

# Early exposure of the rat mammary gland to estrogen and progesterone blocks co-localization of estrogen receptor expression and proliferation

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## Abstract

An early single full-term pregnancy induces a long-lasting protective effect against mammary tumor development in humans and rodents. This protective effect can be mimicked in rats by short-term administration of estrogen and progesterone hormones prior to carcinogen administration. The hormones of pregnancy are able to induce a proliferative block upon carcinogen challenge that is not observed in the age-matched virgin. We wished to determine whether carcinogen is needed to induce a paracrine-to-autocrine shift of proliferation in steroid receptor positive cells or if such a cell population already exists in the age-matched virgin mammary gland. Here we show that estrogen receptor positive (ER+) proliferating

cells are rare in the developing mammary gland of the virgin rat but represent the majority of the proliferating cells in the mature (96-day-old) mammary gland of the virgin rat. As the majority of the proliferating cells before carcinogen challenge were ER positive, the ER+ proliferating cells in the mature mammary gland may represent the target cells for carcinogen-induced transformation. Importantly, prior exposure of the mammary gland to pregnancy levels of estrogen/progesterone blocked this positive association. This ability to block the proliferation of the ER+ cells may be one factor by which pregnancy induces protection against breast cancer.

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## Introduction

An early full-term pregnancy inhibits human breast tumorigenesis (MacMahon *et al.* 1970, 1973, De Waard & Trichopoulos 1988, De Waard 1992, Russo *et al.* 1992, Kelsey *et al.* 1993, Rosner *et al.* 1994). This result is mimicked in rodents by a full-term pregnancy (Gullino *et al.* 1975, Haslam 1979, Russo & Russo 1980, Russo *et al.* 1982) or by exposure to estrogen and progesterone for 21 days (Grubbs *et al.* 1985, 1988, Sinha *et al.* 1988, Sivaraman *et al.* 1998, Guzman *et al.* 1999, Yang *et al.* 1999). One of the earliest consequences of the specific effect of estrogen/progesterone (E/P) is a block to methylnitrosourea (MNU)-induced proliferation at 6–8 days post-carcinogen treatment (Sivaraman *et al.* 1998). The molecular mechanism of this block is not understood.

Cell proliferation is indispensable for normal growth and development of the mammary gland. It has been shown in rats (Russo *et al.* 1999), mice (Brisken *et al.* 1998, 2000, Seagroves *et al.* 2000) and humans (Clarke *et al.* 1997, Shoker *et al.* 1999) that epithelial cell expression of

estrogen receptor (ER) (and progesterone receptor (PR)) and that of proliferation-associated markers are almost mutually exclusive in the normal developing gland. This has been interpreted to mean that steroid receptors regulate proliferation in a paracrine fashion in the normal gland. It has been hypothesized that this organization is altered in the neoplastic state such that steroid receptors regulate proliferation in an autocrine fashion, representing an important pathogenetic step in the development of breast cancer.

In the current study, we have addressed the paracrine-to-autocrine shift hypothesis in our rodent experimental model of mammary tumorigenesis. We wished to determine whether there are increased ER positive (ER+) proliferating cells in animals that have not gone through pregnancy at or after carcinogen challenge and whether pregnancy is able to block this paracrine-to-autocrine shift of steroid receptor-regulated proliferation. We have shown here that a putative population of susceptible ER+ proliferating cells already exists in the mature rat mammary gland, which would likely give rise to tumors upon carcinogen challenge. Most importantly, our results

**Table 1** ER/BrdU analysis of young, adult, carcinogen-treated AMV and E/P-treated involuted rat mammary glands

Group	No. of BrdU (%)	No. of ER (%)	No. of double (%)	No. of DAPI	Expected (%)	O/E	Association	P value
45 day AMV (– TEB)	252 (12.4)	842 (41.3)	50 (2.5)	2037	5.12	0.49	Negative	<0.001
45 day AMV (TEB)	229 (20.9)	581 (53.1)	58 (5.3)	1095	11.1	0.48	Negative	<0.001
96 day AMV	69 (4.5)	481 (31.6)	38 (2.5)	1523	1.42	1.76	Positive	<0.001
96 day E/P	15 (1.0)	503 (32.9)	4 (0.26)	1529	0.329	0.79	—	0.8
96 day AMV (3 day MNU)	77 (2.5)	647 (21.3)	32 (1.05)	3036	0.53	1.98	Positive	<0.001
96 day E/P (3 day MNU)	21 (1.3)	397 (24.9)	1 (0.06)	1594	0.32	0.19	Negative	0.039
96 day AMV (7 day MNU)	174 (4.3)	1132 (28.2)	60 (1.5)	4013	1.22	1.25	Positive	0.06
96 day E/P (7 day MNU)	39 (1.7)	685 (29.1)	8 (0.34)	2354	0.48	0.71	—	0.29

At least five fields were captured per tissue section, at least one proliferating cell was included in each field of capture, and at least five animals were included in each group.

