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Gonadotropin Induced Superovulation Drives Ovarian Surface Epithelia Proliferation in CD1 mice.

Running title: Proliferation of OSE during superovulation

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Abstract

The ovarian surface epithelium (OSE) is a monolayer of cells that surround the ovary and accommodate repeated tear and repair in response to ovulation. OSE cells are thought to be the progenitors of 90% of ovarian cancers. Currently, the total amount of proliferation of the OSE has not been reported in response to one ovulatory event. In this study, proliferation of the OSE was quantified in response to superovulation induced by intraperitoneal injection of pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) in immature 27-day-old CD1 mice using bromodeoxyuridine (BrdU). BrdU incorporation into the OSE cells was measured from the time of hCG injection for a total cumulative label of 12 hours. BrdU incorporation was also measured from the time of PMSG injection for a total label of 60 hours to correlate proliferation with specific gonadotropin stimulation. The OSE proliferation was significantly higher in superovulated animals compared to control mice at all time points. Proliferation was also analyzed in discrete anatomical sections and indicated that OSE covering antral follicles and corpora lutea proliferated more rapidly than OSE distal to follicular growth. Finally, apoptosis was assessed in response to ovulation and virtually no cell death within the OSE was detected. These data demonstrate that the OSE, especially near antral follicles and corpora lutea, proliferates significantly in response to the gonadotropins PMSG and hCG. Therefore, ovarian surface cell division in response to ovulation could contribute to ovarian cancer by proliferation-induced DNA mutations and transformed cell progression.

INTRODUCTION

The ovarian surface epithelium (OSE) is a single cell layer of squamous and cuboidal cells that express both epithelial and mesenchymal characteristics (1). These cells do not contain discrete markers when compared to other tissues derived from coelemic epithelia suggesting that they are less differentiated and more pleuripotent (2). The OSE is important to the integrity of the ovary and serves as the regulated barrier at the time of ovulation. The OSE was originally studied to investigate its contribution to ovarian follicular rupture and the subsequent repair (3, 4). Currently, the OSE receives attention because these cells are considered the progenitors of 90% of ovarian cancers (5). The etiology of the disease and mechanisms by which ovarian cancer cells progress to more dangerous phenotypes are poorly understood.

Ovulation induces a rupture site that is closed through re-epithelialization by the OSE proliferating and then migrating to cover the gap (6, 7). First proposed by Fathalla in 1971, the "incessant ovulation" hypothesis suggests that continual ovulation subjects the OSE to transformation events, which can lead to ovarian cancer (8). Increased occurrence of ovarian cancer is associated with increased ovulatory events in women undergoing infertility treatments (9). Additionally, the risk of ovarian cancer is reduced in women who experience fewer total ovulations either by the use of oral contraceptive pills, pregnancy, late menarche, early menopause, or hysterectomy (10, 11). At the site of ovulation, OSE cells suffer DNA oxidative damage and express the tumor suppressor, p53, showing the potential of ovulation as a stress factor on OSE cells to give rise to a transformed progenitor cell capable of causing a malignant tumor (12). Domestic hens, the only other species besides humans to form ovarian cancer, have an increased risk of this disease directly related to their number of ovulations and increased oxidative damage (13-15). In this proposed ovarian cancer pathway, ovulation induces DNA

damage that escapes endogenous repair mechanisms. After transformation, the increased rate of OSE proliferation, potentially stimulated from ovulation, may contribute to tumor progression (16). The expression of FSH and LH receptors in the OSE reflects on their ability to directly respond the gonadotropins FSH and LH independent of an ovulatory event (17, 18). Here we address the influence of the gonadotropins PMSG and hCG, as well as ovulation, on proliferation of OSE cells in CD1 mice.

Most of the current studies have focused on the proliferative activity of OSE in discrete anatomical areas in relationship to follicles and the hilus at specific times using a pulse of BrdU to label dividing cells during a short window of time. However, the total proliferative activity of the OSE in response to PMSG and hCG stimulation in the mouse has not been reported directly nor has it been analyzed in relation to follicle growth, epithelialization, and formation of the corpus luteum (CL). Additionally, theories regarding increased proliferation near the mesothelium, surrounding the hilus, or in OSE with specific a squamous morphology has not been substantiated using cumulative labeling strategies. We describe the total proliferation of OSE during one superovulation cycle in immature mice, which directly correlates to one ovulatory event without previous follicular rupture to confound results. These studies illuminate the normal proliferative function of the OSE in response to ovulation and are significant for understanding the stress imposed on OSE cells from ovulation and repair during transformation and progression of ovarian cancer.

