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Ovarian epithelial inclusions cysts in chronically superovulated cd1 and smad2 dominant negative mice

Short title: Ovulation and ovarian epithelial cysts

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Abstract
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 intraperitoneal injections of pregnant mare serum gonadotropin (5IU/animal) followed 48 hours later by human chorionic gonadotropin (5IU/animal). Wild-type CD1 mice were utilized 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 six months of age for the total number adjusted 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 Smad2DN transgene and superovulation resulted in an increase in cyst formation compared to 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 (OSE) 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 to unstimulated controls, but the epithelial lined cysts did not progress to cancer over the course of this study.

Introduction

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 post-ovulatory epithelial wound repair (2). Despite carrying out 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 over 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. Prior to ovulation, release of luteinizing hormone (LH) by the pituitary stimulates the OSE adjacent to a preovulatory follicle to induce the secretion of plasminogen activator and this triggers tumor necrosis factor alpha (TNFα) breakdown of the OSE layer (4). The OSE expresses receptors for follicle-stimulating hormone (FSH) and LH (5-8), and elevated amounts of gonadotropins stimulate proliferation of both rat and mouse OSE prior to 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 re-establishing normal surface continuity, it may also be a source of uncontrolled cell growth involved in cyst or tumor formation.

While 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, while 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). Since 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 progression (29-32). Alternative precursor lesions identified in prophylactically removed BRCA 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 transforming growth factor beta (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 Smads 2 and 3 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 alpha 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 alpha 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 if 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 CD-1 mice were obtained through in house breeding lines. Mice were maintained in accordance with the policies of the Northwestern University's Animal Care and Use Committee. Mice were housed and bred in a controlled barrier facility within Northwestern University's Center for Comparative Medicine. Temperature, humidity, and photoperiod (12L, 12D) 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 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 previously described (47). Smad2DN transgene blocks Smads 2 and 3 as demonstrated previously (47, 48).

Experimental Design for Chronic Ovulation

Smad2DN and their normal littermate (NLM) counterparts were genotyped at day 18 and subsequently placed into one of four groups: NLM ovulation suppressed and non-superovulated, NLM superovulated, Smad2DN ovulation suppressed and non-superovulated, 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) (5IU/mosue) (Sigma, St. Louis, MO) 48 hours later once a week starting at age 6 weeks 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 sacrificed and the serum, ovaries, and uteri of all mice were collected. Organs were fixed in 4% paraformaldehyde for 8-12 hours, dehydrated with ethanol, paraffin embedded, and serial sectioned at 4μm. Images were obtained using 10X, 20X, or 40X objectives on a Nikon Eclipse E600 microscope with a Spot camera (Diagnostic Instruments, Sterling Heights, MI). Every 10^{th} 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 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 of sacrifice. The animals were injected with BrdU in order 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 hours on day 25 of life and continuing to label all dividing cells until the time of sacrifice on day 27 for a total of 60 hours. Abridged basal proliferation was quantified in animals labeled with BrdU at 0900 on day 27 of life until sacrifice for a total of 12 hours. Total periovulatory proliferation was defined as the mitosis of OSE occurring from 0900 day 25 until 2100 day 27 for a total of 60 hours in animals injected with PMSG and hCG. Proliferation measured in PMSG and hCG injected animals from 0900 day 27 until sacrifice is defined as periovulatory proliferation and depicts cell division for 12 hours from the time of the hCG injection until sacrifice. These labels apply to both normal littermates and Smad2DN animals. Images were obtained around the perimeter of at least one section per animal using a 20X objective and the ovarian surface was reconstructed using Adobe Photoshop 7.0. 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 then counts for the treatment groups were averaged.