Expected = the percentages of double-labeled cells that would be expected (E) if the two variables were independent was calculated by multiplying the percentage of ER+ and BrdU+ cells and then dividing by 100 for each sample.

Observed/expected; O/E = actual number of dual labeled cells counted/expected calculated as described above.

O/E gives a ratio indication of whether receptor expression and proliferation are negatively or positively associated with each other and the strength of the association. In the former case, values less than 1 are expected and in the latter case, values greater than 1 are expected.

P values for Fisher's exact test for independence.

have demonstrated the decreased frequency of this population of cells in E/P-treated mammary glands.

## Materials and Methods

### Animals

Female Wistar–Furth rats, 35 days old, were purchased from Harlan Sprague–Dawley, Indianapolis, IN, USA. The animals were acclimatized to our animal facility for 10 days prior to experimental manipulations. The animals were kept with unrestricted access to food and water, and housed under conditions of a 12 h light:12 h darkness cycle.

### Experimental regime

The experimental regimen to mimic pregnancy using E/P and preparation and administration of MNU to induce mammary tumors has been described before (Sivaraman *et al.* 1998). Briefly, 45-day-old Wistar–Furth rats were primed with 0.1 ml solution of 2.5 µg estradiol benzoate (E2B) s.c. Three days later the rats were treated with 20 µg estrogen and 20 mg progesterone, delivered in the form of beeswax pellets. After 21 days of hormone stimulation, the mammary glands were allowed to involute for 28 days. On day 96, the animals were administered 50 mg/kg body weight (BW) MNU, i.p. Mammary tissues were collected for experimentation at suitable time-points described below. Control animals received blank beeswax pellets. In the current studies there were five experimental animals in each group and at each time-point.

