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GRANT NUMBER DAMD17-94-J-4254

TITLE: The Physiological Role of Progesterone Receptors in Breast Development and Tumorigenesis

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REPORT DATE: September 1998

TYPE OF REPORT: Final

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 1998	Final (1 Sep 94 - 31 Aug 98)

4. TITLE AND SUBTITLE
The Physiological Role of Progesterone Receptors in Breast Development and Tumorigenesis

5. FUNDING NUMBERS
DAMD17-94-J-4254

6. AUTHOR(S)
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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

Progesterone and estrogen are the key steroidal hormones involved in breast development and tumorigenesis. The effects of progesterone and estrogen are mediated through specific intracellular receptors and the status of these receptors in breast tumors has been used as an important prognostic indicator in determining the probability of disease free survival and response to hormonal therapies. The progesterone receptor (PR) is composed of two isoforms, PRA and PRB which have different transactivation functions *in vitro*. This suggests that these receptors are likely to have different physiological roles in breast development andtumorigenesis. To date no in vivo model exists to address this question. The objectives of this proposal are to establish the collective and individual physiological roles of these receptors in breast development and carcinogenesis *in vivo*.

To achieve the above objective, genetic mouse models have been generated in which the PR status is altered by either a null mutation or selective ablation ofthe A or B forms of the PR. The physiological analysis of these mutant mouse lines provides valuable information on the selective contribution of the PRA and PRB to breast development and tumorigenesis *in vivo*. This information will improve prognostic capabilities with regard to analysis of PR status in breast tumors as well as improved treatment strategies.

14. SUBJECT TERMS
Breast Cancer

15. NUMBER OF PAGES
25

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

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Orla M. Connelly 9/16/98

PI - Signature Date

TABLE OF CONTENTS

V. INTRODUCTION

Problem

Progesterone and estrogen are the main steroid hormones involved in breast development and tumorigenesis and can have both stimulatory and inhibitory effects on carcinogenesis that are both stage and dose dependent. The effects of these hormones are mediated through specific intracellular receptors. However, the specific contribution of these receptors to proliferation, differentiation and tumor growth of mammary tissue remains controversial. The overall objective of this project is to evaluate the physiological role of the progesterone receptor and its individual A and B isoforms in mammary gland development and tumorigenesis. Our approach is to examine the consequences of ablation of the PR A and B proteins on mammary gland physiology and function using PR null mutant mouse models.

Background

Progesterone and estrogen are the principle steroid hormones involved in normal breast development and tumorigenesis (1-3). In the case of mammary gland tumorigenesis, the effects of progesterone and estrogen on carcinogenesis can be both stimulatory and inhibitory and are dose and stage dependent (4). These hormonal effects are mediated by specific high affinity intracellular receptor proteins that are members of a superfamily of related transcription factors (5,6). Binding of steroids to these receptors results in the formation of activated receptor dimers that bind to specific enhancer DNA elements located in the promoter regions of hormone-responsive genes (7,8). The activation or repression of these genes represents the manifestation of the hormonal response.

The mammary gland is the site of milk production and secretion, and in females, is a major site of tumorigenesis (9). Mammary gland development occurs during the fetal, post-natal and adult stages of life (10). The development of the mammary gland occurs primarily post-natally and is directed by a complex signal transduction interplay between hormonal (polypeptide and steroid) and growth factor signals. During pregnancy, progesterone and estrogen promote growth and differentiation of normal mammary tissue by regulating ductal branching, alveolar formation (11) and lobuloalveolar development (12). Studies on the ontogeny of mouse mammary gland responsiveness to ovarian steroid hormones have indicated that receptors for estrogen and progesterone (ER and PR respectively) are present in both stromal and epithelial cells, and begin to exert effects on terminal end bud proliferation at 4 and 7 weeks of age, respectively (13). Furthermore, it now appears that epithelial cells, which can express receptors for estrogen and progesterone, are the major sites of primary mammary carcinomas (14).

Although the general consensus on progestin action in the uterus is that progesterone inhibits the proliferative effect of estrogen and acts as a differentiating hormone, this concept cannot be extended to the breast (3). Considerable evidence has accumulated to implicate progesterone in the proliferation of normal mammary epithelium in virgin animals (15) and in the development of the lobular-alveolar structure in mammary glands of pregnant animals (16). Unlike estrogen action, progesterone is a mitogen, not only in the epithelium of the terminal end buds, but also in the ductal epithelium (17). Depending on the time of administration and the dosage used, progestin agonists have been shown to reverse the anti-tumor effects of the anti-estrogen, tamoxifen, and induce tumor growth (18). The observation that the tumor inhibitory effect of tamoxifen can be reversed by progestin agonists (18) together with the stage and dose dependent carcinogenic activity of progestin agonists (3) suggest that some of the effects of ERs may be mediated by

PRs whose expression is known to be induced by estrogen (19). Taken collectively, the above data supports the proliferative effect of progesterone in normal breast development and in contributing to the oncogenic potential of the breast. Conversely, studies using the carcinogen-induced rat mammary tumor model (20) have shown that early pregnancy (21) or the administration of high doses of progesterone and 17βestradiol (22) shortly after the onset of sexual maturity were effective in reducing the susceptibility of the mammary gland to chemical carcinogenesis. Thus, progesterone appears to have both stimulatory and inhibitory effects on mammary gland tumorigenesis that are stage and dose dependent.

From a clinical standpoint, the estrogen and progesterone receptor status of breast tumors is an important prognostic factor in determining the probability of disease free survival and response to hormonal therapy (2,23). Breast tumors that contain functional ERs and PRs have a higher response to hormonal therapy and higher disease free survival probability (2). However, as tumorigenesis progresses, the disease develops to a state that is characterized by a lack of ERs and PRs and a resistance toward hormonal and cytotoxic therapies.

It has been established that PR is composed of two hormone binding forms *in vivo*, termed PR_A and PR_B (24,25). It is thought that the A and B forms arise as a result of either alternate initiation of translation from a single mRNA (26) or by alternate transcription from promoters within the same gene (27). These receptor isoforms differ only in that PR_B contains an additional stretch of amino acids at the amino terminus of the receptor. Previous experiments have shown that these proteins exhibit different promoter specificities for target gene activation (28) while binding to the same enhancer DNA element (29). Remarkably, recent data have implicated a novel repressor function as well as an activator role for PR_A (30). Depending on the promoter and cell context, PR_A was shown to act as a potent transdominant repressor of PR_B-mediated gene transcription. In addition, the repressor function of PR_A was found to influence the activity of other members of this superfamily of transcription factors which included the glucocorticoid, mineralcorticoid and androgen receptors. Intriguingly, recent transient cotransfection experiments have revealed that PR_B when occupied by progestin antagonists can activate transcription (31). Furthermore, this unusual PR_B mediated antagonist transactivation can be dominantly inhibited by the PR_A isoform. This apparent paradoxical stimulatory action of progesterone antagonists via PR_B, if substantiated, would prompt a reevaluation or the potential efficacy of any chemoprevention strategy involving these 'anti-progestins' in the treatment of breast and uterine cancer.

Although, for two decades, the PR has been shown to be composed of two receptor isoforms, the specific physiological role for each of these two PR subtypes in normal breast development, tumor initiation and progression, has yet to be established. However, the existence of both these receptors in different species and tissues, and the elaborate mechanisms regulating their expression suggest that the absolute and relative levels (receptor status) of PR_A and PR_B in a progestin target cell are critical for the correct cellular response to progesterone and its antagonists. The equimolar expression of both forms of the PR in the same cell would allow the possible formation of two homodimers and one heterodimer (A:A, B:B and A:B). The potential existence of three dimeric forms of PR, each having different transcriptional regulatory specificities, would serve to further expand the repertoire of physiological responses to progesterone. Although breast tissue may contain an overall equimolar ratio of PR_A to PR_B, it is quite possible that different cell types of this tissue, for example epithelial and stromal cells, may have a different ratio which is critical for the normal functioning of these cells. Therefore alterations in the ratio of PR_A to PR_B, would be expected to contribute to an altered susceptibility of these cells to carcinogenesis and have a dramatic effect on the cellular response to progesterone agonists, antagonists, other steroids and growth factors and proto-oncogenes regulated by progesterone.

An additional level of complexity in the involvement of these receptor isoforms in mammary gland development and tumorigenesis arises from influence of growth factors and proto-oncogenes such as epidermal growth factor (EGF), c-myc and cyclin D1 which have been shown to be increased by progestins in cultured human breast cancer cell lines (32). These mitogens may represent "early target" genes for progesterone which may act via autocrine and paracrine mechanisms to influence breast tissue proliferation and differentiation. At this stage, it is not known which of these gene products are modulated by either one or both isotypes of PR.

