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**Title: Progesterone Receptor Isoforms A and B: Temporal and Spatial Differences in Expression During Murine Mammary Gland Development**

Running Title: PR isoform expression in mammary gland

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**Abstract**

Progesterone (P) is a potent mitogen in the mammary gland. Based on studies using cells and animals engineered to express progesterone receptor (PR) isoforms A or B, PRA and PRB are believed to have different functions. Using an immunohistochemical approach with antibodies specific for PRA only or PRB only, we show that PRA and PRB expression in mammary epithelial cells are temporally and spatially separated during normal mammary gland development in the Balb/c mouse. In the virgin mammary gland when ductal development is active the only PR protein isoform expressed was PRA. PRA levels were significantly lower during pregnancy, suggesting a minor role at this stage of development. PRB was abundantly expressed only during pregnancy, during alveologenesis. PRA and PRB colocalization occurred in only a small percentage of cells. During pregnancy there was extensive colocalization of PRB with BrdU and cyclin D1; 95% of BrdU positive cells and 83% of cyclin D1 positive cells expressed PRB. No colocalization of PRA with either BrdU or cyclin D1 was observed at pregnancy. In the virgin gland PRA colocalization with BrdU or cyclin D1 was low; only 27% of BrdU positive cells and 4% of cyclin D1 positive cells expressed PRA. The implication of these findings is that different actions of P are mediated in PRB positive vs. PRA positive cells in vivo. The spatial and temporal separation of PR isoform expression in mouse mammary gland provides a unique opportunity to determine the specific functions of PRA vs. PRB in vivo.

## Introduction

The relative roles of estrogen (E) and progesterone (P) in regulating epithelial cell proliferation of the normal human breast and their contributions to breast cancer risk have been controversial. Originally it was presumed that since P antagonizes E-induced proliferation in the uterus, it would also antagonize E-induced proliferation in the breast (1). However, P in combination with E has more potent proliferative activity than E alone in the adult mammary gland in animal models (monkey and rodent) (2, 3) and in the adult human breast (4). In humans this is the case for premenopausal cycling women and for postmenopausal women receiving hormone replacement therapy (HRT). In postmenopausal women, combined continuous E+P HRT is associated with the highest proliferative index and the highest increase in breast epithelial density when compared to no HRT or E alone HRT (4). Furthermore, a significantly greater breast cancer risk is associated with E+P HRT (5-8). Thus, P can contribute significantly to breast cancer risk.

Progesterone action is mediated through binding to the progesterone receptor (PR). The progesterone receptor (PR) consists of two isoforms, PRA and PRB, which are expressed from a single gene in both humans and rodents (9). Two promoters, one specific for PRA and the other specific for PRB, have been identified for human (10) and rat (11) PR. Initiation of translation at two distinct AUG signals produces the B and A forms of PR. PRB differs from PRA by an amino terminal extension of 164 amino acids. Studies to identify the functional roles of PRA and PRB in the mammary gland have been carried out in vivo using transgenic mice (PRA or PRB transgenes) (12, 13) and PR gene-deleted mice [total PR (PRKO), PRA only (PRAKO) or PRB only (PRBKO)] (14-16). From these studies it has been inferred that PRB is required for alveologenesis during pregnancy. The specific function of PRA has not yet been identified. In vitro studies using cell lines have shown that the unique amino terminal region of PRB encodes a

transactivation function that plays an important role in specifying target genes that can be activated by PRB but not by PRA (17). Therefore, PRA and PRB can have different functions in the same cell, and the activity of the individual isoforms of the receptor may also vary among different types of cells.

The mouse is currently the most extensively studied and best understood model of progesterone action in the normal mammary gland. Genetically altered mice have provided some insights into the functions of the two PR isoforms in mouse mammary gland. These genetically altered mice have an altered mammary gland phenotype (12-16); this suggests that mammary gland development is abnormal. Our approach in the present study was to investigate specific PR isoforms in mammary gland of genetically unaltered, wildtype mice as a function of development.

Biochemical methods to analyze PR isoform expression and function in the mouse mammary gland have provided limited information about the functional roles of PRA and PRB because they do not provide insight into the cellular distribution or colocalization of the isoforms. The most direct approach to address this question is immunohistochemical analysis of PR isoform-specific expression. It was generally assumed that if an anti-PR antibody detected both isoforms in immunoblot analysis, then it also detected both isoforms in immunohistochemical analysis (16, 18, 19). The report of Mote et al. (20) showed that this assumption is not correct. Mote et al. (20) analyzed a panel of 11 anti-human PR antibodies for their ability to detect PRA and/or PRB in human cells engineered to express specific isoforms of PR. To determine antibody specificity, MCF-7 breast cancer cell sublines that express only PRA, only PRB or both PRA and PRB were analyzed (20). By immunoblot analysis, 10 of the antibodies detected both PRA and PRB; only one antibody detected only PRB. By contrast, by immunohistochemistry, eight of the antibodies detected only PRA. These 8 antibodies were

unable to detect PRB in MCF-7 cells expressing only PRB. Two of the antibodies detected both PRA and PRB. Only one antibody detected PRB only.

The findings of Mote el al. demonstrate the importance of using anti-PR antibodies with well defined immunohistochemical PRA or PRB isoform specificity. Previous studies of PR in mouse mammary gland used anti-PR antibodies that had not been characterized for immunohistochemical PR isoform specificity (16, 18, 19). The purpose of the present study was to determine the in vivo expression pattern of PRA and PRB proteins in mouse mammary gland by immunohistochemistry using well characterized, PR isoform-specific antibodies. We have used antibodies that detect only PRA or only PRB by immunohistochemistry in human tissues and have also been shown to have the same isoform specificity in mouse ovary (21). Using these PR isoform-specific antibodies we analyzed PR isoform expression and colocalization in various structures of the normal mouse mammary gland (ducts, end buds, side branches, alveoli) at different developmental stages that are known to exhibit different proliferative and morphological responses to progesterone (22-24). We also investigated colocalization of PRA, PRB, BrdU and cyclin D1.

## Materials and Methods

### Animals

Balb/c female mice from our own colony were the source of mammary glands at the following ages and developmental stages: virgin immature (3 or 6 weeks), virgin adult (10-12 or 17-20 weeks), pregnant (7 or 14 days), lactating (10 days), or postpartum involuting (9 weeks). To simulate mammary gland development during pregnancy, ovary intact virgin mice received subcutaneous beeswax pellets containing 17β-estradiol (20 μg) plus progesterone (20 mg) (E+P) for 13 days. C57BL PRA null mice were obtained from Dr. Orla Conneely (Baylor College of

Medicine). All animal experimentation was conducted in accord with accepted standards of humane animal care, and approved by the All University Committee on Animal Use and Care at Michigan State University.

Immunohistochemistry with anti-PR isoform-specific antibodies

Mouse monoclonal antibodies specific in immunohistochemistry for PRA only (hPRa7; referred to as anti-PRA antibody) or PRB only (hPRa6; referred to as anti-PRB antibody) (20, 21) were a generous gift from Dr. Christine Clark (University of Sydney) or were purchased from Neomarkers (Fremont, CA). Mammary tissues were fixed in 10% phosphate buffered formalin (0.4% sodium phosphate monobasic and 0.65% sodium phosphate dibasic (anhydrous) in 10% formalin) overnight at 4 C, dehydrated, cleared and embedded in paraffin. Five μm sections were mounted onto coverslips to which 3-aminopropyl triethoxysilane (APES) had been applied, and allowed to dry for 24 hours at room temperature. Tissue sections were immersed in 10 mM sodium citrate solution (pH 6.0) and exposed to a combination of heat and pressure for antigen retrieval as previously described (25). The protocol used to detect PRA or PRB in mouse mammary gland was similar to that used in human breast tissue (26) and mouse ovary (21) as described. To block non-specific background staining, sections were incubated with goat anti-mouse IgG Fab fragments (Jackson Laboratories, West Grove, PA) (1:100 in phosphate buffered saline (PBS) containing 1% BSA (1% PBSA), 60 min), rinsed with PBS, and then blocked with normal goat serum (Vector Laboratories, Burlingame, CA) (1:1 dil in PBS, 30 min). Incubation with primary mouse anti-PRA or anti-PRB monoclonal antibody (1:100 dil in PBS/0.5% Triton-X 100) was for 1 hr followed by 30 min with a biotinylated goat anti-mouse antibody (Dako, Carpinteria, CA) (1:400) and ABC reagent (Vector Laboratories, Burlingame, CA). Two PBS rinses were performed between incubation with each antibody. Immunoperoxidase localization of antibody staining was obtained using 3'-3'- diaminobenzidene

(DAB). The sections were counterstained with hematoxylin. Sections were visualized using a Nikon Eclipse 400 microscope and a SPOT RT color camera with SPOT software (Diagnostic Instruments, Sterling Heights, MI).

Double labeling with PRA and PRB isoform-specific antibodies.

