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A Reappraisal of Progesterone Action in the Mammary Gland

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The ovarian hormones estrogen and progesterone and their respective receptors are essential for maintenance of postnatal developmental plasticity of the mammary gland and play a key role in mammary tumorigenesis. Mouse models in which expression of the progesterone receptors was genetically ablated have recently become available. Studies of these models have demonstrated that progesterone is specifically required for pregnancy associated ductal proliferation and lobuloalveolar differentiation of the mammary epithelium, but not for immediate postpubertal ductal morphogenesis. Use of these mice in combination with mammary gland transplantation indicates that developmental regulation by progesterone appears to occur through a paracrine mechanism in which progesterone receptor (PR)[3] positive cells represent a subset of non-proliferating epithelial cells that are capable of directing proliferation and/or differentiation of neighboring receptor negative cells. The hierarchical organization of these receptors in the epithelium and their segregation from proliferating cells is a conserved feature in rodent and human mammary tissue. The identification of paracrine mediators of the progesterone response is now an imminent goal as is the delineation of the individual contributions of the two PR isoforms using similar approaches.

KEY WORDS: Progesterone; mammary gland; PRKO mouse; epithelial-stromal; PR-A and -B.

INTRODUCTION

The evolution and propagation of the eutherian mammal has depended on the ovarian steroid hormone: progesterone. Although the role of progesterone in uterine biology is well recognized, its involvement in mammary gland development and function has been less well understood. However, as described in the ensuing sections, recent studies using rodent models amenable to genetic manipulation together with mammary gland transplantation approaches have begun to unravel the specific developmental role of progesterone in the mammary gland, and have exposed the progesterone-signaling pathway to molecular dissection.

The physiological effects of progesterone are mediated by interaction of the hormone with specific intracellular progesterone receptors (PRs) that are members of the nuclear receptor family of transcription factors (1,2). Progesterone receptors consist of two protein isoforms, termed A and B, that are expressed from a single gene in rodents and humans (3,4). The A and B proteins are produced by initiation of translation at two distinct AUG signals and differ by an amino terminal extension of 128–165 amino acids (depending on species) that is specific to the B protein [the mouse PR (5) is shown in Fig. 1, panel A]. Binding of progesterone to its receptors induces receptor dimerization, binding to specific *cis*-acting DNA elements in the promoter region of specific target genes and recruitment of coactivator proteins and general transcription factors to regulate transcription of responsive target genes [reviewed in (1)]. A significant body of evidence has accumulated in

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[3] Abbreviations: Progesterone receptor (PR); Progesterone and estrogen receptor knockout (PRKO and ERKO respectively); Terminal end bud (TEB).

