Circulating hormones and estrous stage predict cellular and stromal remodeling in murine uterus

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Abstract

The understanding of how estrogen and progesterone (P4) drive uterine remodeling in rodents has largely been based on studies involving administration of exogenous hormones, using steroid receptor-deficient mice, or relying on vaginal smears. In all these cases, the actual serum levels of 17β-estradiol (E2) and P4 are not directly measured, and the relationship between physiological levels of female sex hormones and uterine remodeling in cycling mice has not been fully explored. Here, we measured the circulating levels of E2 and P4 in cycling mice and performed correlation analysis between hormone levels and epithelial and stromal uterine parameters, irrespective of the estrous stage. In parallel, these parameters were analyzed in relation to the more conventional method of vaginal smear classification of estrous stage. We found that circulating P4 inversely correlated with uterine width, luminal epithelial proliferation, stromal apoptosis, and degradation of luminal epithelial basement membrane collagen type-IV. Circulating E2 positively correlated with uterine width, stromal cell proliferation, and collagen type-I content, while it negatively correlated with glandular epithelial proliferation, loss of collagen type-IV surrounding glandular epithelium, and apoptosis in luminal, glandular, and stromal compartments. Our findings indicate that measuring P4 or E2 levels can predict many concurrent cellular and stromal events in the mouse uterus, suggesting that in naturally cycling mice cellular responses to hormone changes are not delayed, but occur very rapidly.

Introduction

In response to systemic changes in the sex steroids, 17β-estradiol (E2) and progesterone (P4), the endometrium of the uterus undergoes extensive remodeling during each reproductive cycle. This involves a repeating synchrony of cellular proliferation, apoptosis, and differentiation, along with extracellular matrix turnover, angiogenesis, and leukocyte infiltration, and occurs in both humans and mice (Whittingham 1981, Evans et al. 1990, Rugh 1990). Unlike humans and primates, menstruation, the physical shedding of the endometrium, is not a feature of the rodent estrous cycle. Owing to the ease of manipulation and genetic tractability, the mouse uterus has been examined extensively as a model to understand human uterine tissue responses to E2 and P4 (Owens & Ashby 2002).

Circulating levels of E2 and P4 produced by the ovaries fluctuate as a result of the reproductive cycle. The murine estrous cycle generally lasts 4–5 days and can be divided into four stages called proestrus, estrus, metestrus, and diestrus. In the mouse, circulating levels of E2 peak prior to ovulation, which occurs at estrus, while P4 levels rise during metestrus and diestrus, and then decline from proestrus to estrus (Walmer et al. 1992, Fata et al. 2001).

Studies of the effects of hormones on the murine uterus generally fall into three groups: those that rely on vaginal smears, where the actual levels of P4 and estrogen are assumed; those that involve exogenously administered hormones to the atrophic uteri of ovariectomized mice with no measurement of serum concentrations; and those that employ genetically modified mice. Various combinations of these approaches have provided important findings, but they do not address how the endogenous circulating levels of E2 and P4 correlate with concurrent uterine tissue changes. To date, only a handful of studies have used a strict regimen to map out the natural circulating changes of these hormones in mice in relation to the four stages of the estrous cycle (Walmer et al. 1992, Dharma et al. 2001, Fata et al. 2001). We have previously reported how circulating...
levels of E₂ and P₄ in cycling mice correlate with mammary gland epithelial and stromal turnover in the adult virgin gland (Fata et al. 2001). Here, we investigated changes in uterine width, cellular (luminal, glandular, and stromal) proliferation and apoptosis, ECM turnover, and inflammation in relation to both circulating E₂ and P₄, as well as to estrous stage classification. Our results provide a comprehensive assessment of how both the circulating levels of E₂ and P₄ correlate with several aspects of the uterine response and provide a comparison of how the more conventional estrous stage classification would group these responses.