Purpose of the Present Work

Based on the above observation, we propose the following hypothesis:
During breast development and tumorigenesis, progesterone mediates its mitogenic effect through two receptor isoforms, PR_A and PR_B. We predict that, in vivo, PR_A and PR_B have distinct physiological effects and that the ratio of PR_A to PR_B is a key determining factor for normal breast development, oncogenic potential and carcinogenesis.

Methods of Approach

We have used a genetic approach to test the above hypothesis. Two fundamental questions regarding the role of progesterone and its receptor in breast development are being addressed. These are: (1) What is the *in vivo* functional significance of progesterone in general breast development? and (2) What is the *in vivo* functional relevance of the A and B forms of PR in normal breast development and tumorigenesis. These questions are being addressed by the physiological analysis of mutant mice deficient in both forms of the receptor (PR_{A+B}-ve) and mouse lines deficient in either the A or B form of the receptor (PR_A-ve and PR_B-ve respectively). The generation of these mouse models is accomplished by the mutation of the endogenous mouse PR gene by homologous recombination (gene targeting) in mouse embryonic stem (ES) cells. Pluripotent ES cells carrying the mutated PR allele are injected into mouse blastocysts where they become the progenitor cells of most of the embryonic tissues including the germ line. Germ line transmission of the mutated PR allele allows the creation of mouse strains that are heterozygous and homozygous for the mutant PR gene.

Progress.
In this section I will summarize our overall progress for the duration of the grant. I will then detail the progress during the past year that is not previously reported or published.

Role of Progesterone Receptors in development of the mammary gland.
Our initial studies on the role of PR in mammary gland development were carried out using PRKO mice in which both forms of the progesterone receptor were ablated by null mutation of the PR gene. Our studies with these mice confirmed previous reports that PR exerts both a proliferative and differentiative role in mammary epithelial development. Ablation of PR resulted in decreased pregnancy associated development and dichotomous branching of the ductal epithelium, a striking absence of terminal end buds and a complete inhibition of lobuloalveolar differentiation in response to exogenous E and P treatment.

Identification of Target Genes that mediate the proliferative and differentiative effects of PR in the Mammary Gland.

Although the specific molecular mechanisms by which PR exerts its effects on mammary epithelium are not known, the phenotype overlaps in part with those recently observed in null mice generated for STAT5a and STAT5b (91) (92), CEBPβ (93) (94), D1cyclin (95) PRL receptor (96), LAR phosphatase (97), paralagous hox9 genes (98) and in mice carrying a spontaneous EGFR mutation (99). Because of the significant overlap and lack of redundancy in the physiological responses to these factors, it is most likely that they participate in convergent rather than distinct non-redundant signalling pathways. Consistent with this hypothesis previous in vitro studies have demonstrated that P can induce the expression of growth factors including EGFR (100), STAT5a and STAT5b proteins (101) and the cell cycle protein cyclin D1 in breast cancer cells(59,102). To test the hypothesis that some of these factors may mediate the proliferative and/or differentiative effects of PR in the mammary gland, we focused on D1cyclin for the following reasons: 1) D1 cyclin is induced by mitogenic stimuli during the G1 phase of the cell cycle and mediates their proliferative effects by activating cyclin dependent kinases to remove the cell cycle block at the G1/S phase checkpoint and allow progression through mitosis; 2) D1 cyclin is induced by progesterone in the G1 phase in T47D breast cancer cells resulting in accelerated cell cycle progression through G1 in these cells; 3) Both D1 induction and cell cycle progression are blocked by progesterone antagonists in these cells; 4) overexpression of D1 in the mammary gland of transgenic mice results in mammary adenocarcinomas and the gene is localized on human chromosome 11q13 in a region that is amplified in 15-20% of mammary carcinomas. Finally and most strikingly, the mammary phenotype of our PR-/- null mutant mice shows significant overlap with that of the D1 cyclin null mutant mice and strongly supports a D1 cyclin mediated proliferative and differentiative response to progesterone.

To determine whether D1 cyclin can selectively mediate the effects of progesterone (P) in the mammary gland, we compared the temporal expression patterns of D cyclin mRNA transcripts in wild-type versus PR-/- null mutant mice after treatment with estrogen (E) alone or E and P. Using Northern naalysis and immunohistochemistry, we demonstrated that D1 cyclin was induced by both estrogen and progesterone in the mammary gland of wild-type mice and that the increase in expression of this gene correlated closely with mammary gland proliferation in the mouse. The induction of D1 cyclin by P was lost in PRKO mice and this loss of induction was closely associated with a decrease in proliferation of mammary epithelial cells. Taken together, these results strongly supported the conclusion that the proliferative effects of PR in the mammary gland are mediated at least in part by induction of D1cyclin (publication #2 below). If these responses prove to be mammary specific, then D1 cyclin may be a primary hormone responsive target whose deregulation mediates mammary specific tumorigenesis.

Contribution of the A and B Isoforms of PR to mammary gland development.

The major focus of our research during the past four years was to generate novel PR mutant mouse strains that would selectively ablate either the A or B isoforms of PR and allow in vivo analysis of the selective functions of each isoform. At the time this effort began, no subtle mutations had been successfully introduced into the genome of mice by homologous recombination and the technologies for generating such mutants were in the development stage. We adopted two alternative approaches to generate these mutants.

The first approach (tag and exchange) involved introduction of a mutant PR vector into embryonic stem cells to 'tag' the progesterone receptor locus followed by a second step in which a selectable marker in the tagging vector is replaced by homologous recombination with a second PR vector that contains the desired mutation. Although theoretically sound, the approach proved technically unfeasable due to a highly inefficient second selection step in the gene targeting. Although a few reports demonstrating success of this technique (in ES cells but not in mice) had been published at the time, we and other investigators abandoned the approach in favor of the more promising CRE-loxP site specific recombination approach. At 18 months into the project, we began to design new vectors to begin the second strategy for generation of PR A or B expressing mice. In the final two years we were successful in generating both lines of mice and report below the results of initial physiological analysis using one of these lines of mice (PRAKO mice that express only the PRB protein). **These analyses provide the first in vivo proof of principle that the PR A and B proteins have different tissue specific physiological functions and that the PR B protein alone is sufficient the elicit normal proliferative and differentiative responses of the mammary epithelium to progesterone.** Finally, in the case of the PRBKO mouse strain, although we have obtained also obtained homozygote mice in which the ATG PRB initiation codon is mutated and express only the PR A protein. These mice are currently being bred to obtain sufficient adult homozygote females for physiological analysis. Hence, this report will focus on the physiological phenotype of PRAKO mice.

Physiological Analysis of PRAKO Mice.

In the previous progress report, I presented data confirming that PRAKO mice express only the PR B protein and measured by Western immunoblot analysis of uterine extracts from PRAKO and wildtype mice. In this report, I will present results from physiological analysis of these mice that demonstrates that the PRB protein functions in a tissue specific manner to mediate some but not all of the reproductive functions of progesterone.

PRAKO mice are infertile and have severely impaired ability to ovulate. Five heterozygote(A+/-) and homozygote (-/-) PRAKO littermate mice were mated with wild type males to examine fertility of PRAKO mice. Fertility studies were initiated at 6 weeks of age and were continued for a period of 2 months. All heterozygotes were fertile and had successful pregnancies. However, despite repeated detection of vaginal plugs, all female PRAKO homozygotes were infertile. To determine whether the infertility was due to an inability to ovulate, we initially treated 6 wild-type (A+/+), A+/- and A-/- mice at 8-10 weeks of age with PMSG (5IU) followed by HCG (5IU) 48 hours later. 24 hours after HCG treatment, oocytes were flushed from the oviduct and counted. In the case of the wild type and heterozygotes all animals ovulated and produced a total of 95 and 90 oocytes respectively. However, only 1 of 6 A-/- mice ovulated and produced only 4 eggs indicating that ovulation is severely impaired if not completely absent in these mice. In order to optimize the superovulation regime, the experiment was repeated with 22-day old immature females and oocytes counted from individual mice of each genotype. These results (Table 1) confirmed normal superovulation in A+/+ and A+/- mice. However, all A-/- mice produced less than 15 oocytes confirming severely impaired but not absent ovulation. This finding is different to that of the PRKO mouse in which superovulation attempts have consistently shown that ovulation is absent (33).

