Uterine Gland Formation in Mice Is a Continuous Process, Requiring the Ovary after Puberty, But Not after Parturition¹

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ABSTRACT

Uterine gland formation occurs postnatally in an ovary- and steroid-independent manner in many species, including humans. Uterine glands secrete substances that are essential for embryo survival. Disruption of gland development during the postnatal period prevents gland formation, resulting in infertility. Interestingly, stabilization of beta-catenin (CTNNB1) in the uterine stroma causes a delay in gland formation rather than a complete absence of uterine glands. Thus, to determine if a critical postnatal window for gland development exists in mice, we tested the effects of extending the endocrine environment of pregnancy on uterine gland formation by treating neonatal mice with estradiol, progesterone, or oil for 5 days. One uterine horn was removed before puberty, and the other was collected at maturity. Some mice were also ovariectomized before puberty. The hormone-treated mice exhibited a delay in uterine gland formation. Hormone-treatment increased the abundance of uterine CTNNB1 and estrogen receptor alpha (ESR1) before puberty, indicating possible mechanisms for delayed gland formation. Despite having fewer glands, progesterone-treated mice were fertile, suggesting that a threshold number of glands is required for pregnancy. Mice that were ovariectomized before puberty did not undergo further uterine growth or gland development. Finally, to establish the role of the ovary in postpartum uterine gland regeneration, mice were either ovariectomized or given a sham surgery after parturition, and uteri were evaluated 1 wk later. We found that the ovary is not required for uterine growth or gland development following parturition. Thus, uterine gland development occurs continuously in mice and requires the ovary after puberty, but not after parturition.

endometrium, female reproductive tract, uterine gland, uterus

INTRODUCTION

Endometrial (uterine) glands are present in all mammalian uteri and develop postnatally in many species, including

rodents. Endometrial glands produce and transport secretions, including leukemia inhibitory factor (LIF) [1] and calcitonin (CALCA) [2], that are essential for embryo implantation. Therefore, the absence of uterine glands results in infertility in a number of different species [1, 3]. Thus, understanding the mechanisms regulating endometrial gland formation and development is critical for establishment and maintenance of pregnancy in the adult.

The fetal müllerian ducts give rise to the oviducts, uteri, cervix, and anterior vagina. At birth, the uterus consists of a simple epithelia surrounded by a mesenchyme. The mesenchyme then differentiates into the two myometrial layers, which surround the stroma. The luminal epithelium (LE) lines the lumen of the uterus. Within 1 wk after birth, the columnar LE forms invaginations into the stroma, which become cuboidal glandular epithelia (GE) [4, 5]. Depending upon the species, endometrial glands undergo varying degrees of coiling and branching. Within 2 wk after birth, the mouse uterus contains histoarchitecture similar to that of a mature animal; however, the number of endometrial glands continues to increase after puberty [4]. Mechanisms regulating neonatal uterine differentiation and uterine gland formation are complex and involve intricate communications between the epithelium and mesenchyme, which are mediated through the composition and distribution of the extracellular matrix, binding of growth factors and their receptors, and expression of steroid hormone receptors [6–9]. Furthermore, a number of genes are known to be involved in uterine gland formation, or adenogenesis, including *Wnt4* [10], *Wnt7a* [11], *Wnt5a* [12], *Wnt11* [13], β -catenin (*Ctnnb1*) [14–16], *Foxa2* [17], *Hoxa11* [18], and *Dicer1* [19, 20].

In many species, including rodents [21, 22] and livestock [23–25], initial endometrial gland formation (adenogenesis) and uterine growth occur in the absence of steroid hormones. Although adenogenesis occurs independently of ovarian and adrenal hormones, inappropriate exposure of the immature female reproductive tract to steroids during developmentally critical periods can result in disruption of developmental events necessary for fertility in the adult. In sheep and pigs, neonatal treatment with estradiol (E2) or progesterone (P4) disrupts normal gland formation [23, 25, 26]. Adult rats treated neonatally with estrogen do not exhibit an estrous cycle and have small ovaries with no corpora lutea and numerous atretic follicles. These mice also possess metaplastic LE, fewer uterine glands, and fibrotic stroma and myometria [27, 28]. The period of greatest sensitivity to estrogen treatment is between Postnatal Day (PD) 1 and PD5 [28]. Because postnatal uterine development is estrogen-independent, administration of P4 during neonatal development decreases epithelial proliferation but stimulates stromal

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DNA synthesis [29]. P4 administration between PD3 and PD9 altered the ability of adult uteri to form deciduomata [30]. This suggests that the critical age of sensitivity of the developing uterus to exogenous steroid hormones is between PD3 and PD10, because endogenous levels of both P4 and E2 increase on PD10 [31].

