



Role of estrogen and progesterone receptors in neonatal uterine cell proliferation in the mouse

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Abstract

The major endocrine regulators of the female reproductive tract are 17 β -estradiol (E2) and progesterone (P4). This review discusses our recent work related to the roles of E2 and P4 and their receptors, estrogen receptor 1 (ESR1) and progesterone receptor (PR), respectively, in the neonatal uterus. Neonatal uterine cells in mice are mitogenically responsive to estrogens, but neonatal ovariectomy does not inhibit pre-weaning uterine cell proliferation, indicating that this process does not require endogenous estrogens. Neonatal uterine cell proliferation could result from ligand-independent growth factor activation of ESR1, or be independent of ESR1 neonatally despite its obligatory role in adult uterine epithelial proliferation. To determine the role of ESR1 in uterine development, we analyzed cell proliferation and uterine gland development (adenogenesis) in wild-type (WT) and *Esr1* knockout (*Esr1*KO) mice postnatally. Our results indicate that pre-weaning uterine cell proliferation and adenogenesis are independent of ESR1, but these processes become dependent on E2/ESR1 signaling for maintenance and further proliferation and uterine growth during puberty. How pre-weaning uterine cell proliferation and adenogenesis occur independently of E2/ESR1 signaling remains unknown, but ligand-independent activation of ESR1 is not involved in this process. The synthetic glucocorticoid dexamethasone (Dex) inhibits luminal epithelial (LE) proliferation in neonatal mouse uteri, but it has been unclear whether Dex effects were mediated by glucocorticoid receptor (GR) and/or PR. We have used PR knockout (PRKO) mice to test whether PR is required for Dex inhibition of LE proliferation. Our results indicate that maximal inhibitory Dex effects on uterine LE proliferation require PR, possibly reflecting Dex crosstalk with PR. Inhibitory effects of Dex and P4 on LE proliferation may also involve GR binding, as indicated by the small but significant inhibition of LE proliferation by both Dex and P4 in PRKO mice.

Keywords: adenogenesis, ESR1, glucocorticoids, uterus.

Introduction

During puberty, females began to exhibit reproductive cycles (menstrual cycles in primates, estrous cycles in other mammals) that involve development and eventually ovulation of one or more oocytes, and then subsequent changes in the uterus and other tissues in preparation for the potential establishment of pregnancy. These reproductive cycles are characterized by regular and pronounced changes in the production and serum concentrations of 17 β -estradiol (E2) and progesterone (P4), the major endocrine regulators of female reproductive function in adults. The developing follicles produce E2 in increasing amounts during the follicular phase (proestrus and estrus) of the estrous cycle. Following ovulation, there is a rapid and sharp fall in E2 production, and then the corpora lutea develop from the remnants of the ovarian follicles and begin secreting high levels of P4 during the luteal phase (metestrus + diestrus) of the cycle.

Many reproductive processes are affected by both E2 and P4, and these steroids often produce opposing actions and can also alter expression of receptors for the other. Thus, the complexity and constant changes in the endogenous steroid hormone environment during the estrous cycle make it problematical to study actions of these hormones in intact animals. This problem has been addressed in part by utilization of the ovariectomized rodent model in many studies that sought to determine the role of E2 and/or P4 in various facets of reproductive tract development or function. Ovariectomy allows removal of endogenous sources of these hormones, and by administering various types of hormone replacement, the actions of these hormones, alone and in concert, can be established in a more controlled and manipulable endocrine environment. The use of this type of approach is well illustrated by the work of Finn, Martin and colleagues in England beginning about a half-century ago (Finn and Martin, 1970; Martin *et al.*, 1973). This group used the ovariectomized mouse model to great effect to definitively establish the effects of E2 and P4 on

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Received: August 11, 2014
Accepted: September 3, 2014



a wide variety of parameters in the uterus, and significant concepts in our present understanding of sex steroid effects can be traced to their pioneering studies.

