Menstrual Cycle-Dependent Variations of Breast Cyst Fluid Proteins and Sex Steroid Receptors in the Normal Human Breast

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Menstrual cycle dependent variations in cytosol levels of estrogen receptor (ER), progesterone receptor (PR), and two breast cyst fluid glycoproteins, gross cystic disease fluid protein (CDP) and nonreceptor progesterone binding protein (PBP), were demonstrated in epithelial-enriched "normal" breast tissues from 56 premenopausal women. Criteria for normalcy were: (1) normal menstrual history; (2) absence of drug use or debilitating disease; (3) normal adrenal, ovarian and pituitary function; and (4) no clinical, gross or histologic evidence of breast disease. Highest mean levels of CDP (8180 ± 2850 ng/mg protein) and PBP (17750 ± 6320 ng/mg protein) were noted during the follicular phase (days 8–14) of the menstrual cycle, while lowest levels of CDP (450 ± 260) and PBP (1810 ± 380) were observed during the luteal phase (days 15–20). Both CDP and PBP had peak values on days 10–12 with a smaller peak on days 20–23. The number of samples with ER > 3 fmole/mg protein (8/14; 57%) and mean ER level (7.5 ± 2.9 fmole/mg protein) were significantly higher during the proliferative phase (days 3–7) than during other menstrual phases. Maximum ER values occurred on days 5–8. The greatest number of samples with PR > 3 fmole/mg protein (5/15, 33%) and highest mean levels of PR (36 ± 27.8 fmole/mg protein) were noted during the follicular phase of the menstrual cycle. These data demonstrate that the normal human breast has menstrual cycle variations of CDP and PBP which may be useful as markers of sex steroid receptor integrity. These data also documented that appreciable ER levels were found during the proliferative phase with low levels at other times in the menstrual cycle.

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THE NORMAL HUMAN BREAST[1] and endometrium[2] have been shown to have distinct variations in morphologic features correlating with the phases of the menstrual cycle. It is now well established that estrogen and progesterone exert their effects by binding to specific, high affinity receptor proteins present in the cytosol of these tissues.[3] Since the menstrual cycle is characterized by cyclic changes in plasma estrogen and progesterone, it is also not surprising that normal human[4,5] and primate[6] endometrium should have menstrual cycle dependent variations of sex steroid receptors. Similar studies in non-primate animals have demonstrated an estrous cycle dependence for levels of both estrogen receptor (ER) and progesterone receptor (PR) in uterus[7,8] and breast.[9]

Despite the abundance of evidence for the presence of ER in human breast carcinoma tissue, there is still controversy concerning estrogen receptors in non-neoplastic human breast tissues. Several investigators have found low levels of ER in breast tissue,[10–12] while others were unable to demonstrate measurable levels of ER.[13–15] Progesterone receptors were shown to be present in tissue obtained from normal breasts[11] and in specimens obtained from "normal" areas adjacent to breast carcinoma.[16] In this latter study, the levels of PR were found to vary with the menstrual cycle.[16]

A number of endometrial[5,17] and breast[11] cytosol proteins appear to be related to sex steroid receptor interaction. Gross cystic disease fluid protein (CDP) and non-

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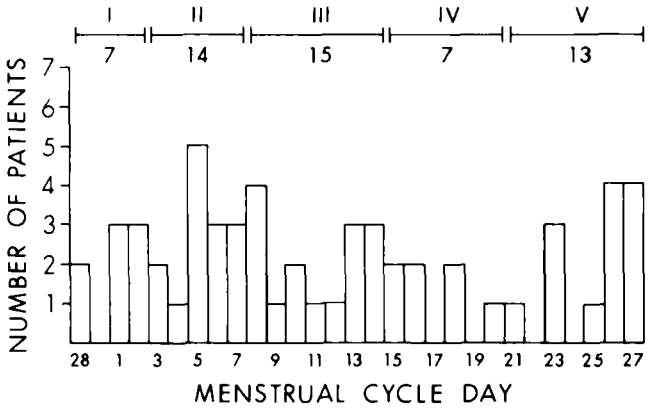


FIG. 1. Distribution of patients by menstrual cycle day. Vertical bars represent the number of patients sampled on that menstrual cycle day. Horizontal rules divide the menstrual cycle into phases. The number of patients per menstrual phase is given below the horizontal rule.

receptor progesterone binding protein (PBP) represent two glycoproteins isolated from breast cyst fluid.[18] These breast cyst proteins are suggested to be related to sex steroids since breast tumor cytosol levels of CDP and PBP correlate with ER and PR levels.[19]

This study evaluates the menstrual cycle dependent variations of breast cyst proteins, CDP and PBP, and sex steroid receptors in breast epithelial tissues obtained from normal premenopausal women.

