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PRINCIPAL INVESTIGATOR: Gopalan Shyamala, Ph.D.

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Our studies reveal that with increasing age mammary glands acquire a greater sensitivity to estrogen and progesterone which becomes very evident at ~8 months of age. The increased sensitivity to estrogen and progesterone is reflected both at the morphological level and in the expression of progesterone receptor. In transgenic mice carrying an imbalance in the native ratio of the two isoforms of progesterone receptor, mammary glands exhibit an altered sensitivity to estrogen and progesterone which is evident at both morphological level and in the pattern of cell proliferation. At the level of progesterone receptor expression, interestingly, the glands of young transgenic mice and ~8 month old mice somewhat resemble each other. These observations indicate that estrogen and progesterone and the two isoforms of progesterone receptor may be the key factors responsible for maintaining the mammary glands of adult females in a quiescent state and a derangement in these mechanisms can trigger inappropriate cell proliferation and hence, carcinogenesis. As such, they provide evidence to the stated hypothesis of this proposal, i.e. there is a need to target estrogen and progesterone and progesterone receptors as critical factors responsible for the observed relationships between the reproductive history and breast cancer risk.

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INTRODUCTION

(a) Background and Significance of the Research

Numerous epidemiological studies spanning nearly two decades have clearly established that, excluding the genetic background, reproductive history is an important and consistent "natural" risk factor associated with breast cancer. In particular, both early menarche and late age at menopause have been shown to be associated with an increased risk. Epidemiological studies have also shown that early (before or around age 20) full term pregnancy offers about a 50% protection against breast cancer but this protection is greatly reduced when pregnancy occurs at a later age and no longer apparent if it takes place after age 34. Taken together, these studies indicate that it is the total length of time between menarche and menopause and/or the total length of time between menarche and first pregnancy which is associated with the risk factor (1-3).

A distinguishing physiological characteristic of the female between the onset of menarche and menopause is the presence of functional ovaries which, during each menstrual cycle synthesize and secrete the female sex steroids, estrogen and progesterone (4,5). Accordingly, the total length of time between menarche and menopause can be translated as the total years to which the normal breast is exposed to estrogen and progesterone. Similarly, estrogen and progesterone are also the principal hormones of pregnancy and progesterone is absolutely essential for the maintenance of pregnancy to full term (6). This taken together with the fact that these ovarian steroids have been implicated in mammary cancer for more than half a century, argues the need to target estrogen and progesterone as key factors responsible for the observed relationships between the reproductive history and the breast cancer risk.

Extensive studies spanning over two decades have clearly established that almost all the effects of steroid hormones, including estrogen and progesterone, are mediated through their respective receptors present in the cells which are responsive to these hormones (7,8). There is also extensive documentation that in female sex organs the synthesis of progesterone receptors (which are required for progesterone action) require estrogen (8). In the human female, the growth of the breast accompanying each menstrual cycle is associated with the luteal phase or the progesterone dominant phase of the cycle (9-14). Similarly

the extensive growth of the breast which accompanies pregnancy also co-relates with the rising levels of progesterone (15). In genetically engineered mice lacking progesterone receptors (mice in which progesterone receptor gene has been "knocked out") there is an arrested development of the breast. This arrested development persists despite the administration of estrogen and progesterone (at doses and length of time) known to be sufficient to cause development in normal mice, analogous to that seen during pregnancy (16).

Among the various classes of steroid receptors, progesterone receptors are somewhat unique in that they exist in two molecular forms commonly referred to as the 'A' and 'B' form (17). In the same cell the 'A' and 'B' forms can have different functions and also the activity of the individual form of the receptor can vary between different types of cells (18, 19). Furthermore, depending on the cell, the 'A' form can either inhibit or enhance the activity of the 'B' form receptor (18). Therefore, to the extent age is a critical factor which influences the reproductive capability of the female, the epithelial cell of the breast in a young female represents a different phenotype from that of the older female. As such the relative activities of the 'A' and 'B' forms of the receptor and the modulation of the activity of the 'B' form by the 'A' form can vary between the "young" and the "old" cells; this, in turn will lend to differences in the type of genes expressed and their magnitude of expression between the "young " and "old" cells. In this context, it is important to note that the 'A' and 'B' forms of progesterone receptor have also been shown to modulate estrogen receptor and estrogen dependent gene expression (20-22). Thus, an alteration in the relative activities of the 'A' and 'B' forms of progesterone receptor can conceivably affect both estrogen and progesterone dependent gene expression. Our hypothesis is that such differential gene expression may in fact be responsible for the age dependency associated with the beneficial effect of pregnancy on breast cancer and the risk associated with the total length of time between menarche and menopause.

We further hypothesize that the relative activities of either the 'A' or the 'B' form or both change due to age and reproductive status and such alterations result in an imbalance in the relative activities of the growth factors and growth inhibitors, a phenomenon often associated with carcinogenesis or initiation of tumor formation. In this context it is noteworthy that while both pregnancy and carcinogenesis represent extensive epithelial cell proliferation, initiated in the relatively quiescent breast of the adult female, only the growth accompanying pregnancy is followed by terminal differentiation of the epithelial cells. Thus, it is

possible that carcinogens can mimic the effects of endogenous cellular factors responsible for the epithelial growth occurring during pregnancy but fail to achieve terminal differentiation, a process perhaps intrinsic only to cells replicating in response to progesterone. Therefore, an investigation of progesterone and hence, progesterone receptor action on normal breast is essential to understand the mechanisms involved in the growth and differentiation of normal breast and this is also a pre-requisite for understanding the role of progesterone in the etiology of breast cancer. This is because cancer arises from the normal breast and its hall mark is unrestricted growth resulting from derangement in the balance between normal growth and differentiation. Therefore, studies on tumor cells, while being informative with respect to their behavior, cannot allow an identification of pathways responsible for giving rise to tumor. To this end, the purpose of the research project is as follows:

(b) Purpose of the research:

1. To examine the effect of age on the ability of normal mammary glands to undergo DNA synthesis in transgenic mice carrying either the 'A' or the 'B' form of progesterone receptor and determine if these exhibit differences from that of normal (wild type) mice.
2. To examine the effect of age on the ability of normal mammary glands to achieve pregnancy dependent differentiation and determine if this differs between the normal (wild type) mice and transgenic mice carrying either the 'A' or the 'B' form of progesterone receptor.
3. To examine the pattern of gene expression in mammary glands of transgenic mice carrying either the 'A' or the 'B' form of progesterone receptor and compare this with that of normal (wild type) mice and also determine if these change as a function of age.