**Materials and Methods**

**Estrous cycle determination**

The murine estrous cycle is 4–5 days long and its four stages are termed proestrus, estrus, metestrus, and diestrus. The stage of estrous was determined by cytological evaluation of vaginal smears as we have described previously (Inderdeo et al. 1996). Food (Harlan Teklad, Madison, WI, USA) and acidified water were provided ad libitum, and a 12 h light:12 h darkness cycle was maintained automatically with lighting changes occurring at 0600 and 1800 h. Vaginal smears from mature virgin female C57BL/6 mice (12–14 weeks old) were obtained twice daily and only those mice undergoing normal estrous cycle changes were included in the study. In this group, vaginal smears were performed at 0900 and 1200 h daily, and mice undergoing a stage change into diestrus, proestrus, or metestrus within this time were killed. This regimen precisely determined the initial onset of the stage and minimized any intra-stage variation. Since mice enter the estrus stage in the early hours of the night, vaginal smears were obtained at 2100 and 0900 h, and those entering estrus overnight were killed immediately (i.e., at 0900 h). To validate the accuracy of stage determination, vaginal smears were again obtained at the time of killing. A total of 35 mice were utilized in the study. In this group, vaginal smears were obtained twice daily and only those mice undergoing normal estrous cycle changes were included in the study. Since mice enter the estrus stage in the early hours of the night, vaginal smears were obtained at 2100 and 0900 h, and those entering estrus overnight were killed immediately (i.e., at 0900 h). To validate the accuracy of stage determination, vaginal smears were again obtained at the time of killing. A total of 35 mice were utilized in the study. Data for each stage were generated from groups consisting of no fewer than seven mice. Mice were cared for in accordance with the regulations established by the Canadian Council for Animal Care under the protocols approved by the Animal Care Committee of the Ontario Cancer Institute, Toronto, Ontario, Canada.

**Hormone assays**

Approximately, 1 ml blood was removed from each mouse while killing, allowed to clot at room temperature for 30 min, then centrifuged at 6000 g for 20 min to prepare serum. Serum samples were stored at −70 °C until processed for E₂ and P₄ measurements (kindly performed by Dr S Tokmakejian, University Hospital, London, Canada) using assays from Bayer Diagnostics according to the manufacturer’s instructions (Taieb et al. 2002). P₄ was measured by a competitive chemiluminescent assay (ACS-180 analyzer; Chiron Diagnostic, Walpole, MA, USA) and E₂ was measured by a competitive enzyme immunoassay on an Immuno-1 analyzer (Bayer Diagnostics). The sensitivities of these E₂ and P₄ assays are 9.4 pg/ml and 0.63 ng/ml respectively.

**Immunohistochemistry**

One uterine horn was removed, fixed for 3 h at 4 °C in 4% paraformaldehyde and embedded in paraffin. Sections were dewaxed in two changes of xylene, rehydrated through an ethanol series, and placed into 3% hydrogen peroxide for 10 min to block endogenous peroxidase activity. Antigen retrieval was performed as follows: slides were rinsed in water and microwaved in 10 mM citrate buffer, pH 6.0 for proliferating cell nuclear antigen (PCNA), F4/80 and anti-neutrophil immunostaining, or tissue was digested for 40 min at 42 °C with pepsin (0.4% in PBS, pH 5.2, Sigma) for laminin and collagen type-IV immunostaining. Slides were then rinsed in PBS, incubated with either rabbit anti-PCNA (1:1000 dilution; Novocastra Laboratories Ltd, New Castle, UK), rabbit anti-laminin (1:500 dilution; Mono-san, Uden, Netherlands), rabbit anti-collagen type-IV (1:500 dilution, Chemicon, Temecula, CA, USA), rat anti-F4/80 (a mouse macrophage marker; Serotec, Raleigh, NC, USA), or rat anti-mouse neutrophil (Serotec) antibodies for 1 h in a humid chamber at room temperature, and then washed in PBS. To detect rat primary antibodies, biotin-conjugated donkey anti-rat immunoglobulin G (IgG; Research Diagnostics, Concord, MA, USA) was applied in the ratio of 1:500 for 30 min at room temperature. Immunohistological staining of apoptotic cells was performed using published protocols (Wijsman et al. 1993). Briefly, sections were dewaxed in two changes of xylene, rehydrated through an ethanol series, and placed into 3% hydrogen peroxide for 10 min to block endogenous peroxidase activity. After rinsing in water for 5 min, slides were treated with 0.4% pepsin (pH 5.2) at 42 °C followed by rinsing in water for 5 min. Slides were equilibrated in buffer A (50 mM Tris–HCl, 5 mM MgCl₂, 10 mM β-mercaptoethanol, 0.005% BSA fraction V, pH 7.5) for 1 h at room temperature. Klenow (1 U/ml; Sigma) and 2.5 μM of each dATP, dCTP, dGTP, and dTTP (Sigma) and incorporated dUTP, biotinylated donkey antibodies, and primary rabbit antibodies were detected using the Level 2 Ultra Streptavidin Detection System, which contains biotinylated goat anti-rabbit antibodies, according to the manufacturer’s instructions (Signet Laboratories Inc., Dedham, MA, USA). Immunostaining was visualized using fresh 3-amino-9-ethylcarbazole substrate (AEC; Sigma). Tissue was
counterstained in Mayer’s hematoxylin, rinsed in PBS, and mounted with Crystal Mount (Biomeda Corp, Foster City, CA, USA).

