Deletion of the transcription factor CCAAT/enhancer binding protein (C/EBP)\(\beta\) results in a severe inhibition of lobuloalveolar development in the mouse mammary gland. Because progesterone receptor (PR) is requisite for alveolar development, the expression of PR was investigated in C/EBP\(\beta^{-/-}\) mice. Unexpectedly, the number of PR-positive cells, as well as the levels of PR mRNA, were elevated 3-fold in the mammary glands of C/EBP\(\beta^{-/-}\) mice. Furthermore, in contrast to wild-type nulliparous mice, in which PR distribution shifted from a uniform to nonuniform pattern between 8–12 weeks of age, C/EBP\(\beta^{-/-}\) mice exhibited uniform PR distribution throughout all stages of mammary development analyzed. No change in C/EBP\(\beta\) mRNA levels was observed in the mammary glands of PR\(^{-/-}\) mice, suggesting that PR acts in a pathway either in parallel to or downstream of C/EBP\(\beta\). The overexpression and disrupted cellular distribution of PR in C/EBP\(\beta^{-/-}\) mice were coincident with a striking 10-fold decrease in cell proliferation after acute steroid hormone treatment, assayed by incorporation of bromodeoxyuridine. In wild-type mice, PR and bromodeoxyuridine-positive cells were adjacent to each other and rarely colocalized. No differences in the level or pattern of PR expression were observed in the uterus, suggesting that C/EBP\(\beta\) influences PR in a mammary-specific fashion. Together, these data suggest that C/EBP\(\beta\) may control cell fate decisions in the mammary gland through the appropriate temporal and spatial expression of molecular markers, such as PR, that induce the proliferation of alveolar progenitor cells via juxtacrine mechanisms. (Molecular Endocrinology 14: 359–368, 2000)

INTRODUCTION

A key question in the study of breast cancer is the mechanism by which hormonally regulated signaling pathways that promote normal development in response to pregnancy become altered to result in aberrant proliferation. Progesterone (P) has been implicated as a mitogen for the mammary gland during pregnancy (1, 2) when alveolar secretory units of the gland bud from a simple tree of ductal epithelium. Since breast tumors are initially steroid hormone dependent, the progesterone receptor (PR) has been extensively used as a molecular marker. Recent reports suggest that the spatial distribution of steroid receptors is critical to normal lobuloalveolar development. For example, while PR and estrogen receptors (ER) colocalize in more than 96% of normal breast epithelial cells, proliferating cells are steroid receptor-negative (3, 4). However, very little is known about the mechanisms that regulate the expression and spatial distribution of PR in the normal breast or in mouse mammary epithelium.

Furthermore, the molecular mechanisms of steroid hormone-mediated regulation of target genes that control normal mammary epithelial cell (MEC) proliferation remain poorly defined. Previous studies have demonstrated that the transcription factor CCAAT/enhancer binding protein \(\beta\) (C/EBP\(\beta\)) is required for normal ductal morphogenesis and for the proliferation and differentiation of mammary epithelial cells in response to estrogen (E) and P during pregnancy (5, 6). C/EBP\(\beta\) belongs to a
family of basic leucine-zipper (bZIP) DNA-binding proteins that regulate transcription by binding as homo- or heterodimers with other C/EBPs to a common nucleotide consensus sequence. C/EBPβ has been implicated as a critical regulator of proliferation vs. differentiation in multiple tissues including liver, adipose tissue, ovary, immune system, and skin (7–11). Alternative translation of the intronless C/EBPβ transcript produces proteins that differ in their activities based on inclusion of the N-terminal transactivation domain. The ratio of activating to repressing protein isoforms is critical in mediating expression of target genes (12). The expression of the dominant-negative C/EBPβ isoform is tightly regulated during mouse mammary gland development (5) and during the progression of breast cancer (13, 14).

Studies in mice lacking the PR have confirmed that PR is required for the initiation of alveolar budding from the ductal tree in response to E+P (15). However, alveolar development can be rescued if PR−/− MEC mixed with PR+/+ MEC are reconstituted in close proximity within the cleared fat pads of RAG1−/− hosts, suggesting a juxtacrine mechanism of PR action (16). Recombination of PR−/− stroma and PR+/+ epithelium indicates that the stroma does not play a critical role in alveolar morphogenesis, further emphasizing the importance of epithelial-epithelial juxtacrine interactions, rather than epithelial-stromal interactions, in PR action (16). Similarly, transplantation of MEC from the C/EBPβ−/− mouse into the cleared fat pads of C/EBPβ+/+ hosts has also demonstrated that C/EBPβ, like PR, acts in an epithelial cell-autonomous manner (5, 6).