BODY

(a) Rationale for studies performed in year 02

During year 01, we devoted our efforts primarily to specific aim #2. To meet this objective, we had originally proposed to breed separate groups of nulliparous females beginning at ~twelve weeks of age and, therefore, at eight week intervals up to fifty weeks of age. To assess differentiation, we proposed to examine wholemount preparations and histological sections of mammary glands from these various groups. However, once funding was obtained and we began to plan our experiments, it occurred to us

that with increasing age, the ability to become pregnant may be compromised which, in turn, may jeopardize the outcome of our experiments. Therefore, we decided to use an alternate experimental protocol which has been widely used to initiate differentiation analogous to that observed during the onset of pregnancy i.e. to ovariectomize the animal and then administer estrogen and progesterone for twenty-one days (23). These studies revealed that with increasing age, there was an increase in the degree of lobulo-alveolar development in response to estrogen and progesterone which was dramatically apparent in mice of ~8 months of age. Thus it appeared that at around 8 months of age, mammary glands might acquire a greater sensitivity to estrogen and progesterone. Since, the age (~8 months) in mice corresponds roughly to the age of a peri-menopausal human female, an increased sensitivity to ovarian steroids can result in relatively higher proliferation of epithelial cells. This, in turn, can increase the chances of both intrinsic mutagenesis and that resulting from exposure to carcinogens and thereby lead to cancer. An increased sensitivity to endogenous steroids also implies that with age there may be an increased sensitivity to environmental estrogens. As such, in the aging female, the higher risk for breast cancer may be at least in part due to an increased sensitivity to estrogen and progesterone for breast cancer. Thus, the observations made in year 01 were very pertinent to meeting the long-term objective of our proposal which was to identify the role of ovarian sex steroids, and in particular, the role of progesterone, in the etiology of breast cancer. However, to definitively conclude that with increasing age there was an increased sensitivity to ovarian steroids, we needed to examine another marker, preferably a biochemical/molecular marker. For this, we chose progesterone receptor (PR) since it can serve as a marker for both estrogen and progesterone action. This is because estrogen can increase PR expression in normal mammary glands (24) and PR is the mediator of progesterone action (25).

b. Experimental Methods:

(1) Steroid treatments: Nulliparous females of different ages were ovariectomized and after two weeks, administered one microgram of estrogen and one milligram of progesterone per day, for a total period of twenty-one days.

(2) Preparation of whole mounts: These were prepared as described previously (26) except for using carmine instead of hemotoxylin.

(3) Analysis for PR: PR was examined using an indirect immunofluorescence assayed with a secondary antibody conjugated to fluorescein isothiocyanate (FITC) (27). The antibody used for analysis of PR was prepared against synthetic peptide corresponding to amino acid residues 376-394, selected from the amino-terminal half of the mouse PR sequence (28); this antibody reacts with both the A and B forms of murine PR (27).

(4) Analysis for Proliferation Cell Nuclear Antigen (PCNA): Immunolocalization assays for PCNA were performed using an antibody conjugated directly to FITC (obtained from DAKO, Inc) using manufacturer's instructions.

(5) Determination of percent positive cells: The number of positive cells was determined by taking photographs of 4,6-diamidino-2-phenylindole (DAPI) and fluorescein labeled tissue sections using Kodak Trichrome ASA400 film. The number of epithelial cell nuclei was determined by counting DAPI stained cells forming ducts. The percent positive cells was determined by counting fluorescein positive cells in parallel photographs.

(c) Results:

1. Effect of age on expression of PR in mammary glands from wild type mice.

In previous studies (done in year 01), the sensitivity of mammary glands to estrogen and progesterone was significantly greater in 30-32 week old mice as compared to twelve or twenty week old mice using the degree of lobulo-alveolar development as an end point. This age dependent difference in the sensitivity of mammary glands to estrogen and progesterone also manifests with respect to PR. As shown in Figure 1 in mammary glands of both 20 and 30 week old females (ovariectomized and treated with estrogen and progesterone), PR was detected in both the single ducts (Panels A and C) and in lobulo-alveolar structures (Panels B and D). However, the intensity of staining in both the single ducts and ducts in lobulo-alveolar structures was greater in 30 week old mice (Compare Panel A with Panel C and Panel B with Panel D). Furthermore, while in the glands of 20 week old females, the intensity of staining was reduced in the lobulo-alveolar units as compared to single ducts (Compare Panel B with Panel A), this did not occur in the glands of 30 week old mice (Compare Panel C with Panel D). Also, in the lobulo-alveolar structures, there were more PR positive cells ($14.7 \pm 2.5\%$) in the 30 week old mouse, as compared to their counterparts in the 20 week old mouse ($9.3 \pm 1.7\%$).

Our laboratory has previously documented that while estrogen can increase the synthesis of PR in mammary glands, progesterone can oppose this action of estrogen by down-regulating PR (29). To this end, the higher level of PR in mammary glands of 30 week old mice could have resulted from either an increased sensitivity to estrogen or a decreased sensitivity to progesterone. To resolve this, we analyzed the mammary glands from mice of different age groups and in these studies, only estrogen was administered to ovariectomized animals. Panel A in Figure 2 shows the basal level of PR in ovariectomized young mice which was similar among the different age groups (data not shown). In all age groups, as expected, with estrogen treatment, there was an increase in the levels of PR; however, this increase was much more in 30 and 40 week old mice as compared to young (10 week old) mice (Compare Panels C and D with Panel B). These data provided definitive evidence for an age dependent increase in sensitivity to estrogen in normal mammary glands.

2. Effect of age on pregnancy dependent regulation of PR in mammary epithelial cells.

The foregoing studies established that (a) using our experimental protocol, chosen to mimic pregnancy dependent development, age had an impact on differentiation (lobulo-alveolar development) and (b) this manifests at ~30 weeks of age. Since 30 week old mice can become pregnant, to verify that our experimental protocol indeed reflected the situation encountered during pregnancy, we next examined the mammary glands of mice during early stages (6-8 days) of pregnancy. We chose this stage because the lobulo-alveolar development achieved due to estrogen and progesterone treatment roughly corresponds to this stage of pregnancy (data not shown). As shown in Figure 3, in the glands of the 30 week old pregnant mouse, the staining intensity for PR was more in both the single ducts (Compare Panel A with Panel C) and in ducts of lobulo-alveolar structures (Compare Panel B with panel D). There were also more PR positive cells in the lobulo-alveolar structures of the 30 week old pregnant mouse (15.7$\pm$2.9%) as compared to 20 week old mouse (5.3$\pm$1.2%). These studies confirmed the results obtained with estrogen and progesterone treatment.

3. Mammary glands of PR-A transgenics exhibit altered sensitivity to estrogen and progesterone.

One of the hypotheses underlying our proposal was that an alteration in the relative activities of the "A" and "B" forms of PR may affect both estrogen and progesterone dependent gene expression. Towards testing this, we had proposed to carry out studies with transgenic mice in which the native ratio

of the two forms of PR had been altered due to introduction of additional "A" form as transgene (30). In these transgenic mice (referred to as PR-A transgenics), mammary glands respond to estrogen and progesterone differently as compared to wild type mice. As shown in figure 4, in ovariectomized mice treated with estrogen and progesterone for 21 days, there is relatively less lobulo-alveolar development in the transgenic mice as compared to control mice (Compare Panel A and B). The status of PR in these mammary glands is shown in Figure 5. As may be seen, the intensity of staining was similar in the single ducts of both transgenic and wild type mice (Compare left hand side of Panel A with Panel B). In the wild type mice, as expected, the intensity of staining was less in the lobulo-alveolar structures and also very few PR positive cells were present (Compare left hand side of Panel A with right hand side). In contrast, in mammary glands of transgenic mice, in the lobulo-alveolar like structures (Panel C) the staining intensity was similar to that seen in the single ducts (Compare Panel B with Panel C). Furthermore, in these lobulo-alveolar like structures (Panel C), there were also far more PR positive cells as compared to wild type mice (Compare Panel B with Panel C).