The use of knockout mouse model systems to study the effects of 17 β -estradiol and progesterone in the female reproductive tract

The classical endocrinology tools of ovariectomy and hormone replacement that were critical to developing our understanding of ovarian hormone function during the 20th century have over the last two decades been augmented by methodologies which now make it possible to more clearly establish the role of steroid receptors such as the main estrogen receptor, estrogen receptor 1 (ESR1), and progesterone receptor (PR), and more recently membrane estrogen receptors (Adlanmerini *et al.*, 2014; Pedram *et al.*, 2014), in various processes within the female reproductive tract. The development of *Esr1* knockout (*Esr1KO*) mice in 1993 by Lubahn, Korach, Smithies and co-workers (Lubahn *et al.*, 1993), and the subsequent development a few years later of another *Esr1KO* mouse that provided more complete elimination of ESR1 by Chambon and colleagues (Dupont *et al.*, 2000), gave researchers a powerful tool to explore the effects of E2/ESR1 signaling in a wide variety of organs and processes and revealed the phenotypic, biochemical and molecular consequences of the lack of expression of ESR1 in both reproductive and non-reproductive organs. This *Esr1KO* mouse was one of the earlier ones developed using the homologous recombination technique and ES cells to introduce transgenic DNA into a mouse line and create knockout animals. In addition to many seminal observations regarding the role of ESR1 in various aspects of female reproduction (Couse and Korach, 1999), work with this mouse also allowed the critical role of ESR1 in male reproductive organs such as the efferent ducts to be revealed (Hess *et al.*, 1997). Research with this mouse also led to a clearer understanding of the role of ESR1 in adipose tissue in both sexes, the immune system, bone and other reproductive and non-reproductive organs (Heine *et al.*, 2000; Deroo and Korach, 2006).

The development of the progesterone receptor knockout (PRKO) mouse (Lydon *et al.*, 1995) shortly after the *Esr1KO* mouse was introduced provided a unique tool for understanding the role of progesterone and progestins in both reproductive and non-reproductive organs. As with the *Esr1KO* mouse, understanding the roles of PR in various reproductive and non-reproductive tissues has been greatly enriched by the use of PR knockout model to address key issues in progestin biology.

This review summarizes two lines of recent work that have generated unique insights into the roles of steroid hormone receptors in the developing mouse reproductive tract. The first study utilized the *Esr1KO*

mouse to determine that ESR1 is not involved in early development of the female reproductive tract, and the second study utilized the PRKO mouse to explore the mechanism of action by which glucocorticoids inhibit uterine epithelial proliferation.

Role of ESR1 in female reproductive tract development during neonatal life

There has been extensive interest in the role of E2 and P4 in the neonatal mouse uterus for many years, and an extensive literature has indicated that the role of E2 in uterine development during the neonatal period differs from that in older animals. The critical role of E2 as the major mitogen for adult uterine luminal epithelium (LE) is well established. Uterine LE proliferation is minimal in ovariectomized adult mice, but administration of E2 induces robust LE proliferation within a day (Finn and Martin, 1970). Conversely, uterine LE proliferation in mice is high neonatally (Ogasawara *et al.*, 1983; Bigsby and Cunha, 1986; Kuhara *et al.*, 1999), then declines steadily during the pre-weaning period. This rapid neonatal proliferation occurs despite low E2 concentrations in the mouse. Indeed, E2 is not necessary for neonatal uterine epithelial proliferation, despite its critical adult role in this process, as shown by seminal work from Ogasawara *et al.* that neonatal ovariectomy does not inhibit pre-weaning uterine growth and epithelial proliferation (Ogasawara *et al.*, 1983) and the demonstration that grafting neonatal uteri into ovariectomized mice did not alter the high proliferative rate of cells in the neonatal uteri (Bigsby and Cunha, 1985).

Although neonatal uterine LE proliferation does not require E2, neonatal E2 treatment induces robust increases in uterine epithelial proliferation (Ogasawara *et al.*, 1983; Bigsby and Cunha, 1986) and ESR1 is present in neonatal mouse uterus (Cunha *et al.*, 1982; Yamashita *et al.*, 1989). If uterine epithelial cells do not require E2 to proliferate, an important question is what regulates the high rate of neonatal proliferation. Extensive evidence has suggested that a number of growth factors may induce ligand-independent activation of ESR1, and this mechanism could drive the normal uterine growth observed following neonatal ovariectomy. Conversely, neonatal uterine growth and development could be entirely independent of ESR1, despite the obligatory role of E2/ESR1 signaling in uterus during puberty and adulthood. We have recently used *Esr1KO* mice to directly determine which of these two paradigms is correct and to establish ESR1's role in uterine epithelial proliferation during the neonatal and pubertal period, and that is one major focus of this review.

