Induction of proliferation in the primate ovarian surface epithelium in vivo

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BACKGROUND: Epithelial ovarian cancer (EOC) is the primary ovarian malignancy affecting women. Proposed etiologies of EOC resist direct testing due to the absence of a suitable animal model, as EOC affects only primates, not other mammals. The role of proliferation in ovarian surface epithelium (OSE) transformation has been suggested but not demonstrated, nor has OSE proliferation been widely reported. We selected the rhesus macaque as a model to evaluate the unique primate OSE in vivo, and to determine whether it can undergo proliferative repair, which may relate to EOC etiology. METHODS: Macaque ovaries were collected at three stages of the cycle. Very late luteal phase ovaries were gently brushed during laparoscopy to remove a portion of the OSE, and ovaries (≤3 per group) were collected 1–4 days later. Ovary samples were also collected from 10 women aged 33–74 years. Ovarian tissue sections were probed with OSE markers (keratin, β-catenin, E- and N-cadherin), proliferation markers [proliferating cell nuclear antigen (PCNA), phosphorylated histone H3 (phospho-H3), and phosphorylated Retinoblastoma (pRb)], or labels of collagen and basement membrane. RESULTS: Brushing partially removed the OSE; did not cause tissue damage/adhesions; elevated the frequency of PCNA, phospho-H3 and pRb in the residual OSE, marking as many as 10–50% of cells in brushed regions (unbrushed areas contained <0.1% positive cells), and; did not induce proliferation in underlying stromal cells. CONCLUSIONS: The OSE can undergo proliferative repair, and thus its normal regulation could contribute to EOC etiology.

Keywords: epithelial ovarian cancer; ovarian surface epithelium; cell proliferation; ovarian malignancy; rhesus macaque

Introduction

Basic premises of epithelial ovarian cancer (EOC) etiology have yet to be evaluated in a relevant in vivo context, in part because EOC is almost unique to women. The ovarian surface epithelium (OSE) gives rise to the vast majority of ovarian cancers in women (Wong and Auersperg, 2003), even though it comprises less than 1/1000th of the whole ovary. EOC is the most common and lethal gynecological malignancy afflicting women, and a 1.8% lifetime risk for ovarian cancer is conspicuously high considering that relatively few cells make up the OSE (Dietl and Marzusch, 1993; King et al., 2001). The early etiology of EOC is poorly understood, and risk factors have been identified primarily on the basis of clinical studies, and less on experimental approaches (Auersperg et al., 2001; Riman et al., 2004). Rigorous testing of hypotheses derived from clinical studies is limited due to the absence of suitable animal models, since EOC does not occur or is exceedingly rare in most species, and attempts to genetically engineer an animal model have only recently met with success (Vanderhyden et al., 2003; Clark-Knowles et al., 2007); consequently, it is unclear what aspects of normal ovarian function are most important to OSE transformation. This absence of information hampers clinical decisions regarding hormone-based therapies, such as infertility protocols and hormone replacement therapy (Auranen et al., 2005; Mahdavi et al., 2006), and impedes progress in EOC risk assessment, detection, prevention and treatment. It is telling that the incidence of and prognosis for EOC have changed little in the last three decades (Bhoola and Hoskins, 2006).

Pregnancy and oral contraceptive use are statistically protective against EOC (Bosetti et al., 2002; Riman et al., 2002; Purdie et al., 2003), suggesting that temporary suspension of...
ovarian function (ovulation and steroid production) may reduce risk. It is generally held that the OSE is ablated at the site of ovulation and that repeated rounds of proliferative repair are linked to transformation (Fathalla, 1971; Berchuck and Carney, 1997; Murdoch and McDonnell, 2002). Furthermore, the local ovarian environment itself poses challenges to the OSE during the normal menstrual cycle: the preovulatory follicle and its glandular successor, the corpus luteum (CL), produce high levels of estrogen and/or progesterone before and following the ovulation-triggering LH surge (Chaffin et al., 1999; Fujiwara et al., 2000), that may act directly on the OSE (Clinton and Hua, 1997; McDonnell and Murdoch, 2001; Rodriguez et al., 2002; Wright et al., 2002). In addition, the local environment at the site of ovulation is pro-inflammatory and contains DNA-damaging factors (Espey et al., 1982; Murdoch, 2005; Rae and Hillier, 2005; Fleming et al., 2006). These conditions may act in concert to promote the development and amplification of harmful mutations within the OSE.