**Histomorphometry**

All histomorphometric analyses were performed blind on tissue sections from one uterine horn. To generate indices for PCNA, apoptotic cells, collagen type-IV degradation, and laminin degradation, counts were performed on luminal epithelium, stromal compartment, and glandular epithelium separately. PCNA and apoptotic cells were scored as either immunohistochemically positive or negative. A minimum of 150 total nuclei were scored for PCNA and apoptosis positivity for luminal epithelium, glandular epithelium, and stroma. Indices were calculated by dividing the number of positive cells by the total number of cells counted, and the result was then multiplied by 100 to derive a percentage index.

To determine the amount of collagen type-IV and laminin degradation on each section, four separate fields were quantified using a Hertz graticule. Both collagen type-IV and laminin immunopositivity were scored as being intact when intersecting with the graticule lines, whereas an absence of immunopositivity was scored as being degraded or remodeled. Percentage degradation for each field was calculated as the number of times immunopositivity was absent divided by the total number of times basement membrane crossed (positively or negatively stained) the graticule line. Indices were generated by averaging the percentage degradation from all four fields.

To measure the thickness of uterine tissue, the uterus was embedded with one edge roughly parallel to the paraffin block face and sectioned until the histology technician neared the mid-sagittal plane, at which point several serial sections were taken for histomorphometry and immunostaining. Hematoxylin- and eosin-stained sections were viewed through a Zeiss Axioplan microscope and digitally captured using a Sony 3 CCD color camera and Northern Eclipse software (EMPIX Imaging, Mississauga, Ontario, Canada). Once captured, the image was displayed on the screen at 10× magnification and four lines perpendicular to the long axis were randomly drawn per uterus (example line shown in Fig. 1B). Measurements along these lines were made for myometrium, endometrium, and total uterine thickness including lumen, and the average thickness for each parameter was calculated.

Sections immunostained for macrophages and neutrophils were assessed for the relative amounts of these cells associated with stroma, glandular epithelium, and luminal epithelium. Sections were graded by a veterinary pathologist on a scale of 0–3, with 0 being no detectable cells and 3 being the maximum number seen. These grades for individual mice were used to examine potential correlations with serum hormone levels.

**Statistical analysis**

To assess whether differences in the indices for proliferation, apoptosis, basement membrane integrity, and mRNA levels observed at specific stages of the estrous cycle were statistically significant, an ANOVA test was performed on the entire group. If the entire group was significant at $P<0.05$, then pairwise comparisons of stage-specific mean values were performed using Fisher’s least significant difference test (LSD). Stages were considered significant if they were in a group that passed the ANOVA test and significantly different from another stage as determined by the LSD test. All values were expressed as the mean ± S.E.M. To determine the degree of linear relationship between various indices, analysis was performed on all mice in the study regardless of estrous stage, and the coefficient of correlation ($r$) was calculated. The degrees of freedom for all correlation analyses were 31 (since two mice were omitted from analyses related to hormone levels due to serum sample problems).