PRAKO Genotype	# oocytes per mouse	# mice
+/+	49+/-10	4
+/-	56+/-12	18
-/-	10+/-5	9

Subsequent histological analysis (Figure 1) of the gonadotrophin stimulated ovaries of these mice removed 24 hours after HCG treatment indicates the presence of several unruptured mature follicles (UF, panel B) in contrast to wild type ovaries (panels A and C) which show the presence of several corpora lutea that are typical of an ovary that has recently ovulated. Analysis of a typical UF (panel D) at high power shows the presence of an intact oocyte that has not necrotized and appears similar to that previously observed in PRKO mice.

Hyperproliferation of uterine epithelium and lack of decidualization in PRAKO mice.
To examine uterine morphological responses to E and P treatment, 6 week old ovariectomized female wild type, PRAKO and PRKO mice (6 per group) were implanted twice with beeswax pellets containing E (20μg) and P (20mg), or with control pellets lacking hormone over a three week period. Uteri were then removed, sectioned and stained with H and E for histological analysis. The results in Figure 2 demonstrate that the luminal epithelium (LE) of treated PRAKO and PRKO mice show a hyperplastic and disorganized morphology compared to wild-type mice. Moderate inflammation was also observed in PRAKO mice. However, this aspect and stromal cell responses need further examination with BrdU labeling and inflammatory marker analysis. Nevertheless, our data to date clearly demonstrate that the A protein is essential for mediation of the antiproliferative effects of progesterone observed in the epithelial compartment of the uterus. Thus, while the B protein is clearly induced by E in uteri of PRAKO mice, PR B cannot block the proliferative effects of E on the epithelium.

We examined the decidual responses of PRAKO mice using estrogen and progesterone and stimulated the left uterine horn with a burred needle. The increases in uterine weight associated with decidualization were then compared in wild type, PRAKO homozygotes, PRAKO heterozygotes and PRKO mice (Figure 3). Both wild type and PRAKO heterozygotes showed a strong decidual response to stimulation, while PRAKO homozygotes and PRKO mice were unresponsive. Thus, the PRA protein is also essential for mediating the uterine decidual responses to progesterone.

Morphological responses of the mammary gland to E and P are normal in PRAKO mice.
Mice from each genotype (6 per group) were administered E and P or control pellets for 3 weeks as indicated above and the inguinal glands were removed and whole mounts stained with hematoxylin to examine developmental responses to E and P. The results in Figure 4 show extensive E+P dependent ductal branching in PRAKO mice that is similar to that observed in wild type mice but is strikingly absent from treated glands of PRKO mice. Although preliminary analysis of these whole mounts suggests that normal terminal end bud development and alveolar differentiation have taken place in the PRAKO glands, this will need to be confirmed with confocal microscopic and marker analysis. Thus, the PRB protein is sufficient to elicit the proliferative responses of the mammary ductal epithelium to progesterone and this process does not require functional expression of the PRA protein. Most important in the context of mammary tumor development, this model now allows us to determine whether selective expression of PRB alters the susceptibility of the mammary gland to tumorigenesis.

Conclusion.
We have demonstrated that PR is essential for pregnancy associated proliferation and differentiation of the mammary gland and we have indentified at least one target gene (D1cyclin) that acts downstream of PR to

mediate the proliferative effects of progesterone. We have also generates novel mouse mutant strains that selectively express either the A or B isoforms of PR. Using one of these strains, we demonstrate that the PRB protein mediates the physiological effects of progesterone in a tissue specific fashion and that the PR A and B proteins exert distinct physiological functions in manifesting the hormonal response to progesterone. This work is currently near completion for publication. Most importantly, we have demonstrated that the PRB protein alone is sufficient to elicit normal mammary gland morphological responses to progesterone. In light of the recent demonstration that transgenic overexpression of PRA in mammary epithelium causes abnormal proliferation of mammary epithelium (34), it will be very important to determine whether the PRA protein alone can induce mammary gland proliferation and differentiation to progesterone and whether changes in the susceptibility to carcinogens occurs with selective expression of either of these proteins. With regard to the latter question, we have initiated cross breeding of both the PRAKO and PRBKO mice to and FEV mouse strain to generate and in-bred mouse strain carrying these mutations so that the carcinogenesis studies can commence. We anticipate that a minimum of 6 generations of in-breeding (18 months) will be required in order to approach 100% strain penetrance. This approach is necessary because of the significant variation in mouse strain tumorigenic responses to carcinogens and the high susceptibility of the 129SV/EV/C57Bl strain (used for generation of all knock-out mice) to succumb to ovarian cancers in response to DMBA and NMU carcinogens used in mammary tumor models.

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Said, T.K., **Conneely, O.M.**, Medina, D., O'Malley, B.W., Lydon, J.P., (1997) Progesterone in addition to estrogen induces cyclin D1 expression in the mammary epithelial cell, in vivo. Endocrinology, 138: 3933-3939.

Mulac-Jericevic, B. and Conneely, O.M. (1998) Mice lacking progesterone receptor A isoform demonstrate tissue specific contribution of the PR isoforms to the reproductive effects of progesterone. Abstract #5 Serono Symposium on Ovulation: Evolving Scientific and Clinical Concepts. Sept. 24-27 Salt Lake City, Utah

Mulac-Jericevic, B., Lydon, J.P., DeMayo, F. and Conneely, O.M. (1997) Physiological Role of Progesterone Receptors in Breast Development. Abstract #145-1, DAMD Breast Cancer Meeting, Washington D.C. Nov.30-31.

Personnel funded by this project during the past four years.

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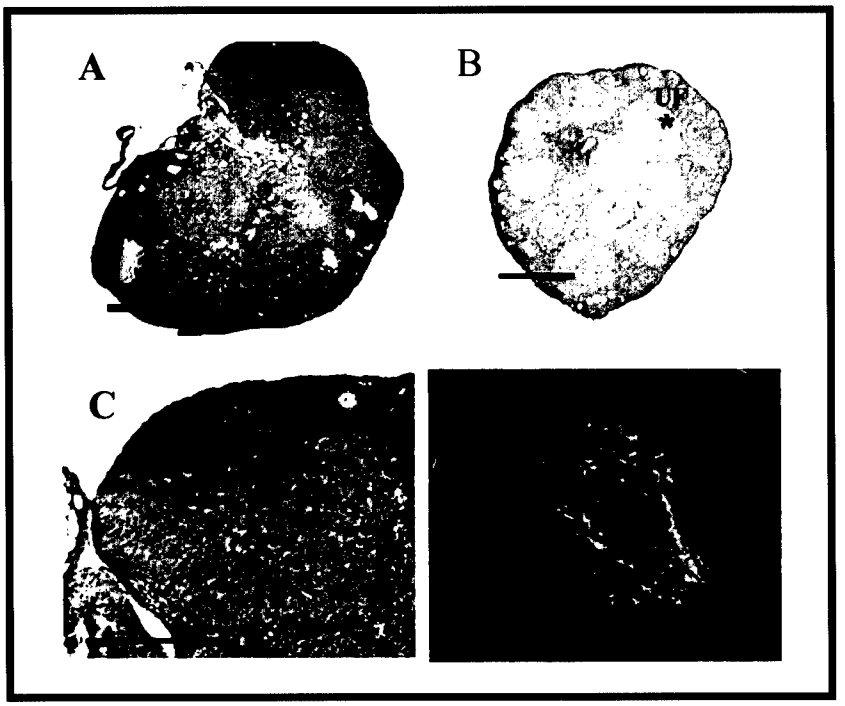

Figure 1. Hematoxylin and eosin stained 5um sections of wild type (WT, panel A and C) and PRAKO (panel B and D) ovaries from mice treated with PMSG (40IU) and HCG (50IU) and removed 24 hours after HCG. Scale bars, A and B (100um); C and D, 20um. Corpora lutea (CL) and unruptured follicles (UF)are indicated by asterix.

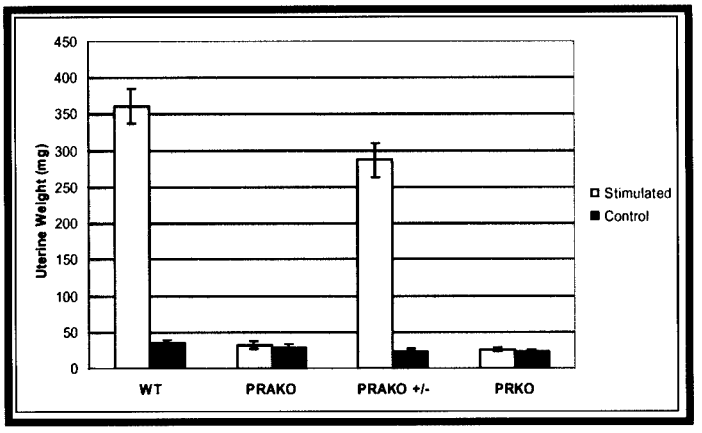

Figure 3. Analysis of uterine responses associated with decidualization stimulus in wild type (wt), PRAKO null mutants (PRAKO), PRAKO heterozygotes (PRAKO +/-) and PRKO null mutant mice. 6 mice were used per group.

