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THE REGULATION AND FUNCTION OF PROGESTERONE RECEPTOR ISOFORMS A AND B IN THE NORMAL MOUSE MAMMARY GLAND

By

Mark Douglas Aupperlee

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ABSTRACT

THE REGULATION AND FUNCTION OF PROGESTERONE RECEPTOR ISOFORMS A AND B IN THE NORMAL MOUSE MAMMARY GLAND

By

Mark Douglas Aupperlee

Progesterone (P) is an important mitogen in the mammary gland, and it has been implicated in increasing the risk of breast cancer. P acts through binding to its cognate nuclear receptor, the progesterone receptor (PR), which exists as two isoforms, PRA and PRB. The expression of PRA and PRB protein throughout BALB/c mouse mammary gland development was performed by immunohistochemistry. PRA was the predominant PR isoform in the virgin mammary gland, but during pregnancy PRA level decreased while PRB level increased to significant levels. PRB was expressed in the majority of proliferating cells during pregnancy, whereas PRA was expressed in few proliferating cells during puberty or pregnancy.

To investigate the hormonal regulation of PR isoform expression and isoform-specific functions, hormone ablation and replacement studies in adult BALB/c mice were performed. Treatment with P produced extensive sidebranching and limited alveologenesis that was enhanced by estrogen (E) + P treatment. PRA expression was increased by E and decreased by P. PRB expression was increased by P or E+P treatment. PRA was the predominant isoform expressed during sidebranching, and proliferation during sidebranching primarily occurred in PRA negative cells. PRB was predominantly expressed in alveoli, consistent with a role in alveologenesis.

In contrast to the BALB/c gland, the pregnant C57BL/6 mouse mammary gland exhibits a delay in sidebranching and alveologenesis. It was hypothesized that this delay was due to a reduced response to P in the C57BL/6 mammary gland. Therefore, mammary gland development and in vivo proliferative responses to E and/or P in BALB/c vs. C57BL/6 mice were analyzed. C57BL/6 glands have reduced sensitivity to P as evidenced by reduced P-induced expression of PRB and Receptor Activator of NF-κB Ligand protein expression, reduced nuclear localization of Id2, and significant differences in nuclear cyclin D1 expression relative to BALB/c glands.

In summary, these studies characterized PR isoform expression throughout development of the normal mouse mammary gland, determined the hormonal regulation of PR isoforms in the adult mammary gland, and established a number of downstream targets of P action in the mammary gland that are influenced by genetic background.

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TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 1

LITERATURE REVIEW

LITERATURE REVIEW

Progesterone and Breast Cancer Risk

According to the 2007 information from the American Cancer Society, breast cancer is the second most common cancer among American women, after skin cancer, and it is the second leading cause of death from cancer in women, after lung cancer (1). The chance of a woman developing invasive breast cancer during her life is about 1 in 8, and the chance that breast cancer will cause a woman's death is about 1 in 35. Thus, it is important to understand the risk factors associated with the etiology and development of breast cancer.

There are a number of reproductive factors that increase or decrease the risk of breast cancer. These factors include the age of onset of menarche, the age at the first full term pregnancy, the age at onset of menopause, and long-term menopausal hormone therapy (2-6). The mechanism for these risk factors is not fully understood, but the ovarian hormones estrogen (E) and progesterone (P) are believed to play an important role. Henderson and Feigelson hypothesized that lifetime exposure to E is a crucial factor increasing breast cancer risk due to the proliferative effects of E on the breast (7). However, recent studies demonstrated that progestins, in combination with estrogens in menopausal hormone replacement therapy (HRT), increase breast risk, whereas E alone HRT is not associated with increased breast cancer risk (8-10). Understanding the mechanisms of P action in the breast is particularly important since progestins are widely used not only in HRT, but also in contraceptives and for suppression of ovarian function in the treatment of certain pathological conditions (11, 12).

Progesterone has been shown to be an important mitogen in rodent models and in the human breast (6, 13). In the adult human breast, DNA synthesis in the breast epithelium, as measured by tritiated thymidine incorporation (14-16) or cell proliferation as measured by PCNA or Ki67 expression (6) is increased during the luteal phase of the menstrual cycle when P levels are highest. The greatest proliferative activity occurs in the terminal duct lobular unit (TDLU), the site of origin of most breast cancers (6). The fact that the highest proliferation during the menstrual cycle occurs during the luteal phase indicates that P in combination with E promotes epithelial cell proliferation. Furthermore, postmenopausal estrogen plus progestin hormonal therapy increases proliferation in the breast above that of estrogen alone hormonal therapy (6). The ability of P to stimulate proliferation in both the adult premenopausal and postmenopausal human breast suggests that progestins have a potential role in the etiology of breast cancer and in the growth of established tumors.

Progesterone Receptor: Structure

P exerts its effects through binding to the progesterone receptor (PR), which is a member of the nuclear steroid receptor family (17, 18). PRs are activated by binding of the ovarian hormone, P, and are classified as ligand-activated transcription factors that regulate gene expression by binding directly or indirectly to DNA (19). When P is not present, the PR is in a complex with several chaperone molecules, including heat shock protein (hsp) 90, hsp70, hsp40, hsp-organizing protein (Hop) and p23 (20). Hsp70 interacts with hsp90 and this complex is involved in opening the progesterone binding cleft of PR. p23 is a 23 kDa protein that binds to PR-hsp90 complexes once the PR has

been converted to the steroid-binding state and helps to stabilize that interaction. Hop interacts with hsp70 and also plays a role in opening the progesterone binding cleft (20). Upon ligand binding, the PR undergoes a conformational change, dimerization, and hsp dissociation.

The PR is expressed as two isoforms, PRA and PRB, which are transcribed from a single gene in both humans and rodents. Translation of PRA and PRB protein initiates at two distinct AUG signals, which produces two proteins that are identical except for an additional N-terminal 164 amino acids on PRB (Fig. 1.1.) (21). The additional unique N-terminal portion of PRB is referred to as the B-upstream segment, or BUS (22).

Regions common to both PRA and PRB include a central DNA binding domain (DBD) and a carboxy-terminal ligand binding domain (LBD) (Fig. 1.1). The LBD of PRA and PRB is thought to bind P with equal affinity. PRA and PRB both contain multiple activation function (AF) and inhibitory elements, which enhance or repress transcriptional activation by association of these domains with transcriptional coregulators (23). A hormone-inducible AF2 is located in the carboxy-terminal LBD (24). Hormone binding induces a conformational change in the PR that allows association of AF2 with cofactors, such as steroid receptor coactivator (SRC) SRC-1 (25, 26), SRC-3, and CBP (27). A constitutively active AF1 is located just upstream of the DBD (24, 28). The unique region of PRB contains a third transcription activation domain, AF3, in addition to AF1 and AF2 that are common to PRA (29, 30). This additional AF3 allows the binding of a subset of coactivators to PRB that progestin-bound PRA is unable to efficiently recruit (31). Another region common to both PRA

Figure 1.1. Progesterone Receptor Structure. PRB and PRA are identical steroid hormone receptors except for an additional 164 amino acids on the N-terminus of PRB. Both PRA and PRB contain a hormone binding domain (HBD), a hinge region (H), a DNA-binding domain (DBD), and an inhibitory domain (ID). Activation functions (AF) are regions that interact with co-activators. PRA and PRB contain a hormone-independent AF1 and a hormone dependent AF2. The unique portion of PRB contains a third activation function, AF3.

and PRB is an inhibitory domain at the N-terminal end of PRA and PRB upstream of AF1 (32). This inhibitory domain is active in PRA, and inhibits the transcriptional activity of AF1 and AF2, but is inactivated in PRB by the addition of the BUS and the AF3 it contains (32). The BUS of PRB is thought to alter the structure of PRB and thus its functional associations with other proteins, which leads to the difference in the transcriptional activity between PRB and PRA (33).

Progesterone Receptor: Regulation of Expression

Studies in both the human (21) and rat (34) have shown that PRA and PRB are produced from separate promoters. It is not known if there are two promoters in the mouse as well. Most of what is known about the PR promoter has been learned through examination of the human PR gene *in vitro* in cell lines. The MCF-7 breast cancer cell line has been used to show that estrogen (E) upregulates expression of PR (35, 36). However, neither the PRA nor PRB promoters contain a canonical estrogen response element (ERE) (21). Further examination of the human PR promoter has identified a number of sites that may mediate the estrogen responsiveness of the PR promoter. A series of studies have found two Sp1 sites in the -80/-34 region (37), a +90 AP-1 site (38), and an ERE half site with two adjacent Sp1 sites from +571 to +595 (39, 40), that confer estrogen receptor (ER)-mediated E responsiveness to the PR gene. In contrast to the +90 AP-1 site that increases transcription of PR, Petz et al. found an additional AP-1 site at +745 that decreases E-mediated transcription of the PR gene (41). The elements of the PR promoter responsible for E-induced expression of PR are fairly well conserved

across species, but to date there have been no published studies examining the PR promoter in mice.

Little is known about regulation of PR isoforms in the normal human breast. A study examining PR in the postmenopausal breast found that PR expression is increased by HRT with E relative to non-HRT users (6). However, that study did not address the regulation of specific PR isoforms. Studies in breast cancer cell lines examining the regulation of PR isoform expression by hormones have produced conflicting results. A study by Graham et al. in T47D cells found that PRB was preferentially stimulated by E, while both PR isoforms were downregulated by P treatment (42). However, a later report by Vienon et al. showed stimulation of both PRA and PRB by E in T47D cells (43). This same report also demonstrated preferential upregulation of PRA by E in MCF-7 cells and preferential upregulation of PRB by E in ZR-75-1 cells (43). Thus, while E upregulates PR expression in breast cancer cells, how E regulates PR isoforms expression in the normal breast or *in vivo* in breast cancer is not well understood.

Studies in mice have also demonstrated an effect of E on PR expression (44, 45). The loss of estrogen production through ovariectomy has been shown to decrease PR levels (45), while the addition of E to an ovariectomized mouse increases PR levels (44, 45). However, the relative effect of hormones on PR isoform expression in the mouse has not been determined.

Progesterone Receptor: Nuclear Activity

When P binds to PR it induces a conformational change that leads to dimerization, localization to the nucleus, binding to a progesterone-responsive element (PRE), and the

recruitment of specific coregulators and general transcription factors (18). PR complexes bound to DNA are able to increase target gene transcription through chromatin remodeling and recruitment of the general transcription machinery to the promoter. PRA and PRB dimers exist in three possible classes: A:A, A:B or B:B (46). The availability of PRA and PRB to form dimers impacts dimer formation: a greater amount of one isoform favors homodimer formation of that particular isoform. As mentioned above, the region unique to PRB alters its transcriptional activity relative to PRA and PRA is capable of inhibiting PRB, and thus the three classes of dimers can have different transcriptional activity. Indeed a study of PRA:PRB heterodimers showed that in pure heterodimers, PRA is a dominant negative inhibitor of PRB (46). *In vitro* studies have shown that the two PR isoforms have very different effects on progestin-responsive promoters (47, 48). PRA is a weaker activator of transcription (24) and in some contexts PRA is also able to inhibit the activity of PRB and other nuclear receptors (48). PRB is a stronger activator of transcription and can have transcriptional activity even in the presence of antagonist (24, 49). The antagonist-bound transcriptional activity of PRB appears to occur without direct binding to a PRE, but rather through interactions with tethering proteins. In contrast to PRB, PRA is not transcriptionally active when bound by antagonist (49).

Recent studies in breast cancer cell lines have shown that PRA and PRB generally regulate different genes (50). Richer et al. used a PR-negative subclone of T47D cells that were engineered to stably express either PRA or PRB to examine genes regulated by PRA and PRB (50). There were 94 genes found to be regulated by P, and 65 of those were regulated only by PRB, 4 were regulated only by PRA, and 25 genes were regulated

by both isoforms (50). These studies demonstrate that PRA and PRB regulate different genes and due to the larger number of genes regulated uniquely by PRB, provide further evidence that PRB is a more potent transcriptional activator than PRA. They also highlight the importance of isoform-specific studies in determining PR function.

Phosphorylation of the PR has also been shown to modulate transcriptional activity (51). In human PR, 14 constitutive and ligand-dependent phosphorylation sites have been identified (51). Six phosphorylation sites are unique to PRB, implying that transcriptional activity of PRB may be regulated differently by cellular kinase pathways (52). Phosphorylation of Serines 81, 162, 190, and 400 is constitutive, even in the absence of hormone (53). Phosphorylation of Serines 102, 294 and 345 is progestin-dependent (51). Serine 294 has been specifically shown to be phosphorylated by mitogen-activated protein kinase (MAPK) (54). In progestin treated T47D cells expressing only PRB, Ser294 phosphorylation by MAPK stimulates proteasome-dependent PR degradation (55). Paradoxically, such downregulation of PR protein coincides with the highest PR transcriptional activity. The authors propose that transcriptional activity of PR is tightly coupled with receptor turnover, which is regulated by ligand-dependent phosphorylation on Ser294. *In vitro*, eight of the 14 sites (Serines 25, 162, 190, 213, 400, 554, 676, and Thr430) are phosphorylated by cyclin A/cyclin-dependent protein kinase 2 (CDK2) complexes (53, 56). The function of PR phosphorylation is not completely understood at this time, but it appears that phosphorylation may affect interactions with co-regulators, influence nuclear localization and receptor turnover, and alter hormone sensitivity (19).

Steroid receptor coactivators (SRC) bind the ligand-binding domain of steroid receptors and enhance the transcriptional activities of steroid receptors. Several studies have established that SRCs may modulate the functional activity of steroid receptors. Studies in doubly transgenic mice expressing an indicator of PR activity and carrying the genetically engineered disruption of either SRC-1 or SRC-3 have shown that the absence of SRC-1 does not impair PR responses to E+P treatment in mammary epithelium, whereas the absence of SRC-3 abrogates the PR responses after E+P treatment in mammary gland (57). In another study, the genetically engineered loss of SRC-2 exclusively in cells expressing PR leads to impaired sidebranching and alveolar formation after E+P treatment (58). Together, these results demonstrate that SRC proteins modulate the physiological function of PR in mammary tissue *in vivo*. In the human breast, SRC-2 is expressed in a distinct punctate nuclear pattern similar to the pattern of PR expression (58). However the role of SRC-2 in the human breast is currently unknown. It remains to be determined if different SRCs act in an isoform specific manner.

The transcriptional activity of PR fluctuates throughout the cell cycle with the highest transcriptional activity occurring during S phase (59, 60). Cyclin A/cyclin dependent kinase 2 (cdk2) complexes have been shown to function as coactivators of PR-dependent transcription. In HeLa cells, cyclin A increases transcriptional activity of PRA and PRB independent of their phosphorylation status (61). In the S phase, the cyclin A/cdk2 complex is recruited to PR bound to DNA and stimulates the additional recruitment of coactivator SRC-1 (59). In the G1 phase when cyclin A is absent, PR transcriptional activity and the recruitment of SRC-1 are diminished.

Interestingly, the majority of genes that are thought to be regulated by P do not contain consensus PR binding sequences or PREs (50). Additionally, there are a number of genes that are regulated by PR expression, but do so independently of P (62, 63). Therefore, despite the depth of understanding of transcriptional activities of PRA and PRB, the mechanism of action for the regulation of particular genes in response to P and PR remains largely unknown. Importantly, the correlation of the regulation of specific genes in response P and PR to changes in cell biology remains to be determined

Progesterone Receptor: Extranuclear Activity

In addition to its activity in the nucleus, PR has been shown to interact with cytokine and growth factor signaling pathways at multiples levels to influence signaling cascades that play important roles in mammary cell proliferation and differentiation. In contrast to genomic effects of P treatment, which are delayed by several minutes to hours, the nongenomic effects of P occur within minutes (19). In T47D cells expressing only PRB, epidermal growth factor (EGF) and P act synergistically on promoters that drive the cell-growth regulatory genes c-fos and p21, neither of which contains a PRE (64). This synergy is believed to be mediated through a MAPK. Human PR has been shown to mediate rapid activation of the Src/Ras/Raf/mitogen-activated protein kinase signaling pathway through a Pro-Xaa-Xaa-Pro-Xaa-Arg motif located in the N-terminal domain of both PRA and PRB (65). Mutation of the PRB DBD removed PR transcriptional activity, but did not affect P-induced c-Src or MAPK kinase activation. Thus, the activation of MAPK signaling pathways is distinct from PR transcriptional activity and is not

dependent upon the DBD of PR. Both PRA and PRB can bind to Src, but only PRB produces strong stimulation of Src kinase activity and downstream effectors.

Another signaling pathway influenced by progesterone involves signal transducers and activators of transcription (Stats) (64). Stat5 is important for normal lobuloalveolar development and lactational function of the mammary gland (66). Nuclear localization, DNA binding and regulation of target genes by Stat5 requires phosphorylation that is induced by growth factors and cytokines, such as prolactin, via Janus kinases. Studies in T47D cells expressing PRB only show that P acts to upregulate Stat5 mRNA and that P/PRB action is also implicated in Stat5 nuclear localization. This is believed to occur through a direct interaction of Stat5 and PR, and it is believed that Stat5 is translocated to the nucleus as a companion with PR (64).

Progesterone Receptor: Detection of Expression

PR expression is primarily measured by immunoblots, real-time RT-PCR, and immunohistochemistry. Of these methods, real time RT-PCR analysis is the most sensitive for detection of PR isoforms. However, various studies have used primers that claim to be specific for PRB mRNA, but are directed to the 5' untranslated region (UTR) of PRA mRNA, which overlaps with the PRB reading frame (67-69). In fact, such primers amplify both PRB and PRA mRNAs since the 5' UTR of PRA mRNA overlaps with the PRB reading frame. Thus, the design of PRB-specific primers should be directed against the 5'UTR of PRB mRNA. Additionally, when designing primers that detect both PRA and PRB mRNA, the sequences between PRB and PRA translation start sites should be used, instead of the sequences located in the DBD. Primers located in the DBD

may amplify PRC in addition to PRB and PRA mRNAs. For quantitative analysis, it is additionally important that there be similar amplification efficiency of the primers used to detect PRB vs. primers used to detect total PR (PRA+PRB) transcripts.

Biochemical methods to analyze PR isoform expression, such as immunoblot analysis and immunoprecipitation, may provide important information about PR isoform molecular sizes and post-translational modifications. They can provide quantitative analysis of expression levels when used for homogeneous cell cultures, such as isolated primary mammary epithelial cell cultures or breast cancer cell lines. However, when used to quantify PR levels in protein extracts of whole mammary gland, an important confounding factor is the unknown contribution of stromal proteins to the total protein in extracts. This is particularly relevant in mammary tissues that exhibit changes in overall epithelial content, such as the pubertal gland versus adult virgin gland versus pregnant mammary gland. A further limitation to biochemical methods is sensitivity of detection. For example, in the mouse whole mammary gland samples the dilution of PR present in mammary epithelial cells by the stromal cell component that lacks PR often results in PR levels below the limit of detection (70, 71).

Immunohistochemistry with antibodies that are specific for PRA or PRB is a suitable method to determine the cell type-specific expression, intracellular distribution, and colocalization of PR isoforms within the same cells. In some cases, interpretation of studies of PR isoform expression has been confounded by lack of information about the specificity of the antibody used to detect only PRA, only PRB or both PRA and PRB. The study by Mote et al. in human breast tissue and cells has shown that of 11 antibodies generated against human PR, 10 detect both PRA and PRB and 1 detects only PRB by

immunoblot analysis (72). However, in immunohistochemical analysis 8 of the antibodies tested have detected only PRA, and 2 detect both PRA and PRB. Only one of the antibodies tested has been specific for PRB. Prior to that study it has often been assumed that antibodies that detect both PRA and PRB by immunoblot also detect both isoforms by immunohistochemistry. Since a number of the commercially available anti-PR antibodies detect only PRA or both PRA and PRB, the interpretation of immunohistochemical analyses which draw conclusions about specific PR isoform expression must be viewed in this context.

Studies in the adult human premenopausal breast using immunoblots have shown that PRA and PRB are expressed in a 1:1 ratio (73). Immunohistochemical studies in human breast (74), mouse (45) and rat mammary gland (75) showed that PR expression is confined to the luminal epithelium. PR isoforms are expressed unevenly in different cell types within the mammary gland. Analysis of PR isoform specific expression in the normal human premenopausal breast has revealed that both PRA and PRB are expressed in luminal cells and colocalized in the same cells (74). The average proportion of PR positive epithelial cells in the normal human premenopausal breast is about 10-20%, although individual ducts and lobules varied between 0 and 90% PR positivity (74). PR isoforms have not been detected in human or mouse mammary stroma by immunohistochemistry (45, 74).

Mammary Gland Development: Overview

Mammary gland development has been studied to learn more about the role of hormones *in vivo* in the normal breast and how hormones are involved in the etiology of

breast cancer. The development of the mammary gland is unique because the majority of mammary gland development occurs postnatally. With the exception of the emergence of a primitive mammary epithelial rudiment established in the midgestational embryo, mammary gland development primarily occurs in two distinct phases that are marked by the onset of puberty and pregnancy (76). In humans, the mammary gland is present in two breasts on the chest wall. In contrast, the mouse generally develops five pairs of mammary glands. The first pair is located in the neck region near the salivary glands. The second pair and third pair are located on the chest wall and are separated by a thin layer of muscle. The fourth pair is located on the abdominal wall, and is the mammary gland that is most commonly studied. The fifth pair is located in the inguinal region (77). The mammary gland epithelium originates at the nipple and extends into the mammary fat pad. In the human, a more complex structure is present that radiates out from the nipple into five to ten ducts. The mouse contains a single duct that forms five to ten secondary ducts.

The mammary gland is composed of numerous cell types that compose an epithelial and a stromal component (Fig. 1.2). The ductal epithelium consists of two distinct cell types: luminal epithelial cells that line the ducts and lobules, and myoepithelial cells that form the contractile network surrounding the luminal epithelium. Separating the epithelial component of the mammary gland from the stroma is a layer of basement membrane. Immediately adjacent to this layer are fibroblasts, which interact with the epithelium and secrete factors that influence epithelial cell proliferation and migration. The adipocytes in the stroma comprise the major cell type of the mammary fat

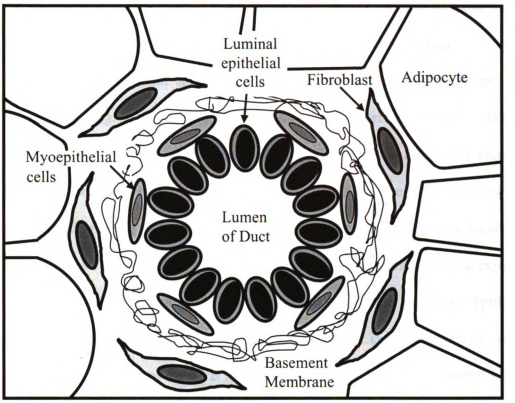

Figure 1.2. Mammary gland cell types. In this cross section of a mammary gland duct, the luminal epithelial cells line the lumen of the duct and are directly surrounded by a layer of contractile myoepithelial cells. The epithelium is separated from the stroma by a layer of basement membrane. Fibroblasts secrete the basement membrane and are located adjacent to the basement membrane. Adipocytes fill the majority of the fat pad. Not shown, but also present in the mammary gland, are cells found in blood and lymphatic vessels or nerve bundles and wandering cells of the immune system.

pad. The stroma is also composed of a number of other cell types, such as those found in blood and lymphatic vessels, nerve bundles and cells of the immune system.