When we labeled with either anti-PRA or anti-PRB antibody alone, virgin and pregnant mammary glands yielded the same isoform-specific staining patterns whether detection was by immunoperoxidase or immunofluorescence. However, when we double-labeled virgin or pregnant mammary gland with the anti-PRA antibody plus anti-PRB antibody, the PRA- and PRB-specific patterns were not maintained and all PR positive cells were positive for both PRA and PRB. We overcame this antibody staining artifact in double labeling experiments, by using a rabbit polyclonal anti-PR antibody, SC#538 (Santa Cruz Biotechnology, Santa Cruz, CA) that we demonstrated in this study recognizes only PRA (see Fig. 7). With this method the PR isoform-specific patterns were maintained in double labeling experiments. After antigen retrieval, sections were incubated overnight at 4 C with SC#538 (1:400 in 2% PBSA), rinsed twice with PBS, and incubated with goat anti-rabbit antibody conjugated to Alexa 488 (green), (Molecular Probes, Eugene, OR) (1:100 in PBS, 30 min). Sections were then blocked with goat anti-mouse IgG Fab fragments (Jackson Laboratories, West Grove, PA) (1:100 in 1% PBSA, 60 min), blocked with normal goat serum (Vector Laboratories) (1:1 in PBS, 30 min), and incubated overnight at 4 C with mouse monoclonal primary antibody (anti-PRB,1:50 in PBS-0.5% Triton-X 100). PRB localization was detected with goat anti-mouse secondary antibody conjugated to Alexa 546 (red) (Molecular Probes, Eugene, OR) (1:100 in PBS, 30 min). In some experiments the fluorochromes used to detect PRA and PRB were reversed. Nuclei were counterstained with TOPRO-3 Iodide (blue) (Molecular Probes, Eugene, OR) and sections were visualized and images captured using a Zeiss Pascal laser scanning confocal microscope.

Immunoblot analysis

In the 6-week-old virgin mammary gland there is a high ratio of stroma to epithelium. To overcome the problem of dilution of epithelial cell proteins, mammary epithelial cells were obtained from pooled mammary glands of seven 6-week-old mice and enriched by an enzymatic dissociation method used to obtain epithelial cells for primary culture, as previously described (27). Whole mammary glands were obtained from 14-day pregnant mice. Uteri were obtained from 6-week-old virgin mice. Whole mammary glands or uteri were minced and homogenized in PEMTG buffer (50 mM potassium phosphate pH 7.0, 10 nM EGTA, 10 mM sodium molybdate, 12 mM thioglycerol, 10% glycerol) (1ml/gm mammary tissue, 0.5 ml/uterus) containing protease inhibitor cocktail (Sigma, St. Louis, MO) using a Polytron homogenizer. Epithelial cells were sonicated in 400 µl PEMTG buffer. Homogenates were centrifuged at 14000g for 30 min and supernatants were used for immunoblots. Mammary gland extract (35 µl) or uterine extract (15 µl) was mixed with NuPAGE LDS sample buffer and NuPAGE Sample Reducing reagent (Invitrogen, Carlsbad, CA) according to the manufacturer instruction and boiled for 10 min at 70º C. Protein samples were resolved on 4-20%NuPAGE Bis-Tris gel (Invitrogen, Carlsbad, CA) under denaturing conditions and transferred onto Protran nitrocellulose membranes (Schleicher&Schuell, Keene, NH). Membranes were treated with Qentix Western Blot Signal Enhancer (Pierce, Rockford, IL), blocked in 5% milk in Tris-Buffered Saline with 0.5% Tween-20 overnight at 4ºC and incubated with primary antibodies for at least 2 hrs at room temperature. To detect PR, mouse monoclonal anti-human PR hPRa7 (dil 1:100) or hPRa6 (dil 1:100) (Neomarkers, Fremont, CA) or rabbit polyclonal anti-human PR SC#538+SC#539 (dil 1:100 for each) (Santa Cruz Biotechnology, Santa Cruz, CA) primary antibodies were used. The combination of SC#538+SC#539 was used for immunoblot analysis of pregnant mammary gland in an attempt to enhance detection of PRA, because PRA expression was reduced during

pregnancy. The secondary antibodies were horseradish peroxidase labeled sheep anti-mouse antibody (dil1:2000) (Amersham, UK) or donkey anti-rabbit antibody (dil 1:2000) (Santa Cruz Biotechnology, Santa Cruz, CA), respectively. After 1 hr incubation with secondary antibodies membranes were washed, incubated with Super Signal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) and exposed to X-ray film for 2-10 min.

Colocalization of PRA , PRB, cyclin D1 and BrdU

For these studies mouse monoclonal anti-BrdU antibody (provided as a kit from Amersham Biosciences, Piscataway, NJ) and mouse monoclonal anti-cyclin D1 antibody (Cell Signaling Technology, Beverly, MA) were used. After antigen retrieval tissue sections were incubated overnight at 4°C with mouse monoclonal anti-PRA or anti-PRB antibody. PRA or PRB localization was detected with goat anti-mouse secondary antibody conjugated to Alexa 546 (red) (Molecular Probes, Eugene, OR) (1:100 in PBS, 30 min). Sections were then blocked with goat anti-mouse IgG Fab fragments (Jackson Laboratories, West Grove, PA) (1:200 in 1% PBSA, 60 min), blocked with normal goat serum (Vector Laboratories) (1:1 in PBS, 30 min), and incubated for one hour at RT with anti-BrdU antibody or overnight at 4 C with the anti-cyclin D1 antibody ( 1:200 in 2% PBSA). BrdU and cyclin D1 localization were detected with a biotinylated goat anti-mouse secondary antibody (Dako, Carpinteria, CA) (1:400 in PBS, 30 min), which was recognized by streptavidin-conjugated Alexa 488 (green) (Molecular Probes, Eugene, OR) (1:100 in PBS, 45 min).

For all dual immunofluorescent labeling, nuclei were counterstained with TOPRO-3 Iodide (blue) (Molecular Probes, Eugene, OR) and sections were visualized and images captured using a Zeiss Pascal laser scanning confocal microscope.

PR quantitation and statistical analyses

Sections treated for PRA and/or PRB detection by immunoperoxidase or immunofluorescence methods were quantitated for the number of PRA and/or PRB positive cells with the aid of a light microscope (immunoperoxidase) or from captured images (immunofluorescence). Three to 10 mice per developmental stage were analyzed; a minimum of 1000 total cells and 3 independent sections per mouse were analyzed. PR positive cells are expressed as a percentage of total epithelial cells counted. Results are expressed as mean ± SEM and differences are considered significant at $P < 0.05$ by using Student's t test or ANOVA where appropriate.

**Results**

Immunoperoxidase localization and quantitation of PRA at different stages of mammary gland development

The earliest age examined for PRA expression was 3 weeks of age. At this age ovarian cycles have not yet started and the pre-pubertal mammary gland exists as a small epithelial rudiment similar to the one present at birth; the percentage of PRA positive cells was 55 ± 2% (Fig. 1). By 6 weeks of age, ovarian cycles have started and in the pubertal 6-week-old virgin mammary gland 58 ± 3% of mammary epithelial cells were PRA positive (Fig. 1). At 6 weeks of age, the PRA positive cells were observed in end buds (Fig. 2A, E) and in ducts (Fig 2B, F). PRA positive cells in end buds were localized in the internal layer of cells; the cap cell layer of end buds was negative for PRA (Fig. 2E). At 10-12 week of age the mammary glands of most mice had grown to the limits of the fat pad; however the glands of some mice (23%) still contained endbuds. In the mammary glands of 10 to 12-week-old virgin mice, the percentage of PRA positive epithelial cells in ducts was 50 ± 2 % (Figs. 1, 2C,G), which was not significantly different from 3- or 6-week-old virgin. We also examined the effect of estrus cycle stage on PRA

expression; no difference in the percentage of PRA positive cells was observed at estrus vs. diestrus (52 ± 3% estrus vs. 51 ± 4% diestrus). At 17-20 weeks of age, in all cases, endbuds were no longer detected and the ductal tree had grown to the limits of the mammary fat pad. The percentage of PRA positive cells decreased significantly to 28 ± 3 % ($p < 0.05$) (Fig.1). At 7 days of pregnancy PRA was detected in 25 ± 1 % of cells (Fig.1). However, at 14 days of pregnancy, PRA was detected in only 11 ± 2 % of ductal epithelial cells and 6.0 ± 0.3% of alveolar cells ($p< 0.001$)(Figs. 1, 2D,H). No PRA positive cells were detected during lactation (Fig. 1). After lactational involution, at 9 weeks post-weaning, PRA was detected in 12 ± 1% of the ductal cells and 10 ± 1% regressed alveolar cells (Fig. 1). Notably, the percentage of PRA positive cells was significantly lower after pregnancy compared to age-matched virgin mice at 17- 20 weeks of age ($p <0.01$) (Fig. 1). Antibody staining of PRA was always localized to the nucleus of epithelial cells and was not detected in myoepithelial cells or stromal cells at any of the developmental stages studied (Fig. 2).

Immunoperoxidase localization and quantitation of PRB at different stages of mammary gland development

No PRB positive cells were detected in 3, 6, 10-12 or 17-20 week-old virgin mammary glands (Figs. 3, 4A,B,D,E). During pregnancy, no PRB was detected at 7 days, but PRB was abundantly expressed by 14 days, in 48 ± 4% of epithelial cells (Fig. 3). PRB was localized mainly in alveolar cells (Fig. 4C,F). PRB staining was seen in both the cytoplasm and nucleus of epithelial cells (Fig. 4F). PRB was not detected in myoepithelial cells or stromal cells (Fig. 4F). No PRB was detected in the lactating mammary gland. After lactational involution PRB staining was observed in 6 ± 1 % of cells in remaining alveolar structures (Fig. 3); less than 1% of ductal cells were PRB positive.

PR isoform specificity of antibodies for immunohistochemistry

Although Mote et al.(20) had already demonstrated PRA and PRB isoform specificity of monoclonal anti- PRA (hPRa7) and anti-PRB (hPRa6) antibodies, respectively, we sought further confirmation using PRA gene-deleted mice (PRAKO) (15). Fig. 5A shows that no staining was detected with the anti-PRA antibody in virgin 8-week-old PRAKO mice, whereas PRA positive cells were detected in wildtype 8-week-old virgin mice. PRAKO mice cannot become pregnant; however, E+P treatment induces pregnancy-like lobuloalveolar development (15). Fig. 5A also shows that no PRA staining was detected in E+P-treated PRAKO mice, whereas PRA staining was detected in E+P-treated wildtype mice.

