Overlapping and Distinct Expression of Progesterone Receptors A and B in Mouse Uterus and Mammary Gland during the Estrous Cycle

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In rodents, progesterone receptors (PRs) A and B have different and often nonoverlapping roles, and this study asked whether different activities of the PR proteins in mouse are related to differences in their expression in reproductive tissues. The individual expression of PRA and PRB was determined immunohistochemically in mammary gland and uterus during the estrous cycle or in response to endocrine manipulation. In the mammary gland, PRA and PRB were colocalated in PR+ epithelial cells, with little change during the estrous cycle. In the uterus, PRA was not detected in luminal epithelium at any stage of the cycle, and PR+ luminal cells expressed only PRB. In the stroma and myometrium, PRA and PRB levels fluctuated with cyclical systemic hormone exposure. Observation of functional end points suggested that augmented stromal and/or myometrial PRA in proestrus inhibited estrogen receptor expression and epithelial proliferation. Colocational PRA and PRB was hormonally regulated, and ovariectomy did not reproduce the expression of PRA and PRB in the uterus during the estrous cycle. Whereas PRB was the only PR in the luminal epithelium in cycling mice, ovariectomy restored PRA expression, resulting in PRA-PRB colocation. In stroma and myometrium, PRA and PRB were colocalated in PR+ cells, but ovariectomy reduced PRA levels more than PRB, resulting in PRB-only-expressing cells. This study has shown that nonoverlapping PRA and PRB expression in the uterus, in particular the lack of PRA, and expression of PRB only in the luminal epithelium throughout the estrous cycle, is likely to contribute to the distinct roles of PRA and PRB in the adult mouse.

THE OVARIAN HORMONE progesterone plays an essential role in mediating the profound changes associated with the female reproductive cycle (1). In the uterus, progesterone opposes estrogen-mediated proliferation and reverses its proinflammatory effects, while stimulating stromal cell proliferation and differentiation as a prelude to implantation (2). In the mammary gland, progesterone directs ductal side branching and lobuloalveolar development and is critically involved in the complex changes in mammary gland function during pregnancy, lactation, and involution (3).

Progesterone mediates its effects via two progesterone receptors (PRs), PRA and PRB, which are transcribed from two different promoters and are identical except that PRA is N-terminally truncated (4). In normal human tissues in vivo, including breast (5) and uterus (6), all PR-positive epithelial cells coexpress PRA and PRB at similar levels. PR is known to form dimers on activation, and the PRA:PRB heterodimer is the likely active species in the human because the two proteins colocalize to nuclear foci in normal tissues in vivo (7).

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Abbreviations: ER, Estrogen receptor; FITC, fluorescein isothiocyanate; PBT, PBS and Triton X-100; PR, progesterone receptor; PRAKO, PRA null; PRBKO, PRB null; TXR, Texas red.

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different activities of PRA and PRB in the mouse may be mediated by separate expression of PRA and PRB in the mouse reproductive system, rather than their colocation as seen in the human. There is evidence from biochemical studies that this may be the case. Whereas PRA and PRB are both present in the mouse uterus and mammary gland, there is an overall predominance of PRA in mouse tissues (15, 16). PRA levels in the mouse uterus have been shown to be higher than those of PRB by photoaffinity labeling and immunoblot analysis, with a relative PRA:PRB expression of 3:1 (16), suggesting that the two PR proteins are unequally expressed. PRA and PRB are also expressed in different cells in the mouse ovary (17), but there is no information on the mammary gland or the uterus.

To determine the likely predominant molecular PR species mediating progesterone action in the mouse, information is needed on the individual expression of mouse PRA and PRB in the physiologically relevant context of the estrous cycle. Hormonal control of PR in the rodent has largely been studied by ovariectomy followed by hormone replacement. However, this regimen may not reproduce the cyclical fluctuations in estrogen and progesterone levels that occur over the short time frame of the natural estrous cycle (18–20), and PR fluctuations in this complex hormonal environment are poorly understood. PR regulation in the uterus of cycling rats has been described (21), but there is limited information in mice. Moreover, the individual expression of PRA and PRB in the uterus and mammary gland, during the estrous cycle in mice, has not been explored.