Immunohistochemistry

All reagents were obtained from Vector Laboratories, Inc. (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 minutes on high and 7 minutes on low followed by cooling in solution for 20 minutes. Slides were washed in Tris-buffered saline (TBS) with Tween [20mM Tris, 500mM NaCl, 0.1%Tween 20 (pH 7.4)]. Tissues were blocked for 15 minutes in 3% hydrogen peroxide (Fisher Scientific) followed by avidin and biotin according to manufacturer's instructions. Slides were incubated in 10% serum of the secondary antibody host in 3% bovine serum albumin in TBS for 1 hour at room temperature. After blocking, slides were incubated overnight at 4°C in primary antibody in 3% BSA-TBS-10% serum. Slides were rinsed 3 times for 5 minutes in TBS-Tween and then incubated at room temperature for 1 hour in secondary antibody in 3% BSA-TBS. After washing slides in TBS-Tween, ABC reagent was added and incubated for 30 minutes at room temperature. Slides were then washed in TBS and antigen-antibody-horseradish peroxidase complex was visualized using diaminobenzidine (DAB) reagent for 3 minutes. For the phospho-Smad2 and Smad3 antibodies, the method of enzyme detection was the tyramide signal amplification (TSA) florescein kit (Perkin-Elmer, Wellesley, MA) used with biotinylated anti-rabbit secondary at 1:400 dilution (Vector Labs, Burlingame, CA). 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) cytokeratin 8 (CK8 TROMA-1 antibody (rat); 1:50; Developmental Studies Hybridoma Bank, Iowa City, IA) phospho-Smad2 (3101 (rabbit); 1:50; Cell Signaling, Boston, MA), Smad3 (rabbit, 1:200; Invitrogen, Carlsbad, CA), phospho-histone

3 (9764 (rabbit), 1:100; Cell Signaling, Boston, MA), estrogen receptor alpha (sc-543 (rabbit), 1:100; Santa Cruz Biotechnologies, Santa Cruz, CA), progesterone receptor (sc-538 (rabbit), 1:100; Santa Cruz Biotechnologies, Santa Cruz, CA), and Müllerian Inhibiting Substance (sc-6886 (goat), 1:50; Santa Cruz Biotechnologies, Santa Cruz, CA) and they were incubated overnight at 4°C with ovary sections. The following secondary antibodies were incubated with their respective sections for 1 hour at room temperature: biotinylated anti-sheep (1:200), biotinylated anti-goat (1:200), biotinylated anti-rabbit (1:200), and biotinylated anti-rat (1:200) antibodies.

Statistical Analysis

All numerical data was analyzed using ANOVA followed by secondary paired t-tests using the Bonferonni correction factor. For data representing a percentage, a chi-square analysis was performed using the Piersen's correction factor and then grouped using a correspondence analysis. Significance of the data was determined as $p < 0.05$.

Results

Chronic Superovulation of Smad2DN Mice Increased Cyst Number Compared to CD1 Animals.

In order to study the impact of repeated ovulatory events, CD1 mice and Smad2DN transgenic mice were subjected to chronic superovulation. The four groups analyzed are as follows: normal littermates (NLM), normal littermates superovulated (NLM SO), transgenic mice (TX), and transgenic mice superovulated (TX SO). Superovulation was induced by injecting animals beginning at six weeks of life with 5IU/animal PMSG followed 48 hours later with an injection of hCG (5IU/mouse) once per week. Cysts were systematically evaluated in superovulated and control ovaries to examine if the process of superovulating mice until six months of life would induce cystic lesions (**Figure 1A**). Ovarian epithelial cysts were confirmed by immunostaining the adjacent slide with cytokeratin 8 antibody. Epithelial cysts were defined as those expressing CK8 (**Figure 1B**). The impact of superovulation increased the average amount of cysts formed in normal littermate animals as compared to control mice, but the difference did not reach significance.

Ovaries collected from Smad2DN transgenic mice were similarly evaluated for an increase in cyst formation from chronic superovulation. The average cyst number for transgenic animals was higher than normal littermates as previously reported (**Figure 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 chronic superovulation and Smad2DN transgenic expression results in an increase in cyst number compared to control.