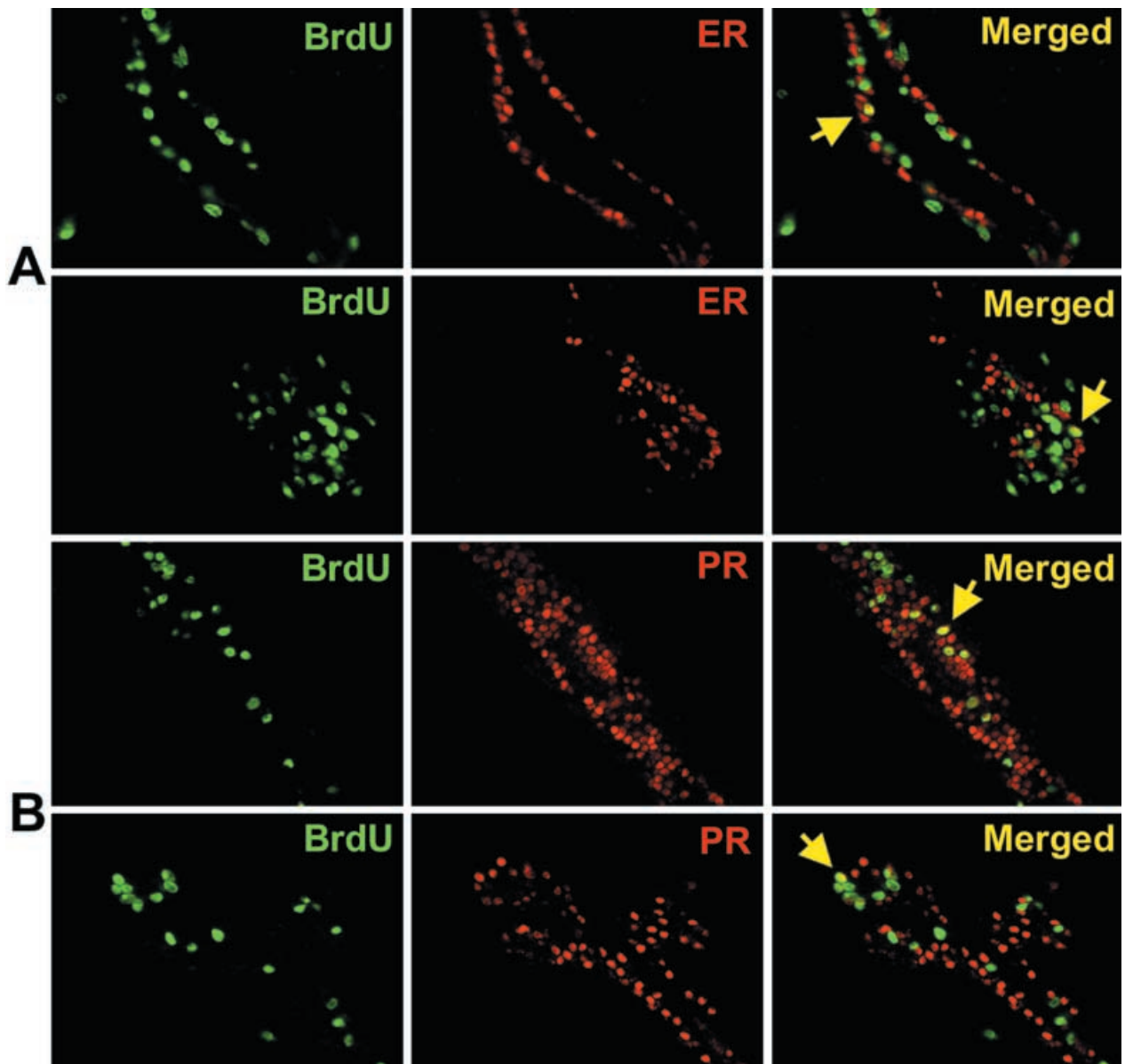
### Collection of tissues

Mammary gland tissues were collected from untreated, E/P-treated and carcinogen-treated animals. The no. 4 abdominal glands were collected from 45- and 96-day-old

virgin rat mammary glands, from 96-day-old E/P-treated/involuted mammary glands and from age-matched virgin (AMV) and hormone treated/involuted mammary glands 3 days and 7 days post MNU administration. The tissues were processed as described below for dual immunofluorescent labeling.

### Double immunofluorescence labeling

Animals were injected i.p. with bromodeoxyuridine (BrdU; 30 mg/kg BW; Sigma Chemical Co., St Louis, MO, USA) 2 h prior to their being killed. Tissues were fixed in chilled 4% paraformaldehyde in phosphate-buffered saline for 2 h. Paraffin sections (5–7 µm) were cut onto Probe-On Plus charged slides (Fisher Scientific, Pittsburgh, PA, USA). Sections were deparaffinized, hydrated through graded ethanols and subjected to microwave antigen retrieval in 10 mM citrate buffer, pH 6.0 (Katoh *et al.* 1997). The tissues were blocked in 5% normal goat serum. For ER/BrdU immunofluorescent labeling, sections were incubated sequentially with ERα antibody (1:300; MC-20, Santa Cruz, CA, USA), biotinylated goat anti-rabbit IgG (1:500; Vector Laboratories, Inc., Burlingame, CA, USA), Texas Red (TR)-conjugated streptavidin (1:250; Jackson ImmunoResearch Lab., West Grove, PA, USA), anti-BrdU antibody (1:50; Roche Diagnostic Corporation, Roche Biochemicals, Indianapolis, IN, USA) and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (1:20; Pierce, Rockford, IL, USA). For PR/BrdU dual immunofluorescence labeling the same protocol was used but a PR antibody (1:100; DAKO Corporation, Carpinteria, CA, USA) was used. All procedures were done at room temperature except the primary antibody incubations, which were carried out at 4 °C, overnight. Slides were



**Figure 1** Pattern of distribution of ER<sup>+</sup> and PR<sup>+</sup> cells in relation to proliferating cells in the virgin rat mammary gland: 45-day-old female Wistar–Furth rats were injected with BrdU 2 h before they were killed. The no. 4 abdominal glands were excised and fixed in 4% paraformaldehyde. (A) Mammary glands stained simultaneously for ER (TR – red) using a rabbit polyclonal anti-ER antibody and for BrdU (FITC – green) using a mouse monoclonal anti-BrdU antibody. (B) Mammary glands were stained simultaneously for PR (TR – red) using a rabbit polyclonal anti-PR antibody and for BrdU (FITC – green) using a mouse monoclonal anti-BrdU antibody. Green and red images are superimposed in the panels indicated ‘merged’. Arrows indicate cells in which ER/PR and BrdU are co-localized.

counterstained with 4',6-diamidino-2-phenylindole (DAPI) (1:1000 of a 1 mg/ml solution; Sigma Chemical Co.) and mounted in Vectashield mounting medium (Vector laboratories, Inc.).

#### Cell counting and analysis

Images were captured using a Zeiss Axioskop microscope equipped with appropriate fluorescence filter sets

coupled to a Hamamatsu C5810 CCD camera (Hamamatsu Corp., Bridgewater, NJ, USA) and processed using Adobe Photoshop 4.0 (Adobe Systems, Inc., San Jose, CA, USA). At least five individual fields were captured using a  $\times 20$  lens and at least five animals were used in each experiment. Cells were counted using the Image Tool software (UTHSCSA, for Windows, version 2.0).

### Statistical analysis

Data obtained in the co-localization (ER vs BrdU) studies were analyzed using the Fisher's exact test for independence. Since separate analyses of animals of the same age and exposure history gave similar results, only the combined analyses are shown. The strength of the positive or negative association between ER positivity and proliferation (BrdU positive) was expressed as the ratio (observed/expected; O/E) of the observed proportion of cells staining for both ER and BrdU (O) to the proportion expected if the two characteristics were statistically independent ( $E = \%(\text{ER}+) \times \%(\text{BrdU}+)/100$ ). BrdU positivity and ER+ are treated as two separate assessments. Values greater than 1 indicate a positive association, values less than 1 indicate a negative association and values near 1 indicate no association (independence). Fisher's exact test was used to determine *P* values.