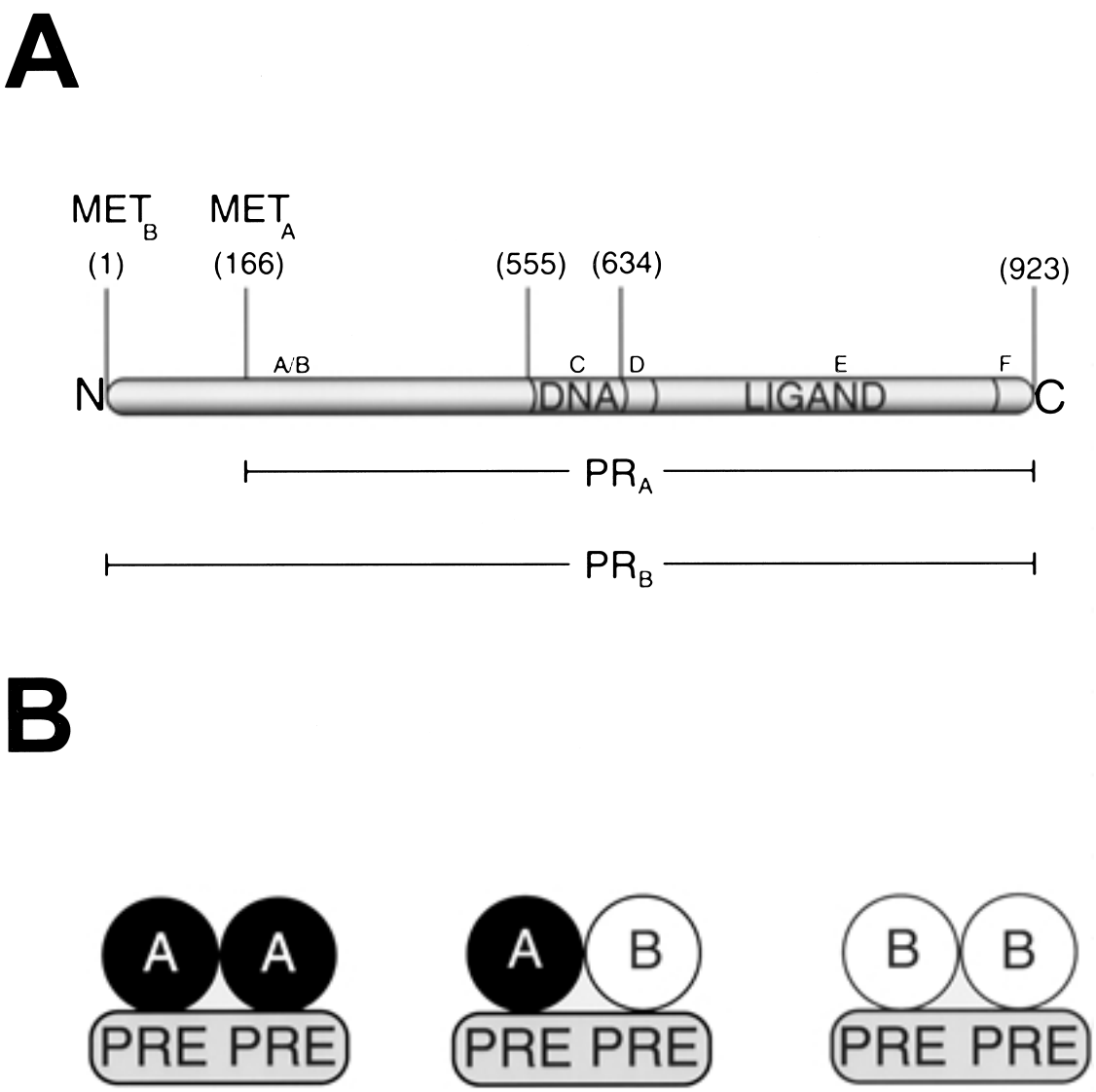


Fig. 1. The progesterone receptor gene encodes two receptor isoforms: PR-A and PR-B. Panel A. The progesterone receptor contains a long N-terminus region (A/B), a short DNA binding domain (C) and hinge region (D), a ligand binding domain (E) and a short C-terminus (F). The PR comprises two receptor isoforms, PR-A and PR-B. PR-B is structurally identical to PR-A, except for an N-terminal extension that varies depending on the species. The mouse PR is depicted; numbers in parenthesis denote amino acid number; MET_B and MET_A are the initiating methionines for PR-B and PR-A respectively. Panel B. Assuming coexpression of PR-B and -A within the same cell, the possible PR homo- and hetero-dimers that can occur are denoted. Each PR dimer type would be expected to elicit a different transactivational response; PRE is an acronym for progesterone response element.

recent years demonstrating that while both the A and B protein bind progesterone and the same DNA elements, they interact differently with some coactivators and have different promoter and cell specific transcription activation properties [reviewed in (6)]. These findings have significant implications with regard to the complexity of progesterone signaling *in vivo*. If expressed in the same cells, the A and B proteins can dimerize and bind to DNA as three species: A:A or B:B homodimers or A:B heterodimers (Fig. 1, panel B). The specific contribution of each of these species to the regulatory effects of progesterone will depend on the differential transactivation properties contributed to these complexes by the B

specific domain. Based on the distinct transactivation properties of PR-A and -B proteins observed *in vitro*, the relative expression of these isoforms in the mammary gland is likely to have a significant impact on the functional responses to progesterone.

In this review, we will first summarize the essential role of steroid hormones in maintaining postnatal developmental plasticity of the mammary gland. We will then describe the recent advances in our understanding of the specific roles of progesterone in regulating mammary gland morphogenesis and tumorigenesis that have emerged through genetic ablation of PR expression in mice and mammary gland transplantation experiments. Finally, we will discuss how the different functional activities of the PR-A and -B proteins observed *in vitro* are likely to contribute to the complexity of progesterone signaling within the gland.

OVARIAN STEROIDS AND MAMMARY GLAND DEVELOPMENT

Despite over fifty million years of divergent evolution between the human and rodent, mammary gland development in both mammals is remarkably similar; thus within this phylogenetic Class, the rodent model has been the experimental system of choice to define the general principles of mammary gland development. In particular, our recent ability to genetically manipulate the mouse has provided unparalleled opportunities to investigate the molecular and cellular mechanisms that underlie normal mammary gland development as well as mammary tumorigenesis, in an *in vivo* context.

For all eutherian mammals, mammary gland development occurs predominantly postnatally, and is directed by a complex signal transduction interplay between endocrine hormones and locally acting growth factors [reviewed in (7,8)]. The adult mammary gland consists primarily of a secretory epithelium consisting of a branching ductal architecture embedded in an adipose stromal compartment (also known as the fat pad) that provides an inductive environment (from the embryonic stage to the adult) in which the epithelium develops and functions. Mammary gland morphogenesis occurs in distinct developmental stages depending on the age and reproductive state of the animal. In the prepubertal mouse, as with the human, the mammary gland contains a rudimentary primary epithelial duct with limited side branching that extends a short distance from the nipple into the mammary fat pad. Following puberty, cells localized in the bulbous shaped terminal end buds (TEB) at the distal ends of the mammary ducts undergo extensive mitotic activity that results in both the elongation and bifurcation of mammary ducts to the periphery of the fat pad. Once the ductal network extends to the limits of the mammary fat pad, the TEB regress and mammary epithelial ductal growth is arrested until pregnancy. The second round of mammary epithelial proliferation occurs in response to the hormones of pregnancy, and subsequent epithelial differentiation is manifested by the development of secretory alveolar structures that progressively occupy the remaining fat pad during pregnancy, parturition and the onset of lactation. Following removal of the suckling stimulus at weaning, milk protein-secreting alveoli undergo apoptic-mediated reductive remodeling termed involution, and by four weeks of this post lactational epithelial regression, the mammary gland developmental pathway is essentially complete.

The entire developmental program is controlled by the combined action of ovarian steroids and peptide hormones, such as prolactin [reviewed in (7,8)]. Progesterone and estrogen are the principle steroid hormones involved in normal breast development and tumorigenesis (9). Early ductal outgrowth observed postpuberty is strongly controlled by the cyclic rise in ovarian estrogen. Deletion of this hormone in mice by a null mutation of the aromatase gene responsible for its synthesis [the aromatase knockout (ARKO) mouse, (10)] results in a rudimentary hypoplastic epithelium that is typical of a prepubertal mammary phenotype. Null mutations of each of the two receptor genes [estrogen receptor-α (ER-α) and estrogen receptor-β (ER-β), (11,12)] known to mediate the genomic effects of estrogen have demonstrated that the ER-α receptor alone is both necessary and sufficient to mediate the morphogenic effects of estrogen on the mammary epithelium. Further, embryonic tissue recombination experiments using wild type and ER-α knock-out (ERKO) mice suggest that loss of ER-α in the embryonic stromal but not epithelial compartment results in inhibition of ductal outgrowth and a rudimentary ductal structure that lacks terminal end buds (13). This study suggests that the role of ER-α in early ductal proliferation and branching morphogenesis may involve a paracrine signaling system initiated by estrogen dependent stromal derived signals acting on epithelial cells to promote their proliferation. However, because this investigation was limited to embryonic tissue, extrap-

olation of these suggestions on ER-α action to the mammary gland of the pubescent and adult mouse must be tempered.

The studies summarized have underscored the essential role of both estrogen and ER-α in ductal proliferation and branching that occurs during puberty. The PR, however, is a downstream molecular target for ER action (discussed later), and as such, the ERKO and ARKO mouse models were unable to define the specific role of progesterone in this organ system. The adoption of a similar genetic approach to specifically ablate expression of the PR in mice has allowed us to delineate the specific effects of progesterone signaling on mammary gland development.