Although a model of EOC that invokes OSE cell death and proliferation is straightforward in principle, it has not been confirmed that the OSE engages in these activities during normal ovarian function. Few studies have attempted to identify proliferation within the human OSE in vivo, and these generally report negligible levels (Werness et al., 1999; Heller et al., 2003; Piek et al., 2003; Slot et al., 2006). No studies have examined human OSE proliferation in relation to ovulation or the local ovarian environment. Likewise studies have not established a necessity for OSE proliferation in vivo nor identified the extent of ovulation-induced OSE ablation in human, although reports suggest OSE clearance at the site of ovulation in sheep (Murdoch, 1998; Murdoch et al., 1999; Murdoch and Martinchick, 2004) and some effect of ovulation and cyclicity on OSE proliferation in rodents (Tan and Fleming, 2004; Gaytan et al., 2005; Burdette et al., 2006). If the human OSE undergoes some degree of ablation and repair, its restoration may be temporally and spatially restricted during the ovulatory cycle; thus, it may be the case that researchers have yet to examine ovaries from women during phases when OSE proliferation occurs, leaving the role of proliferation equivocal.

The rhesus monkey, Macaca mulatta, has a reproductive physiology similar to human, including cycle length, hormone profiles, and ovarian structure (Stouffer et al., 1993; Hibbert et al., 1996; Wu et al., 2000). We have studied cultured OSE cells derived from human and macaque ovaries and found similarities between these cells in vitro that distinguish them from non-primate OSE cell cultures, including the expression of cadherin isoforms and receptors that mediate cell responsiveness to extracellular cues (Wright et al., 2002; Hobson et al., 2003). Some of these common features may be relevant to EOC, especially in light of the observation that among mammals only primates develop EOC (Moore et al., 2003).

Here we extend our in vitro characterization of the rhesus OSE in vivo, examine the proliferative activity of the macaque OSE associated with ovaries that have dominant structures (i.e. a preovulatory follicle or a steroidogenically active CL), and evaluate the potential to experimentally induce proliferation by the macaque OSE by partial ablation. Ovaries were collected from the follicular and luteal phases, and analysed using antibodies against cadherin isoforms, β-catenin and keratin that distinguish OSE cells from adjacent cell types. Antibodies against proliferating cell nuclear antigen (PCNA), phosphorylated histone H3 (phospho-H3) and phosphorylated Retinoblastoma (pRb) were used as indicators of proliferative activity within the OSE. Additional ovaries from the very late luteal phase with no dominant structures were gently brushed to remove a portion of the surface epithelium. This phase was selected to minimize the possible effects on the OSE of a large preovulatory follicle or an active CL. These ovaries were collected 1, 2 or 4 days later and analysed histologically and with markers for the OSE, indicators of proliferation, and an antibody against activated nuclear factor-kappa B (NFkB), to assess potential tissue trauma (Tergaonkar, 2006). These studies demonstrate similarities between the human and macaque OSE in vivo that include gene expression and a general lack of proliferative activity. In addition, the ability to induce a proliferative response in the OSE by partial ablation provides a model to investigate the mechanisms controlling OSE proliferation in vivo, which may contribute to our understanding of pathological OSE growth in ovarian cancer progression.

Materials and methods

In vivo protocols and tissue collection

Rhesus monkeys were cared for and housed by the Division of Animal Resources at the Oregon National Primate Research Center (ONPRC), and all experimental protocols were approved by the ONPRC Institutional Animal Care and Use Committee. Adult female rhesus monkeys aged 6–9 years were monitored for menstrual cyclicity. Visual inspection established the time of menses, and serum concentrations of estradiol and progesterone were assayed to accurately track entry into the luteal phase, where serum progesterone levels are typically 1–5 ng/ml (Zelinski-Wooten et al., 1998). Serum hormone concentrations were determined by specific electrochemoluminescent assay using a Roche Elecsys 2010 Analyzer in the Endocrine Services Laboratory of the ONPRC (Young and Stouffer, 2004). Ovaries were collected from (1) the follicular phase days 8–11 (n = 3 ovaries, from three monkeys), when a dominant follicle selected to ovulate was clearly visible, but prior to the LH surge, (2) the luteal phase days 5–10 (n = 3 ovaries, from three monkeys) when progesterone levels are high (determined to be 1–5 ng/ml, versus follicular phase values of <0.2 ng/ml), or (3) the very late luteal phase when a functional dominant structure was not present and serum progesterone levels were determined to be less than 0.5 ng/ml, to reduce the possible effects of dominant ovarian structures and their products on the OSE subsequent to experimental manipulation. In all instances, serum estradiol levels were verified to be 10–90 pg/ml, below peak values that occur prior to ovulation, which can exceed 800 pg/ml.