This study was designed to determine whether PRA and PRB were expressed in the same cells in mouse uterus and mammary gland during the estrous cycle, to determine the individual regulation of PRA and PRB by steroid hormones and to correlate this with functional end points such as estrogen receptor (ER) expression and cell proliferation. It is likely that a number of factors contribute to the different activities of PRA and PRB in the mouse, including complex interactions with multiple transcriptional cofactors, but it is also feasible that differential expression of the two PR isoforms also contributes.

**Materials and Methods**

**Cycling and ovariectomized BALB/c mice**

Cycling and ovariectomized BALB/c mice were housed in humidity- and temperature-controlled rooms with a 12-h light, 12-h dark cycle (lights on from 0600–1800 h) with food (meat-free rat and mouse pellets; Specialty Feeds, Glen Forrest, Western Australia, Australia) and water provided ad libitum. BALB/c mice were handled and ovariectomies performed according to the guidelines of the Animal Research Act, 1985, and the National Health and Medical Research Council Australian Code of Practice for Care and Use of Animals for Scientific Purposes, 6th edition, 1997. All experiments were approved by the Institutional Animal Care and Ethics Committee.

Vaginal smears were taken daily to monitor progression through the estrous cycle (22) in 10-wk-old, virgin, female, BALB/c mice. Mice exhibiting two consecutive, 4- to 5-d cycles were killed at 1000 h on each day of the estrous cycle (proestrus, estrus, metestrus, and diestrus) and also at 2200 h in proestrus (n = 3/stage). Bilateral ovariectomies were performed on adult, virgin, BALB/c mice (8 wk old). Cycling and ovaritectomized mice were anesthetized with ketamine (100 mg/g body weight) and xylazine (10 mg/g body weight), and their uter i and mammary glands were removed and immediately fixed in neutral buffered formalin and paraffin embedded. Sections were cut at 2 μm and mounted onto SuperFrost Plus slides (Menzel-Glaser, Braunschweig, Germany), which were coated with Movers albumin adhesive (23). Slides were air dried at 37 °C for 72 h and stored at 4 °C until use.

**PRA null (PRAKO) and PRB null (PRBKO) mice**

PRAKO and PRBKO mice were generated as previously described (9, 10). Food (PicoLab Rodent Diet 20; Purina Mills Inc. Richmond, IN) and water were provided ad libitum. Wild-type, PRAKO, and PRBKO mice (C57BL/129SV) were ovariectomized at 6 wk and then rested for 2 wk. Mice (n = 2/treatment) were given daily sc injections of sesame oil (vehicle), sesame oil solution of estrogen (100 ng) or estrogen (100 ng) + progesterone (1 mg) for 4 d. To analyze mammary gland response to estrogen and progesterone, mice were implanted with beeswax pellets (vehicle) or pellets containing hormones (20 μg of estrogen and 20 mg of progesterone) on d 1 and 10, and glands were collected on d 21. Animal studies were approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine and were in accordance with procedures outlined in the National Institutes of Health Guidelines for Care and Use of Laboratory Animals.

**Transfected cell lines**

Primary antibody specificities were established using previously described (6) breast cancer cell lines transfected with either PRA only (MCF-7/M11/PRA) or PRB only (MDA-MB-231/PRB). Cells were fixed in formalin and paraffin embedded. The cell blocks were prepared as described previously (6).

**Primary antibodies**

Preliminary experiments were performed to determine the optimal dilution and incubation times for each primary antibody (data not shown) and to select antibodies that detect PR in the mouse uterus and mammary gland (Table 1). The commercially available PR antibodies tested were: Dako, rabbit polyclonal, catalog no. A 0098 (Dako Inc., Carpinteria, CA); NeoMarkers, clone SP2, catalog no. RM-9102 (NeoMarkers Inc., Fremont, CA); Affinity BioReagents, clone PR-AT 4.14, catalog no. MAI-411 (Affinity BioReagents, Golden, CO); Novocastra, clone 16, catalog no. NCL-PR-312, clone SAN27, catalog no. NCL-PR-B, and clone 16 and

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SAN27, catalog no. NCL-PGR-AB (Novocastra Laboratories Ltd., New-
castle, UK).