MATERIALS AND METHODS

Animals for Proliferation Study

Female CD-1 mice, age 25 days, 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 of 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*.

Experimental Design of Proliferation Study

Control mice were injected intraperitoneally with phosphate-buffered saline at 0900 hours. Superovulated mice were injected intraperitoneally with 5IU of pregnant mare serum gonadotropin at 0900 and 5IU of human chorionic gonadotropin 48 hours later diluted in PBS (Sigma, St. Louis, MO). Injections containing 5-bromo-2-deoxyuridine (BrdU) were given intraperitoneally at 100mg/kg either at the time of the first and second hormone injection or solely with the second hormone injection (Sigma, St. Louis, MO). Upon first injection of BrdU, water was changed to contain 0.8mg/ml BrdU to allow for continuous labeling (**Figure 1A**). Each experimental group contained n=7 animals. Mice were sacrificed using CO_2 asphyxiation and cervical dislocation. Ovaries, including fat pad, bursa, oviduct, and partial uterine tube, were collected at 18:00, 12 hours following the second hormone injection. Ovaries were fixed in 4% paraformaldehyde for 8-12 hours, dehydrated with ethanol, paraffin embedded, and serial sectioned at 4μm.

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 and then cooled 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. Slides were counterstained using hematoxylin. Control slides received serum block instead of primary antibody.

Antibodies

The primary antibodies against BrdU (BrdU antibody (sheep); 1:50 dilution; Abcam, Cambridge, MA), and cytokeratin 8 (CK8 TROMA-1 antibody (rat); 1:50; Developmental Studies Hybridoma Bank, Iowa City, IA) 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) and biotinylated anti-rat (1:200) antibodies. Tunel staining was performed according to the manufacturer's protocol (Deadend Fluorometric Tunel

System, Promega, Madison, WI). The slides were mounted with Vectastain Mounting Medium containing DAPI and coverslipped.

Imaging and Counts

Images were obtained around the perimeter of at least one section per animal using a 20X objective on a Nikon Eclipse E600 microscope (Diagnostic Instruments) with a Spot camera and reconstructed using Adobe Photoshop 7.0. After reconstruction and printing of the image, the perimeter boundaries of the given image were defined. Two separate investigators, blinded to the conditions, independently counted the total number of cells and the total number of positively stained cells (**Figure 1C**). Counts for each investigator were averaged then counts for treatment groups were averaged. Regional counts were completed similarly by partitioning off surface epithelium surrounding follicles with an antrum, the hilus of ovaries when present in the section, distal segment of OSE not overlying any follicles, and in superovulated animals over a CL (**Figure 1B**).

Statistical Analysis

Total label and regional labels for the distal, follicle and hilus regions were analyzed using 3-factor analysis of variance, with genotype, labeling time and hormone status as the 3 factors. Regional labels for the CL were analyzed using two-factor analysis of variance, with genotype and labeling time as the two factors. In all analyses, tests of main effects were followed by pairwise t-test comparisons with p value significant at 0.05.

RESULTS

Cumulative Proliferation of OSE in Response to Superovulation

In order to investigate proliferative changes of OSE cells in response to ovulation, BrdU incorporation of the OSE was quantified in mice that were superovulated (**Figure 1A**). 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 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 ovulatory 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 post-ovulatory proliferation depicts cell division for 12 hours from the time of the hCG injection until sacrifice. While the timing of ovulation post hCG varied slightly from animal to animal, the time point of sacrifice was optimized at 12 hours post hCG (data not shown). Ovulation was confirmed in hormone-injected animals based on the presence of CL.

Proliferation of the OSE was detected all around the circumference and in all sections analyzed. The incorporation levels of BrdU in granulosa cells of developing follicles served as an internal positive control to monitor proliferation. OSE cells of both squamous and cuboidal m morphologies were found to have proliferated in all of the ovaries analyzed (**Figure 1C**). OSE proliferation in the abridged basal animals reached 37.5% after 12 hours of labeling and 45.1% in the total basal group after 60 hours of labeling (**Figure 2**). The basal proliferation rate includes the contribution from endogenous gonadotropins that stimulated the growth of large antral

follicles found in every section obtained from the control mice used in the study, suggesting some role for FSH in proliferation of the OSE (19). Stimulation from PMSG and hCG in the total ovulatory animals resulted in a significant increase in the amount of proliferating OSE cells (72.8%) as compared to the total basal group (45.1%). Post-ovulatory animals were injected with BrdU at the time of hCG in order to label the proliferation that occurred from the time of follicular rupture until repair. The proliferation rate of the OSE in the post-ovulatory group (56.4%) was significantly higher than in abridged basal mice (37.5%). The rate of proliferation in response to hCG (56.4%) in the post-ovulatory animals did not differ significantly from the rate of proliferation measured from total ovulation (72.8%). If the contribution from hCG as measured in the post-ovulatory mice was subtracted from the cumulative labeling index of the total ovulatory animals, the contribution of PMSG to OSE proliferation was 16.4%.