4. Validation of PCNA as a marker for mammary epithelial proliferation.

Specific aim #1 of our proposal was to examine the effect of age on the ability of normal mammary glands to undergo DNA synthesis, which was selected as a marker for cell proliferation. For this we had proposed to examine the incorporation of radioactive thymidine by the epithelial cells. Recently, our institution has been under considerable pressure to use non-radioactive experimental approaches due to the prohibitive cost of disposing radioactive waste and also reduced availability of disposal sites. Therefore, to comply with the institutional guidelines, we chose to use PCNA as a marker for cell proliferation; PCNA is an essential component of the DNA replication machinery (31). Since our laboratory did not have previous experience with this experimental approach, initially we examined if this assay could be used with confidence in our studies. As shown in Figure 6 with our technique, PCNA was localized in the nuclei of mammary epithelial cells and the relative number of cells staining for PCNA followed the expected patterns. As such, in the mammary glands of pre-pubertal six week old mice, in which cell proliferation is known to be high in the terminal end bud structures (26, 32), large number ($27.7 \pm 5.5\%$) of cells stained for PCNA (Panel A). In the same glands, the mature duct (Panel B) had very few

(5.2±2.2%) PCNA positive cells. Panel C shows the lack of staining with the deletion of antibody and establishes the specificity of staining.

In the mammary ducts of young (10-18 weeks old) adult females, which are known to be relatively quiescent, very few (3.2 ±1.0%) of ductal cells stained for PCNA (Fig. 7, Panel A). In the mammary glands of pregnant females, in which epithelial cell proliferation had resumed, several PCNA positive cells were detected (Panel B). Also, interestingly, in the mammary glands of pregnant mice, a larger proportion (19.8±4.1%) of cells stained in the ducts (Panel B), as compared to cells in the lobulo-alveolar units (9.9±1.6%); (Panel C). It is well known that as normal cells achieve differentiation, their proliferative capacity decreases and this is most likely the underlying reason for the presence of fewer PCNA positive cells in the lobulo-alveolar units as compared to cells in single ducts. Regardless, the data shown in Figures 6 and 7 clearly established the validity of using PCNA as a marker for mammary epithelial cell proliferation.

5. Effect of age on PCNA expression.

Analysis for the pattern of PCNA staining in 30 week old pregnant mouse revealed 18.7±1.6% of cells staining in the ducts; this was similar to that observed in the ducts of 10 week old pregnant females (19.8±4.1%) discussed in the foregoing section. However, in the 30 week old pregnant mouse, as compared to the 10 week old pregnant mouse (9.9±1.6%; discussed in the foregoing section), a larger proportion (15.1±2.9%) of cells in the lobulo-alveolar units stained for PCNA.

6. Analysis for PCNA staining in the mammary glands of PR-A transgenic mice.

We have reported that in PR-A transgenic mice mammary morphogenesis is abnormal (29). In particular, mammary glands of these mice contain dysplasias indicating an imbalance in epithelial cell replicative homeostasis. Indeed, as shown in Figure 8, in the mammary glands of adult young mice, in the absence of pregnancy, a large number of PCNA positive cells were detected both in ducts and in other abnormal structures (Panels A-C).

CONCLUSIONS

In year 01, we established that with increasing age, there was an increase in lobulo-alveolar development in response to estrogen and progesterone which became readily apparent in mice beginning at ~8 months of age. During this past year, we have confirmed this observation using PR as a biochemical

marker for both estrogen and progesterone action. We have also established, using PR as a marker, that during pregnancy, there is an altered sensitivity to estrogen and progesterone in mammary glands of 30 week old mice, as compared to glands of pregnant mice of younger age. Furthermore, using PCNA expression as a marker for epithelial cell proliferation, we have also demonstrated that in the mammary glands of 30 week old mice, there may be more proliferative cells in the lobulo-alveolar structures as compared to their counterparts in younger females. A higher proportion of proliferative cells in the glands of 30 week old females also provide additional evidence for the increased sensitivity of these mammary glands to estrogen and progesterone. Most importantly, they suggest that the glands of older females may be more susceptible to carcinogensis, since it is well known that cellular proliferation is a pre-requisite for transformation of normal cells to a tumor phenotype (33). As such, to date, all our studies on the effect of age on the sensitivity of mammary glands to estrogen and progesterone argue strongly that in the aging female, who is at a higher risk for breast cancer, an increased sensitivity to estrogen and progesterone may be a contributing factor.

Our studies, so far, have also revealed that an imbalance in the expression and hence, activities of the two forms of PR can have adverse consequences with respect to mammary gland morphogenesis. Indeed in the mammary glands of young non-pregnant females carrying an imbalance in the native ratio of the two forms of PR (PR-A transgenics) there is a marked increase in the number of PCNA staining cells. Also, interestingly, in these young transgenic mice, there were far more PR positive cells in the lobulo-alveolar like structures as compared to their wild type counterpart and hence, resembling the mammary glands of old wild type mice. These data, therefore, provide evidence to our hypothesis that an imbalance in the expression/activities of the two forms of PR may be crucial for maintaining the epithelial cell replicative homeostasis in the adult female. Furthermore, they also provide indirect evidence for our hypothesis that the relative activities of the two forms of PR may change with age since the glands of older wild type females and younger PR-A transgenics somewhat resemble each other. It has been reported that in some human mammary tumors, there is an altered expression of the two forms of PR (34) but at present the precise consequence of this phenomenon is unknown. It has also been shown that in mammary tumor cells, the "A" and "B" forms may differ with respect to their activities (35). All these data lead us to conclude that estrogen and progesterone and the two isoforms of PR are key factors maintaining the

mammary glands of adult females in a quiescent state and an alteration in their activities may be responsible for the observed relationships between the reproductive history, age, and breast cancer risk.