The in-house antibodies used to detect PR in this study were raised against human PR (24) and recognize murine PR by immunoblot analysis (15, 16, 25, 26). The mouse antihuman PR monoclonal antibody hPRa6 detects only PR by immunoblot (24) and immunohistochemical staining (6, 27). The mouse antihuman PR monoclonal antibody hPRa7 detects PRA and PRB by immunoblot (15, 24) but only PRA by immuno-
operoxidase staining of formalin-fixed tissue due to hindrance of the PRB epitope on formalin-fixation (27). The selectivity of hPRa7 for PRA in formalin-fixed tissue has also been demonstrated in cell lines ex-
pressing only PRA or PRB (27) and by dual immunofluorescence (6).

ER was detected using NeoMarkers rat monoclonal antibody, clone H226, catalog no. Ab-22, and proliferation (Ki67) was detected using Dako rat monoclonal antibody, clone TEC-3, catalog no. M7249.

Antigen retrieval

A combination of heat and pressure was used for antigen retrieval, as described previously (28). Briefly, immediately before staining, sec-
tions were dewaxed, rehydrated to distilled water, and placed in 0.01 M sodium citrate solution (pH 6.0). Slides were heated in a Tuttnauer 2540 EKA autoclave at 121°C, 15 lb/square inch for 30 min.

Immunoperoxidase staining

Sections were placed in 3.0% (vol/vol) hydrogen peroxide for 5 min, washed, incubated for 1 h with goat Fab fragment to mouse IgG [50 mg/ml in 1% BSA/PBS (Cappel Antibodies, ICN Biomedical, Auro-
ra, CA)], and then for 30 min with normal goat serum (Hunter Antiseras, Jesmon, New South Wales, Australia), diluted 1:1 in PBS. Primary antibodies were diluted in PBT [PBS/0.5% Triton X-100 (Amresco, Solon, OH)], hPRa6 and hPRa7 were used at 1:40 and 1:80, respectively, and incubated for 2 h, whereas the Dako PR primary antibody (Dako) was diluted 1:100 and incubated for 4 h. The Dako primary antibody was diluted 1:100 and incubated overnight, and the Ki67 antibody (Dako) was diluted 1:30 and incubated 1 h. Primary antibody incubation was followed by 30 min incubation with either biotinylated goat antimouse or goat antirabbit antibody (diluted 1:100 and 1:300 in PBS, respectively; Dako) or a goat antirat antibody (diluted 1:100 in PBS; Abcam Ltd., Cambridge, UK) and then for 30 min incubation with streptavidin-biotinylated peroxidase (reconstituted according to the manufacturer’s instructions (Zymed, South San Francisco, CA), or in the case of hPRa7 antibody with streptavidin-horseradish peroxidase diluted 1:100 in PBS (Dako)). PR proteins were visualized using 3,3‘-diaminobenzidine (Dako). The con-
trol sections were treated in the same way except for the replacement of the primary antibody with PBT.

Analysis of PR isoform expression by light microscopy

Stained sections were analyzed using an BX-40 microscope (Olym-
pus, Tokyo, Japan) at ×600 magnification. The PR content was analyzed for each cell type over the entire section and digital images captured using a SPOT CCD camera (SciTech, Victoria, Australia). The images presented are representative of three experiments for which the same results were obtained. For fluctuations in PR expression during the estrous cycle the intensity of staining was graded as follows: –, no staining; –/+; very weak staining; +, weak staining; ++ moderate staining; +++, strong staining; and +++++, intense staining. An overall score for each cycle phase or treatment group (n = 3) was obtained by taking the predominant score from the mice in that group. Results are shown schematically (see Fig. 4) by creating a fourth-order polynomial trend line (Excel 2000; Microsoft, Redmond, WA).

Dual-immunofluorescent staining

Dual-immunofluorescent staining was based on the methodology described previously (6) with the following modification: before incu-
bation with normal goat serum, sections were incubated for 1 h with goat Fab fragment to mouse IgG (as for immunoperoxidase staining de-
scribed above). Briefly, to detect PRB, sections were incubated with hPRa6 diluted 1:40 in PBT, with a biotinylated goat antimouse antibody (Dako), and Texas red (TXR)-avidin (Molecular Probes Inc., Eugene,

OR). To block sites of potential cross reactivity between the two staining sequences, sections were incubated overnight with goat antimouse IgG Fab (50 mg/ml in 1% BSA/PBS; Cappel Antibodies, ICN Biomedical). To detect PRA, sections were incubated with hPRa7 diluted 1:80 in PBT, with a biotinylated goat antimouse antibody (Dako) and fluorescein isothiocyanate (FITC)-avidin (Calbiochem, Sydney, New South Wales, Australia). Under dual-fluorescent excitation, PRB proteins that were labeled with TXR appeared orange/red; PRA proteins, labeled with FITC, appeared green; and nuclei expressing both PRA and PRB at comparable levels were yellow. The protocol was validated by dual-
imunofluorescent staining of transfected cells expressing both PRA and PRB or expressing only PRA or PRB (6).