### Results

In the mammary gland, proliferation is regulated by the levels of estrogen and progesterone. Therefore, in this study we examined the relationship between steroid receptor expression and proliferation in the normal adult virgin rat mammary gland and the effect early exposure to E/P had on this relationship. We have used a dual immunofluorescent labeling approach to detect steroid receptor and proliferation. The analysis was performed on paraffin-embedded tissue using an antigen-retrieval protocol in glands derived from 45-day-old virgin, 96 AMV, 96-day E/P-treated, 3-day and 7-day carcinogen-treated AMV and E/P rats. Table 1 summarizes the data and all the statistical analysis. It is clear from Table 1 that proliferation in the AMV is higher than in the E/P-treated gland and all time-points examined, namely, 96 days, 3 days post-carcinogen and 7 days post-carcinogen. To our surprise the 96-day-old virgin rat mammary gland showed a significant number of BrdU positive cells (4.5%). This is different from that which we reported earlier (Sivaraman *et al.* 1998) and we attribute this difference to the following reasons. (1) These experiments were designed specifically to find BrdU+ cells to examine for double labeling. Therefore our search was biased to include BrdU+ areas and was not a random analysis. (2) We examined both distal and proximal areas of the gland while the original study reported that the two portions of the gland are not equivalent with respect to the extent of proliferation. (3) Animals are likely to be in different estrous states but animals housed in the same cage are normally in the same estrous cycle. Therefore we cannot directly compare this study to our earlier study because the criteria/parameters were different. Importantly, however, the proliferation in the AMV group was significantly higher than in the E/P-treated group 7 days post MNU

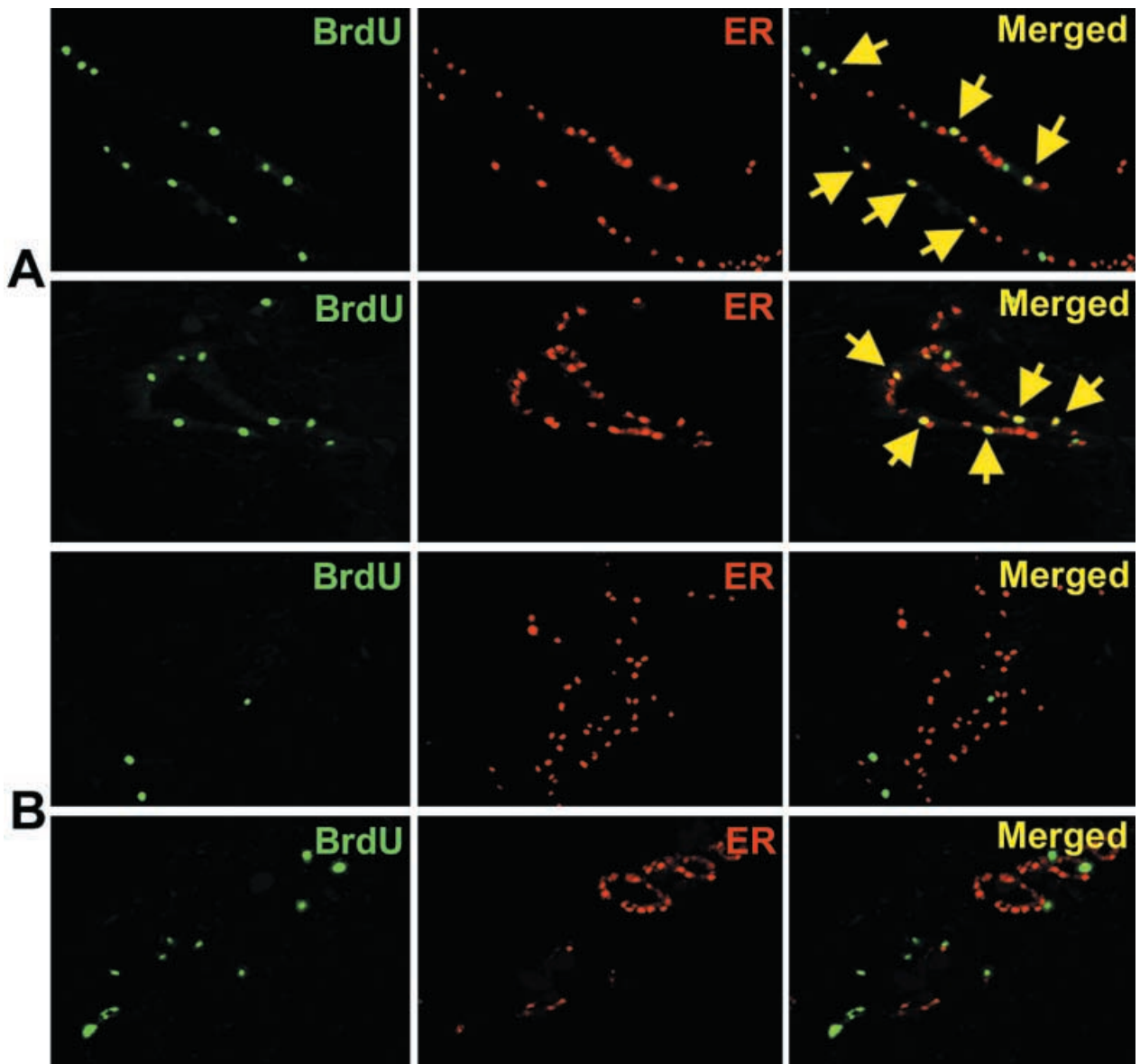
( $P < 0.001$ ) and corroborates our earlier finding (Sivaraman *et al.* 1998).