PROGESTERONE RECEPTOR KNOCK-OUT MOUSE

Delineating the Role of Progesterone

To directly address the physiological importance of PR function in the murine mammary gland as well as gain insight into progesterone's functional interrelationship not only with estrogen, but also with prolactin and locally acting growth factors known to influence mammary gland development, a progesterone receptor knockout (PRKO) mouse model was generated in which the functional activity of both forms of the PR (PR-A and PR-B) were simultaneously ablated through gene targeting techniques (14).

Initial mammary gland whole mount analysis revealed that the ductal architecture of the adult PRKO mammary gland was similar to that of the age-matched wild type virgin (Fig. 2; panels A and B). Importantly, the virgin PRKO mammary gland was not a phenocopy of the ERKO or ARKO mammary defect, demonstrating the importance of estrogen rather than progesterone in mammary gland development that occurs between the stages of puberty and the adult virgin.

However, in response to exogenous estrogen and progesterone treatment, comparative whole mount analysis revealed that the adult PRKO mammary gland failed to develop the typical pregnancy-associated epithelial ductal morphogenesis that consists of extensive side-branching with attendant interductal lobuloalveolar development (Fig. 2; panels C–F). These initial gross morphological investigations, in addition to recent molecular analysis (15), unequivocally demonstrated both a proliferative and a differentiative involvement for progesterone and its receptor in this tissue. To address the argument that this simple steroid treatment was insufficient to rescue the PRKO mammary phenotype, recent mammary gland transplantation experiments were performed in which the PRKO mammary gland was transplanted *in toto* into a wild type mouse (16). In concordance with our initial observations, the PRKO mammary defect was not rescued during pregnancy, despite the exposure of the transplanted gland to the full spectrum of pregnancy hormones.

Because epidemiological and experimental studies have demonstrated that an early first pregnancy lowers breast cancer risk whereas nulliparity or a late first pregnancy increases this risk (17,18), understanding the cellular and molecular mechanisms by which progesterone induces mammary gland proliferation and differentiation during pregnancy is a major focus of mammary gland research.

TOWARDS A CELLULAR MECHANISM OF ACTION FOR PROGESTERONE IN THE MAMMARY GLAND

The PRKO mammary phenotype represents a critical *in vivo* validation of the importance of progesterone in the induction of mammary gland epithelial proliferation and differentiation that is required for the formation of ductal and alveolar structures during pregnancy. In the embryo and the adult, normal mammary epithelial ontogenesis is dependent on a reciprocal molecular dialogue between the epithelial and stromal cellular compartments (19). Similar to the prostate and uterus (20,21), the existence of epithelial-stromal interactions in the mammary gland has potentially important clinical implications for certain disease states like breast cancer. The aberrant cellular proliferation and loss of steroid hormonal regulation that often occurs in mammary cancers could conceivably be associated with a change or a loss in normal regulatory interactions between mammary stromal and epithelial cells (20). Elucidating these reciprocal cellular interactions can be facilitated by a clear understanding of the spatiotemporal expression of PRs in the mammary gland and by the gland's intrinsic ability to recapitulate all the ductal and alveolar structures from a fragment of primary ductal epithelium transplanted into a isogenic stroma: the mammary gland transplantation technique (22).

Two different approaches to study the spatiotemporal expression of PR in the mammary gland