Uterine development is a dynamic process that continues during the reproductively viable period of a female's life. This is particularly the case in menstruating species that undergo endometrial regeneration with every menstrual cycle. However, regeneration of uterine layers occurs following parturition in species with invasive placentation. This postpartum endometrial regeneration, or involution, is poorly understood in most species [32–38]. In mice, regeneration of the entire endometrium must occur at the sites of implantation. At parturition, uterine glands are largely absent, particularly in the implantation sites. Histoarchitecture of the mouse uterus returns to a nonpregnant state in less than a week (Gonzalez, Huang, and Behringer, unpublished observations). Both E2 and P4 levels are reduced following parturition [39]. The role of ovarian hormones in postpartum uterine involution is unknown.

We hypothesize that endometrial gland formation and development occur over an extended time rather than only during a defined postnatal period. Thus, we have investigated uterine gland development in the presence of exogenous steroid hormones as well as in the absence of endogenous ovarian hormones during endometrial gland development. Brief neonatal exposure to E2 or P4 delay gland formation until after puberty. This delay may be regulated via disruption of uterine *Ctnnb1* and expression of estrogen receptor alpha (ESR1) by the epithelia and subepithelial stroma in prepubertal mice. Indeed, absence of *Ctnnb1* and *Esr1* appear to be permissive for adenogenesis. Overall, three periods exist in which endometrial glands can form and develop in the life span of a mouse: neonatal, postpubertal, and postpartum. The ovary is required for both uterine growth and gland development after puberty, but not after parturition. Thus, endometrial gland formation and development is a process that occurs over the life span of mice, is regulated via multiple mechanisms, and is sensitive to exogenous ovarian steroid hormones.

MATERIALS AND METHODS

Mice

All hormone treatment studies were performed using Swiss Webster outbred mice (Model: SW; Taconic). *Esr1* was deleted in mice by crossing mice containing floxed exon 3 of *Esr1* (C57BL/J background) to *Sox2-cre* mice (*Tg(Sox2-cre)IAmc/J*; The Jackson Laboratory) to create *Ex3Esr1KO* mice, as described previously [40]. All experimental animals were maintained in accordance with the Institutional Animal Care and Use Committee of the M.D. Anderson Cancer Center or National Institute of Environmental Health Sciences and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Experimental Design

Effect of steroid hormone administration on uterine histology. To determine the effect of extending the endocrine environment of pregnancy on uterine gland formation, neonatal mice were treated with steroid hormones (Fig. 1A). Pups were randomized at birth to minimize differences between litters. Beginning at PD0 (birth), female pups were weighed and administered daily subcutaneous injections of E2 (8 µg/kg body wt; E8875; Sigma-Aldrich), P4 (50 µg/g body wt; P0130; Sigma-Aldrich), or oil for 5 or 10 days. Dosage of hormones was selected based upon previous reports [40–42]. To determine uterine histology and abundance of endometrial glands before puberty, female mice were hemihysterectomized at 3 wk of age (weaning). Five weeks later (at 8 wk of age), the remaining uterine horn was collected (Fig. 1B). This allowed uterine histology and endometrial gland abundance to be determined within the same animal. For all experiments in the present study, body weight was

determined, and uterine horn length was measured from the uterotubal junction to the uterine bifurcation at collection time. Uteri were dissected and fixed in 4% paraformaldehyde in PBS for 24 h. Due to the histological similarities between the 5- and 10-day hormone-treated tissues, 5 days of treatment were considered to be sufficient to induce a phenotype and were used for all remaining experiments.

To determine the short-term effects of steroid administration on the uterus, a similar experiment was performed. Beginning at PD0 (birth), female pups were weighed and administered daily subcutaneous injections of E2 (8 µg/kg body wt), P4 (50 µg/g body wt), or oil for 5 days. On P10, reproductive tracts were dissected from these mice.

*Role of *Esr1* during uterine gland development.* The role of *Esr1* during uterine gland development is largely unknown. *Ex3Esr1KO* mice were collected at PD10, after the onset of adenogenesis. Uteri were dissected and fixed in 4% paraformaldehyde in PBS for 24 h and stored in 70% ethanol for histological evaluation.

Effect of ovariectomy and steroid hormone administration on uterine histology. This experiment was designed to determine if the ovary is necessary for uterine gland formation. The previous experiment (Fig. 1A) established that 5 days of treatment with steroid hormones were sufficient to alter uterine histology and decrease endometrial gland abundance. Thus, postnatal female pups were weighed and administered daily subcutaneous injections of E2 (8 µg/kg body wt), P4 (50 µg/g body wt), or oil for 5 days. At weaning, female mice were hemihysterectomized, and both ovaries were removed. The remaining uterine horn was collected at 8 wk (Fig. 1B).