Invaginations typically occur in human ovaries with age due to 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 previously been reported to be location dependent after chronic ovulation (16). In order 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 (**Figure 2A and 2B**).

For each treatment group, the percentage of the cysts in each location are reported in **Figure 2A**. An average increase in the percentage of cysts in the middle of the ovary was seen with superovulation in combination with transgenic 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.

Superovulation and Cyst Area

Cyst area varied greatly between genotypes and treatment groups. In order 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 **Figure 3**. The average area of unstimulated transgenic mice cysts was significantly larger than superovulated normal littermate cysts using a paired students 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 due 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 transgene 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 if an increase in proliferation of the OSE resulted from a single superovulation event (**Figure 4A**). Animals injected with BrdU to label all cells that had synthesized the DNA during the time of superovulation revealed that Smad2DN animals have the same increase in proliferation rate in response to PMSG and hCG as do CD1 mice (**Figure 4B**). Therefore, the increase in cyst rate in superovulated transgenic animals is likely not due 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 to the ovarian surface. Two methods were used to compare proliferation. First, chronically ovulated animals were injected with BrdU 24 hours before sacrifice and an immunostain directed against BrdU was used to mark cells that had divided at any time during the 24 hours after the injections. Secondly, an antibody directed against phosphorylated-histone 3 was utilized to mark cells dividing at the time of sacrifice. Using both methods, there was no difference in the amount of proliferating cells in the ovarian cysts of transgenic mice as compared to NLM regardless of superovulation (**Figure 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 as compared to the OSE (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 lab previously reported that the OSE cells of Smad2DN transgenic mice have significantly less Smad2 phosphorylation as compared to their normal littermates due 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 transgenic mice as compared to normal

littermates. These data would suggest that eliminating activin or TGFβ signaling through Smad2 is permissive for cyst formation. In order to investigate these mechanisms of cyst formation, the amount of Smad2 phosphorylation was compared between cysts and the ovarian surface in both normal and transgenic animals. 60% of unstimulated transgenic animals had OSE that did not display Smad2 phosphorylation as previously reported, whereas only 30% of normal littermates had undetectable phosphorylation. In all cases, the ovarian cysts themselves were found to have more Smad2 phosphorylation as compared to the ovarian surface of the same ovary analyzed (**Figure 6** top and middle panel). These data suggest that once epithelial ovarian cysts form in both normal and transgenic 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 transgenic and normal animals was previously found to express MIS thus explaining why Smad2 phosphorylation is reduced in the transgenic animals (47). To correlate this reacquisition of Smad2 signaling with MIS expression, ovarian cysts were stained for MIS. In the normal littermate cysts MIS expression was retained, while in the Smad2DN animals, MIS expression was lost. The loss of MIS expression suggests that the cysts of Smad2DN mice lose MIS promoter expression and thereby regain Smad2 phosphorylation (**Figure 6** bottom panel). 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 than 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 transgenic alteration and superovulation, the hormone receptor status was investigated to determine if 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 than normal surface epithelia. Using an antibody directed against ERα, estrogen receptors were detected in all of the cysts analyzed regardless of genotype or superovulation (**Figure 7** top panel). 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.

In order 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 (**Figure 7** bottom panel). However, the expression of progesterone receptors in the cyst was found to be constant, while OSE expression was only detected in ~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 normal littermates and transgenic 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 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 tumorogenesis 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 transgenic mice had significantly more cysts than unstimulated normal littermates. 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 transgenic mice generated more cysts than in unstimulated normal littermates. 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 knock-out 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 (Rb) 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 immuno-deficient 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 OSE represent a naïve 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 alpha (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 calretenin (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 as compared to 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, calretenein, or inhibin-α.