Coupled with the marked inhibition of lobuloalveolar development, a transient decrease in proliferation of C/EBPβ−/− epithelium transplanted into the cleared fat pads of C/EBPβ+/+ mice has been observed during pregnancy (6). Based on these observations, the expression and localization of PR and the relationship to proliferation were determined in wild-type and C/EBPβ−/− mice over the course of mammary gland development. These studies revealed that C/EBPβ acts either upstream of, or parallel to, PR in the normal mammary gland to regulate proliferation in response to steroid hormones in a mammary-specific fashion. Unexpectedly, the lack of C/EBPβ in the mammary gland resulted in increased levels of PR mRNA per cell and an increase in the total number of PR-positive (PR+) MEC compared with wild-type controls. Furthermore, the cellular distribution of PR shifted in wild-type mice from a uniform pattern at 6–8 weeks of age to a nonuniform pattern by 11–12 weeks of age. In contrast, at all stages of development analyzed, C/EBPβ−/− mice exhibited a uniform pattern of cellular distribution of PR. The increased expression and mislocalization of PR in C/EBPβ−/− mice was concomitant with a marked inhibition of epithelial cell proliferation in response to E+P treatment. These results support the hypothesis that C/EBPβ controls cell fate decisions of putative alveolar progenitor cells through regulation of the expression and cellular distribution of molecular markers such as PR.

RESULTS AND DISCUSSION

Increased Expression of PR in the C/EBPβ−/− Mammary Gland

The expression and spatial distribution of PR were examined by indirect immunofluorescence (IF) in mammary tissue biopsied from intact mature virgin mice (at 11 weeks to 8 months of age) and in the contralateral mammary glands of the same cohort of females after acute E+P treatment. Acute steroid treatment was chosen to simulate alveolar development in early pregnancy when alveoli begin to proliferate from ductal progenitor cells. A marked impairment of alveolar development was observed in C/EBPβ−/− mice in response to E+P (Fig. 1A, c–d). Mice lacking C/EBPβ possessed only a simple network of enlarged ducts as reported previously (5). Surprisingly, these studies revealed an increase in the percentage of PR+ MEC in C/EBPβ−/− compared with −/+ mice (Fig. 1A, b and f vs. a and e). In mature virgins, the percentage of PR+ MEC was approximately 2.5-fold greater in C/EBPβ−/− mice (Fig. 1B). After acute E+P exposure, a 3-fold increase in PR+ MEC was observed in the contralateral glands of the C/EBPβ−/− mice (Fig. 1C). In C/EBPβ+/+ mice, the percentage of PR+ MEC in both treatment groups averaged 25% (Fig. 1, B and C), and, in general, individual cells in C/EBPβ−/− mice stained less intensely for PR than in mice lacking C/EBPβ. No specific IF signal corresponding to PR was detected in mammary glands isolated from mice lacking PR (PRKO) mice (data not shown).

The elevated number of PR+ MEC in C/EBPβ−/− mice also corresponded with a change in their spatial distribution from the normal scattered pattern (20) to a more uniform pattern in C/EBPβ−/− mice (Fig. 1A, a and e vs. b and f). The increased expression of PR in individual MEC, as well as the increased percentage of MEC expressing PR protein, was confirmed at the mRNA level by in situ hybridization (Fig. 1D, a and b). Similar to PR protein, PR mRNA is expressed in a nonuniform pattern in C/EBPβ−/− mice (>11 weeks of age) and in a uniform pattern in C/EBPβ+/+ mice. Therefore, the lower levels of PR IF staining observed in wild-type animals do not result from lack of sensitivity during antibody staining. The combined results of these analyses demonstrate that up-regulated expression and altered distribution of PR in the absence of C/EBPβ corresponds to an inability to initiate alveolar development. The defect in alveolar development in C/EBPβ−/− mice is MEC autonomous. Since the stroma does not contribute to the defects ob-
served in alveolar development, they may result from a deficiency in epithelial-epithelial juxtacrine signaling pathways. Recently Shyamala et al. (17) demonstrated the involvement of such interactions in PR-regulated development where transgenic mice overexpressing PR-A lose cell-cell junctions and expression of E-cadherin along cell borders. In light of this observation, we analyzed the expression of E-cadherin in C/EBPβ−/− mice. Immunostaining for E-cadherin revealed appropriate basolateral localization in MEC of C/EBPβ−/− mice (data not shown), suggesting that any juxtacrine-mediated signaling mechanism dependent on this type of cell-cell interaction should be intact despite the absence of C/EBPβ.