Effect of steroid hormone administration on fertility. This experiment was performed to determine the fertility of mice exposed postnatally to steroid hormones. Both E2 and P4 treatments resulted in fewer endometrial glands, but the effect of a decreased number of glands on fertility is unknown. Postnatal female pups were weighed and administered daily subcutaneous injections of E2 (8 µg/kg body wt), P4 (50 µg/g body wt), or oil for 5 days. These mice were not subjected to any surgical manipulation. At 8 wk, females were placed in cages with Swiss Webster males in a 1:1 ratio and monitored for presence and frequency of litters and litter size. After 12 wk with the male, mice were separated, and females were collected 2 wk after parturition.

Determination of whether the ovary is necessary for postpartum uterine regeneration. This experiment was designed to establish the involvement of the ovary during postpartum uterine gland formation. During pregnancy and parturition in mice, endometrial glands are absent in the implantation sites and regenerate rapidly for maintenance of subsequent pregnancies. Mature Swiss Webster females were bred to Swiss Webster males to establish pregnancy. On the day of parturition (Postpartum Day [PPD] 0.5), adult females were ovariectomized or received a sham surgery (*n* = 4 per group). On PPD8, female reproductive tracts were collected and evaluated for histomorphology and presence of endometrial glands. Uterine horn length was measured from the uterotubal junction to the uterine bifurcation following fixation.

Histology and Morphometry

Sections of paraffin-embedded tissues (section thickness, 5 µm) were stained with hematoxylin and eosin. To determine uterine gland abundance, endometrial gland cross-sections were counted in sections (five slides for each animal with six sections per slide) of the uterus from the same mice at 3 and 8 wk of age from each treatment group (*n* = 4–9 in each group) in a blind experiment. Uterine gland cross-sections per uterine horn were calculated by determining the number of cross-sections in 1 µm and then multiplying by uterine horn length. Images of uterine cross-sections (*n* = 3 per mouse) at 3 and 8 wk were taken using a Nikon Eclipse 80i Upright microscope and NIS Elements AR 3.1 imaging software (Nikon). These images were used to determine uterine area with Image J v1.41 software (<http://rsbweb.nih.gov/ij/index.html>). Uterine area was calculated with Image J software via an outline of the various uterine regions, including myometrium, endometrium, and uterine lumen. The area of the uterine lumen was subtracted from uterine and endometrial areas for accuracy.

Immunofluorescence

The following primary antibodies were used for immunofluorescence: phospho-histone-H3 (H3S10ph; 1:250; clone 3H10; Millipore), CTNNB1 (1:50; Santa Cruz Biotechnology, Inc.), smooth muscle alpha-actin (ACTA; 1:600; A2547; Sigma-Aldrich), and ESR1 (1:50; M7047; DAKO). Immunofluorescence was performed using paraffin-embedded uterine tissue sections (thickness, 5 µm). Briefly, slides were deparaffinized and rehydrated to PBS. Antigen unmasking was performed with boiling 0.05 M Tris buffer (pH 9.0) for 20 min. Nonspecific binding was blocked with 10% normal goat serum in PBS and 0.05% Tween-20 (PBST) for 30 min at

FIG. 1. Uterine gland development is delayed when the endocrine environment of pregnancy is extended. The experimental design to determine the effects of neonatal hormone treatment on postnatal and post-pubertal uterine gland development is shown (A), as is the histological comparison of mice at 3 and 8 wk of age that were treated neonatally with oil, E2, or P4 (B–G). Tissues were obtained from the same animal at different time points (i.e., B and E). Gland numbers were obtained from uterine cross-sections at 3 and 8 wk (H). Arrowheads indicate gland development in E2- or P4-treated mice at 8 wk. An asterisk (*) indicates significantly fewer endometrial glands in hormone-treated mice compared to age-matched, oil-treated mice ($P < 0.0001$). Data are shown as the mean $\pm$ SEM. myo, myometrium; St, stroma. Bar = 100 μ m.

room temperature. Slides were then incubated with the primary antibodies in 10% goat serum in PBST overnight at 4°C, washed, and incubated with Alexa Fluor-conjugated secondary antibodies (1:600; Molecular Probes) for 45 min. Slides were counterstained with Vectashield mounting media with 4',6-diamidino-2-phenylindole (Vector Laboratories Inc.) and cover-slipped.