To control for nonspecific staining, adjacent sections were stained as above, except the primary antibody was replaced with PBT (1) in place of both primary antibodies to control for nonspecific staining and 2) to replace the second sequence primary antibody to ensure no cross-re-
activity between the two staining sequences. Normal human myome-
trium served as a positive control and was stained as above for both PRA and PRB.

Analysis of PRA and PRB colocation by dual-immunofluorescent microscopy

Sections stained by dual immunofluorescence for PRA and PRB were examined using a BX 40 microscope (Olympus) fitted with filters to detect both TXR (BP 545–580) and FITC (BP 450–480) fluorescence simultaneously and each of the two fluorochromes separately. The whole section was examined in detail under both individual fluor-
ochrome excitations and also using the dual filter to identify the local-
ization of each PR isoform. Individual digital images for each fluoro-
chrome were captured using a SPOT CCD camera (SciTech, Victoria, Australia). Because the objective of the dual-immunofluorescent staining was to determine whether PRA and PRB were colocated, signal to noise ratios of the digital images were optimized (SPOT advanced soft-
ware version 4.1; Diagnostic Instruments, Sterling Heights, MI), and optimized images were merged (SPOT advanced software version 4.1; Diagnostic Instruments).

Results

Selective detection of PRA and PRB in the mouse

This study examined PRA and PRB expression in cycling mouse uterus and mammary gland (Fig. 1) and uterus and mammary glands from ovariectomized and hormone-
treated wild-type, PRAKO and PRBKO mice (Figs. 2 and 3). Few studies have examined the individual expression of PRA and PRB in mouse tissue by immunohistochemistry; there-
fore, a panel of commercial and in-house antibodies was tested for PRA or PRB selectivity in the mouse. Most of the antibodies tested recognized human PR in cell lines expressing PRA only or PRB only, and a number of antibodies were selective for human PRA or PRB (Table 1). However, few of these antibodies detected mouse PR (Table 1). Only three antibodies detected mouse PR: hPRa6, recognizing PRA; hPRa7, recognizing PRA; and Dako, recognizing both PRA and PRB (Table 1). The selectivity of hPRa6 and hPRa7 for human PR and PRA, respectively, has been demonstrated previously (27), and their cross-reactivity with mouse PR as demonstrated on immunoblot (15, 25) was confirmed by immunohistochemistry in this study. The Dako antibody detected both human PRA and PRB and mouse PR and was used to validate the selectivity of hPRa6 and hPRa7 for mouse PRA and PRB.

The selectivity of the hPRa6 and hPRa7 antibodies for PRB and PRA, respectively, in the mouse was demonstrated in PRAKO and PRBKO mice (Figs. 2 and 3). The hPRa6 antib-
body did not stain PRBKO uterus or mammary gland, nor
did the hPRA7 antibody stain PRAKO tissues. The staining with hPRA6 and hPRA7 in the PRAKO and PRBKO tissues was confirmed using the Dako antibody, which gave comparable staining to the human PRA antibodies (Figs. 2 and 3). Consistent with published evidence that PRA is more abundantly expressed than PRB in mouse uterus and mammary gland (16), revealing PRB immunoreactivity necessitated the use of the highly sensitive streptavidin-biotinylated peroxidase complex method, whereas PRA was revealed using the less sensitive streptavidin-horseradish peroxidase method.

Only PRB is expressed in luminal epithelium, whereas both PRA and PRB are expressed in stroma and myometrium of cycling mouse uterus

In the luminal epithelium of the uterus, PRB was the only PR expressed at all estrous cycle stages (Fig. 1A). There was no PRA expression in the luminal epithelium at any cycle stage (Fig. 1A). Occasional very weak PRA expression in the glandular epithelium was noted in proestrus evening but not at any other time (not shown). Overall there was little cycle-related fluctuation in PRB expression, and it persisted at moderate to high levels at all cycle stages (Fig. 1A). The lack of dramatic fluctuation of PRB in the luminal or glandular epithelium during the cycle suggests that PRB was not sensitive to cyclical hormonal regulation in the epithelium.