Chronic superovulation of mice generated a higher incidence of cyst formation but did not result in ovarian cancer formation within six 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. Secondly, human epithelial ovarian cancer often forms metastasis in the peritoneal cavity and ascites, which may be fundamentally different in the mouse due 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 EOC involves the process of inflammation at ovulatory sites, yet mice ovulate multiple follicles at once and still fail to spontaneously develop EOC. 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 transgenic animal models that lack a functional activin and TGFβ signal either due to inhibin overexpression or Smad2DN as compared to 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 as compared to the ovarian surface of transgenic 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 alpha 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 as compared to ovarian surface, and this is consistent with the cycling rat (11). Therefore, progesterone does not seem to be directly reducing cyst formation as the receptor is more highly expressed in cysts as compared to the surface. The expression of hormone receptors might allow this model to be used to determine if excess of estrogen as in hormone replacement therapy increases the incidence of cyst formation or whether anti-estrogens and aromatase inhibitors block cyst formation. Also the expression of hormone receptors may explain cyst persistence as 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 <1000 sq um 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 transgenic 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.

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Figure Legends

Figure 1. Epithelial cyst adjusted number in wild type and Smad2DN mouse ovaries after chronic superovulation. **A.** The data shown represents the average number of cysts per animal as measured in both ovaries on every 10^{th} slide through serial sections and the standard error from the mean. The significant differences ($p<0.05$) are indicated by "*" symbol. **B.** Immunohistochemical analysis of cyst cell type and origin. Sections adjacent to H&E stained tissue where cysts were located were stained with the epithelial marker CK8. Cysts from each treatment group acquired with 200X magnification after staining. Black arrows indicate cyst lining.

Figure 2. Epithelial ovarian cyst location in mouse ovaries. **A**. Data represents 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.

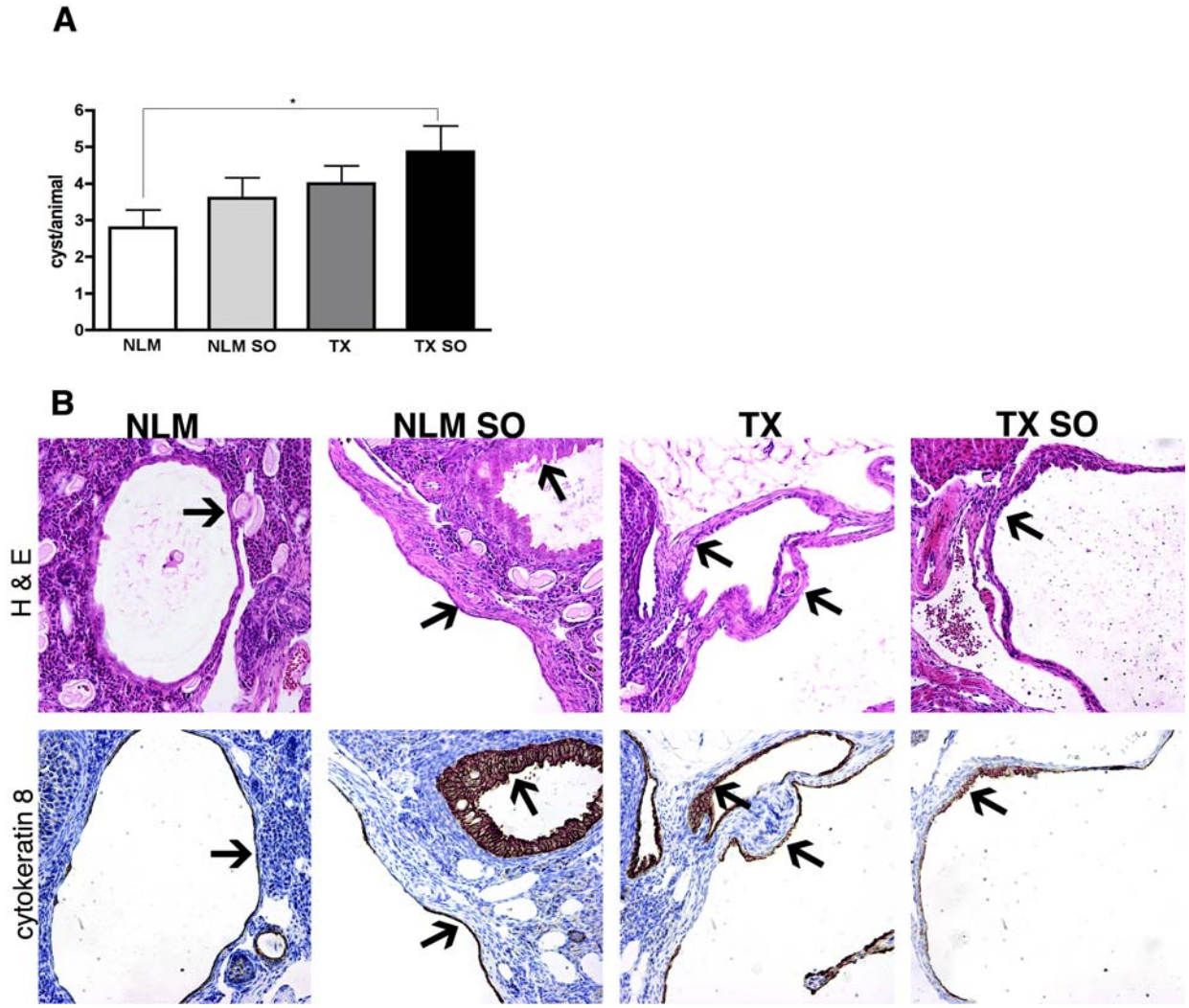
Figure 3. Superovulation and cyst area. **A**. Cysts were followed through serial sections and cyst area was calculated at the widest measured diameter. The data represents the average total cyst area at its widest point from all cysts observed in each group and the standard error from the mean. The significant differences ($p<0.05$) are indicated by "*" symbol.