Ovary collection was performed on anesthetized animals during laparoscopy (Xu et al., 2005). In OSE ablation experiments, both ovaries from each female were brushed during laparoscopy using a cytology brush. Time 0 ovaries were collected immediately after brushing, whereas others were collected at 1, 2 or 4 days later during subsequent laparoscopies with females undergoing up to three laparoscopies. Initially, a single ovary was collected at each time point; however, since no change in PCNA expression in the OSE was observed at Day 1, additional collections focussed on
Days 2 and 4, to obtain a final number of ovaries (n) of three (Days 0 and 4), one (Day 1) or two (Day 2), collected from five monkeys. As the OSE is highly susceptible to accidental removal, ovaries were collected with extreme attention to preserving the ovarian surface. Brushing was digitally recorded for later identification of the region targeted for OSE removal.

Human ovarian samples were collected intraoperatively after having obtained signed informed consent (OHSU IRB 9921) from women undergoing primary surgery for benign gynecologic disease, initial ovarian cancer staging procedure, or risk reducing oophorectomy due to increased genetic risk of ovarian cancer. Samples were collected from 10 patients aged 33–74 years, with three premenopausal patients between Day 3 and 10 of the follicular phase.

Human and macaque tissue was immediately fixed for 24 h at 4°C in formaldehyde (38% solution, Fisher Scientific; Pittsburg, PA, USA) diluted 1:1 in phosphate-buffered saline (PBS)–Triton (0.6% Triton X-100, 1.8 mm NaH2PO4, 8.4 mm Na2HPO4 and 175 mm NaCl) and adjusted to pH 9 with NaOH. Tissue was then maintained in 10% sucrose for 24 h then transferred to Optimal Cutting Temperature compound (OCT)-filled embedding molds. Prior to freezing, ovaries were inspected grossly for evidence of tissue damage and oriented to ensure that sectioning occurred transverse to the axis of brushing. Frozen blocks were sectioned at 10 μm in the Imaging and Morphology Core Laboratory at the ONPRC using an American Optical microtome.

### Tissue analysis
Ovarian tissue was stained with hematoxylin or Masson’s Trichrome stain (IMEB Inc., San Marcos, CA, USA), to selectively label cell nuclei and connective tissues. For immunohistochemical analysis, sections were heated in distilled water to 80°C for 25 min, then blocked in PBS–Triton plus 1% calf serum, and all subsequent washing and antibody incubations were carried out in PBS–Triton. The following primary antibodies were applied overnight at 4°C, diluted 1:1000: cytokeratin (DAKO Corp.; Carpinteria, CA, USA); N-cadherin, E-cadherin and β-catenin (Transduction Laboratories; Lexington, KY, USA); phospho-Ser807/811 of Rb and phospho-Ser276 of the p56 subunit of NFKB (Cell Signalling Technology, Inc.; Danvers, MA, USA); phospho-H3 (Upstate Biotechnology; Lake Placid, NY, USA); laminin (Millipore; Billerica, MA, USA); and PCNA (Chemicon; Temecula, CA, USA). Phosphatase-conjugated secondary antibodies were used (Kirkegaard & Perry Laboratories; Gaithersburg, MD, USA), and signal was detected using a NBT/BCIP liquid substrate (NitroBlue Tetrizolium, Bromo-Chloro-Indolyl Phosphate) premixed solution (Kirkegaard & Perry Laboratories). As a negative control for nonspecific antibody labeling, secondary antibodies were applied in the absence of primary antibodies and reacted.