Fig. 5B shows that PRB staining was observed in 12-week-old E+P-treated PRAKO mice with the anti-PRB antibody, and the pattern of staining was the same as seen in wild type E+P-treated mice. No PRB staining was detected in 8-week-old virgin PRAKO or wildtype mice (Fig. 5B). These results demonstrate that the anti-PRB antibody detects PRB only in PRAKO mice (under conditions of simulated pregnancy) similar to wildtype mice. Thus, the staining patterns obtained in PRAKO mice confirmed the specificity of the anti-PRA antibody to detect only PRA and the specificity of the anti-PRB antibody to detect only PRB.

Immunoblot analysis of PRA and PRB expression

The apparent absence of PRB in virgin mammary gland, based upon immunohistochemistry, was explored further by immunoblot analysis, using an antibody that detects both PRA and PRB isoforms (Fig. 6A). As expected both PRA and PRB were detected in mouse uterus immunoblot (lane 1) which is known to express both isoforms (28). By contrast, this antibody detected only PRA in virgin mammary gland (Fig. 6A, lane 2). PRB was not detected in virgin mammary gland using antibody specific for PRB (hPRa6) ( Fig. 6B, lane 2); the same antibody detected PRB in mouse uterus (Fig. 6B, lane 1). These findings are consistent

with our immunohistochemical finding of only PRA in virgin mammary gland, and indicate that absence of PRB is not due to epitope masking.

The low level of PRA in pregnant mammary gland, based on immunohistochemistry, was explored further by immunoblot analysis using antibody that detects both isoforms (Fig. 6B). Immunoblot analysis showed only a PRB band (Fig. 6B, lane 2), consistent with immunohistochemistry showing a predominance of PRB over PRA. Failure to detect a PRA band is likely due to the low percentage of PRA positive cells in pregnant mammary gland.

Immunofluorescence colocalization of PRA and PRB

The different patterns of PRA and PRB expression observed during pregnancy suggested that PRA and PRB are present in different cells. To test this hypothesis we undertook colocalization studies with the anti-PRA and anti-PRB antibodies. Immunofluorescent labeling of virgin and pregnant mammary glands with either anti-PRA or anti-PRB antibody alone yielded the same isoform-specific staining patterns that were obtained by immunoperoxidase detection. However, when we double-labeled virgin or pregnant mammary gland with the anti-PRA antibody plus anti-PRB antibody, the PRA- and PRB-specific patterns were not maintained and all PR positive cells were positive for both PRA and PRB. To overcome this artifact, we sought to identify another antibody that was specific for only PRA in immunohistochemistry.

The pattern of PRA expression that we observed in the virgin mammary gland with the anti-PRA antibody was similar to antibody staining patterns reported by others who used the SC#538 anti-PR antibody (18). This led us to surmise that the SC#538 antibody might in fact be PRA-isoform specific. To directly test this hypothesis, we carried out double labeling experiments with the anti-PRA antibody plus SC#538 antibody, and used immunofluorescence confocal microscopy to investigate colocalization of the antibodies. The results presented in Fig. 7A show complete colocalization of the anti-PRA antibody with the SC#538 anti-PR antibody in

the virgin mammary gland. In the 14-day pregnant gland (Fig. 7B) the SC#538 antibody also showed complete colocalization with the anti-PRA antibody and the same low level of expression (relative to the virgin) that was observed with the monoclonal anti-PRA antibody (Fig 2D,H). PRA was exclusively localized in the nucleus with the SC#538 antibody in both virgin and pregnant mammary glands. Thus, it appears that the SC#538 antibody is specific for the PRA isoform in immunohistochemistry. The SC#538 has also been shown to be specific for PRA in immunohistochemistry in human cells (20).

Having established the specificity of SC#538 to detect only PRA, we carried out colocalization studies of PRA and PRB in double labeling experiments with SC#538 and the anti-PRB antibody. At 14 days of pregnancy, three subsets of cells were found: cells that expressed PRA only, PRB only, or both PRA and PRB (Fig. 8A, B). Forty-three percent of cells were positively labeled for PRB (Fig. 8A). Of the PRA positive cells (8%), about half were also positive for PRB (Fig. 8A). Thus, colocalization of PRA and PRB occurred in only 4% of cells during pregnancy.

PR isoform expression and colocalization with cyclin D1 or BrdU

A role for P has been implicated in ductal development in the virgin mammary gland (19). PRB and cyclin D1 are required for alveologenesis during pregnancy (16, 29). Epithelial cell proliferation is common to both ductal development and alveologenesis. Having found that PRA and PRB are present in different cells and at different stages of mammary gland development, it was of interest to determine how PR isoform expression was related to proliferation and cyclin D1 expression. To accomplish this, mammary glands were obtained from 14 day pregnant and 6-week-old mice injected with a pulse of BrdU 2 hours prior to sacrifice, to label cells in S-phase. Tissue sections were double labeled with anti-BrdU plus anti-PRA antibody or with anti-BrdU antibody plus anti-PRB antibody. Additional tissue sections were also double labeled with

anti-cyclin D1 antibody plus anti-PRA or anti-PRB antibody. Immunofluorescence confocal microscopy was used to determine the colocalization of PRA and/or PRB with BrdU or with cyclin D1.

In the pregnant mammary gland 16% of cells were BrdU positive at 2 h post BrdU injection, and 46% of cells were PRB positive (Fig. 9A). Fifteen percent of cells were BrdU and PRB positive; thus 95% of BrdU positive cells were PRB positive (Figs 9A,10A). In pregnant mammary gland PRA and BrdU were not colocalized in the same cells (Figs. 9A,10B).

We also analyzed PRA and BrdU colocalization in the 6 week-old, virgin mammary gland. We chose this age and stage of development because there is extensive proliferation and a high percentage of PRA positive cells in the virgin mammary gland. We found that 15% of cells were BrdU positive, 56% of cells were PRA positive and 4% were PRA and BrdU positive (Fig. 9B). Thus only 27% of BrdU positive cells were PRA positive and only 7% of PRA positive cells were BrdU positive. Most BrdU positive cells were located in the cap cell layer of end buds (Fig. 10D), which is a region of the end bud that is devoid of PRA positive cells (Fig. 2A,E). Fewer BrdU positive cells were present in ducts (Fig. 10C).

In the pregnant mammary gland 56% of cells were cyclin D1 positive and 49% were PRB positive (Fig. 11A). Forty-six percent of cells were positive for both PRB and cyclin D1; thus 83% of cyclin D1 positive cells were PRB positive and 94% of PRB positive cells were cyclin D1 positive (Figs.12A). There was no colocalization of PRA with cyclin D1 (Figs.11A, 12B).

In the 6-week-old virgin mammary gland the percentage of cyclin D1 positive cells was significantly less than in pregnant mammary gland (18% vs. 56%; $p<0.05$) (Fig 11A,B). Fifty-four percent of cells were PRA positive, and cyclin D1 and PRA were colocalized in 1% of cells; thus 4% of cyclin D1 positive cells were also PRA positive and 2% of PRA positive cells

were cyclin D1 positive (Fig. 11B). Fig. 12C illustrates colocalization in a duct that is cyclin D1 positive. Many ducts had no cyclin D1 positive cells, yet PRA was highly expressed (Fig. 12D).

**Discussion**

The results presented in this paper demonstrate that PRA and PRB expression are temporally and spatially separated during murine mammary gland development. Only PRA was highly expressed in the immature and adult virgin mammary gland. By contrast, PRB was seen only during pregnancy, mainly in alveolar epithelial cells. During pregnancy, the majority of PR positive cells contained only PRB and colocalization of PRA and PRB occurred in a small proportion of epithelial cells. During pregnancy PRB colocalized extensively with the proliferation marker BrdU and with cyclin D1. In contrast PRA did not colocalize with BrdU or cyclin D1 during pregnancy and was infrequently colocalized with BrdU or cyclin D1 in the virgin gland. The implication of these findings is that different actions of P are mediated in PRB positive vs. PRA positive cells in vivo.

Progesterone action in the virgin mammary gland: predominant role of PRA

In the 6-week-old immature virgin gland while 54 % of epithelial cells were PRA positive, only 2% of PRA positive cells were cyclin D1 positive and only 4% of PRA positive cells were BrdU positive. These results indicate that the majority of PRA positive cells were not in S-phase during our 2 hour labeling period. Most BrdU positive cells were in the cap cell layer of end buds, which is recognized to be a major growth point. The cap cell layer was devoid of PRA positive cells, supporting the concept that PRA positive cells do not constitute the major pool of proliferating cells. We cannot rule out the possibility that P may play a role in proliferation via a paracrine mechanism in which PRA positive cells produce a factor that affects the proliferation of neighboring PR negative cap cells.

Proliferation leading to ductal elongation occurs via cap cell proliferation and is mediated by E and growth factors such as EGF, HGF and IGF-I (3, 30). The requirement for E is supported by the complete absence of ductal elongation in ERα gene-deleted mice (31). In contrast, ductal elongation does occur in total PR gene-deleted (PRKO) mice (14). These results indicate that the presence of PR is not an absolute requirement for ductal elongation in the virgin gland.