In uterine stroma and myometrium, both PRA and PRB were detected during the estrous cycle (Fig. 1A shows PRA; PRB not shown). Cyclical fluctuations in both PRA and PRB in the stroma and myometrium were apparent, with expression being highest in proestrus morning and lowest in proestrus evening (Fig. 4). Fluctuations of PRA and PRB were greatest in the stroma, and PRA was persistently expressed at moderate to high levels in myometrium (Fig. 1A shows PRA; PRB not shown; Fig. 4).

ER expression and proliferation in the cycling mouse uterus

ER expression was highest in all tissue compartments in diestrus and proestrus morning (Fig. 4), consistent with the timing of the major serum estrogen peak in rodents (19). ER decreased in all compartments in proestrus evening, when serum progesterone is maximal, and there was a second small increase in metestrus. Proliferation, as measured by Ki67 staining, was detected only in the epithelium in proestrus morning and was low or absent in other cell types and at other cycle phases (Fig. 4).
**PRA and PRB expression in cycling mouse mammary gland**

In the mammary gland, PRA and PRB were both expressed in scattered ductal epithelial cells (Fig. 1B). No stromal staining of PR was detected. There were no major cycle-related fluctuations in PRA or PRB levels in the mammary gland (Fig. 1B, proestrus morning and estrus shown), suggesting that these were expressed at physiologically maximal levels at all cycle stages.

**Hormone ablation altered PRA distribution in the mouse uterus and reduced PR expression in the mammary gland**

In cycling mice PRA was totally absent from the luminal epithelium at all cycle phases. Ovariectomy restored PRA expression in the luminal epithelium (Fig. 1A). PRB expression was only mildly reduced in the luminal epithelium after ovariectomy (Fig. 1A), suggesting that in the epithelium, PRB expression was less sensitive than PRA to regulation by ovarian hormones.

In the uterine stroma and myometrium of cycling mice, there were cycle-related fluctuations in PRA or PRB levels (Fig. 1A; PRA and PRB, Fig. 4). In particular, maximal PRA and PRB expression early in proestrus (Fig. 4) was consistent with the timing of the major peak of rodent serum estrogen in proestrus morning (19), and decreased PRA and PRB expression in proestrus evening (Fig. 4) was coincident with the serum progesterone peak at that time (20). Ovariectomy resulted in altered PRA and PRB expression in stroma and myometrium, confirming the role of serum hormones in these observed changes. Stromal and myometrial expression of PRA was markedly reduced by ovariectomy (Fig. 1A), whereas there was less change in PRB (not shown).

In the mammary gland, ovariectomy reduced the overall levels of PR, although the scattered staining patterns of PRA and PRB were the same as those observed in cycling mice (Fig. 1B).

**Hormone treatment alters PRA and PRB expression**

The involvement of ovarian hormones in regulating PRA and PRB expression, as evidenced by the estrous cycle effects on PRA and PRB expression and the effect of ovariectomy, was confirmed by hormone treatment of ovariectomized mice. In the ovariectomized uterus, PRA was decreased by estrogen in the epithelium (Fig. 2, wild type), whereas PRB expression in the epithelium of the estrogen-treated ovari-
ectomized uterus was similar to that in vehicle-treated animals (Fig. 2, wild type). In myometrium (not shown) and stroma (Fig. 2, wild type), PRA was increased by estrogen, suggesting that the proestrus rise in PRA was mediated by the serum estrogen peak at that time. Estrogen treatment also increased PRB in stroma (Fig. 2, wild type) and myometrium (not shown). Estrogen + progesterone treatment down-regulated PRA and PRB expression to undetectable levels in all compartments of the uterus (Fig. 2, wild type).

In the mammary gland, estrogen + progesterone treatment of ovariectomized animals for 21 d resulted in a strong induction of PRA (Fig. 3, wild type). PRB expression in the mammary gland was modestly increased by estrogen + progesterone (Fig. 3, wild type).