Unstained, stained and immunohistochemically labeled tissue sections were visualized using an inverted Olympus IMT-2 microscope with Nomarski differential interference contrast filters (Olympus; Center Valley, PA, USA). Digital images were captured with an Olympus Microfire camera connected to an Apple iMac G4 (Apple; Cupertino, CA, USA) operating PictureFrame 2.0 (Optromtics; Goleta, CA).

Three-dimensional reconstruction of the brush effects on the OSE was performed by analysing six cohorts of serial sections collected at intervals, with each cohort separated by approximately 500 μm. Sections were subdivided into 15 radial segments, each spanning approximately 1 mm along the ovarian surface. Each segment was evaluated for the amount of OSE persisting after brushing, the dominant OSE cell morphology present and the expression of PCNA or pRb within the residual OSE. Sections were aligned using internal ovarian tissue landmarks.

### Statistical analysis
Changes in the expression of markers for proliferation following partial OSE ablation were analysed by analysis of variance of log-transformed data, followed by Student’s t-test or Mann–Whitney rank sum test where appropriate. A Pearson’s Coefficient of Correlation was calculated to determine whether proliferation within the OSE correlated with the time following partial ablation. Chi-square analysis was used to determine whether OSE brushing had an effect on OSE cell morphology. Statistical software used was SigmaStat 2.0 and Microsoft Excel X. A value of P < 0.05 was considered significant.

### Results

#### Antigen detection in the primate OSE
In agreement with in vitro results, rhesus monkey and human OSE were positive for keratin, β-catenin and N-cadherin in vivo (Fig. 1A–F), and no variation in staining was evident that would suggest variable expression based on cycle phase or age of donor. In addition these antigens did not reveal a distinction between rhesus monkey and human OSE, with the exception that rhesus monkey OSE showed a weaker N-cadherin signal that did not uniformly label the OSE. In contrast to reports in vitro and in human tissue sections (Auersperg et al., 1994; Maines-Bandiera and Auersperg, 1997; Wright et al., 2002), both rhesus monkey and human OSE expressed robust levels of E-cadherin (Fig. 1G–H).

The basis for the transition from E- and N-cadherin in vivo to solely N-cadherin expression in vitro is unknown. Each of these four markers was sufficient to distinguish the primate surface epithelium from adjacent cell types, although β-catenin and keratin antibodies labeled the OSE most uniformly and were therefore used as the primary identifying markers in this study.

#### Distribution and proliferative potential of the OSE in relation to dominant ovarian structures
Archived tissue samples are available that represent ovaries at various stages of the menstrual cycle; however, they are notoriously of little value for accurately depicting the OSE, since these samples are typically missing large portions of the surface epithelium as a result of poor handling. Ovaries for this study that were collected in a manner to preserve the OSE showed it to be continuous at follicular phase days 8–11 and luteal phase days 5–10 (Fig. 2). No evidence of OSE ablation was found near, i.e. within 1 mm of, the dominant follicle selected to ovulate (Fig. 2A–B), nor was the ovarian surface in the vicinity (within 1 mm) of the active CL void of OSE cells (Fig. 2D–E). The distribution of keratin and β-catenin labeling gave no indication that the growing follicle or CL disrupted OSE cell–cell contacts or affected cell morphology, which was primarily columnar-to-cuboidal, as compared to OSE present at other areas of the ovarian surface. In addition, PCNA expression and pRb were absent or negligible in the OSE regardless of its proximity to the dominant follicle or CL (Fig. 2C and F; pRb not shown). No PCNA or pRb was detected in human OSE from follicular phase (n = 3) or postmenopausal (n = 7) samples.
Effect of brushing on the ovary and OSE removal

Ovaries collected immediately after brushing (Day 0) appeared grossly normal, with no signs of tissue trauma, scarring, bleeding, or adhesions, and no increase in NFkB activation was detected within the residual OSE or underlying tissue. Although the ovary was generally unaffected by brushing, the surface epithelium was effectively targeted for removal in regions that had been brushed (Fig. 3). The absence of OSE was evident even in unstained sections (Fig. 3A–B), and markers clearly distinguished between areas that had intact, partial, or entirely ablated OSE (Fig. 3C–D). These markers demonstrated that the cells found on the ovarian surface were OSE cells, not stromal cells that had been exposed by brush elimination of the extracellular matrix under the OSE layer, and this was supported by Masson’s Trichrome stain which revealed an intact matrix at the ovarian surface where the OSE had been brushed (Fig. 3E–F), and labeling for the basement membrane component laminin similarly showed the