The mouse is the most studied and best understood model of mammary gland development (78) (Fig. 1.3). At birth the mammary gland exists as a small rudiment of epithelium in the form of a ductal tree and for the first few weeks after birth, mammary gland growth is isometric, or proportional to increases in body size (79). With the onset of estrus cycles during the prepubertal growth period, extensive proliferation localized to club-like structures called terminal end buds (TEB) (Fig. 1.4) drives allometric expansion of the epithelium to fill the fat pad. Allometric growth of the mammary epithelium generally commences at around 31 days of age (80). The TEB is composed of two cell types. The outermost layer of the TEB is composed of cap cells, which interact with the surrounding stroma through a thin basal lamina. The cap cells have a high proliferation rate with very little apoptosis (81). Cap cells are thought to be progenitors of myoepithelial cells, which are characterized by their expression of myosin (82-84). Cap cells lack polarity and an organized cytoskeleton and are loosely adherent to one another (85). The interior of the TEB is filled with body cells, which are thought to be more luminal epithelial cell-like (81). In a TEB, the cap cell layer directly abuts the fat pad. The neck of the end bud acquires more differentiated cells as ducts are formed. As the ductal epithelium matures, extracellular matrix and fibroblasts are present in the surrounding stromal compartment.

Hormones and growth factors control ductal proliferation at the terminal end bud as well as bifurcation and trifurcation that lead to the formation of secondary and tertiary branches. As the ductal network is formed, significant apoptosis occurs in the body cells

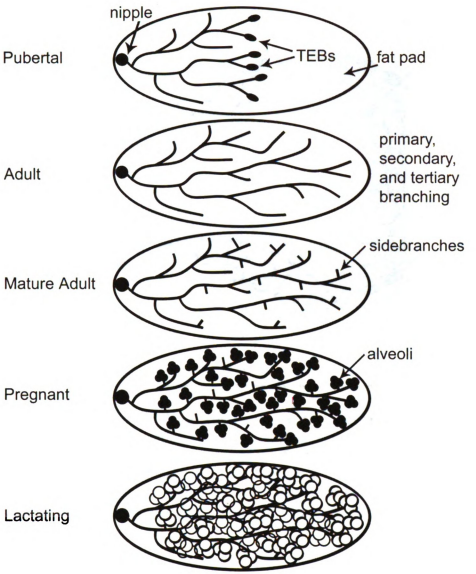

Figure 1.3. Mouse mammary gland development. With the onset of puberty, the production of estrogen and progesterone by the ovaries promotes ductal development, which is driven by proliferation localized to bulbous structures called terminal end buds (TEBs) at the ends of ducts. In the adult mammary gland, the ductal epithelium has formed a network of primary, secondary, and tertiary ducts that extend to the limits of the fat pad. In the mature adult mammary gland, successive estrous cycles induce the formation of sidebranches that increase in number over time. Increased levels of hormones, such as estrogen and progesterone, and the peptide hormone prolactin drive proliferation and alveologenesis during pregnancy. The mammary gland achieves full differentiation during lactation, when the mature luminal epithelial cells produce and secrete milk into the alveoli that travels through the ductal network to the nipple. Upon removal of the suckling stimulus, involution occurs and the mammary gland regressed to a prepregnant-like state.

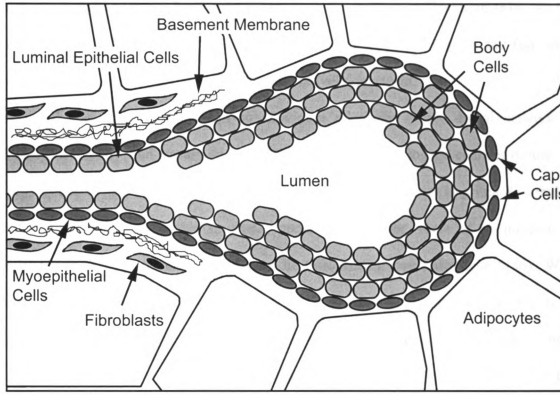

Figure 1.4. Terminal End Bud structure in the pubertal mammary gland. Illustration of a longitudinal section through a terminal end bud (TEB) showing the cap cell layer and body cells at the leading edge of the TEB. Apoptosis in body cells farthest away from the cap cells is critical for lumen formation. In the neck region of the end bud, basement membrane and fibroblasts line the epithelial compartment that now consists of differentiated luminal epithelial and myoepithelial cells.

of the TEB that is critical for normal ductal structure and lumen formation (81). As the mammary epithelium reaches the limits of the fat pad, end buds regress and form duct ends, and the mammary gland becomes proliferatively quiescent.

The adult mammary gland consists of a relatively simple ductal network of primary, secondary, and tertiary ducts that has minimal morphological changes during estrus cycles (Fig. 1.3) (77). In contrast, the adult human breast is primarily lobular, with extensive lobuloalveolar structures present. However, there is an increase in proliferation in the mammary gland during diestrus, when P levels are highest (86). With each estrus cycle, the increase in proliferation is associated with the formation of sidebranches, which are small branches off the secondary and tertiary ducts. These sidebranches are the sites of alveolar formation. Successive estrus cycles over the lifetime of the mouse increase sidebranching and alveolar content (Fig. 1.3) (86).

With the onset of pregnancy, levels of E and P increase (87) and the mammary gland undergoes a second stage of proliferation. Proliferation in response to pregnancy induces extensive sidebranching and alveologenesis. At mid-pregnancy (~day 14.5) extensive alveoli are present throughout the mammary gland (Figure 1.3). Alveoli, the milk-producing units of the mammary gland, are composed of luminal epithelial cells that secrete lipids and proteins into a central lumen. The luminal epithelial cells of the alveoli are surrounded by a basket-like structure of myoepithelial cells that surround the alveolus. By day 16.5 of pregnancy, the alveoli have formed clusters, and dilate by the pressure of secretions produced from the epithelial cells (88). The mammary gland achieves full differentiation upon lactation (Fig. 1.3). Lactation is distinguished by two stages (reviewed in (88)). The first stage begins at mid-pregnancy and is marked by a

sustained increase in the expression of genes involved in the synthesis of milk proteins such as β-casein, lactalbumin, and whey acidic protein (WAP). During the first stage, intracellular lipid droplets are visible. The second stage of lactation occurs around parturition (day 21) and during this stage the expression of milk proteins increases further, the tight junctions between epithelial cells in the alveoli close, and the cytoplasmic lipid droplets and casein are secreted into the alveolar lumen. Secreted lipids and milk proteins travel through the ductal system to the nipple.

Mammary gland involution, an apoptotic-driven process, occurs upon removal of the suckling stimulus, and the mammary gland regresses and loses alveolar structures. However, the mammary gland remains permanently altered following full lactational differentiation, as some residual alveolar structures and an increase in sidebranching remain relative to the nulliparous mammary gland.

The Role of Estrogen in the Mammary Gland

Ductal elongation during pubertal development is largely dependent on E and locally acting growth factors (89), while P may also play a role (80). Initial experiments using ovariectomy to remove endogenous E production demonstrated an essential role for ovarian hormones in ductal development and the formation of TEB structures (85). In ovariectomized pubertal mice treated with E or progestins, E plays the essential role in ductal elongation (90). Implantation of E pellets directly adjacent to the epithelium of pubertal ovariectomized mice stimulates end bud formation only near the implants (91). Interestingly, the overall changes in serum E levels are relatively minor during the

pubertal period when end buds are forming and active proliferation is present (80), suggesting that factors other than E are also required for TEB formation.

E acts in the mammary gland through binding to its receptor, the estrogen receptor (ER). There are two estrogen receptors, ERα and ERβ (18). Similar to PR, estrogen receptors generally act as ligand-activated transcription factors that regulate gene expression by binding directly or indirectly to specific sites in the DNA (18). ER is also thought to reside in the cell membrane or cytoplasm, and can initiate rapid responses through interaction with various signaling pathways, such as the mitogen-activated protein kinases (92). Studies examining the localization of ERα in end buds demonstrated that nuclear ER was concentrated in stromal cells around end buds, but was not present in the rapidly dividing cap cells (93).

A role in mammary gland development for E acting through ERα, has been established using the ERα knockout (αERKO) mouse (94, 95). ERa is thought to be the predominant receptor required to mediate the effects of E in the mouse mammary gland (96). The mammary glands of adult αERKO mice appear similar to the glands of a newborn. They fail to develop TEBs and ductal elongation fails, suggesting that ERα is essential for ductal development (94, 95). Initial studies using tissue recombination of mammary epithelium and stroma from wild-type (WT) and αERKO mice produced contradictory results. An initial study using transplantation of neonatal tissue suggested a necessary role for stromal ERα, whereas epithelial ERα was dispensable (94). A later study using isolated adult mammary epithelial cells from WT and αERKO mice injected into epithelial-free fat pads showed that both stromal and epithelial ERα are required for mammary gland development (97, 98). These results demonstrated that neonatal and

adult mammary tissues use a different tissue-specific role for ERα. However, later studies have shown that the genomic αERKO mice used in these studies are hypomorphic for ERα (99). In these mice the ERα gene was disrupted through insertion of a neomycin resistance gene into the first coding exon, but alternative splicing produced a variant ERα protein that retained substantial ERα function (99). Therefore, circulating prolactin levels were reduced in these mice (95) and restoration of prolactin was able to normalize development. Additionally, treatment with exogenous E+P was able to induce ductal elongation and TEB formation in these αERKO mice (98).

More recent studies using MMTV-Cre-mediated ablation of ERα that produces no detectable ERα transcript have shown that epithelial ERα is also required for ductal elongation and branching (100). In these same studies the use of whey acidic protein (WAP)-Cre-mediated deletion of ERα reduced ductal sidebranching, alveologenesis, and ductal dilation associated with pregnancy, suggesting that ERα may be not only important for normal ductal development during puberty, but also plays an essential role in alveologenesis during pregnancy (100). It is likely that both stromal and epithelial ERα mediate proliferation in the mammary gland and this proliferation is induced through a paracrine mechanism.

In contrast to ERα, which is expressed in a small subset of epithelial cells, ERβ is expressed in 70% of epithelial cells (101). Despite the high levels of ERβ in the mammary gland, ERβ gene deleted mice develop normal ductal structures compared to wild-type littermates. However, despite normal ductal development, mammary glands from pregnant ERβ gene deleted mice display increased proliferation and decreased

organization of epithelial cells during lactation compared to wild-type mammary glands (102). These findings suggest a role for ERβ in the terminal differentiation of the mammary gland during lactation.

Local Mediators of Estrogen Action

During ductal growth, there are complex interactions between growth factors and hormones leading to end bud formation and ductal elongation. Epidermal Growth Factor (EGF) has been shown to be an essential mediator of the E response in ductal elongation. Ankrapp et al. used neutralizing antibody to EGF to block E-induced stimulation of end buds (103). Previous studies used ovariectomized mice treated with slow-release EGF implants to demonstrate that EGF could substitute for E in the stimulation of end buds (104). The EGF receptor (EGFR), a member of the ErbB receptor tyrosine kinase family (105), is present on both epithelial and stromal cells (106). EGFR knockout mice display inhibition of ductal growth and suggest an essential role for EGF in ductal elongation (107). Additionally, transplant experiments placing EGFR -/- ducts into wild-type stroma and vice versa, showed that while epithelial EGFR was dispensable for ductal outgrowth, stromal EGFR was essential for ductal development (107). It has also been hypothesized that stroma-derived EGF may regulate E-inducible PR in the mammary epithelium (78).

Amphiregulin is the major EGFR ligand expressed during puberty, and is regulated by E (108). Amphiregulin is most highly expressed during puberty relative to other EGFR ligands, and its expression localizes to epithelial cells in ducts and TEBs of the pubertal mammary gland (109). Amphiregulin, like many other EGFR ligands, is expressed as an inactive precursor molecule, and members of the ADAM family of

metalloproteinases have been shown to be responsible for the cleavage and activation of amphiregulin *in vitro* (110). Studies using ADAM17 -/- mice revealed that ADAM17 plays a crucial role in mammary morphogenesis by releasing amphiregulin from mammary epithelial cells (111). Recent studies have demonstrated that amphiregulin is induced by E acting through ERα and that amphiregulin is required to induce proliferation in the mammary epithelium. Similar to epithelial expression of ERα, amphiregulin expression in the epithelium is necessary for normal epithelial cell proliferation, TEB formation, and ductal development during puberty (108). Finally, these studies also showed that amphiregulin is an important paracrine mediator of E function during pubertal ductal development.

Hepatocyte Growth Factor (HGF), or scatter factor, has also been shown to be involved in ductal elongation in the mammary gland (112). Interactions between stroma and mammary epithelium have been studied using a minimally supplemented, serum-free, three-dimensional collagen gel primary culture system (113). Using this system, it has been shown that ER-expressing fibroblasts mediate E-induced epithelial cell proliferation through HGF (114). Direct treatment of epithelial cells with E does not produce a morphological or proliferative response. The effect of HGF on P-dependent proliferation and alveologenesis was also examined using this system, and it was shown that while HGF by itself induces tubule formation, HGF + progestin treatment reduces tubule formation and induces the formation of multiluminal alveolar-like structures similar to those seen *in vivo* in response to E+P (115). Additionally, treatment with HGF + progestin also produces a synergistic increase in proliferation. In order to examine the role of endogenous HGF in ductal development and alveologenesis *in vivo*, pellets

containing neutralizing antibody to HGF were implanted into the mammary glands of pubertal or adult mice (113). In pubertal mice, HGF neutralizing antibody limited ductal elongation, and in adult mice, ductal sidebranching was reduced by the anti-HGF antibody. Therefore, it appears that HGF plays an important role in mediating the effects of E in the pubertal and adult gland, and that P interacts with HGF.

The Role of Progesterone in the Mammary Gland

The pubertal mammary gland is generally less sensitive to P than the adult mammary gland (116), and thus the primary role of P in mammary gland development is in the adult during pregnancy, when P is essential for the induction of sidebranching and alveologenesis (78). Studies in pubertal wild-type mice have shown that acute treatment with E+P has only a minimal additional effect on proliferation and does not produce sidebranching or alveologenesis, suggest that the pubertal mammary gland is less sensitive to P than to E (90).

A requirement for P in the sidebranching response has been shown using ovariectomized mice (89). In ovariectomized *adult* mice, acute treatment with E has only a minor proliferative effect. In contrast, acute treatment with E + P significantly increases proliferation resulting in sidebranching and the start of alveologenesis (90). Thus, maturation of the gland in adulthood is accompanied by the acquisition of responsiveness to P. Ovariectomy decreases PR expression and E treatment upregulates PR expression (45). However, isoform-specific regulation of PR has not been determined. In the adult mammary gland immunoblot studies have been used to demonstrate that the ratio of PRA to PRB is about 3:1 (117). Immunohistochemical

studies of PR expression have not distinguished between PR isoforms, but suggest that PR expression is localized to a subset of mammary epithelial cells (45, 118, 119). In the adult gland treated with estrogen plus progesterone, it has been hypothesized that estrogen upregulates PR expression and that proliferation is mediated by progesterone acting through PR (78). It is thought that the early sidebranching and alveologenesis response to pregnancy is primarily mediated by P acting through PR and that later development of the alveoli is directed by prolactin (PRL) binding to the prolactin receptor (PRLR) (120).

In order to examine the role of PR isoforms in mammary gland development, mice lacking both PR isoforms (PRKO), lacking PRA (PRAKO), or lacking PRB (PRBKO) have been generated (71, 121, 122). The importance of P acting through PR for sidebranching and alveologenesis has been demonstrated using the total PRKO mouse (121). Tissue recombination studies using PRKO tissues revealed that epithelial PR signaling is required for sidebranching and alveologenesis (123). In these same studies chimeric epithelium composed of PR-/- cells in close vicinity to PR wild-type cells went through complete alveolar development. Labeling of PR -/- epithelium in the chimeric mixture showed that these cells contributed to alveolar development, suggesting that P acts through a paracrine mechanism on a subset of mammary epithelial cells during alveologenesis. Additionally, these results suggest that PR expression is only necessary in a subset of epithelial cells for normal alveologenesis. Selective ablation of PRA protein in the PRAKO mouse demonstrated that PRA is not essential for normal mammary gland ductal development and alveologenesis. The PRAKO mouse does contain severe abnormalities in ovarian and uterine function, so the role of PRA in

alveologenesis was studied through E+P treatment (122). PRB ablation did not effect ovarian or uterine responses, but the mammary gland phenotype was similar to that of the PRKO mouse (71). Thus, studies using the PRAKO and PRBKO mouse demonstrated that PRB is essential for sidebranching and alveologenesis during pregnancy, whereas the functional role of PRA was not determined (71, 122). While E plays a predominant role in ductal elongation, careful examination of the PRKO mouse revealed a slight delay in ductal elongation, suggesting that P also plays a role in ductal development during puberty (124).

Transgenic mice overexpressing either PRA or PRB have also been used to examine the role of PRA and PRB in the mammary gland (125, 126). These contain either excess PRA (PRA transgenic) (126) or excess PRB (PRB transgenic) (125) driven by a cytomegalovirus (CMV) promoter. PRA transgenic mice contain extensive sidebranching in the adult mice, and also exhibit ductal hyperplasia and a disorganized basement membrane. PRB transgenic mice were characterized by inappropriate alveolar growth, as well as an inability of the ducts to completely fill the fat pad. Thus, the PRB transgenic mouse further suggests a role for PRB during alveologenesis in the mammary gland, whereas the PRA transgenic suggests a role for PRA in sidebranching. However, expression of PR is usually only present in a subset of cells, and the inappropriate targeting of PRA or PRB to cell types that usually do not express PR may confound the influence of PRA or PRB overexpression on mammary gland phenotype.

In summary, P is important for sidebranching and alveologenesis during pregnancy and these responses appear to be mediated through epithelial PRA and PRB. A specific role for PRB in alveologenesis has been ascribed, but the role of PRA in the

mammary gland remains unclear. No studies have addressed the developmental and hormonal regulation of PR isoforms during mammary gland development.

Local Mediators of Progesterone Action

There are a number of target genes that have been shown to mediate the responses of P *in vivo*. Msx-2, a homeobox containing transcription factor, has been shown to be upregulated by P in T47D breast cancer cells (50) and plays a role in P-induced branching during the peripubertal period (127). However, Msx-2 does not appear to be involved in P-induced side branching and alveolar budding during pregnancy (127).

Calcitonin (CT), a peptide hormone involved in calcium homeostasis, has also been shown to be regulated by P and is produced in luminal epithelial cells in the mammary gland (128). Calcitonin acts through binding to the CT Receptor (CTR) C1a subtype, which is a membrane-spanning G protein-coupled receptor (129). In the mammary gland, the CTR is localized to myoepithelial cells, suggesting that CT produced in luminal epithelial cells may influence myoepithelial cell proliferation and organization (128). However, no direct link between CT and mammary gland proliferation or organization has been established.

In response to P, Wnt-4 is released as a secreted factor that binds to the Frizzled receptor (130). Originally, Wnt-4, along with Wnt-2, Wnt5a, Wnt5b, Wnt-6, and Wnt-7, was shown to be expressed in the mammary gland, and Wnt-4 expression was reduced by ovariectomy (131). In the canonical pathway, Wnt-4 binding to the Frizzled receptor leads to the activation of β-catenin through stabilization, which causes accumulation in the nucleus (132). Once in the nucleus, β-catenin is also able to activate transcription of

cyclin D1 (133). Overexpression of Wnt-4 in mammary epithelial cells was examined through the use of a recombinant retrovirus to constitutively express Wnt-4 in mammary epithelial cells transplanted into virgin animals (134). Wnt-4 overexpression results in increased ductal sidebranching and the appearance of alveolar-like structures in these virgin animals. The degree of mammary development in the epithelium overexpressing Wnt-4 is similar to the development found after 10 days of pregnancy, which suggests that Wnt-4 plays a role in mammary gland development during early pregnancy. In agreement with this hypothesis, Wnt-4 knockout mice have been used to demonstrate an essential role for Wnt-4 in the early sidebranching response to pregnancy in the mammary gland (130).

Receptor Activator of Nuclear Factor kappa B (NFκB) Ligand (RANKL) has been shown to be upregulated by P (71) and is essential for alveologenesis. RANKL is produced by epithelial cells and secreted as a paracrine factor that can affect other epithelial cells in an autocrine or paracrine manner through binding to Receptor Activator of NFkB (RANK) (135). Studies using RANKL and RANK gene deleted mice have shown an essential role for RANKL and RANK in the differentiation of mammary alveolar cells during pregnancy. In the absence of either RANKL or RANK, alveologenesis and lactation in the mammary gland is deficient (135). Binding of RANKL to RANK induces the phosphorylation and degradation of IKKα and the subsequent activation of NFkB (136). There are NFkB binding sites on the cyclin D1 promoter and NFkB activation leads to upregulation of cyclin D1 expression (137).

It has also been shown that RANKL is able to induce the nuclear translocation of Id2, a basic helix-loop-helix (bHLH) inhibitor. RANKL does not affect Id2 protein

levels, but increases nuclear levels of the protein through phosphorylation of serine 5. Interestingly, this nuclear translocation and accumulation of Id2 is impaired in RANKL gene deleted mice (138). Id proteins, which lack a DNA binding domain but contain a helix-loop-helix motif, act as negative regulators of bHLH transcription factors through heterodimerization with bHLH partners (139). Once in the nucleus, Id2 is also capable of repressing the cyclin-dependent kinase inhibitor (CDKI), p21, and thus is thought to play a positive role in cell growth and cell cycle progression (138). While Id2 and cyclin D1 both stimulate cell cycle progression and tumorigenesis, overexpression of cyclin D1 does not rescue the defect of Id2 gene deficient mice in lobuloalveolar development (140). Thus, the production of RANKL in response to P treatment may affect cell cycle regulation through cyclin D1 and/or Id2.

