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Remodeling of the Cervix and Parturition in Mice Lacking the Progesterone Receptor B Isoform[1]

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ABSTRACT

Withdrawal of progestational support for pregnancy is part of the final common pathways for parturition, but the role of nuclear progesterone receptor (PGR) isoforms in this process is not known. To determine if the PGR-B isoform participates in cervical remodeling at term, cervices were obtained from mice lacking PGR-B (PGR-BKO) and from wild-type (WT) controls before or after birth. PGR-BKO mice gave birth to viable pups at the same time as WT controls during the early morning of Day 19 postbreeding. Morphological analyses indicated that by the day before birth, cervices from PGR-BKO and WT mice had increased in size, with fewer cell nuclei/area as well as diminished collagen content and structure, as evidenced by optical density of picrosirius red-stained sections, compared to cervices from nonpregnant mice. Moreover, increased numbers of resident macrophages, but not neutrophils, were found in the prepartum cervix of PGR-BKO compared to nonpregnant mice, parallel to findings in WT mice. These results suggest that PGR-B does not contribute to the growth or degradation of the extracellular matrix or proinflammatory processes associated with recruitment of macrophages in the cervix leading up to birth. Rather, other receptors may contribute to the progesterone-dependent mechanism that promotes remodeling of the cervix during pregnancy and in the proinflammatory process associated with ripening before parturition.

birth, cervix, inflammation, macrophage, neutrophil, parturition, progesterone/progesterone receptor, ripening

INTRODUCTION

Progesterone is essential to sustain pregnancy while withdrawal of its trophic actions initiates parturition [1–4]. Classic nuclear progesterone receptors (PGRs) mediate the effects of progestational agents to maintain pregnancy, whereas antagonists of these receptors induce birth. PGR-A and PGR-B are ligand-binding isoforms of the nuclear PGR [5, 6]. Mice lacking both receptor isoforms (PGRKO) or only PGR-A (PGR-AKO) have a variety of reproductive anomalies, including inability to ovulate, uterine hyperplasia, and inflammation, as well as defects in uterine implantation [7, 8]. As a consequence of the essential role for PGR-A in ovulation, PGRKO or PGR-AKO mice are infertile [9]. By contrast, mice lacking the PGR-B isoform (PGR-BKO) are reproductively competent and deliver viable pups [10]. Proliferation and hyperplasia of the uterus are functions attributed to PGR-B through its putative role as a transcriptional activator of progesterone-responsive genes [7, 9]. These PGR-B-mediated activities may be opposed by actions of PGR-A based upon in vitro evidence that PGR-A can function as a dominant repressor of PGR-B actions [11, 12]; however, net genomic effects are likely to be cell and tissue specific as well as PGR-B:PGR-A ratio dependent. Given the variety of progesterone-mediated actions in tissues and specific roles served by PGR isoforms [1, 13], little known is known about PGR isoforms in processes that remodel the cervix with pregnancy or in preparation for parturition.

As pregnancy progresses, progesterone concentrations increase in circulation, achieving a peak in rodents several days before birth while remaining at or near the peak level in peripartum women [14–16]. As term approaches, remodeling of the cervix is characterized by reduced density of cell nuclei, degradation of extracellular matrix collagen content and structure, and immigration of immune cells [17–20]. By the day before birth, immune cells have immigrated into the stroma and perimetrium of the cervix in mice [21, 22] and rats [23, 24]. Evidence in primates also suggests that these characteristics of inflammation are part of the process that remodels the cervix [25–27]. Although PGR-B may not be essential for maintaining pregnancy or for parturition in mice, whether the PGR-B isoform regulates inflammatory processes that remodel the cervix is not known. The present study addressed the hypothesis that the PGR-B isoform mediates recruitment of immune cells into the prepartum cervix but not remodeling of the extracellular matrix in preparation for birth. Findings suggest that a PGR-mediated mechanism other than that related to PGR-B remodels and ripens the cervix for parturition.