**Results**

**Stage-specific histological features of cycling mouse uteri**

Mouse uteri were collected at discrete times within each estrous cycle stage to minimize intra-stage variation (see Materials and Methods for determination of stage). Sagittal sections through the approximate center of mouse uteri revealed distinct histological morphologies for each stage of estrous cycle (Fig. 1A–D). At estrus, there was a pronounced increase in extracellular fluid throughout the endometrium when compared with all other stages (Fig. 1B). The profile of the luminal surface at low power appeared jagged at metestrus (Fig. 1C), but was relatively smooth at all other stages. The uterine lumen was collapsed at diestrus (Fig. 1D) and at proestrus there was typically an undulating endometrium with projections of endometrial tissue into the lumen ending in a pointed tip (Fig. 1A).

**Serum E$_2$ and P$_4$ levels at each stage of the estrous cycle**

Serum E$_2$ and P$_4$ levels vary throughout the estrous cycle, as shown in Fig. 1E and F. The intra-assay variation was 10.0% for E$_2$ and 5.66% for P$_4$. E$_2$ levels are significantly higher during estrus than all other stages ($P<0.05$, ANOVA) and remain at baseline levels from metestrus to proestrus. P$_4$ levels fluctuate more than E$_2$ levels during the estrous cycle, and are significantly higher during diestrus and lower during estrus when compared with all other stages ($P<0.05$, ANOVA).
Uterine thickness as a function of estrous stage, and serum E₂ and P₄ levels

The relationship between uterine thickness and serum E₂ and P₄ levels was examined by correlation analysis. Total uterine thickness (outer myometrium to outer myometrium; line in Fig. 1B) directly correlated with E₂ levels (r = 0.40, P < 0.05; Fig. 1G), and inversely with P₄ (r = -0.56, P < 0.005; Fig. 1H). Based on the stage of the cycle (Fig. 1I), total uterine thickness at estrus was significantly greater than all other stages (P < 0.01), with metestrus being significantly greater than both proestrus and diestrus (P < 0.05). When endometrial and myometrial thicknesses were examined individually, correlation analysis yielded no correlation between either E₂ or P₄ and endometrial or myometrial thickness. However, when mice were grouped by stage, the endometrial thickness was significantly greater at estrus than at all other stages (0.71 ± 0.06 mm vs. 0.40 ± 0.04 mm, P < 0.01).

Cyclical proliferation and apoptosis in uterine luminal epithelium, glandular epithelium, and stromal cells

Using PCNA immunohistochemistry, proliferation indices were obtained for luminal epithelium, glandular epithelium, and stromal cells (Fig. 2A–F, Supplementary Fig. 1, which can be viewed online at www.reproduction-online.org/supplemental/). For luminal epithelium, no significant difference in proliferation was found when assessed as a function of estrous stage (Fig. 2B). In contrast, high proliferation significantly correlated with low P₄ (r = -0.49, P < 0.05; Fig. 2i). Although a positive trend was observed between high luminal epithelial proliferation and high E₂ (r = 0.26, data not shown), this relationship was not statistically significant. Proliferation in the glandular compartment contrasted with that in luminal epithelium, in that it was highest at proestrus and metestrus, both of which were significantly higher than proliferation at estrus and diestrus (Fig. 2D; P < 0.005). When analyzed as a function of hormone levels, glandular proliferation index negatively correlated with E₂ levels (r = -0.36, P < 0.05; Fig. 2i), while no correlation was seen with P₄ (data not shown). Finally, stromal cell proliferation was elevated only at estrus (P < 0.005). In contrast to proliferation in the epithelial compartments, stromal proliferation positively correlated with E₂ levels (r = 0.51, P < 0.005; Fig. 2ii). Conducting individual correlation analyses between hormone levels and proliferation in different cellular compartments for each stage of the estrous cycle showed no significant relationships (Supplementary Table 1, which can be viewed online at www.reproduction-online.org/supplemental/).