Cyclin D1 has been proposed as a mediator of P-induced proliferation because levels of cyclin D1 expression increase in response to P treatment and the increase is associated with increased proliferation. Additionally, while E alone is able to increase cyclin D1 levels and P further increases cyclin D1 levels, the increased level induced by P is lost in the PRKO mouse, suggesting that P acting through PR is responsible for increased cyclin D1 expression (141). Cyclin D1 is involved in cell cycle activation through binding and activating Cdk4 and Cdk6 in the G1 phase of the cell cycle. Cdk4 and Cdk6, in turn, phosphorylated their downstream target, the retinoblastoma protein Rb. Upon phosphorylation, pRB is inactive, allowing release of E2F and other transcription factors, which activate the transcription of S-phase genes and cell cycle progression (142). Studies examining the localization of cyclin D1 have demonstrated that nuclear accumulation of cyclin D1 is required for S-phase entry (143). Cyclin D1 is

the primary D-type cyclin expressed in the mouse mammary gland, and its expression is thought to primarily be controlled by mitogens. *In situ* hybridization has been used to demonstrate a lack of cyclin D2 and D3 expression (144). Cyclin D1 gene deleted mice develop normally through puberty, although a detailed examination of ductal elongation has not been performed. During pregnancy, cyclin D1 gene deleted mice exhibit dramatically reduced lobuloalveolar development (145, 146). As mentioned above, increased cyclin D1 expression is linked to the Wnt4 pathway through β-catenin activation and it is linked to the RANKL pathway through NFκB activation (133, 136). However, the exact mechanism of how P increases cyclin D1 levels is not known.

The Role of Pituitary Hormones in the Mammary Gland

Ductal elongation in the mammary gland requires the presence of E, but it also requires the presence of the pituitary. Historical studies in ovariectomized mice showed that the addition of growth hormone (GH) plus E was more effective at stimulating ductal development than either hormone alone (147). More recently, mammary glands from GH receptor gene deleted mice have been shown to fail to undergo ductal elongation (148). GH acts on the mammary gland through local expression of insulin-like growth factor-I (IGF-I), which has been shown by IGF-I substitution for the pituitary gland in promoting ductal development and through increased IGF-I levels in response to GH treatment (reviewed in (149)).

The pituitary hormone prolactin (PRL) has been called the master controller of alveologenesis and lactogenic differentiation (88). Transplant studies using prolactin receptor knockout mice (PRLR -/-), have shown that in the absence of PRL signaling, a

fully branched ductal system still develops and sidebranching and the formation of alveolar buds occur in response to pregnancy. However, there is no lobuloalveolar development and functional differentiation of mammary epithelial cells (150). Adding support to the importance of PRL in alveologenesis is the similar phenotype of both janus 2 kinase (JAK2) and Signal transducer and activator of transcription 5a/b (Stat5a/b) gene deleted mice to the PRLR -/- mammary gland (151-154). JAK2 and STAT5 are both principal downstream mediators of PRL signaling (88).

There are a number of local factors thought to play a role in mediating PRL action in the mammary gland. Insulin-like growth factor 2 (IGF-2) has been shown to be increased by PRL treatment both *in vivo* and in primary cultures of mouse mammary epithelial cells (155). The addition of IGF2 to PRLR -/- mice restored alveologenesis, and this is thought to occur through cyclin D1 because IGF-2 treatment induces cyclin D1 expression (155). RANKL is also thought to be a potential mediator of PRL effects in the mammary gland. STAT5a, an important mediator of PRL signaling, has been shown to increase expression of RANKL (156). In BALB/c mice, E+P treatment induces expression of activated nuclear STAT5a, which is thought to be activated by PRL (157). Therefore, PRL may have effects in the mammary gland that are indirect through IGF-2 or RANKL or direct through activation of JAK2 or STAT5.

PRL also plays a role in the induction of P, as shown by reduced levels of P in PRLR gene deleted mice (158). Treatment of PRL gene deleted mice with P results in sidebranching, but not formation of alveolar buds (159). Additionally, P treatment of PRLR gene deleted mice induces sidebranching, but not alveolar bud formation (120).

The Influence of Mouse Strain on the Mammary Gland

As described above, the mouse mammary gland has frequently been used as a model for elucidating the role and function of P during normal mammary gland development. In addition, it has been used to study the role of P in the etiology of mammary cancer (160-162). Most *in vivo* studies of P action in genetically unaltered mice have been carried out using BALB/c mice (80, 90, 116, 127, 163-166). Studies showing that P-responsiveness is acquired when PR becomes inducible by E (90, 116), that P is involved in branching (80, 127, 165), that P stimulates proliferation in the mammary gland (164), and that P leads to sidebranching and alveologenesis in the adult (90) have all been performed in BALB/c mice. More recently, however, insights into PR isoform functions have been obtained from studies of total PR, PRA, or PRB-deficient mice in a mixed C57BL/6 × 129SV genetic background (71, 121, 122). In fact, most gene deleted mouse models used to study mammary gland development have been created using this mixed genetic background (144, 150, 155, 167, 168).

Interestingly, strain-specific differences in mouse mammary gland development (169), responsiveness to hormones, and susceptibility to carcinogenesis have been reported (170). For example, C57BL/6 mice exhibit reduced P-induced sidebranching and alveologenesis, and reduced susceptibility to carcinogen- and medroxyprogesterone acetate-induced tumorigenesis compared to BALB/c mice (171, 172). There have been few studies that examine the reduced hormonal responsiveness in the C57BL/6 mammary gland. It has been reported that differences between C57BL/6 mice and mouse strains exhibiting a more highly branched pattern may be attributed to different contributions of the stroma (173). Additionally, a recent report suggests that the stroma plays a crucial

role in strain-specific differential hormone responsiveness (171). However, primary culture of mammary epithelial cells from C57BL/6 mice have been shown to be less responsive to treatment relative to BALB/c mammary epithelial cells (Leipprandt & Haslam, unpublished observations), suggesting that the epithelium contains differences between strains as well. Therefore, it remains to be determined what factor(s) are responsible for differences in hormone responsiveness and tumorigenesis between mouse strains.

It is important to consider mouse strain when examining the role of P and PR in the normal mouse mammary gland, and a comparison of different mouse strains may produce important information on P action *in vivo*.

Conclusion

Progesterone has been well established as an important mitogen in the mammary gland. Numerous studies using genetically altered mice have identified P acting through the PR as essential for sidebranching and alveologenesis in response to pregnancy. However, little is known about PR isoform expression and function in the genetically unaltered mouse mammary gland. The relative expression level and localization of PRA and PRB protein throughout mammary gland development are not known. While a functional role for PRB in alveologenesis has been described, a role for PRA in the mammary gland has not been established. Both the developmental and hormonal regulation of PRA and PRB in the mouse mammary gland are not known. Finally, the downstream targets of PRA signaling have not been clearly elucidated. In order to more carefully examine P action in genetically unaltered mice, the more hormonally sensitive

BALB/c mouse and the less sensitive C57BL/6 mouse were used to examine potential downstream mediators of P in the mammary gland. The research presented in this thesis establishes the developmental regulation of expression and localization of PRA and PRB, proposes a function for PRA in early sidebranching, provides novel insight into the regulation of PRA and PRB *in vivo*, and demonstrates that genetic background may play an important role in determining P responses.

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CHAPTER TWO

PROGESTERONE RECEPTOR ISOFORMS A AND B: TEMPORAL AND SPATIAL DIFFERENCES IN EXPRESSION DURING MURINE MAMMARY GLAND DEVELOPMENT

Note: The contents of this chapter have been published in Aupperlee M.D., Smith K.T., Kariagina A., and Haslam S.Z. Progesterone receptor isoforms A and B: temporal and spatial differences in expression during murine mammary gland development. Endocrinology. (2005) Aug;146(8):3577-88.

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. 2.7). 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 70° 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 4°C 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.

Images in this dissertation are presented in color.

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. 2.1). 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. 2.1). At 6 weeks of age, the PRA positive cells were observed in end buds (Fig. 2.2A, E) and in ducts (Fig 2.2B, 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. 2.2E). 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. 2.1, 2.2C,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. 2.1). At 7 days of pregnancy PRA was detected in 25 ± 1 % of cells (Fig. 2.1). However, at 14 days of pregnancy, PRA was detected in only 11 ± 2 % of

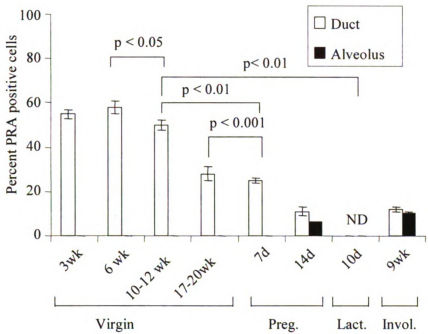


Figure 2.1. Quantitation of PRA at different stages of mammary gland development. Immunoperoxidase localization of PRA was carried out using anti-PRA antibody on tissue sections from 3, 6, 10-12 or 17-20-week-old virgin, 7 or 14 day pregnant, 10-day lactating mice, and at 9 weeks post weaning (lactational 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.

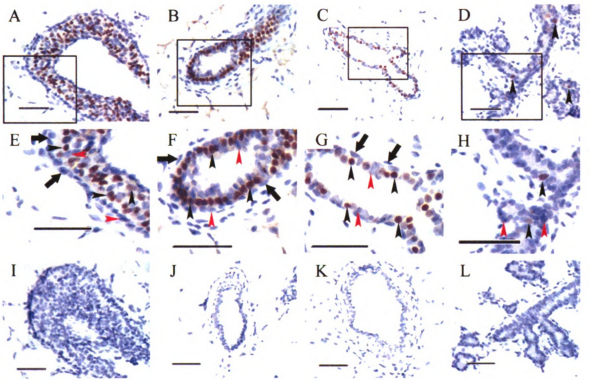


Figure 2.2. Immunoperoxidase localization of PRA at different stages of mammary gland development. Representative sections from 6-week-old immature (A, E end bud, B,F duct), 12-week-old adult (C, G duct), and 14 day pregnant (D, H alveoli) mouse mammary gland were treated with anti-PRA antibody (A-H) 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).

ductal epithelial cells and 6.0 ± 0.3% of alveolar cells ($p < 0.001$)(Figs. 2.1, 2.2D,H). No PRA positive cells were detected during lactation (Fig. 2.1). 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. 2.1). 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. 2.1). 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. 2.2).

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. 2.3, 2.4A,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. 2.3). PRB was localized mainly in alveolar cells (Fig. 2.4C,F). PRB staining was seen in both the cytoplasm and nucleus of epithelial cells (Fig. 2.4F). PRB was not detected in myoepithelial cells or stromal cells (Fig. 2.4F). 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. 2.3); less than 1% of ductal cells were PRB positive.

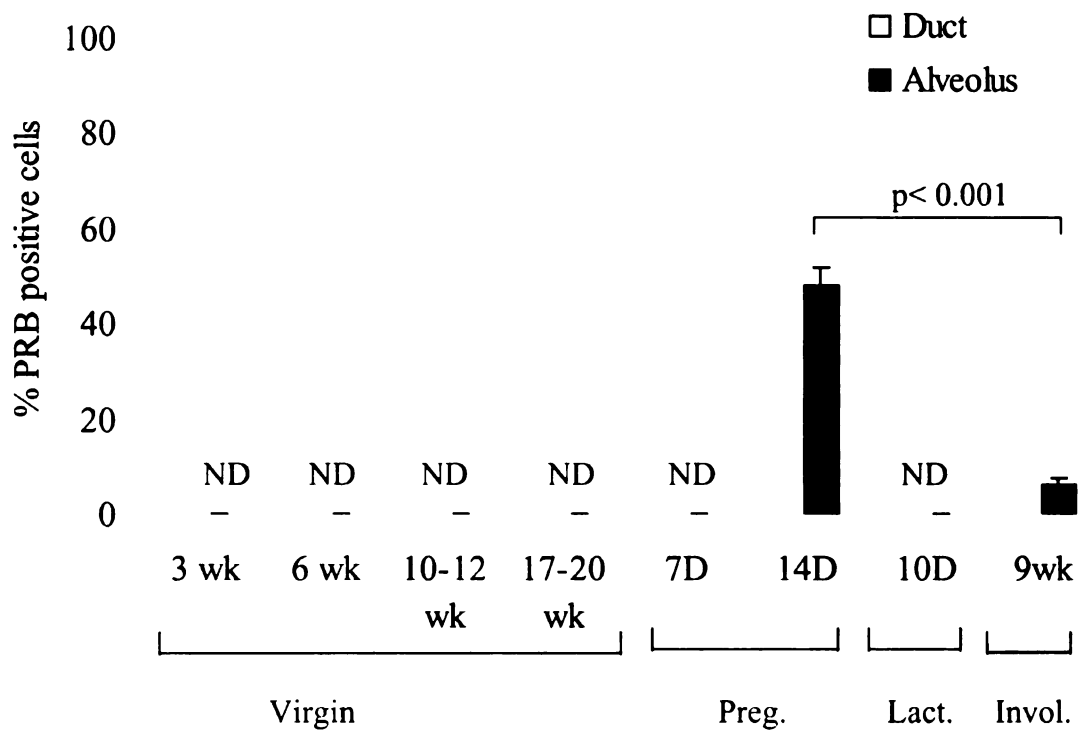


Figure 2.3. Quantitation of PRB at different stages of mammary gland development. Immunofluorescence localization of PRB was carried out using anti-PRB antibody on tissue sections from 3, 6, 10-12, or 17-20-week-old virgin, 7 or 14 day pregnant, 10-day lactating mice, and at 9 weeks post weaning (lactational 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 ($p<0.001$).

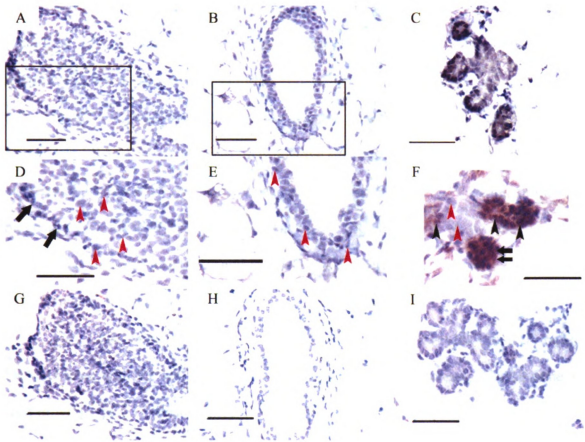


Figure 2.4. Immunoperoxidase localization of PRB at different stages of mammary gland development. Representative sections from 6-week-old immature (A, D end bud), 12-week-old adult (B, E duct), and 14 day pregnant (C, F alveoli) mice were treated with anti-PRB antibody (A-F) as described in the Methods 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).

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. 2.5A 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. 2.5A 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. 2.5B 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. 2.5B). 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.

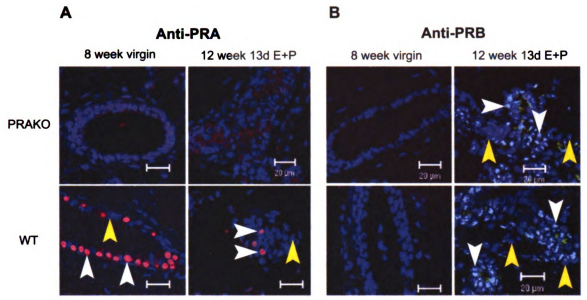


Figure 2.5. Immunodetection of PRA and PRB in wild type vs. PRA null mice. Immunofluorescence localization of (A) PRA or (B) PRB was carried out on sections from 8-week-old virgin or 13 day E+P-treated 12-week-old virgin wild type (WT) and PRA null (PRAKO) mice. Antibody staining was carried out with 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).

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. 2.6A). 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. 2.6A, lane 2). PRB was not detected in virgin mammary gland using antibody specific for PRB (hPRa6) (Fig. 2.6B, lane 2); the same antibody detected PRB in mouse uterus (Fig. 2.6B, 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. 2.6B). Immunoblot analysis showed only a PRB band (Fig. 2.6B, 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

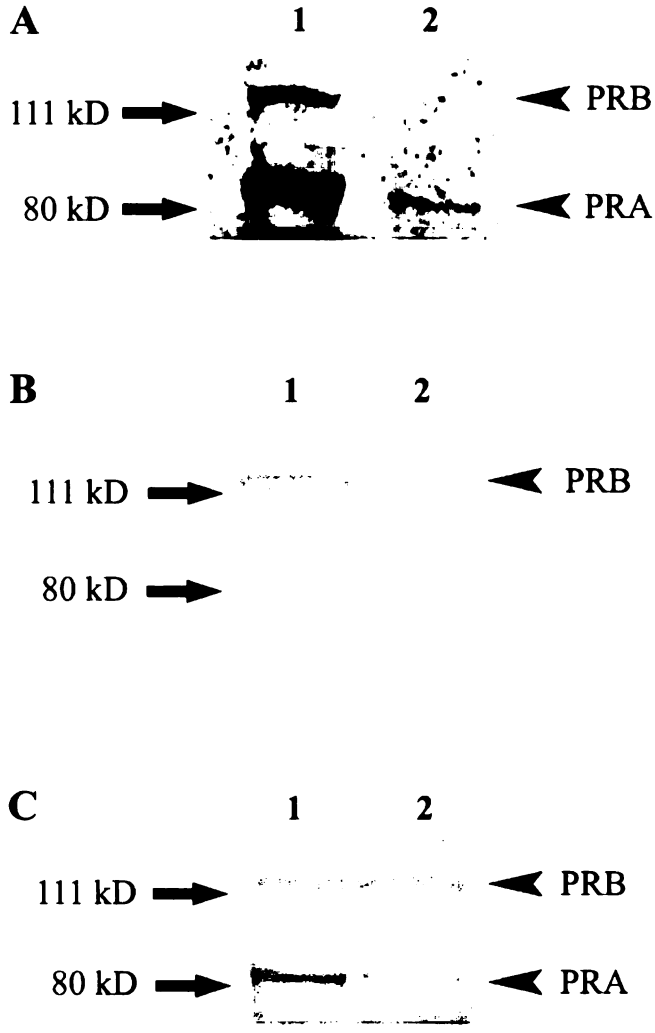


Figure 2.6. Immunoblot analysis of PR in mammary gland. A. Extracts from uterus (lanes 1) and isolated epithelial cells from 6-week-old mammary glands (lane 2) were subjected to SDS-PAGE and blots were probed with hPRa7 anti-PR antibody as described in the Methods section. PRA was detected a single band 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).

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. 2.7A 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. 2.7B) 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 2.2D,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).

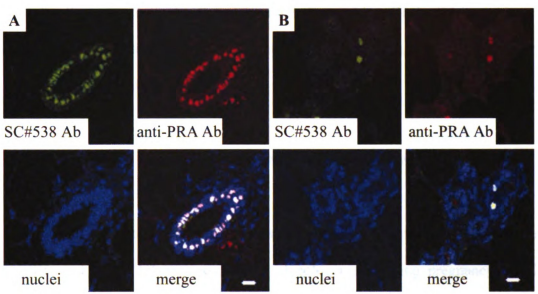


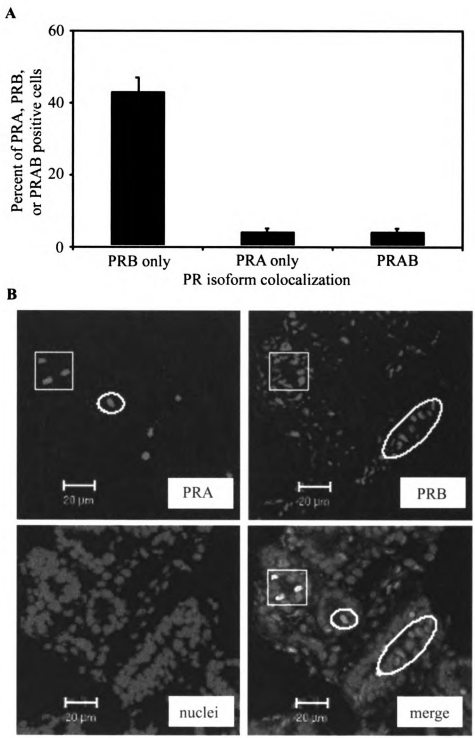
Figure 2.7. Immunodetection of PRA by SC#538 anti-PR antibody. Tissue sections from (A) 6-week-old virgin or (B) 14 day pregnant mammary glands were double labeled with anti-PRA antibody (red nuclei) and SC#538 antibody (green nuclei); nuclei were counterstained with TOPRO-3 (dark blue). In the virgin 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)

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. 2.8A, B). Forty-three percent of cells were positively labeled for PRB (Fig. 2.8A). Of the PRA positive cells (8%), about half were also positive for PRB (Fig. 2.8A). 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.

Figure 2.8. 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).


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

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. 2.9A). Fifteen percent of cells were BrdU and PRB positive; thus 95% of BrdU positive cells were PRB positive (Figs 2.9A, 2.10A). In pregnant mammary gland PRA and BrdU were not colocalized in the same cells (Figs. 2.9A, 2.10B).

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. 2.9B). 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. 2.10D), which is a region of the end bud that is devoid of PRA positive cells (Fig. 2.2A,E). Fewer BrdU positive cells were present in ducts (Fig. 2.10C).

In the pregnant mammary gland 56% of cells were cyclin D1 positive and 49% were PRB positive (Fig. 2.11A). 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 (Fig. 2.12A). There was no colocalization of PRA with cyclin D1 (Figs. 2.11A, 2.12B).

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%; $p<0.05$) (Fig2.11A,B). Fifty-four percent of cells were PRA positive, and cyclin D1 and PRA

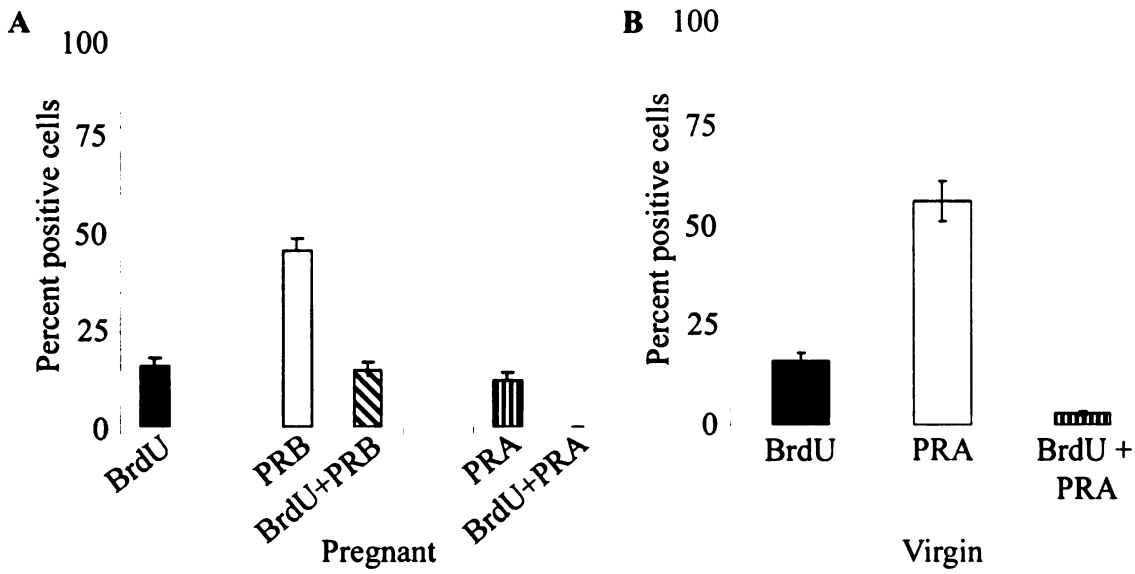


Figure 2.9. Quantitation of colocalization of PRB or PRA with BrdU in pregnant and virgin mammary glands. Dual immunofluorescence detection of PRB or PRA and BrdU was carried out on tissue sections from (A) 14 day pregnant and (B) 6-week-old virgin mammary glands and visualized by laser scanning confocal 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.