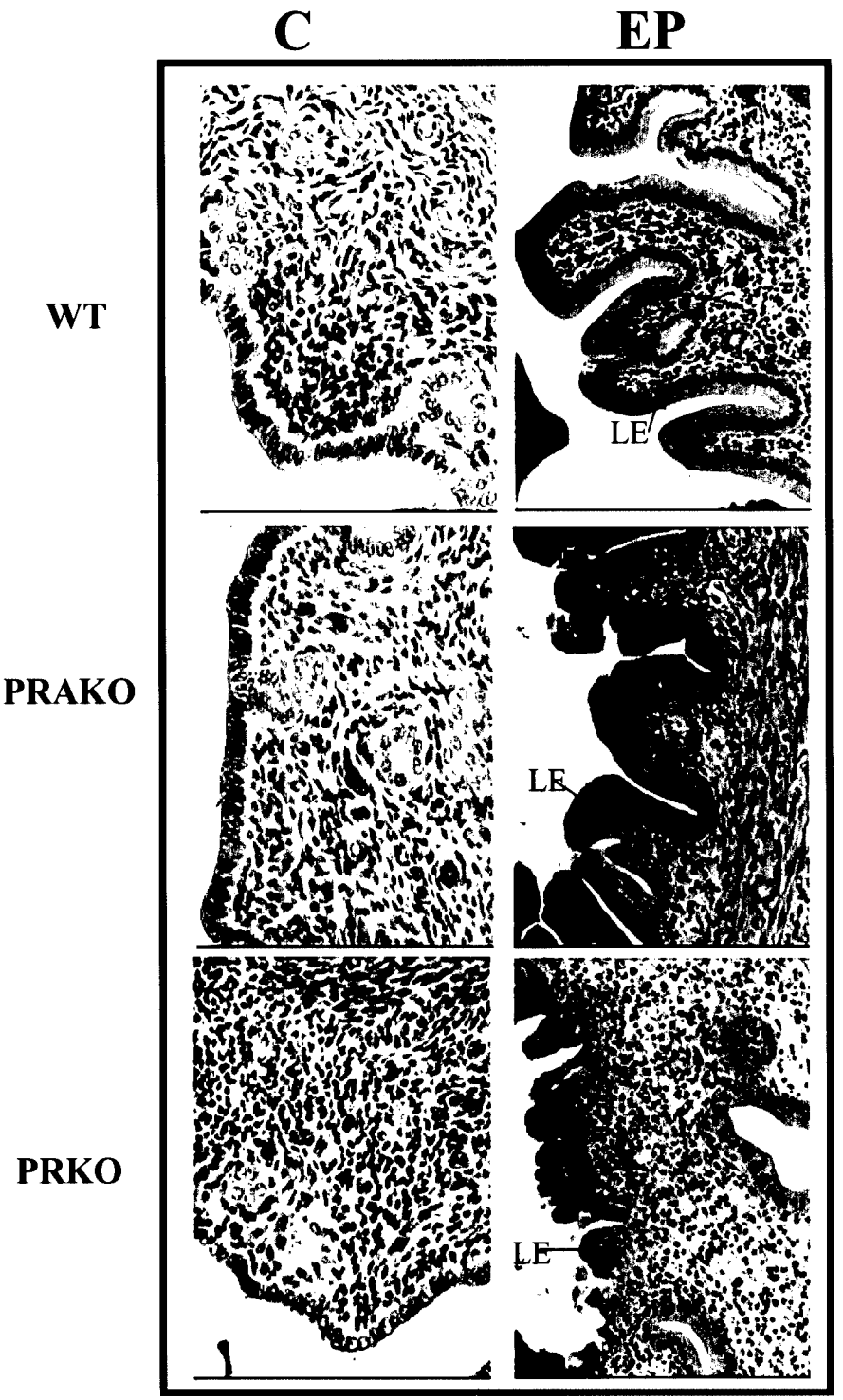

Figure 2. Histological analysis of H and E stained sections (5um) from control (C) and E+P treated (EP) uteri from WT, PRAKO and PRKO mice. Magnification, 20X.

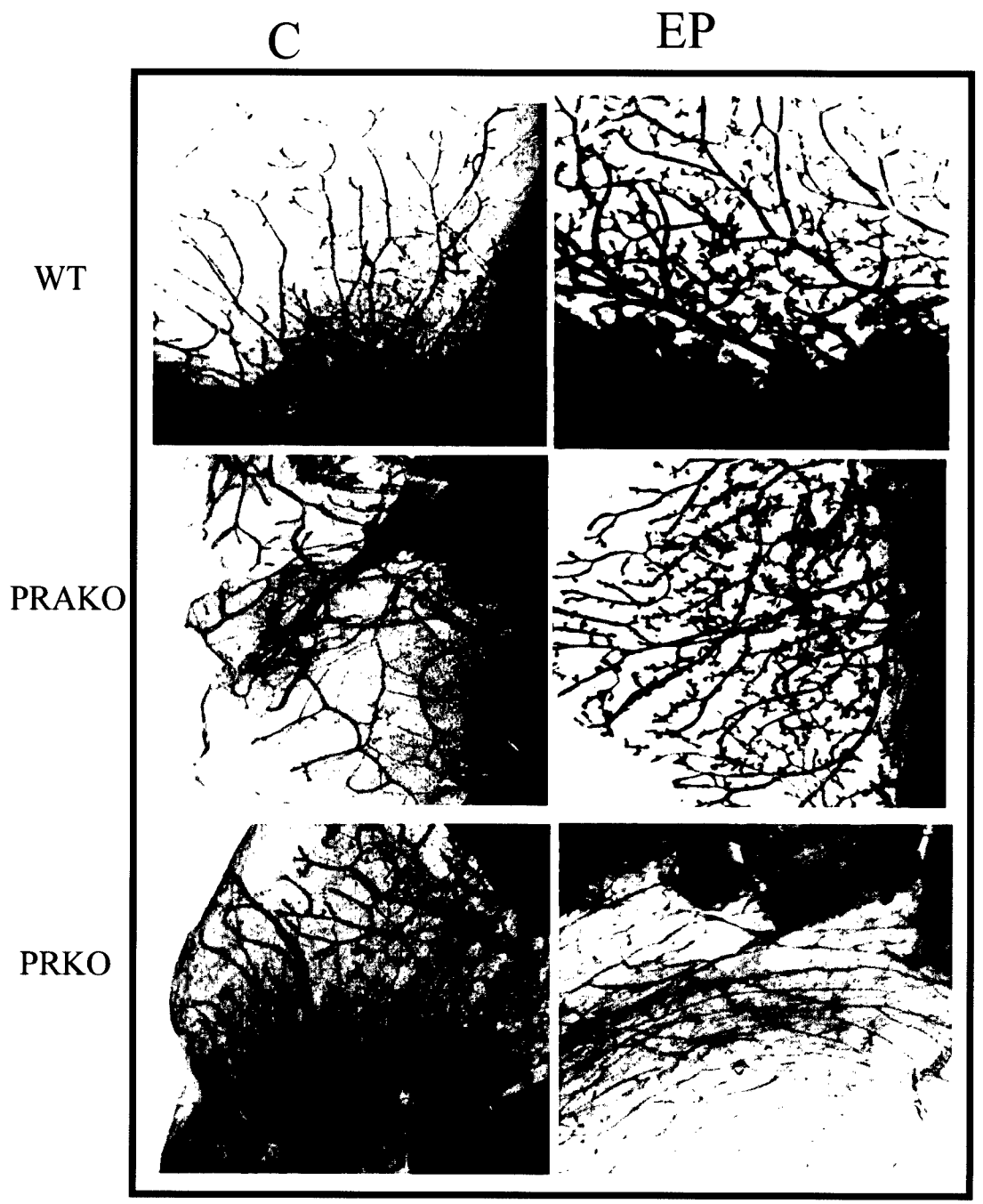

Figure 4. Morphological responses of the mammary gland of WT, PRAKO and PRKO mice to E+P treatment. Hematoxylin staqined whole mounts from the inguinal gland are shown. Magnification, 5X.