Apoptosis was measured by in situ DNA end labeling, and indices were obtained for luminal epithelium, glandular epithelium, and stromal cells (Fig. 3A–F, Supplementary Table 2, which can be viewed online at www.reproduction-online.org/supplemental/).
Supplementary Fig. 2, which can be viewed online at www.reproduction-online.org/supplemental/). There was significantly higher apoptosis in both luminal epithelium (P < 0.05) and stromal cells (P < 0.005) at metestrus when compared with all other stages. It should be noted that the percentage of apoptotic cells in luminal epithelium is approximately tenfold greater than the other two cell compartments. When analyzed as a function of serum E2 and P4 hormones, we found that increasing concentrations of serum E2 inversely

![Figure 2](image)

**Figure 2** Proliferation of cell types in the mouse uterus during the estrous cycle. PCNA immunopositive nuclei (arrowheads) are shown in representative sections from (A) proestrus, (C) metestrus, and (E) estrus. Percentage of PCNA-positive nuclei are shown for (B) luminal epithelium, (D) glandular epithelium, and (F) stroma. Significant differences in proliferation between stages were evident in glandular epithelium (D) and stroma (F). Significant correlations were found between proliferation in luminal epithelium and progesterone (i), glandular epithelium and 17β-estradiol (ii), and stromal cells with 17β-estradiol (iii). Examples of low and high stromal labeling can be seen in A and E respectively, and C and E show the appearance of low and high gland labeling. Scale bar = 50 μm for all. Abbreviations are the same as given in Fig. 1 (*P < 0.05).

![Figure 3](image)

**Figure 3** Apoptosis of cell types in the mouse uterus during the estrous cycle. Immunopositive apoptotic nuclei, indicated by arrowheads in luminal epithelium (A), glandular epithelium (C), and stroma (E), were detected by DNA end labeling. Images (A, C and E) are from metestrus. Apoptotic indices are shown for luminal epithelium (B), glandular epithelium (D), and stroma (F). Significantly high levels of apoptosis were seen at metestrus in both luminal epithelium (B; P < 0.05) and stroma (F; P < 0.05). Significant correlations were found between 17β-estradiol and apoptosis in luminal epithelium (i), glandular epithelium (ii), and stromal cells (iii). Examples of low and high stromal apoptosis can be seen in C and E respectively. Scale bar = 50 μm for all. Abbreviations are the same as given in Fig. 1(*P < 0.05).
correlated with apoptosis in all cell compartments (Fig. 3i–iii), while P4 levels inversely correlated only with the stromal compartment (Table 1). Again, individual correlation analyses between hormone levels and apoptosis in different cellular compartments for each estrous stage did not yield any significant relationships (Supplementary Table. 1, which can be viewed online at www.reproduction-online.org supplemental/).

**Cyclical stromal changes in murine uteri**

Changes in the stromal compartment are intimately related to those at the cellular level. Using histomorphometry, we quantified the percentage degradation of the basement membrane components collagen type-IV and laminin throughout the estrous cycle based on loss of immunopositivity. Collagen type-IV was located in the basement membranes of luminal epithelium, glandular epithelium, and blood vessels (Fig. 4A, B, D and E). At estrus, there was a pronounced loss of collagen type-IV in basement membrane of the luminal epithelium (Fig. 4B; arrowheads), when compared with all other stages (Fig. 4C; P<0.05). Although we also observed a clear loss of collagen type-IV in the glandular epithelium, there was no significant difference found between stages. We also noted a complete absence of degradation in blood vessel collagen type-IV (data not shown). Statistical analysis revealed inverse correlations between the loss of luminal collagen type-IV with P4 and the loss of glandular collagen type-IV with E2 levels (Table 1). Laminin immunopositivity was located in identical areas as that of collagen type-IV (Fig. 4G, H, J and K). Loss of laminin was maximal at estrus for both luminal and glandular epithelia, but did not significantly differ from other stages (Fig. 4I). Laminin degradation was not detectable surrounding blood vessels (data not shown). No correlation was seen between loss of laminin and hormone levels in either compartment. Collagen type-I immunopositivity was primarily found within the endometrium between stromal cells. Using computer-assisted analysis, the total amount of collagen type-I immunopositivity was calculated. We found a approximately tenfold reduction in the amount of this matrix protein within the endometrium from estrus to metestrus. At estrus, the entire endometrium contained abundant collagen type-I (Fig. 4M), whereas at metestrus, the endometrium was almost devoid of this matrix protein (Fig. 4N). The amount of collagen type-I at estrus within the stromal endometrial compartment was significantly greater than any other stage, while the amount of collagen type-I at metestrus was significantly lower than any other stage (Fig. 4O; P<0.0005). Irrespective of the estrous stage, we found that higher E2 levels directly correlated with the amount of endometrial collagen type-I (Table 1).