In these studies, uteri from WT and homozygous *Esr1KO* mice were immunostained for MKi67, a proliferative marker, at various ages between birth and weaning at day 22 (Nanjappa *et al.*, 2014, University of Florida, Gainesville, FL, USA; submitted). Previous studies have shown that after a



nadir of proliferation at about postnatal day 22, uterine cells start to proliferate and uterine weight increases again at about day 29 (Ogasawara *et al.*, 1983), presumably as a result of increased E2 secretion between days 26 and 29 of life (Safranski *et al.*, 1993; Mayer *et al.*, 2010). Therefore, in a second set of experiments, we also examined uterine development and cell proliferation during this pubertal period and in adulthood.

Uterine cell proliferation is independent of ESR1 during the pre-weaning period

Proliferation of uterine LE in WT mice was high neonatally, then decreased to low levels at weaning (day 22), consistent with earlier results (Fig. 1). Critically, LE proliferation was similar in *Esr1*KO and WT mice from day 2 to day 22 (Fig. 1), suggesting that ESR1 is dispensable for neonatal LE proliferation. At days 29 and 35, LE proliferation was higher in WT compared to *Esr1*KO mice, and then by day 60 LE proliferation was minimal in *Esr1*KO mice (Nanjappa *et al.*, 2014, University of Florida, Gainesville, FL, USA; submitted). This suggests that during pubertal life WT mice are showing increasing LE proliferation in response to increasing endogenous E2 levels produced by ovaries, while the uterine epithelium is becoming increasingly dependent on E2 for proliferation, which accounts for the continued declines in LE proliferation in *Esr1*KO mice. This transition culminates in adult mice being obligatorily dependent on E2/ESR1 signaling for LE proliferation, as shown by the minimal proliferation in *Esr1*KO mice by 60 days of age.

Similar to LE, stromal proliferation was highest neonatally then decreased gradually, reaching low levels by day 22 (Fig. 1). Importantly, stromal proliferation in *Esr1*KO mice was similar to WT mice, suggesting that ESR1 is not required for stromal cell proliferation in the neonatal period.

The neonatal mouse uterus at birth consists of simple LE surrounded by undifferentiated mesenchyme, but by day 6, LE begins to invaginate and differentiate into glandular epithelium (Cooke *et al.*, 2012). During subsequent life, the glandular epithelium continues to proliferate and the glands continue to enlarge until adulthood. Critically, in the *Esr1*KO uterus, gland development (adenogenesis) was comparable to that seen in WT uterus at all ages up to day 22 postnatal (Fig. 1), suggesting that neonatal adenogenesis does not require ESR1. Furthermore, the glandular epithelium proliferation that drives adenogenesis was also comparable in WT and *Esr1*KO uteri in the pre-weaning period. These findings are consistent with the observation of Stewart *et al.*, who reported that gland development in mice lacking *Esr1* was comparable to WT mice at 10 days of age, indicating that the early stages of adenogenesis occur in the absence of ESR1 (Stewart *et al.*, 2011).

The number of glands in murine uterus

increases rapidly from 3 weeks to 8 weeks in uteri of intact mice (Stewart *et al.*, 2011). However, gland number and size in *Esr1*KO mice did not continue to increase during this period, and by day 35 postnatal the glands had undergone partial regression in the *Esr1*KO mice and were far less extensive than controls at this age (Nanjappa *et al.*, 2014, University of Florida, Gainesville, FL, USA; submitted). Thus, E2 signaling through ESR1 is necessary for maintenance of existing uterine glands and further increases in gland number and size during and after puberty.

Consistent with our data showing similar patterns of cell proliferation and adenogenesis in *Esr1*KO uteri, overall growth and development of *Esr1*KO uteri was normal until day 22 postnatal (Fig. 1). However, homozygous *Esr1*KO uterus then began to differ grossly from WT counterparts at days 29 and 35, and this became more apparent by day 60 (Nanjappa *et al.*, 2014, University of Florida, Gainesville, FL, USA; submitted). These whole mount studies corroborate the data and conclusions generated from analysis of cell proliferation and adenogenesis in these animals.