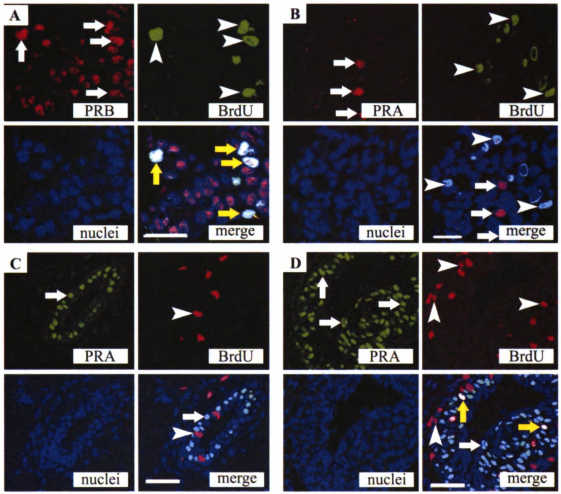


Figure 2.10. Detection of colocalization of PRB or PRA with BrdU in pregnant and virgin mammary glands. Dual immunofluorescence detection was carried out in (A,B)14 day pregnant or (C,D) 6-week-old virgin mammary gland using anti-PRB (A) or anti-PRA (B,C,D) antibodies and TOPRO-3 nuclear stain and were 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).

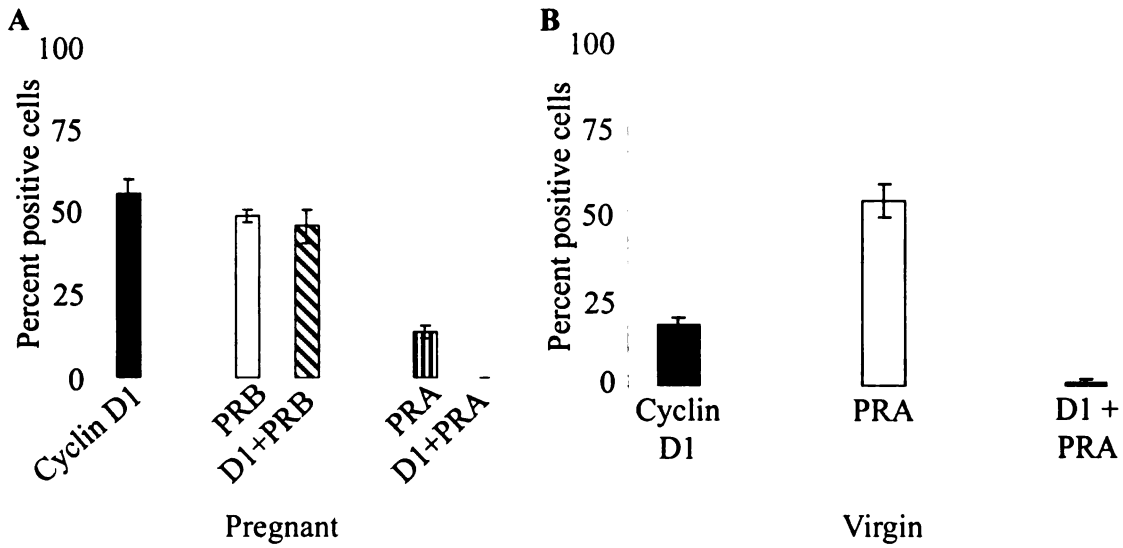


Figure 2.11. Quantitation of colocalization of PRB or PRA with cyclin D1 in pregnant and virgin mammary glands. Dual immunofluorescence detection of PRB or PRA and cyclin D1 was carried out on tissue sections from (A) 14 day pregnant and (B) 6-week-old virgin mammary glands and visualized by laser scanning 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.

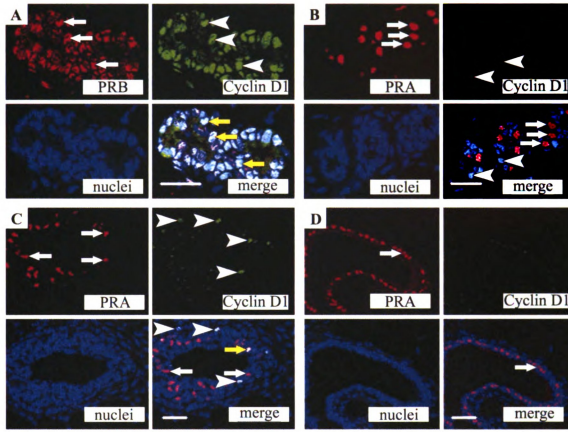


Figure 2.12. Detection of colocalization of PRB or PRA with cyclin D1 in pregnant and virgin mammary glands. Dual immunofluorescence detection of PRB or PRA and cyclin D1 was carried out on tissue sections from (A,B) 14 day pregnant and (C,D) 6-week-old virgin mammary glands and visualized by laser scanning 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)

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. 2.11B). Fig. 2.12C illustrates colocalization in a duct that is cyclin D1 positive. Many ducts had no cyclin D1 positive cells, yet PRA was highly expressed (Fig. 2.12D).

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. 2.4C, 2.8B,C) or in alveolar structures of adult E+P-treated mice (Fig. 2.5B). 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. 2.1,2.3).

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.2.7) (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. 2.4F). 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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CHAPTER 3

DIFFERENTIAL HORMONAL REGULATION AND FUNCTION OF PR ISOFORMS IN NORMAL ADULT MOUSE MAMMARY GLAND

Note: The contents of this chapter have been published in Aupperlee, M.D. and Haslam, S.Z. Differential hormonal regulation and function of PR isoforms in normal adult mouse mammary gland. Endocrinology. (2007) May;148(5):2290-300.

ABSTRACT

In normal mouse mammary gland, the mitogenic action of progesterone (P) is mediated by two progesterone receptor (PR) isoforms, PRA and PRB. PRA is predominantly expressed in the adult virgin, and PRB is predominantly expressed during pregnancy. To investigate hormonal regulation of PR isoform expression and isoform-specific functions in vivo, adult ovariectomized BALB/c mice were treated for 3, 5, or 10 days with estrogen (E), P, or E+P. Using an immunohistochemical approach with isoform-specific antibodies, we investigated hormonal regulation of PRA and PRB and their functional roles in proliferation and morphogenesis. Significant E-induced proliferation was only observed after 5 days at the distal tips of ducts; there was no sidebranching or alveologenesis. P induced proliferation that resulted in sidebranching and alveologenesis, but E+P treatment produced more proliferation sooner and more extensive sidebranching and alveologenesis. PRA levels were increased by E and decreased by P. Increased PRB levels were induced by treatment with P or E+P and coincided with the formation of alveoli. PRA was the predominant PR isoform expressed during sidebranching, and colocalization of PRA with BrdU revealed that proliferation of PRA positive and negative cells were responsible for P-induced sidebranching. PRB was the predominant PR isoform expressed during alveologenesis, and colocalization of PRB with BrdU showed that both PRB positive and negative cells proliferated during alveolar expansion. These results demonstrate different hormonal regulation of PRA and PRB levels in vivo and suggest that P can induce proliferation through either PRA or PRB via direct and paracrine mechanisms.

INTRODUCTION

Progesterone (P) plays an important role in regulating proliferation and differentiation in the normal mammary gland (1). Proliferation is regulated by P in the adult human breast (2) as well as in rodent and monkey models (1, 3). Progesterone acts through binding to its cognate nuclear receptor, the progesterone receptor (PR), and PR exists as two isoforms, PRA and PRB, which are identical except for a 164 amino acid N-terminal extension on PRB. PRA and PRB can regulate different genes and have different functions (4). Studies using PRB knockout mice, PRB transgenic mice, and immunohistochemical analysis of PRB expression in wild-type mice indicate a role for PRB in alveologenesis (5-7). The role of PRA in the mammary gland is less clear, but studies with PRA transgenic mice and immunohistochemical analysis of PRA expression in wild-type mice indicate that PRA may be involved in ductal development and sidebranching (7, 8).

To date, PR expression and progesterone action in the normal mammary gland have been most extensively studied in the mouse model. PRA and PRB expression are temporally and spatially separated during murine mammary gland development from puberty through pregnancy, lactation, and involution (7). Our previous studies of developmental expression of PR isoforms found that PRA is predominantly expressed in the virgin mouse mammary gland, which is primarily composed of ducts, whereas expression of PRB is induced in alveolar structures upon lobuloalveolar development during pregnancy along with a concomitant decrease in PRA expression (7). Additionally, PRA and PRB are infrequently expressed in the same cell. In contrast, in

the normal adult premenopausal human breast PRA and PRB are coexpressed in the same cells and the ratio of PRA:PRB in individual PR positive cells is 1:1 (9). The pattern and level of PRA and PRB expression at other stages of human mammary gland development are not known. However, breast cancers exhibit an altered PRA:PRB ratio with a higher PRA:PRB ratio associated with less differentiated and more aggressive tumors (9). The mechanism(s) that regulate the relative expression of PRA and PRB in breast cancer is not known.

Little is currently known about the hormonal regulation of PRA and PRB expression in the normal breast. Studies using human breast cancer cell lines have shown that E induces expression of PR, and P has been shown to downregulate expression of PR (10, 11). However, it is not clear how individual PR isoforms are regulated. It has been reported that ovariectomy reduces PR expression in the mouse mammary gland (12). While a role for estrogen (E) in regulation of PR has been determined (12, 13), isoform-specific regulation of PRA and PRB has not been examined. The effects of E and/or P on PR isoforms in vivo in the normal human breast have not been well studied. Since alterations in PRA:PRB ratios are associated with breast cancer progression (9), understanding the normal regulation of PRA and PRB may provide insight into the deregulation that occurs in breast cancer.

Biochemical analyses of PR expression, such as immunoblots, can provide useful information about the molecular sizes of protein isoforms or protein post-translational modifications. However with regard to analysis of whole mammary gland extracts, immunoblots can be limited in their sensitivity and accuracy for PR detection and quantitation due to the dilution of epithelial proteins by stroma-derived proteins. This

occurs because PR is expressed in the epithelial compartment of the gland, whereas the stromal compartment is PR negative (4, 7). This is particularly relevant for quantitation of relative expression of PRA or PRB in mammary tissues that exhibit changes in overall epithelial content, such as after ovariectomy versus treatment with pregnancy levels of E + P. In the first case, the gland exists as a rudimentary ductal system with a predominance of stroma, whereas after E+P treatment there is a proliferative expansion of the epithelium in the form of sidebranches and alveoli and an overall increase in the ratio of epithelium to stroma. Thus, the same amount of protein from mammary gland extracts obtained at different physiological states represents different amounts of mammary epithelium.

In the present study, we used an immunohistochemical approach with antibodies specific for PRA or PRB (7, 14) to examine the hormonal regulation of PRA and PRB and the roles of PRA and PRB in mediating proliferative and morphological responses in the adult mouse mammary gland. One advantage of this approach is that it allowed analysis and quantitation of the cellular distribution of PR isoforms and their colocalization with proliferation markers. We found that PRA expression was increased by E and decreased by P. The initial proliferative response to P, leading to sidebranching, was mediated by PRA. Proliferation and PRB expression were induced by P alone, but were accelerated and enhanced by the combination of E+P. Induction of PRB expression coincided with decreased PRA levels and the onset of alveologenesis. Analysis of ERα expression revealed that only PRA was extensively colocalized with ERα. These studies demonstrate differences in the hormonal regulation of PRA and PRB and isoform-

specific roles in mediating proliferation and differentiation in the normal mouse mammary gland.

MATERIALS AND METHODS

Animals:

Mammary glands were obtained from adult (19 to 22-week-old) BALB/c female mice purchased from Harlan (Indianapolis, IN). Hormone treated adult virgin mice were ovariectomized (OVX) and one week after OVX animals were injected for 3, 5, or 10 days with saline control (C), 17-β-estradiol (E) (1 μg/injection), progesterone (P) (1 mg/injection), or E+P (1 μg + 1 mg respectively/injection) administered subcutaneously. Two hours prior to sacrifice mice were injected with 5-bromo-2'-deoxyuridine (BrdU) (70 μg/g of body weight) to label proliferating cells. 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. Mammary tissues were fixed and processed as whole mounts (15) or formalin fixed and paraffin-embedded for immunohistochemistry as previously described (7).

Immunohistochemistry with anti-PR isoform specific antibodies:

The protocol used to detect PRA and PRB was the same as previously described (7). Tissue sections were treated with a combination of heat and pressure for antigen retrieval and then blocked with goat anti-mouse IgG Fab Fragments (Jackson ImmunoResearch Laboratories, West Grove, PA) [1:100 in PBS containing 1% BSA (1% PBSA), 60 min], blocked with normal goat serum (Vector Laboratories, Burlingame,

CA)(1:1 in PBS, 30 min), and then incubated with primary antibody against PRA (hPRa7, Neomarkers, Fremont, CA) (1:50 in PBS/0.5% Triton X-100, overnight, 4 C) or against PRB (hPRa6, Neomarkers) (1:50 in PBS/0.5% Triton X-100, overnight). Sections were rinsed with PBS/0.5% Triton X-100 and the primary antibody was recognized by goat anti-mouse antibody conjugated to Alexa 488 (Molecular Probes, Eugene, OR) (1:200 in PBS, 30 min).

When immunostaining to recognize PRA, nuclei were counterstained with 4',6-diamidino-2-phenylindole, dilactate (DAPI) (Molecular Probes) (1:10,000 in H_2O), and sections were visualized and images captured using a Nikon inverted epifluorescence microscope (Mager Scientific, Dexter, MI) with MetaMorph software (Molecular Devices Corporation, Downington, PA). When immunostaining to recognize PRB, nuclei were counterstained with TOPRO-3 Iodide (Molecular Probes) (1:1000 in fluorescent mounting media) and sections were visualized and images captured using a Zeiss Pascal laser scanning confocal microscope (Zeiss, Thornwood, NY).

Colocalization of PRA or PRB with BrdU, cyclin D1, or ERα:

Double labeling of PRA or PRB with BrdU and cyclin D1 was performed as previously described (7). Briefly, after antigen retrieval, sections were blocked and incubated with anti-PRA or PRB antibody (overnight, 4 C) as described above.

For colocalization with BrdU, sections were first stained for PRA or PRB, and then PRA or PRB localization was detected with a goat anti-mouse secondary conjugated to Alexa 488 (Molecular Probes) (1:200 in PBS, 30 min). Next, sections were blocked

with goat anti-mouse IgG Fab fragments (Jackson Immunoresearch Laboratories) (1:100 in 1% PBSA, 60 min), blocked with normal goat serum (Vector Laboratories) (1:1 in PBS, 30 min) and incubated for 60 min at room temperature with anti-BrdU antibody (kit from Amersham Biosciences, Piscataway, NJ). BrdU localization was detected with a biotinylated goat anti-mouse secondary (Dako, Carpinteria, CA) (1:400 in PBS, 30 min), which was recognized by streptavidin-conjugated Alexa 546 (Molecular Probes) (1:100 in PBS, 30 min).

For colocalization with cyclin D1, sections were first stained for PRA or PRB, and then PRA or PRB localization was detected with a goat anti-mouse secondary conjugated to Alexa 546 (Molecular Probes) (1:200 in PBS, 30 min). Sections were then blocked with 2% PBSA for 30 min and incubated overnight at 4 C with rabbit polyclonal anti-cyclin D1 antibody (Biosource, Camarillo, CA) (1:100 in 2% PBSA). Cyclin D1 localization was detected with a goat anti-rabbit antibody conjugated to Alexa 488 (Molecular Probes) (1:200 in PBS, 30 min).

For colocalization with ERα, sections were first stained for PRA or PRB, and then PRA or PRB localization was detected with a goat anti-mouse secondary conjugated to Alexa 488 (Molecular Probes) (1:200 in PBS, 30 min). Next, sections were blocked with goat anti-mouse IgG Fab fragments (Jackson Immunoresearch Laboratories) (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 anti-ERα antibody (NCL-L-ER-6F11) (Novocastra, Newcastle, United Kingdom). ERα localization was detected with a biotinylated goat anti-mouse secondary (Dako, Carpinteria, CA) (1:400 in

PBS, 30 min), which was recognized by streptavidin-conjugated Alexa 546 (Molecular Probes) (1:100 in PBS, 30 min).

For all dual-immunofluorescence labeling, nuclei were counterstained with DAPI (Molecular Probes) (1:10,000 in H_2O), and sections were visualized and images captured using a Nikon inverted epifluorescence microscope (Mager Scientific, Dexter, MI) with MetaMorph software (Molecular Devices Corporation, Downington, PA).

Quantitation of fluorescence and statistical analyses:

Sections treated for detection of PRA, PRB, BrdU, or cyclin D1 by immunofluorescence methods were quantitated for the number of positive luminal epithelial cell nuclei from captured images using MetaMorph software. Positive nuclei displayed staining above luminal epithelial cytoplasmic background. To analyze fluorescence intensity, the average pixel intensity of all positively stained nuclei within the ductal epithelium was determined. Images were thresholded to exclude background fluorescence and gated to include intensity measurements only from positively staining epithelial cells. Six mice per treatment group were analyzed; a minimum of 1000 total cells and three independent sections per mouse were analyzed. Results are expressed as mean +/- SEM, and differences are considered significant at $P < 0.05$ by using Student's *t* test.

Images in this dissertation are presented in color.

RESULTS

The most dramatic changes in mammary gland morphology, proliferation and PRA and PRB expression occur in the adult mammary gland in response to pregnancy (7). Thus, we treated adult ovariectomized mice with pregnancy levels of estrogen (E), progesterone (P) or E + P for a total of 3, 5, or 10 days, and studied the hormonal regulation of PRA and PRB expression and their relationship to hormonal regulation of proliferation and alveolar morphogenesis.

Morphological responses of the mammary gland to hormone treatments

Morphological responses to ovariectomy and hormonal treatments are shown in Fig. 3.1. Ovariectomy and control treatment resulted in a reduction in the size of the ducts and duct ends, and mammary gland morphology was similar after ovariectomy and 3, 5, or 10 days of control treatment. Treatment with E produced a transient enlargement of the distal tips of ducts and dilation of ducts; this response was maximal after 5 days and decreased by 10 days. Treatment with E+P produced morphological changes that increased with treatment length. After 3 days of E+P, sidebranching and some dilation of the ducts were observed. Treatment for 5 days with E+P produced more extensive sidebranching and the start of alveologenesis, as defined by the presence of multi-luminal structures at the ends of sidebranches. A close examination of sidebranches revealed bulb shaped structures that appeared to pinch off into alveolar units. After 10 days of E+P treatment, there was more extensive alveolar development; all the sidebranches produced lobular structures with multiple alveoli. After 3 days of treatment with P alone,

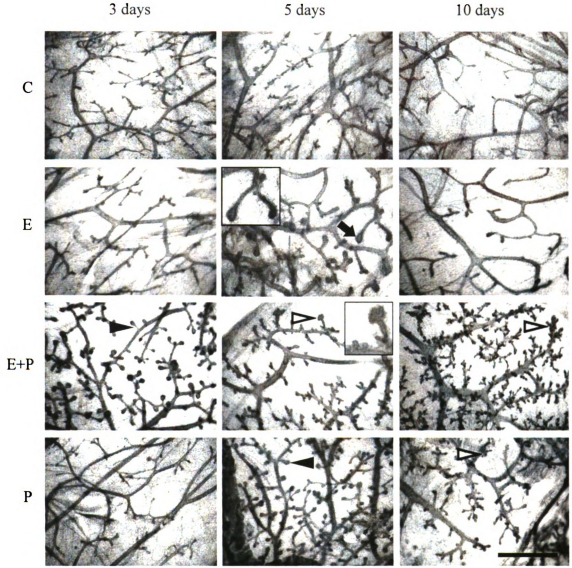


Figure 3.1. Morphological response of the mammary gland to hormone treatment. Mammary gland whole mounts were prepared from adult BALB/c ovariectomized mice treated for 3, 5, or 10 days with saline (control, C), estrogen (E), progesterone (P) or E + P. Proliferation in response to E occurred after 5 days at the distal tips of ducts (black arrow indicates the distal tip of a duct), but the distal tips regressed by 10 days. A higher magnification inset of stimulation of the distal tips at the ends of ducts is shown for 5 days E. After 3 days of E+P or 5 days of P, sidebranching was present (black arrowheads). Alveologenesis started after 5 days of E+P or 10 days of P (open arrowheads). A higher magnification inset of alveologenesis is shown for 5 days E+P. Expansion of alveoli occurred after 10 days of E+P (open arrowhead) (scale bar, 1 mm).

no change in morphology was visible. However, after 5 days of P treatment, there was extensive sidebranching throughout the mammary gland. Treatment with P for 10 days produced the start of alveologenesis. These results show that treatment with P alone caused sidebranching and the initiation of alveologenesis. Sidebranching and alveologenesis were accelerated and enhanced by the addition of E in the E+P treated mice.

Proliferative responses to hormone treatments

The morphological changes described above are associated with proliferation. To determine the relationships among hormone treatments, proliferation, and changes in morphology, mice were treated with a pulse of 5-Bromo-2'-deoxyuridine (BrdU) two hours prior to sacrifice. Since the mammary epithelium is composed of two cell types, luminal epithelial cells and myoepithelial cells, double labeling with BrdU and α-smooth muscle actin (SMA), a specific marker of myoepithelial cells, was carried out to determine the proliferative response in the two mammary cell types.

Analysis of proliferation in response to the various hormone treatments is shown in Fig. 3.2A,B. No proliferation was observed in luminal or myoepithelial cells in ovariectomized, control-treated animals. Treatment with E produced a significant but transient increase in proliferation after 5 days; proliferation occurred in luminal epithelial cells and myoepithelial cells and was specifically localized to the distal tips of ducts.