**Distribution of inflammatory cell populations**

Immunostaining for neutrophil and macrophage-specific markers in mouse uteri revealed a large population of these cells that fluctuated with the estrous cycle. Most notably, neutrophils were present within the luminal epithelial layer at metestrus (Fig. 5B), whereas at estrus (Fig. 5A), proestrus, and diestrus they were present only within the stroma. Macrophages also showed a stage-dependent shift in distribution. Macrophages were present in the myometrium and occasionally in the deep endometrial stroma during diestrus (Fig. 5C), metestrus, and estrus, whereas during proestrus macrophages were also located adjacent to, but not within, the luminal epithelium (Fig. 5D). Semi-quantitative analysis of neutrophil and macrophage distributions revealed no significant correlations between inflammatory cells and hormone levels (Table 1).

**Discussion**

A number of studies have shown that, in the intact mouse, the uterus is highly responsive to both estrogen and P4 (Quarmby & Korach 1984, Evans et al. 1990, Tibbetts et al. 1998). The results presented here define how the mouse uterus undergoes distinct changes in uterine width and histological appearances (Fig. 1A–D and G–I), proliferation (Fig. 2), apoptosis (Fig. 3), extracellular matrix (ECM) remodeling (Fig. 4), and leukocyte infiltration (Fig. 5) during the estrous cycle.

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**Table 1** Summary table of correlations between cyclical uterine changes and 17β-estradiol (E2), progesterone (P4), or stage of estrous cycle for the same parameters.

<table>
<thead>
<tr>
<th>Change in uterus</th>
<th>E2</th>
<th>P4</th>
<th>Estrous stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uterine width</td>
<td>+</td>
<td>–</td>
<td>+ Estrus + metestrus</td>
</tr>
<tr>
<td>Luminal proliferation</td>
<td>n.s.</td>
<td>n.s.</td>
<td>+ Proestrus + metestrus</td>
</tr>
<tr>
<td>Glandular proliferation</td>
<td>+</td>
<td>n.s.</td>
<td>+ Estrus</td>
</tr>
<tr>
<td>Stromal proliferation</td>
<td>–</td>
<td>n.s.</td>
<td>+ Metestrus</td>
</tr>
<tr>
<td>Luminal apoptosis</td>
<td>–</td>
<td>n.s.</td>
<td>+ Metestrus</td>
</tr>
<tr>
<td>Glandular apoptosis</td>
<td>–</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>Stromal apoptosis</td>
<td>–</td>
<td>–</td>
<td>+ Metestrus</td>
</tr>
<tr>
<td>Luminal collagen type-IV degradation</td>
<td>–</td>
<td>n.s.</td>
<td>+ Estrus</td>
</tr>
<tr>
<td>Glandular collagen type-IV degradation</td>
<td>–</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>Luminal laminin degradation</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>Glandular laminin degradation</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>Collagen type-I abundance</td>
<td>+</td>
<td>n.s.</td>
<td>+ Estrus – metestrus</td>
</tr>
<tr>
<td>Luminal neutrophils</td>
<td>n.s.</td>
<td>n.s.</td>
<td>+ Metestrus</td>
</tr>
<tr>
<td>Luminal macrophages</td>
<td>n.s.</td>
<td>n.s.</td>
<td>+ Proestrus</td>
</tr>
</tbody>
</table>

+, Positive correlation or significantly higher than other stages; –, inverse correlation or significantly lower than other stages; n.s., not significant. All the indicated correlations have a P<0.05.
and how these changes correlate with the natural levels of circulating hormones. Determining the stage of the mouse estrous cycle by vaginal smear analysis requires interpretation of cell types present in the smear. Although this method provides pertinent information, estrous stage determination is somewhat subjective and affected by mouse-to-mouse variations within each stage. To minimize intra-stage variations, we collected uterine tissue that represented the earliest transition from one stage to the next (see Materials and Methods section). As a complement to vaginal cytology, we also measured serum E₂ and P₄ levels for every mouse, which permitted testing of correlations irrespective of estrous stage. Together, these two approaches yielded a comprehensive analysis of natural hormone–uterine dynamics, allowing comparisons with other studies that employ the less physiologic techniques of genetic and/or hormone manipulations.