Figure 1: Markers to identify the OSE are common to rhesus macaque and human
Ovarian tissue was collected from rhesus monkeys in the follicular (n = 3) and luteal (n = 3) phases, and from premenopausal follicular phase (n = 3) and postmenopausal women (n = 7). Tissue was fixed then frozen and sectioned. Tissue sections were probed with antibodies against human antigens that clearly distinguish OSE cells from neighboring cells in rhesus monkey (A, C, E, G) and human (B, D, F, H) tissue sections. Antibodies are keratin (A–B), β-catenin (C–D), N-cadherin (E–F), and E-cadherin (G–H). Secondary antibody alone (I, J) was used as a negative control, and hematoxylin staining shows OSE nuclei (K, L). Scale bar = 20 µm

Figure 2: The OSE (black arrows) is present near dominant ovarian structures but is not proliferatively active
Rhesus macaque ovaries from the follicular (A–C) or luteal (D–F) phase have a single dominant follicle selected to ovulate or a steroidogenically active corpus luteum (CL) (labeled in A and D). Keratin antibody labeled the OSE (A–B, D–E) and showed an intact epithelium in the vicinity of the dominant follicle and CL. Proliferation was not detected in the OSE as evidenced by a lack of PCNA reactivity (C, F) in the OSE. Scale bar = 100 µm (A, D) or 20 µm (B–C, E–F)
underlying tissue was largely intact (Fig. 3G–H). Ovaries collected on Days 1, 2 and 4 were indistinguishable from time 0 ovaries in regards to gross tissue quality and the absence of signs of trauma, scarring, bleeding or adhesions. Likewise, no evidence of NFkB activation or damage to the underlying matrix was seen in ovaries collected on Days 1, 2 and 4.

**Reconstruction of the effects of brushing on OSE retention, morphology and proliferation**

We examined the overall effects of brushing on the entire OSE by analysing multiple tissue sections from different regions of the ovary and subdividing them into arc segments (Fig. 4A). Individual arc segments were scored on the basis of OSE remaining (scored as 0, undetected; 1, fewer than 10 cells; 2, less than 50% intact; 3, over 50% intact; or 4, fully intact), the predominant OSE cell morphology (squamous, cuboidal or columnar), and the number of PCNA- or pRb-positive OSE cells. Brushing resulted in a clear pattern of OSE ablation, limited to one face of the ovarian surface (Fig. 4B and C). This pattern showed thoroughly brushed areas with few to no residual OSE cells, separated from fully intact OSE by transitional zones. These transitional zones contained partially ablated OSE and reached several millimeters in length, as seen in single sections and in reconstructed sequential sections; i.e. within and across sections.

The distribution of PCNA and pRb was examined in ovaries collected 1, 2 and 4 days after brushing (Fig. 4D and E, show PCNA data from two reconstructed ovaries at Days 2 and 4, respectively; pRb not shown). PCNA- and pRb-positive cells were found only in or immediately adjacent to regions where the OSE had been partially ablated and were absent in areas away from the brushed regions. In segments containing labeled cells, the number of labeled nuclei ranged from 1 to 10, with the percentage of positive nuclei ranging from 1 to 50%. The wide variation in the percentage of positive nuclei is highly dependent on the number of residual OSE cells in each given segment (with a range of 0–200). Although it is unknown whether the level of PCNA expression would have increased beyond Day 4, there is a significant ($P < 0.05$) increase in the level of PCNA or pRb detected within the OSE by Day 4, both in the number of PCNA- and pRb-positive OSE cells per tissue section analysed (Fig. 5A) and in the number of arc segments in each section that contain PCNA- or pRb-positive cells (Fig. 5B). Notably, when the analysis is restricted to comparing the unbrushed faces of ovaries, there is no significant difference in antigen frequency (not shown), indicating that the upregulation of proliferative indicators is restricted to sites of OSE ablation. Furthermore, when the total number of labeled cells in the analysed sections from each ovary are counted, a significant ($P < 0.001$) positive correlation is seen between the number of PCNA- or pRb-expressing cells and the time of OSE removal.