Regional Proliferation of the Mouse OSE from Superovulation

In order to elucidate whether proliferation of the OSE was occurring in discrete areas in response to follicular development, several anatomical regions of the ovary were selected and counted for the percent of proliferating cells. The schematic in **Figure 1B** describes the areas of ovarian surface cells analyzed. The areas selected for evaluation included the OSE directly tandem to the hilus, antral follicles, corpora lutea in superovulated animals, and an area distal from follicular development. At least five areas were counted in separate animals to generate the average percent of dividing cells localized around each structure, and the labeling percentage was analyzed between treatment groups. A site distal from follicular growth was chosen to compare the influence of follicle expansion on OSE cells to a region not impacted. The OSE surrounding distal areas did not differ significantly between any of the ovaries indicating that when follicular maturation is not influencing OSE, the difference in proliferation is not

significant (**Figure 3A i.-v.**). These distal areas may be impacted by a variety of factors making OSE proliferation appear uniform independent of hormone treatment, although the label increased according to the amount of time BrdU was being incorporated. The proliferation of OSE adjacent to antral follicles matched the same relative change in proliferation surrounding the entire ovarian surface (**Figure 3B i.-v.**). These data indicate that OSE are partially influenced by the proximity of an expanding follicle to divide. The OSE counts adjacent to follicular development in the total ovulatory group (81.8%) had significantly higher amounts of proliferation than the total basal group (41.2%), and this difference was also significant between the post-ovulatory and abridged basal groups. The increased OSE cell division stimulated by the gonadotropin injections near antral follicles suggests that OSE respond to systemic hormone administration. Finally, the OSE cells covering the hilus region were not different with the exception of those cells in the total basal group, which were labeled significantly less than the other three groups (**Figure 4 i.-v.**).

Epithelialization of Corpora lutea after Follicular Rupture

The OSE proliferation adjacent to corpora lutea of animals in the total ovulatory group (83.5%) as compared to the post-ovulatory group (60.0%) was significantly higher providing evidence that some of the OSE proliferation around a CL occurs in the time prior to its formation when the follicle is growing (**Figure 5A-5B**). Previous investigators have reported that a corpus luteum takes several days to fully re-epithelialize after follicular rupture (7). In order to confirm that the measurements obtained in this study reflected the presence of OSE around superovulated ovaries, the known OSE marker, cytokeratin 8, was used to distinguish ovarian surface cells. The antigen was detected around the entire ovarian surface and not in any of the follicular compartments (**Figure 5C-5D**). The superovulated mouse ovaries were lined with CK8 antigen

positive cells indicating that OSE had covered over the area of rupture within the 12 hours after hCG. These data imply that the covering over of the CL by epithelium occurs rapidly within minutes to hours of the initial formation of the CL from superovulation.

Mouse OSE Do Not Undergo Apoptosis in Response to Superovulation

One possible mechanism monitoring cell levels after proliferation of the ovarian surface may be apoptosis. Apoptosis of the OSE might allow damage incurred during ovulation to be cleared thereby reducing the risk of developing mutations over time. In order to evaluate this possibility, ovaries from unstimulated and superovulated animals were stained with TUNEL to quantify the amount of apoptosis in OSE cells. Granulosa cells in atretic follicles served as an internal positive control that the stain was accurately marking apoptotic cells, while blood vessels demonstrated autofluorescence. The unstimulated immature ovaries had no apoptotic OSE cells (**Figure 6A-6B**). Superovulated ovaries also lacked any signs of apoptosis in the OSE (**Figure 6C-6D**).

DISCUSSION

In this study, we have demonstrated three significant findings regarding the proliferation of OSE cells in response to superovulation using cumulative labeling. First, OSE cells in superovulated mice proliferate more than those in unstimulated animals providing evidence that gonadotropin stimulation influences cell division. Second, the proliferation of the OSE in response to gonadotropins occurs primarily within certain anatomical regions of the ovary, primarily near antral follicles and corpora lutea as compared to regions distal from follicular development and occurs both prior to and during tear and repair. Finally, the amount of apoptosis occurring in OSE cells was investigated and no evidence was found of programmed

cell death at the time points analyzed either in unstimulated or superovulated ovaries. This study advances the understanding of the normal proliferative function of the OSE in response to each gonadotropin during ovulation with special attention to the OSE overlying distinct anatomical regions.