Figure 4. BrdU incorporation into the OSE over the total surface area of the ovary following 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 7 ovaries. The shown data represent the least square mean (%) of total proliferation and the standard error from the mean. The significant differences ($p<0.05$) are between groups labeled with "a vs. b".

Figure 5. Ovarian inclusion cysts undergo rapid proliferation. The top panel shows phospho-histone 3 positive cells lining the cysts undergoing proliferation. The bottom panel shows BrdU incorporation into the epithelial lined cysts. Sections were stained with DAB and counterstained with hematoxylin. Black arrows indicate cyst lining.

Figure 6. Smad2 phosphorylation of OSE compared to the lining of epithelial cysts. Images in top panel depict the phosphorylation of Smad2 in the ovarian surface epithelium from ovaries acquired in each treatment group. Ovarian surface labeled with a white arrow. Images in middle panel depict phosphorylation of Smad2 in cysts determined to be of epithelial origin. Cysts lining are labeled with a red arrow. The bottom panel represents Müllerian inhibiting substance immunostained with DAB and counterstained with hematoxylin demonstrating the loss of gene activation from the endogenous MIS promoter in cysts of transgenic mice. Red arrows point towards cyst lining.

Figure 7. Ovarian inclusion cysts express hormone receptors. The top panel shows the estrogen receptor alpha expression in epithelium lining inclusion cysts found in each treatment group. The bottom panel depicts progesterone receptor expression in epithelium lining of inclusion cysts from each treatment group. Black arrows point toward inclusion cyst lining.