0013-7227/97/$03.00/0
Endocrinology

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ABSTRACT

Previous investigations, *in vitro*, have demonstrated that progestins can induce the transcription of the cell cycle regulator, cyclin D1, thereby suggesting that cyclin D1 may mediate, at the molecular level, the proposed mitogenic effects of progesterone during mammary epithelial cell proliferation. To extend these initial studies into an *in vivo* context, comparative cyclin D1 Northern and immunohistochemical analyses were performed on mammary gland tissue isolated from wild type (WT) females as well as from the recently reported progesterone receptor knockout (PRKO) mouse model. Northern analysis revealed that estrogen induced cyclin D1 expression, 5- to 7-fold over control levels, both in the WT and PRKO female. Immunohistochemistry demonstrated that, for both test groups, the number of mammary epithelial cells expressing cyclin D1 increased significantly as compared with control values, in response to estrogen. In the case of estrogen plus progesterone treatment, Northern analysis revealed that, in the WT gland, cyclin D1 transcription increased approximately 3-fold over estrogen induced levels, an increase that was paralleled by an equivalent increase in the number of mammary epithelial cells expressing cyclin D1. Conversely, under the same hormone regimen, the PRKO mammary gland did not exhibit a further increase in cyclin D1 induction over estrogen only levels. Finally, these studies not only demonstrate that in the mammary epithelial cell, both estrogen and progesterone can induce the expression of cyclin D1 but also show that this induction correlates with mammary gland proliferation in the mouse. (*Endocrinology* **138:** 3933–3939, 1997)

MAMMARY gland development is regulated by the interplay of systemic hormones, local growth factors, and the reciprocal relay of cell-cell interactions between the epithelium and the surrounding stroma (1). Until recently, it was generally assumed that the normal proliferation of the mammary gland epithelium, as well as the initiation and progression of mammary tumorigenesis, were dependent on the ovarian steroid, estrogen (E). This assumption was based largely on the established E-induced proliferative effects on the endometrial luminal and glandular epithelial cell; conversely, progesterone (P), based on its antiestrogenic effects in the endometrium, was assumed, by extension, to have antiproliferative effects in the mammary gland, (reviewed in Ref. 2 and references therein).

Although a number of previous rodent studies have implicated P-induced proliferative effects in the murine virgin (3) and pregnant (4, 3) mammary gland, as well as during mammary tumorigenesis in the rat and mouse (5, 6, 7), current reports exist suggesting that in the human gland P exhibits insignificant proliferative effects (8).

To define further the role of P in murine mammary gland proliferation and differentiation, we recently generated a progesterone receptor knockout (PRKO) mouse model in which the functional activity of the progesterone receptor (PR) was ablated through gene targeting techniques (9). Comparative whole mount analysis of mammary glands isolated from the ovariectomized PRKO and WT female, previously treated with exogenous E and P, revealed a striking phenotype in mammary epithelial ductal development and differentiation in the PRKO mouse. Specifically, the PRKO mammary gland failed to develop the typical pregnancy-associated epithelial ductal morphogenesis that consists of extensive dichotomous branching with attendant interductal lobuloalveolar development (1). These initial gross morphological studies unequivocally demonstrated a proliferative role, in addition to a differentiative role, for P in this tissue.

The downstream molecular targets and mechanisms by which P exerts these proliferative effects in the mammary gland epithelium are unknown. Previous studies in cultured T-47D cells have revealed that exogenous P can induce the transcription of the gene for cyclin D1, a cell cycle regulatory protein (10). Although these *in vitro* studies did not demonstrate that P-induced cyclin D1 expression resulted in sustained cell proliferation, these results were, nonetheless, the first to provide preliminary support for the proposal that the proliferative effects of P observed in the murine mammary epithelia *in vivo* (9) may be mediated, in part, by influencing cell cycle progression through modulation of cyclin D1 expression. In support of this proposal, the mammary gland phenotype of the cyclin D1 null mutant mouse (11, 12) exhibited a striking similarity to the PRKO mammary phenotype (9). Furthermore, recent cyclin D1 *in situ* localization studies on the normal murine mammary gland have demonstrated that the highest levels of cyclin D1 expression occur

Received March 7, 1997.
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* This work was supported by grants from the National Institutes of Health, HD-07858 (to B.W.O.) and CA-11944 (to D.M.); and from the Department of Defense, DAMD17-94-J-4254 (to O.M.C.).

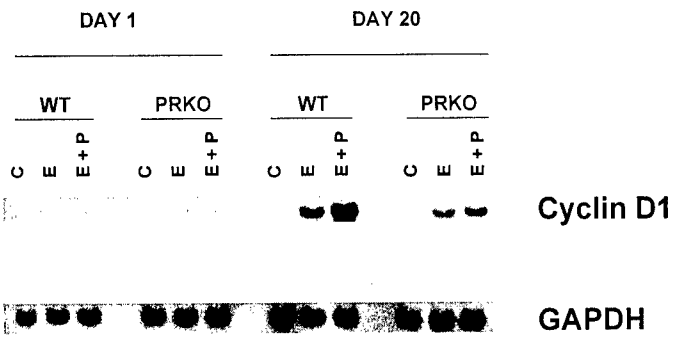

FIG. 1. Cyclin D1 mRNA induction in the WT and PRKO mammary gland, in response to sesame oil (C); estrogen (E); or estrogen plus progesterone (E + P), at day 1 and after day 20 of treatment. Following an overnight exposure to x-ray film, filters containing the cyclin D1 signal were stripped and subsequently probed with GAPDH. Typical GAPDH signals (see above) were achieved after 1.5 h of autoradiography. Using densitometric analysis GAPDH was used to normalize for variations in signal intensity. Each lane of the above Northern result represents an individual mouse and this result was typical of five other Northern blots that were performed in which the RNA samples were derived from a different set of individual mice, in each case.

during midpregnancy (13), a time period that correlates with the highest levels of serum P (14). Together, these observations implicate extensive overlapping functions between PR and cyclin D1 in mammary gland development and suggest that, during pregnancy, cyclin D1 may mediate, in part, the P-induced proliferative signal in the murine mammary gland.

To substantiate these observations in an *in vivo* context, we evaluated the comparative levels of cyclin D1 induction in the PRKO and WT type mouse, both at the RNA and protein level to determine whether E and/or P can modulate cyclin D1 expression in the proliferating murine mammary gland.

Materials and Methods

Animals and steroid hormone treatment

Two test groups, the 12-week-old PRKO and aged matched WT female mouse were used in these experiments. Two weeks before steroid hormone treatment (see below), animals in both test groups were bilaterally ovariectomized. Mammary glands were stimulated to proliferate with either a daily sc injection of either 1 μg of E or 1 μg of E plus 1 mg of P (E + P) for either one or 20 day(s) as described previously (9). Corresponding controls for both test groups at each time point consisted of daily administration of sesame oil (hormone vehicle). For Northern and histological analysis (see below), at each time point, six mice per test group were used for each hormone treatment. A corresponding number of control treated mice were also used. In all cases, animals were euthanized by anesthetizing the animal with a triple anesthetic combination: (ketamine: 37.5 mg/ml; xylazine: 1.9 mg/ml; and acepromazine: 0.37 mg/ml) (5 μl of anesthetic per gram of body weight). Finally, all animal surgical procedures and experimentation, described herein, met with the highest humane animal care in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals.

Northern analysis

At a given time point (see above), animals were killed and both inguinal glands were removed and pooled, before total RNA was isolated using the RNAzol B extraction method (Cinna/Biotecx, Laboratories Inc., Houston, TX). Fifteen micrograms of total RNA were electrophoresed through a denaturing 2.2 M formaldehyde gel of 1.2% agarose before transfer to Zetaprobe GT membranes (BioRad Laboratories, Hercules, CA) that were subsequently hybridized with a [α-^{32}P] dCTP radiolabeled random primed murine cyclin D1 probe. The full-length mouse cyclin D1 complementary DNA, (CYL-1), (15), was used as probe template, which was kindly provided by Dr. Charles J. Sherr. Subsequent hybridization and washing conditions have been previously described (16). To control for unequal loading and transfer of RNA, filters were routinely hybridized with a probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). To quantitate for cyclin D1 messenger RNA (mRNA) induction, densitometric analysis was performed on filters containing hybridization signals for cyclin D1 and subsequently for GAPDH using a Betascope 603 blot analyzer (Betagen, Inc., Waltham, MA).