Cumulative labeling catalogues all cellular divisions in response to gonadotropins and accurately represents all growth from one ovulatory event. Previous experiments using mice and rats to measure the proliferation of OSE may have underestimated the total amount of proliferation in response to ovulation by using PCNA or short pulses of BrdU (7, 20, 21). However, in this study, between 85-90% of all OSE had divided in response to ovulation when measured over a 60-hour period. Superovulation has been suggested to overestimate the amount of proliferation occurring in mice due to the increased number of follicles recruited, but this paradigm ensures that only one ovulatory cycle is measured and that each gonadotropin's individual contribution can be assessed. Cumulative labeling also helps to delineate the morphology of dividing cells and reveals that proliferating cells may alternate between a cuboidal type while dividing, when they stain positive for PCNA, but later become either cuboidal or squamous as were depicted in these results (7). This study by using cumulative labeling illustrates the proliferative potential of the OSE during one ovulatory event and demonstrates the dynamic change in OSE morphology regardless of cell division.

The data from this study demonstrates that proliferation of the OSE is related to both follicle proximity as well as systemic gonadotropin stimulation. For example, the rate of proliferation of OSE adjacent to antral follicles and CL exceeded that of areas distal to follicles consistent with previous findings (7). Also, proliferation of OSE near antral follicles in post-ovulatory and total ovulatory groups was higher than those surrounding antral follicles in the total basal and

294 abridged basal groups indicating that the systemic influence of gonadotropins must contribute to
295 the increased proliferation of the OSE. Previously, other investigators concluded that the
296 mesothelium is the local anatomical target of proliferation from ovulation; however, cumulative
297 labeling depicted sporadic staining throughout the OSE indicating that the entire ovarian surface
298 is capable of cell division (22). Because our study and others have demonstrated a regional
299 increase in proliferation, especially with respect to the position of antral follicles and corpora
300 lutea as compared to distal areas in the ovary, these data strongly support the idea that both local
301 and systemic factors contribute to OSE proliferation and may offer an explanations why cultured
302 cells do not always proliferate in response to gonadotropins (23-26). Therefore, studying the
303 proliferation of the OSE with relation to anatomical regions and the administration of
304 gonadotropins in vivo reveals that both follicular growth as well PMSG and hCG influence OSE
305 cellular division.

306 Because the ovary must repair the surface after each ovulation, proliferation was primarily
307 thought to occur post rupture in response to the wound, allowing the ovary to heal the exposed
308 area. Our study supports a contribution of PMSG toward proliferation and suggests that
309 proliferation occurs prior to ovulatory induced wounds. Consistent with our findings, other
310 reports have provided evidence of proliferation from PMSG, independent of wounding, and in
311 sites distant from follicular rupture (20, 21, 25, 27). In general, the cumulative labeling index of
312 cells covering antral follicles was the same as OSE covering corpora lutea when compared
313 against the same relative labeling time. In addition, the proliferation of OSE cells covering
314 corpora lutea was higher in animals labeled for 60 instead of 12 hours directly reflecting the pre-
315 wounding proliferation. Our studies help confirm and extend the concept that proliferation

316 occurs in response to both PMSG and hCG and that follicular growth as well as tear and repair
317 contribute to cell division of the OSE.

318 The expression of FSH and LH receptors in the OSE reflects on their ability to directly
319 respond to the gonadotropins FSH and LH suggests that stimulation of these receptors provide
320 the mechanism for proliferation in this study (17, 18). Isolated OSE from rabbits in the estrous
321 phase proliferate in response to hCG and LH, but not FSH (25). Human cancer-derived OSE
322 respond to LH, FSH, estradiol, and testosterone in vitro by proliferating while progesterone
323 growth inhibits these cells (24). However, primary OSE scrapes from pre- and post-menopausal
324 women indicate that FSH growth inhibits the epithelium and LH had no influence on the
325 proliferation of OSE (26). Although the role of FSH and LH receptors on proliferation of the
326 OSE after ovulation is poorly understood, recent studies concluded that FSH receptors are more
327 highly expressed in cancer as compared to normal human OSE and that overexpression of the
328 FSHR activates oncogenic pathways (28, 29). Investigations into the expression of LH and FSH
329 receptors in the mouse would be facilitated by the development of new antibodies. Validating
330 that OSE proliferate in response to gonadotropins because of receptor activation is an important
331 future experimental objective.