We next determined the correlation between the frequency of ER+ cells with the frequency and distribution of BrdU+ cells. PR+ proliferating cells were also examined in a 45-day-old virgin and the 7-day carcinogen-treated mammary gland. The results are illustrated in Figs 1–3.

Figure 1A and B depicts the dissociation of ER and PR expression with proliferation in a 45-day-old virgin mammary gland respectively. ER+ proliferating cells represent a numerically small population in a 45-day-old rat mammary gland and comprise 5.3% of the cells in the terminal end buds (TEB) and 2.5% of the cells in large ducts, small ducts and lobular structures of the mammary gland. Co-localized cells were predominantly found in small ducts and TEBs. The results support a negative association between proliferation and ER positivity. The percentage of co-localized cells was greater than that reported for 55-day-old rats by Russo *et al.* (1999) where the percentage of dual-labeled cells in TEBs was 1.01% and that in alveolar buds and lobules 0.43%. While both tritiated thymidine and BrdU are specific S-phase markers, the higher value might be attributed to the longer exposure time to the label (2 h for BrdU vs 1 h for  $^3\text{H}$ -thymidine), a difference in rat strains used or just increased sensitivity of detecting incorporated BrdU compared with radiolabeled DNA. Figure 1B depicts the dissociation of PR expression and proliferation. In a 45-day-old rat mammary gland, while approximately 47% of the luminal epithelial cells express PR, only 2.6% of the PR+ cells were proliferating. Thus ER+ and PR+ proliferating cells are a small population in the young virgin rat mammary gland.

We next examined proliferation in adult virgin and E/P-exposed rat mammary glands where TEBs are absent. The proliferation in the E/P-treated and AMV gland at 96 days was significantly different (1% and 4.5% respectively,  $P < 0.001$ ); however, the percentages of ER+ cells in the two groups were comparable (32.9% and 31.6% respectively). More importantly, the frequency of dual-labeled cells between the two groups was significantly different;  $P \leq 0.001$  (Table 1). The percentage of dual-labeled cells in the 96-day-old AMV was 2.5% and that in the E/P-treated 96-day-old animal 0.26% (Table 1). Thus it appears that co-localization of ER and proliferation is abolished by hormone stimulation prior to carcinogen exposure.