TABLE 1. Cyclin D1 mRNA induction by estrogen (E) and estrogen plus progesterone (E+P) in the mammary epithelial cell following day 1 and day 20 of hormone treatment[a].

Treatment group	Day 1	Day 20
WT (C)	1.00	1.04 ± 0.12[a]
WT (E)	1.02 ± 0.18[a]	6.14 ± 1.20[b]
WT (E+P)	0.98 ± 0.17[a]	22.10 ± 4.14[b,c]
PRKO (C)	1.04 ± 0.12[a]	1.07 ± 0.13[a]
PRKO (E)	0.98 ± 0.15[a]	5.73 ± 1.32[b]
PRKO (E+P)	1.01 ± 0.15[a]	5.93 ± 1.41[b]

Five independent northerns were scanned by densitometry and normalized to the GAPDH signal before cyclin D1 induction for a given treatment group was expressed as an average fold increase (± SD) relative to the wild-type control group WT (C) treated with sesame oil after day 1 of treatment. Groups with superscript *a* are not significantly different from WT (C) at day 1; groups with different superscripts from each other are significantly different ($P < 0.001$). Statistical analysis was done by paired Student's *t* test.

Immunohistochemistry

Administration of 5-bromo-2-deoxyuridine (BrdU). Two hours before sacrifice, each animal received an ip injection of BrdU (70 μg of BrdU (Sigma)/g BW). Following BrdU labeling, animals were killed and both inguinal glands were dissected out, carefully flattened on glass slides, fixed in 10% buffered formalin for 8 h, followed by a 3-min wash in ordinary tap water, before long-term storage in 70% ethanol. Following fixation, mammary tissue was embedded in paraffin before being sectioned (4 μm) for either standard hematoxylin and eosin staining, or for immunohistochemical staining (see below).

BrdU and cyclin D1 immunostaining

Before immunostaining, tissue sections were deparaffinized and blocked as described earlier (17). BrdU immunohistochemistry was performed using the Cell Proliferation Kit from Amersham Life Science Inc. (Arlington Heights, IL) and by following the manufacturer's protocol. For each tissue section, cell counting consisted of counting the number of BrdU staining cells in a random field of 1000 cells. The average number of BrdU staining cells in a given tissue section was obtained by taking the average obtained from counting three separate fields of 1000 cells per section. Representative sections from each inguinal gland were used in these studies.

Following deparaffinization and blocking, cyclin D1 immunostaining was performed by incubating sections with a rabbit anticyclin D1 polyclonal antibody (Upstate Biotechnology Inc., Lake Placid, NY) (1:50 dilution) for 30 min, in a humidified chamber, at 40 C. Sections were subsequently washed three times in Tris buffer (Tris-HCl, pH 7.5, 0.9% sodium chloride and Tween-20) before incubation with an antirabbit biotinylated second antibody (1:500 dilution) for 15 min, at 40 C. Following three washes with Tris buffer, sections were incubated with the Vectastain ABC reagent (Vector Laboratories Inc., Burlingame, CA) (1:80 dilution) for 12 min, at 40 C. After three washes in Tris buffer, tissue sections were incubated in 3,3'-diaminobenzidine (Vector Laboratories Inc.) for 8 min, in the dark, at room temperature. Sections were subsequently counterstained with 0.1% methyl green for 20 seconds, followed by two washes with distilled water, before sequential dehydration in 95%, 100% ethanol, and xylene. Finally, sections were mounted with

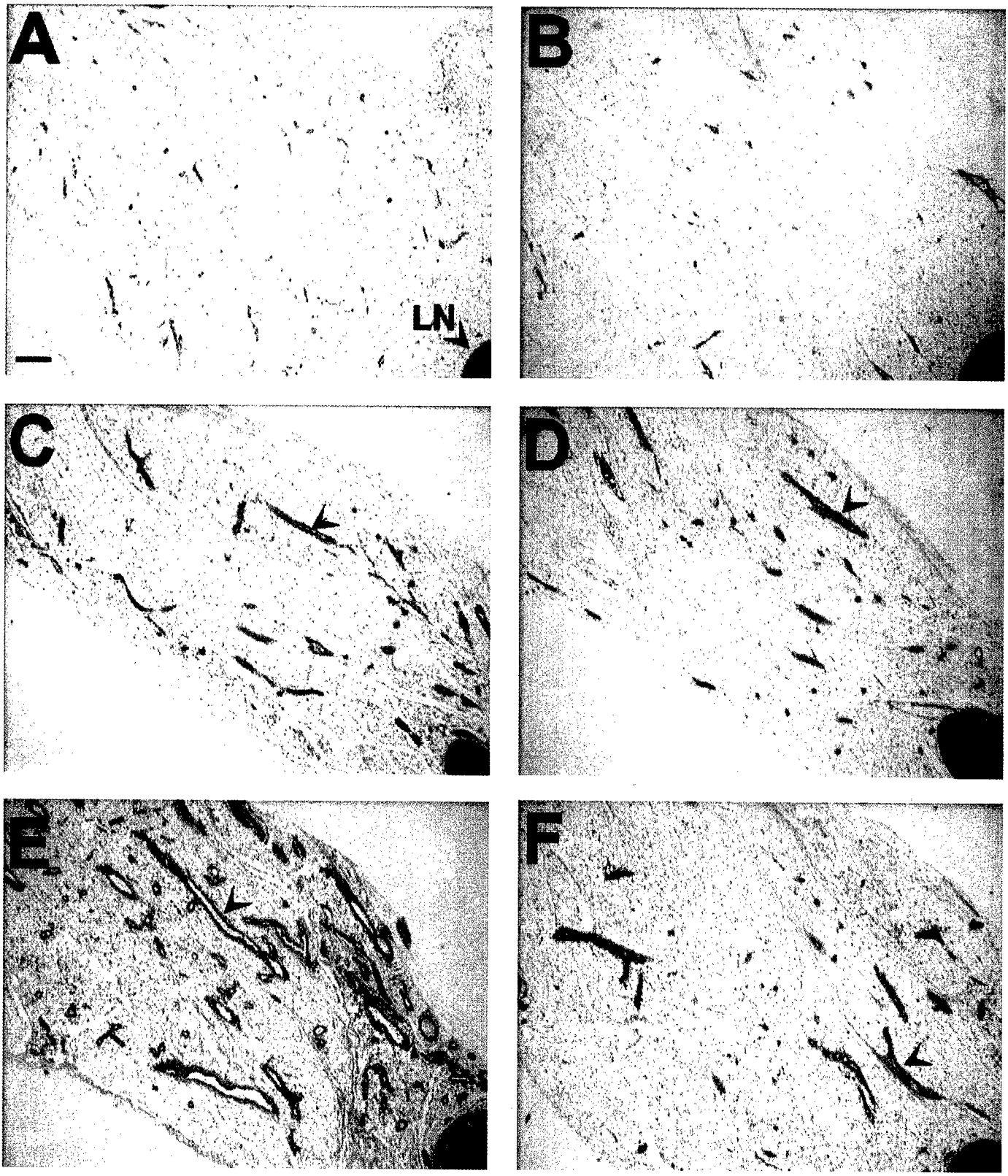

FIG. 2. Mammary epithelial ductal proliferation in the WT and PRKO ovariectomized mouse, after 20 days of hormone treatment. The panels show histological sections of the inguinal fat pad with the lymph node (LN) proximal to the nipple as a reference point. A and B, Lack of ductal proliferation in the WT and PRKO mouse respectively, after treatment with sesame oil (control). C and D, Typical transverse sections of mammary glands derived from WT and PRKO mice respectively, following E treatment alone. Note the increase in the number of ductal structures (*arrowhead*) in both test groups, as compared with corresponding controls (A and B). E and F, Degree of ductal proliferation in the WT and PRKO mammary gland, following E + P treatment. Note the striking increase in epithelial ductal proliferation in the WT (*arrowhead*) as compared with the PRKO gland. All sections (4 μm) were stained with hematoxylin and eosin, the *scale bar* represents 300 μm.