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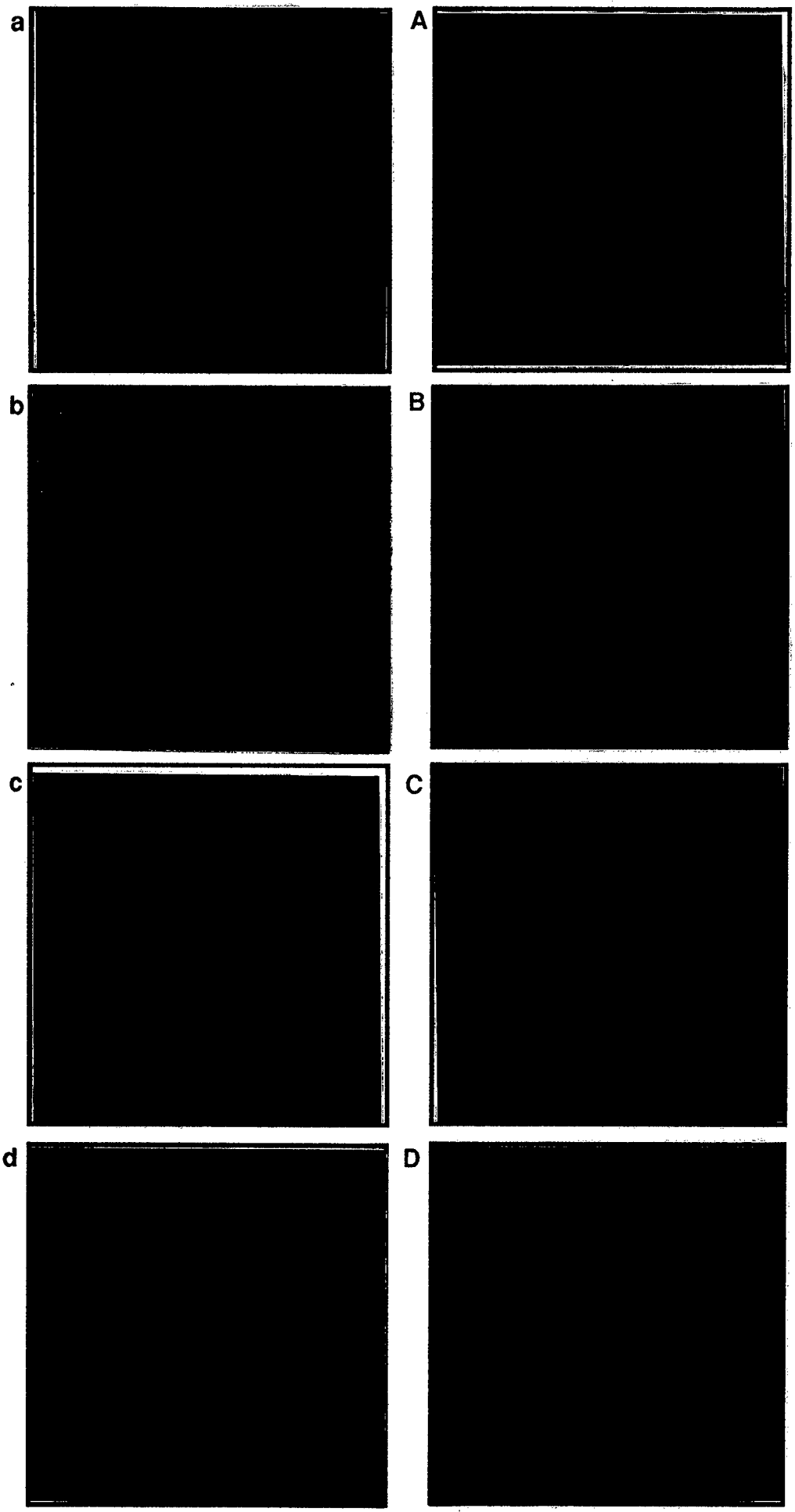
a
A
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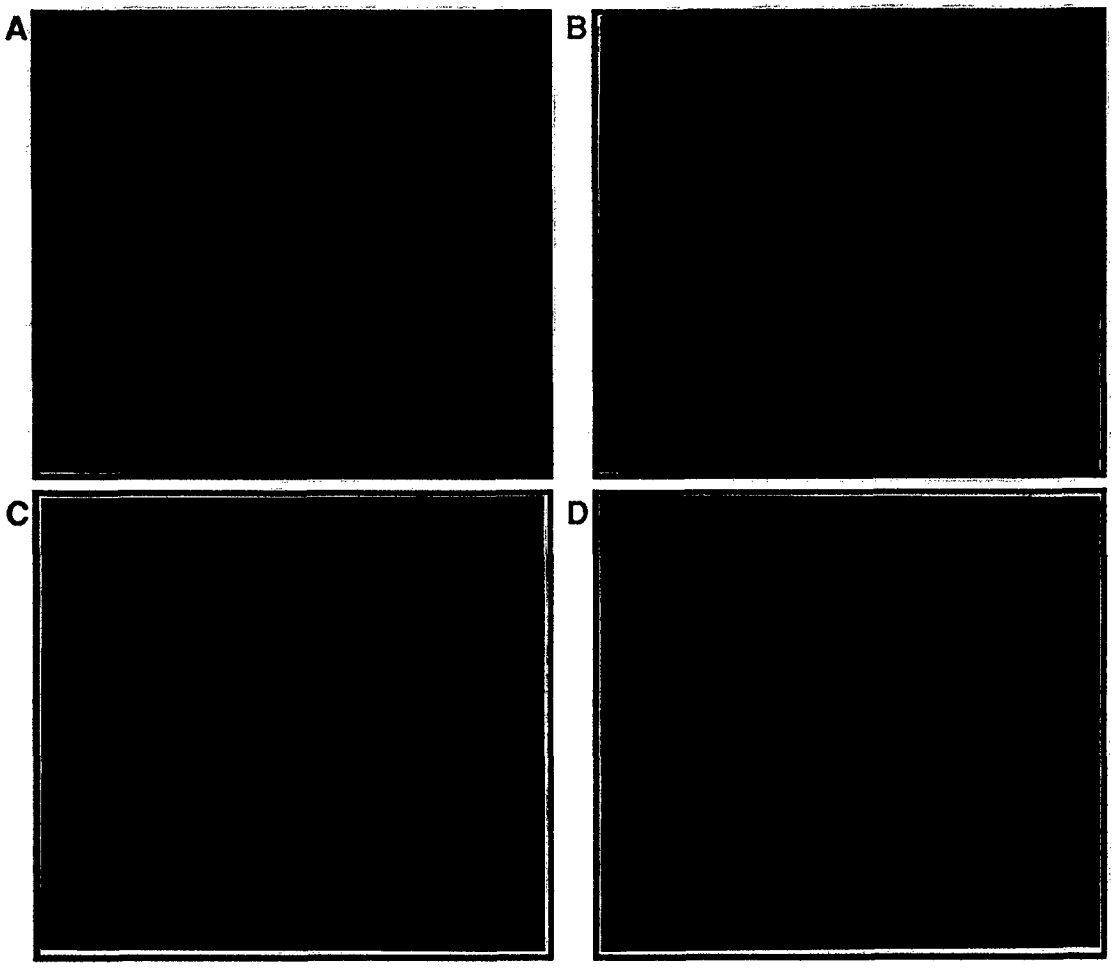
D

Legend for Figure 1

Analysis for immunoreactive PR in mammary glands of 20 week and 30 week old mice.