332 Because the ovary exhibits a large amount of proliferation in the surface epithelium in
333 response to ovulation, it may eliminate some cells to compensate for this massive cellular
334 division. In order to investigate whether cells might be shed through apoptosis, we performed a
335 TUNEL stain on immature and superovulated ovaries. Virtually no apoptosis was demonstrated
336 in either set of ovaries, which is consistent with previous morphological evaluations (21).
337 Although sheep OSE apoptosis is regulated by estrogen and progesterone, in the current study,
338 mouse OSE do not appear to undergo apoptosis *in vivo* (12). Future investigations may

339 determine if the OSE undergo necrosis, are shed during the ovulatory process, or if the cells
340 undergo an epithelial to mesenchymal transition and become incorporated into the stroma (2).
341 Therefore, most OSE cells are not eliminated through apoptotic mechanisms in superovulated
342 mice.

343 One hypothesis regarding ovarian cancer is that an increased number of ovulations
344 contribute to the formation of transformed cells. Gonadotropins involved in ovulation were
345 investigated in the current study to determine their role in stimulating OSE proliferation. The
346 gonadotropins responsible for ovulation may stimulate both cancer initiation and progression by
347 stimulating OSE cellular proliferation. Mechanistically, investigators have speculated that
348 transformation events are incurred during ovulation when reactive oxygen species form DNA
349 adducts in the OSE (12, 30). Also, rat OSE cells continuously proliferated in culture eventually
350 become transformed (31, 32). The current study demonstrates that OSE proliferate significantly
351 in response to follicular growth and administration of the gonadotropins PMSG and hCG. The
352 contribution of ovulation on OSE proliferation as measured in the mouse may help to explain
353 how repeated ovulation in humans might act to transform OSE and progress ovarian cancer.

354 In conclusion, cumulative BrdU labeling revealed that the ovarian surface epithelium rapidly
355 proliferates in response to superovulation in immature mice. The total amount of proliferation in
356 the OSE appears to most strongly correlate with the proliferation rate of the OSE that are tandem
357 to antral follicles and CL. Both PMSG and hCG contributed to proliferation that could occur
358 prior to or during ovulation. Apoptotic clearing of damaged OSE cells was not detected in
359 superovulated or unstimulated animals using TUNEL stain. Therefore, ovulation dramatically
360 increases the rate of OSE proliferation without simultaneously increasing programmed cell death

potentially creating a system where damaged cells would be retained and could contribute to the formation of transformed cells.

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FIGURE LEGENDS

Figure 1. Schematic of hormone and BrdU injection times, anatomical regions surveyed, and morphologies observed. **A,** Time intervals are shown for labeling incorporation of BrdU, injections of PMSG and hCG, and time of sacrifice. **B**, Representative superovulated ovary indicating discrete anatomical regions counted. **C**, Morphological characterization of cuboidal and squamous OSE. Examples of cells stained positive for BrdU indicated by arrow and negative cells labeled with an arrowhead.

Figure 2. BrdU incorporation into the OSE over the total surface area of the ovary following gonadotropin stimulation with PMSG and hCG. 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 3. BrdU incorporation into the OSE covering areas distal from folliculogenesis or tandem to antral follicles. **Ai.,** The shown data represent the least square mean (%) of distal proliferation and the standard error from the mean. **Aii.-v.,** BrdU in the OSE of distal areas stained with DAB and counterstained with hematoxylin from **ii.** abridged basal, **iii.** post-ovulatory, **iv.** total basal, and **v.** total ovulatory treatment groups. **Bi.,** The shown data represent the least square mean (%) of proliferation in areas near antral follicles and the standard error from the mean. The significant differences ($p<0.05$) are between groups labeled with "a" vs. "b". **Bii.-v.,** BrdU in the OSE of cells surrounding antral follicles stained with DAB and counterstained with hematoxylin from **ii.** abridged basal,

iii. post-ovulatory, **iv.** total basal, and **v.** total ovulatory treatment groups. Cells positive for BrdU indicated by an arrow, cells negative for BrdU labeled with an arrowhead, and proliferating granulosa cells within antral follicles labeled with a red arrow.