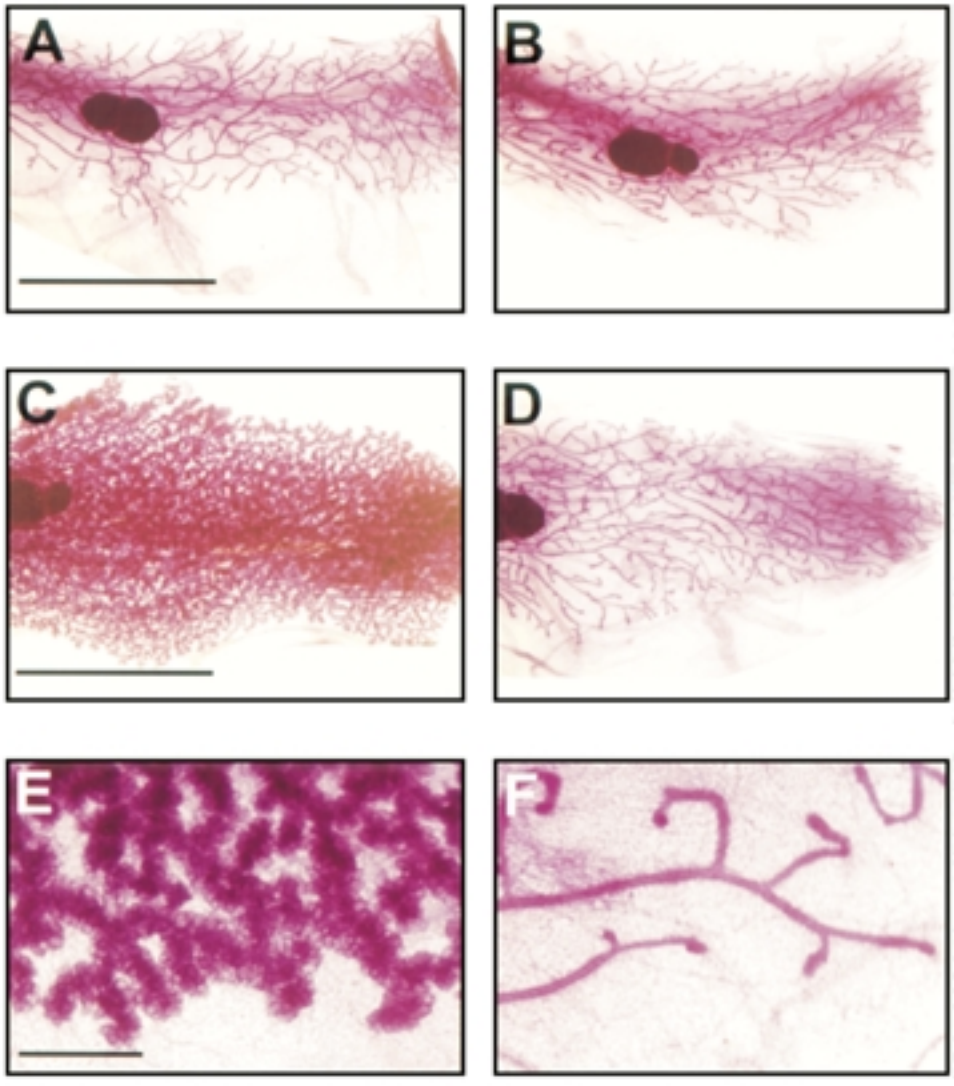


Fig. 2. Ablation of progesterone receptor function manifests as a defect in mammary gland ductal branching and alveologenesis. Adult virgin wild type (panel A) and PRKO (panel B) mammary glands show a similar ductal morphology; scale bar in panel A is 5 mm. The PRKO mammary phenotype is clearly evident when wild type (panel C) and PRKO (panel D) are treated for three weeks with estrogen and progesterone. Note the absence of extensive side branching and alveologenesis in the hormone-treated PRKO (panel D) as compared to wild type (panel C). Panels E and F are higher magnifications of C and D respectively; scale bar in panel E is 500 μm.

have provided conflicting results: Early binding studies using R5020 demonstrated progestin binding sites in both the epithelial and stromal compartments that were distinct with respect to their biochemistry and ontogenesis (23,24). However, more recent immunohistochemical analyses support an exclusively epithelial distribution of receptors that are undetectable in the stromal compartment (25–28). The discrepancy in PR localization observed using these two techniques could be attributed to either poor specificity of the R5020 binding method or to insufficient sensitivity of the anti-PR antibodies. However, recent analysis of PR expression in newly generated PR knock-in mice using Lac-Z as a reporter gene demonstrate an exclusively luminal epithelial localization for PR that is in complete agreement with data

observed from immunohistochemical studies (J. P. Lydon, unpublished observations). The epithelial localization of PR in the mammary gland is surprising in light of its regulation by estrogen and the previous demonstration that ER-α is localized to both the stromal and epithelial compartments of the gland (13). Taken together, these observations suggest that the regulation of PR by estrogen may be compartment specific in the mammary gland.

Most importantly, support for the functional involvement of epithelial rather than stromal derived PRs in mediating progestin dependent mammary morphogenesis has been obtained through the use of the PRKO mouse in combination with mammary gland transplantation approaches to produce mammary gland recombinants that were devoid of PR in either the epithelial, stromal, or both compartments (16).

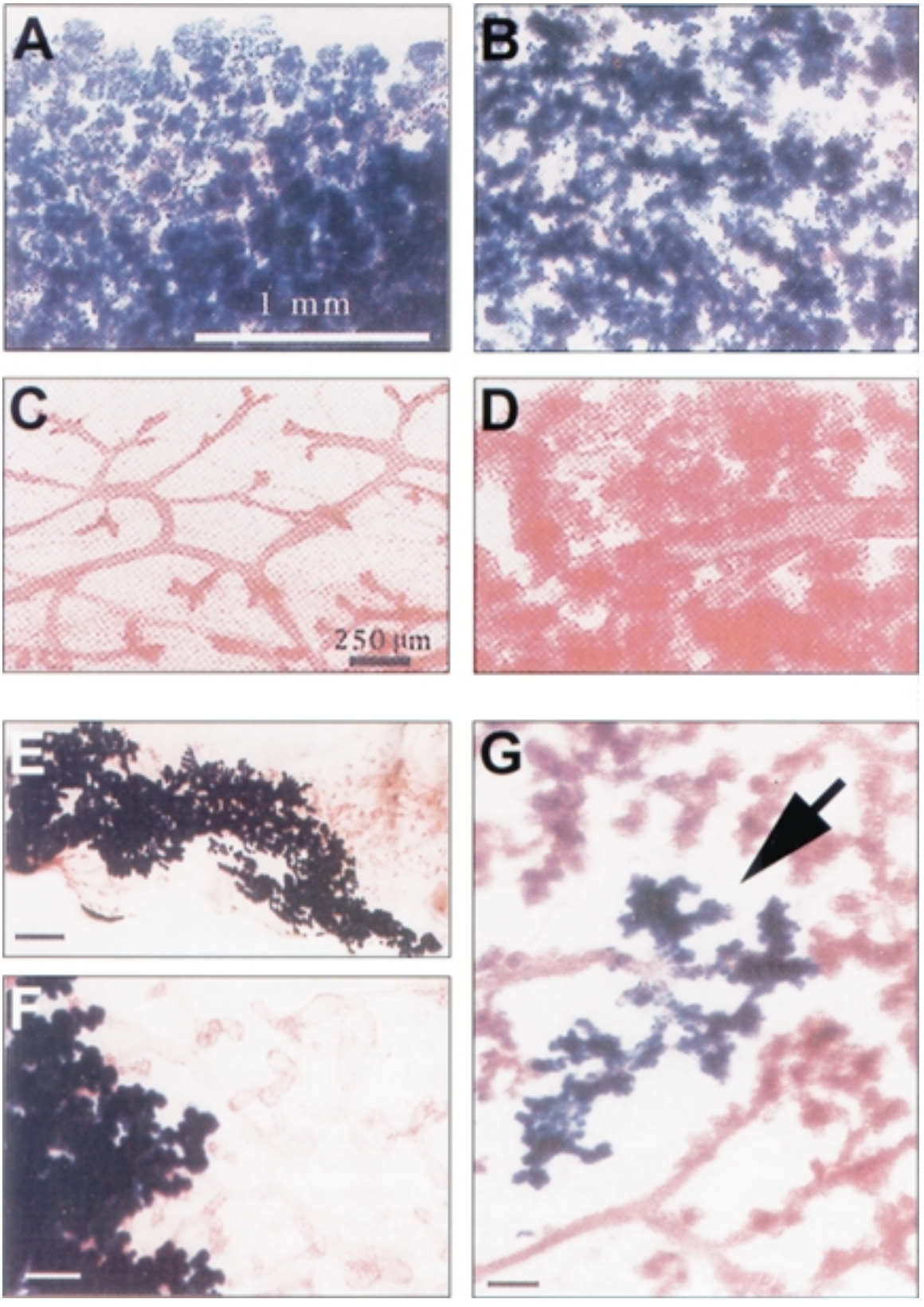
To determine whether PR mediated responses within the mammary stroma contribute to ductal side branching and alveologenesis, wild type epithelium was transplanted into the PRKO stroma, previously divested of PRKO epithelium. Following the pregnancy of the host animal, whole mount analysis revealed that this mammary tissue recombinant developed normally, suggesting mammary stroma was not the primary target for progesterone-initiated proliferative and differentiative responses in the mammary gland (Fig. 3; panels A and B).