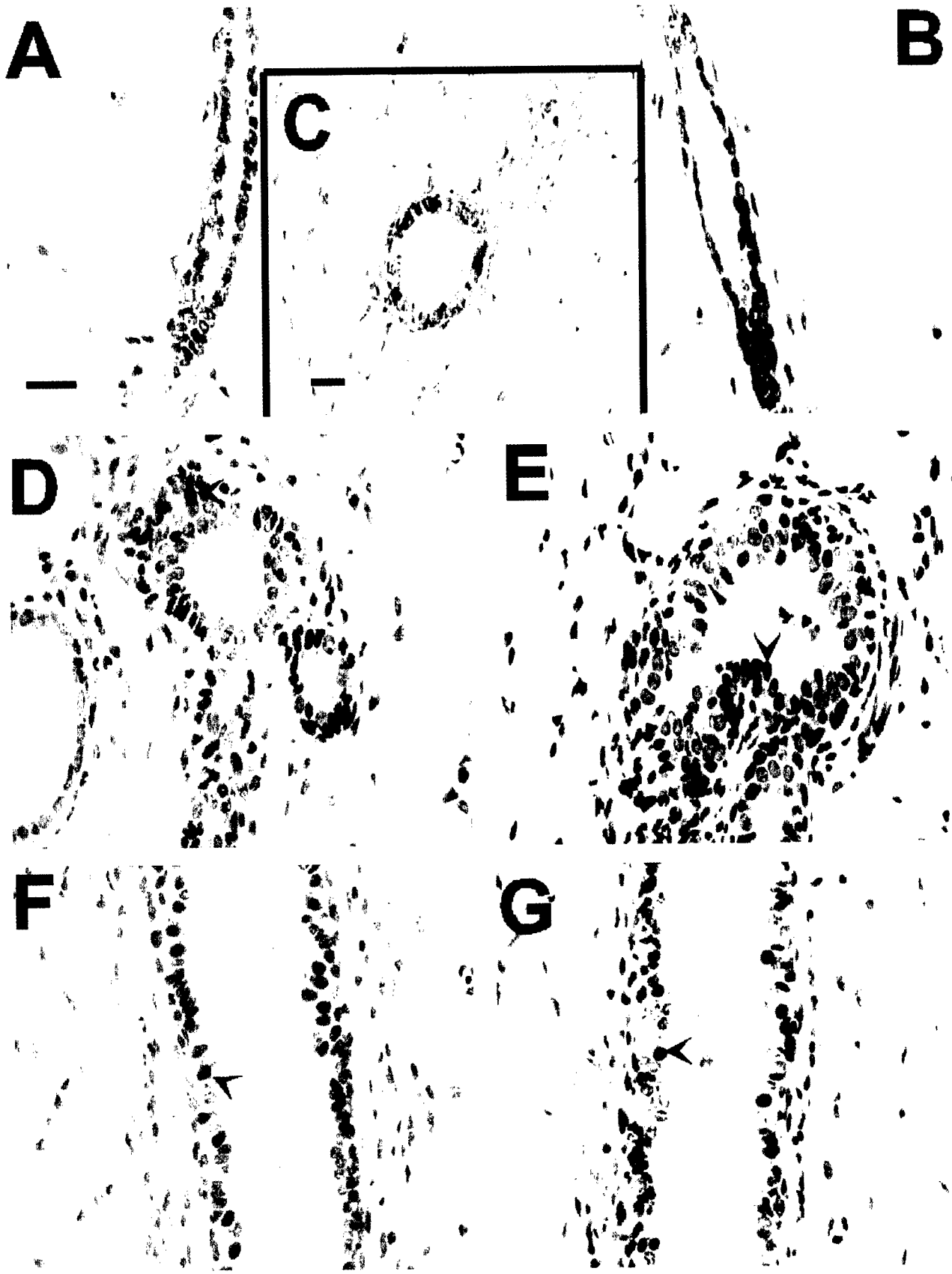

FIG. 3. Cyclin D1 immunohistochemistry of mammary glands derived from ovariectomized WT and PRKO mice, following 20 days of hormone treatment. Mammary glands isolated from WT (A) and PRKO (B) animals did not exhibit cyclin D1 protein induction, following sesame oil administration (control). C, Degree of background staining, in the absence of primary antibody, of epithelial ducts of a WT gland treated with

Permount and coverslipped. Control sections consisted of a similar protocol as above, except that the primary antibody was excluded. The average number of cells expressing cyclin D1 per section were scored as described above. For cell counting, cyclin D1 immunostaining was classified as either low or high intensity; only high intensity immunopositive cells were scored in this study.

Results

Induction of cyclin D1 transcription by E and P

To determine whether cyclin D1 transcription was modulated by E and/or E + P treatment, Northern analysis was performed on mammary tissue RNA isolated from the WT and PRKO female. Figure 1 shows that after 1 day of either E or E + P treatment, the level of cyclin D1 expression did not significantly differ between the WT and PRKO treatment groups. Following 20 days of hormone treatment, in the case of the WT and PRKO mouse, E alone was shown to induce cyclin D1 expression 5- to 7-fold over control values in both WT and PRKO mice (Table 1). In the case of E + P stimulation, for the WT gland, cyclin D1 RNA levels were further augmented 3- to 4-fold over levels attained by E stimulation alone. Although the levels of cyclin D1 induction in the E-treated PRKO mouse did not differ significantly from the E treated WT, P failed to increase the level of cyclin D1 transcription over E only values in the PRKO mouse.

Cyclin D1 protein induction in response to E and P

To determine whether the induction of cyclin D1 mRNA by E and P was reflected in a corresponding increase in the number of cells expressing cyclin D1 proteins, cyclin D1 immunohistochemistry was performed on mammary tissue sections after 20 days of hormone treatment. Representative mammary tissue sections, stained with hematoxylin and eosin, revealed that in the case of controls for both test groups, the number and size of mammary epithelial ducts was small (Fig. 2, A and B). In contrast, 20 days of daily E administration resulted in a significant and equivalent increase in the number of epithelial ducts in both the WT and PRKO test groups (Fig. 2, C and D). In the case of E + P treatment, the WT test group exhibited an additional increase in the number and size of epithelial ducts as compared with the corresponding PRKO test group, which did not reveal a further increase in ductal number over E only values (Fig. 2; compare panels E and F).

Cyclin D1 immunostaining revealed that the control glands for both WT and PRKO groups did not express cyclin D1 (Fig. 3, A and B); C shows, in the absence of primary antibody, the background staining of epithelial ducts of WT glands previously treated with E + P for 20 days. In the case of E stimulation, cyclin D1 expression was shown to be exclusively in the nucleus of the mammary epithelial cell in both test groups (Fig. 3, D and E). Cell counting demonstrated that the percentage of epithelial cells expressing cyclin D1 was approximately equivalent for both E treated WT and PRKO test groups (see Table 2). E + P treatment significantly increased the number of cells expressing cyclin D1 in the WT mammary gland (Fig. 3F and Table 2). Under the same hormone regimen, the PRKO mammary gland did not exhibit an additional increase in the number of cyclin D1 expressing cells, as compared with E treatment alone (Fig. 3, compare panels G and E; and Table 2).

TABLE 2. Cyclin D1 protein induction by estrogen (E), and estrogen plus progesterone (E+P), in the mammary epithelial cell.

Treatment group	Average number of cells expressing cyclin D1 per 1000 cells
WT (C)	ND
WT (E)	77 ± 9.3[a]
WT (E+P)	277 ± 15.4
PRKO (C)	ND
PRKO (E)	71 ± 8.1
PRKO (E+P)	80 ± 9.1

ND, Not detected.
[a] Mean ± SD (n = six animals).

BrdU immunohistochemistry was performed to evaluate the location and number of mammary gland cells in S-phase and undergoing active cell division during this hormone treatment. Figure 4 demonstrates the complete absence of BrdU immunostaining in the glands of WT and PRKO control groups (A and B). Following E treatment, cells containing BrdU immunoreactivity were detected at equivalent levels in the WT and PRKO mammary epithelial cell (Fig. 4, C and D; and Table 3). In this case, the number of BrdU containing cells corresponded closely to the number of cyclin D1 expressing cells (compare Tables 2 and 3). E + P treatment resulted in a significant increase in the number of cells staining for BrdU in the WT mammary gland (Fig. 4E and Table 3). In contrast, the PRKO mammary gland did not exhibit any further increase in BrdU immunoreactivity. Cell counting revealed a close correspondence between the number cells staining for BrdU and the number of cells expressing cyclin D1 in the E + P treated WT and PRKO test groups (compare Tables 2 and 3).

Discussion

To evaluate the selective effects of E and P on cyclin D1 expression in the proliferating murine mammary gland, ovariectomized WT and PRKO females were treated with either E or E + P for 20 days. In the case of E + P treatment, we have previously shown that this hormone regimen can elicit a morphological pregnancy phenotype in the WT mammary gland (9). In the studies described herein, E treatment alone induced both cyclin D1 RNA and protein levels in the WT and PRKO mammary gland. This result confirms recent *in vitro* studies in MCF-7 cells that have shown that exogenous E can induce cyclin D1 expression (18), as well as activate the resulting cyclin D1-cdk4 complex (18, 19); antiestrogens were shown to reverse this effect (19). Our immunohistochemistry studies revealed that cyclin D1 was expressed exclusively in the ductal epithelium in agreement with recent cyclin D1 localization studies (13).