Sequential actions of E2 and P4 prepare the uterus for successful implantation and pregnancy. Rising E2 levels during proestrus induce LE proliferation and higher serum P4 levels during diestrus inhibit E2-induced LE proliferation. This same P4 inhibition of E2-induced uterine LE proliferation can be observed in ovariectomized rodents (Finn and Martin, 1970). These processes occur through PR, and P4 does not inhibit LE proliferation in PRKO mice (Fernandez-Valdivia *et al.*, 2010). However, in neonatal mice, we showed that P4 produces similar inhibitions of neonatal uterine epithelial proliferation in WT and *Esr1*KO mice (Nanjappa *et al.*, 2014, University of Florida, Gainesville, FL, USA; submitted). These results indicate that P4 inhibition of epithelial proliferation, like uterine cell proliferation and overall development, does not involve or depend on ESR1 during the pre-weaning period.

In the absence of ESR1, the factors that drive neonatal uterine development are still unclear. A wide variety of growth factors (fibroblast growth factors, keratinocyte growth factor, insulin-like growth factors and epidermal growth factor) and their receptors are expressed in the developing uterus (Bossert *et al.*, 1990; Falck and Forsberg, 1996; Kuhara *et al.*, 1999; Hu *et al.*, 2004; Masui *et al.*, 2004). Although our results show unequivocally that growth factor activation of ESR1 in a ligand-independent manner is not responsible for neonatal uterine growth, as had been suggested previously, these growth factors may act independently of ESR1 to regulate neonatal uterine growth. Furthermore, during this period there are complex changes in the expression of various secreted products (Wnt5a, Wnt7a), enzymes and transcription factors (Hoxa10, Hoxa11, Hoxd10, MSX-1) that may regulate neonatal uterine development totally independent of E2/ESR1 signaling (Hu *et al.*, 2004).