Mammary glands from 20 week old (Panels a, A and b, B) and 30 week old (Panels c, C and d, D) mice, ovariectomized and treated with estrogen and progesterone daily for 21 days, were analyzed for PR using an indirect immunolfuorescence assay. The green color in Panels A-D correspond to immunoreactive PR localized in the nuclei. Panels a-d show nuclei in the same sections stained blue with 4,6-diamidino-2-phenylindole.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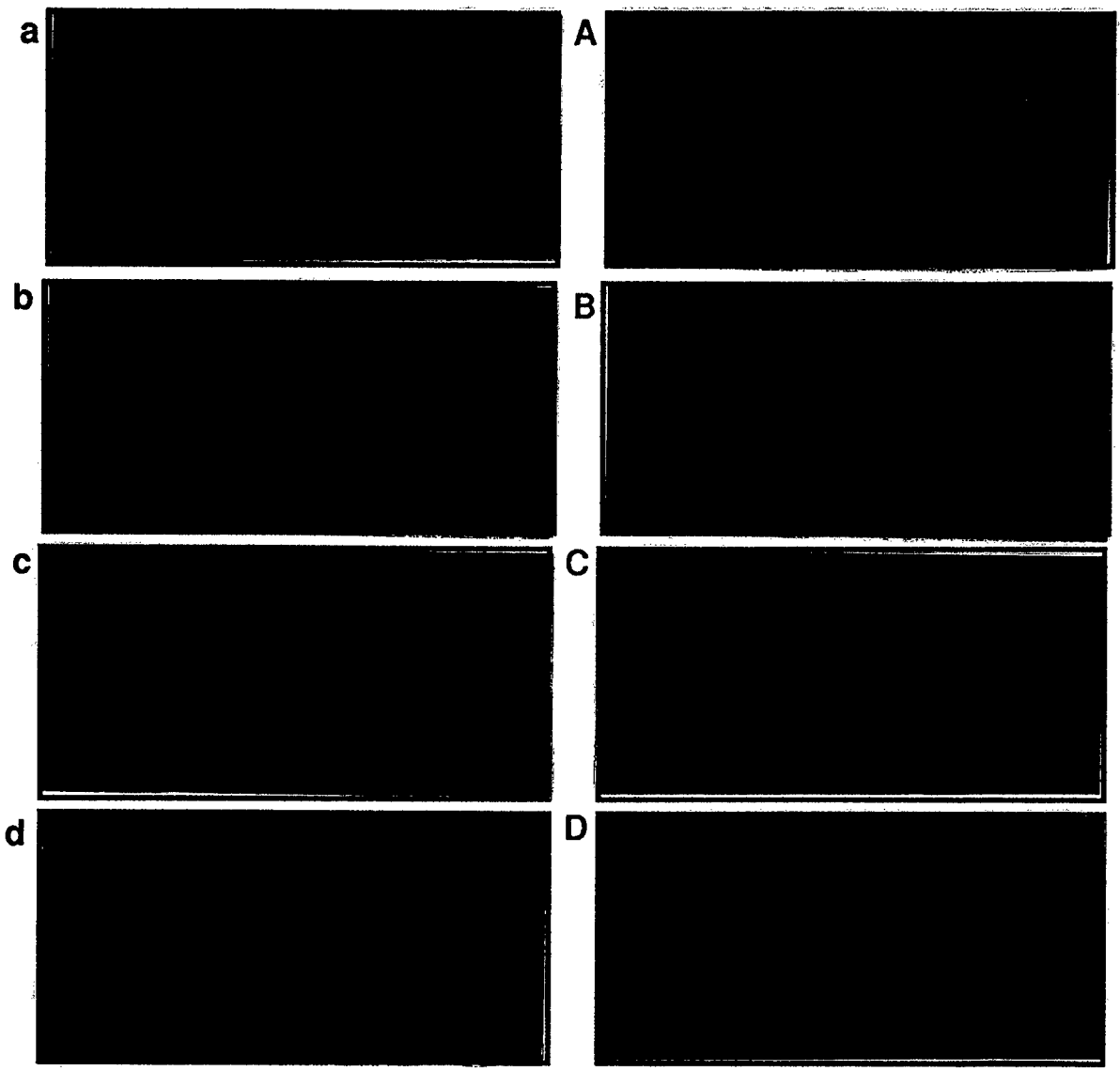
Legend for Figure 2

Effect of age on estrogen dependent expression of PR in mammary glands.

Panel A: Mammary glands from ovariectomized 10 week old mouse. Panel B: Mammary glands from 10 week old ovariectomized mouse treated with estrogen. Panel C: Mammary glands from 30 week old ovariectomized mouse treated with estrogen. Panel D: Mammary glands from 40 week old ovariectomized mouse treated with estrogen. Green color in each panel corresponds to immunoreactive PR.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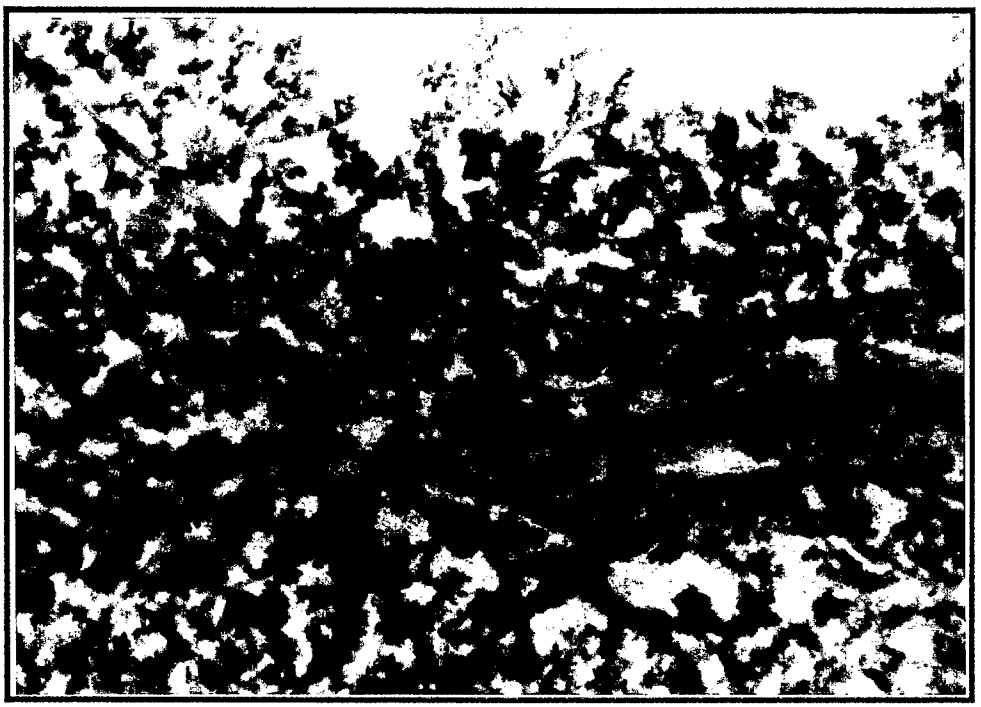
Legend for Figure 3

Expression of PR in mammary glands of pregnant mice:

Panels A-D show immunoreactive PR (green color) while panels a-d show nuclei stained blue in the same sections. Panels a, A and b, B show the single duct and ducts in lobulo-alveolar structures, respectively, in the mammary glands of 20 week old pregnant mouse. Panels c, C and d, D show the single duct and ducts in lobulo-alveolar structures, respectively, in the mammary glands of 30 week old pregnant mouse.