Materials and Methods

Patient Selection

One hundred and seventy-three women underwent subcutaneous mastectomy for indications other than neoplasia at Duke University Medical Center from August 1978 to December 1980. Each patient was interviewed preoperatively using a standardized protocol. Fifty-six of the 173 patients met the following criteria and were included in the present study: adequate epithelial lobular units in dissected material to derive sufficient protein extraction for receptor and cyst protein analysis, history of regular menses with accurate recall of menstrual dates, absence of hormone use, medications, or debilitating disease which might influence pituitary–ovarian function. In addition, paraffin-embedded hematoxylin and eosin-stained sections from each case were reviewed. Specimens containing simple fibrocystic disease greater than 10% of lobular–alveolar units involved or those with epithelial proliferative changes were eliminated from the study. A majority of women had histories of 28-day menstrual cycles; those with other than 28-day cycles had menstrual dates normalized to a 28-day cycle.

Menstrual Phases

We have described morphologic criteria which allow reproducible categorization of the normal human breast into five distinct menstrual phases:[1] proliferative: days 3–7; follicular phase of differentiation: days 8–14; luteal phase of differentiation: days 15–20; secretory: days 21–27; and menstrual: days 28–2. Patients were segregated into the above phases based on the interval in days between last menses and operation and the histology of their breast tissues (Fig. 1). There was no statistically significant differences among phases with respect to age or parity. The mean age of the patients was 35 years (range, 15–51 years).

Cytosol Preparation and Receptor Assays

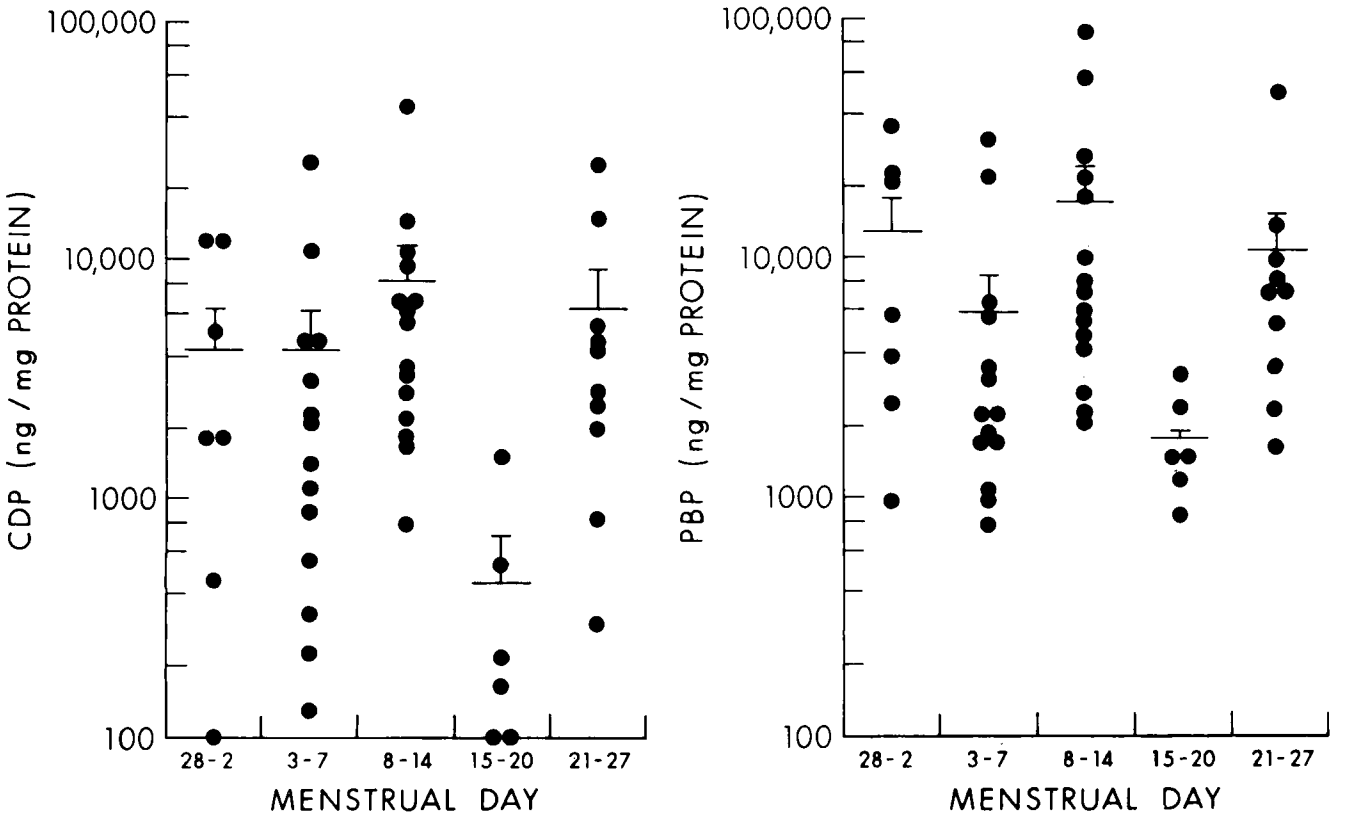
Breast specimens were transported from the operating room on ice, rapidly sectioned in the saggital plane at 3 mm intervals, then examined with a stereomicroscope under trans and epi-illumination in order to dissect lobular–alveolar units.[20] Tissues for assay were washed in buffer (0.05 M Tris, 0.05 M Hepes, 0.1M thioglycerol, 0.1M EDTA, pH 7.4 at 4°C). Tissue cytosols were prepared as previously described.[21] Briefly, tissue samples were frozen in liquid nitrogen, pulverized and homogenized. Homogenates were centrifuged at 12,000 × *g* for 10 minutes followed by ultracentrifugation at 104,000 × *g* for 30 minutes. Cytosols were coded then stored at −80°C until analyzed. Estrogen and progesterone receptors were analyzed by multiconcentration titration dextran-coated charcoal (DCCA) and sucrose density gradient (SDGA).[21]