Organogenesis during embryonic development results from the net effect of the precise spatial patterning of proliferation and apoptosis. Similarly, postnatal ductal development in the mammary gland can be considered to be the result of spatially organized proliferation and apoptosis. Proliferation occurs in the cap cell layer of the endbud, giving rise to a multilayered internal mass of cells below the cap cell layer (32). Formation of the ductal lumen requires the removal of this internal cell mass. Apoptotic cells have been observed in this internal layer of cells of the end bud (32), suggesting that apoptosis may play a key role in lumen formation in ducts. We have previously shown in vitro that mammary organoids derived from virgin mammary gland respond to the synthetic progestin, R5020, by forming a lumen (30). Treatment of organoids with R5020 induces apoptosis that is spatially localized within mammary organoids and centrally within luminal structures; R5020 does not induce proliferation in these organoids (30). Based on these observations we have hypothesized that one of the actions of P in mammary gland development is to facilitate lumen formation through P-induced apoptosis (30). In the present study we showed that only PRA was expressed in the virgin gland, and within endbuds PRA positive cells were localized in the internal layer of cells. This raises the possibility that one way that P promotes ductal development in the virgin gland, at least in part is by facilitating lumen formation through a pro-apoptotic mechanism mediated by PRA. The observation that ductal development can occur in total PR deleted as well as PRA gene-deleted

mice indicates that there are additional mechanisms that promote lumen formation, and that these mechanisms are operative in PR gene-deleted mice and may compensate for the lack of PR.

Progesterone action in pregnancy: predominant role of PRB

PRB positive cells were seen only in mammary glands of pregnant mice (Figs. 4C, 8B,C) or in alveolar structures of adult E+P-treated mice (Fig. 5B). In pregnant mice PRB was abundantly expressed and the PRB positive cells were localized mainly in alveolar structures. We found extensive colocalization of PRB with BrdU and cyclin D1 in pregnant mammary gland. This indicates that PRB positive cells are in the proliferative pool of cells and express cyclin D1. Our results indicate that PRB has the primary role in inducing alveologenesis. Other studies have inferred the same conclusion based upon different approaches, namely, that there is no defect in alveologenesis in the PRAKO mouse (15), that there is a lack of alveologenesis in the PRBKO mouse (16), and that precocious alveologenesis occurs in PRB over-expressing transgenic mice (13).

In contrast to PRB, there was no PRA colocalization with either BrdU or cyclin D1 in the pregnant gland, suggesting that PRA positive cells do not constitute the major proliferative pool in the pregnant mammary gland. These observations do not discount the possibility that PRA nevertheless plays a role in pregnancy since expansion of the epithelium and sidebranching are detected as early as day 7 of pregnancy (unpublished observations, Aupperlee & Haslam), when 27% of the epithelial cells were PRA positive and none were PRB positive (Figs.1,3).

Previous studies have reported a lack of colocalization of PR with markers of proliferation (16, 33). However, in those studies the PR isoform specificity of the antibody used (DAKO A0098) was not identified. We have determined that the DAKO A0098 anti-PR antibody colocalizes with PRA and not with PRB. This was determined in studies carried out as shown for the SC#538 anti-PR antibody (Fig.7) (unpublished observations, Aupperlee &

Haslam). Our own studies using PR isoform-specific antibodies demonstrate a lack of colocalization of PRA with BrdU in pregnant mammary gland, but we find extensive colocalization of PRB with BrdU and cyclin D1 in pregnant mammary gland.

The lack of PRA and PRB staining during lactation is in agreement with previous reports of the absence of specific P ligand binding and lack of detectable PR mRNA in lactating mouse mammary gland (28, 34). Although PRA positive cells were detected again post-involution, the percentage of PRA positive cells never returned to the pre-pregnancy virgin level. This was not due to aging since the percent of PRA positive cells was significantly higher in 20-week-old virgin mammary glands than age-matched parous mice. A low level of PRB (6 % PRB positive cells) was detected in alveolar structures after lactational involution, but not in age-matched virgin mammary gland. These results demonstrate that expression of both PRA and PRB is permanently altered by pregnancy. Pregnancy is protective against carcinogen-induced mammary tumors in mice and rats (35). Our results show two important changes caused by pregnancy: a reduction in PRA positive cells relative to age-matched virgins, and presence of PRB post lactation relative to the virgin state. Further studies to elucidate the specific functional roles of PR isoforms in the mammary gland before, during and after pregnancy may provide new insights about the mechanism(s) underlying differences in susceptibility to tumorigenesis of virgin vs. parous mice.

PR isoform subcellular localization and progesterone action

Previous studies using cell lines have shown that if expressed in the same cells, PRA and PRB proteins can dimerize and bind to DNA as three different species: AA or BB homodimers or AB heterodimers (9). The specific contribution of each of the dimers to the effects of P may be dependent on the transactivation properties contributed to the complexes by the PRB-specific domain. It has also been reported that PRB transcriptional activity is inhibited by PRA. During

pregnancy we found that the vast majority of PRB positive cells contained only PRB and only a small percentage of cells (4%) contained both PRA and PRB. Our results indicate that the prevailing situation in the mouse mammary gland is that cells contain AA or BB homodimers, and that the potential for AB heterodimer formation is limited to a small number of cells during pregnancy. This suggests that in the mouse, heterodimer formation does not play a major role in progesterone action in the mammary gland.

In our study, PRB was detected primarily in the nucleus and in some cells faintly in the cytoplasm (see Fig. 4F). In contrast, using the anti-PRA and SC#538 anti-PR antibodies, PRA was detected only in the nucleus. We cannot rule out the possibility that there may also be a cytoplasmic form of PRA not detected by the PR antibodies we used. PR localization in the mammary gland in both cytoplasm and nucleus has been detected using a PR antibody of unknown PR isoform specificity (18) and in human T47D breast cancer cells overexpressing either PRA or PRB (36). It is conceivable that different anti-PR antibodies may detect epitopes that are exposed on the cytoplasmic, nuclear, or both forms of the receptor.

PR isoform expression in the human vs. mouse

In the human breast, immunohistochemical analysis of PR isoform expression has been carried out on normal tissue from premenopausal cycling women (26). In that study PRA or PRB expression and colocalization were determined by dual immunofluorescence with the same antibodies used herein (26). PRA vs. PRB were expressed at a ratio of 1:1, and patterns of expression were similar. The proportion of PR positive cells was 10-20% with marked variability throughout a section, with PR positivity in individual ducts or lobules ranging from 0-90%. Dual immunofluorescence studies revealed uniform colocalization of PRA with PRB (26). Our study indicates an interesting difference in PR isoform expression between the mouse and the human mammary gland. In the mouse there is PRB expression only during pregnancy, and

colocalization of PRA with PRB occurs in only a small percentage of cells. One possible explanation for this difference may be the predominance of a ductal organization of mammary epithelium in the adult non-pregnant mouse. This is particularly true in Balb/c strain mice, used in our study. In contrast, in the adult non-pregnant human there is a higher ratio of lobules to ducts. Studies of PRB null mice have shown that PRB expression is required for alveologenesis and lobule formation (16). Therefore, PRB expression may be a defining characteristic of mammary lobule formation and/or maintenance and may explain why PRB positive cells are more abundant in the human breast. In this regard, the maintenance of some alveolar structures in mouse mammary gland after pregnancy may also be due to the continued, albeit reduced, expression of PRB after pregnancy. Analysis of other mouse strains, such as the C3H strain, which develop a more lobular morphology in the virgin state (compared to Balb/c strain) may provide additional insights into the relationship between alveolar morphogenesis and PRB expression. It is also important to note that PR isoform expression in the human has only been studied in the adult premenopausal breast. There is no information on PR isoform expression at other stages of human breast development such as puberty or pregnancy. It remains to be seen what analysis of these other stages may reveal about the pattern of PR isoform expression and/or colocalization in the human breast. Clearly more information is needed about PR isoform expression in the human breast.

Understanding the specific functions of PRA and PRB isoforms in vivo is critical to understanding their respective roles in the normal breast and in the etiology of breast cancer. The spatial and temporal separation of PRA and PRB isoform expression in mouse mammary gland offers a unique opportunity to explore further the specific functions and mechanisms of action PRA vs. PRB in vivo.

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**Figure Legends**

Figure 1. Quantitation of PRA at different stages of mammary gland development. Immunoperoxidase localization of PRA was carried out using anti-PRA antibody on tissue sections from 3, 6, 10-12 or 17-20-week-old virgin, 7 or 14 day pregnant, 10-day lactating mice, and at 9 weeks post weaning (lactational involution) as described in the Methods section. The values represent the mean ± SEM from 3-5 mice per group with a minimum of 1000 cells per mouse analyzed. PRA positive cells decreased significantly with age (3 and 6 wk > 10-12 wk > 17-20 wk) in virgin mice and further during pregnancy and lactation (7d > 14 d > lact). The 9 week involuted mammary gland had fewer PRA positive cells than age-matched virgin mammary gland (17-20-week-old) ($p< 0.01$). No PRA staining was detected (ND) during lactation.

Figure 2. Immunoperoxidase localization of PRA at different stages of mammary gland development. Representative sections from 6-week-old immature (A, E end bud, B,F duct), 12-week-old adult (C, G duct), and 14 day pregnant (D, H alveoli) mouse mammary gland were treated with anti-PRA antibody (A-H) as described in the Methods section; control sections without antibody (I immature end bud, J immature duct, K adult duct, L pregnant alveoli). Higher magnification images of boxed areas in A-D are shown in E-H. Brown stained PRA positive nuclei are indicated by black arrowheads and PRA negative cells by red arrowheads. End bud cap cells (E) or myoepithelial cells (F, G) are indicated by arrows (Scale bar, 50 μm).

Figure 3. Quantitation of PRB at different stages of mammary gland development. Immunofluorescence localization of PRB was carried out using anti-PRB antibody on tissue sections from 3, 6, 10-12, or 17-20-week-old virgin, 7 or 14 day pregnant, 10-day lactating mice,

and at 9 weeks post weaning (lactational involution) as described in the Methods section. The values represent the mean ± SEM from 3-5 mice per group with a minimum of 1000 cells per mouse analyzed. No PRB staining was detected (ND) in the virgin mammary gland (3, 6, 10-12, or 17-20-week old), in the 7 day pregnant mammary gland or during lactation. PRB was detected at 14 days of pregnancy and in a smaller percentage of cells in the 9 wk involuted mammary gland ($p<0.001$).