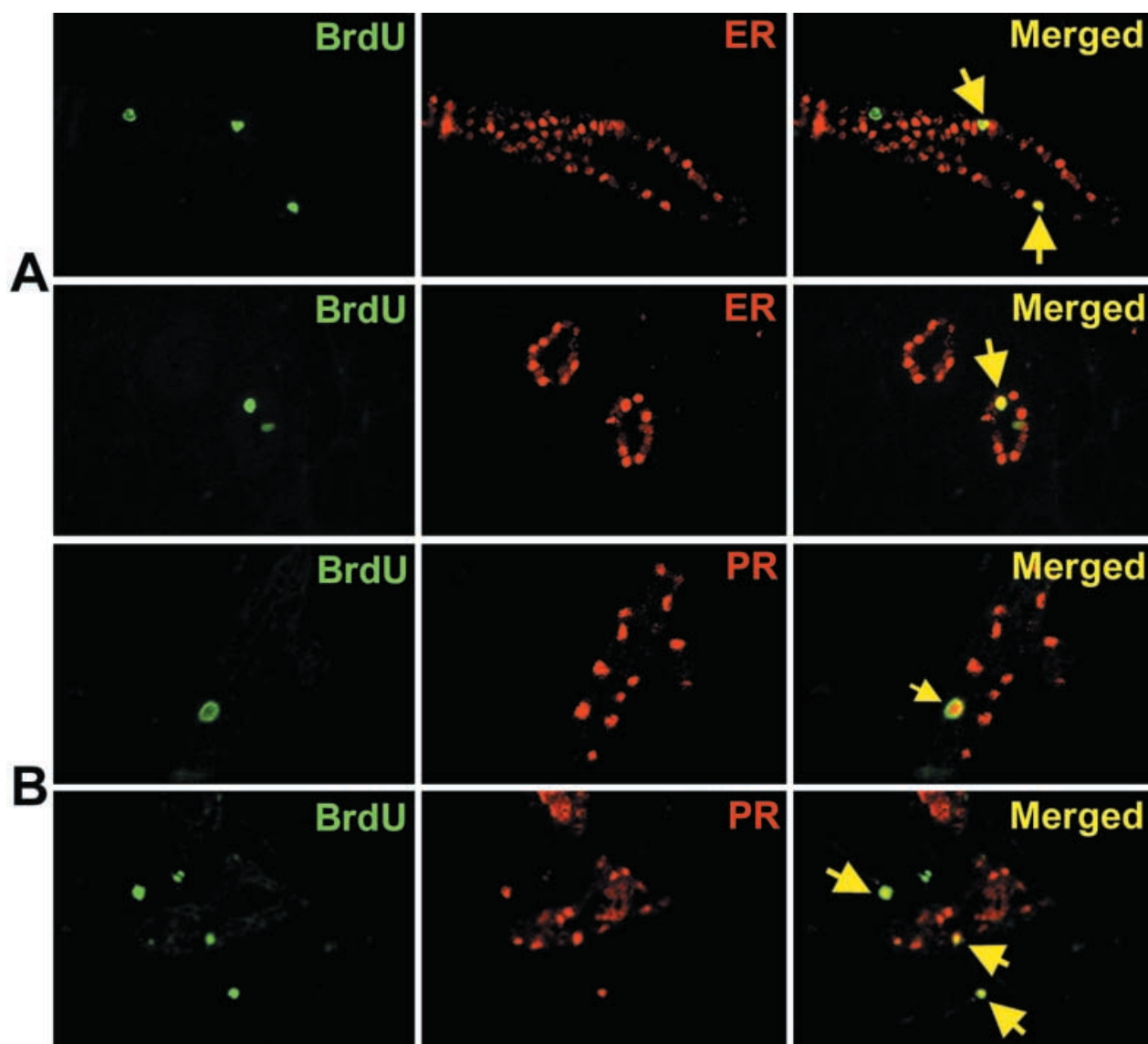
Figure 2A clearly depicts the increased presence of ER+ proliferating cells in the AMV 3 days after carcinogen challenge (1.05%). The percentage of such dual-labeled cells in the glands that have been exposed to E/P remained low (0.06%, Fig. 2B). Thus, prior exposure to hormones blocks the appearance of ER+ proliferating cells in the mammary gland while, in the adult gland, a significant portion of the proliferating cells are ER positive.



**Figure 2** Absence of ER+ proliferating cells in E/P+ treated rat mammary glands 3 days after MNU administration: 45-day-old rats were primed with E2B for 3 days. They were then administered beeswax pellets containing 20 µg estrogen and 20 mg progesterone for 21 days to mimic pregnancy. After 28 days of gland involution (96 days), the animals were administered 50 mg/kg BW MNU. Ninety-six-day-old AMV rats, treated with blank beeswax pellets were also administered 50 mg/kg BW MNU and served as control animals. Three days later the animals were injected with BrdU 2 h before being killed. The no. 4 abdominal glands were excised and fixed in 4% paraformaldehyde. Tissues were embedded in paraffin, sectioned and stained for ER and BrdU as detailed in Fig. 1. (A) AMV and (B) E/P-treated rats. Green and red images are superimposed in the panels indicated 'merged'. Arrows indicate cells in which ER and BrdU are co-localized.

Figure 3 confirms the presence of ER+ (Fig. 3A) and PR+ (Fig. 3B) proliferating cells in the AMV 7 days after carcinogen exposure. Approximately 1.5% of the ER+ cells and 1.0% of the PR+ cells were proliferating in the AMV and only about 0.34% of the ER+ and 0.23% of the PR+ cells were proliferating in the E/P-treated mammary glands.