A

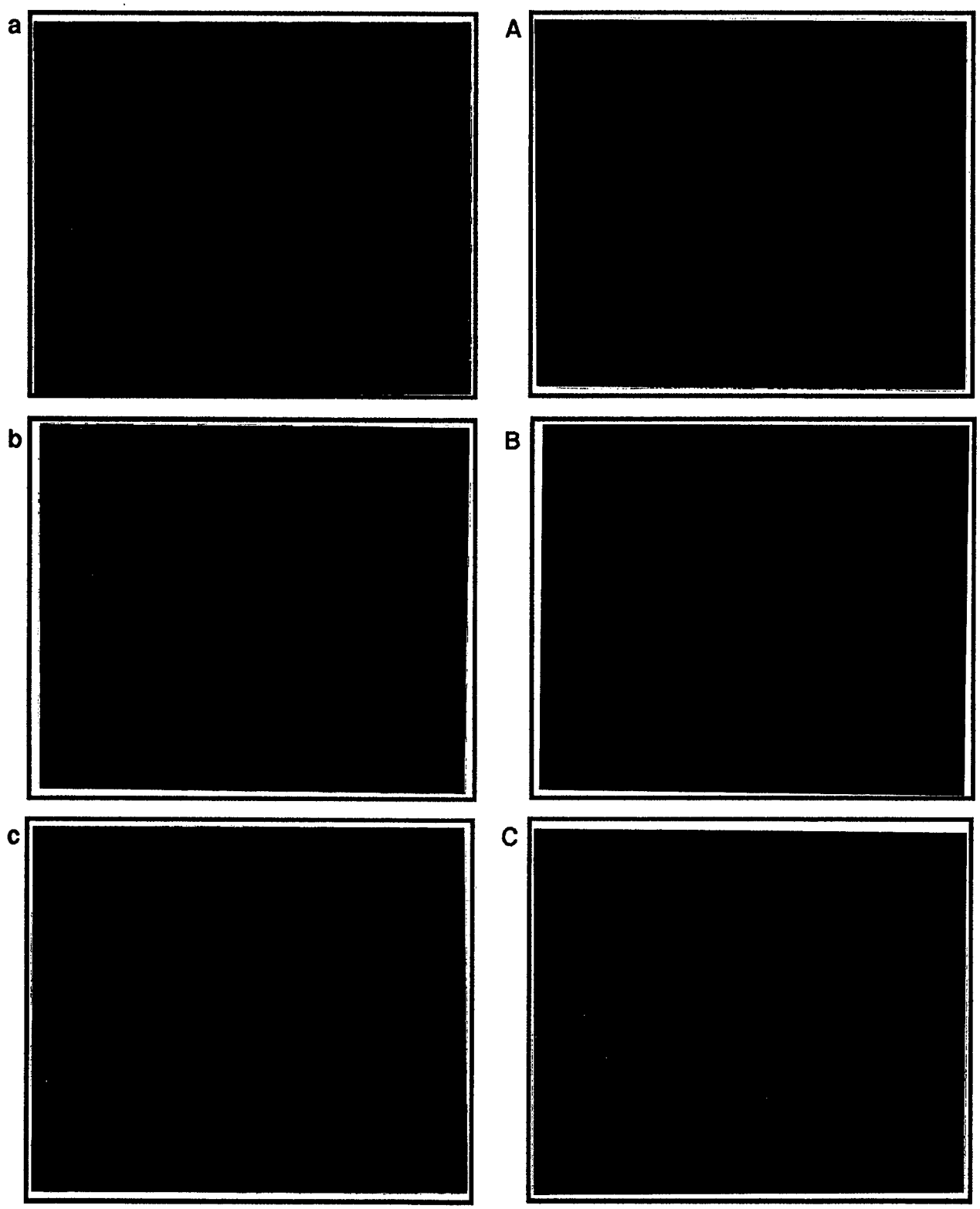


B

Legend for Figure 4

Photomicrographs of mammary gland whole mounts from young mice, ovariectomized and treated with estrogen and progesterone for 21 days.

Panel A: Mammary gland from wild type mouse. Panel B: Mammary gland from PR-A transgenic mouse.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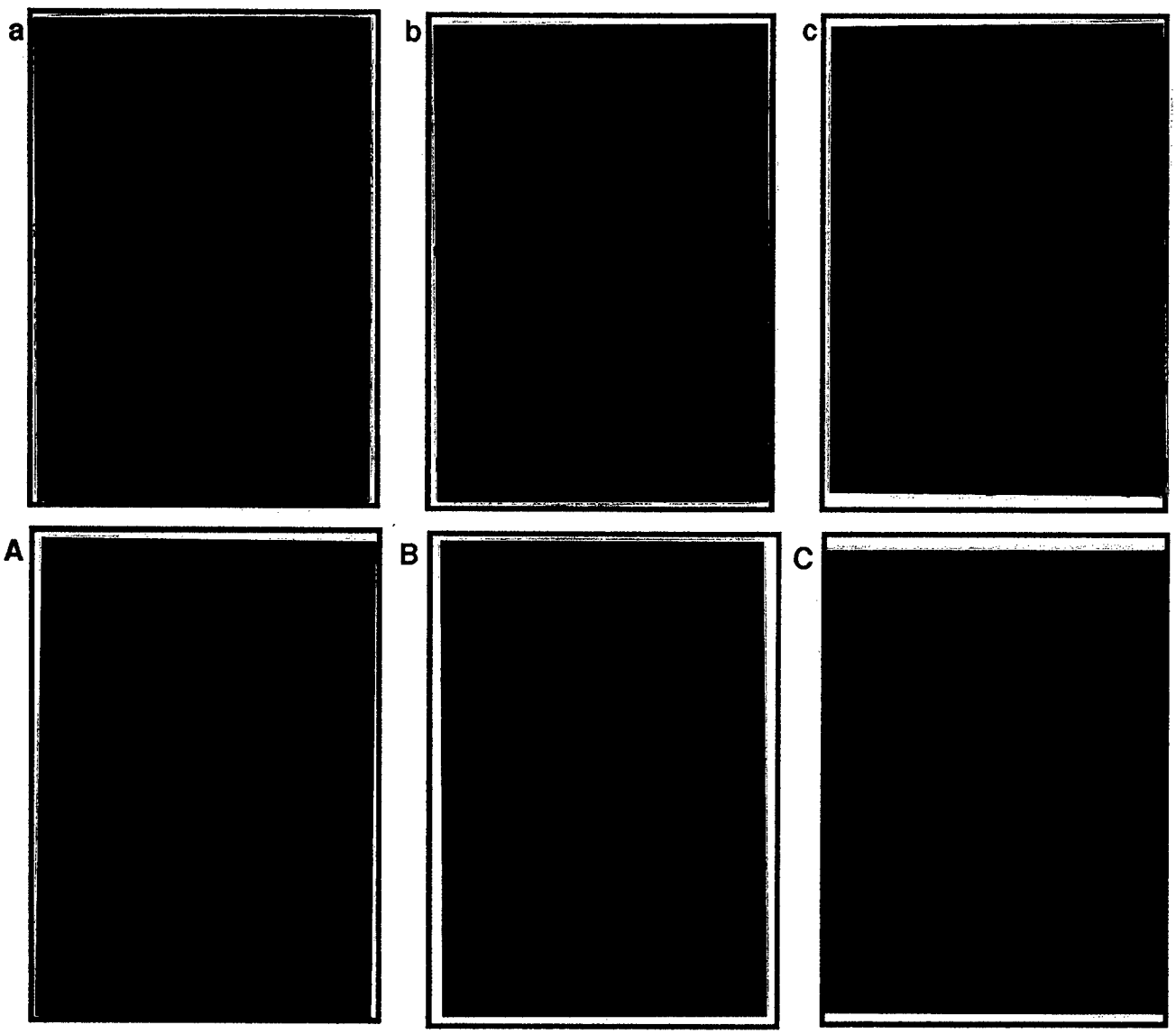
Legend for Figure 5

Analysis for PR in mammary glands of PR-A transgenic and control wild type mice.