Figure 4. Immunoperoxidase localization of PRB at different stages of mammary gland development. Representative sections from 6-week-old immature (A, D end bud), 12-week-old adult (B, E duct), and 14 day pregnant (C, F alveoli) mice were treated with anti-PRB antibody (A-F) as described in the Methods section; control sections without antibody (G immature, end bud; H adult, duct; I pregnant, alveoli). Higher magnification images of boxed areas in A,B are shown in D,E and a higher magnification of pregnant mammary gland is shown in F. Brown stained PRB positive nuclei (F) are indicated by black arrowheads and PRB positive cytoplasmic staining (F) by double arrows; PRB negative nuclei by red arrowheads. End bud cap cells (D) are indicated by arrows (Scale bar, 50 μm).

Figure 5. Immunodetection of PRA and PRB in wild type vs. PRA null mice. Immunofluorescence localization of (A) PRA or (B) PRB was carried out on sections from 8-week-old virgin or 13 day E+P-treated 12-week-old virgin wild type (WT) and PRA null (PRAKO) mice. Antibody staining was carried out with anti-PRA antibody (red nuclei) or anti-PRB antibody (light blue nuclei); nuclei were counter-stained with TOPRO-3 (dark blue nuclei). Positive staining is indicated by white arrowheads and negative nuclei are indicated by yellow arrowheads. (Scale bar, 20 μm).

Figure 6. Immunoblot analysis of PR in mammary gland. A. Extracts from uterus (lanes 1) and isolated epithelial cells from 6-week-old mammary glands (lane 2) were subjected to SDS-PAGE and blots were probed with hPRa7 anti-PR antibody as described in the Methods section. PRA was detected a single band at 91 kD in uterus and isolated epithelial cells (lanes 1,2); PRB was detected as a single band at 119 kD in uterus only (lanes 1). B. Extracts from uterus (lanes 1) and isolated epithelial cells from 6-week-old mammary glands (lane 2) were subjected to SDS-PAGE and blots were probed with hPRa6 anti-PR antibody, which detects only PRB, as described in the Methods section. PRB was detected as single band at 119 kD in uterus ( lane 1); no PRB was detected in isolated epithelial cells (lane 2). C. Extracts from uterus (lanes 1) and whole 14-day pregnant mammary glands (lane 2) were subjected to SDS-PAGE and blots were probed with a mixture of SC#538 and SC#539 anti-PR antibodies described in the Methods section. PRA was detected a single band at 91 kD in uterus only (lane 1); PRB was detected as a single band at 119 kD in uterus and mammary gland (lanes 1, 2).

Figure 7. Immunodetection of PRA by SC#538 anti-PR antibody. Tissue sections from (A) 6-week-old virgin or (B) 14 day pregnant mammary glands were double labeled with anti-PRA antibody (red nuclei) and SC#538 antibody (green nuclei); nuclei were counterstained with TOPRO-3 (dark blue). In the virgin and pregnant gland the anti-PRA and SC#538 antibody staining show complete colocalization and are visualized as white nuclei in the merged images. (Scale bar, 20 μm)

Figure 8. Colocalization of PRA and PRB in pregnancy. Dual immunofluorescence detection of PRA and PRB was carried out and visualized by laser scanning confocal microscopy as

described in the Methods section. A. Quantitation of PRA and PRB colocalization; the values represent the mean ± SEM of the percentage of epithelial cells expressing one isoform only (PRA or PRB) or both isoforms (PRAB);values were obtained using 5 mice with a minimum of 1000 cells per mouse analyzed. B. Photomicrograph of PRA and PRB colocalization. PRA (green nuclei) and PRB (red nuclei); nuclei were counterstained with TOPRO-3 (blue nuclei). Three subsets of PR positive cells are seen in the merged image: those expressing both isoforms (white nuclei in square), PRA only (green nucleus in circle) or PRB only (red nuclei in oval). (Scale bar, 20 μm).

Figure 9. Quantitation of colocalization of PRB or PRA with BrdU in pregnant and virgin mammary glands. Dual immunofluorescence detection of PRB or PRA and BrdU was carried out on tissue sections from (A) 14 day pregnant and (B) 6-week-old virgin mammary glands and visualized by laser scanning confocal microscopy as described in the Methods section. A minimum of 1000 cells were counted for each antibody combination tested i.e., PRB and BrdU or PRA and BrdU in pregnant mammary gland and PRA and BrdU in virgin mammary gland. The values represent the mean + SEM from 3-5 mice with a minimum of 1000 cells per mouse analyzed.

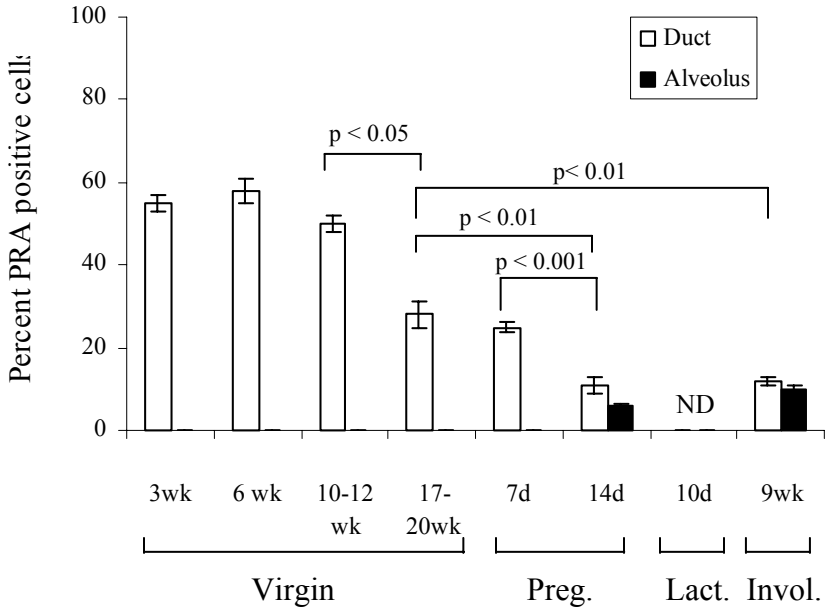
Figure 10. Detection of colocalization of PRB or PRA with BrdU in pregnant and virgin mammary glands. Dual immunofluorescence detection was carried out in (A,B)14 day pregnant or (C,D) 6-week-old virgin mammary gland using anti-PRB (A) or anti-PRA (B,C,D) antibodies and TOPRO-3 nuclear stain and were visualized by laser scanning confocal microscopy as described in the Methods section. A. PRB (red nuclei, white arrows) and BrdU (green nuclei, white arrowheads) staining were extensively colocalized (white nuclei, yellow arrows) in merged

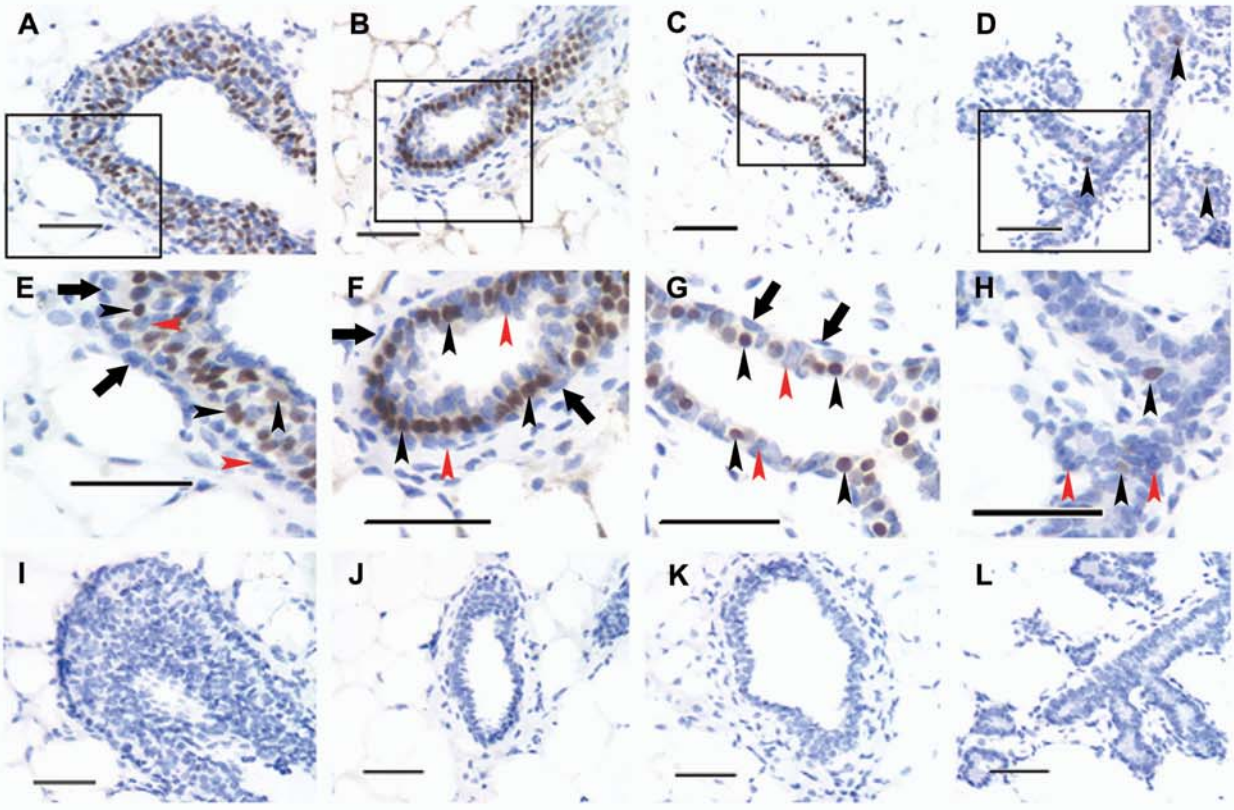
images. B. PRA (red nuclei, white arrows) and BrdU (green nuclei, white arrowheads) staining did not colocalize in merged images and were seen as red (white arrows) and light blue (white arrowheads) nuclei. C. In 6-week-old virgin mammary gland duct PRA (green nuclei, white arrow), BrdU (red nuclei, white arrowhead) staining did not colocalize in merged images and were seen as red (white arrowheads) and light blue (white arrows) nuclei. D. In 6-week-old mammary gland end bud most PRA (green nuclei, white arrow), BrdU (red nuclei, white arrowhead) staining did not colocalize and in merged images and were seen as red (white arrowheads) and light blue (white arrows) nuclei. End bud cap cells were prominently labeled by BrdU (red nuclei, white arrowheads). Instances of colocalization of PRA and BrdU are seen in merged image as white nuclei (yellow arrows). (Scale bar, 20μm).