In the present study, only the data for ER/BrdU were statistically analyzed since estrogen is the primary mitogenic hormone in the mammary gland. Table 1 summarizes in detail the percentage of cells staining for BrdU, ER, dual-labeled cells and the percentages of double-labeled cells that would be expected if the two variables were independent. We have used the O/E

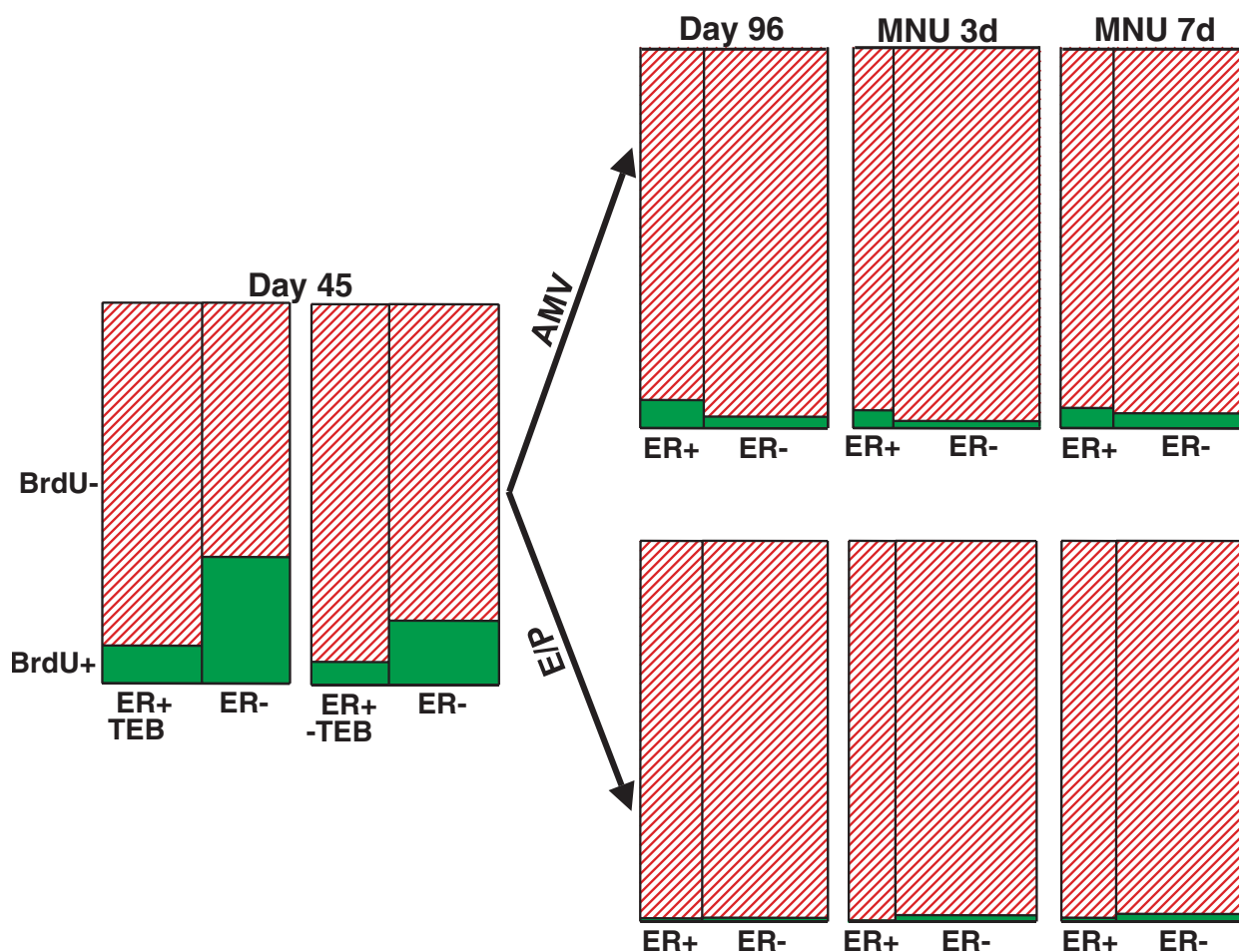


**Figure 3** Co-localization of steroid receptors and proliferation upon carcinogen challenge: 96-day-old rats were administered 50 mg/kg BW MNU. Seven days later, the animals were injected with 30 mg/kg BrdU 2 h before they were killed. The no. 4 abdominal glands were excised and fixed in 4% paraformaldehyde. Tissues were embedded in paraffin, sectioned and stained either for (A) ER and BrdU or (B) PR and BrdU by double immunofluorescent labeling (green – BrdU, red – PR/ER). Green and red images are superimposed in the panels indicated 'merged'. Arrows indicate cells in which ER/PR and BrdU are co-localized.

ratio to indicate whether the two markers are positively, negatively or not associated with each other (ratio of  $>1$ ,  $<1$  or  $\approx 1$  respectively) and the strength of the association.

The O/E ratio for the mammary gland of a 45-day-old virgin was 0.48 for the TEBs and 0.49 for the remaining parts of the gland, revealing that receptor expression and proliferation are negatively associated. Thus, in the virgin rat mammary gland, receptor expression and proliferation are primarily mutually exclusive events.

Similar analysis was carried out in the AMV and E/P-treated glands at 96 days, and 3 and 7 days after MNU. On all of the 3 days the AMV always showed a positive association between receptor expression and proliferation (96 days O/E=1.76, 3 day MNU O/E=1.98, 7 day MNU O/E=1.25). In sharp contrast, the E/P-treated gland always showed a numerically negative association between the two markers (96 days O/E=0.81, 3 day MNU O/E=0.19, 7 day MNU O/E=0.71), although only day 3 was statistically significant.