Marked Changes in PR Expression and Localization Occur during Normal Mammary Gland Development in Virgin Mice

Intact mice of at least 11 weeks of age were initially studied because younger animals (7–9 weeks old) ex-
hibited considerably less alveolar development after E+P treatment (18). Therefore, it was hypothesized that the maximal responsiveness of a nulliparous female to E+P may correlate with the expression and/or spatial distribution of PR in the mammary gland. The expression of PR during normal mammary development in nulliparous mice was determined by IF at 6, 8, and 12 weeks of age in intact C57BL/6 mice. This period of development includes two phases of ductal proliferation: 1) penetration of the fat pad by ducts from 3 to 8 weeks of age through proliferation at distal tips within specialized structures known as terminal end buds (TEBs), and 2) cessation of proliferation between 9 and 12 weeks of age as ducts approach the edges of the fat pad and TEBs disappear. PR was present in almost every MEC of previously formed ducts in 6- to 8-week virgins (Fig. 2c) and was more concentrated in the inner cell layers of the TEBs than in the outer, more proliferative cells (Fig. 2a) (19). However, by 12 weeks of age, the pattern of PR distribution in the majority of the ducts was restricted to a subset of MEC (Fig. 2e), as previously reported (20). When mammary glands from 8-week C/EBPβ+/− and −/− mice were stained for PR, the expression and localization of PR were very similar to the uniform pattern observed in the C57BL/6 8-week-old female mice (data not shown, and Fig. 2c). Therefore, between 8 and 12 weeks of age, as wild-type female mice approach the age of maximal response to exogenous E+P, the cellular distribution of PR changes dramatically.

These observations suggest that the temporally regulated switch in spatial distribution of PR from a uniform to a scattered pattern may be required to facilitate maximal alveolar proliferation in response to steroid hormones. At 6–8 weeks of age, when PR is expressed uniformly in a majority of the epithelial cells, the MEC population may not be spatially defined to assume an alveolar cell fate program. At this age, the ducts have not yet reached the edges of the fat pad so this mechanism may exist to reduce extensive premature alveolar development before formation of the entire ductal tree. Mice at 6–8 weeks of age are more refractory to the acute effects of E+P (18), although wild-type females impregnated as early as 5 weeks of age are capable of some alveolar development. Sub-

![Fig. 2. PR Localization Changes Markedly during Normal Virgin Mammary Development](https://academic.oup.com/mend/article-abstract/14/3/359/2747851/C-EBP-CCAAT-Enhancer-Binding-Protein-Controls-Cell-by-guest-on-15-September-2017)
sequent restriction of PR to a subset of MEC in the mammary gland of the mature nulliparous female depends on C/EBPβ, since aberrant localization and expression of PR persists in C/EBPβ+/− females as old as 32 weeks of age and in C/EBPβ−/− females treated acutely or chronically with E+P. Once pregnancy is initiated in wild-type mice, growth factors may act as juxtacrine mediators to stimulate proliferation of alveoli from the adjacent steroid receptor-negative cells.

Although the results demonstrate that the shift in PR localization occurs in C/EBPβ+/+ nulliparous mice between 8 and 11–12 weeks of age, it should be noted that the spatial distribution of PR may be differentially temporally regulated in other strains of mice. For example, mammary glands of pure C57BL/6 mice do not form alveolar buds or fine side branching in response to the estrus cycle, and they exhibit minimal alveolar development in response to exogenous hormone treatment compared with other strains of mice such as C3H (21). However, C/EBPβ−/− females, which are maintained as a mixed strain, do exhibit alveolar budding from the ducts as early as 9 weeks of age, and in these females PR is expressed in the nonuniform pattern (T. Seagroves and J. Rosen, personal observations). Haslam has reported that the responsiveness to P is acquired at 7 weeks of age in Balb/C females; therefore, it would be interesting to determine whether the spatial distribution of PR was coincident with response to P in this strain of mice (18). Although some strains of mice do exhibit minimal MEC alveolar budding as virgins, there are relatively few alveoli present in virgin compared with pregnant females, and there are relatively low levels of proliferation of MEC until the onset of pregnancy or the administration of exogenous hormones.