Table 1 summarizes the cyclical uterine parameters examined in this study and their correlations to serum E₂ and P₄ levels, in conjunction with significant differences between each estrous cycle stage. Serum levels of E₂ measured...
correlated with proliferation and/or apoptosis in most cell types, but did not correlate with as many extracellular matrix changes, while \( P_4 \) levels correlated with only a few parameters. \( E_2 \) is reported to induce proliferation in both luminal and glandular epithelia, while \( P_4 \) is thought to directly counter these effects (reviewed in Emons et al. 2000, Henderson & Feigelson 2000). Although we found a significant inverse correlation between \( P_4 \) levels and luminal epithelial proliferation (Fig. 2i), \( P_4 \) did not correlate with low glandular proliferation. \( P_4 \) is thought to stimulate uterine stromal cell proliferation (Martin et al. 1973b, Lydon et al. 1995), but we found instead that it correlated with low stromal cell apoptosis. The reported proliferative effects of \( E_2 \) on luminal and glandular epithelia were not found in our study. In contrast, \( E_2 \) correlated with low apoptosis in all the three cellular compartments examined (Fig. 3i–iii). The mitogenic effect of unopposed estrogen exposure on glandular epithelium is considered a major factor in the development of type I endometrial cancer in both humans (Key & Pike 1988, Emons et al. 2000, Henderson & Feigelson 2000, Ellenson & Wu 2004) and rodent models (Vollmer 2003). Our findings show that in naturally cycling mice, serum \( E_2 \) levels do not correlate with glandular proliferation, but may instead act to protect glandular endometrial cells from apoptosis.

Several parameters examined in our study showed correlations with hormone levels as well as specific estrous stages (Table 1). Uterine width is greatest at estrus, which coincides with the highest levels of \( E_2 \) and lowest levels of \( P_4 \), and these correlations are to be expected. Likewise, glandular proliferation was high at stages other than estrus and correlated with low serum \( E_2 \); stromal proliferation was greatest at estrus and correlated with high \( E_2 \); and stromal apoptosis was greatest during metestrus and correlated with both \( E_2 \) and \( P_4 \), which are relatively low at this stage. In contrast, some parameters showed correlations with either \( E_2 \) or \( P_4 \), but were not associated with a particular stage of estrous (luminal proliferation, glandular apoptosis, and glandular collagen type-IV degradation; Table 1). Likewise, we found distinct differences in the location of uterine macrophages and neutrophils as a function of estrous stage, but not as a function of \( E_2 \) or \( P_4 \) levels (Table 1). Estrous stage-associated shifts in macrophage location have previously been reported for mouse (Pollard et al. 1998, Shimada-Hiratsuka et al. 2000) and rat (Kachkache et al. 1991), and the migration of neutrophils into the luminal epithelium at metestrus has been well documented (Corbeil et al. 1998). Although \( E_2 \) and \( P_4 \) are often regarded as having pro- and anti-inflammatory effects respectively on mouse uterus (Martin et al. 1973a, 1973b, De & Wood 1990, Hunt et al. 1998, Tibbetts et al. 1998), the effects of \( E_2 \) and \( P_4 \) have never been tested in normal cycling mice. Our finding that neutrophil and macrophage migrations are not correlated with hormone levels suggests that in a physiological setting, these cells are influenced more by cyclical structural changes in the endometrium than by serum hormone levels themselves.

The changes that define the transition from one stage of estrous to the next in the mouse occur over a mere 24 h, requiring a rapid cellular response to hormonal...
fluctuations. Indeed, the first wave of DNA synthesis following exogenous E₂ administration to ovariec-tomized mice begins at 10 h post-injection and peaks at 16 h (Hewitt et al. 2003). Furthermore, it is now recognized that very rapid E₂ signaling that does not require target gene transcription can occur, leading to activation of the phosphoinositol-3-kinase pathway and stimulation of ERK and p38 MAP kinases (Kelly & Levin 2001, Warner & Gustafsson 2006). Our findings show that concurrent hormone levels alone can predict the state of many measurable uterine parameters, suggesting that in naturally cycling mice these cellular responses to hormone changes are not delayed, but occur very rapidly. A few parameters did not correlate with levels of either hormone. It is possible that these aspects of uterine remodeling have a delayed response to hormone fluctuations, such that serum levels may not reflect their concurrent state.