**PRA and PRB regulation by ovarian hormones in PRAKO and PRBKO mice**

The individual regulation of PRA and PRB by ovarian hormones was confirmed in mice null for one PR protein. In the uterus of PRAKO mice, PRB expression was increased by estrogen in the stroma (Fig. 2) and myometrium (not shown), consistent with the observations in ovariectomized wild-type mice (Fig. 2). In PRBKO mice, estrogen caused the same decrease in epithelial PRA and increase in stromal (Fig. 2) and myometrial (not shown) PRA as observed in ovariectomized wild-type mice (Fig. 2). Estrogen + progesterone treatment of PRAKO and PRBKO mice reduced PRA and PRB to undetectable levels in all tissue compartments, as observed in wild-type mice (Fig. 2).

In the mammary gland of PRAKO mice, PRB expression was the same as in wild-type mice and estrogen + progesterone treatment caused the same modest increase in expression (Fig. 3). In the mammary gland of PRBKO mice, PRA expression was the same as in wild-type mice and estrogen + progesterone treatment caused the same strong increase in expression (Fig. 3).

**Colocation of PRA and B in mouse tissues**

In the uterine stroma and myometrium and in the mammary gland, where both PRA and PRB are expressed, the question of whether PRA and PRB localized in PR-positive cells was addressed using dual immunofluorescence. In the cycling mouse uterus, as expected, PRA and PRB colocalized in the stroma (Fig. 5A, proestrus morning) and myometrium (not shown), and there were a number of cells expressing PRB only. Consistent with our demonstration that PRB was the only PR expressed in the luminal epithelial cells (Fig. 1A), there was no colocation of PRA and PRB in luminal epithelial cells (Fig. 5A, proestrus morning).

Ovariectomy altered the colocation of PRA and PRB. In the luminal epithelium of ovariectomized mice, there was strong PRA expression and therefore colocation of PRA and PRB (Fig. 5A, ovex, vehicle), whereas there was no colocation in cycling mice (Fig. 5A, proestrus morning). By contrast, because ovariectomy reduced PR expression in the stroma, the colocation of PRA and PRB observed in the stroma of cycling mice (Fig. 5A, proestrus morning) was largely ablated by ovariectomy (Fig. 5A, ovex, vehicle). Treatment of ovariectomized mice with estrogen for 4 d restored the predominant expression of PRB in the luminal epithelium and the lack of PRA and PRB colocation in these cells (Fig. 5A, ovex, estrogen treatment). Estrogen treatment also resulted in increased PRA and PRB in the stroma, and in estrogen-treated animals, there was colocalization of PRA and PRB in the stroma (Fig. 5A, ovex, estrogen treatment) and myometrium (not shown), as had been observed in cycling mice (Fig. 5A, proestrus morning). In the ovariectomized mouse uterus, therefore, PRA and PRB were colocalized in epithelium but not stroma, by contrast with cycling mice in which PRA and PRB were colocalized in stroma but not epithelium. Estrogen treatment of ovariectomized mice restored the colocation of PRA and PRB in the stroma and myometrium observed in cycling mice and also the lack of colocation of PRA and PRB in epithelium.
In the cycling mammary gland, there was colocation of PRA and PRB (Fig. 5B, cycling). The same pattern of colocation was observed in ovariectomized mice (Fig. 5B, ovex). Estrogen/progesterone treatment increased the number of PRA- and PRB-expressing cells, as shown in Fig. 3, but the overall pattern of colocation (not shown) was the same as in vehicle-treated ovariectomized mice (Fig. 5B, ovex).

**Discussion**

**Luminal epithelial cells in the uterus lack PRA throughout the estrous cycle**

There was a striking lack of PRA in the luminal epithelium of the uterus, with PRA never being detected in these cells during the estrous cycle. PRB was expressed at similar levels in all phases, with no marked fluctuations, and PRB was the only PR in the luminal epithelium. By contrast, in the stroma and myometrium, PRA and PRB were both expressed at levels that fluctuated in line with the fluctuating levels of serum hormones during the estrous cycle. The demonstration of PRA and PRB in the uterus in adult virgin mice by immunohistochemistry provides the first information on the overlapping and also the distinct expression of the individual PR proteins in this tissue. These immunohistochemical data are in agreement with previous demonstrations of PRA and PRB expression in adult virgin mouse uterus by biochemical techniques (15, 16), and importantly this study has revealed the cell-specific localization of PRA and PRB, not detectable using biochemical approaches.