**Mitosis in the OSE**

To verify that the residual OSE was engaging in a true mitotic response, beyond transitioning from G1 to S phase, we labeled sections from ovaries collected 0 or 4 days after brushing with an antibody against phospho-H3, as a direct indicator of mitotic activity (Brenner et al., 2003). The OSE of Day 0 ovaries had no detectable phospho-H3, whereas Day 4 ovaries showed detectable phospho-H3 in the OSE, although in far fewer cells than were positive for PCNA or pRb (data not shown). The number of phospho-H3-positive OSE cells was low (<10% of the number of PCNA-positive cells in serially adjacent sections, with no sections containing more than three phospho-H3-positive OSE cells) and it was only detected in regions showing the greatest amount of proliferative activity.
(i.e. where PCNA was at the highest levels), suggesting this marker may not accurately reflect the scope of the proliferative response even though it most accurately indicates mitotic activity. The increase in phospho-H3-positive cells was not statistically significant (Mann–Whitney rank sum test; $P = 0.132$), but demonstrates the OSE can undergo mitosis.

Figure 4: A comprehensive analysis of OSE presence, morphology, and proliferative potential was performed using multiple sequential tissue sections from brushed ovaries.

Individual tissue sections were divided into arc segments equivalent to 24°, or about 1 mm along the ovarian surface (schematized in A). Each arc segment was scored on the basis of predominant OSE morphology (see Table I) and the amount of residual OSE after brushing. Residual OSE was scored 0–4, with 0, entirely absent; 1, fewer than 10 cells; 2, mostly absent; 3, mostly present and; 4, intact (roughly 200 cells). OSE presence is shown for six aligned sections, separated by 500 μm, from a single ovary collected 2 days after brushing (B). The identical data are graphed in a 3-D representation (C). In order to overlay the number of PCNA positive cells present in each arc segment, the graph in (C) was rotated, and the number of positive cells is included on the relevant bar in white (D). A similar analysis of an ovary collected 4 days after brushing shows elevated numbers of PCNA-positive OSE cells in the region of brushing (E).
Cell morphology in response to brushing

Normal primate OSE cell morphology can be squamous, cuboidal or columnar with the predominant form being cuboidal to columnar (Fig. 6A and B). In regions that contained an intact, unbrushed OSE the predominant morphology was columnar (53%) or cuboidal (45%), with the remainder (2%) appearing squamous. Brushing the ovarian surface caused a Significant \( P < 0.05 \) following \( \chi^2 \) analysis) shift in the distribution of OSE morphological types in brushed regions (Table I summarizes data from Day 4 ovaries). In those segments showing signs of partial ablation, few OSE cells retained a columnar morphology (Fig. 6C–F). Only 3% of brushed segments exhibited a predominantly columnar OSE; most were cuboidal (49%) or squamous (48%). The percentage of predominantly squamous segments was higher among those segments with the least amount of residual OSE. Of those segments that contained fewer than 10 residual OSE cells most (85%) were squamous.

Cell morphology of PCNA-positive cells was consistent with the predominant morphology of the nearest PCNA-negative cells (Fig. 6). Although the morphology of PCNA-positive cells was more cuboidal-to-squamous than intact OSE, this shift was not clearly dependent on the proliferative status of the OSE but was more related to the degree of OSE ablation. OSE cells that expressed PCNA were mostly cuboidal (66% of 192 counted from Day 4 sections), with fewer that were squamous (31%). Chi-square analysis shows that the morphological profile of PCNA-positive cells is distinct from the profile of OSE cells in areas with the greatest ablation or OSE, i.e. fully intact (OSE scored as 1, 2 and 4, respectively; see Fig. 4 legend). In contrast, when compared with the morphological profile of OSE cells where the OSE is mostly intact (OSE scored as three), there is no significant \( P > 0.05 \) difference in morphological profiles. Indeed when the total expected number of cells from all regions showing some ablation are pooled (Table I), there is no statistical distinction between the morphological distribution of these cells in comparison with all PCNA-positive cells \( P = 0.238 \).

These data indicate that OSE proliferation correlates more closely with brushing than with specific cell morphology.

