Impact of progesterone receptor on cell-fate decisions during mammary gland development

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Mammary epithelium contains lineage-limited progenitors that give rise to cells that form distinct morphological structures, ducts vs. lobules, depending on the endocrine status of the female. Progesterone signaling through progesterone receptor (PR) is essential for lobulo-alveolar development that accompanies pregnancy, but not for ductal growth accompanying puberty. PR exists in two molecular forms, A and B, and an imbalance in the native ratio of the two isoforms can lead to alterations in PR signaling. Indeed, as we reported previously, in transgenic mice carrying additional A form of PR, mammary development is abnormal, characterized by excessive lateral ductal branching. This suggests that alterations in PR signaling may have important consequences to mammary development, particularly with regard to ductal vs. alveolar growth. To test this further, we created transgenic mice carrying additional B form of PR and report that mammary development in these mice is also abnormal, characterized by inappropriate alveolar growth. More importantly, these mammary glands, on serial transplantation, undergo a premature arrest in ductal growth without any alteration in the potential for lobulo-alveolar growth. Such an arrest in ductal growth does not occur with transgensics carrying additional A form of PR. These studies, therefore, provide strong evidence to indicate that PR signaling may be of paramount importance for appropriate cell-fate decisions during normal mammary development, and also that this requires a regulated expression of the two isoforms.

Abbreviations: PR, progesterone receptor; CMV, cytomegalovirus; ER, estrogen receptor; PRL, prolactin; PRLR, PRL receptor.

Materials and Methods

Construction of Transgenic Mice. To generate transgenic mice carrying an excess of the B form of PR, we used a binary transgenic system. In this system, the GAL-4 gene, driven by the murine cytomegalovirus (CMV) promoter (CMV-GAL-4 mice), served as the transactivator of the PR-B gene, carrying four GAL-4 binding sites (UAS) (UAS-PR-B mice). Crossing the CMV-GAL-4 mice with UAS-PR-B mice resulted in bigenic mice carrying additional B form of PR (PR-B transgenics), and report that mammary development in these mice is also abnormal, characterized by inappropriate alveolar growth. More importantly, in mammary glands of PR-B transgenics, there is a premature arrest in the ability of ducts to fill the fat pad without any alteration in the potential for lobulo-alveolar growth, a phenomenon that does not occur with PR-A transgenics.
mice were identified initially by Southern blot analysis, and, once
the founder lines were established, they were routinely screened
by PCR using tail DNA, as described (16). Transgene and
endogenous PR expression was examined by reverse transcrip-
tion-coupled PCR as described (16).

Whole-Mount Preparation and Histological Analysis. The entire num-
ber 4 inguinal mammary glands were removed, fixed in Carnoy’s
solution (acetic ethanol) at room temperature, and processed as
described (16).

Tissue Transplantation. Mammary fat pads devoid of epithelium
were prepared according to the cleared fat pad technique of
DeOme et al. (5, 18) by using 21-day-old mice. Mammary tissue
fragments from donor mice (~1.5 mm³) were implanted within the
cleared fat pads, and the mammary glands of hosts were
examined at specific times after transplantation.

Analysis for PR Expression. PR mRNA levels in mammary glands
were estimated by using total cellular RNA and RNase protec-
tion assay as described (19). The probe used for the detection of
PR mRNA was generated by linearizing the plasmid mPR17 (17)
with Xmn1 and transcribing with T3 polymerase to yield a 369-bp
fragment. For examining the immunolocalization of PR, an
indirect immunofluorescence assay using an antibody prepared
against mouse PR was used as described (20).

Results

Analyses for Transgene and Total PR Expression. Fig. 1iii shows the
analyses for transgene expression in the mammary glands of
CMV-GAL-4 and bigenic mice. As shown, PR-B transgene expres-
sion was found in mammary glands of bigenic mice (Fig. 1iii, lane
4) and not in glands of monogenic mice carrying only the Gal-4 gene
(Fig. 1iiA, lane 6). Gal-4 gene expression was found in glands of both
bigenic and monogenic mice carrying only the Gal-4 gene (Fig. 1iiB,
lanes 4 and 6). As expected, endogenous PR expression also was
found in the glands of both monogenic and bigenic mice (Fig. 1iiiC,
canes 2, 4, and 6). Analysis for total PR mRNA (Fig. 1iii) revealed an
increase in the mammary glands of bigenic mice (Fig. 1iii, compare A with B) and thus confirmed the overexpression of PR.
Immunolocalization studies (Fig. 1iv) also clearly revealed an
increase in PR in the mammary epithelial cells of PR-B transgenics,
as compared with transgene-negative control littermates (Fig. 1iv,
compare A with B).

Ductal Elongation But Not Alveolar Growth Is Compromised in Mam-
mary Glands of PR-B Transgenics. Initial whole-mount analyses of
mammary glands of adult (10- to 14-wk-old) PR-B transgenics
did not reveal any dramatic differences as compared with
transgene-negative littermates. However, in contrast to wild-
type littermates, in approximately 20% of mice, even at 20 wk of
age, the fat pad was not completely filled. Also, in some of these
 glands, in certain regions, there was no lateral branching. More
significantly, even in the absence of fat pad filling, these glands
did not contain any end-buds (data not shown), indicating that,
on overall, there was a cessation in growth; end buds represent sites
of active proliferation in the growing ducts (4).