MATERIALS AND METHODS

Animals

Adult mutant and wild-type (WT) mice from the same genetic background (B6x129/SVE) were provided by one of the authors (E.M.A.) from a colony at the Baylor College of Medicine to serve as breeders at Loma Linda University. The initial group of mice consisted of homozygotic PGR-BKO males and heterozygotic females with one PGR-B allele for gene transcription. As previously described, the CRE-*loxP* gene targeting approach was used in embryonic stem cells to introduce a point mutation into the *Pgr* gene at the ATG codon encoding Met I [10]. Homozygotic male and female progeny were bred so that time-dated pregnant PGR-BKO females could be used for the present study. To confirm PGR-BKO status, the genotype of every mouse in

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the present study was determined from a tail tissue clipping (EmBark Scientific, previously known as MendelWorks). Mice were housed in a temperature- and humidity-controlled vivarium room with a 12L:12D photoperiod, lights-on at 0700 h, and food and water provided ad libitum. Animal care followed guidelines set by the National Institutes of Health, and experimental procedures were approved by the Loma Linda University School of Medicine Institutional Animal Care and Use Committee.

Cervical Processing and Analysis

The WT and PGR-BKO mice were killed on the morning of Day 15, 18, or 19 postbreeding (Day 19 = day of birth [~2–4 h postpartum]). Nonpregnant (NP) mice in estrus or metestrus, based upon assessment of vaginal smears, served as additional controls. The cervix was removed, postfixed overnight, embedded in paraffin, sectioned (thickness, 10 μm), and processed as previously detailed [28, 29]. Briefly, sections of cervix from each mouse were stained with picrosirius red to identify collagen and cross-link structure. Other sections were processed by immunohistochemistry to stain macrophages (BM8 antibody, 1:800; Bachem Americas, Inc.) or neutrophils (7/4 antibody, 1:100; AbD Serotec), then counterstained with hematoxylin to identify cell nuclei. As previously discussed [29], these antibodies are widely used and specific for differentiated immune cells. Cross-reactivity of the 7/4 antisera with naïve monocytes is lost with differentiation into macrophages as cells migrate into tissue. For analyses of collagen content and structure of the extracellular matrix, the optical density of polarized light from type I collagen birefringence in picrosirius red-stained sections of cervix was normalized to the cell nuclei density for each mouse to account for the hypertrophy of tissue in pregnant mouse cervix. To enumerate resident immune cells, an automated image-analysis protocol was used to count BM8-labeled macrophages or 7/4-labeled neutrophils. Briefly, a photomicrograph of the field of view in the microscope was captured, and using Image ProPlus 6 software (Media Cybernetics), cells were enumerated based on criteria related to the color of specific immunohistochemical stain for each pixel. These settings were in the eye-dropper mode (degree of sensitivity = 5, color index = 3, minimum number of pixels = 4) and count/size mode (object pixel connections = 8, smoothing = 0, fill holes checked, area range = 3–330). Photomicrographs were reviewed to assure that each cell count met criteria that colored areas were in association with a single cell nucleus. Macrophage numbers per field were independently confirmed by direct visual counts in a random survey of sections by multiple coauthors blinded to group designations in the experimental design. Three cervical sections per mouse and eight nonoverlapping 10×10 grid placements per section (i.e., 152 100 μm^2) were analyzed. As before, data were an average of replicate analyses in the three sections normalized to cell nuclei density for each individual to account for hypertrophy and hyperplasia of the reproductive tract tissue, with the mean ± SEM per group determined.

Statistics

Data were normally distributed and individual comparisons made by one-way ANOVA or Student *t*-test using GraphPad Prism (GraphPad Software). Following ANOVA, Tukey test was used for individual comparisons. In a few instances, Levene test for homogeneity of variance was significant, and data were analyzed using the Kruskal-Wallis test. Data are presented as the mean ± SEM, with $P < 0.05$ considered to be significant.

RESULTS

Effects of PGR-B Ablation on Parturition

The WT and PGR-BKO mice gave birth in the early morning hours of Day 19 postbreeding. The presence of pups within 2 h of lights-on was comparable to that of other murine strains in our previous reports. An average range of three to eight pups per litter was found postpartum in WT and PGR-BKO mice. Postmortem, the number of implantation sites in the uterus matched the number of pups in each cage. Thus, litter size, lack of fetal mortality, duration of pregnancy, and timing of birth were similar in WT and PGR-BKO mice.