**Discussion**

The biology of the normal primate OSE is poorly understood and the basis for its high rate of transformation evades rigorous analysis. During normal ovarian function, ovulation compromizes the OSE and putative re-growth of the OSE, in addition to other factors associated with the local ovarian environment, may contribute heavily to OSE transformation (Fathalla, 1971; Adams, 2001; Murdoch and Martinchick, 2004; Gubbay et al., 2005; Wright et al., 2005). However, prior reports and data presented here indicate the normal human OSE is almost exclusively an inert epithelium, lacking clearly detectable PCNA and pRb. In addition, we found no evidence that the macaque OSE undergoes significant proliferation or ablation in the presence or absence of dominant ovarian structures; i.e. we detected negligible levels of PCNA and pRb in normal macaque OSE. Thus, it appears widespread proliferation of the surface epithelium is not required to accommodate an expanding follicle or CL. However, it will be important to analyse additional ovaries from other stages of the menstrual cycle to assess the presence of growing epithelium at critical...
by 4 days, with a significant correlation between the time of activity until at least 2 days after brushing, and this increased surface epithelium did not express markers of proliferative repair (Gillett et al., 1994). We found that after brushing the surface epithelium of ovaries in the absence of a dominant structure, the surface epithelium did not express markers of proliferative activity until at least 2 days after brushing, and this increased by 4 days, with a significant correlation between the time of brushing and the increase in markers for proliferation (PCNA and phosphor-Rb, but not phospho-H3). The presence of these markers was restricted to regions of the ovarian surface that had been brushed, and no enhancement was seen in regions far removed from the site of brushing. These data indicate the OSE is uniformly competent to proliferate, and that a subpopulation of OSE precursor cells is unnecessary for repair. The low number of phospho-H3-positive OSE cells is consistent with the brief presence of phospho-H3 in mitotic cells and with the doubling time of cultured rhesus OSE cells; i.e. by Day 4 following OSE ablation these cells may not have reached M-phase, with only a few exceptions. As a result of partial ablation, the general morphology of the OSE shifted from cuboidal/columnar to cuboidal/squamous. This shift in morphology accompanied the increase in apparent proliferative activity, but these changes were not causally linked; rather, the shift in morphology was more closely associated with the degree of OSE ablation. It may be that the continuity of the OSE monolayer is critical for the maintenance of normal morphology, so that cell proliferation is dependent on the continuity of the epithelium directly, and cell morphology coincidentally. In this regard, OSE morphology may relate to ovulatory effects, as suggested in mouse (Burde et al., 2006; Gotfredson and Murdoch, 2007), and not proliferative or pathological potential (Gillett et al., 1991; Maines-Bandiera and Auersperg, 1997).

Unrestrained proliferation by EOC cells is a hallmark of tumor malignancy, yet the occurrence of proliferation within the normal OSE or in the early stages of OSE pathology has not been widely observed (Slot et al., 2006). Understanding the circumstances that promote or inhibit OSE growth in vivo may be invaluable in advancing our knowledge of how these processes may be perturbed in a way that places the human OSE at a uniquely high risk of transformation.

The rhesus monkey model system developed here is a viable alternative to other laboratory models, owing to critical shared times (e.g. pre- and post-ovulation) or regions (e.g. the follicle apex) of the ovary.

Here, we manipulate the primate OSE to assess its potential for growth. Although not a model for the biochemical and structural events that may affect the OSE during ovulation (Murdoch and McDonnel, 2002; Gubbay et al., 2004; Wong and Leung, 2007), partial ablation via brushing provides a direct approach to induce a proliferative response and may simulate other forms of ovarian damage that would necessitate repair (Gillett et al., 1994). We found that after brushing the surface of ovaries in the absence of a dominant structure, the surface epithelium did not express markers of proliferative activity until at least 2 days after brushing, and this increased by 4 days, with a significant correlation between the time of