Figure 11. Quantitation of colocalization of PRB or PRA with cyclin D1in pregnant and virgin mammary glands. Dual immunofluorescence detection of PRB or PRA and cyclin D1 was carried out on tissue sections from (A) 14 day pregnant and (B) 6-week-old virgin mammary glands and visualized by laser scanning confocal microscopy as described in the Methods section. The values represent the mean + SEM from 3 mice per group (virgin and pregnant) with a minimum of 1000 cells per mouse analyzed for each antibody combination tested i.e., PRB and cyclin D1 or PRA and cyclin D1 in pregnant mammary gland and PRA and cyclin D1 in virgin mammary gland.

Figure 12. Detection of colocalization of PRB or PRA with cyclin D1 in pregnant and virgin mammary glands. Dual immunofluorescence detection of PRB or PRA and cyclin D1 was carried out on tissue sections from (A,B) 14 day pregnant and (C,D) 6-week-old virgin mammary glands and visualized by laser scanning confocal microscopy as described in the Methods

section. Nuclei were counterstained with TOPRO-3 (A-D, blue). Examples of PRB (A; red nuclei) or PRA (B,C,D; red nuclei) positive cells are indicated with white arrows, and examples of cyclin D1 positive cells (A, B,C; green nuclei) are indicated with white arrowheads. PRB and cyclin D1 colocalization is seen as white nuclei in the merged image (A) and examples are indicated with yellow arrows. In pregnant mammary gland (B) there was no colocalization of PRA and cyclin D1 in the merged image and PRA positive nuclei stain red (white arrows) and cyclin D1 positive nuclei stain light blue (white arrowheads). In virgin mammary gland (C) when colocalization of PRA and cyclin D1 was observed it was seen as white nuclei in the merged image; examples are indicated with yellow arrows. (D) An example of a virgin duct without cyclin D1 positive cells. (Scale bar, 20μm)


Percent PRA positive cells
100
80
60
40
20
0
Duct
Alveolus
p < 0.05
p< 0.01
p < 0.01
p < 0.001
ND
3wk
6 wk
10-12 wk
17-20wk
7d
14d
10d
9wk
Virgin
Preg.
Lact.
Involution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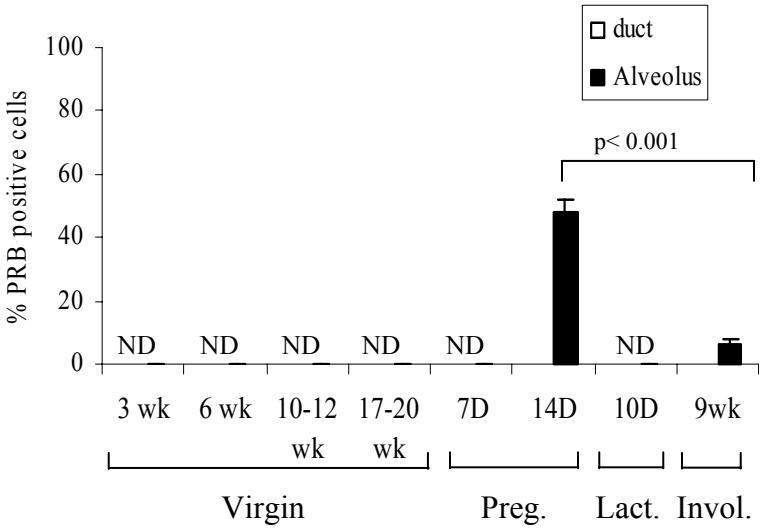
duct
Alveolus
% PRB positive cells
100
80
60
40
20
0
p< 0.001
ND
ND
ND
ND
ND
ND
3 wk
6 wk
10-12 wk
17-20 wk
7D
14D
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9wk
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Preg.
Lact.
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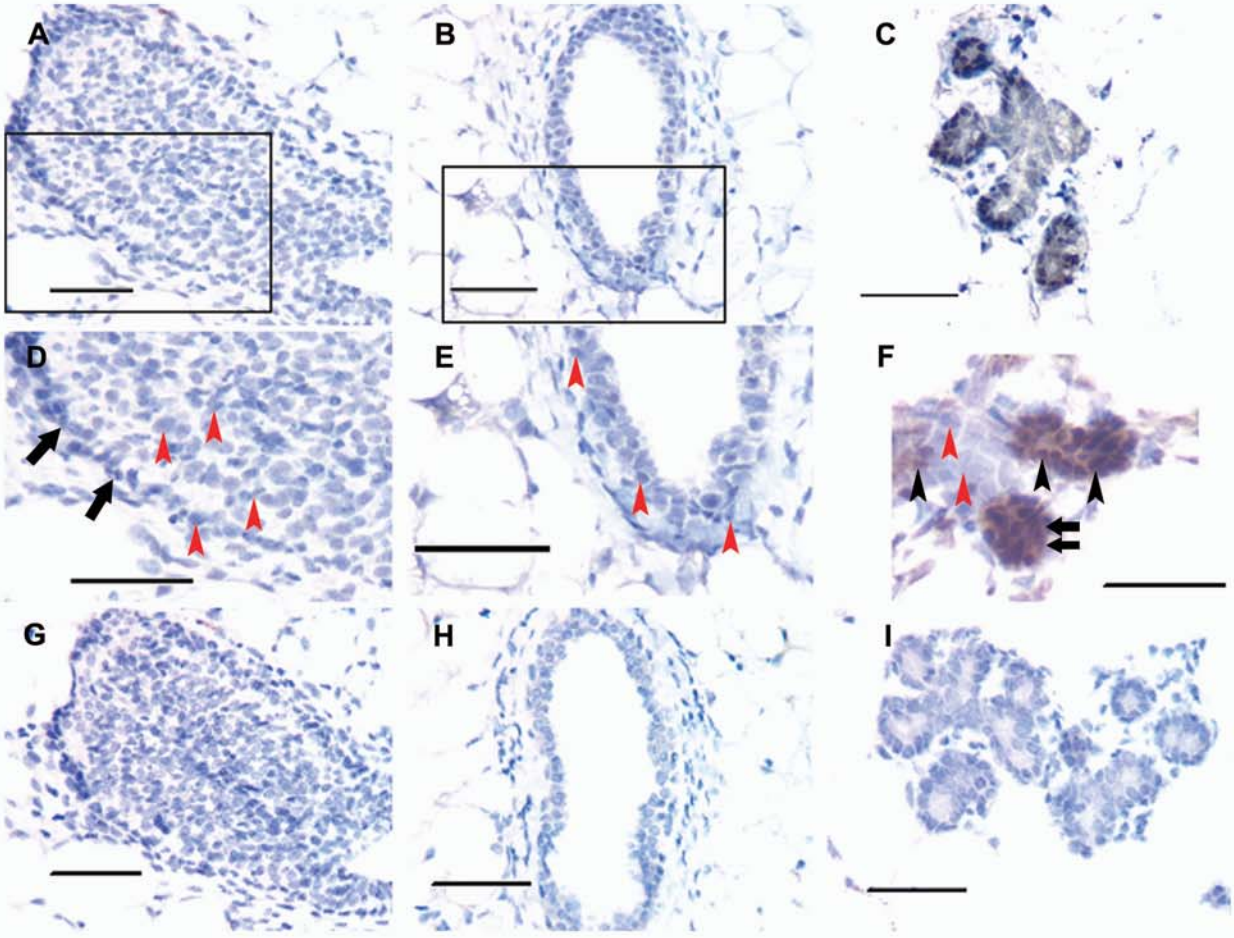
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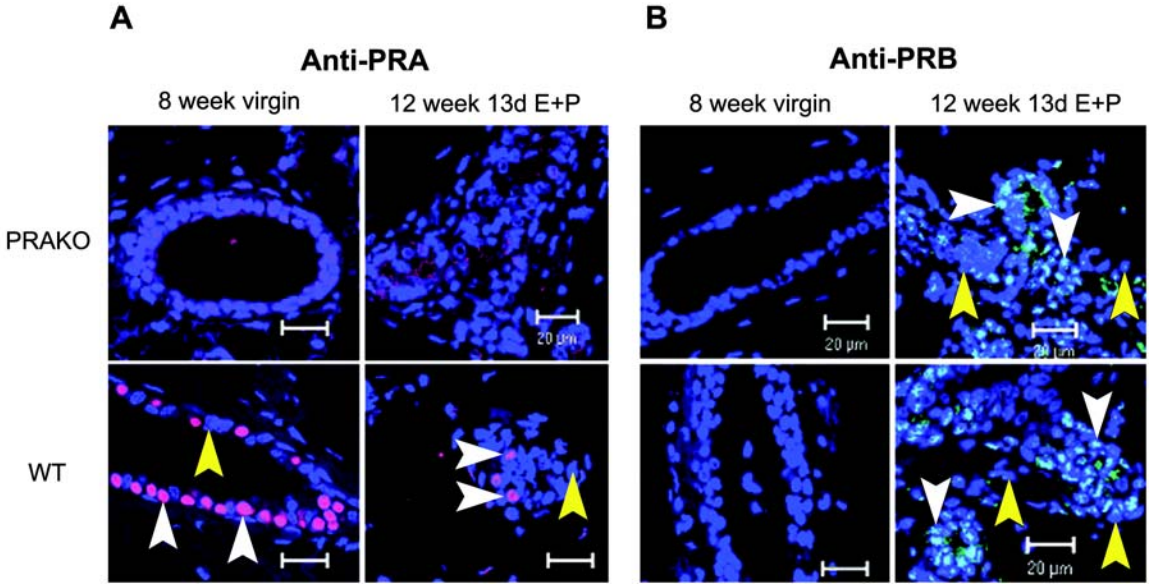
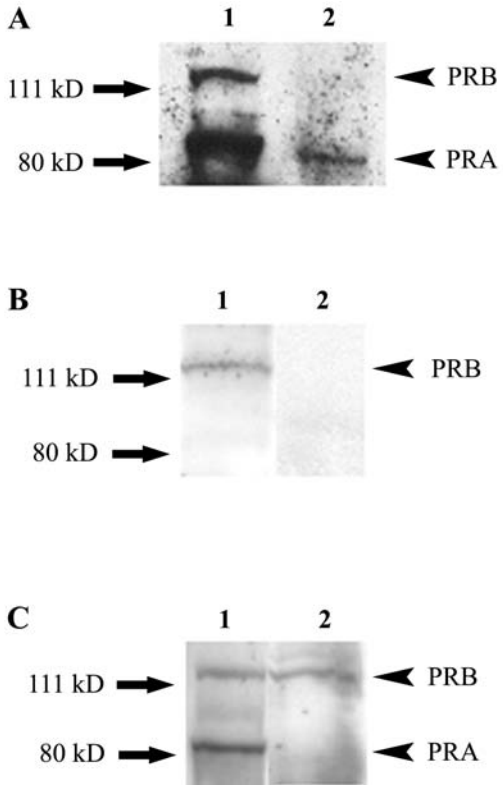