Effects of PGR-B Ablation on Cervical Remodeling

Morphological changes in the cervix with pregnancy were similar in control and mutant mice. The luminal epithelium in the cervix from WT and PGR-BKO mice on Day 15 postbreeding was thicker, with more intracellular space surrounding fewer cell nuclei in the stroma, in each microscopic field of view compared to that in cervical sections from NP mice. The numbers of cell nuclei per area of cervix decreased by Day 15 postbreeding, significantly so in WT mice, and in both WT and PGR-BKO mice on Day 18 postbreeding as well as in postpartum mice relative to NP mice (Fig. 1, top). This decrease in cell nuclei density was accompanied by a reduced content and organization of collagen in the prepartum and postpartum cervix. In NP mice, collagen fibers in the cervix were darkly stained with picrosirius red, and fibrils were tightly packed in a striated arrangement. In sections of cervix from all pregnant mice, whether from WT or PGR-BKO genotypes, the intensity and area of birefringence was reduced, collagen fibrils were loosely packed, and pockets with light stain were evident. The optical density of polarized light, inversely related to collagen content and structure, increased in sections of cervix from all pregnant WT and PGR-BKO mice by Day 18 postbreeding relative to that in NP mice (Fig. 1, bottom). Thus, absence of PGR-B expression did not interfere with growth and remodeling of the extracellular matrix in the peripartum cervix.

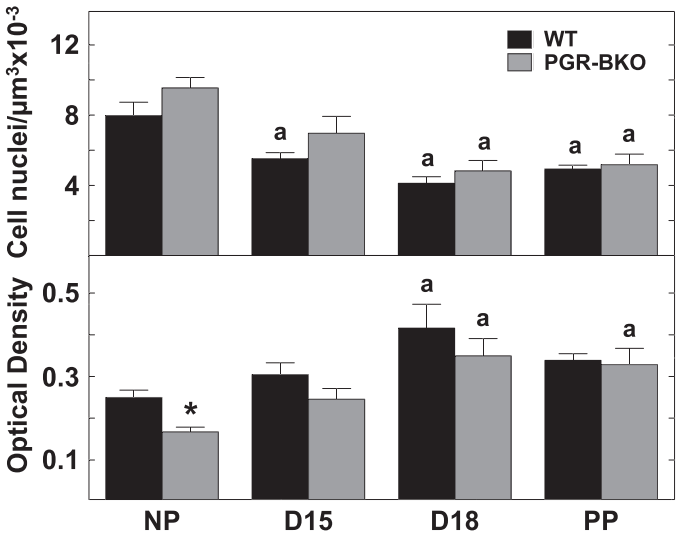

FIG. 1. **Top**) Number of cell nuclei per area in the cervix in WT (dark, n = 6–10 per group) and PGR-BKO (open, n = 3–6 per group) mice on Day 15 (D15) or Day 18 (D18) of pregnancy or postpartum on Day 19 postbreeding (PP) compared to that in NP mice. Reduced cell nuclei per area indicates hypertrophy of cervix with pregnancy. **Bottom**) Optical density of picrosirius red-stained sections cervix from WT (n = 6–7 per group) controls and PGR-BKO (n = 3–6 per group) mice before or after birth and from NP controls. Optical density of polarized light was normalized to cell nuclei density and is inversely related to collagen content and structure. Data are presented as the mean ± SEM. $^{a}P < 0.05$ vs. NP, same strain, by ANOVA, $^{*}P < 0.05$ vs. WT, same day, by Student *t*-test.