There is relatively little literature examining normally cycling mice and these studies rely exclusively on vaginal cytology. Most other studies on cyclical changes in the murine uterus examine the effects of exogenous hormones 7-14 days following ovariectomy, when uterine E₂ and P₄ receptor expressions are highly deregulated (Graham & Clarke 1997, Kurita et al. 2001) or used hormone receptor-deficient mice. Actual serum levels of E₂ and P₄ post-injection are rarely measured, and these rather severe manipulations may lead to unambiguous data and strong conclusions that have little basis in normal physiology. Uterine estrogen receptor α (ERα) mRNA expression has been reported to fluctuate as a function of the estrous cycle, and decrease to baseline levels 2 days after ovariectomy (Bergman et al. 1992). In complete contrast, another study (Carley et al. 2003) reported that uterine ERα expression significantly increased following ovariectomy when compared with sham-operated mice, while ERβ levels remained unchanged. In a study of mouse intestinal macrophages, the number of ERα-positive macrophages varied with the stage of estrous, and ovariectomy resulted in fewer ERα-positive macrophages (Kawano et al. 2004). Thus, ovariectomy itself can lead to a change in the expression of ERα and the subsequent response to exogenous hormone treatment, obscuring significant cyclical effects of the estrous cycle. Using vaginal cytology to stage mice, we found that most parameters examined were significantly different at one stage of the cycle from the other three (Table 1). Increased numbers of luminal epithelial cells during proestrus/estrous (combined) stages when compared with metestrus and diestrus have been reported (Evans et al. 1990), but this study did not examine proliferation directly. In our study, mean luminal proliferation was higher for estrus than the other stages (Fig. 2A), but the difference was not significant. We found that luminal cell apoptosis was very low during estrus (Fig. 3B), which could result in an overall increase in the number of luminal cells when coupled with the relatively high luminal cell proliferation at this stage. This same study (Evans et al. 1990) and another (Dharma et al. 2001) found low luminal cell numbers and high apoptosis respectively at metestrus, similar to our findings (Fig. 3B), and high stromal cell apoptosis during metestrus. Although no differences in glandular epithelial cell numbers were found by Evans et al. (1990), this is likely due to their combining proestrus and estrus stages since we found large differences in this parameter between proestrus and estrus (Fig. 3D).

Previously, we have reported on the cellular and morphological changes that occur in the mouse mammary gland as a function of the normal estrous cycle and endogenous E₂ and P₄ (Fata et al. 2001). Together, these studies reveal several major differences between mammary gland and uterus in their response to hormones and stage of estrous cycle. In the mammary gland, epithelial proliferation and apoptosis correlated with serum P₄ levels and not with E₂, whereas in the uterus these are mainly associated with E₂ (Table 1). Diestrus differed from all other stages in terms of mammary morphology, proliferation, and apoptosis, but this was not the case in the uterus for any parameter. The dramatic difference in hormone responsiveness of these two cyclically changing female reproductive tissues may have implications for therapies that alter hormone actions. Estrogen receptor-modulating drugs such as tamoxifen are used as a powerful adjuvant therapy for breast cancer treatment, but increase the risk of endometrial cancer (Jordan 2004). The weakly pro-estrogenic effect of tamoxifen in the uterus is now recognized as the reason for the paradoxical effect of anti-estrogens on endometrial cancer promotion (Pritchard 2001), and the association of E₂ with low apoptotic rate in glandular cells may be playing a role in this.

In summary, this work highlights the correlation between changing circulatory levels of estrogen and P₄ and the multiple parameters that undergo measurable changes in the murine uterus over the relatively short estrous cycle. Instead of simplifying the interplay between changing hormone levels and uterine structure, our study illustrates the complex, dynamic nature of uterine physiology in the mouse.

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