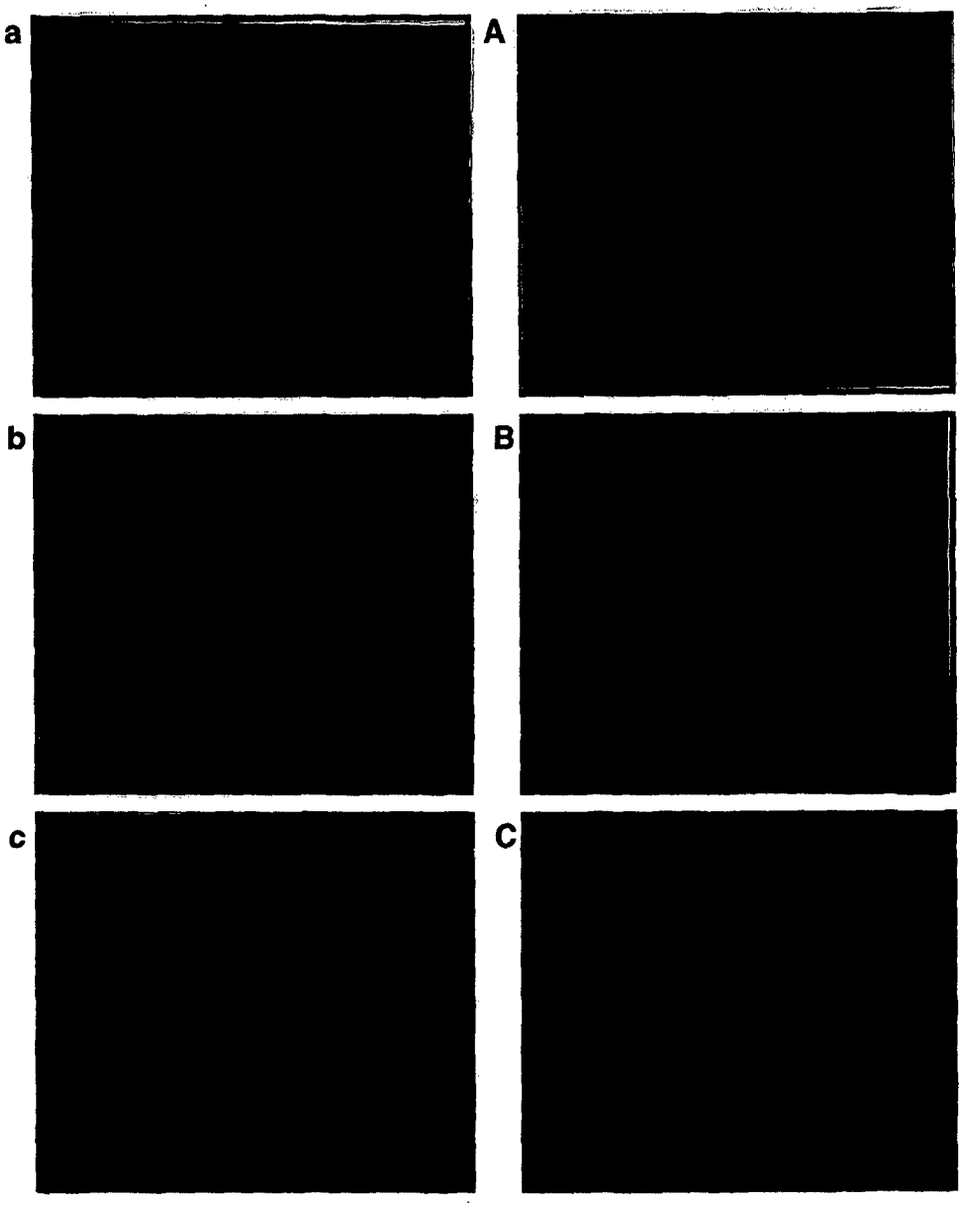
Mice were ovariectomized and treated with estrogen and progesterone daily for 21 days prior to analysis for PR. Panels A-C show immunoreactive PR (green color) while Panels a-c show nuclei (blue color) in the same sections. Panels a, A: Mammary glands from wild type mouse. Panels b, B: single duct from the mammary glands of PR-A transgenic mouse. Panels c, C: Lobulo-alveolar like structures in the mammary glands of PR-A transgenic mouse.


a
b
c
A
B
C

Legend for Figure 6

Analysis for PCNA expression in mammary glands of pre-pubertal mouse:

Panels A-C show PCNA staining (green color) while Panels a-c show nuclei (blue color) in the same sections. a, A: End bud structures. b, B: Mature duct. c, C: Antibody control.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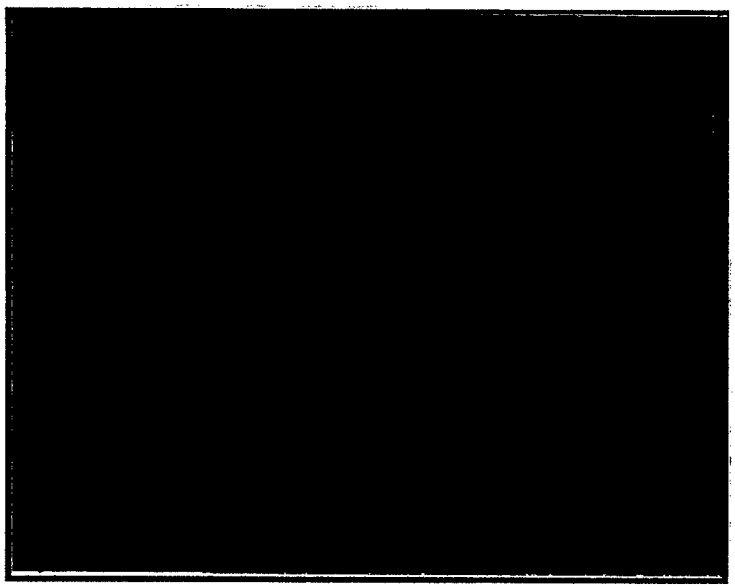
Legend for Figure 7