Surprisingly, 3 days of treatment with P alone also increased proliferation of luminal and myoepithelial cells relative to OVX controls. Luminal epithelial cell and

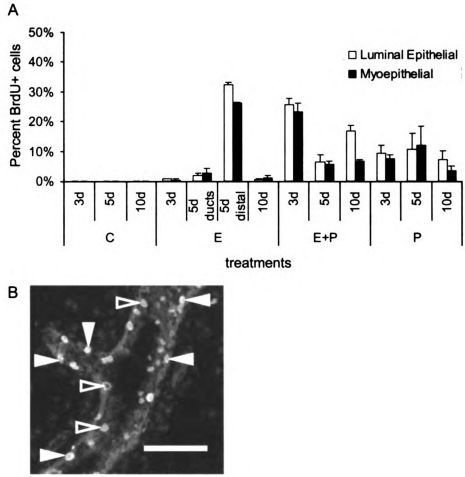


Figure 3.2. Cell type specific proliferation in response to hormone treatment. Dual immunofluorescence detection of BrdU and α-SMA was performed on mammary gland sections from adult OVX mice treated for 3, 5, or 10 days with saline (control, C), estrogen (E), progesterone (P), or E+P. A. Quantitation of the percent BrdU positive myoepithelial cells and luminal epithelial cells was determined. Proliferation after 5 days of E treatment was significantly increased only in the distal tips of ducts. Proliferation was highest after 3 or 10 days of E+P treatment. Treatment with P produced sustained proliferation after 3, 5, or 10 days of treatment. Proliferation was induced in both luminal and myoepithelial cells. The values represent the mean ± SEM from three to five mice per group with a minimum of 1000 cells/mouse analyzed. B. After 3 days of E+P treatment, proliferating cells were recognized using an anti-BrdU antibody (teal), myoepithelial cells were distinguished by anti-α-SMA staining (red), and nuclei were counterstained with DAPI (blue). Proliferation occurred in both myoepithelial (solid white arrowheads) and luminal epithelial cells (open arrowheads) (scale bar, 25 μm).

myoepithelial cell proliferation were also observed in ducts, sidebranches and alveoli after 5 and 10 days of P treatment. From 7-11% in luminal epithelial cells were BrdU positive (BrdU+) and 4-12% myoepithelial cells were BrdU+ between 3 and 10 days of treatment.

Overall, the greatest proliferation throughout the gland was observed in response to E+P treatment. Proliferation occurred in ducts, sidebranches and alveoli. After 3 days of E+P treatment, 26% of luminal epithelial cells were BrdU+ and 23% of myoepithelial cells were BrdU+. Proliferation decreased after 5 days of E+P treatment to 7% in luminal epithelial cells and 6% in myoepithelial cells. After 10 days of E+P treatment, proliferation increased and 17% of luminal epithelial cells in both ducts and alveolar structures were BrdU+; a smaller percentage of myoepithelial cells (7%) were BrdU+.

These results demonstrate that treatment with E alone produced a transient burst of proliferation in luminal and myoepithelial cells that was restricted to duct ends. In contrast, treatment with P alone produced a low level of sustained proliferation in myoepithelial and luminal epithelial cells throughout the 10 day treatment period. Thus, P by itself, in the absence of E, was capable of inducing proliferation in both cell types. Treatment with E+P resulted in a biphasic proliferative response that was high at day 3, decreased at day 5, and was high again at 10 days of treatment. Notably, E enhanced overall proliferation when combined with P.

Hormonal regulation of PRA

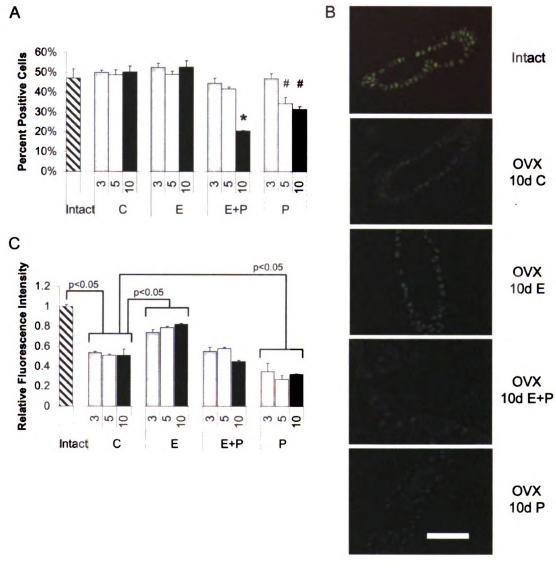
The hormonal regulation of PRA was investigated by immunofluorescence staining with antibody specific for PRA. The effects of ovariectomy and hormone

treatments on the percentage of PRA positive (PRA+) cells are shown in Fig. 3.3A. Ovariectomy did not change the percentage of PRA+ cells compared to the ovary intact control. Similarly, no effect of treatment with E on the percentage of PRA+ cells was observed. To determine if the cellular content of PRA was affected by the various treatments, analysis of immunofluorescence staining intensity was performed using software that measures pixel intensity in positive cells (Fig. 3.3B, C). This analysis revealed that the level of PRA protein was significantly decreased by ovariectomy compared to ovary-intact controls. Treatment with E increased PRA content in PRA+ cells relative to ovariectomized controls, but did not restore PRA content to the level of the ovary-intact control. Treatment with P for 5 or 10 days significantly reduced the percentage of PRA+ cells and significantly decreased PRA levels below that of ovariectomized controls. Treatment for 10 days with E+P produced the largest decrease in the percentage of PRA+ cells. Treatment with E + P did not increase PRA content above that in ovariectomized controls. These results demonstrate that E upregulates the levels of PRA, whereas P downregulates PRA levels and blunts upregulation by E.

The relationship between PRA level and proliferation during morphogenesis

PRA and PRB are detected only in luminal epithelial cells (7); therefore, analysis of the relationship between PR isoforms and proliferation was determined in luminal epithelial cells. The relationship between PRA, progesterone, and proliferation was examined in E+P or P treated mice by studying the colocalization of PRA with the proliferation marker BrdU by dual immunofluorescence (Fig. 3.4). After 3 days of E+P treatment, 41% of luminal epithelial cells were PRA+, 25% were BrdU+ and 4% were

Figure 3.3. Hormonal regulation of PRA expression in the adult mouse mammary gland. PRA was detected by immunofluorescence with an anti-PRA antibody on mammary gland sections from adult intact or ovariectomized mice treated for 3, 5 or 10 days with control (C), estrogen (E), progesterone (P) or E+P. A. Quantitation of the percent PRA positive luminal epithelial cells. The values represent the mean ± SEM from three to five mice per group with a minimum of 1000 cells/mouse analyzed. *, $p<0.05$ 10 day E+P was significantly less than all other groups. #, $p<0.05$ 5 and 10 day P were significantly less than intact, all C and E treated groups and 3d E+P group ($p<0.05$). B. Representative sections of PRA immunofluorescence staining from adult intact or ovariectomized mice treated for 10 days with C, E, P, and E+P. The percent PRA positive cells decreased in the E+P or P treated group. PRA intensity was highest in the intact mouse and following E treatment of ovariectomized mice and lowest in the P treated ovariectomized mice (scale bar, 25 μm). C. Quantitation of PRA immunofluorescence staining intensity. Values represent the mean pixel intensity in PRA positive cells ± SEM from three to five mice per group with a minimum of 1000 cells/mouse analyzed. Intensity of PRA staining was significantly decreased after ovariectomy and treatment with E significantly increased the intensity of PRA staining compared to ovariectomized controls. Treatment with E+P did not increase PRA staining and treatment with P alone further decreased the intensity of PRA staining compared to the ovariectomized control groups.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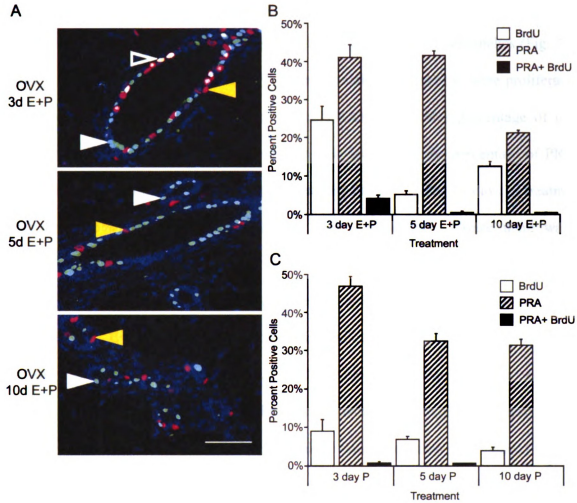

Figure 3.4. Localization of PRA in proliferating cells. A. Dual immunofluorescence detection of PRA and BrdU was performed on mammary gland sections from adult ovariectomized mice treated for 3, 5, or 10 days with E+P or P alone. In the representative images shown, PRA+ cells are teal, BrdU+ cells are pink, PRA and BrdU+ cells are white, and nuclei counterstained with DAPI are blue. After 3 days of E+P, a small population of proliferating cells express PRA (open arrowhead), whereas after 5 or 10 days of E+P most proliferating cells are PRA negative (yellow arrowhead). Examples of cells expressing PRA only are indicated with white arrowheads (scale bar, 25 μm). B. Quantitation of the percent PRA+ cells, BrdU+ cells, and colocalization of PRA and BrdU in E+P treated mammary glands. C. Quantitation of the percent PRA+ cells, BrdU+ cells, and colocalization of PRA and BrdU in P treated mammary glands. The values represent the mean ± SEM from three to five mice per group with a minimum of 1000 cells/mouse analyzed.

positive for both PRA and BrdU. Of the total proliferating cells, 16% expressed PRA (Fig. 3.4A, B). Thus, a subpopulation of PRA+ cells, in addition to PRA negative (PRA-) cells, was proliferating coincident with the formation of sidebranches (Fig. 3.1). After 5 days of E+P treatment, only 5% of the luminal epithelial cells were proliferating and now only 5% of proliferating cells were PRA+. The lower percentage of cells positive for both PRA and BrdU was not due to a decrease in the percentage of PRA+ cells since 42% of luminal epithelial cells were still PRA+. After 10 days of treatment with E+P, 13% of luminal epithelial cells were proliferating. Since there was a significant increase in the total number of epithelial cells after 10 days of E+P treatment (Fig. 3.1), the total number of proliferating cells was likely higher at 10 days compared with 5 days of E+P. The percentage of PRA+ cells was significantly reduced to 20%, and only 3% of BrdU+ cells were PRA+. The reduced percentage of PRA+ cells and reduced colocalization of PRA and BrdU coincided with increased alveologenesis.

Overall, proliferation induced by P alone was lower than that induced by E+P (Fig. 3.4C). The percentage of BrdU+ cells was 9%, 7%, and 4% after 3, 5, and 10 days of P treatment, respectively. In P-treated mice, the development of sidebranches was delayed and observed only after 5 days of treatment compared with after 3 days of E+P treatment. This is likely due to the lower amount of proliferation induced by P alone. BrdU incorporation in PRA+ cells was lower than observed with E+P treatment. After 3 or 5 days of P treatment, only 2% of proliferating cells were PRA+. After 10 days of P treatment when alveolar development was observed, no PRA+ cells were proliferating. Taken together, the results obtained in P and E+P treated mice suggest that alveolar development and expansion are not associated with the proliferation of PRA+ cells.

Nuclear localization of cyclin D1 is associated with the induction of cell cycle progression toward S-phase and cyclin D1 expression is believed to be regulated by P (16). Additionally, cyclin D1 expression is believed to be required for alveologenesis during pregnancy (17). Thus, we also examined colocalization of PRA with cyclin D1 after treatment with E+P or P by dual immunofluorescent labeling (Fig. 3.5). After 3 days of E+P treatment, nuclear cyclin D1 was expressed in 51% of luminal cells, 42% of cells were PRA+, and 42% of cyclin D1 positive (cyclin D1+) cells were also PRA+ (Fig. 3.5B). After 5 days of E+P treatment, the percentage of cells expressing nuclear cyclin D1 was reduced to 34% and only 13% of these cyclin D1+ cells were PRA+. Thus, while the percentage of PRA+ cells after 3 or 5 days E+P treatment was similar, colocalization of PRA with cyclin D1 significantly decreased after 5 days of treatment. Following 10 days of E+P treatment, nuclear cyclin D1 was expressed in 37% of luminal epithelial cells and only 3% of cyclin D1+ cells were PRA+. Thus, PRA colocalized with cyclin D1 during sidebranching after 3 days of E+P treatment, but not during maximal alveologenesis observed after 10 days of E+P treatment.

In mice treated with P alone for 3 days, the percentage of cyclin D1+ cells (37%) was lower than observed with E+P treatment and 29% of cyclin D1+ cells were PRA+ (Fig. 3.5C). The percentage of cyclin D1+ cells and colocalization with PRA did not change significantly after 5 or 10 days of treatment and PRA colocalized with cyclin D1 with a similar frequency at 3, 5 and 10 days of P treatment.

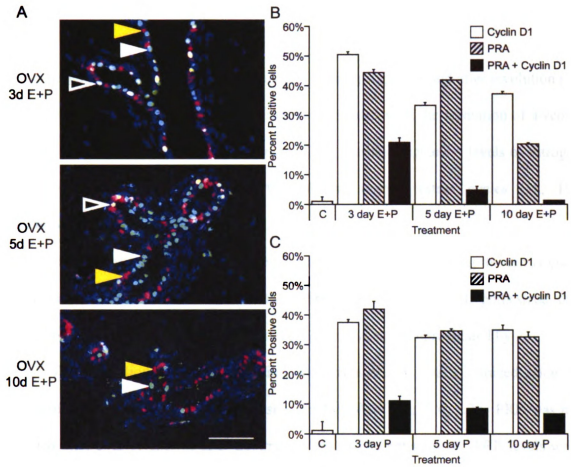

Figure 3.5. Colocalization of PRA and cyclin D1. A. Dual immunofluorescence detection of PRA and cyclin D1 was performed on mammary gland sections from adult ovariectomized mice treated for 3, 5, or 10 days with E+P or P alone. In the representative merged images PRA+ cells are teal, cyclin D1+ cells are pink, PRA and cyclin D1+ cells are white, and nuclei counterstained with DAPI are blue. Specific examples of PRA+ cells (white arrowhead), cyclin D1+ nuclei (yellow arrowhead), and colocalization of PRA and cyclin D1 (open arrowhead) are shown. Colocalization of PRA with cyclin D1 was greatest after 3 days E+P and decreased after 5 and 10 days of E+P treatment (scale bar, 25 μm). B. Quantitation of percent PRA+ cells, cyclin D1+ cells, and colocalization of PRA and cyclin D1 in E+P treated mammary glands. The percent cyclin D1+ cells increases in E+P treated mammary glands relative to saline control treatment. C. Quantitation of percent PRA+ cells, cyclin D1+ cells, and colocalization of PRA and cyclin D1 in P treated mammary glands. The percent cyclin D1+ positive cells increases in E+P treated mammary glands relative to saline control treatment. The values represent the mean ± SEM from three to five mice per group with a minimum of 1000 cells/mouse analyzed.

Hormonal regulation of PRB

High levels of PRB are detected mainly during pregnancy and after involution (7). PRB expression during pregnancy is primarily associated with the formation of alveolar structures, and PRB levels are lower in ducts (7). During pregnancy, levels of estrogen and progesterone are much higher than during normal estrus cycles (18, 19). Additionally, these high hormone levels are maintained during pregnancy, so there is greater continuous exposure to hormones during pregnancy than during the estrus cycle in the adult virgin (18). Based on these previous findings, we hypothesized that regulation of PRB would differ from that of PRA and would be upregulated by P.

The regulation of PRB expression was examined in ovariectomized mice by immunofluorescence using an antibody specific for PRB (Figs. 3.6, 3.7). PRB was not detected after ovariectomy or after treatment with E (data not shown). PRB levels were increased by 5 days of E+P or by 10 days of P treatment, but at a very low level, so accurate determination of the percent positive cells was not feasible (Fig. 3.6). PRB levels rose to a clearly detectable level in 25% of epithelial cells only after 10 days of E+P treatment (Fig. 3.7). Thus, PRB was only detected after prolonged treatment with P or E+P. Additionally, the increase in PRB levels coincided with the appearance of alveolar structures (Fig. 3.1).

The relationship between PRB level and proliferation during morphogenesis

Colocalization of PRB with proliferation was determined by dual immunofluorescence with antibodies specific for PRB and BrdU. This analysis was

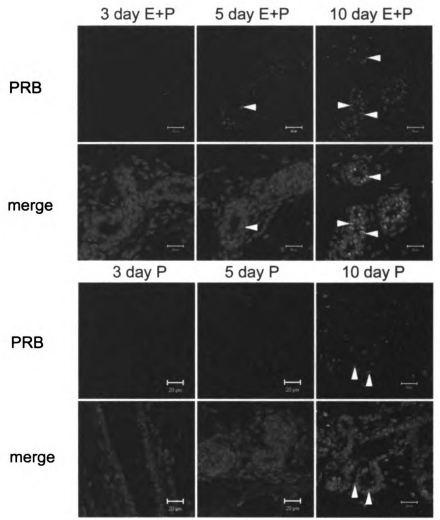

Figure 3.6. Hormonal regulation of PRB expression in the adult mouse mammary gland. Immunofluorescence detection of PRB was performed on mammary gland sections from adult OVX mice treated for 3, 5, or 10 days with C, E, P or E+P. PRB was only detected after 5 days E+P or 10 days of P treatment. PRB (green nuclei, white arrowheads) was faintly detected after 5 days of E+P or 10 days of P treatment, but was most strongly expressed after 10 days of E+P treatment. Nuclei were counterstained with DAPI (blue)(scale bar, 20 μm).

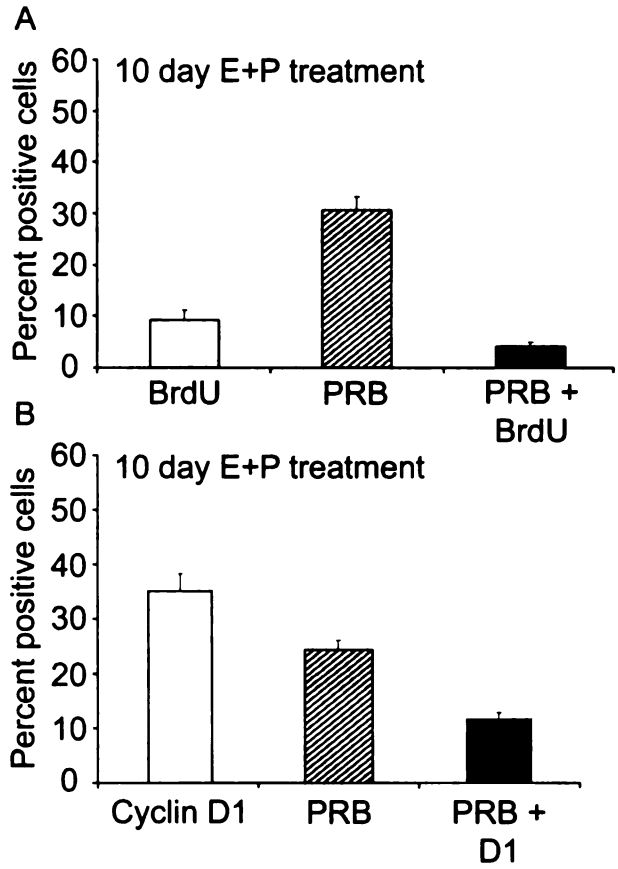

Figure 3.7. Colocalization of PRB with BrdU or cyclin D1. Dual immunofluorescence detection of PRB colocalization with BrdU or with cyclin D1 was performed on mammary gland sections from adult OVX mice treated for 10 days with estrogen + progesterone (E+P). A. Quantitation of percent PRB+ cells, BrdU+ cells and colocalization of PRB and BrdU. B. Quantitation of percent PRB+ cells, cyclin D1+ cells and colocalization of PRB and cyclin D1. The values represent the mean ± SEM from three to five mice per group with a minimum of 1000 cells/mouse analyzed.

carried out only in the 10 day E+P treatment group because this was the only treatment that resulted in clearly detectable and quantifiable levels of PRB (Fig. 3.7A). After 10 days of E+P treatment, 10% of luminal epithelial cells were BrdU+, 30% were PRB+ and 49% of the BrdU+ cells were PRB+. Thus, about half of proliferating cells were PRB+ and since increased PRB levels coincided with the development of alveoli, this suggests that alveoli are formed by proliferating PRB+ cells. This is in contrast to the finding that only 3% of proliferating cells were PRA+ during the time of maximal alveolar development at 10 days of E+P treatment (Fig. 3.4B)

Dual immunofluorescence detection of PRB and cyclin D1 was also performed in the 10 day E+P-treated mammary gland (Fig. 3.7B). Nuclear cyclin D1 expression was detected in 35% of luminal epithelial cells and 30% of cyclin D1+ cells were PRB+. This is in contrast to the 7% of cyclin D1+ cells that were PRA+ (Fig. 3.5B). Thus, after 10 days of E+P treatment PRB was the predominant isoform expressed and was more frequently colocalized with BrdU and cyclin D1 than PRA. Taken together, the frequent colocalization of PRB with BrdU and with cyclin D1 at the time of extensive alveolar development further suggests that PRB+ cells proliferate to form alveoli.

Colocalization of PRA and PRB with ER

As shown above, the levels of both PRA and PRB were regulated by estrogen, which acts through binding to the estrogen receptor (ER). Estrogen increased PRA levels and enhanced P-induced increase in PRB levels. ERα, and not ERβ, is required for ductal development in the mammary gland (20, 21). ERβ appears to play a role during lactation,

when neither PRA nor PRB are expressed (21). Thus, to further address the role of E in the regulation of PR isoforms, we used dual immunofluorescence to analyze ERα expression with PRA or PRB expression.

ERα was expressed in all the treatment groups through day 10 (Fig. 3.8A). However, intensity of staining with anti-ERα antibody was lower in E and E+P treated mammary glands than in control or P- treated glands, indicating that E downregulates ERα levels (Fig. 3.8A). Notably, PRA and ERα were coexpressed in the same cell under all treatment conditions (Fig. 3.8B). Increased PRB levels coincided with decreased ERα levels and the majority of PRB+ cells were ERα negative (Fig. 3.8B). The co-expression of ERα and PRA suggests that E acting through ERα may directly regulate PRA expression. Conversely, the lack of significant co-expression of PRB with ERα suggests that E acts indirectly to enhance P-induced upregulation of PRB.

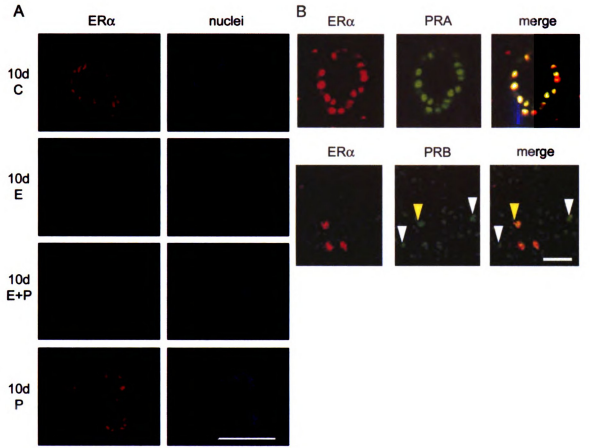

Figure 3.8. Colocalization of PRA and PRB with ERα. Dual immunofluorescence detection of ERα and ERα colocalization with PRA or with PRB was performed on mammary gland sections from adult OVX mice treated for 3, 5, or 10 days with saline control (C), E, P, or E+P. A. Representative immunofluorescence images of ERα staining. ERα (red) expression was detected in all groups, but was decreased by treatment with E or E+P. Nuclei were counterstained with DAPI (blue)(scale bar, 25 μm). B. Representative images of PRA (green) or PRB (green) colocalization with ERα (red) are shown. In merged images, colocalized cells are yellow. There was a high degree of PRA and ERα expression colocalization. The majority of PRB positive cells (white arrowheads) did not colocalize with ERα. Instances of PRB and ERα colocalization are shown with yellow arrowheads (scale bar, 20 μm).