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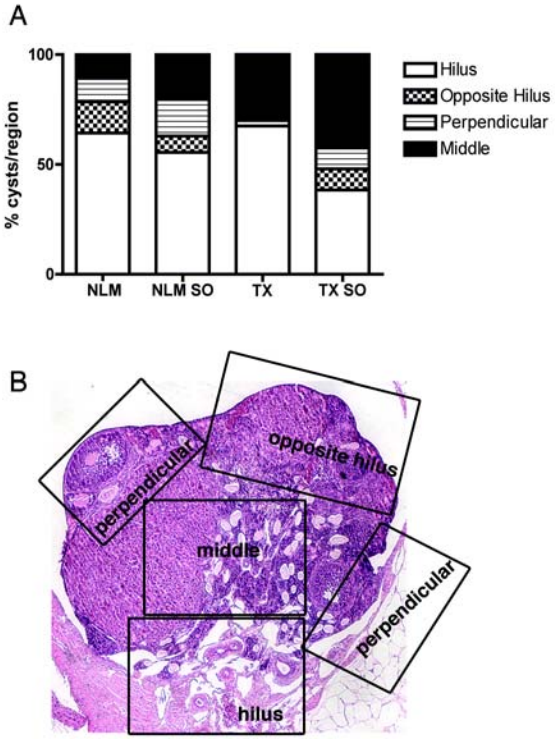
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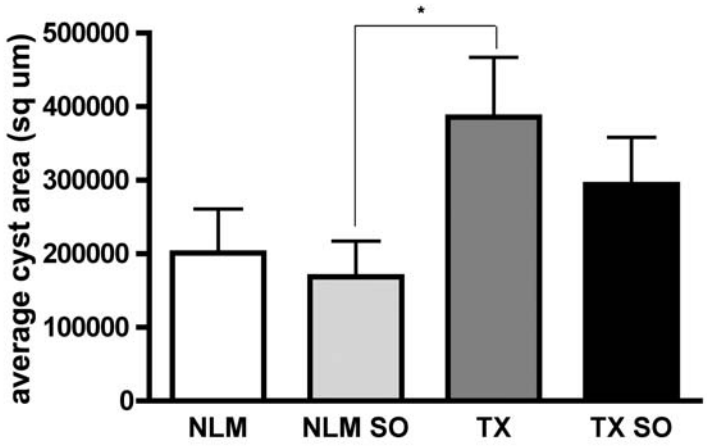
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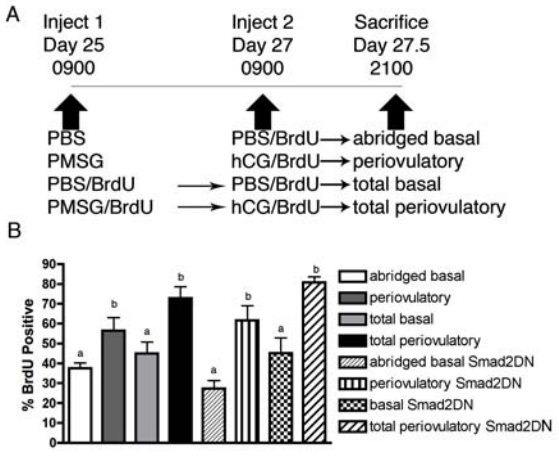
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PBS
PMSG
PBS/BrdU
PMSG/BrdU
PBS/BrdU → abridged basal
hCG/BrdU → periovulatory
PBS/BrdU → total basal
hCG/BrdU → total periovulatory
B
% BrdU Positive
0
10
20
30
40
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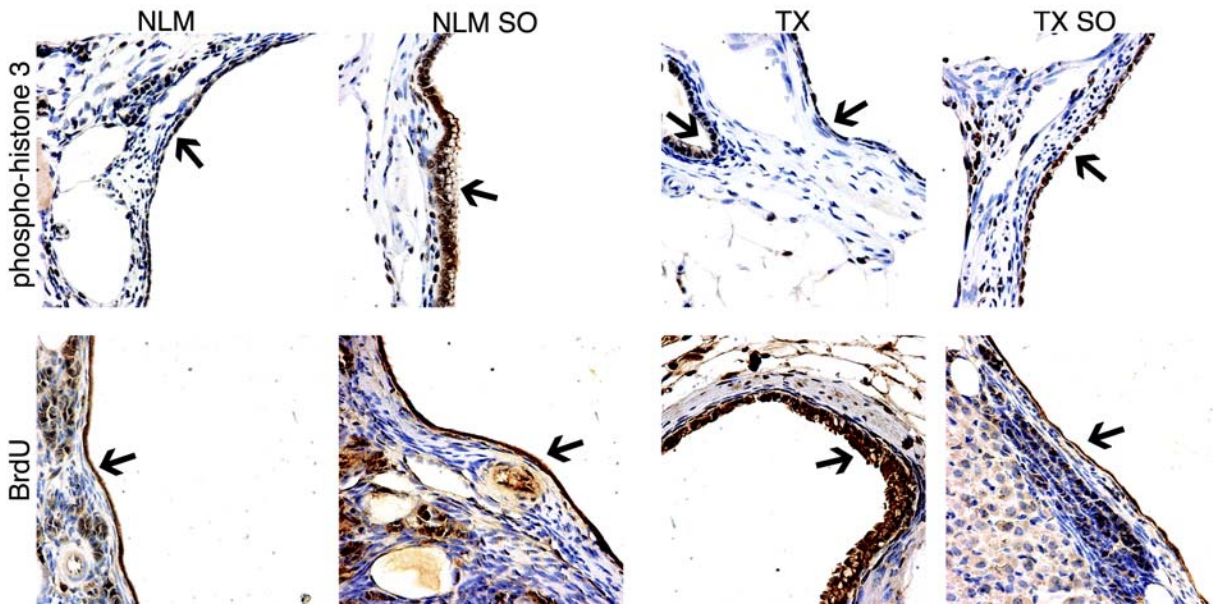
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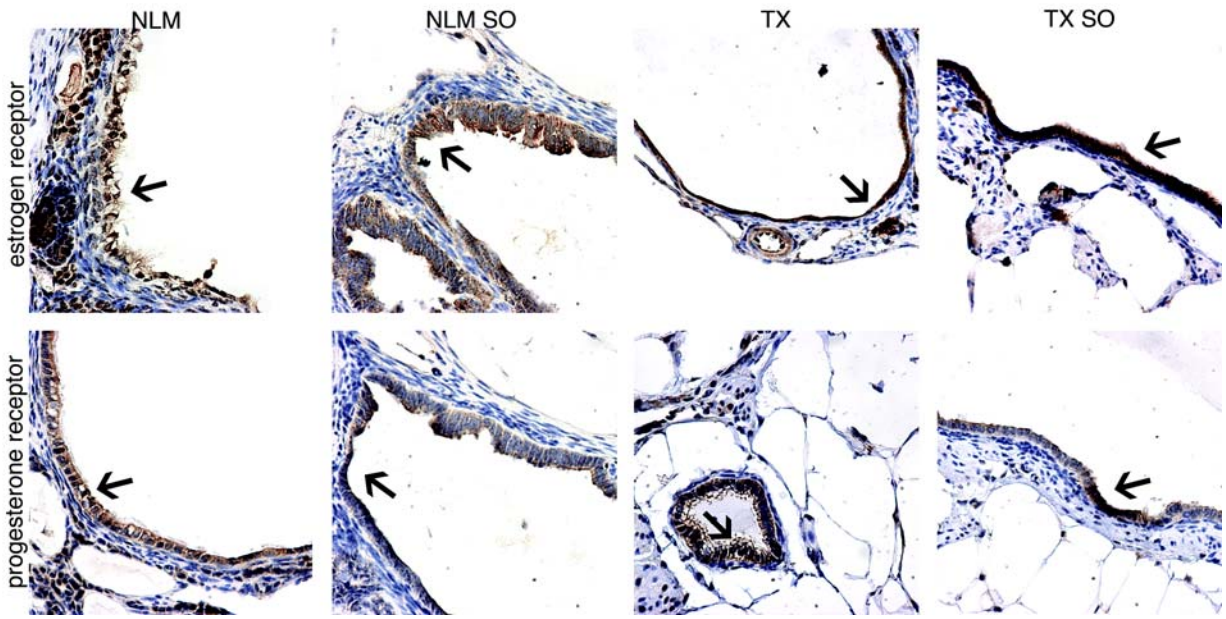
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