Effects of PGR-B Ablation on Census of Immune Cells in the Cervix

BM8-stained cells were found in the stroma, around blood vessels, in the submucosal epithelium, and to a limited extent, in the epithelium of the cervix of all mice (Fig. 2, top). Dark brown-stained cell bodies and processes were typically associated with a violet counterstained cell nucleus. Brown-colored areas not associated with a violet counterstained cell nucleus (presumably macrophage pseudopodia cell fragments) were not counted. During pregnancy, the luminal epithelium

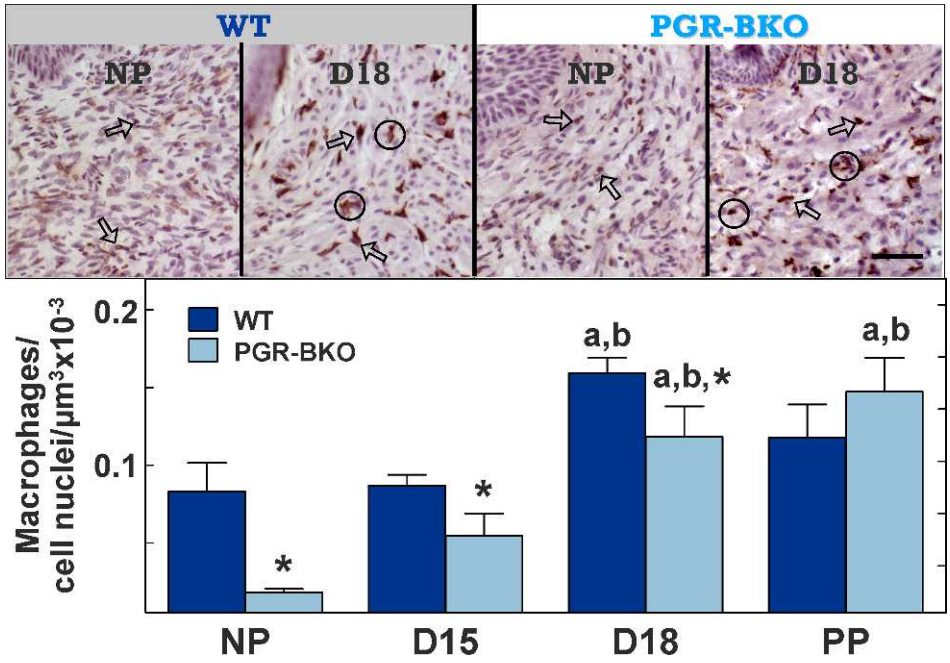

FIG. 2. **Top**) Photomicrographs of macrophages in the cervix of NP or Day 18 (D18) WT and PGR-BKO mice. Dark BM8-labeled cells contrast with pale counterstained cell nuclei (brown vs. violet, respectively). Arrows point to examples of individual cells, whereas clusters of macrophages are within circles. Scale bar = 50 μm. **Bottom**) Number of macrophages (mean ± SEM, n = 3–6 per group per strain) in the cervix from WT and PGR-BKO mice. Data are normalized to cell nuclei to adjust for tissue hypertrophy. D15, Day 15; PP, postpartum. [a]$P < 0.05$ vs. NP, same strain, by ANOVA, [b]$P < 0.05$ vs. D15, same strain, by ANOVA, *$P < 0.05$ vs. WT, same day, by Student *t*-test.

thickened while more macrophages were present in the expanded stroma and between smooth muscle cells in the cervix from WT and PGR-BKO mice (Day 18 postbreeding shown; Fig. 2, top). Thus, the distribution of BM8-stained macrophages was similar in the cervix of WT and PGR-BKO mice and comparable to that of controls in our previous reports for other strains of mice.

Analyses indicated that more macrophages were present in the cervix by the day before birth in WT and PGR-BKO mice. Numbers of macrophages similarly increased in the cervix of both WT and PGR-BKO mice by Day 18 postbreeding compared to the cervix from NP or Day 15 mice (WT: $df = 3$, $F = 4.02$; PGR-BKO: $df = 3$, $F = 20.3$; ANOVA, $P < 0.05$) (Fig. 2, bottom). Fewer macrophages were present in the cervix of PGR-BKO mice compared to WT mice in the NP, Day 15, and Day 18 groups. Postpartum, the census of resident macrophages in the cervix of PGR-BKO mice remained elevated compared to that in NP or Day 15 mice. Thus, in both WT and PGR-BKO mice, a 2-fold increase in resident macrophages was found between Day 15 and the day before birth (Day 18 post breeding) [30].