Statistical Analyses

Least-squares ANOVA using the general linear models procedures of the Statistical Analysis System (SAS Institute, Inc.) was used to analyze the iterations of data in the uterine gland counts and area measurements. Gland counts and area measurements were analyzed for effect of time and hormone treatment group as determined using the LSMEANS/PDIFF option of the Statistical Analysis System. A Student *t*-test was used to compare gross morphology, including body weight and uterine horn length. Two-tailed, unpaired *t*-tests were performed on the data from the breeding trial. The SEM was used to compare error between experiments. For statistical analyses, $\alpha = 0.05$ was used to determine significance.

RESULTS

Effect of Hormone Treatment on Adenogenesis and Uterine Histology

To determine the effect of neonatal hormone treatment on uterine gland formation, mice were treated with oil, E2, or P4 for 5 days after birth. At 3 and 8 wk of age, endometrial glands were abundant in the uteri of oil-treated mice. In contrast, treatment of neonatal pups with either E2 or P4 substantially reduced the number of endometrial glands (Fig. 1). At 3 wk, uterine glands were largely absent from the endometrium of hormone-treated mice. However, these same mice at 8 wk had uterine glands present, but in less abundance. Quantification of endometrial glands per uterine cross-section revealed fewer glands in both E2- and P4-treated mice compared to oil-treated mice at both 3 and 8 wk of age ($P < 0.0001$). Within each

FIG. 2. Hormone treatment alters epithelial differentiation and cell proliferation as early as PD10. Histological analysis (A–C) and immunofluorescence localization of H3S10ph (D–F) and ACTA (G–I) in the uteri of oil-, E2-, and P4-treated mice at PD10 are shown. White arrows (E) indicate proliferating cells; asterisks (H, I) indicate disorganization of E2 smooth muscle layers and ACTA within the P4 endometrium. IC, inner circular myometrium; myo, myometrium; OL, outer longitudinal myometrium; St, stroma. Bar = 100 μ m. J) H3S10ph-positive cells within the epithelial layer were quantitated; * $P < 0.0001$.

treatment group, uterine gland cross-section number increased between 3 and 8 wk of age ($P < 0.0001$). The absence of endometrial glands at 3 wk and their presence at 8 wk in hormone-treated mice suggests that uterine gland genesis can occur after 3 wk of age.

In addition to the reduced number of uterine glands, the hormone-treated mice exhibited differences in uterine morphology. The myometrial-endometrial border on the mesometrial side of the uterus of P4-treated mice was indistinct. As early as PD10, ACTA can be detected within the stroma specifically in this region (Fig. 2I). However, no morphological or proliferative differences were detected in epithelial, stromal, or other myometrial cells from P4-treated mice. In contrast, a number of morphological differences, including myometrial disorganization (Fig. 2H), hyperplastic GE (Fig. 1F), and increased density of LE cells (Fig. 1C), were found in E2-treated mice. Treatment with E2 also increased epithelial proliferation at PD10 ($P < 0.0001$; Fig. 2J). This corroborates previous reports of postnatal treatment with E2 [27, 28]. Analysis of ovarian morphology at 8 wk revealed small ovaries

with no corpora lutea in the E2-treated mice (data not shown), as reported previously [27]. Ovaries from P4-treated mice were similar to those from oil-treated mice and contained numerous follicles and corpora lutea (data not shown). Overall, P4 treatment resulted in fewer changes in uterine and ovarian morphology than E2 treatment.

Mechanisms Regulating the Delay in Gland Formation

Mice with *Ctnnb1* stabilized in the postnatal uterus have fewer endometrial glands than control mice [15], whereas mice with *Ctnnb1* stabilized in the uterine mesenchyme-derived tissues exhibit a delay in endometrial gland development similar to that found in the present study (Stewart and Behringer, unpublished observations). Thus, CTNNB1 expression was evaluated in the present study (Fig. 3). CTNNB1 was expressed by the cell membrane of epithelial cells, but not the stromal cells, of oil-treated mice at 3 wk (Fig. 3A). Abundant CTNNB1 was localized to the nucleus and cytoplasm of epithelial and stromal cells of the hormone-treated mice (Fig. 3,

FIG. 3. CTNNB1 and ESR1 are more abundant in the endometrium of hormone-treated mice. Immunofluorescence localization of CTNNB1 and ESR1 in the uteri of oil-treated (A, D, G, and J), E2-treated (B, E, H, and K), and P4-treated (C, F, I, and L) mice at 3 and 8 wk are shown. Gland development is normal in *Ex3Esr1*KO mice at PD10 (N) compared with wild-type (WT) controls (M). White arrows indicate CTNNB1 in the shallow stroma (B and C) and ESR1 in the LE and shallow stroma (H and I) at 3 wk. No differences were observed at 8 wk. myo, myometrium; St, stroma. Bar = 50 μ m (A–L) and 100 μ m (M and N).