The converse experiment was performed in which PRKO epithelium was transplanted into wild type stroma. Despite a full-term pregnancy, whole mount analysis showed that the absence of PR in the epithelium resulted in the typical PRKO mammary phenotype (Fig. 3; panels C and D). This result unequivocally demonstrated that the epithelial cellular compartment is the primary target for progesterone action in the mammary gland. Recent reciprocal mammary gland transplantation approaches applied to the CAAT enhancer binding protein beta (CEBP-β), cyclin D1 and prolactin receptor knockout mouse models have revealed that the mammary epithelium is also the primary target for these important regulatory proteins (29–32). In apparent contrast to these observations, mammary transplantation experiments using the ERKO mouse have demonstrated that the stromal rather than epithelial derived ER population is necessary for ductal proliferation in the neonatal gland (13); however, the adult gland was not examined in this study.

These PRKO mammary gland transplantation experiments provided a unique insight into the functional exclusivity of epithelial-derived PRs in pregnancy-associated mammary morphogenesis. However, these investigations did not address the question of whether all epithelial cells are required to express the PR as a prerequisite for normal ductal side-branching and alveologenesis. This question was prompted by data emerging from immunohistochemical experiments that revealed a nonuniform expression pattern for PR in the luminal epithelial cellular compartment of the mammary gland of the adult murine virgin (25–28).

To answer this question, mammary epithelial cells derived from the PRKO mouse were mixed with equivalent cells from the wild type (16). To differentiate between wild type and PRKO epithelial contributions, the wild type or the PRKO was back-crossed to the ROSA26 mouse in which the *lacZ* gene is expressed in all epithelial cells; ROSA26 derived cells stain blue with X-gal, a chromogenic substrate for β-galactosidase. The resultant chimeric epithelial cell mixture was injected into a cleared stromal compartment of the wild type gland followed by transplanta-

Fig. 3. The mammary epithelial cellular component is the primary target for progesterone receptor action. In the PRKO stroma, wild type epithelium can undergo normal epithelial proliferation and differentiation in response to pregnancy hormones (panel A). Panel B is a positive control in which wild type epithelium is transplanted into wild type stroma. Wild type mice consisted of the ROSA 26 (β-galactosidase$^+$) mouse line. Transplantation of PRKO epithelium into wild type stroma (panel C) could not rescue the PRKO defect despite exposure to pregnancy hormones. Panel D is a positive control in which wild type epithelium is transplanted into wild type stroma; note the pregnancy-induced ductal branching and alveologenesis. The mere coexistence of fragments of wild type and PRKO epithelium within the same wild type stroma could not rescue the PRKO phenotype following pregnancy (panels E and F). The wild type epithelium is blue (ROSA 26 (β-galactosidase$^+$)) whereas the PRKO epithelium is red; scale bars in panels E and F are 2 mm and 200 μm respectively. Following pregnancy, PRKO mammary epithelial cells (ROSA 26 (β-galactosidase$^+$)) can contribute to alveologenesis when mixed with wild type epithelial cells; panel G (scale bar: 200μm). This result suggests that (through paracrine effects) PR positive cells can affect neighboring PR negative (PRKO) cells to contribute to alveologenesis and ductal side branching. Adapted from Brisken et al. (16).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tion of the recombinant gland to a wild type host. Following pregnancy, whole mount and immunohistochemical analysis revealed that PRKO epithelial cells could contribute to alveolar structures (Fig. 3; panel G), and functioned normally as judged by the expression of the milk protein: β-casein. Thus, PRKO derived epithelial cells can contribute to both the proliferative and differentiative responses to progesterone when placed in close apposition with PR positive cells.

The following conclusions can be drawn from these observations. First, at least two distinct luminal epithelial cell lineages exist in the mammary gland: cells that score positive or negative for PR expression. Indeed, apart from the adult mouse, mammary epithelial cells, expressing PR, have been identified in the embryonic and prepubertal stages of development, suggesting that the fate of these cells may be specified early for specialized functions later in the adult (25) and (J. P. Lydon, unpublished observations); these functions may consist of initiating alveologenesis and ductal side branching. This proposal is supported by the fact that although PR expression occurs early in mammary development, loss of PR function as in the PRKO does not impact normal mammary morphogenesis until pregnancy. Second, because only PR negative cells in close association with PR positive cells can contribute to ductal side branching and alveologenesis (16), paracrine-signaling pathways between these cell types is hypothesized to occur. Third, although lacking PR-positive cells, the PRKO mammary gland still retains those PR negative cells that are responsive to PR-mediated paracrine signaling, again suggestive of distinct epithelial cell populations that have evolved separately to either express PR or to be receptive to paracrine signals induced by PR.

These conclusions have been recently substantiated and further extended by studies on human and rat mammary tissue that revealed a segregation between those epithelial cells expressing both ER and PR and those cells undergoing proliferation (33–35), and Fig. 4, panel A. Close scrutiny of the immunohistochemical results revealed that the majority of proliferating cells were distinct from, but in close apposition to steroid receptor positive cells, again hinting at a paracrine action between steroid receptor positive 'sensor' cells and a sub-population of steroid receptor negative cells, but mitotically competent, 'effector' cells. These observations do not discount the possibility that steroid receptor positive cells may eventually commit to cell division with attendant loss of steroid receptor expression (temporal segregation); the reverse scenario could also be contemplated.