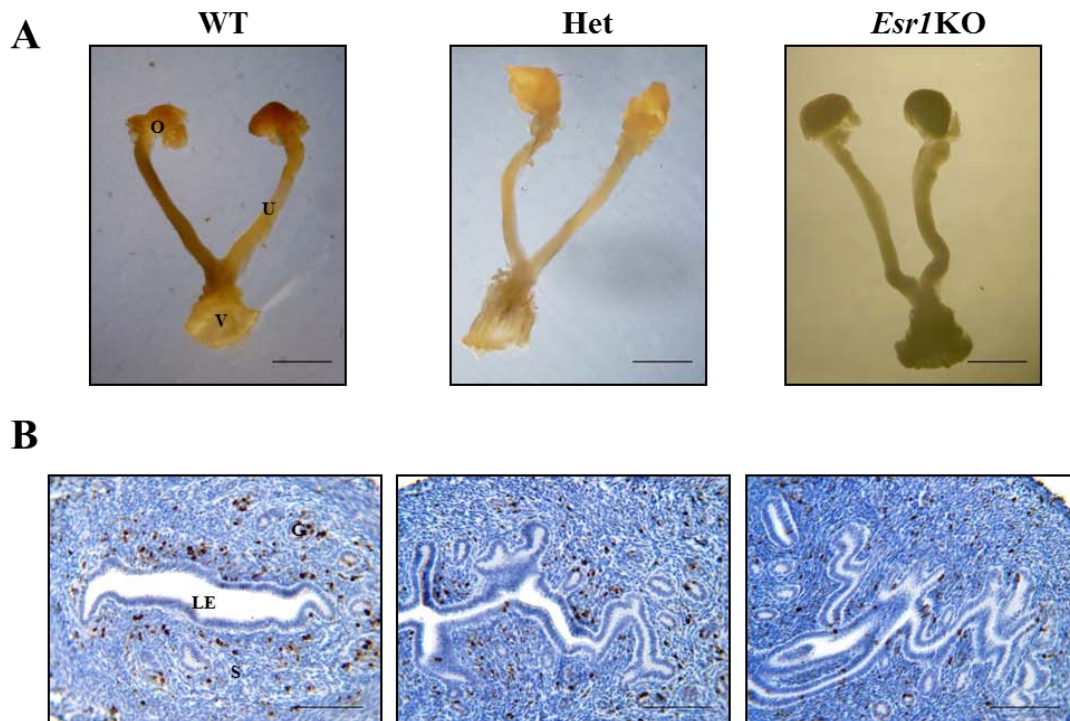


Figure 1. Representative whole mounts (A) and histology (B) of uterus from wild-type (WT), heterozygote (Het) and *Esr1*KO mice at 22 days of age. The overall size and thickness of the uterine horns of *Esr1*KO was comparable to WT and Het animals at day 22 (A). Uteri from day 22 females were immunostained for the cell proliferation marker MKI67 and counterstained with hematoxylin (B). Luminal epithelium (LE) and stroma (S) showed minimal to modest proliferation in all the groups. Note that gland (G) development at day 22 is similar in the WT, Het and *Esr1*KO uteri. Critically, both cell proliferation and adenogenesis in the *Esr1*KO uterus is similar to WT and Het up to day 22, suggesting that ESR1 is not required for neonatal uterine development before day 22. All whole mount and histological images are at the same magnification, with the magnifications bars in panel A = 2.5 mm and in panel B = 100 μ m. O = ovary, U = uterus, V = vagina.

Mechanism of the inhibitory effect of P4 and glucocorticoids on uterine epithelial proliferation during neonatal life

Progesterone inhibits many actions of E2 in the mammalian uterus, including E2 induction of LE proliferation in ovariectomized mice (Martin *et al.*, 1973). Progesterone treatment of neonatal mice results in a similarly strong inhibition of LE proliferation (Bigsby and Cunha, 1985). As mentioned above, this signature P4 effect requires nuclear PR and is not seen in PRKO mice (Fernandez-Valdivia *et al.*, 2010).

The synthetic glucocorticoid dexamethasone (Dex) also exhibits a wide range of anti-estrogenic activities (Rhen *et al.*, 2003; Rhen and Cidlowski, 2006; Whirlledge and Cidlowski, 2013). Dex administration inhibits uterine LE proliferation at high doses in both neonates (Bigsby and Cunha, 1985) and ovariectomized, E2-stimulated adults (Markaverich *et al.*, 1981; Stewart *et al.*, 1983). Dexamethasone has an affinity for glucocorticoid receptor (GR) that exceeds that of endogenous glucocorticoids such as cortisol and corticosterone (Funder *et al.*, 1973; Bigsby and Young, 1993), and is far more potent in terms of glucocorticoid

activity than endogenous glucocorticoids, which is the basis for its clinical utility. The ability of Dex to inhibit uterine epithelial proliferation obviously could involve signaling through GR, which is expressed in the uterus (Bigsby and Young, 1993). However, Dex also has some affinity for PR, and it is possible that the antiproliferative effects of Dex in the uterus could be partially or completely mediated by signaling through PR. We have recently used PRKO mice to determine whether PR and/or GR are required for inhibitory effects of Dex on LE proliferation in uterus using PRKO mice (Nanjappa *et al.*, 2014, University of Florida, Gainesville, FL, USA; submitted). This work has produced some important insights into the roles of both PR and GR in the neonatal uterus, and is the second focus of this review.

Inhibitory effect of glucocorticoids on uterine epithelial proliferation may involve both progesterone and glucocorticoid receptor

The approach of these studies was simple, and involves administration of either P4 or Dex at the dose of 40 μ g/g to day 5 female WT and PRKO pups, in

which both PR-A and PR-B, the two main forms of nuclear PR, are deleted. Twenty-four hours later, by examining Dex effects on neonatal uterine LE proliferation in the presence or absence of PR, the requirement of PR for inhibitory activity of Dex can be determined. Results of these types of studies then provide unequivocal data indicating that PR is or is not essential for Dex effects on uterine LE proliferation.