It is well established that mammary epithelial cells will grow
when transplanted into de-epithelialized (cleared) fat pads of syngeneic hosts (5). Indeed, using this in vivo cell transplantation technique, epithelial progenitor cells with three distinct developmental potentials have been identified in mouse (10). Similarly, this technique also has been used successfully to demonstrate epithelial cell senescence in mammary glands (7, 21). Therefore, to ascertain whether mammary glands of PR-B transgenics were indeed growth compromised, serial transplantation studies were performed. Fig. 2 shows the growth patterns of representative outgrowths derived from serial transplantations, whereas Fig. 3 shows the relative ability of the various outgrowths to repopulate the fat pad. Although in the majority of first-generation outgrowths, growth was not compromised significantly (Fig. 3), in some transplants, the ducts did not extend to fill the fat pad (Fig. 2Ad). In contrast to first-generation outgrowths, a significant number of outgrowths from second generation did not fill the fat pad (Fig. 2Ab), whereas, in third-generation transplants, growth was extremely limited (Fig. 2Ac). In contrast, as reported previously (7, 21), third-generation transplants of wild-type tissue were able to repopulate the fat pad to full capacity (Figs. 2Ca and 3). The limited growth observed with transplants of PR-B transgenics was intrinsic to the tissue and not the result of host-derived factors, because it manifested readily even when tissues were propagated in transgene-negative females. For example, the transplant with limited growth (Fig. 2Ad) had been propagated in PR-B transgene-negative females. Also, in all mice, in contrast to the very limited growth observed with the transplants, the fat pads of host mammary glands were filled with ducts (data not shown). To examine if lobulo-alveolar growth also was compromised in PR-B transgenics, mice carrying third-generation transplants were mated, and mammary glands of these pregnant mice were examined for their growth potential. As shown in Fig. 2Ad, although these transplants still did not fill the fat pad with ducts, they did display lobulo-alveolar development.

In previous studies (16), we had shown that mammary glands of PR-A transgenics also have abnormal development, but had not examined the behavior of these tissues on serial transplantation. Therefore, it was necessary to determine whether the limited mammary ductal growth in PR-B transgenics was specifically caused by the introduction of additional B form or by alterations in PR signaling, arising from the overall imbalance in the native ratio of A/B forms, which was amplified by transplantation. As shown in Figs. 2B and 3, in contrast to PR-B transgenics, mammary glands from PR-A transgenics could be easily transplanted up to three generations without any significant impairment in their ability to repopulate the fat pad. Furthermore, these outgrowths also maintained the phenotype of the donors characterized by excessive and abnormal side branching (compare Fig. 2Ba with Bb–Bd); for comparison, the pattern and degree of side branching in an age-matched wild-type nulliparous mouse also is shown in Fig. 2Cb.

Finally, we also verified that, in the case of both PR-A and PR-B transgenics, transgene expression was intact in the outgrowths. Also, the behavior of the glands from the two genotypes was observed with more than one founder line of UAS-PR-B transgenics (data not shown).
Morphological and Histological Analyses. To further define the mammary phenotype of PR-B transgenics, both morphological and histological analyses were performed on the serial outgrowths carried in both nulliparous and pregnant hosts. As expected, in outgrowths of wild-type mice, mammary ducts, on cessation of growth, terminated in smooth blunt ends (Fig. 4Aa).

In contrast, in mammary outgrowths of PR-B transgenics, carried in nulliparous mice, bulbous structures were present at the end of some mature ducts and also in the interductal spaces (Fig. 4Ab–Ad). These structures were also more numerous in second- and third-generation outgrowths as compared with first generation (compare Fig. 4Ab with Ac and Ad). Histological analyses revealed that these bulbous structures at the termini of ducts and in interductal spaces represented regions containing clusters of acini (Fig. 4B).

Histological analyses of outgrowths of PR-B transgenics carried in pregnant hosts revealed that lobular growth was achieved by these transplants. However, the structure of the lobules was somewhat abnormal. As such, as shown in Fig. 5, in contrast to the glands of the pregnant host (Fig. 5D–F), the lobules in outgrowths of PR-B transgenics (Fig. 5C and E) formed compact acini and frequently were embedded in a highly cellular connective tissue. Also, these structures were somewhat disorganized with limited secondary and tertiary ductal branching. The alveoli were also less differentiated, as revealed by a lack of cytoplasmic lipid vacuoles seen with the host gland (Fig. 5F).

Fig. 5 also shows that, in the transplants of PR-B transgenics, carried in both nulliparous (Fig. 5A) and pregnant (Fig. 5B and E), hosts contain many mitotic figures. A notable feature of the alveoli in outgrowths of PR-B transgenics was that several of these mitotic figures were abnormal (Fig. 5B).