A
Anti-PRA
8 week virgin
12 week 13d E+P
PRAKO
WT
20 µm
B
Anti-PRB
8 week virgin
12 week 13d E+P
20 µm
20 µm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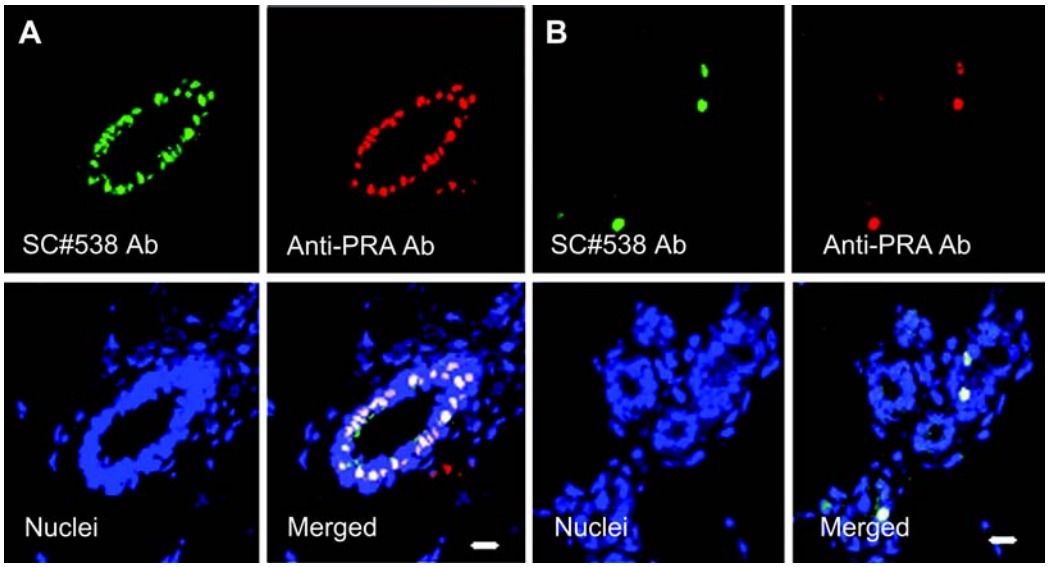
80 kD
PRB
PRA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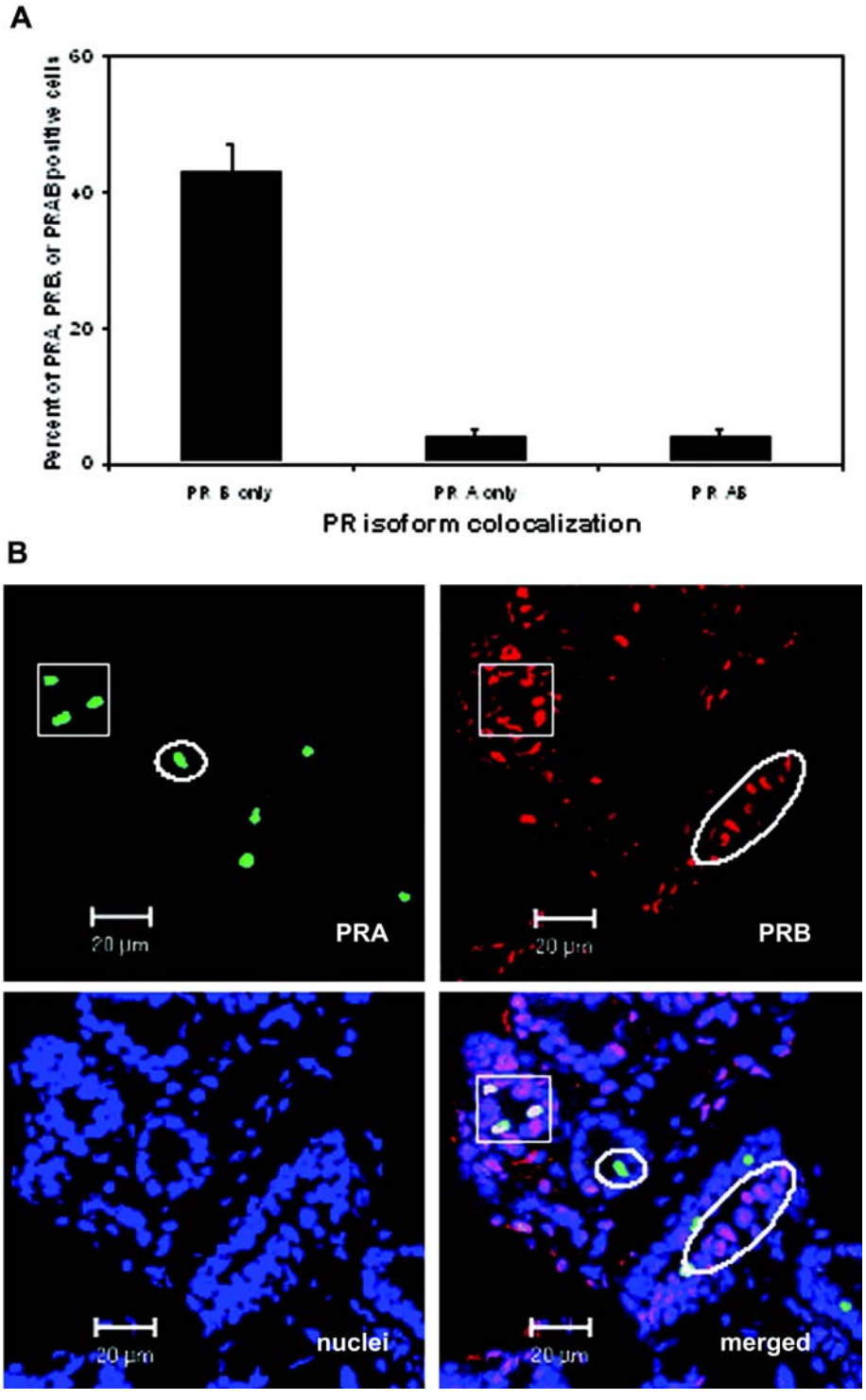
Anti-PRA Ab
B
SC#538 Ab
Anti-PRA Ab
Nuclei
Merged
Nuclei
Merged


A
Percent of PRA, PRB, or PRAB positive cells
60
40
20
0
PR B only
PR A only
PR AB
PR isoform colocalization
B
20 µm
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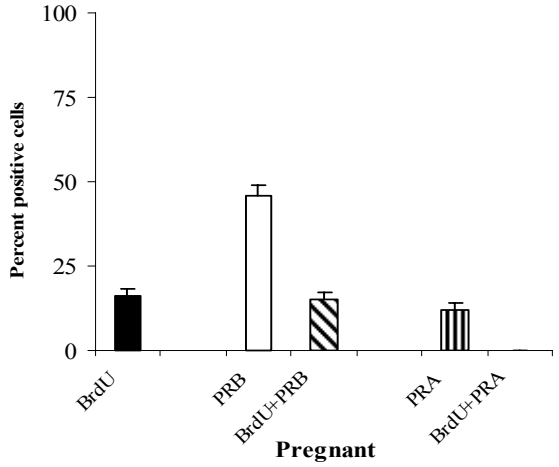
20 µm
PRB
20 µm
nuclei
20 µm
merged