As for macrophages, cells labeled with the 7/4 antibody were similarly distributed in stroma and perimetrium of the cervix; however, residency varied with genotype. In WT mice, the number of neutrophils increased in the cervix by the day before birth (Day 18 postbreeding) compared to that in the cervix from NP or Day 15 mice ($df = 3$, $F = 9.62$; ANOVA, $P < 0.05$) (Fig. 3, bottom). By contrast, neutrophils remained at levels found in NP mice in all PGR-BKO groups. Thus, no

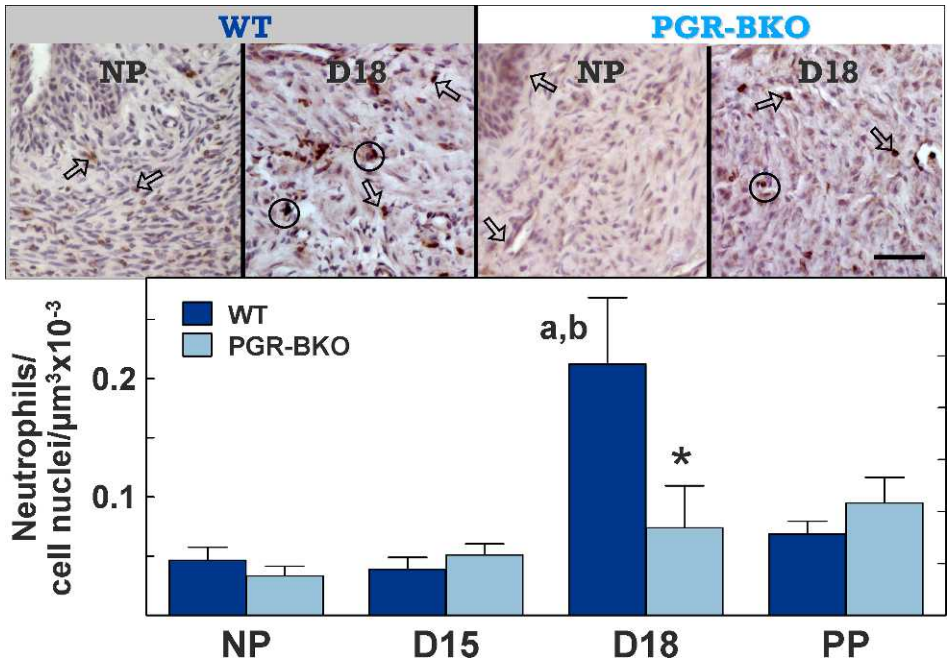

FIG. 3. **Top**) Photomicrographs of 7/4-labeled neutrophils in the cervix of NP or Day 18 (D18) WT and PGR-BKO mice. Arrows point to examples of individual cells, whereas clusters of macrophages are within circles. Scale bar = 50 μm. **Bottom**) The census of neutrophils (mean ± SEM, n = 3–6 per group per strain) in the cervix from groups of WT and PGR-BKO mice. Data are normalized to cell nuclei to adjust for tissue hypertrophy. D15, Day 15; PP, postpartum. [a]$P < 0.05$ vs. NP, same strain, by ANOVA, [b]$P < 0.05$ vs. D15, same strain, by ANOVA, *$P < 0.05$ vs. WT, same day, by Student *t*-test.

prepartum increase in neutrophils occurred in the cervix of prepartum PGR-BKO mice.

DISCUSSION

As pregnancy nears term in mammals, the cervix remodels and then ripens as a requirement for parturition and the birth of viable offspring. The present study indicates that the PGR-B isoform does not mediate aspects of remodeling related to growth and hypertrophy of cells in the cervix with pregnancy (i.e., reduced cell nuclei density, reduced extracellular collagen, and by the day before birth, increased presence of immune cells). These morphological characteristics define, in part, remodeling in the rodent cervix [22, 28] and occur when progesterone in circulation is at or near peak (i.e., Days 15–17 postbreeding in mice [2–4 days before birth]) [31, 32]. The characteristics of hypertrophy as well as reduced collagen content and structure in the cervix are dependent upon the actions of progesterone, as evidenced by effects on cervical growth and morphology after prolonged treatment of NP mice with gonadal steroids [28]. Moreover, this phase of remodeling occurs well before the shift to a contractile phenotype by the uterine myometrium sometime late on Day 18 of pregnancy, the day before birth in mice [33]. Findings in the present study suggest that PGR-B is not the sole mediator for progesterone to maintain pregnancy, because PGR-BKO and WT mice had same duration of pregnancy, no evidence of dystocia was found, and labor produced viable pups. These results concerning cervical remodeling and the birth process extend previous findings that neither ovarian nor uterine functions are impaired by the absence of PGR-B [10].