Figure 4. BrdU incorporation into the OSE adjacent to the hilus. **i.,** The shown data represent the least square mean (%) of proliferation adjacent to the hilus and the standard error from the mean. The significant differences ($p<0.05$) are between groups labeled with "a" vs. "b". **ii.-v.,** BrdU in the OSE of cells surrounding the hilus stained with DAB and counterstained with hematoxylin from **ii.** abridged basal, **iii.** post-ovulatory, **iv.** total basal, and **v.** total ovulatory treatment groups. Cells positive for BrdU indicated by an arrow and negative cells labeled with an arrowhead

Figure 5. BrdU incorporation into the OSE tandem to the corpus luteum of superovulated mice. **A,** The shown data represent the least square mean (%) of proliferation near CL and the standard error from the mean. The significant differences ($p<0.05$) are between groups labeled with "*". **B,** BrdU in the OSE of cells surrounding the corpus luteum stained with DAB and counterstained with hematoxylin from superovulated animals. Cells positive for BrdU indicated by an arrow and negative cells labeled with an arrowhead. **C,** The corpus luteum of superovulated animals stained with the OSE marker, cytokeratin 8, demonstrated re-epithelialization of the OSE within 12 hours post hCG injection at 60X (**C**) and 100X (**D**).

Figure 6. Mouse OSE does not undergo apoptosis. OSE from ovarian sections were fixed and stained with TUNEL to detect apoptosis. Granulosa cells of atretic follicles serve as an internal positive control. Blood vessels autofluoresce. Nuclei were counterstained with DAPI. Images were taken at 60X for non-superovulated animals (**A**) and 100X (**B**) and at 60X for superovulated animals (**C**) and 100X (**D**).

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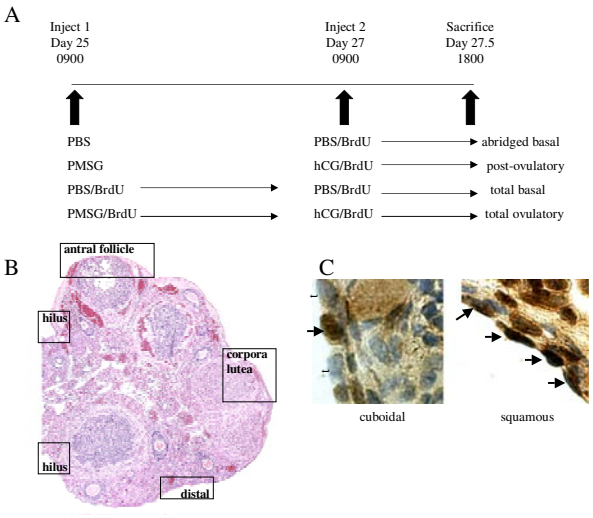
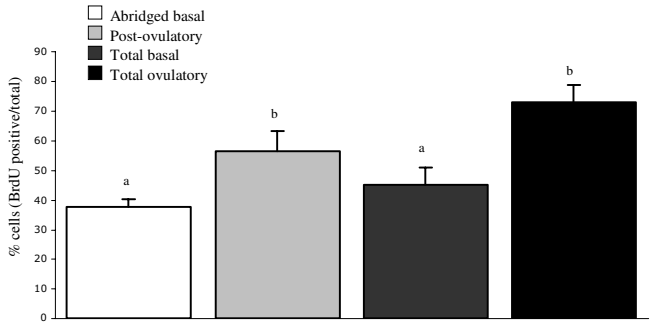
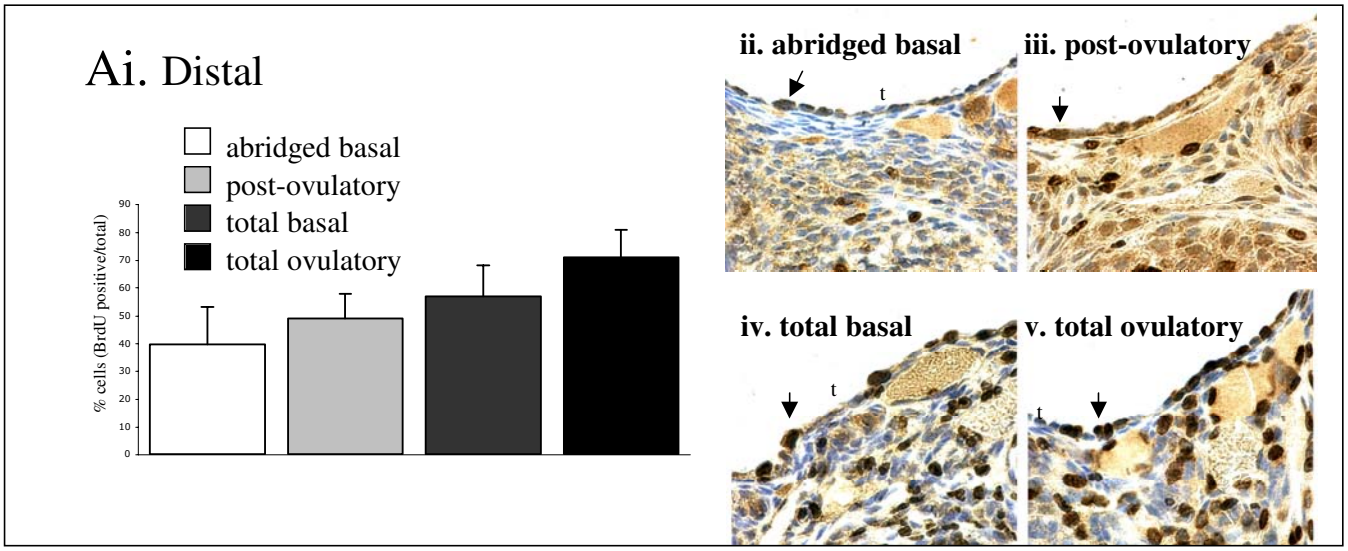
Burdette Figure 1