**A**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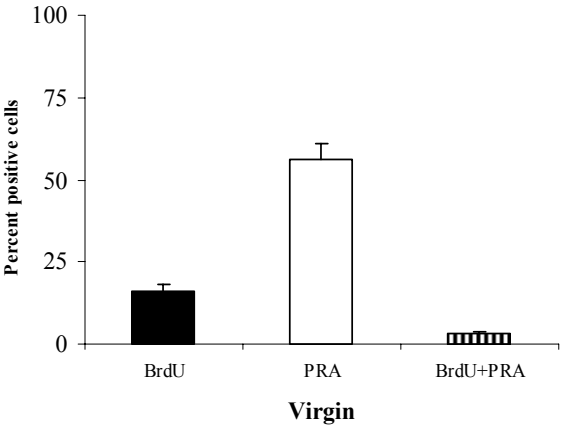
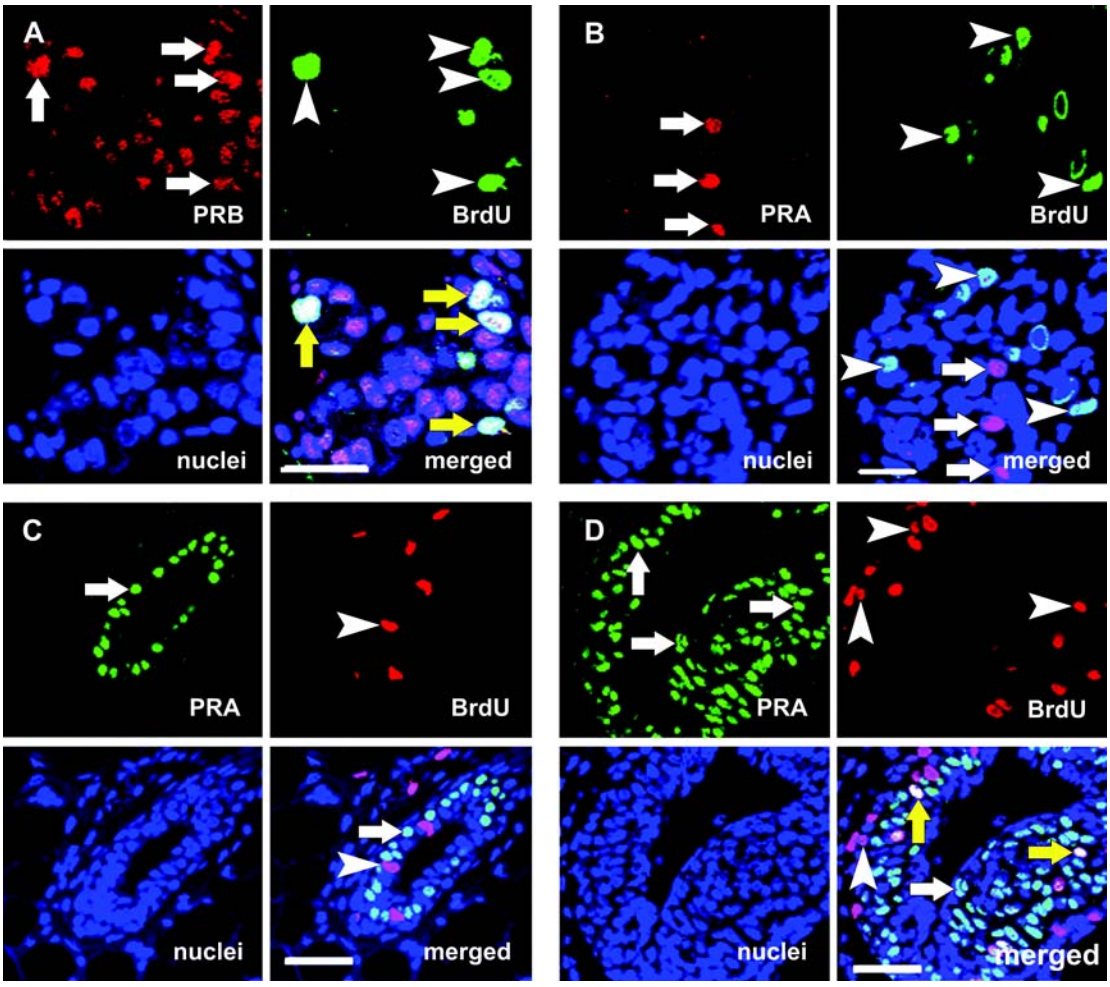
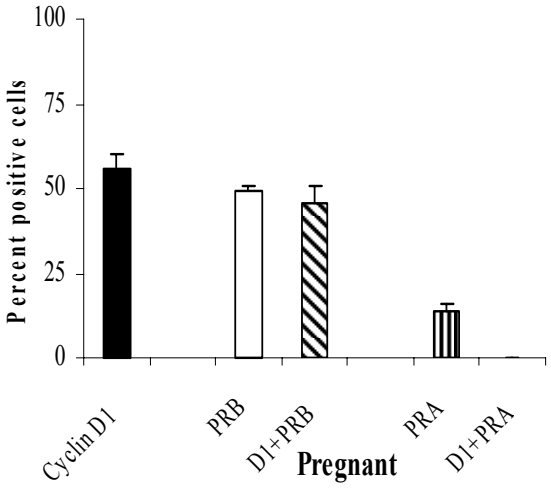
25
0
Percent positive cells
BrdU
PRB
BrdU+PRB
PRA
BrdU+PRA
Pregnant

**B**

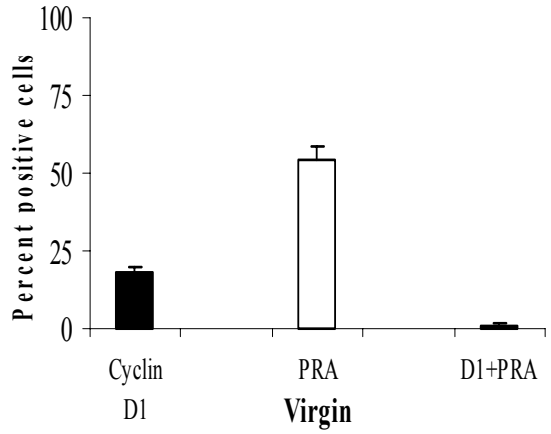

100
75
50
25
0
Percent positive cells
BrdU
PRA
BrdU+PRA
Virgin

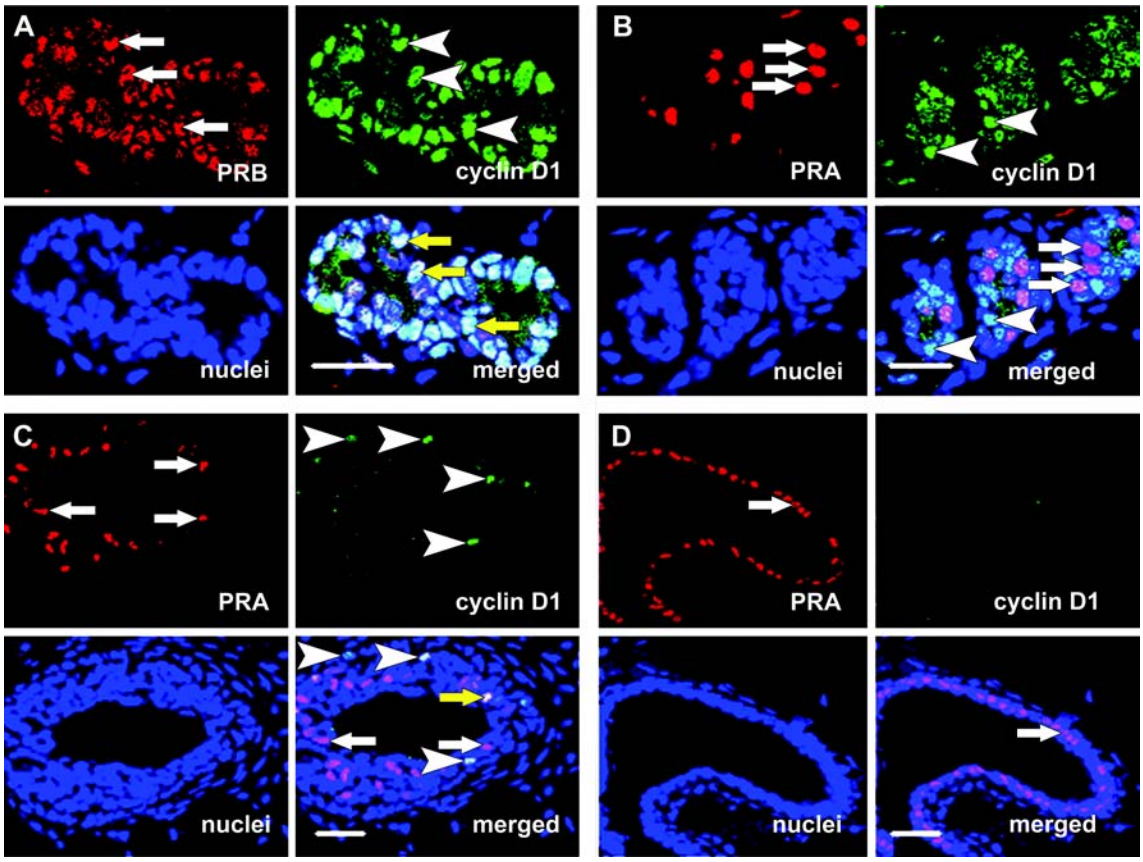

A
PRB
BrdU
nuclei
merged
B
PRA
BrdU
nuclei
merged
C
PRA
BrdU
nuclei
merged
D
PRA
BrdU
nuclei
merged

**A**


100
75
50
25
0
Percent positive cells
Cyclin D1
PRB
D1+PRB
PRA
D1+PRA
Pregnant

**B**


100
75
50
25
0
Percent positive cells
Cyclin D1
PRA
D1+PRA
Virgin


A
PRB
cyclin D1
nuclei
merged
B
PRA
cyclin D1
nuclei
merged
C
PRA
cyclin D1
nuclei
merged
D
PRA
cyclin D1
nuclei
merged