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<th>OSE presence</th>
<th>Predominant cell morphology (percent of segments/median expected cell number)</th>
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<tr>
<td></td>
<td>Squamous</td>
<td>Cuboidal</td>
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<tr>
<td>1</td>
<td>85/60</td>
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<td>2</td>
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<td>3</td>
<td>24/756</td>
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<td>1, 2 and 3</td>
<td>48/1604</td>
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<td>4</td>
<td>2/460</td>
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<td>Total</td>
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Arc segments were scored on the basis of amount of residual OSE after brushing (0, entirely absent; 1, fewer than 10 cells; 2, mostly absent, <100 cells; 3, mostly present, >100 cells, and; 4, fully intact, ≥200 cells) and predominant cell morphology. Row ‘1, 2 and 3’ lists pooled data from all segments that showed any degree of ablation. On the basis of the index of OSE presence, the expected median of cell number and total expected cell number from the analysed sections (n = 12) are listed. Chi-square analysis shows a significant (P < 0.05) change in the distribution of morphological types between intact (Score 4) and all categories of partially ablated OSE (Scores 1, 2 and 3), when comparing both the predominant morphology of arc segments and the median expected cell number.

Figure 6: OSE cell morphology and PCNA expression following partial removal, Days 2 and 4
Unbrushed OSE (black arrows) appeared continuous and normal, with predominantly cuboidal (A) or columnar (B) morphology. Columnar cells frequently had papillae (arrowhead). PCNA staining of cells was absent or negligible in unbrushed OSE (A–B), in contrast to brushed OSE (C–F, white arrows). After brushing, PCNA was detected in spans of OSE that appeared mostly intact (C–D) or obviously impacted by brushing (E–F). Cell morphology of PCNA-positive cells was rarely columnar (3%); however, the shift to a squamous/cuboidal morphology was also seen in adjacent cells, reflecting the effects of physical ablation, not proliferative activity specifically. Scale bar = 20 μm
features with human that are not held in common with other species. Human and rhesus have 4 week cycle lengths, are monovulatory, exhibit similar gonadotropin regulation of ovarian function, and have oviductal rupture sites several millimeters in diameter—in contrast with rodent rupture sites which are only about 150 μm in diameter (Tan and Fleming, 2004). It is unknown what specific influence these factors may have on the OSE of differing species, nor how they relate to the absence of EOC in common laboratory and domestic animals. In addition, experiments that specifically target the OSE for physical ablation may not be feasible in rat and mouse due to the membranous bursa which surrounds the whole rodent ovary. Importantly, these data demonstrate that the primate OSE is accessible to partial elimination using a cytology brush, and that this procedure does not result in the formation of adhesions, general ovarian trauma, or destruction of the collagen matrix of the tunica albuginea that normally resides beneath the OSE. The laminin component of the basement membrane also seemed generally intact, and while this study did not examine interactions between the OSE and basement membrane, components of which mediate cellular activities such as motility and proliferation (Monniaux et al., 2006), it will be of interest to determine whether disruption of the basement membrane affects the ultimate regrowth of the OSE following ovulation or experimental ablation, a process that requires a greater time frame than examined in the current study.

The experiments here demonstrate that the primate OSE does indeed have the potential to engage in proliferative repair, and also establish a model to investigate how the primate OSE may be normally regulated. The current study examines the ability of the OSE to engage in proliferative activity following manipulation in the late luteal phase, when no dominant structures are present and the general hormonal milieu may be in its least influential state. It is important to recognize that this study is limited by examining a proliferative response at only a single phase of the cycle (the late luteal) and a similar response to brushing might not occur during other phases, or in specific regions of the ovary in relation to dominant ovarian structures. In future studies, this model may be used to manipulate the OSE at distinct phases of the menstrual cycle and during ovulation, by precisely controlling the timing of ovulation (Young et al., 2003), to determine whether local ovarian factors and events positively and/or negatively regulate OSE growth, cell cycle arrest, or survival. More broadly, this model has the potential to examine OSE cell regulation in response not only to naturally occurring cues, but also to pharmacologically interesting reagents delivered systemically or directly to the ovary by intraovarian infusion (Patton et al., 1990; Xu et al., 2005).

No definitive role for the primate OSE has been established. It is unknown whether the OSE is a necessary component of ovarian function, primarily timely ovulation or whether aberrations in the OSE contribute to ovarian defects, including polycystic ovary syndrome and infertility. Because the basis for some of these conditions, as well as EOC, may be unique to primates, further studies to develop the rhesus monkey ovarian model and OSE manipulations may provide valuable insight into future directions for the prevention and treatment of an array of conditions relating to human health and reproduction.

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