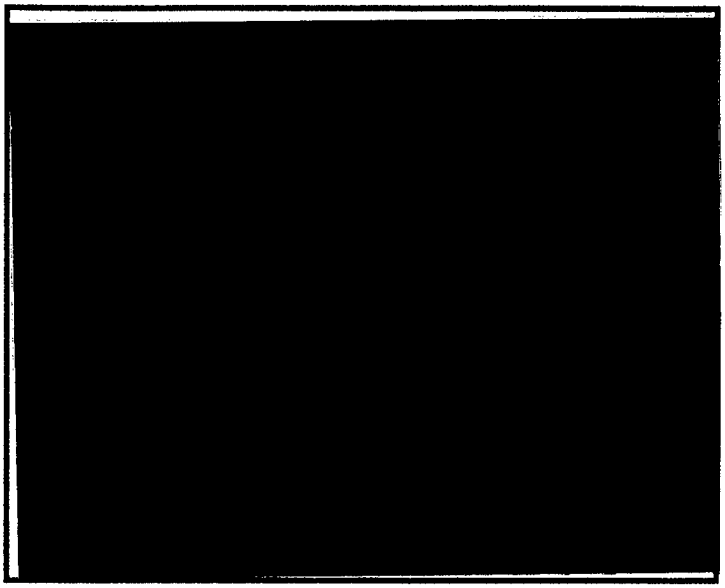
Analysis for PCNA expression in mammary gland of adult mouse

Panels A-C show PCNA staining (green color) while panels a-c show nuclei (blue color) in the same sections. Panels a,A: Mammary glands of adult nulliparous mouse. Panels b, B: Single duct in the mammary glands of 6 day pregnant mouse. Panels c, C: lobulo-alveolar structure in the mammary glands of 6 day pregnant mouse.

a



A



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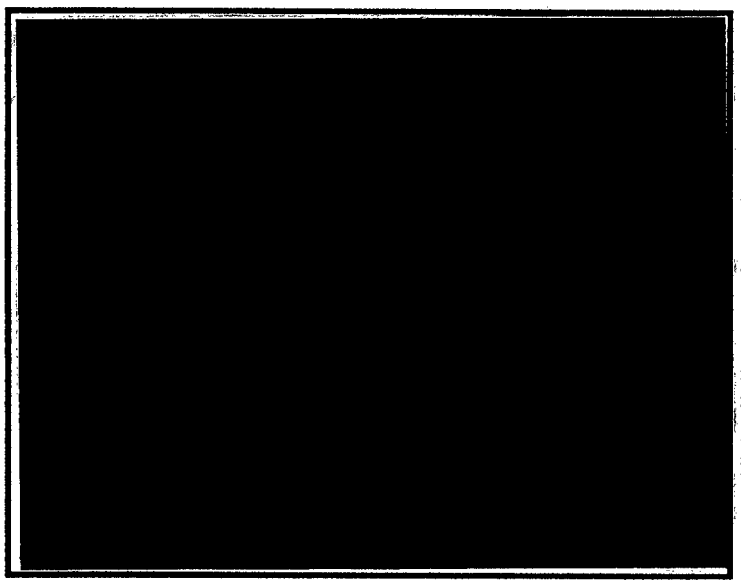

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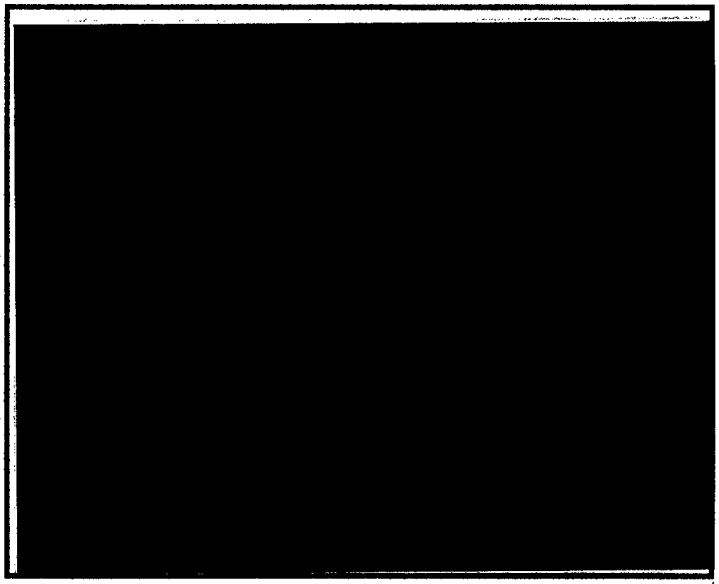

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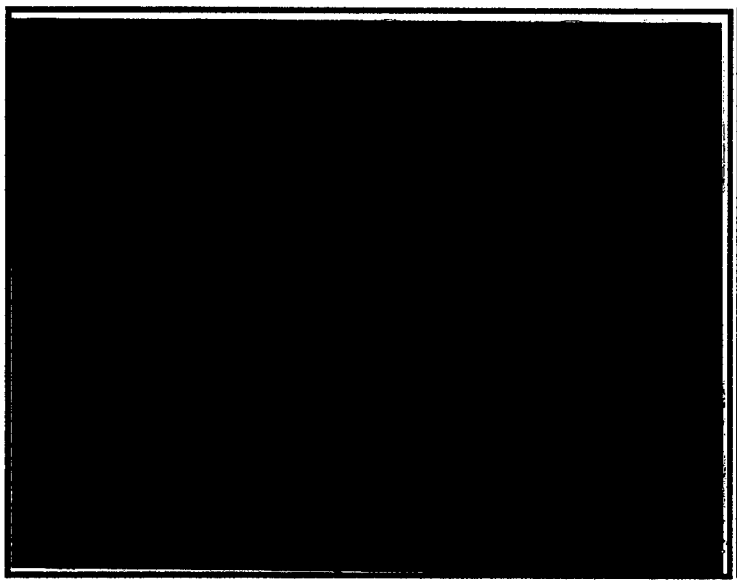

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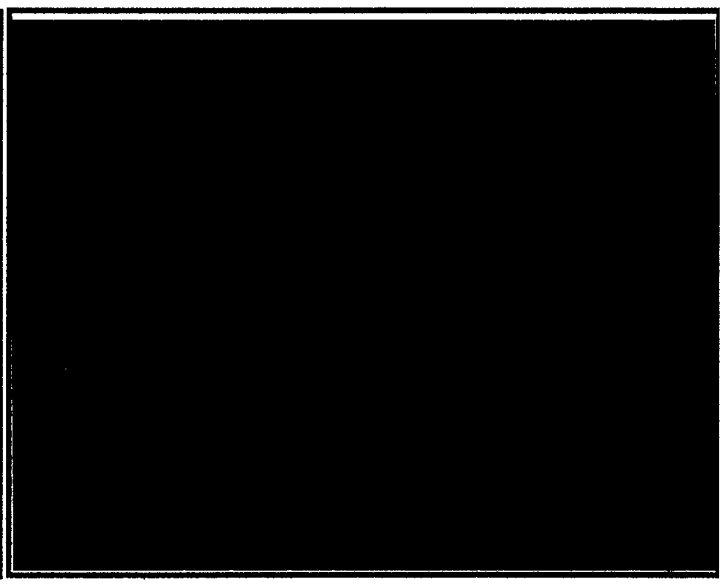

Legend for Figure 8

Analysis for PCNA expression in mammary glands of PR-A transgenic mice.

Panels A-C show PCNA staining (green color) while panels a-c show nuclei (blue color) in the same sections.

List of Personnel Receiving Pay from this Effort

1. Dr. G. Shyamala

2. Dr. Xinli Yang

3. Dr. Kenji Murata

4. Ms. Sharianne G. Louie

Received 2/8/00

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US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

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