarii and epoophoron. Pathology 32:77–83
65. **Pon YL, Auersperg N, Wong AS** 2005 Gonadotropins regulate N-cadherin-mediated human ovarian surface epithelial cell survival at both post-translational and transcriptional levels through a cyclic AMP/protein kinase A pathway. J Biol Chem 280:15438–15448
66. **Murdoch WJ, Van Kirk EA** 2002 Steroid hormonal regulation of proliferative, p53 tumor suppressor, and apoptotic responses of sheep ovarian surface epithelial cells. Mol Cell Endocrinol 186:61–67
67. **McMullen ML, Cho BN, Yates CJ, Mayo KE** 2001 Gonadal pathologies in transgenic mice expressing the rat inhibin α-subunit. Endocrinology 142:5005–5014
68. **Bamberger C, Scharer A, Antsiferova M, Tychsen B, Pankow S, Muller M, Rulicke T, Paus R, Werner S** 2005 Activin controls skin morphogenesis and wound repair predominantly via stromal cells and in a concentration-dependent manner via keratinocytes. Am J Pathol 167:733–747
69. **Wankell M, Munz B, Hubner G, Hans W, Wolf E, Goppelt A, Werner S** 2001 Impaired wound healing in transgenic mice overexpressing the activin antagonist follistatin in the epidermis. EMBO J 20:5361–5372
70. **Wankell M, Werner S, Alzheimer C** 2003 The roles of activin in cytoprotection and tissue repair. Ann NY Acad Sci 995:48–58

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**