E + P for 20 days. However, following E treatment, both WT (D) and PRKO (E) mammary glands revealed a significant number of cyclin D1 immunoreactive ductal epithelial cells (*arrowhead*). E + P treatment resulted in a further increase in the number of cyclin D1 expressing cells in the WT (F) but not in the PRKO (G) mammary gland. All sections were lightly counterstained with 0.1% methyl green. *Scale bars* in A and C represent 25 μm; the *scale bar* shown in A should be used as a reference magnification for the histology represented in panels B, D, E, F, and G.

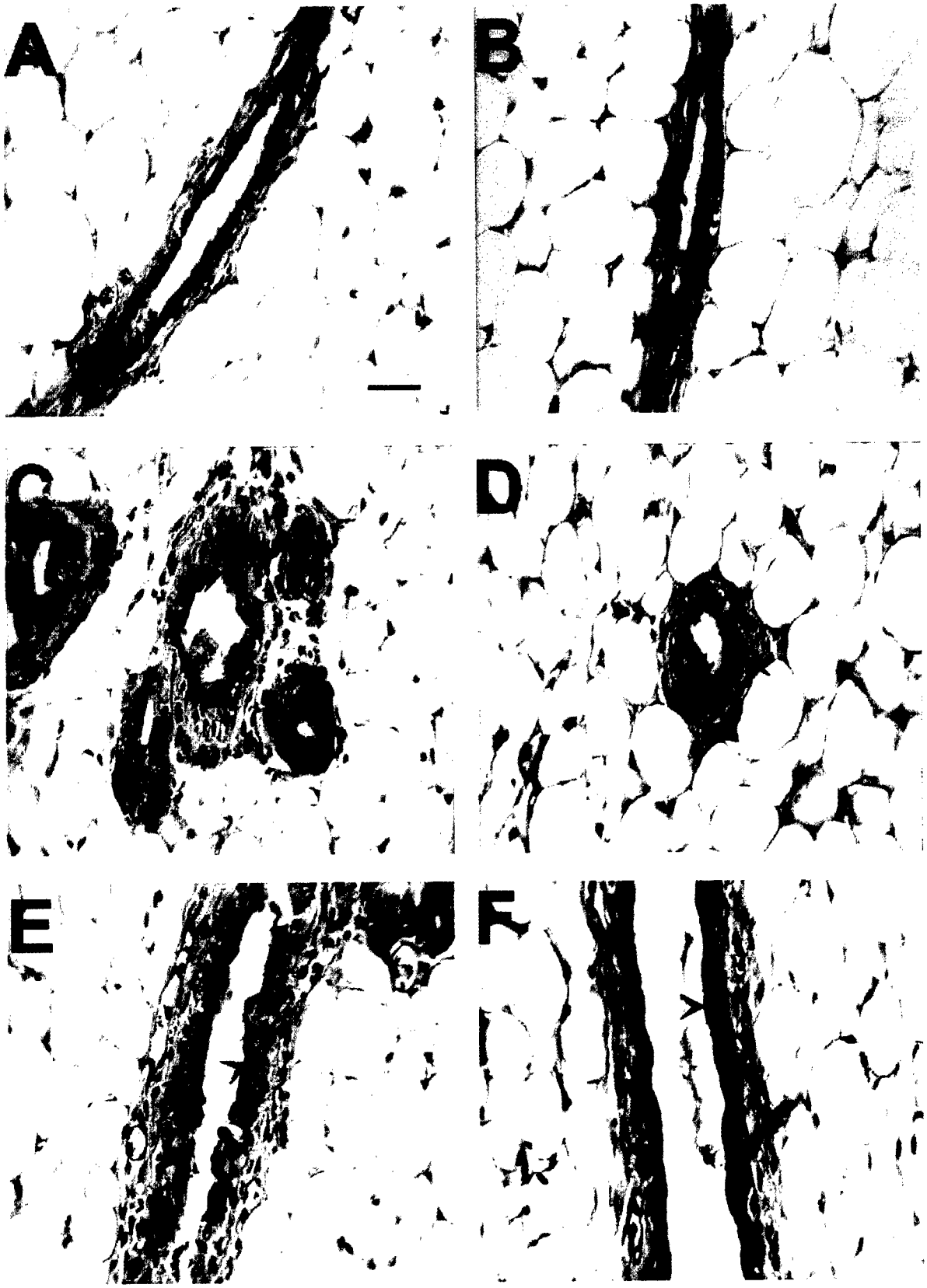

FIG. 4. BrdU incorporation into mammary epithelial cells of the WT and PRKO mammary gland, following 20 days of hormone treatment. Cells staining for BrdU incorporation were not detected in the WT (A) nor the PRKO (B) mammary gland, following sesame oil treatment (controls). Administration of E resulted in the appearance of a significant number of BrdU containing cells both in the WT (C) and PRKO (D) mammary epithelial cell layer. E + P treatment induced a further 3- to 4-fold increase in the number of BrdU staining cells for WT (E) mammary glands but not for the PRKO gland (F). Tissue sections were routinely lightly stained with hematoxylin following BrdU immunocytochemistry; *scale bar*, 25 μm.

TABLE 3. Mammary epithelial cells in S-phase following estrogen (E), and estrogen plus progesterone (E+P) treatment.

Treatment group	Average number of cells that stained for BrdU per 1000 cells
WT (C)	ND
WT (E)	104 ± 10.2[a]
WT (E+P)	313 ± 14.6
PRKO (C)	ND
PRKO (E)	99 ± 9.9
PRKO (E+P)	96 ± 9.7

ND, Not detected.
[a] Mean ± SD (n = six animals).

In the case of E + P treatment, the inclusion of P resulted in a 3- to 4-fold further increase in the number of epithelial cells expressing cyclin D1. In contrast, the addition of P did not further increase the number of cells expressing cyclin D1 in the PRKO mammary gland, thereby underscoring a requirement for PR. Unlike previous *in vitro* investigations, which failed to show a close correspondence between P-induced cyclin D1 induction and cell proliferation (10, 20), the *in vivo* studies described herein establish a strong correlation between P-stimulation of cyclin D1 expression and mammary epithelial cell proliferation. Obviously, a future research goal will be to unequivocally prove that the P-induced proliferative effects observed *in vivo* are dependent on cyclin D1 expression. As with most studies involving knockout mouse models, it could be argued that the PRKO mammary phenotype may be due, in part, to removal of PR function from progestin-target tissues other than the mammary gland. We have recently employed the mammary gland transplantation technique to address this question (21) and have shown that PRKO mammary epithelia transplanted into epithelia-free WT mammary stroma exhibits the same phenotypic responses to E and E + P as the intact PRKO gland, suggesting that the PRKO mammary gland phenotype is due to removal of PR function exclusively from the mammary gland.

In conclusion, although the proliferative effects of P on cultured breast cancer cells (10) and in the human mammary gland (8, 22) have yet to be established, we provide *in vivo* support for a significant proliferative role for P, in addition to E, in the murine mammary gland. Northern and immunohistochemical analyses revealed that cyclin D1 induction was stimulated by E and was further augmented by P. These observations suggest that induction of cyclin D1 could be responsible for coupling the E and/or the P extracellular signal(s) to the nuclear components of the cell cycle clock responsible for orchestrating mammary epithelial cell progression through the G1 phase of the cycle. Indeed, recent studies have shown that through direct physical association, cyclin D1 can specifically stimulate ER transactivation that, in turn, might induce PR expression (23). In combination with the studies described herein, these observations suggest an important cycle of regulation between the ER, the PR, cyclin D1, and the ER/cyclin D1 complex, the perturbation of which would be predicted to lead to undesirable mammary epithelial cell proliferation. Future investigations will consist of determining (a) whether those mammary epithelial cells exhibiting proliferation also express cyclin D1, ER and PR; and (b) whether ER and PR regulate cyclin D1 expression by direct interaction with promoter elements on the cyclin D1 gene, or indirectly, through intermediary factor(s) that have yet to be identified.

Acknowledgments

We extend special thanks to Marisela Mendoza, Gouqing Ge, and Liz Hopkins for their technical expertise. The secretarial assistance of Laura Birkens is gratefully acknowledged.

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