DISCUSSION

We have previously reported that PRA is predominantly expressed in the virgin gland, whereas PRB is predominantly expressed during pregnancy. In this report we have examined the hormonal basis for the differential expression of the two PR isoforms. Since the greatest proliferative and morphological responses to P occur during pregnancy we have focused our study on the mature, adult mammary gland and the effect of pregnancy levels of E and P on PR isoform levels. Additionally, we have analyzed the relationship between regulation of PR isoform expression, proliferation and alveolar development.

PRA is upregulated by estrogen and downregulated by progesterone

We found that while ovariectomy did not affect the percentage of PRA+ cells, it dramatically reduced the level of PRA protein. PRA levels could be restored by treatment with E. In contrast, treatment with P alone or E+P caused a reduction in the percentage of PRA+ cells. Additionally, P treatment decreased PRA levels below that observed after ovariectomy. Furthermore, when P was combined with E, it blunted the upregulation of PRA by E. These results are in agreement with previous studies that have shown estrogenic regulation of PR in the adult virgin mouse mammary gland (12) and our results demonstrate that PRA is the predominant isoform that is regulated by estrogen. Based on in vitro studies, progestins are reported to downregulate PR (11). Our studies

confirm downregulation of PR levels by P and show that this effect is specific for the PRA isoform in the adult virgin mouse mammary gland.

PRA mediates sidebranching

Alveolar development proceeds through a specific sequence of proliferative and morphological events. During pregnancy, the earliest event in alveolar development is the production of ductal sidebranches. Our studies indicate that ductal sidebranching can be effectively induced by P in ovariectomized mice and does not require estrogen. We have previously shown that PRA is the predominant isoform expressed in the nulliparous mouse mammary gland (7), and a recent study using the same anti-PRA antibody confirmed this result (22). Since ductal sidebranching is induced at a time when PRA is the predominant isoform expressed we conclude that this process is mediated by progesterone acting through PRA.

Interestingly, sidebranching can be induced by E+P treatment in the PRA gene-deleted mouse (PRAKO) (23). The PRAKO mouse studies were carried out in the C57Bl/6 genetic background. C57Bl/6 adult wild-type mice have less developed mammary glands when compared to other strains, such as BALB/c (24). Additionally, the C57Bl/6 strain is less responsive to hormones than the BALB/c strain in which our studies were carried out (25). In particular, we have found C57Bl/6 mice to be much less responsive to progesterone than BALB/c mice and exhibit delayed sidebranching during pregnancy (Aupperlee & Haslam, unpublished observations). Therefore, it is possible that additional mechanisms that promote ductal sidebranching may be operative in the

C57Bl/6 strain and might explain the lack of a phenotype in the C57BL/6 PRAKO mouse.

Ductal sidebranching is accelerated in mice treated with E+P and since E increases PRA levels, it is likely that E contributes to ductal sidebranching through its positive effect on the level of PRA. Three days of E+P treatment produced the greatest colocalization of PRA with BrdU and PRA with nuclear cyclin D1, indicating that a subset of PRA+ cells were proliferating in response to E+P treatment at this time. However, in both P and E+P treated mice, the majority of cells that proliferate and form sidebranches were PRA-. The delayed development of sidebranches in P-treated mice most likely reflects the overall lower proliferation observed after P treatment compared with E+P treatment. Interestingly, although a significant percentage of PRA+ cells colocalized with nuclear cyclin D1 after P treatment, only a small percentage of PRA+ cells were BrdU+. This suggests that overall fewer PRA+ cells proliferated after P treatment. Alternatively, it is possible that treatment with P alone leads to slower progress through the G1 phase of the cell cycle, which is reflected by the difference in colocalization with PRA and nuclear cyclin D1, a G1 phase marker, or BrdU, an S phase marker.

PRB upregulation by progesterone

We found that PRB was expressed at a detectable level only after sustained exposure to P. E alone did not result in upregulation of PRB; however, E accelerated the upregulation by P. The earliest detection of significant PRB levels coincided with the

development of alveoli at 5 days of E+P or 10 days of P treatment. Why prolonged treatment with P was required to obtain increased PRB levels is not entirely clear. The coincident timing of the decreased levels of PRA, the initiation of alveologenesis, and PRB upregulation suggest that these events are linked.

One possible explanation for the requirement of prolonged P treatment to upregulate PRB is that PRA inhibits PRB expression. In this case, one would expect that the upregulation of PRB by P would occur when PRA levels are at their lowest. However, this explanation is not compatible with the observation that PRA levels are decreased faster and to a lower level after treatment with P alone (Fig. 3.1A), yet PRB expression is upregulated later and less robustly in P treated glands compared with E+P treated glands (Fig 3.6).

There are two significant differences between P and E+P treated glands that may affect the upregulation of PRB: 1) the overall lower amount of proliferation and 2) the longer time required for ductal sidebranching and the development of alveoli to occur in the P treated glands compared with E+P treated glands. It has been hypothesized by others that the adult virgin mammary gland contains progenitor cells that give rise to three different cell lineages: ductal luminal cells, alveolar luminal cells, and myoepithelial cells (26). It is possible that the cells that proliferate to form the sidebranches in response to P are derived from progenitor cells committed to the alveolar luminal cell lineage and that it is a property of these cells to express PRB and form alveoli. Once PRB expression is induced then P acting through PRB may form a positive regulatory loop to further increase PRB expression and the expansion of alveolar cells.

The role of estrogen and ERα

Estrogen upregulation of PRA was correlated with the co-expression of ERα and PRA within the same cells. Based on this observation it is likely that E acting though ERα regulates PRA through a direct mechanism in PRA+ cells. We also observed that E downregulated ERα, which may indicate that decreases in PRA following prolonged E+P treatment are partially due to a loss of ERα. Surprisingly, we found that ERα was not expressed in the majority of PRB+ cells. Although E enhanced the upregulation of PRB, it does not appear to be due to a direct, ER-mediated effect in PRB+ cells. We propose that E may facilitate the upregulation of PRB through the maintenance of PRA and lead to the enhancement of sidebranching and the expansion of the putative alveolar cell lineage in which PRB is then induced. Additionally, we considered the possibility that E could enhance PRB upregulation indirectly through a systemic effect by increasing plasma prolactin (Prl) levels. However, treatment of ovariectomized adult mice for 5 days with Prl alone had no stimulatory effect on mammary gland morphology. Also, the morphology of the mammary gland after treatment for 5 days with P+Prl was not different from that observed after treatment with P alone (unpublished observations, Aupperlee & Haslam).

Progesterone induces sidebranching and alveologenesis through direct and paracrine mechanisms

It has been previously reported that in the virgin mouse mammary gland proliferating cells are PR negative (5, 27). This has been interpreted to mean that P induces proliferation through a paracrine mechanism(s) (5, 27). In the present study, at least 84% of the cells proliferating at the time of ductal sidebranching were PRA- (Fig. 3.4B). These results suggest that P acting on PRA+ cells may induce a paracrine factor that stimulates the proliferation of PRA- cells. Studies by others have implicated Wnt4 as a paracrine mediator of P-induced sidebranching (28). Thus, our study provides further evidence for a paracrine mechanism of P action and indicates that this mechanism is operative in PRA+ cells during sidebranching. We also have made the novel observation that in addition to increasing proliferation in epithelial cells, P also increased proliferation in myoepithelial cells. Proliferation of myoepithelial cells was observed during both ductal sidebranching and alveologenesis. Since myoepithelial cells lack both PRA and PRB, this indicates that their proliferation is mediated through an indirect effect of P. The mechanisms operative in P-induced proliferation of PR- luminal epithelial cells and myoepithelial cells are currently under investigation.

Cyclin D1 expression has been shown to be regulated by progesterone (16) and is believed to be essential for alveolar development leading to lactation (17). We have shown that cyclin D1 levels were increased by treatment with P or E+P. The highest percentage of cyclin D1+ cells was observed after 3 days of E+P treatment and coincided with the development of sidebranches. Colocalization of cyclin D1 with PRA and

colocalization of PRA with BrdU were also highest at this time point. However, 60% of cyclin D1+ cells were PR-. These results also indicate that cyclin D1 may be regulated by P in PRA- cells through a paracrine mechanism(s) and that the upregulation of cyclin D1 by P in both PRA+ and PR- cells promotes the proliferation that produces sidebranching.

During alveologenesis and alveolar expansion at 5 or 10 days of E+P treatment, colocalization of PRA with cyclin D1 or BrdU was significantly decreased. The decrease in colocalization of PRA with cyclin D1 after 5 or 10 days of E+P treatment is similar to the decreased colocalization during extensive alveolar expansion at day 14 of pregnancy (7). In contrast, cyclin D1 and PRB were highly colocalized after 10 days of E+P during alveolar expansion and similar to the level of colocalization at day 14 of pregnancy (7). Of the total cyclin D1+ cells, 40 % were PRB+, less than 5% were PRA+ and about 55% of cyclin D1+ cells were PR-. This suggests that the upregulation of cyclin D1 by P in both PRB+ and PR- cells likely promotes the proliferation required for alveolar expansion. Thus, P acting on either PRA+ or PRB+ cells appears to produce indirect effects on PR- cells that promote sidebranching and alveologenesis, respectively.

Estrogen enhances progesterone-induced sidebranching and alveologenesis

We propose the following 2 models that integrate the effects of E and P to explain the observed differences in PRA and PRB expression, proliferation, ductal sidebranching and alveologenesis in P vs. E+P treated mice. The first model describes the sequence of events resulting from E+P treatment. In this case, we propose that the high proliferation index observed after 3 days of E+P treatment is due to the combination of 1) robust

stimulation of proliferation of PR- cells by paracrine factors induced by P in PRA+ cells, and 2) proliferation of a subpopulation of PRA+ cells, both facilitated by maintenance of PRA levels by E. Longer treatment with E+P, after 5 and 10 days, results in the downregulation of ERα by E that together with P leads to downregulation of PRA. Proliferation of putative alveolar progenitor cells in sidebranches and sustained P exposure leads to the increase in PRB levels. An earlier increase in PRB, by 5 days, results in earlier and more extensive alveolar development.

The second model describes the sequence of events resulting from treatment with P alone. The lower amount of proliferation after 3 days of P treatment is due to 1) a less robust paracrine induction of proliferation in PRA- cells, and 2) reduced proliferation of PRA+ cells, both due to the lower level of PRA in the absence of the E. Thus, in the absence of E, 5 days of P treatment are required to produce an amount of ductal sidebranching comparable to that observed by 3 days of E+P. Subsequent to the reduced proliferation of the putative alveolar progenitor cells during ductal sidebranching, upregulation of PRB and alveologenesis are delayed.

In summary, we have shown that PRA and PRB are differentially regulated. PRA is upregulated by E and downregulated by P whereas PRB is upregulated by P. ERα colocalizes with PRA and this suggests that E directly upregulates PRA through an ER mediated mechanism. PRB does not colocalize significantly with ER and if E has a role in PRB regulation it likely occurs through an indirect mechanism. The proliferative and morphological changes in the mammary gland that occur during pregnancy were mimicked in our experiments by continuous treatment with either P or E+P. We have shown that P acting on PRA+ cells caused ductal sidebranching by promoting

proliferation of both PRA+ and PRA- cells. This was followed temporally by the induction of PRB and P action in PRB+ and PRB- cells to cause the formation and expansion of alveoli.

ACKNOWLEDGEMENTS

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CHAPTER FOUR

THE MAMMARY GLAND RESPONSE TO ESTROGEN AND/OR PROGESTERONE: DIFFERENTIAL REGULATION OF PROLIFERATION IS GENETICALLY DETERMINED

Note: The contents of this chapter have been submitted for publication in Endocrinology. Aupperlee M.D., Drolet A.A., Durairaj S., Schwartz R.C., and Haslam S.Z. The mammary gland response to estrogen and/or progesterone: differential regulation of proliferation is genetically determined.

ABSTRACT

Progesterone (P) promotion of proliferation in the normal mammary gland is implicated in the etiology of mammary cancer in mice and humans. BALB/c and C57BL/6 mice exhibit strain-specific differences in mammary gland development and response to hormones that may be determining factors of susceptibility to mammary carcinogenesis. To elucidate the basis for these inherent differences, we analyzed mammary gland development and in vivo proliferative responses to estrogen (E) and/or P. C57BL/6 mice exhibited a hypoplastic ductal phenotype in the virgin gland and delayed alveolar development during pregnancy. In comparison to BALB/c mammary glands, we found that C57BL/6 glands exhibited reduced sensitivity to P as evidenced by reduced P-induced expression of progesterone receptor isoform B and Receptor Activator of NF-κB Ligand protein expression, reduced nuclear localization of Id2, and significant differences in nuclear cyclin D1 expression. In contrast, E responsiveness was greater in C57BL/6 than in BALB/c glands. These observations suggest that in human populations with heterogeneous genetic backgrounds, individuals may respond differentially to the same hormone, and thus, genetic diversity may have a role in determining the effects of P in mammary tumorigenesis.

INTRODUCTION

Progesterone (P) plays an important role in the proliferation and differentiation of the normal mammary gland in rodents and humans (1-3). P is also implicated in the etiology of breast cancer and breast cancer progression (4-7). Breast cancer risk is increased in women receiving combined estrogen plus progestin menopausal hormone replacement therapy (HRT) (3, 8-12). Recent decreases in breast cancer incidence have been attributed to reduced use of HRT (13). P acts through binding to its cognate steroid receptor, the progesterone receptor (PR), which exists as two isoforms, PRA and PRB, that are functionally distinct transcriptional regulators (14-16). Alteration in the ratio of PRA to PRB has been associated with breast cancer progression (4-6). The dysregulation of PR expression associated with BRCA-1 and BRCA-2 mutations (17, 18) further highlights the potential importance of progesterone signaling pathways in the etiology of breast cancer.

The mouse mammary gland is a frequently used model system for elucidating the role of P in normal development and function, as well as in the etiology of mammary cancer. Many studies of progesterone action were carried out using BALB/c mice (1, 19-27). Additional insights into PR isoform functions were obtained from studies of total PR-, PRA-, or PRB-deficient mice in a mixed C57BL/6 × 129SV genetic background (28-30). These studies show that PRB is essential for alveologenesis, whereas the specific function(s) of PRA in mammary gland development are not well defined.

Mouse strain-specific differences in mammary gland development (31), response to hormones, and susceptibility to carcinogenesis have been reported (32) C57BL/6 mice

exhibit reduced P-induced sidebranching and alveologenesis, and susceptibility to carcinogen- and medroxyprogesterone acetate-induced tumorigenesis compared to BALB/c mice (33, 34). These findings suggest that an analysis of P action in different mouse strains may provide important information about mechanism(s) of P action in the normal mammary gland and in mammary cancer development

We have analyzed mammary gland development and in vivo responses to exogenous hormones in wild-type BALB/c and C57BL/6 mice because these mouse strains have been widely used to study P action in normal mammary gland development and in mammary tumorigenesis (1, 19-30, 35). We found a hypoplastic ductal phenotype in the virgin gland and delayed alveolar development during pregnancy in C57BL/6 mice. We found reduced P-induced PRB and RANKL protein expression, reduced nuclear localization of Id2, and significant differences in nuclear cyclin D1 expression between the two strains. These findings indicate alternative mechanisms of P-regulated proliferation that are reflected in strain-specific differences in mammary development. The identification of strain-specific determinants of progesterone-regulated proliferation has implications for the role of genetic diversity in determining the effect of P in mammary tumorigenesis.

MATERIALS AND METHODS

Animals:

Mammary glands were obtained from BALB/c (Harlan, Indianapolis, IN) and C57BL/6 (Jackson Laboratory (Bar Harbor, ME) pubertal (6-wk-old), adult (19 to 22-wk-old), and 7-, 10-, and 14-day pregnant mice. Adult virgin mice were ovariectomized (OVX) and one week later were injected subcutaneously for 3, 5, or 10 days with saline control (C), 17-β-estradiol (E) (1 μg), progesterone (P) (1 mg), or E+P (1 μg E + 1 mg P). Two h prior to gland removal, mice were injected with 5-bromo-2'-deoxyuridine (BrdU) (70 μg/g of body weight). Mammary tissues were fixed and processed as whole mounts (36) or paraffin-embedded for immunohistochemistry as previously described (19).

Mixed C57BL/6 × 129SV genetic background cyclin D1 $^{-/-}$ (D1 $^{-/-}$) mice were a gift from Dr. Piotr Sicinski (Dana Farber Cancer Institute, Boston, Massachusetts). To generate D1 $^{-/-}$ mice in a BALB/c genetic background, these mice were backcrossed to BALB/c mice for 6 generations. All animal experimentation was conducted according to standards approved by the All University Committee on Animal Use and Care at Michigan State University.

Immunofluorescence:

The protocol used to detect PRA, PRB, ERα and Stat5a, using anti-PRA (1:50; hPRa7), anti-PRB (1:50; hPRa6) (Neomarkers, Fremont, CA)), anti-Stat5a (1:300; BD

Biosciences, San Jose, CA), or anti-ERα (1:10; Novocastra, Newcastle, United Kingdom) antibodies,was described previously (19). Primary antibodies were detected by goat anti-mouse antibody conjugated to Alexa 488 (Molecular Probes, Eugene, OR). To detect RANKL, sections were first stained for PRA and than incubated with goat anti-RANKL (R&D Biosystems, Minneapolis, MN) (1:500 in PBS/0.5% Triton X-100, overnight, 4° C). RANKL antibody was detected by rabbit anti-goat antibody conjugated to Alexa 488. Id2 was detected with rabbit anti-Id2 (1:50; Santa Cruz Biotechnology, Santa Cruz, CA), followed by goat anti-rabbit antibody conjugated to Alexa 488. For PRA, RANKL, Id2, or STAT5a immunostaining, nuclei were counterstained with 4',6-diamidino-2-phenylindole, dilactate (DAPI) (1:10,000; Molecular Probes). For PRB immunostaining, nuclei were counterstained with TOPRO-3 Iodide (1:1000; Molecular Probes), and sections were visualized and images captured using a Zeiss Pascal laser scanning confocal microscope (Zeiss, Thornwood, NY).

Dual Immunoflourescence:

Double labeling of PRA with BrdU or cyclin D1 was described previously (19). For colocalization with BrdU, sections were first stained for PRA, followed by goat anti-mouse antibody conjugated to Alexa 488. After blocking with goat anti-mouse IgG Fab fragments (1:100; Jackson Immunoresearch Laboratories) sections were incubated with anti-BrdU antibody (kit from Amersham Biosciences, Piscataway, NJ) detected with a biotinylated goat anti-mouse secondary antibody (1:400; Dako, Carpinteria, CA) followed by streptavidin-conjugated Alexa 546 (1:100). For colocalization with cyclin

D1, rabbit polyclonal anti-cyclin D1 (1:100; Biosource, Camarillo, CA) was used. Cyclin D1 was detected with a goat anti-rabbit antibody conjugated to Alexa 488. Nuclei were counterstained with DAPI. Sections were visualized and images captured using a Nikon inverted epifluorescence microscope (Mager Scientific, Dexter, MI) with MetaMorph software (Molecular Devices Corporation, Downington, PA).

Western blot detection of RANKL and Id2:

Whole mammary glands obtained from OVX and hormone treated BALB/c and C57BL/6 mice were homogenized in HB [HB : 50mM Tris-HCl (pH 7.2), 6 mM $MgCl_2$, 1 mM EDTA, 10% sucrose (w/v), protease inhibitors (2.5 μg /ml leupeptin, 0.5 mM phenylmethylsulfonyl fluoride in DMSO, 5 μg/ml aprotinin, and 5 μg/ml antipain] (0.5ml HB /100mg tissue), and centrifuged at 2000 x *g* for 5 min at 4° C; the supernatant was used as the cytoplasmic extract. For nuclear extracts, the pellet was washed twice in 0.5 ml HB, centrifuged at 2000 x *g* for 5 min at 4^0C, and resuspended in HPB (HPB:20 mM HEPES [pH 7.9], 25% glycerol (v/v), 420 mM NaCl, 1.5 mM MgCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, protease inhibitors) by vigorous vortexing for 15 min at 4^0 C. After centrifugation at 14,000 x *g* for 15 min at 4^0C, supernatants were frozen at -80^0 C. Western transfers were then performed as previously described (37). Detections were performed with anti-RANKL (BioLegend Co., San Diego, CA; Cat.# 510007)(1:1000 dilution) or anti-Id2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA; SC-489)(1:1000 dilution), using horseradish peroxidase-conjugated anti-goat IgG (Promega, Madison,

WI) (1:10,000 dilution) or anti-rabbit IgG (Promega) (1:10,000 dilution), and Western Lightning Chemiluminescence Reagent *Plus* (Perkin Elmer, Waltham, MA).

Quantitation and statistical analyses:

BrdU, PRA, ERα or cyclin D1 were quantitated for the number of positive luminal epithelial cell nuclei from captured images using MetaMorph software as previously described (1). To analyze fluorescence intensity the average pixel intensity of all positively stained nuclei within the ductal epithelium was determined. Image thresholds were set to exclude background fluorescence and gated to include intensity measurements only from positively staining epithelial cells. Background staining in epithelial cells was determined through setting thresholds to exclude positive staining, and the level of positive staining above background was calculated by subtracting background fluorescence intensity. A minimum of 3 mice per treatment group and a minimum of 1000 cells in three independent sections per mouse were analyzed. Results are expressed as mean ± SEM, and differences are considered significant at $P < 0.05$ by using Student's *t* test or ANOVA as appropriate.

Images in this dissertation are presented in color.

RESULTS

C57BL/6 mouse mammary glands exhibit a delay in sidebranching compared to BALB/c mice

Ductal development, the degree of ductal branching, and end bud number and size were similar between the two strains in 6-week-old pubertal glands (Fig. 4.1). The adult BALB/c mammary gland contained a well-arborized ductal tree with sidebranching, whereas the C57BL/6 mammary gland was comprised of a simple ductal network with little sidebranching. During pregnancy, sidebranching and alveologenesis were delayed in the C57BL/6 mouse and less extensive at 7 and 10 days of pregnancy compared to the BALB/c mouse. However, by 14 days, the degree of sidebranching and alveologenesis in the two strains was indistinguishable.