Deletion of C/EBPβ Inhibits Alveolar Proliferation from Ductal Progenitor Cells

Recent studies have suggested the steroid receptor-positive cells are not the proliferative population of cells in the normal human breast (3, 4). These observations led to the hypothesis that the increased number of PR⁺ MEC in C/EBPβ−/− mice might prevent alveolar development if steroid receptor expression and proliferation are mutually exclusive. Analysis of bromodeoxyuridine (BrDU)-labeled MEC and their association with PR revealed that in C/EBPβ−/− mice an inverse relationship existed between the expression of PR by MEC and their proliferation (Fig. 3A and Fig. 1C vs. Fig. 3C). Very few MEC exhibited positive BrDU staining in C/EBPβ−/− mice (1.5%, decreased 10-fold compared with C/EBPβ+/− mice), whereas a majority of MEC were PR⁺ (68%). In contrast, in C/EBPβ+/− controls, 15.8% of cells were in S-phase, and PR was expressed in a subset of ductal MEC, approximately 25%, in the nonuniform pattern observed previously (Figs. 3Aa, 3C, and 1C). As in the human breast, the steroid receptor-positive and proliferating cells rarely colocalized to the same cell in the mammary glands of either C/EBPβ+/+ (Fig. 3B, a and b) or −/− mice (data not shown). In the normal gland, the PR⁺ and BrdU⁺ MEC were adjacent to each other, suggesting that PR regulates alveolar proliferation in a juxtacrine fashion. These unique observations of perturbed PR⁺ and proliferating MEC populations in C/EBPβ−/− mice are, therefore, consistent with recent data for the human breast wherein steroid receptor-negative MEC are the proliferative cells in the normal mammary gland of sexually mature females (3, 4). In addition, the results indicate that this aspect of steroid receptor physiology of the mouse is similar to the human.

Alterations in PR Expression in Mice Lacking C/EBPβ Are Specific to the Mammary Gland

Northern blot analyses revealed that PR mRNA levels were increased 3- (6.9- and 3.5-kb transcripts) to 5-fold (8.7-kb transcript) in the mammary glands of virgin and acutely stimulated C/EBPβ−/− mice as compared with those from C/EBPβ+/+ mice, after normalization to cyclophillin (Fig. 4A). In contrast, after acute E+P treatment, the expression of PR in the uterus was not changed significantly by deletion of C/EBPβ. When double IF was performed on sections of uterus isolated from the same animals, no differences in the pattern or expression of PR or the amount of proliferation were noted between genotypes (data not shown). These results are intriguing since opposing effects of steroid hormones and their antagonists have been reported for the mammary gland and uterus (22). Since C/EBPβ is expressed in both the mammary gland and in the uterus (data not shown), it is possible that C/EBPβ may interact with a mammary gland-specific transcriptional coregulator to achieve this tissue-specific response.

Increased levels of circulating P during pregnancy normally result in the down-regulation of PR mRNA and protein in MEC (23). Since PR is not down-regulated in mature mice treated acutely with E+P, mice were then treated chronically (21 days) with E+P to determine whether longer exposure to E and P would decrease the level of PR expression. However, in mammary glands of C/EBPβ−/− mice treated chronically with E+P, the 6.9- and 3.5-kb PR transcripts remained elevated approximately 3-fold compared with C/EBPβ+/+ controls (Fig. 4B).

Therefore, no significant difference in the percentage of PR⁺ cells or the level of PR mRNA for either genotype was observed before and subsequent to administration of E+P. These results are consistent with previous observations that administration of E+P does not alter the overall percentage of epithelial cells expressing PR (24), suggesting that alveolar progenitor cells are permanently marked in the sexually mature female. A 3-fold induction of PR mRNA after E treatment has been reported in the mammary glands of ovariectomized mice (23). Since P suppresses the inductive effect of E on PR mRNA during pregnancy (25), the combinatorial effect of E+P may not signifi-
In summary, the overexpression of PR is persistent in C/EBPβ−/− mice whether or not they are administered exogenous E1P and whether they are treated acutely or chronically with E1P. The deletion of C/EBPβ impairs the normal spatial distribution and expression of PR in a temporal fashion independent of the levels of circulating E1P.