The findings on PRA and PRB distribution in the uterus of the cycling mouse are new insights in this species. A previous study examined PR expression in the rat uterus during the estrous cycle (21) but not PRA and PRB individually. In the cycling rat, PR was not detectable in the luminal epithelium, in contrast with our demonstration of PRB in those cells throughout the cycle in the mouse. The discrepancy may be due to differences in PR expression in epithelial cells between rats and mice, but it may be due equally to technical limitations of the PR antibodies available. A proportion of PR antibodies fail to detect PRB in formalin-fixed tissues (27), even if the two proteins are detectable by immunoblot. Staining of frozen tissue sections is likely to avoid the fixation-related failure of PR antibodies to detect PRB (27) but is a less...
sensitive technique that may fail to detect the relatively lower PRB levels in rodent tissues, compared with PRA (16).

**PRA and PRB expression in ovariectomized mouse uterus does not mimic expression in the estrous cycle**

In the cycling mouse uterus, only PRB was expressed in the luminal epithelium, whereas in ovariectomized mice both PRA and PRB were present in these cells. The lack of PRA in the luminal epithelium throughout the estrous cycle was attributable to estrogen-mediated suppression of its expression because ovariectomy resulted in the appearance of PRA in the luminal epithelium, and estrogen treatment of ovariectomized mice resulted in PRA loss. By contrast, PRB expression in the luminal epithelium was largely unchanged during the cycle and after ovariectomy and was not markedly affected by estrogen treatment of ovariectomized mice, demonstrating that PRB in the luminal compartment was relatively insensitive to endocrine regulation during the cycle.

These findings demonstrated that PRA and PRB are divergently regulated by estrogen in luminal epithelial cells of the mouse uterus, with PRA being decreased and PRB persisting on estrogen treatment. This is at odds with an earlier study that concluded total PR expression in the luminal epithelial compartment of the mouse was decreased by exposure to estrogen (29). The previous study used the same Dako antibody used in this study, which detects both PRA and PRB. Differences between this and the previous study are methodological because shorter antigen retrieval times before immunohistochemistry were used in that study. This may have reduced the sensitivity of detection of PRB, which is known to be expressed at overall lower levels than PRA in the uterus (16). In both human (28) and mouse (current study) tissues, detection of PRB requires extensive antigen retrieval, and the shorter antigen retrieval times in the previous study (29) may explain the lack of detection of PR on estrogen exposure.

In the stroma and myometrium, cycle-related changes in PRA and PRB were consistent with similar regulation of both PRA and PRB in stroma and myometrium by hormonal manipulation. This was supported by the demonstration that ovariectomy reduced levels of both proteins in the stroma and myometrium, and estrogen administration to ovariectomized animals restored their levels. The hormonal regulation of PRA and PRB throughout the cycle in the stroma and myometrium in mice is in keeping with the previously demonstrated fluctuations in total PR levels in the rat, mediated by estrogen and progesterone in those compartments (21).

**PRA and PRB expression and endocrine regulation in mammary epithelial cells**

In the mammary gland, overlapping PRA and PRB expression was demonstrated, with both PRA and PRB being expressed in scattered epithelial cells only within the ductal structures. There were modest or no fluctuations in PRA and PRB expression during the estrous cycle, demonstrating the relative lack of systemic endocrine control of PR expression in adult virgin mammary gland.

The demonstration of PRA and PRB by immunohistochemistry in this study is consistent with previous demonstrations of both PR isoforms in the virgin adult mouse mammary gland by photoaffinity labeling, immunoprecipitation, and immunoblot analysis, using partially purified tissue preparations (15, 16). PR levels were consistently lower by an order of magnitude in the mammary gland than in the uterus (16), and PRA was consistently higher than PRB, supporting our observations in this study. The published data and findings of the current study, that PRB is expressed in the adult mouse virgin mammary gland, are in contrast with a recent report failing to detect PRB in this tissue (30). That report used the same antibody to detect PRB as used in the present study but at a considerably higher dilution, and because published biochemical data suggest relatively low levels of PRB in the mammary gland (16), it is possible that failure to detect PRB in that study (30) could be due to differences in sensitivity of the methods used.