Differences in hormone-induced proliferation and morphology between strains

We considered that delayed sidebranching and alveologenesis in the C57BL/6 mammary gland might be due to differential responsiveness to P. Since the rate of mammary gland development during early pregnancy was a major difference between the two strains, we examined the effect of E and/or P doses commonly used to induce pregnancy-like alveologenesis in OVX adult mice (1).

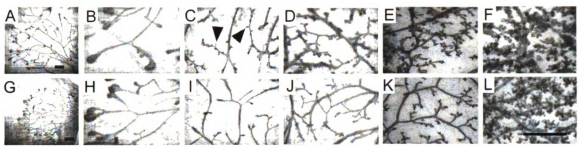


Figure 4.1. Mammary gland development in BALB/c and C57BL/6 mice. Mammary gland whole mounts were prepared from 6-wk-old immature (A, B, G, H), 20-wk-old adult (C, I), 7 d (D, J), 10 d (E, K), and 14 d (F, L) pregnant BALB/c (A-F) and C57BL/6 (G-L) mice as described in Materials and Methods. Lower (A, B) and higher magnification (G, H) images are shown for 6-wk-old immature mammary glands; all other images are higher magnification images to show sidebranching and alveologenesis. *Black arrowheads* indicate examples of sidebranching in the adult BALB/c mouse (C). Scale bar =1 mm.

Response to estrogen

In both strains, OVX resulted in reduced size of ducts and duct ends compared to intact animals (Fig 4.2A). Treatment with E caused enlargement of the distal tips of ducts and ductal dilation in both strains that was maximal after 5 days and decreased by 10 days. Notably, C57BL/6 mice maintained more enlarged distal tip structures after 10 days of treatment. (Fig. 4.2B)

To determine the relationship between E-induced morphological changes and proliferation, BrdU incorporation was examined by immunohistochemistry (Fig 4.2C). E treatment produced a significant increase in proliferation in both strains that was maximal after 5 days and was specifically localized to the distal tips of ducts. While the percentage of BrdU positive (BrdU+) cells in enlarged distal tips was similar in C57BL/6 and BALB/c mice after 10 days, there were significantly fewer enlarged distal tip structures in the BALB/c mammary gland (Fig 4.2A, B).

Response to progesterone

P treatment produced sidebranching and alveologenesis in the OVX BALB/c mammary gland that was maximal after 10 days treatment (Fig 4.2D). P treatment of C57BL/6 mice produced neither sidebranching nor alveologenesis. In BALB/c mice, P treatment increased the percentage of BrdU+ cells in ducts after 3 days and in ducts, sidebranches, and alveoli after 5 and 10 days (Fig 4.2E). In C57BL/6 mice, P treatment produced minimal proliferation, which was restricted to ducts.

Figure 4.2. Effect of E or P on morphology and proliferation in the BALB/c vs. C57BL/6 mammary gland. **A.** Representative mammary gland whole mounts from intact and OVX 3d C, and 5d and 10d E-treated BALB/c and C57BL/6 OVX mice. *White arrowheads* indicate E stimulation of the distal tips of ducts. Scale bar = 1 mm. **B.** Quantitation of stimulated distal tips of ducts. The number of stimulated distal tips was counted per square cm for BALB/c and C57BL/6 adult OVX mice treated for 5 or 10 days with E. A stimulated distal tip was defined as having an area ≥ 0.003 mm^2, which represents the area of an unstimulated duct end. The number of stimulated distal tips in the BALB/c was significantly less than in the C57BL/6 after 10 d E ($P < 0.05$). **C.** Quantitation of proliferating luminal epithelial cells after E treatment. The percentage of BrdU+ cells in ducts and distal tips (DT) was determined by immunofluorescent detection of BrdU in mammary gland sections from adult BALB/c and C57BL/6 OVX mice treated with 10 d C and 3, 5, or 10 d E. **D.** Representative mammary gland whole mounts from adult BALB/c and C57BL/6 OVX mice treated with P for 5 or 10 days. *White arrowheads* indicate sidebranching in the BALB/c gland. Scale bar = 1 mm. **E.** Quantitation of the proliferating luminal epithelial cells after P treatment. Immunofluorescent detection of BrdU was performed on mammary gland sections from adult BALB/c and C57BL/6 OVX mice treated with 10 d C and 3, 5, or 10 d P. The values for B, C and E represent the mean ± SEM from three mice per treatment group with a minimum of 1000 cells per mouse analyzed.

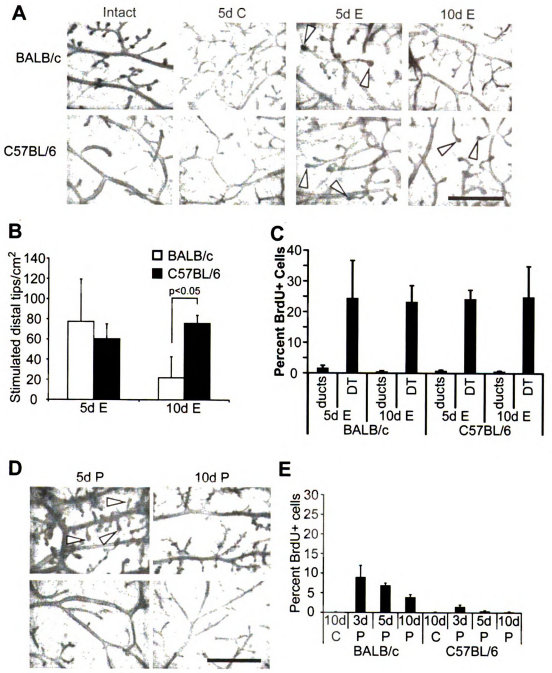
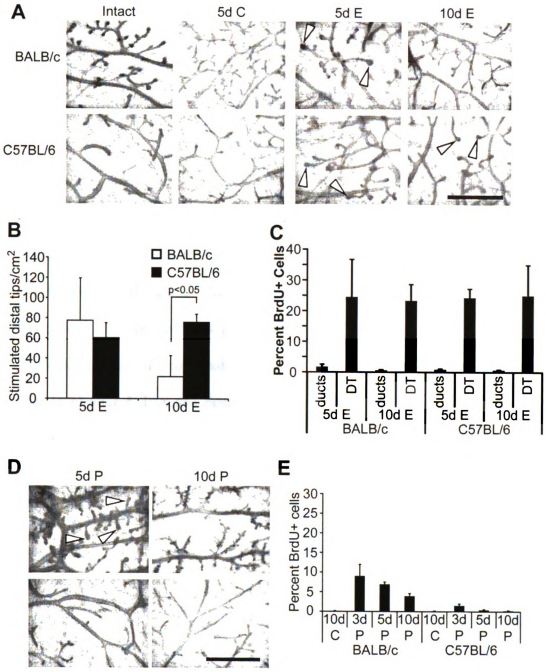

A
Intact
5d C
5d E
10d E
BALB/c
C57BL/6
B
Stimulated distal tips/cm2
BALB/c
C57BL/6
p<0.05
5d E
10d E
C
Percent BrdU+ Cells
ducts
DT
5d E
10d E
BALB/c
C57BL/6
D
5d P
10d P
E
Percent BrdU+ cells
10d C
3d P
5d P
10d P
BALB/c
C57BL/6

Figure 4.2. Effect of E or P on morphology and proliferation in the BALB/c vs. C57BL/6 mammary gland. **A.** Representative mammary gland whole mounts from intact and OVX 3d C, and 5d and 10d E-treated BALB/c and C57BL/6 OVX mice. *White arrowheads* indicate E stimulation of the distal tips of ducts. Scale bar = 1 mm. **B.** Quantitation of stimulated distal tips of ducts. The number of stimulated distal tips was counted per square cm for BALB/c and C57BL/6 adult OVX mice treated for 5 or 10 days with E. A stimulated distal tip was defined as having an area ≥ 0.003 mm^2, which represents the area of an unstimulated duct end. The number of stimulated distal tips in the BALB/c was significantly less than in the C57BL/6 after 10 d E ($P < 0.05$). **C.** Quantitation of proliferating luminal epithelial cells after E treatment. The percentage of BrdU+ cells in ducts and distal tips (DT) was determined by immunofluorescent detection of BrdU in mammary gland sections from adult BALB/c and C57BL/6 OVX mice treated with 10 d C and 3, 5, or 10 d E. **D.** Representative mammary gland whole mounts from adult BALB/c and C57BL/6 OVX mice treated with P for 5 or 10 days. *White arrowheads* indicate sidebranching in the BALB/c gland. Scale bar = 1 mm. **E.** Quantitation of the proliferating luminal epithelial cells after P treatment. Immunofluorescent detection of BrdU was performed on mammary gland sections from adult BALB/c and C57BL/6 OVX mice treated with 10 d C and 3, 5, or 10 d P. The values for B, C and E represent the mean ± SEM from three mice per treatment group with a minimum of 1000 cells per mouse analyzed.


A
Intact
5d C
5d E
10d E
BALB/c
C57BL/6
B
Stimulated distal tips/cm2
BALB/c
C57BL/6
p<0.05
5d E
10d E
C
Percent BrdU+ Cells
ducts
DT
5d E
10d E
BALB/c
C57BL/6
D
5d P
10d P
E
Percent BrdU+ cells
10d C
3d P
5d P
10d P
BALB/c
C57BL/6

Response to estrogen + progesterone

In BALB/C mice, E+P treatment produced extensive sidebranching and alveologenesis that was maximal by 10 days (Fig. 4.3A). Even after 10 days of treatment, very little sidebranching or alveologenesis was observed in C57BL/6 mice. The C57BL/6 mammary gland mainly displayed enlarged duct ends, similar to the response observed after E treatment alone (Fig 4.3A).

A high percentage of BrdU+ cells were present in both ducts and sidebranches in the E+P-treated BALB/c gland. The percentage of BrdU+ cells was maximal after 3 days, decreased after 5 days, and was increased again after 10 days (Fig 4.3B). In the C57BL/6 gland, maximal proliferation was also observed after 3 days, was maintained at a higher level after 5 days and decreased after 10 days. Proliferation in the C57BL/6 gland was localized to ducts and enlarged distal tips, and was similar to the response to E alone.

PRA and PRB regulation in the C57BL/6 and BALB/c mammary gland

PRA is the predominant isoform expressed in the adult virgin mouse mammary gland and is thought to mediate the initial stages of sidebranching (1, 33). In the C57BL/6 gland, there were significantly more PRA positive (PRA+) cells (58% C57BL/6 vs. 43% BALB/c; $p<0.05$) (Fig 4.4A). There was no difference in the percentage of PRA+ cells between the two strains after OVX or in E-treated glands. P or E+P treatment for 5 or 10 days decreased the percentage PRA+ cells in the BALB/c gland

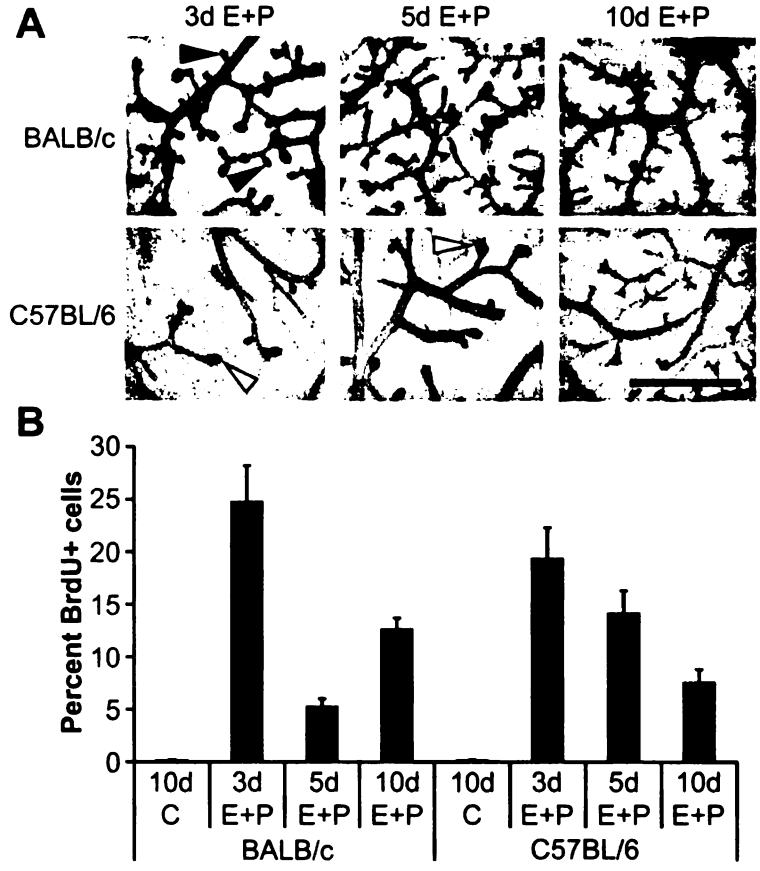


Figure 4.3. Effect of E + P treatment on morphology and proliferation in the BALB/c vs. C57BL/6 mammary gland. A. Representative mammary gland whole mounts from adult BALB/c and C57BL/6 OVX mice treated with E+P for 3, 5 or 10 days. *Black arrowheads* indicate sidebranching in the BALB/c gland. *White arrowheads* indicate stimulation of the distal tips of ducts in the C57BL/6 gland. Scale bar =1 mm. **B.** Quantitation of proliferating luminal epithelial cells. Immunofluorescent detection of BrdU was performed on mammary gland sections from adult BALB/c and C57BL/6 OVX mice treated with 10 d C and 3, 5, or 10 d E+P. The values represent the mean ± SEM from three mice per group with a minimum of 1000 cells per mouse analyzed.

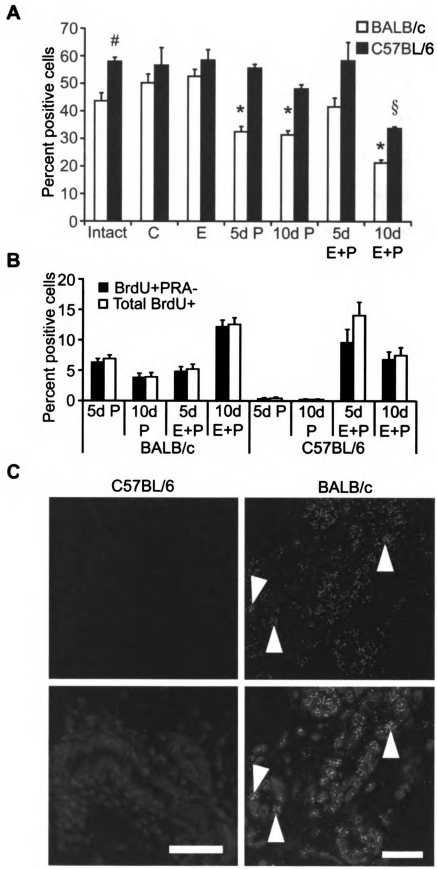
($p<0.05$). However, in the C57BL/6 gland, PRA+ cells were significantly decreased only after 10 days E+P treatment ($p<0.05$). Examination of the fluorescence intensity of PRA immunostaining, as a semiquantitative measure of PRA level, revealed no difference in PRA levels per cell between the two strains under any treatment (data not shown), indicating that the decrease of PRA+ cells in the BALB/c gland was not due to preferential downregulation of PRA protein, but rather was due to the dilution of PRA+ cells by proliferation of PRA negative (PRA-) cells. Indeed, the majority of proliferating cells in the P or E+P-treated BALB/c glands were PRA- (Fig. 4.4B). In contrast, significant proliferation in the C57BL/6 gland occurred only after E+P treatment. However, significantly fewer PRA- C57BL/6 cells proliferated compared to BALB/c cells.

Induction of PRB expression occurs in response to P or E+P treatment of OVX adult BALB/c mice and is correlated with alveolar development (Aupperlee & Haslam 2007). In the C57BL/6 mammary gland, no PRB expression was detected after either 10 days P or E+P treatment (Fig. 4.4C); the lack of PRB expression corresponded with a lack of alveologenesis (Fig. 4.3B).

RANKL is not induced by P alone in C57BL/6 mice

Since most proliferating cells in the BALB/c mammary gland were PRA-, we considered that P-induced proliferation, as has been previously suggested, must occur through a paracrine mechanism (29, 38). Receptor Activator of NF-κB Ligand (RANKL) has been implicated as one such paracrine mediator of P-induced proliferation (29).

Figure 4.4. Hormonal regulation of PRA and PRB expression in the BALB/c vs. C57BL/6 mammary gland. A. Quantitation of the percentage PRA+ luminal epithelial cells. PRA was detected by immunofluorescence in mammary gland sections from adult intact or OVX BALB/c and C57BL/6 mice treated for 5 or 10 d with control (C), E, P, or E+P. PRA+ cells were significantly less abundant in 5 and 10 d P and 10 d E+P-treated groups than in intact, C, E, and 5 d E+P-treated groups in the BALB/c mammary gland (*, $P<0.05$). PRA+ cells were significantly more abundant in intact C57BL/6than in intact BALB/c glands (#, $P<0.05$). PRA+ cells were significantly less abundant in 10 d E+P-treated C57BL/6 glands than in all other C57BL/6 groups (§, $P<0.05$). **B.** Quantitation of the percentage BrdU+ and BrdU+/PRA- luminal epithelial cells. Dual immunofluorescent detection of PRA and BrdU was performed on mammary gland sections from adult OVX BALB/c and C57BL/6 mice treated for 5 and 10 d with P or E+P. The values (A, B) represent the mean ± SEM from three mice per group with a minimum of 1000 cells per mouse analyzed. **C.** Immunofluorescent detection of PRB was performed on mammary gland sections from adult BALB/c and C57BL/6 OVX mice treated for 3, 5, or 10 d with C, E, P, or E+P. Representative PRB staining in 10 d E+P-treated C57BL/6 and BALB/c mammary gland. PRB (green nuclei, *white arrowheads*) was detected in the BALB/c mammary gland after 5 or 10 d E+P, and 10 d P treatment. Nuclei were counterstained with DAPI (blue). PRB was not detected in the C57BL/6 mammary gland under any treatment condition. Scale bar = 25 μm.


A
Percent positive cells
BALB/c
C57BL/6
Intact
C
E
5d P
10d P
5d E+P
10d E+P
B
Percent positive cells
BrdU+PRA-
Total BrdU+
5d P
10d P
5d E+P
10d E+P
BALB/c
C57BL/6
C
C57BL/6
BALB/c

Since treatment with P by itself was insufficient to induce proliferation and sidebranching in the C57BL/6 gland, we considered that this might be due to a lack of RANKL induction. Little RANKL expression was detected after OVX (Fig 4.5A) or E treatment in both mouse strains (data not shown). RANKL expression was induced by P treatment in the BALB/c gland, but not in the C57BL/6 gland (Fig 4.5A,B). RANKL expression was induced in both BALB/c and C57BL/6 mice after 5 days E+P treatment, but was more strongly induced in BALB/c mice. RANKL and PRA were colocalized in the same cells in both strains (Fig 4.5B).

Cyclin D1 is regulated differently in C57BL/6 and BALB/c mammary glands

Another mediator of P-induced proliferation is cyclin D1 (D1), which increases in expression and nuclear localization in response to P treatment (1, 39). D1 is also downstream of RANKL signaling in the mammary gland (40). The lack of P-induced proliferation or induction of RANKL in C57BL/6 mice suggested that nuclear localization of D1 might also be decreased or absent. Surprisingly, nuclear D1 was present in a significantly higher percentage of cells in the OVX C57BL/6 gland (Fig. 4.6A) and there was a significantly higher percentage of cells co-expressing nuclear D1 and PRA (Fig. 4.6B). E treatment did not affect nuclear D1 levels in either strain (data not shown). Nuclear D1 was increased in PRA- cells only after E+P treatment and coincided with an E+P-induced increase in RANKL (Fig. 4.5B).

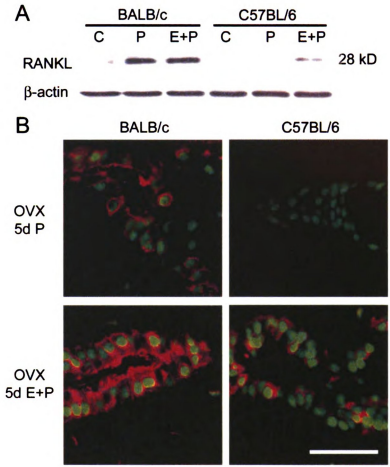


Figure 4.5. Hormonal regulation of RANKL expression in the BALB/c vs. C57BL/6 mammary gland. A. Immunoblot analysis of RANKL in mammary glands from adult OVX BALB/c and C57BL/6 treated for 5 d with C, P, or E+P. Cytoplasmic extracts from mammary gland were subjected to SDS-PAGE and RANKL detected in a western blot as described in *Materials and Methods*. RANKL was detected as a 28 kDa species following P and E+P treatment in the BALB/c mammary gland, but only after E+P treatment in the C57BL/6 mammary gland; β-actin served as a loading control. **B**. Dual immunofluorescent detection of RANKL (red) and PRA (green) in mammary gland sections from adult BALB/c and C57BL/6 OVX mice treated for 5 d with P or E+P. RANKL was expressed in the cytoplasm (red) in PRA+ cells (green nuclei). Scale bar= 25 μm.

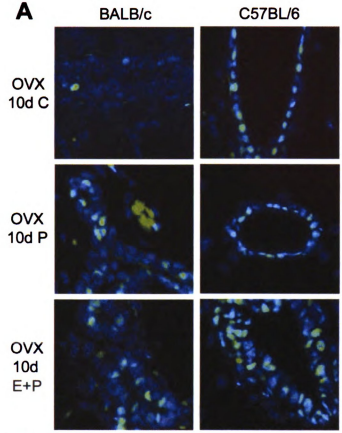
In the BALB/c gland, both P and E+P treatment increased nuclear D1 expression, indicating that nuclear localization of D1 was P-dependent (Fig. 4.6). Increased nuclear D1 expression occurred predominantly in PRA- cells (Fig. 4.6B). We hypothesize that RANKL is a paracrine mediator of increased D1 nuclear localization in PRA- cells in both BALB/c and C57BL/6 glands.

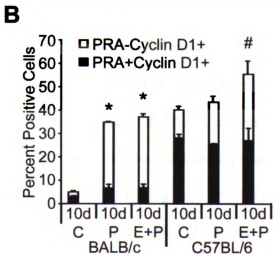
Based on gene deletion studies in the C57BL/6 ×129SV mixed genetic background, D1 is considered to be required for alveologenesis during pregnancy (41-43). However, P is also essential for alveologenesis (28). Since we found that the C57BL/6 gland is less responsive to P and expresses high levels of nuclear D1, we sought to determine the relative importance of P and D1 for alveologenesis in BALB/c vs. C57BL/6 glands during pregnancy. To address this, we generated BALB/c $D1^{-/-}$ mice by backcrossing C57BL/6 × 129SV $D1^{-/-}$ mice into the BALB/c genetic background. In the pregnant BALB/c $D1^{-/-}$ gland, there was extensive alveolar development, although not as extensive as in the wild-type BALB/c gland (Fig. 4.6C). Despite extensive alveolar development, BALB/c D1 $^{-/-}$ mice were lactation impaired and failed to nurse their pups. C57BL/6 × 129SV D1 $^{-/-}$ mice also exhibit impaired lactation, but alveolar development is also significantly impaired relative to BALB/c $D1^{-/-}$ (41-43).