Fig. 3. Inhibition of Alveolar Proliferation in C/EBPβ−/− Mice Acutely Stimulated with E1P

A, Double IF detection of PR (TR) and BrdU (FITC) was performed on paraffin sections of mammary glands of C/EBPβ+/+ (a) and −/− (b) mice. The green arrow in Aa indicates background FITC signal consistently observed in blood vessels of mammary glands. B, Localization of PR+ and BrdU+ cells in a duct (a) and alveoli (b) from mammary glands of C/EBPβ−/− mice. The white arrow in Bb indicates a rare colocalizing cell. C, MEC proliferation (% BrdU+ cells ± SEM) in C/EBPβ−/− (solid bar) and C/EBPβ−/− (hatched bar) mice.

Deletion of PR Has No Effect on C/EBPβ mRNA Levels

To determine whether C/EBPβ acts upstream or downstream of PR, the level of C/EBPβ mRNA was quantitated in mammary glands of individual PR−/− mice by Northern blotting. At 14 weeks of age (untreated adult virgin) or after 21 days of treatment with E1P, no significant change in C/EBPβ mRNA levels was detected after correction for loading by cyclophilin (Fig. 4C). As previously reported, C/EBPβ mRNA levels increase in response to steroid hormones (lanes 1–4 vs. 5–8) (6). Therefore, PR appears to be regulated either in parallel or downstream of C/EBPβ via either direct or indirect mechanisms.

A Testable Model of the Juxtacrine Mechanisms of Alveolar Morphogenesis

The increased expression and uniform localization of PR observed in MEC of virgin mice lacking C/EBPβ persisted upon stimulation with E1P and correlated
with an inhibition of alveolar development. These observations are opposite to the anticipated decrease in PR in the mammary epithelium of mice lacking C/EBPb given that alveolar development is impaired in both PR−/− and C/EBPb−/− mouse models.

Based on these results, and those reported previously by other laboratories (3, 4, 15, 16, 24), we propose a model in which two cell populations may coexist in the normal mammary gland in a delicate balance; the spatially restricted, steroid receptor-positive cells and the subset of steroid receptor-negative cells that will proliferate in response to juxtacrine signals generated by pregnancy (Fig. 5). The aberrant uniform expression of PR in C/EBPb−/− MEC may disrupt this balance. The increased proportion of PR+ MEC could effectively decrease the steroid receptor-negative “target” subpopulation capable of proliferation in response to pregnancy-associated signals. The steroid receptor-negative population of MEC may be growth arrested due to exclusive expression of cyclin-dependent kinase inhibitors (CKIs) such as p27 (R. Clarke and E. Anderson, personal communication).

Although it is proposed in this model that growth factors are synthesized in the steroid receptor-positive cells and are secreted to act on adjacent cells, further experiments are required to substantiate this proposal. In some breast cancers, or in MEC lines derived from breast tumors, alterations in this temporal and spatial program may result in the utilization of autocrine signaling pathways in the steroid receptor-pos-
C/EBPβ is not restricted to the overexpression and altered cellular distribution of PR, leading to the hypothesis that C/EBPβ plays a more global role in cell fate decisions. First, recent experiments have indicated that deletion of C/EBPβ also results in the up-regulation of several other key players implicated in alveolar morphogenesis, including estrogen receptor (ERα), and PRL receptor (PrlR) (31, 32) (T. Seagroves, S. Grimm, R. Hovey, B. Vonderhaar, and J. Rosen, personal observations). Second, suppressive subtraction hybridization screens have identified several novel genes as well as a molecular marker of stratified epithelium that are up-regulated in response to deletion of C/EBPβ (T. Seagroves, S. Grimm, and J. Rosen, personal observations). The observed changes in the expression and patterning of these multiple genes occur in nulliparous, cycling females in a temporal fashion. By 8 weeks of age in most mouse strains, TEBs are no longer present and the MECs within ducts are essentially quiescent. Therefore, the dramatic switch in the patterning of these factors does not appear to be dependent upon a partitioning mechanism requiring cell division.