The fact that this spatial organization for ER and PR positive cells has been conserved between the human and rodent mammary gland strongly supports an evolutionarily conserved cellular mechanism of action by which cells expressing these receptors impact the functional activity of neighboring cells to induce ductal morphogenesis. How and why such a cellular patterning evolved in the mammary epithelium is currently a matter of conjecture; however, perturbations of such an important cellular arrangement would be predicted to have adverse consequences for normal mammary gland development. Indeed, it has been reported that in many breast tumors, the majority of ER and PR expressing cells also undergo proliferation (33), clearly at odds with the earlier paracrine signaling pathways that are operative in the normal gland. Further, they suggest that the development of breast cancer may involve a switch in steroid dependent regulation of proliferation from a paracrine to intracrine/autocrine mechanism. The possible modes of PR action within the luminal epithelial cell are summarized in Fig. 4, panel C.

Based on these studies, it will be interesting to determine whether a nonuniform spatial organization is adopted by mammary epithelial cells that express other important modulators of mammary morphogenesis and function. Indeed, the steroid receptor coactivator-1 (SRC-1), a coactivator for certain members of the nuclear receptor superfamily (36), was shown to be expressed in mammary epithelial cells in a nonuniform spatial arrangement; these cells, however, were distinct from those cells expressing ER and PR (37). Considering that SRC-1 has been shown to be a coactivator for ER and PR, this result was surprising, and suggests that at least in the normal mammary gland, ER and PR do not require this coactivator to exert their effects. Recent characterization of the SRC-1 knockout mouse revealed a mammary defect (38), indicating that SRC-1 may either interact with other nuclear receptors and/or participate in novel regulatory pathways to elicit mammary epithelial ductal branching and alveologenesis.

Although mammary gland transplantation and immunohistochemical experiments have uncovered an unanticipated mechanism of action for PR in the mammary gland, these investigations have also raised questions for future studies. First, do mammary epithelial cells that express the PR comprise a stem cell