Our results indicated that the full inhibitory effect of Dex requires PR (Nanjappa *et al.*, 2014, University of Florida, Gainesville, FL, USA; submitted). This conclusion was reached using data showing that the striking inhibitory effects of Dex on uterine LE proliferation in WT mice were greatly diminished in mice lacking PR (Fig. 2). As expected, the full inhibitory effect of P4 was also lost in PRKO mice. Despite the clear results that the normal inhibitory effect of Dex on uterine epithelial proliferation requires PR, exactly why PR is obligatory is not yet clear.

One possible interpretation of these results would be that the loss of PR could have had an inhibitory effect on GR expression in the uterus, and impaired GR expression could preclude a normal anti-mitogenic response to Dex. Although a dependence of GR expression on PR has not been reported previously, this idea cannot be rejected *a priori*, and could account for the experimental results. To test this hypothesis, we examined GR expression by both immunohistochemistry, as well as Western blot analysis in neonatal uterus. Our results indicated that the neonatal PRKO uterus expressed quantitatively and qualitatively normal GR in both epithelial and stromal cells (Nanjappa *et al.*, 2014, University of Florida, Gainesville, FL, USA; submitted). These results clearly demonstrate that GR expression in PRKO mice is normal and that the lack of the full Dex

effect in PRKO mice is not a consequence of impaired GR expression in PRKO uteri.

An obvious potential explanation for the lack of the full inhibitory effect of Dex on neonatal uterine LE proliferation in PRKO mice is that in normal animals Dex could bind and signal through PR to inhibit epithelial proliferation. Thus, absence of Dex signaling through PR in PRKO neonates could account for its reduced anti-mitogenic effects. This hypothesis is consistent with previous reports that Dex has some affinity for PR in binding assays (Issar *et al.*, 2006), although this affinity is not surprisingly less than that of P4 for PR and is far less than the affinity of Dex for GR. In addition, the ability of glucocorticoids to signal through PR has been demonstrated in breast cancer cells, and this same type of cross-talk may be a factor in the normal inhibitory effects of Dex on uterine LE proliferation (Leo *et al.*, 2004).

One somewhat unexpected result that was obtained from our studies of Dex and P4 effects in the neonatal PRKO uterus was that both Dex and P4 had inhibitory effects in the PRKO mouse. These effects were far less than seen in WT mice. For example, Dex decreased the LE labeling index in WT mice by over 50% compared to vehicle-treated controls, while in PRKO mice LE labeling indices were decreased by only 13% in Dex-treated neonates compared to vehicle-treated controls. Similarly, P4 treatment produced a slight (approximately 4%) but significant decrease in labeling index in PRKO mice. These results suggest PR is required for the major portion of both P4 and Dex effects on uterine epithelial proliferation, but that both Dex and P4 may also be able to signal through GR to inhibit uterine epithelial proliferation, and in PRKO mice this effect must be totally independent of PR.