The analysis of PR as a marker of alveolar cell fate in wild-type and C/EBPβ−/− mice has provided novel insight into mechanisms controlling normal mammary gland lobuloalveolar development. First, in mice that still contain TEBs, the uniform distribution of genes that will later control lobuloalveolar development may serve to inhibit the premature formation of alveoli. Second, the switch to a nonuniform pattern of distribution of these molecular markers that occurs between 8 and 12 weeks of age may facilitate the maximal proliferation of alveolar progenitor cells induced by steroid hormones. Third, an exact cellular address for each individual gene, such as PR, may be required to activate proliferation of a neighboring epithelial cell to prevent autocrine stimulation of proliferation. Disruption of any of these mechanisms may result in either inhibition of proliferation of MEC, as observed in the C/EBPβ−/− mouse model, or result in mammary tumors. Unraveling the molecular mechanism of the C/EBPβ-mediated switch in cellular distribution of these genes awaits further gene discovery and continued investigation of the cellular distribution and/or colocalization of molecular markers in additional knockout mouse models.

**MATERIALS AND METHODS**

**Animals**

C/EBPβ−/− mice were originally provided by Dr. Valeria Poli (70% C57BL/6:20% 129-Sv:10% MF-1). C57BL/6 mice (Taconic Farms, Inc., Germantown, NY) were used to perform IF analysis of PR expression during virgin development (n = 3 per age group). PR−/− samples were isolated from mice in the 129-Sv/C57BL/6 background. Animal studies were conducted in accord with NIH standards for the care and use of experimental animals.
Steroid Treatment Protocols

One inguinal mammary gland from each ovary-intact, mature virgin C/EBP\textsuperscript{b}/\textsuperscript{−/−} mouse was used for IF analysis. Mammary tissues were collected from a subset of the cohorts of mice and three additional mice per genotype were prepared according to the manufacturer's instructions. Total RNA (RNAzol B, Tel-Test, Friendswood, TX) and poly (A) mRNA were isolated. Northern Blot Analysis

Northern Blot Analysis

Total RNA (RNAzol B, Tel-Test, Friendswood, TX) and poly (A)-selected RNA (PolyATract, Promega Corp., Madison, WI) were prepared according to the manufacturer's instructions. Mammary tissues were collected from a subset of the cohorts of C/EBP\textsuperscript{b}/\textsuperscript{−/−} mice (30). After blocking in 5% BSA/0.5% Tween-20 for 4 h at RT, sections were incubated simultaneously with anti-BrdU-fluorescein isothiocyanate (FITC)-conjugated antibody (1:5-1:10; Becton Dickinson and Co., Franklin Lakes, NJ) and a rabbit polyclonal antigen retrieval in 10 mM citrate buffer, pH 6.0 (33). After hybridization, the blots were washed in 10% dextran sulfate, 2% SDS, 100 μg/ml salmon sperm DNA, 1 mg/ml yeast soluble RNA, 100 μm dithiothreitol at 55 C. Riboprobes for PR were transcribed from a 395-bp fragment corresponding to nt 2383-2778 of the mouse PR cDNA (34) subcloned into PCRScript. Hybridization buffer containing 35S-labeled cRNA probe (5 x 10\textsuperscript{7} cpm/μl) was added to sections that were then incubated in a humidified chamber overnight at 55 C. Coverslips were removed in 2× SSC, 50% formamide for 20 min and the sections were washed in 2× SSC, 50% formamide at 60 C for 30 min. Digestion with RNAase A (20 μg/ml) was performed before washes with 2× and 0.1× SSC at 37 C. Sections were exposed to emulsion (NTB-2, Eastman Kodak Co., Rochester, NY) for 4 weeks and then counterstained with Nuclear Fast Red.

REFERENCES


In Situ Hybridization

Sections prepared as described above were treated with 0.2 M HCl, digested with proteinase K (5 μg/ml), postfixed in 4% paraformaldehyde, and acetylated (0.25% acetic anhydride in 0.1 M triethanolamine buffer, pH 8.0. Sections were pre-hybridized for 1 h in hybridization buffer [50% formamide, 0.75 μ NaCl, 0.075 μ Na, citrate (5× SSC), 10% dextran sulfate, 2% SDS, 100 μg/ml salmon sperm DNA, 1 mg/ml yeast soluble RNA, 100 μm dithiothreitol] at 55 C. Riboprobes for PR were transcribed from a 395-bp fragment corresponding to nt 2383-2778 of the mouse PR cDNA (34) subcloned into PCRScript. Hybridization buffer containing 35S-labeled cRNA probe (5 x 10\textsuperscript{7} cpm/μl) was added to sections that were then incubated in a humidified chamber overnight at 55 C. After thorough washing, sections were exposed to emulsion (NTB-2, Eastman Kodak Co., Rochester, NY) for 4 weeks and then counterstained with Nuclear Fast Red.

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