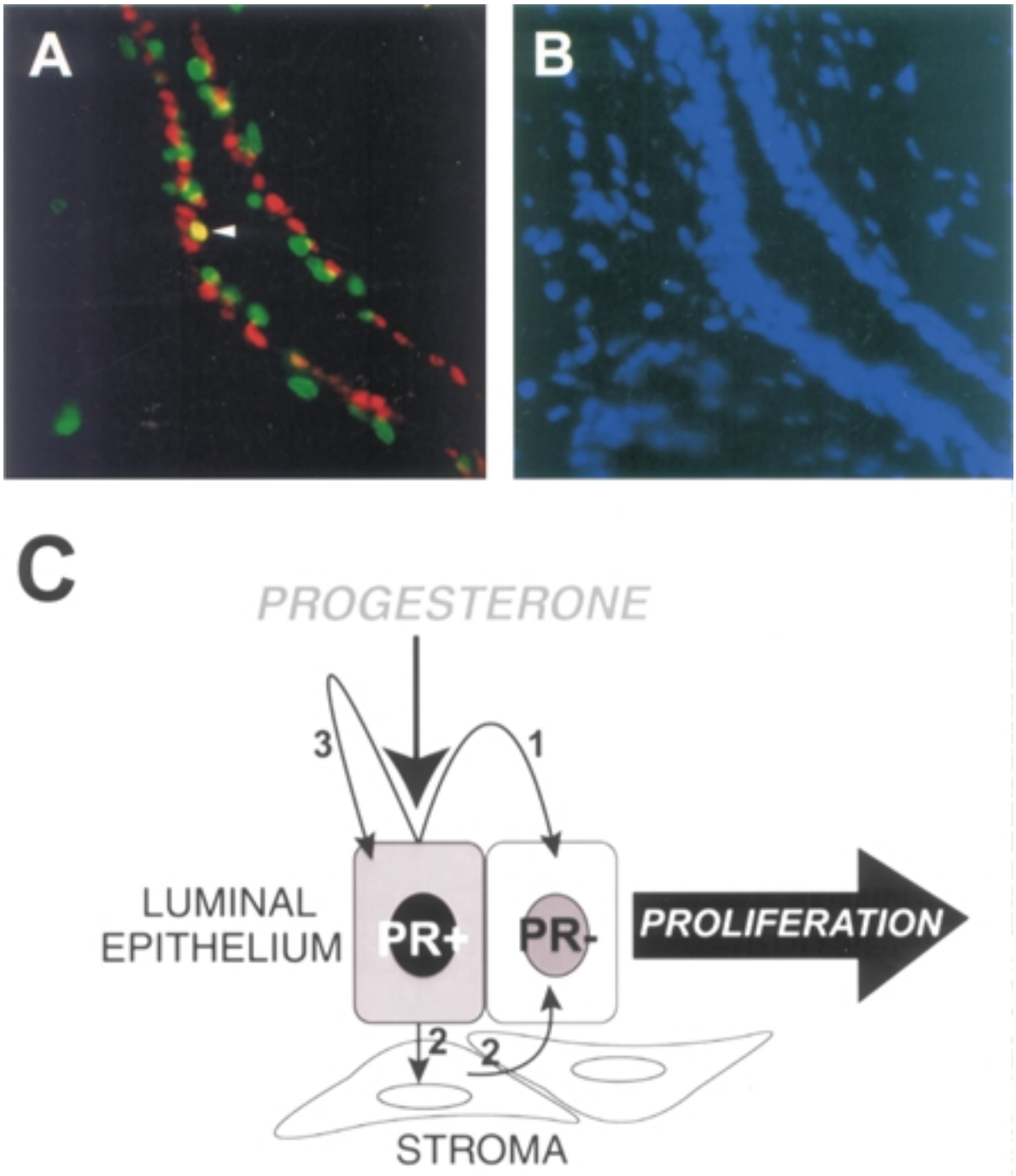

Fig. 4. In the normal mammary gland, the majority of epithelial cells that express PR are segregated from proliferating cells. Panel A: epithelial cells within the mammary gland of a 45 day old rat that express PR and/or incorporate 5-bromo-2-deoxyuridine (BrdU) were detected by indirect immunofluorescence; cells in S-phase of the cell cycle incorporate BrdU. Epithelial cells expressing PR are red (secondary antibody: Texas Red conjugated goat anti-rabbit antibody) whereas cells incorporating BrdU are green (anti-BrdU antibody, conjugated with fluorescein-isothiocyanate (FITC)). Proliferating cells that also express PR are shown in yellow (arrow). Panel B: in parallel, all nuclei in this field were detected with 6-diamidino-2-phenylindole (DAPI), and appear blue. Magnification is 400X. Panel C: possible pathways by which progesterone-initiated signaling can influence PR negative mammary epithelial cells to proliferate. Pathway 1 denotes a paracrine-signaling pathway through which PR positive epithelial cells communicate with juxtaposed PR negative epithelial cells; this pathway predicts the existence of paracrine factors (possibly secretory) that deliver the progesterone signal to neighboring cells. Pathway 2 is similar to 1 except that the stromal cellular compartment is a necessary mediator of the progesterone paracrine signal. Pathway 3 represents an autocrine or intracrine pathway by which the progesterone signal would be directed back to the cell of origin to elicit proliferation. This scenario might occur in a minority of cells in the normal gland (perhaps a preneoplastic cell population) (panel A (arrow)) and/or in the majority of PR positive cells in breast cancer (33).

class? This question may be answered, in part, by examining these cells using electronmicroscopy to determine whether this cell type exhibits the cellular characteristics for stem cells that have recently been described using this technique (39). If mammary epithelial cells expressing PR were identified as stem cells, the spatial organization of these cells (affector/sensor cells) and their close juxtaposition to proliferating cells (effector cells) would draw parallels to a similar cellular organization that has been reported for other self renewing tissues, such as the epidermis and hair follicle (40–42).

Considering the evolutionary importance of this cellular organization for epithelial cells that express PR in the adult virgin gland, how this cellular organization is reinstated following pregnancy, lactation, and involution will be a challenging question for the future. A further question will be to address the issue of whether embryonic, pre- and pubertal stages of mammary gland development also manifest the nonuniform organization of PR-expressing epithelial cells as observed in the adult virgin. If this cellular organization is shown to be exclusive to the adult virgin, it may suggest that at the onset of pregnancy, this cellular arrangement has specifically evolved to ensure that extensive side branching and attendant alveologenesis occurs in a spatially ordered manner.

Finally, a key question to understanding PRs molecular mechanism of action in the mammary gland will be to identify the genes involved in the paracrine signaling pathway(s) between mammary epithelial cells positive and negative for PR expression. Recent elegant studies by Brisken and coworkers have provided compelling evidence that *wnt-4* may play a pivotal paracrine role in dispatching the progesterone signal in the mouse mammary gland (43). Since the arrival of functional genomics, with the availability of knockout mouse models, and an increasing number of gene discovery approaches, we should be confident that more of these paracrine-signaling molecules will be identified in the not too distant future.

PROGESTERONE RECEPTOR'S INVOLVEMENT IN MAMMARY TUMORIGENESIS

In addition to its role in normal mammary gland development, the proposed involvement of progesterone in mammary gland tumorigenesis has formented much discussion. Epidemiological studies have revealed a close correspondence between breast cancer risk and the exposure of the mammary gland to cyclical levels of ovarian sex steroids that occurs during the reproductive years of premenopause [reviewed in (44)]. This correlation is further supported by clinical studies in which inhibition of such steroidal exposure, for example after bilateral oophorectomy, markedly reduced breast cancer risk (45,46); an early menopause and a late menarche have also been shown to exhibit similar beneficial effects (17,47). Based on these observations, the increase in breast cancer observed with advancing age is hypothesized to result from ovarian sex steroid-induced proliferation of the mammary epithelial cell which, throughout the reproductive years, provides a temporal window of opportunity for the accumulation of genetic changes resulting in breast cancer in later life. Because a significant measure of breast cancer risk is linked with the cyclical exposure of the mammary epithelial cell to ovarian sex steroids, breast cancer prevention treatments based on ablating ovarian steroidogenesis have been considered (17).

Based on this hypothesis, to investigate breast cancer etiology, without considering the involvement of progesterone, would be a failure in perspective. However, progress in our understanding of breast cancer in relation to progesterone exposure has been stymied due to our inability to mechanistically dissect *in vivo* the individual and integrative roles of estrogen and progesterone in this cancer. The contentiousness of the issue has been further exacerbated by the numerous conflicting reports concerning the influence of synthetic progestins on mammary tumorigenesis in rodents (48–50). Nevertheless, a number of studies have linked progesterone to the progression of certain carcinogen induced and transplantable rat mammary tumors, these studies also suggest that progesterone may play a role in spontaneous tumors of the murine mammary gland (50–55). However, progesterone can be both stimulatory and inhibitory depending on the time of progesterone exposure in relation to carcinogen treatment suggesting a degree of caution in ascribing a role for progesterone in mammary gland tumorigenesis.

Studies with cultured human breast cancer cells have shown that depending on the dosage and duration of treatment, progesterone can exert both growth stimulatory and inhibitory effects [reviewed in (56)]. Because recent *in vitro* studies have identified a cross-communication pathway between progesterone and growth factor/cytokine family members

(57), it has been hypothesized that, following one round of proliferation, the initial pulse of progesterone acts as a primer for the actions for secondary factors involved in either proliferative, differentiative, or apoptotic pathways. The commitment to any one of these subsequent pathways would be influenced by the type of cross-talk between progesterone and growth/cytokine signaling pathways which in turn would be determined by the dosage and duration of subsequent progesterone exposures.

Recently, the PRKO mouse in combination with the chemical carcinogen-treated mammary tumor model was used to evaluate the functional relevance of progesterone-initiated intracellular signaling in mammary gland tumorigenesis (27). Carcinogen-treated PRKO mice exhibited a significant reduction in mammary tumor incidence as compared to wild types; mammary tumors arose in 12 (60%) of 20 wild type mice compared with 3 (15%) of 20 PRKO mice by 44 weeks after the initial carcinogen treatment. Despite the complexity of progesterone's involvement in mammary tumorigenesis, these results underscored the specific importance of the PR (as distinct from ER) as an obligate mediator for those intracellular signaling pathways that are essential for the initiation of murine mammary tumors induced by chemical carcinogens.