Id2 localization is regulated differently in BALB/c versus C57BL/6 mammary gland

In response to RANKL signaling, Id2 is translocated to the nucleus (44). Nuclear localization of Id2 is required for mammary gland proliferation (44). Mice overexpressing D1, but deficient in Id2 exhibit a defect in mammary epithelial cell

Figure 4.6. Hormonal regulation of cyclin D1 in the BALB/c vs. C57BL/6 mammary gland. Dual immunofluorescent detection of PRA and cyclin D1 was performed on mammary gland sections for adult BALB/c and C57BL/6 OVX mice treated for 10 d with C, P, or E+P. **A.** P or E+P treatment increased nuclear expression of cyclin D1 (green nuclei) in the BALB/c mammary gland, whereas nuclear expression of cyclin D1 was elevated in all C57BL/6 treatment groups. Nuclei were counterstained with DAPI (blue nuclei). Scale bar = 25 μm. **B.** Quantitation of percentage cyclin D1+ PRA- and PRA+D1+ luminal epithelial cell nuclei. Total nuclear cyclin D1+ cells in 10 d P and 10 d E+P-treated BALB/c glands is significantly greater than in 10 d C glands (*, $P<0.05$). Total nuclear cyclin D1+ cells in 10 d E+P-treated C57BL/6 glands is significantly greater than in 10 d C or P-treated glands (#, $P<0.05$). The values represent the mean ± SEM from three mice per group with a minimum of 1000 cells per mouse analyzed. C. Representative whole mounts from adult and 1 d postpartum (1d pp) BALB/c Cyclin D1 $^{-/-}$ and 1 d postpartum wild-type BALB/c mice. Note significant alveolar development in 1 d postpartum BALB/c Cyclin D1 $^{-/-}$ gland.


A
BALB/c
C57BL/6
OVX
10d C
OVX
10d P
OVX
10d
E+P


B
PRA-Cyclin D1+
PRA+Cyclin D1+
Percent Positive Cells
70
60
50
40
30
20
10
0
*
*
#
10d
C
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E+P
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C
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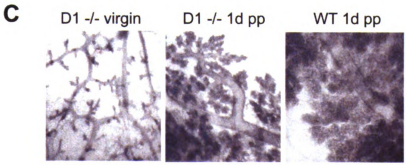
E+P
BALB/c
C57BL/6


C
D1 -/- virgin
D1 -/- 1d pp
WT 1d pp

proliferation (45). We hypothesized that reduced RANKL levels would lead to reduced Id2 nuclear localization in the C57BL/6 mammary gland, and that the activation of Id2 by RANKL was also required for proliferation.

In the BALB/c gland, P or E+P treatment for 5 or 10 days decreased cytoplasmic localization and correspondingly increased nuclear localization of Id2 compared to OVX controls (Fig 4.7A). In the C57BL/6 gland, only treatment with E+P decreased cytoplasmic and increased nuclear localization of Id2 (Fig 4.7A). E+P treatment also increased the level of nuclear Id2 to a greater extent in the BALB/c gland (Fig 4.7B).

Differences in ERα expression between C57BL/6 and BALB/c

The above results indicate that the proliferative effect of E was more pronounced in C57BL/6 OVX mice, as evidenced by sustained proliferation and enlargement of duct ends. This led us to examine ERα expression in the two strains. There were 10% more ERα+ cells ($p < 0.05$) in the C57BL/6 gland in ovary intact animals (Fig. 4.8A). As previously reported for the BALB/c gland (1), ER colocalized with PRA in the C57BL/6 gland (Fig 4.8B). However, the level of ERα expression per cell, as determined by the intensity of immunofluorescent staining with anti-ERα antibody, was 1.7-fold higher in the C57BL/6 mammary gland (Fig. 4.8C). ERα levels were decreased by treatment with E by itself or with E+P in both strains. However, ERα levels were higher in C57BL/6 glands after E or E+P treatment.

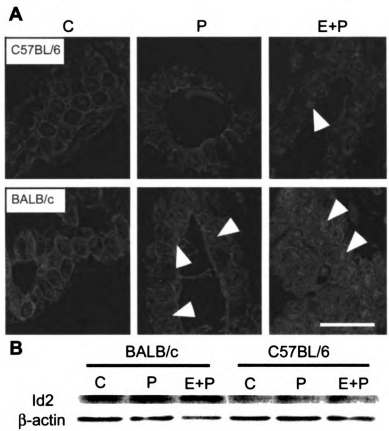
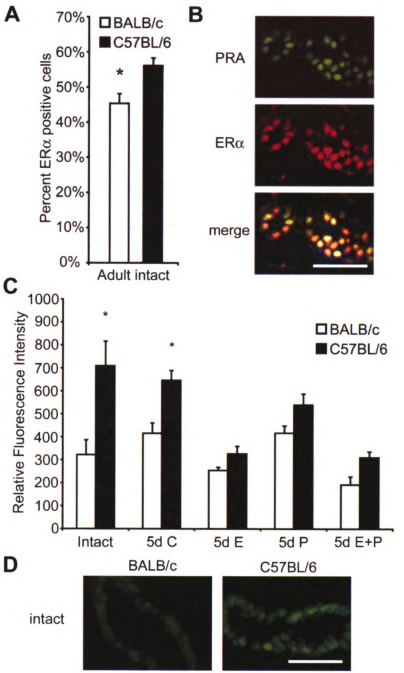


Figure 4.7. Hormonal regulation of Id2 in the BALB/c vs. C57BL/6 mammary gland. A. Immunofluorescent detection of Id2 in mammary gland sections from adult BALB/c and C57BL/6 OVX mice treated for 5 d with C, P, or E+P. Nuclear localization of Id2 (green nuclei, white arrowheads) was increased by 5 d P and E+P treatment in the BALB/c and 5d E+P treatment in the C57BL/6 mammary gland. Scale bar = 25 μm. **B**. Immunoblot analysis of Id2 in mammary glands from adult BALB/c and C57BL/6 OVX mice treated for 5 d with C, P, or E+P. Nuclear extracts from mammary glands were subjected to SDS-PAGE and Id2 detected in a western blot as described in *Materials and Methods*. Nuclear Id2 was detected as a single 15 kDa species and increased in abundance following E+P treatment in the BALB/c and C57BL/6 mammary gland.

Figure 4.8. Expression and hormonal regulation of ERα in BALB/c and C57BL/6 mammary glands. Immunofluorescent detection of ERα was performed on mammary gland sections from adult BALB/c and C57BL/6 intact or OVX mice treated for 5 d with C, E, P, or E+P. **A.** Quantitation of the percentage ERα positive luminal epithelial cells in intact C57BL/6 and BALB/c mammary gland. The values represent the mean ± SEM from three mice per group with a minimum of 1000 cells per mouse analyzed. The percent ERα+ cells in the BALB/c gland was significantly lower than in the C57BL/6 gland (*, $P < 0.05$). **B.** Representative images of PRA (*green nuclei*), ERα (*red nuclei*), and ERα and PRA colocalization (*yellow nuclei)* in the C57BL/6 mammary gland. S*cale bar* = 25 μm. **C.** Quantitation of ERα immunofluorescence staining intensity. Values represent the mean pixel intensity in ERα-positive cells ± SEM from three mice per group with a minimum of 1000 cells per mouse analyzed. Control treated C57BL/6 intact and OVX mice have more ERα than BALB/c mice (*, $P < 0.05$; ANOVA). **D.** Representative ERα immunofluorescence staining of mammary glands from adult BALB/c and C57BL/6 intact mice. S*cale bar* = 25 μm.


A
70%
60%
50%
40%
30%
20%
10%
0%
BALB/c
C57BL/6
Percent ERα positive cells
*
Adult intact
B
PRA
ERα
merge
C
1000
900
800
700
600
500
400
300
200
100
0
Relative Fluorescence Intensity
*
*
BALB/c
C57BL/6
Intact
5d C
5d E
5d P
5d E+P
D
BALB/c
C57BL/6
intact

Estrogen, Stat5a expression, and RANKL induction in BALB/c and C57BL/6 mammary glands

As shown above, C57BL/6 mice required E in addition to P for the induction of RANKL and Id2 nuclear localization. In contrast, P alone was sufficient for these effects in the BALB/c gland, although E enhanced the effects of P. Signal transducer and activator of transcription 5a (Stat5a) has been shown to increase expression of RANKL (46). In BALB/c mice, E+P treatment induces expression of activated nuclear Stat5a (47). Because both E and P were required to induce RANKL in C57BL/6 mice, we hypothesized that E played a critical role in RANKL induction through activation of Stat5a. We found that E treatment increased nuclear Stat5a in the C57BL/6 gland (Fig. 4.9A) and there was an overall increase in Stat5a expression after E+P treatment (Fig 4.9B). Additionally, E+P treatment induced RANKL colocalization with Stat5a (data not shown).

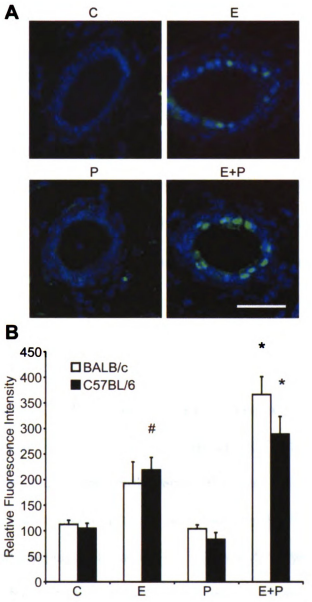


Figure 4.9. Hormonal regulation of Stat5a in BALB/c and C57BL/6 mammary glands. **A**. Immunofluorescence detection of Stat5a in mammary gland sections from adult BALB/c and C57BL/6 OVX mice treated for 3 d with C, E, P, or E+P. Representative images showing Stat5a expression (*teal*) in the 3d C, E, P, and E+P-treated C57BL/6 mammary gland; nuclei were counterstained with DAPI (dark blue). *Scale bar* = 25 μm. **B**. Quantitation of Stat5a immunofluorescence staining intensity. Values represent the mean ± SEM pixel intensity in nuclear Stat5a-positive cells from three mice per group with a minimum of 1000 cells per mouse analyzed. Stat5a intensity is increased in BALB/c and C57BL/6 mammary glands by 3d E+P treatment relative to OVX controls (*, $P < 0.05$). Stat5a intensity is increased in C57BL/6 mammary glands by 3d E treatment relative to OVX controls (#, $P < 0.05$).

DISCUSSION

Progesterone, in conjunction with E and other hormones and growth factors, is required for the extensive sidebranching and alveologenesis that occurs during pregnancy. Previous studies indicate that P promotes ductal sidebranching and alveologenesis through the induction of PRB (1, 19), increased RANKL expression (29), and increased expression of nuclear cyclin D1 (1, 19, 29). We observed a significant delay in ductal sidebranching and alveologenesis during pregnancy in C57BL/6 mice compared to BALB/c mice. To understand the basis for this difference, we have compared hormonal regulation of PRB, RANKL, Id2 and cyclin D1 in C57BL/6 vs. BALB/c mammary glands.

We found that the C57BL/6 gland was less responsive to P than the BALB/c gland, as evidenced by the lack of P-induced PRB and RANKL expression, reduced Id2nuclear translocation, and reduced proliferation. However, RANKL expression, Id2 nuclear translocation, and proliferation could be induced by E+P, suggesting a greater dependence on E for alveologenesis in the C57BL/6 mouse. In contrast, P by itself could induce PRB and RANKL expression, proliferation, and alveologenesis in the BALB/c gland, indicating a greater responsiveness to P.

Studies of C57BL/6 cyclin D1 $^{-/-}$ mice show cyclin D1 expression and its nuclear localization to be critical for alveolar development (41-43). In the BALB/c gland, nuclear cyclin D1 expression was dramatically decreased after ovariectomy, but could be increased by P treatment. Nuclear cyclin D1 levels were increased predominantly in PRA- cells, and were associated with P- or E+P-induced proliferation in PRA- cells.

Surprisingly, we found high levels of nuclear cyclin D1 even after OVX in the C57BL/6 gland. Notably, nuclear cyclin D1 was predominantly expressed in PRA+ cells. While treatment with P did not affect nuclear cyclin D1 levels, treatment with E+P led to an increase in nuclear cyclin D1 in PRA- cells; most proliferation induced by E+P also occurred in PRA- cells. These results demonstrate very different inherent patterns of nuclear cyclin D1 expression and regulation between the two strains, and further demonstrate reduced sensitivity to P in the C57BL/6 gland.

Deletion of the cyclin D1 gene in the C57BL/6 genetic background results in a lack of alveologenesis during pregnancy (42, 43). In contrast, BALB/c cyclin D1 $^{-/-}$ mice exhibited extensive alveologenesis during pregnancy. Viewed in the context of the reduced responsiveness to P that we found in C57BL/6 mice, these results suggest that impaired alveologenesis in C57BL/6 cyclin D1 $^{-/-}$ mice is likely the result of inherent strain-specific reduced responsiveness to P rather than the lack of cyclin D1 per se. However, both BALB/c and C57BL/6 cyclin D1 $^{-/-}$ mice were lactation-deficient, in agreement with previous reports that cyclin D1 plays a specific role in lactational differentiation in addition to its cell cycle regulatory function (41-43).

The proliferative effect of E was more pronounced in C57BL/6 than in BALB/c mice, as evidenced by sustained E-induced enlargement of and proliferation in the distal tips of ducts. Furthermore, E+P-treated C57BL/6 glands exhibited enlarged distal tips similar to those seen with E alone, indicating increased responsiveness to E and reduced responsiveness to P. We found 10% more ERα+ cells and higher levels of ERα per cell in C57BL/6 glands, consistent with their greater sensitivity to E. Similar to our results, Montero Girard et al (33) also found that virgin C57BL/6 mice exhibited a hypoplastic

ductal phenotype and reduced morphological response to P compared to BALB/c mice. However, opposite to our findings, they reported that the percentages of ERα+ and PRA+ cells were greater in BALB/c glands compared to C57/BL/6 glands. One possible explanation for the difference in results lies in the methods used for immunodetection. In the Montero Girard et al. (2007) study, no antigen retrieval was used prior to antibody staining. Using the same antibodies, we and others have found poor detection of ERα or PRA without antigen retrieval (48)(Aupperlee & Haslam unpublished observations). Thus, it is possible that the different results obtained for the percentages of ERα+ and PRA+ cells might be attributed to differences in detection with or without antigen retrieval.

In C57BL/6 mice, E is also critically required in addition to P for alveologenesis. In the C57BL/6 gland, induction of RANKL, a major P-induced paracrine factor promoting alveologenesis (29), also required E in addition to P. In this regard, Stat5a has also been shown to increase RANKL expression (46). Stat5a expression colocalizes with RANKL and it has been suggested that colocalization of the two proteins may be functionally linked (47). E+P treatment also increases Stat5a activation. However, co-treatment of E+P with bromocryptine, which blocks prolactin secretion, only increases cytoplasmic Stat5a indicating that prolactin is required for activated nuclear Stat5a (47). Since E increases prolactin levels, we hypothesize that one way that E contributes to alveologenesis in C57BL/6 mice is through increased prolactin secretion, leading to activation of Stat5a and Stat5a-dependent induction of RANKL. In support of this hypothesis, we found that E and E+P increased nuclear Stat5a levels in the C57BL/6

gland. Further studies are warranted into the detailed mechanism(s) by which E contributes to ductal sidebranching and alveologenesis in the C57BL/6 mouse.

The phenotypic differences between BALB/c and C57BL/6 cyclin D1 $^{-/-}$ mice highlight the importance of inherent strain-specific differences when interpreting the results of genetic manipulations. In this regard, the inherent reduced responsiveness to P of the C57BL/6 strain indicates that this may not be the best strain for studying P-mediated responses, such as sidebranching and alveologenesis. Additionally the variable genetic contribution of mixed genetic backgrounds (i.e. C57BL/6 X 129SV) often used for gene deletion studies may yield inconsistent outcomes, thus confounding the interpretation of a phenotype. However, this problem can be overcome by backcrossing genetically modified mice into a pure genetic background with a well-defined wildtype phenotype.

In summary, we have demonstrated that the BALB/c and C57BL/6 mouse strains differ significantly in their proliferative and morphological responses to estrogen and progesterone. We have identified differences in the progesterone-mediated regulation of several factors: PRB, cyclin D1, RANKL, and Id2. These differences shed light on the basis for the reduced response to P in the C57BL/6 mouse strain. The fact that two genetic backgrounds in the same species differ so significantly in E and P responses indicates that caution is required in generalizing mechanisms of hormone action on the basis of studies in a single mouse strain. These results also suggest that in human populations with heterogeneous genetic backgrounds, individuals may respond differentially to the same hormone through inherent differences in their regulation of downstream signaling pathways. For example, the association of combined estrogen +

progestin hormonal therapy with increased breast cancer risk may be associated with a genetic background in human populations that reflects increased sensitivity to P. These differences may apply to hormonal regulation in both the normal breast and in breast cancers. The differences in expression level and regulation that we have observed for elements in the progesterone-regulated proliferative pathways of mouse mammary epithelial cells suggest their potential utility as biomarkers for the assessment of P-sensitivity in the human breast, as well as their potential as novel biomarkers for breast cancer susceptibility.

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CONCLUDING REMARKS

Progesterone is an important mitogen in the mammary gland. It is critical for normal lobuloalveolar development and has also been implicated in the etiology of breast cancer. Progesterone acts through binding to the progesterone receptor (PR), which exists as two isoforms, PRA and PRB. In this thesis, I set out to examine the developmental regulation of expression and localization of PRA and PRB in the mouse mammary gland, to determine the hormonal regulation of PRA and PRB *in vivo* in the mouse mammary gland, and to examine the mechanism of progesterone action in mouse strains with different genetic backgrounds.

While PR expression in the mouse mammary gland has been previously examined, the developmental regulation of PRA and PRB remained unknown. In chapter 2, I demonstrated that protein expression of PRA and PRB in the developing BALB/c mammary gland is temporally and spatially separated. PRA is the primary PR isoform expressed in the virgin mammary gland, whereas PRB is the primary isoform expressed upon pregnancy. In the virgin mammary gland PRB is not detected, and thus cells containing PR express only PRA. In the pregnant mammary gland when both PRA and PRB are expressed, PRA and PRB expression generally do not colocalize. These results in the mouse contrasted with published reports of complete PRA and PRB co-expression in the human premenopausal breast, and suggested that the separation of PRA and PRB expression during mouse mammary gland development offered a unique opportunity to examine the role of PRA during ductal development and sidebranching and the role of PRB during alveologenesis. Initial analysis during puberty and pregnancy suggested that

PRA primarily mediates proliferation via a paracrine mechanism, whereas during pregnancy PRB may mediate proliferation via a direct mechanism.

The results presented in chapter 2 demonstrated that PRA level decreases during pregnancy, whereas PRB level increases. The regulation of PRA and PRB protein expression *in vivo* is poorly understood, but it has generally been suggested that estrogen upregulates PR and progesterone downregulates PR. In chapter 3, I demonstrated that in the BALB/c mammary gland PRA level is increased by estrogen and decreased by progesterone. In contrast, PRB level is increased by progesterone and is further increased by estrogen + progesterone. The results presented in chapter 3 also reveal potential functional roles for PRA and PRB in the mammary gland. The increase in PRB expression is associated with alveolar formation, consistent with a role of PRB in alveologenesis. PRA expression is associated with ductal sidebranching, suggesting that PRA-mediated proliferation plays a role in the formation of sidebranches. Additionally, PRA-mediated proliferation during sidebranching occurs primarily in PRA negative cells, whereas PRB-mediated proliferation during alveologenesis is present in both PRB positive and negative cells, confirming the conclusion from chapter 2 that PRA and PRB mediate proliferation via different mechanisms.

The results from chapter 2 and chapter 3 suggested that PRA mediates proliferation by a paracrine mechanism. In order to further explore the role of PRA in mediating progesterone-induced proliferation, two adult strains of mice, BALB/c and C57BL/6 were analyzed for their responses to progesterone. In chapter 4 of this dissertation, I presented data showing that C57BL/6 mice have reduced sidebranching prior to pregnancy and a delay in alveologenesis during pregnancy compared to BALB/c

mice. I showed that the lack of sidebranching is due to adult C57BL/6 mice having a reduced morphological and proliferative response to progesterone. When I examined potential downstream signals of progesterone, I found reduced progesterone-induced PRB expression in the C57BL/6 mammary gland. In chapter 4, I also presented results that identified a paracrine mechanism for progesterone action in BALB/c mammary glands. RANKL expression increases in PRA positive cells in response to progesterone, which was associated with increased nuclear localization of Id2 and nuclear localization of cyclin D1 in PRA negative cells. The reduced sensitivity of the C57BL/6 mammary gland to progesterone correlated with reduced progesterone-induced PRB and RANKL expression, reduced nuclear localization of Id2, and significantly different regulation of cyclin D1 expression. These results are the first to provide mechanistic insight into the difference in hormonal responsiveness between mouse strains.

In conclusion, the findings presented in this dissertation provide novel insight into the developmental and hormonal regulation of PRA and PRB, and thus provide a framework for further study of PRA and PRB function. Currently, PRA and PRB expression in the human breast has only been examined in premenopausal women. The temporal and spatial separation of PRA and PRB expression during mouse mammary gland development highlights the importance of similar developmental studies in the human breast. Additionally, PRA and PRB are differentially regulated by hormones in the mouse. Future studies are planned to examine PRA and PRB expression in breast tissue from postmenopausal women receiving hormone replacement therapy with estrogen alone or with estrogen + progesterone. Thus, it remains important to translate these findings in the mouse into a greater understanding of progesterone action in the

human breast. It is expected that there will be changes in PR isoform expression associated with certain developmental states in the human breast, such as puberty and pregnancy, and that these alterations will be associated with increased proliferation.

The results presented in this dissertation also significantly advance our understanding of progesterone mechanism of action in genetically unaltered mice and highlight the importance of genetic background in determining hormone responsiveness. The identification of markers of progesterone action that are differently regulated in two different mouse strains suggests that similar markers of progesterone action need to be found in the human breast. Due to the genetic heterogeneity present in human populations, it is likely that a range of responsiveness to progesterone exists. In order to better understand the role of progesterone in the normal human breast and in the etiology of breast cancer, it is important to consider the role of genetic background in influencing progesterone responsiveness. The contribution of progesterone to the etiology of breast cancer may be determined through greater insight into progesterone responsiveness.