**Figure 4** Graphical representation of ER BrdU labeling. The large boxes represent the total epithelial cells counted. The hatched red represents BrdU negative cells, the green represents BrdU+ cells. The area included in a given color is directly proportional to the percent of cells for that character. Thus, the green area in the ER+ cells in the 45-day-old rat represents 5.3% in the TEBs and 2.5% in the remaining parts of the gland of all cells counted in that group. The rat mammary gland has four populations of epithelial cells, namely, ER+ proliferating and non-proliferating populations and ER negative proliferating and non-proliferating populations. A focus on the green boxes (BrdU+) shows the relative changes in ER+ proliferating cells with gland development and with carcinogen treatment. E/P=estrogen/progesterone; d=day.

## Discussion

In the current study, we have shown that ER+ proliferating cells are rare in the developing mammary gland of a young virgin rat and the two markers, receptor expression and proliferation, are disjointed events. These results are consistent with the current hypothesis that ER-dependent regulation of proliferation is likely to be paracrine in the developing mammary gland of the young virgin rat. In contrast, in the mature gland of the virgin rat, although fewer cells are proliferating, the percentage of dual-labeled cells was significantly increased and the two markers were positively associated. In fact, over half of the proliferating cells are receptor positive in a 96-day-old virgin rat mammary gland. Positive association was

observed before carcinogen challenge. Thus, contrary to common belief, mammary carcinogenesis does not necessarily involve a paracrine-to-autocrine switch in the regulation of proliferation by steroid receptors. Instead, the age-dependent proportionate increase in ER+ proliferating cells suggests that the autocrine pathway is already present in the mammary gland and the ER+ proliferating cells provide a likely target cell population for carcinogen-induced transformation. Finally, and most importantly, prior exposure of the mammary gland to pregnancy levels of estrogen and progesterone resulted in a failure of the mammary gland to attain a positive association between ER expression and proliferation. This might be one mechanism by which pregnancy induces protection against breast cancer, i.e. by blocking the ability

of the ER<sup>+</sup> cells to respond to an abnormal proliferative stimulus.

Our report is novel in that we have studied the responses of a mature mammary gland to carcinogenesis while earlier studies relating refractoriness induced by hormones have looked at young virgin mammary glands. The study of steroid receptors and their association with proliferation could clarify some features regarding the early phases of chemical carcinogenesis-induced neoplastic initiation and progression in the breast. Initiated breast cells could express a different regulation of proliferative activity and steroid receptor expression from the very beginning rather than as a result of a modulation during neoplastic progression. This relation may be different in an E/P-exposed mammary gland that is refractory to carcinogen-induced tumorigenesis. We have shown here that early exposure to E/P in fact is able to block the autocrine regulation of proliferation by steroid receptors.

Figure 4 summarizes graphically the main results. In a 45-day-old virgin rat mammary gland there are two populations of epithelial cells, namely, an ER<sup>+</sup> population and an ER<sup>-</sup>amplifying population whose proliferation is regulated by the ER<sup>+</sup> population in a paracrine fashion. With maturation of the gland and the attainment of a predominately quiescent proliferative state, ER<sup>+</sup> cells represent the major population of proliferating cells (Fig. 4, upper panel). Upon MNU exposure, the ER<sup>+</sup> cells respond with a proliferative burst that is maintained for 7 days and also results in the recruitment of ER<sup>-</sup> cells into the proliferating pool. Therefore, with maturation, proliferation is decreased in the transient amplifying population until the next need for rapid enlargement of the epithelial cell population, e.g. pregnancy.

The observation that hormones of pregnancy block the co-localization of ER expression and proliferation (shown in Fig. 4, lower panel) suggests that these hormones are able to alter the cell fate of mammary epithelial cells such that the putative susceptible population of mammary epithelial cells is abrogated by prior hormone exposure. We hypothesize that prior hormone stimulation results in a blocked proliferative response of ER<sup>+</sup> cell population to MNU. The susceptible ER<sup>+</sup> population capable of proliferation continues to exist in the AMV and is the likely target of transformation upon carcinogen exposure. The molecular pathways that are responsible for the proliferation block and its regulation by E/P are unknown at the present but represent a fertile area for investigation.

Co-expression of steroid receptor co-activators with steroid receptors and loss of the expression of tumor suppressor genes are likely mechanisms by which a disproportionate increase in the co-expression of steroid receptor and proliferation occurs with maturation of the mammary gland. Studies of the molecular pathways by which exposure to hormones result in maintaining the normal relationship between ER and cell proliferation could lead to the identification of molecules that mediate

this process and alternative strategies in the prevention of breast cancer.

## Acknowledgement

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