B and C). The antibody used in these experiments detects both cytoplasmic and nuclear localization of CTNNB1. This up-regulation of CTNNB1 in the stroma may contribute to the delay in gland formation. At 8 wk of age, no differences in CTNNB1 expression were detectable between the treatment groups (Fig. 3, D–F).

Expression of *Esr1* is regulated by steroid hormones and has been linked with uterine gland development in other species [26]. In the present study, ESR1 was abundantly expressed in the LE and shallow stroma of the hormone-treated mice, with little to no expression in the oil-treated mice (Fig. 3, G–I). No differences in *Esr1* expression were detectable between the treatment groups at 8 wk (Fig. 3, J–L). Indeed, mice lacking *Esr1* (*Ex3Esr1*KO mice) exhibit normal gland development compared with wild-type mice at PD10 (Fig. 3, M and N). This suggests that an absence of *Esr1* and *Ctnnb1* is permissive for endometrial gland development.

Effects on Fertility

Endometrial glands and their secretions, such as LIF and CALCA, are necessary for implantation and pregnancy [1, 2]. Thus, a breeding trial was performed with adult, intact females treated postnatally with oil, P4, and E2. Mice were observed daily for the presence of vaginal plugs at the onset of the breeding trial. Vaginal plugs were not detected in any of the E2-treated mice, but were observed in all oil- and P4-treated mice, in the breeding trial. At 8 wk of age, mice treated with E2 ($n = 6$) exhibited edema of the external genitalia, which was either mild or severe (data not shown). The females with severe edema ($n = 3/6$) were removed from the breeding trial after 4 wk. For all other mice, the breeding trial lasted for 12 wk. No differences in fertility were found between oil- and P4-treated mice (Table 1). This is consistent with the fact that postnatal P4 treatment of ewe lambs did not alter the hypothalamic-

TABLE 1. Reduced glands in progesterone (P4)-treated mice do not affect fertility.

Treatment	No. of females	No. of pups	No. of litters	Average no. of pups per litter	Average no. of litters per female
Oil	8	266	20	13.3 $\pm$ 0.36	2.5 $\pm$ 0.19
E2	6	0	0	0	0
P4	9	257	22	11.68 $\pm$ 0.69	2.44 $\pm$ 0.18

pituitary-gonadal axis in sheep [43]. The E2-treated females were infertile, as described previously [27].

Following parturition, the endometrium, including the uterine glands, regenerates. Therefore, after the end of the breeding trial, uterine glands per cross-section were quantified (Fig. 4D). Both E2- and P4-treated mice had fewer endometrial glands than oil-treated mice ($P < 0.001$; Fig. 4, A–C). The uterine glands from the oil- and P4-treated mice appeared to be similar, whereas the glands from the E2-treated mice were hyperplastic (Fig. 4B). No differences in uterine gland number were found between the E2- and P4-treated mice ($P = 0.12$), despite the fact that the E2-treated mice did not undergo endometrial regeneration like the oil- and P4-treated mice. However, the P4-treated mice had normal fertility, suggesting a threshold number of endometrial glands is sufficient to support pregnancy.

Effects of Ovariectomy on Uterine Growth and Gland Development

Endometrial gland development occurs both postnatally and postpubertally, as shown above. To determine whether the postpubertal period of gland development requires the ovary, as well as to establish any beneficial or detrimental effects the postnatal hormone treatment might have, mice were ovariectomized at puberty (Fig. 5A). At 3 wk of age, oil-treated uteri exhibited numerous endometrial glands, whereas both E2- and P4-treated uteri contained few to no glands ($P < 0.0001$; Fig. 5, B–D). The oil-treated mice from this experiment exhibited a greater number of uterine glands per cross-section at 3 wk of age than those in the previous experiment, which were hemihysterectomized (14.2 vs. 9.3, $P < 0.001$; Figs. 1H and 5H). At 8 wk of age, following ovariectomy, uteri appeared to

be histologically identical in size and stage of development to those at 3 wk of age, suggesting that growth retardation had occurred (Fig. 5, E–G). Uterine growth, as measured by uterine horn length (Supplemental Fig. S1J, all Supplemental Data are available online at www.biolreprod.org) and uterine area (Supplemental Table S1) were reduced in all mice in the present study.