Burdette Figure 2




Ai. Distal
abridged basal
post-ovulatory
total basal
total ovulatory
% cells (BrdU positive/total)
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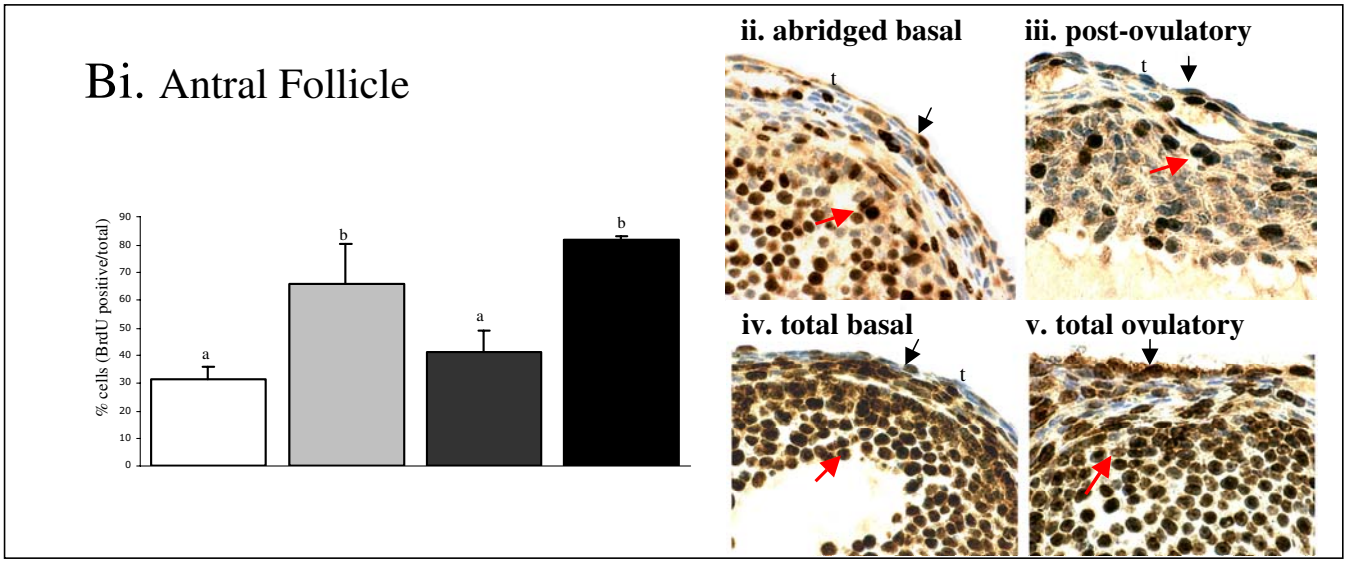
10
20
30
40
50
60
70
80
90
ii. abridged basal
iii. post-ovulatory
iv. total basal
v. total ovulatory
t