As pregnancy nears term, progesterone withdrawal appears to be critical to the parturition process. This long-standing concept from Csapo and Wiest [4] is consistent with observations that blockade of myometrial activity in most mammals, including rodents, is eliminated with decreased concentrations of progesterone in maternal circulation [1] or, in species that lack a systemic decline, a functional local withdrawal of progesterone [2, 34]. By contrast, mice may be underappreciated as models for prepartum changes in the cervix, because as in primates and guinea pigs when pregnancy nears term, the level of progesterone in circulation is sustained during the period when the cervix remodels. Serum progesterone concentrations are at or near peak between Day 15 and Day 17 of pregnancy in mice when remodeling accelerates. Levels of progesterone in circulation and in the cervix subsequently decline to half those of the peak by the day before birth (Day 18 of pregnancy in mice) [35, 36] but still far exceed those of the estrous cycle peak and the saturation threshold for tissue PGRs [11, 37, 38]. Thus, across species, remodeling and ripening of the cervix occur when progesterone concentrations are well above levels that activate genomic PGR. Possible mechanisms, other than a fall in systemic progesterone concentrations, may mediate a local withdrawal of progesterone actions that, common to all mammals, facilitates cervical hypertrophy, extracellular collagen matrix degradation, and recruitment of immune cells, including changes in steroid metabolism in the cervix [32, 39]. With little known about PGR isoforms in the uterus or cervix of rodents during pregnancy, an alternative local mechanism for progesterone to elicit normal growth, extracellular matrix remodeling, and recruitment of macrophages in the cervix before parturition may be to increase expression and activation of the PGR-A isoform [34, 40, 41] or regulate PGR coactivators [42] via nongenomic actions.

The present study also confirms previous findings in other murine strains that more macrophages are present per area in the cervix by the day before birth than earlier in pregnancy. A significantly lower number of macrophages in the cervix of NP and prepartum pregnant PGR-BKO mice versus WT controls has no effect on cervical remodeling or ripening. In addition, the present study is the first report, to our knowledge, of a coincident increase in the presence of neutrophils in the prepartum cervix of WT mice at term. Findings support previous observations in the cervix of women during parturition that immigrating neutrophils appear to transit from venules into stroma and perimetrium as well as to degranulate and induce collagenolysis [17]. However, in the cervix of prepartum PGR-BKO mice, collagenolytic processes that remodel the extracellular matrix of the cervix in preparation for birth were not affected by the lack of recruitment of neutrophils. These findings do not exclude the possibility that activation of a limited number of resident immune cells may drive increased production of prostaglandins, proinflammatory cytokines, and collagen degradation in the prepartum cervix at term [25, 43, 44] or are important for inflammation-induced preterm cervical remodeling [29, 45]. Based upon the present findings in PGR-BKO mice, the PGR-B isoform is unlikely to be involved in such activational cascades that overcome the progestational protection of peak progesterone in circulation.

In summary, our findings do not support the hypothesis that the PGR-B isoform mediates cellular growth, degradation of extracellular collagen, or recruitment of macrophages as components of an inflammatory process that remodels and ripens the cervix in preparation for birth. Absence of PGR-B in the pregnant dam and fetuses, resulting from mating with a PGR-BKO male, does not interfere with physiological and inflammatory processes associated with pregnancy, cervical remodeling, or parturition. However PGR-B may influence recruitment of macrophages and neutrophils in the prepartum cervix, evidence clearly indicates that a lack of the PGR-B isoform does not interfere with the mechanism that remodels the cervix in preparation for parturition.

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