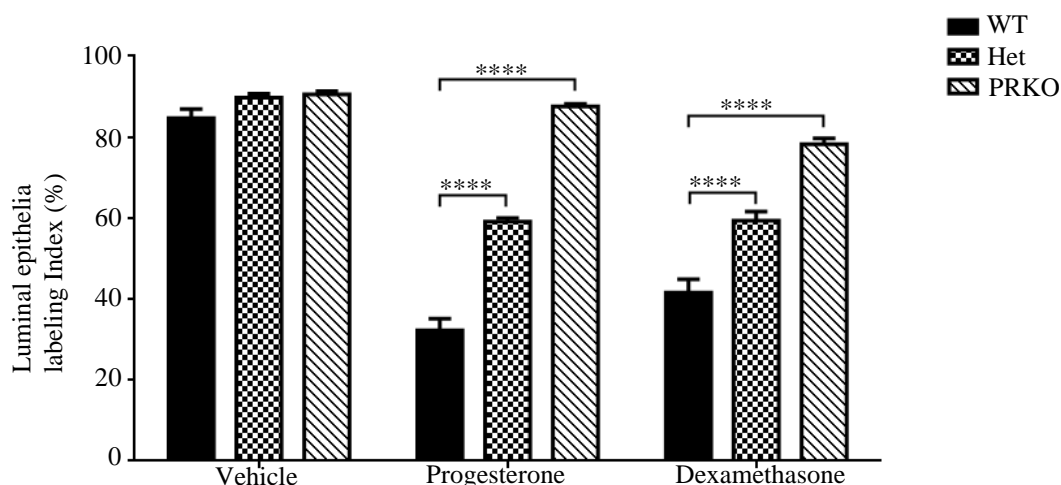


Figure 2. Effects of progesterone (P4) and dexamethasone (Dex) on neonatal uterine epithelial proliferation in wild-type (WT), heterozygote (Het) and PRKO mice. The inhibitory responsiveness to both Dex and P4 at 40 µg/g diminishes with reduced amount of PR. Both P4 and Dex maximally inhibited uterine luminal epithelial proliferation in WT ($PR^{+/+}$) mice. Clear inhibitory effects of P4 and Dex were also seen in heterozygous ($PR^{+/-}$) mice, although the inhibition was less pronounced than in WT mice. In PRKO ($PR^{-/-}$) mice, the large decreases produced by P4 and Dex in WT mice were not seen, although both of these treatments still produced small but significant decreases in LE proliferation.



Conclusions and future directions

Our results indicate that uterine cell proliferation and growth in neonatal mice do not require E2/ESR1 signaling, then there is a gradual transition to E2/ESR1-dependent uterine growth and epithelial proliferation around the time of puberty. Recently, E2/ESR1 signaling was shown to be necessary for post-pubertal uterine development in humans. A young woman with a homozygous missense mutation in the ligand-binding domain of ESR1 showed clinical signs of a hypoplastic uterus, delayed puberty, elevated levels of E2 and markedly enlarged multicystic ovaries. These reproductive effects mirror those seen in ESR1 null mice, indicating that E2/ESR1 signaling is required for post-pubertal uterine growth in humans (Quaynor *et al.*, 2013). Whether some phase or potentially all of pre-pubertal uterine development is also independent of E2/ESR1 signaling is likely based on our mouse results, but remains to be definitively established.

The striking changes in the control of uterine epithelial proliferation during puberty raise the critical question as to what drives this change from E2/ESR1-independent to E2/ESR1-dependent uterine development during the pubertal period. Understanding this key developmental transition would shed light on not only the molecular events that are involved in both the E2/ESR1-independent and -dependent phase of uterine development, but also various uterine pathologies involving aberrant epithelial proliferation.

The results from our studies involving the mechanism of Dex inhibition of uterine epithelial proliferation suggest that the major portion of the Dex effect is dependent on the presence of PR. Direct binding of Dex to PR is likely to be an important component of this, but our results do not allow us to conclude that this is the sole mechanism responsible for the Dex effect. In addition, we have for the first time provided data indicating that the GR may partially mediate the inhibitory effects of Dex, as well as PR, on uterine epithelial proliferation. The picture that is emerging is that both receptors may play a role not only in the Dex inhibition of uterine epithelial proliferation under experimental conditions, but also in normal effects of both P4 and glucocorticoids in intact animals.

Future studies in this area will be directed toward elucidating GR's role in the uterus and various processes regulated by glucocorticoids and P4 in this organ. One of the central conclusions reached by these studies, that GR may play a role in regulating changes in LE proliferation in response to glucocorticoids or P4, could be further tested and refined through analysis of uteri lacking GR expression. Although the global GR knockout is lethal, the crossing of mice in which GR has been floxed in conjunction with mice that express Cre-recombinase driven by promoters such as that for PR, that are ubiquitously expressed in the uterus, would allow the GR gene to be specifically excised in cells that

expressed PR in the uterus and other organs. Thus, a conditional uterine knockout of GR could be created with deletions of GR in all PR-expressing cells in the uterus. These animals would allow further experiments that would corroborate and extend some conclusions reached in our previous study (Nanjappa *et al.*, 2014, University of Florida, Gainesville, FL, USA; submitted), but also provide unique information that could only be obtained from the use of GR conditional knockout animals and could not be obtained from experiments involving PRKO mice. Our group and others are presently pursuing this experimental approach, and this should provide another tool that will further refine our understanding of the exact mechanism by which Dex inhibits uterine LE proliferation and the relative roles of GR and PR in the inhibitory effects of both Dex and P4 in the uterus.

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