Analyses for Estrogen Receptor (ER) and Prolactin Receptor (PRLR). Studies on ER-null mutant mice have shown that ER is essential for the ductal growth accompanying puberty (22). Analyses for the steady-state levels of ER gene expression, as described (19), did not reveal any significant differences in the mammary glands of PR-B transgenics, as compared with transgene-negative littermates (data
Fig. 5. Photoimages of histology of transplanted and host mammary epithelium. (A) The alveolar buds at the terminal end of a transplant of a mammary gland from a PR-B transgenic in a nulliparous host. Note that the cross section shows the unusual branching structure (compare with whole-mount image in Fig. 4Ac). Also note the high number of mitotic figures (arrows). (B) The complex irregular branching terminal end buds with a highly cellular stroma (arrow S) in a (generation III) transplant of mammary gland from a PR-B transgenic in a midpregnant host. Note the numerous normal and abnormal mitotic figures including a sunburst mitotic figure (Upper Inset) and a tri-polar mitotic figure (Lower Inset). (Scale bar = 0.100 mm.) (C) The pattern of compact lobules (arrow) in a mammary transplant (generation III) from a PR-B transgenic in a midpregnant host. Compare this pattern with the whole mount in Fig. 2Ad. (D) The pattern of normal lobules (arrowhead) with extended ducts in the mammary gland in the same mid-pregnant host. Note the relative length and distribution of the branching ducts (space bars = 0.1 mm). (E) The morphology of the compact lobule in the PR-B transplant at a higher magnification of the upper image seen in C. Note the rosette of undifferentiated alveoli clustered around the terminal duct. The epithelium has a high number of mitotic figures. The acini are embedded in a highly cellular, fibrotic connective tissue. (F) The detail of the normal differentiation of the lobules in a midpregnant host. Note the relative distribution of the acini and the length of the terminal ducts. Note also the relatively large number of cytoplasmic lipid vacuoles (arrow) and the lack of mitotic figures (space bars = 0.1 mm).
not shown). It is well established that, in addition to progesterone signaling through PR, signaling through prolactin (PRL) is also essential for lobulo-alveolar growth (2). Indeed, in both PRL- and PRL-null mutant mice, lobulo-alveolar growth is impaired (23, 24). Therefore, it was possible that, in transplants of PR-B transgenics, carried in nulliparous hosts, there was an increase in PRLR expression. However, analyses for PRLR expression, as described (25), in both primary glands and serial outgrowths of PR-B transgenics, carried in nulliparous mice, did not reveal any detectable increase in PRLR expression (data not shown).

**Discussion**

In the present studies, we have documented that mammary development is abnormal in transgenic mice carrying additional B form of PR. A striking feature of the mammary glands of PR-B transgenics is that, on serial transplantation, they have limited capacity for ductal growth, apparent as early as the second generation; this is intrinsic to the tissue and not because of host-derived factors. In contrast, glands of PR-A transgenics do not undergo a similar arrest in ductal growth. Mammary glands of wild-type mice also can be easily propagated well beyond three generations without a significant loss in ductal growth (ref. 7 and Fig. 3). Therefore, the loss in mammary ductal growth observed in PR-B transgenics results from the introduction of additional B form of PR. Mammary transplants of PR-B transgenics carried in nulliparous hosts also contain acini, a feature not seen in the host glands. Also, despite a robust lobulo-alveolar growth in the transplants of PR-B transgenics, they have very limited lateral ductal branching and do not achieve functional differentiation, i.e., lack of cytoplasmic lipid vacuoles. In contrast, in mammary glands of PR-A transgenics, there is excessive lateral branching, even in the absence of pregnancy, and this phenotype persists on serial transplantation. Taken together, these observations suggest that the primary effect of PR signaling in adult females may be to direct the mammary epithelial cells toward a particular developmental fate. If this were so, it also could help to explain why, on serial transplantation, mammary glands of PR-B transgenics lose their capacity for ductal elongation.

In a series of comprehensive studies, Daniel and colleagues (5, 7, 21) have demonstrated that, on serial transplantation, mammary glands of wild-type mice eventually can lose their capacity for ductal elongation, similar to that seen with PR-B transgenics, except with the critical difference that this occurs very rapidly with PR-B transgenics. The availability of new space for ductal elongation, similar to that seen with PR-B transgenics, results from the introduction of additional B form of PR. Mammary transplants of PR-B transgenics carried in nulliparous hosts also contain acini, a feature not seen in the host glands. Also, despite a robust lobulo-alveolar growth in the transplants of PR-B transgenics, they have very limited lateral ductal branching and do not achieve functional differentiation, i.e., lack of cytoplasmic lipid vacuoles. In contrast, in mammary glands of PR-A transgenics, there is excessive lateral branching, even in the absence of pregnancy, and this phenotype persists on serial transplantation. Taken together, these observations suggest that the primary effect of PR signaling in adult females may be to direct the mammary epithelial cells toward a particular developmental fate. If this were so, it also could help to explain why, on serial transplantation, mammary glands of PR-B transgenics lose their capacity for ductal elongation.

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