Endometrial glands were quantified at both 3 and 8 wk of age within the same animals. Compared to 3 wk, fewer endometrial glands were observed at 8 wk in the oil-treated mice ($P < 0.0001$), no difference was found in the E2-treated mice ($P = 0.47$), and more glands were noted in the P4-treated mice ($P < 0.0001$; Fig. 5H). The decrease in the number of endometrial glands in oil-treated mice after puberty suggests that postpubertal endometrial gland development is dependent upon the ovary. Postnatal hormone treatment appeared to have a protective effect on postpubertal endometrial gland development, because no decrease was found in the number of glands at 8 wk in these two treatment groups, as was seen in the oil-treated mice. Despite having more uterine glands at 8 wk than at 3 wk of age, the P4-treated mice continued to have fewer glands overall than the oil group at both 3 and 8 wk of age (Fig. 5H). In all groups, very little gland development occurred in the absence of the ovary, indicating its importance for adenogenesis during this period.

Requirement of the Ovary for Postpartum Uterine Regeneration

Uterine gland development has been studied in a number of different species, but little is known about uterine regeneration following parturition. Following embryo implantation in mice, the uterine LE and GE are ablated, and the stroma undergoes

FIG. 4. P4-treated mice were fertile despite having fewer glands. Uterine histology of mice following the breeding trial (A–C) is shown, as are uterine gland numbers (D) from mice treated with oil, E2, or P4 neonatally and placed with males for 12 wk at maturity. Data are shown as the mean $\pm$ SEM. St, stroma. Bar = 100 μ m. * $P < 0.001$.

FIG. 5. Uterine growth and gland development require ovarian hormones after puberty. The experimental design to determine the role of the ovary and steroid hormones on postpubertal gland development (A) is shown, as is the histological comparison of mice at 3 and 8 wk of age that were treated neonatally with oil, E2, or P4 and ovariectomized at 3 wk (B–G). Tissues were obtained from the same animal at different time points (i.e., B and E). Arrows indicate uterine glands. Uterine gland numbers were quantified (H), and an asterisk (*) indicates significant differences in gland number within the same treatment groups ($P < 0.001$). Data are shown as the mean $\pm$ SEM. myo, myometrium; St, stroma. Bar = 200 μ m.

decidualization at the implantation sites. After parturition, the uterus must undergo rapid regeneration to prepare for a subsequent pregnancy. To determine whether postpartum endometrial gland development requires the ovary, dams were ovariectomized at PPD0.5 following parturition, and the uteri were evaluated 8 days later (Fig. 6A). Uterine histology was not altered in the ovariectomized mice (Fig. 6B), and uterine horn length was similar in all mice ($P = 0.6$) (Fig. 6C). Furthermore, the number of endometrial glands was decreased in the ovariectomized mice compared to mice receiving a sham surgery ($P < 0.001$) (Fig. 6D), but uterine glands had regenerated within the endometrium. This indicates that postpartum uterine regeneration occurs independently of the ovary and ovarian hormones.

DISCUSSION

Neonatal Hormone Treatment Delays Uterine Gland Development

Treatment of neonatal mice with either E2 or P4 for 5 or 10 days initially blocks uterine gland development before puberty, resulting in an absence of glands at 3 wk of age. However, at 8 wk of age, the same mice have endometrial glands. This suggests that different mechanisms regulate endometrial gland development before and after puberty. This is likely due to the role of the ovary and ovarian hormones in this process. Postnatal uterine gland formation occurs independently of ovarian and/or adrenal hormones [21, 22]. The onset of uterine gland formation occurs around PD6, and the ovary begins producing detectable levels of steroid hormones on PD10 [31].

FIG. 6. Uterine growth and gland regeneration occur independently of ovarian hormones following parturition. The experimental design (A) and histological comparison (B) of dams subjected to either sham surgery (Sham) or ovariectomy (Ovx) at PPD0.5 and collected at PPD8 are shown. Uterine horn length (C) and gland numbers (D) were quantified. No difference was found in uterine horn length ($P = 0.6$), but fewer glands were found in Ovx uteri. Data are shown as the mean $\pm$ SEM. St, stroma. Bar = 100 μ m. * $P < 0.001$.

After puberty, levels of ovarian hormones are higher and are regulated via the hypothalamic-pituitary-gonadal axis. This change in ovarian hormone status appears to alter the mechanisms regulating gland development, even though both permit gland formation.

Postnatal P4 treatment appears to directly affect uterine development without altering the hypothalamic-pituitary-gonadal axis, because ovarian morphology was normal, vaginal plugs were observed following mating to males, and pregnancy could be established and maintained. In contrast, E2 treatment does affect the hypothalamic-pituitary-gonadal axis, because ovarian morphology was altered, no vaginal plugs were detected, and mice were infertile. Thus, the delay in endometrial gland formation in E2-treated mice may be due to the ovarian phenotype rather than directly due to the E2 treatment. This does not appear to be the case with the P4-treated mice, as shown previously with sheep treated postnatally using a synthetic progestin [43].