Collectively, these studies have prompted a revaluation in our understanding of progesterone's participation in mammary tumor progression that may have consequences not only for the current use of progestins in contraception and postmenopausal hormone replacement, but also in the design of diagnostic approaches and/or therapies for the future treatment and prevention of breast cancer.

Delineating the Specific Role of the PR-A and -B

While the PRKO mouse provides a powerful tool to define the role of progesterone in mammary epithelial cell proliferation, differentiation and tumorigenesis, the specific contribution of the PR-A and -B isoforms to these activities remains to be addressed. As indicated in the introduction, significant evidence has accumulated indicating that the PR-A and -B proteins are functionally distinct when examined *in vitro*. First, when expressed individually in tissue cultured cells, PR-A and -B display different transactivation properties that are specific to both cell type and target gene promoter used [reviewed in (6)]. These findings suggest that A and B may regulate different physiological target genes in response to progesterone and that each protein may display different transactivation capabilities in different target tissues. Second, when the A and B proteins are coexpressed in cultured cells in cell and promoter contexts in which agonist bound PR-A is inactive, the protein can act as a dominant repressor of PR-B activity (58,59). These findings suggest that PR-A has the ability to diminish overall progesterone responsiveness of specific target genes in specific tissues. This repressor capability, which appears to be a selective property of the A protein, extends not only to PR-B but also to other steroid receptors. Thus, PR-A has been shown to inhibit estrogen, glucocorticoid and mineralocorticoid receptor dependent gene activation presumably through competition for common limiting coactivators (60). Third, the A and B proteins also respond differently to progestin antagonists [reviewed in (6)]. While antagonist bound PR-A is inactive, antagonist bound PR-B can be converted to a strongly active transcription factor by modulating intracellular phosphorylation pathways (61–63). Finally, transrepression of ER activity has been observed in the presence of either protein when bound to antagonists (59,60,64,65).

Both the PR-A and -B proteins are expressed in the mammary glands of rodents and humans. In the case of the mouse, the expression of PR-A predominates over PR-B by a 2:1 ratio in the virgin gland and throughout pregnancy (32,66). In normal human breast tissue, the ratio of PR-A and -B is equimolar while this ratio is significantly altered in some breast tumors (67). It is unclear at the present time whether alterations in the ratio of PR-A to PR-B can contribute to an altered susceptibility of these cells to carcinogenesis. Conceivably, such alterations could have a dramatic effect on the cellular response to progesterone agonists and antagonists as well as the growth factors and proto-oncogenes regulated by progesterone.

Most importantly, recent reports have provided the first *in vivo* demonstration that disruption of PRA/B ratios by overexpression of either the PR-A or PR-B protein in the mammary gland of transgenic mice results in impaired mammary gland development. As a consequence of PR-A overexpression, mammary glands exhibited increased ductal branching and hyperplasia and most interestingly, an abnormal disruption of the organization of the basement membrane and decreased cell-cell adhesion (68). Interestingly, PR-B overexpression resulted in limited ductal elongation and branching;

alveolar growth was unaffected (69). These findings provide strong evidence that a regulated expression of these receptor isoforms is critical for the mammary gland to respond appropriately to progesterone. However, in light of our recent studies and those of others, the mammary defects observed in PR-A and -B transgenic mice could also be explained by inappropriate targeting of PR-A and -B expression to epithelial subtypes that normally would not express PR, but may be competent to proliferate. Thus, the indiscriminate targeting of these receptor isoforms to the mammary gland would breach the cellular segregation rules that apply to normal epithelial cell growth, resulting in a scenario reminiscent of the inappropriate co-localization of steroid receptor expression and proliferation observed in cells of breast tumors.

CONCLUSIONS

Studies during the past five years have led to significant advances in our understanding of the specific role of progesterone in the mammary gland. The combined use of improved detection methods (PR specific antibodies) to detect PRs *in situ*, genetic manipulation of PR expression in mice and classical mammary gland transplantation technologies have begun to expose the progesterone signaling pathway to molecular dissection. Together, these approaches have defined a specific role for progesterone in pregnancy associated ductal epithelial proliferation and lobuloalveolar differentiation within the normal mammary gland as well as tumorigenesis in response to carcinogen challenge. Further, they have demonstrated that PRs are expressed in the adult mammary gland in a nonuniform subset of epithelial cells, most of which are nonproliferative. These receptors appear to regulate both epithelial cell proliferation and differentiation in the normal gland by a paracrine mechanism in which proliferation and differentiation of PR negative cells is controlled by paracrine factors released from neighboring PR positive cells. The hierarchical organization of PRs within epithelial cells appears to be a key conserved feature of rodent and human mammary epithelial cells that may underscore normal progesterone dependent regulation of pregnancy associated developmental plasticity of the gland. The observation that a high number of human breast tumors show a direct association between PR and ER expression and proliferation suggests that disruption of the normal paracrine relationship between receptor positive and proliferating cells may contribute to abnormal steroid dependent signaling and may be an important feature of tumorigenesis. A key objective in future studies will be to gain insight into how the cellular pattern of PR positive cells is specified and maintained during mammary gland development and to identify the paracrine effectors of progesterone dependent epithelial morphogenesis.

Finally, despite a wealth of *in vitro* evidence supporting a distinct role of the PR-A and -B proteins in mediating transcriptional responses to progesterone, our knowledge of the individual contributions of each of these isoforms to progesterone physiology is still in its infancy. We have recently begun to address this question by producing a second generation of PR knockout mice in which either the PR-A or -B isoforms have been selectively ablated. Our initial analysis of these mice provides definitive evidence that the PR-A and -B proteins contribute to the reproductive functions of progesterone in a different tissue specific manner (manuscript in preparation). These models promise to provide exciting new information on the selective contribution of PR-A and -B to mammary gland development as well as to mammary tumorigenesis. Further, information derived from these analyses may ultimately lead to novel tissue specific chemotherapeutic approaches to the treatment of breast cancer.

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