Bi. Antral Follicle
% cells (BrdU positive/total)
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b
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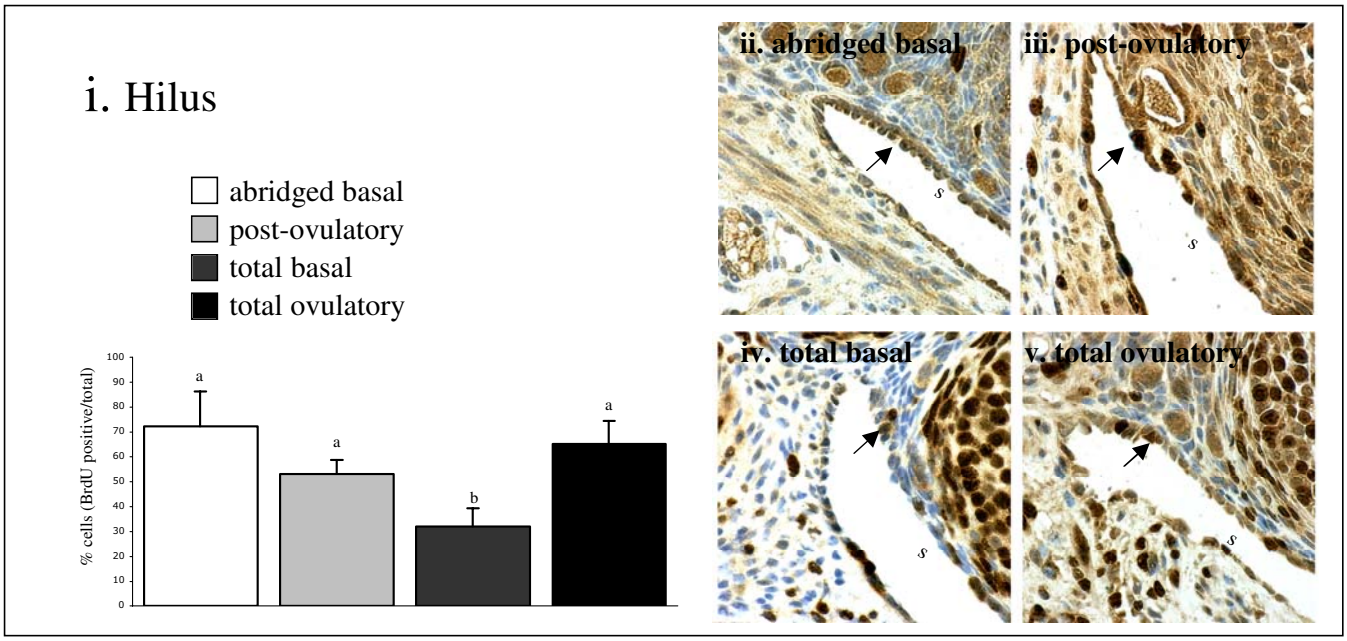
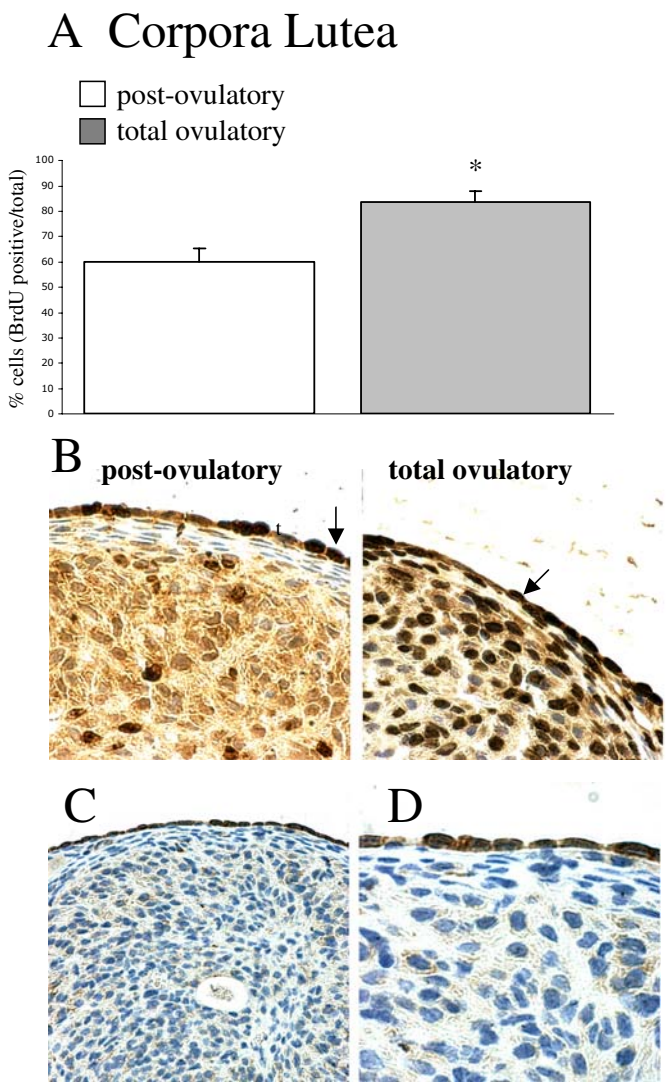
b
ii. abridged basal
iii. post-ovulatory
iv. total basal
v. total ovulatory
t

Burdette Figure 4




A Corpora Lutea
post-ovulatory
total ovulatory
% cells (BrdU positive/total)
100
90
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post-ovulatory
total ovulatory
C
D

Burdette Figure 6