Previous studies in livestock species have shown that endometrial glands can only form during a specific postnatal period. If gland development is prevented by postnatal treatment with exogenous steroid hormones, these animals will never form glands [43]. In mice, this is not the case. Mice can begin to develop endometrial glands during the postnatal period as well as after puberty. In fact, the number of endometrial glands continues to increase in nulliparous mice with age (Stewart, Gonzalez, and Behringer, unpublished observations). Furthermore, endometrial regeneration following parturition involves uterine gland development as well. Overall, this suggests that endometrial gland development is a dynamic process that can initiate and continue over the life span of the animal. Three predominant periods of gland development occur: neonatal, postpubertal, and postpartum. The postpubertal period encompasses the entire nonpregnant

life span of the animal, and the postpartum period reoccurs with each subsequent pregnancy.

Mechanisms Regulating the Delay in Uterine Gland Formation

The WNT signaling is essential for normal uterine development and uterine gland formation. A number of *Wnt* genes are expressed in the uterus, including *Wnt4*, *Wnt5a*, *Wnt7a*, *Wnt7b*, *Wnt11*, and *Wnt16* [13]. Deletion of *Wnt5a* and *Wnt7a* prevent gland formation from occurring [11, 12]. Canonical WNT signaling is mediated via *Ctnnb1*, which plays a critical role in formation and function of the female reproductive tract [15, 44, 45]. Endometrial gland development occurs normally in mice with conditional deletion of *Ctnnb1* in müllerian duct mesenchyme-derived tissues (i.e., uterine stroma and myometria), albeit in reduced numbers [14]. However, conditional deletion of *Ctnnb1* postnatally in both the epithelium and stroma prevented uterine gland formation [15]. This suggests that epithelial *Ctnnb1* is required for postnatal gland formation, but that stromal *Ctnnb1* is not. Similarly, low levels of CTNNB1 are expressed in the uterus of oil-treated mice, and an increase in abundance of CTNNB1 in the stroma is found in hormone-treated mice, corresponding with a delay in gland formation. Conditional stabilization of *Ctnnb1* in the müllerian duct mesenchyme delayed gland development (Stewart and Behringer, unpublished observations), similar to the observations in the present study. However, conditional stabilization of *Ctnnb1* postnatally in both the epithelium and mesenchyme caused endometrial gland hyperplasia [15]. This suggests that mesenchymal CTNNB1 influences epithelial gland formation via a paracrine factor. The increased abundance of CTNNB1 is only present before puberty and coincides with the absence of uterine glands in

the hormone-treated mice. After puberty, CTNNB1 levels return to normal, and gland development begins. These results, combined with those of the present study, suggest that an increase in stromal *Ctnnb1* is associated with a delay in gland formation, but that an increase in epithelial *Ctnnb1* stimulates gland formation.

Expression and localization of steroid hormone receptors are involved in uterine morphogenesis. The hormonal sensitivity of the mesenchyme-derived tissues (i.e., stroma and myometria) is more significant than that of the epithelia during reproductive tract development [46]. That is, response to a hormone may not require the presence of receptors within the responding epithelial cell if they are associated with hormonally sensitive mesenchymal cells. The communication between mesenchymal cells and epithelial cells is mediated via growth factors and their receptors [46, 47]. This was recently demonstrated by the use of uterine epithelial-specific *Esr1* ablation [48]. Previous reports have described ESR1 expression in uterine stromal cells at birth and also in the epithelia by PD4. Developing endometrial glands highly express *Esr1* on PD7 to PD8 [49]. The present study found low to no expression of *Esr1* in oil-treated mice, but abundant *Esr1* in the epithelial and subepithelial stroma of both E2- and P4-treated mice, at PD10. This corresponds with previous reports, in which postnatal treatment with estrogenic compounds increased ESR1 in the endometrium [7]. Levels of ESR1 return to normal in the hormone-treated mice by 8 wk of age, coinciding with the onset of gland development.

The specific role of *Esr1* in the postnatal, developing uterus is largely unknown. An effective animal model available to study the role of *Esr1* in endometrial gland development is the *Ex3Esr1*KO mouse. These mice exhibit a normal onset of postnatal uterine gland development at PD10. In combination with the increased abundance of ESR1 in the hormone-treated mice, this suggests that little to no expression of *Esr1* in the uterine stroma and LE is essential for uterine gland formation. In fact, we hypothesize that conditionally overexpressing *Esr1* in the perinatal uterine mesenchyme and/or epithelia would delay or prevent gland formation. So why is *Esr1* expressed in the developing uterus at all? Before PD10, low to no circulating ovarian hormones are found [31]. This suggests that ESR1, first in the stroma and the LE and developing GE, may be activated by growth factors binding to their receptors. Even low levels of ESR1 expression make the developing uterus extremely sensitive to endocrine disruption from steroid hormones, allowing estrogenic compounds to directly target the uterus rather than working at the level of the hypothalamus or pituitary.