**Ovarian hormone control of PRA and PRB colocation in cycling mouse**

In cycling animals, serum ovarian hormones regulated the colocation of PRA and PRB in the uterus. In the luminal epithelium, cyclical estrogen exposure resulted in absence of PRA, and this prevented colocation of PRA and PRB in these cells at any stage of the estrous cycle. Ovarian ablation resulted in the appearance of PRA in the luminal epithelium and the colocation of PRA with PRB in these cells. In light of the demonstration in this study, that colocalization of PRA and PRB in the luminal epithelium never occurs in virgin adult animals in vivo, endpoints of PR action in the epithelium observed in ovariectomized animals may need to be viewed with caution. Whereas estrogen administration restores the relative expression of PRA and PRB in luminal epithelium seen in the cycling animal, the colocation induced simply by ovarian ablation may lead to different PR regulation of gene expression and different functional outcomes in the luminal epithelium than those occurring physiologically.

In the stroma and myometrium, PRA and PRB were co-located in PR-positive nuclei, demonstrating the potential for their interaction in mediating progesterone action in these cells. Ovarian ablation decreased PRA expression in the stroma more markedly than that of PRB, so in ovariectomized mice there was less colocation of PRA and PRB than in the intact cycling mice. Taken together, our results in the uterus suggest that ovariectomy followed by endocrine manipulation does not reproduce the physiological context of PRA and PRB expression and colocation observed in the intact mouse. In the mammary gland of cycling animals, PRA and PRB were colocalized within the same nuclei and ovarian ablation did not markedly alter the proportion of cells with colocalized PRA and PRB.

**Functional implications of distinct PR isoform expression patterns**

The distinct expression of PRA and PRB in the luminal epithelium is likely to have functional consequences. PRA is required to inhibit ER-mediated proliferation in the mouse uterus (8), and because proliferation during the estrous cycle
was detected only in the luminal epithelium in proestrus morning and PRA was lacking from these cells at all cycle stages, we conclude that augmented stromal and/or myometrial PRA in proestrus was responsible for inhibition of epithelial proliferation. This substantiates previous findings, from tissue recombination experiments, that stromal PR is both essential and sufficient to mediate the inhibition of estrogen-induced proliferation in mouse uterine epithelium (31). This study on PR isoform expression in adult mice upholds the view that PRA and PRB expressed in different cell compartments may be mediating different aspects of the proliferative and differentiating program regulated by progesterone in the mouse uterus. In support of this, the continued expression of only PRB in luminal epithelium may mediate differentiated function throughout the estrous cycle, including secretion into the lumen at estrus, and leukocyte infiltration later in the cycle (32). The coexpression of PRA and PRB in the mammary gland and the lack of regulation during the estrous cycle are consistent with a role for both proteins in the virgin mammary gland.

The unequal expression of PRA and PRB in the mammary gland (16) and the expression of PRA and PRB in different cells in the mouse ovary (17) and mouse uterine luminal epithelium (this study) contrast with the expression of PRA and PRB in human tissues. In normal human adult breast and uterus, all PR-positive epithelial cells coexpress PRA and PRB at similar levels (5, 6). The relative levels of PRA and PRB become disrupted early in malignancy, and in invasive breast cancers, a predominance of PRA is common (5). The alteration of coexpression of PRA and PRB to a predominance of PRA is associated with changed PR signaling, including changes in cell cytoskeleton and migration properties and gene expression (14, 33, 34). Specifically, loss of one isoform confers progestin sensitivity to genes that are not normally targets of PR, and this is supported by the observation that cells containing only one PR isoform regulate more genes than those containing both (14). These data in human tissues suggest that fidelity of response to progesterone is maintained in part by concordant expression of PRA and PRB in normal target cells and that loss of concordant expression results in aberrant progesterone response. In the mouse by contrast, PRA and PRB are unequally expressed, with PRA normally being more highly expressed than PRB. Moreover, there is nonoverlapping expression of PRA and PRB in the luminal epithelial cells of the uterus, and the lack of colocalization of the two PR isoforms strongly suggests the homodimer to be an active species in those cells. This study demonstrates that unequal and nonoverlapping expression of PRA and PRB may be one mechanism contributing to the different activities of PR proteins in the mouse, whereas colocalization and cooperative activity of PRA and PRB are likely to be responsible for PR action in the human.

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