In sheep, postnatal treatment of ewe lambs with a synthetic progestin prevents uterine gland formation, resulting in a uterine gland knockout phenotype [25, 50, 51]. Ablation of gland formation in sheep involves a down-regulation of *Esr1* in the uterine LE [52]. Treatment of ewe lambs with E2 also prevents gland formation and causes a decrease in stromal ESR1 [53]. These findings are different from observations in mice, in which an increase in epithelial and stromal ESR1 is observed. Female pigs, or gilts, treated postnatally with E2 have altered endometrial gland development as well as increased endometrial ESR1 expression [23, 54]. Furthermore, administration of an antiestrogen to neonatal gilts from birth inhibits uterine gland formation [26]. High levels of ESR1 expression are detected in the emerging uterine glands of the developing rodent, ovine, and porcine uterus [26, 49, 55]. Overall, endometrial ESR1 expression appears to be a key regulator of endometrial gland formation in a number of species, albeit using different mechanisms.

Fewer Uterine Glands Do Not Prevent Fertility

Uterine glands produce and transport secretions that are essential for peri-implantation embryo development. As mentioned previously, a number of genes are known to be involved in uterine gland formation. If these genes are knocked out, the mice are infertile [11, 15, 17–20]. Furthermore, if uterine gland formation is compromised in other species via endocrine disruption, animals are also infertile [3]. The hormone-treated mice in the present study exhibit a delay in uterine gland formation. The glands do not form during the postnatal period; instead, they form during the postpubertal period, resulting in fewer glands overall in mature animals. This reduction in uterine glands does not result in infertility, or even subfertility, in P4-treated mice. The infertility in E2-treated mice is due to the ovarian phenotype rather than the decrease in uterine glands [27]. Thus, a threshold number of endometrial glands is required for embryo survival, implantation, and decidualization. This is further illustrated by the restoration of the ability to undergo decidualization in *Foxa2*-null females, which have reduced endometrial glands, by the intrauterine administration of LIF [17]. Only a minimal amount of LIF and other uterine secretions is necessary for pregnancy. Large numbers of endometrial glands may be less critical after implantation in mice due to the short peri-implantation period and the invasive implantation in this species. Endometrial secretions are more essential in species with extended peri-implantation periods, such as sheep, pigs, and cows. In fact, uterine gland secretions may be an important supplement to hematotrophic nutrition during mid to late pregnancy in domestic livestock [56–58] as well as humans [59, 60]. In humans, endometrial glands likely support a primary role in conceptus nutrition during the first trimester of pregnancy, because the apposition of maternal and fetal tissues is being established [59, 60]. This would suggest that having fewer endometrial glands may result in more severe effects on fertility in humans.

Following parturition, the uterine endometrium undergoes regeneration, which includes formation of glands. P4-treated mice still have fewer glands than oil-treated mice after parturition (~20 wk of age), but more than P4-treated mice at 8 wk of age. This is significant, because the endometrium underwent regeneration two to three times in the P4-treated mice during this period, suggesting that the hormonal effect of the postnatal P4 treatment is reduced during uterine involution. Another possibility is that the mechanisms regulating uterine gland formation during postpartum regeneration differ from postnatal uterine development.

Ovarian Requirement for Uterine Growth and Gland Development

Rats ovariectomized and/or adrenalectomized on PD6 underwent normal postnatal uterine gland formation, but they also exhibited reduced uterine growth [22]. The postnatal increase in both P4 and E2 is required for uterine growth to occur. In the present study, mice that were ovariectomized at 3 wk of age, before reaching puberty, did not undergo uterine gland development or normal uterine growth. In contrast, both uterine gland development and uterine growth following parturition do not involve ovarian hormones. This further illustrates the different mechanisms regulating uterine gland development during the separate periods of adenogenesis.

During the proliferative phase of the menstrual cycle in humans and nonhuman primates, low circulating levels of P4 and E2 occur, and *Esr1* is expressed by both the epithelia and

stroma [61, 62]. These are the conditions in which the endometrial glands regenerate in humans following endometrial sloughing during menstruation. This period of gland development requires ovarian E2 for both endometrial gland development as well as uterine growth, making it similar to the postpubertal period of gland development in mice. Understanding the cellular and molecular mechanisms regulating postpubertal gland development in mice may lead to greater understanding of this process in humans.

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