Cytosol CPD and PBP Assays

Cytosol assays were performed by direct radioimmunoassay. Rabbit anti-CDP antibody, cytosol sample (0.05 ml) and ^{125}I-labeled CDP were incubated together at 4°C for 18 hours. The antibody was precipitated by centrifugation with solid-phase (KYNAR) goat anti-rabbit serum, supernatant decanted and the precipitate counted in a gamma well counter.[22] In a similar fashion, PBP was determined by rabbit anti-PBP antibody, cytosol sample (0.01 ml) and ^{125}I-labeled PBP.

Statistical Methods

Data was stored and analyzed using CLINFO (a clinical data analysis system, Bolt, Beranek and Newman, Boston, MA). Descriptive statistics, two-tailed *t* test, one-way ANOVA, chi-square and Wilcoxon rank sum tests were used to evaluate significance from programs resi-



FIGS. 2A AND 2B. Cytosol levels of breast cyst proteins in the normal human breast. Cytosol levels of CDP and PBP are divided by menstrual phases (see text). (A, left) levels of CDP. (B, right) Levels of PBP. Horizontal line: mean; vertical line: SEM.

dent in CLINFO. The Kruskal-Wallis test[25] was used to evaluate rank sums for three or more groups.

Results

Menstrual Cycle Distribution: Cyst Fluid Proteins

The distribution of breast cyst proteins by phase of menstrual cycle demonstrated a significant difference for CDP and PBP levels during the menstrual cycle ($P < 0.005$, Kruskal-Wallis test) (Figs. 2A and 2B). In contrast to ER, CDP and PBP had highest levels during the follicular phase of differentiation, days 8–14; lowest levels during luteal differentiation and intermediate levels at other times (Table 1). There was a sharp peak in CDP and PBP values when levels for each day were pooled with adjacent days of the menstrual cycle to calculate a running average with approximately five samples per point (Fig. 3). Maximum levels for both CDP and PBP were observed from days 10 to 14 coincident with the plateau of PR values and 3–5 days later than maximum estrogen receptor levels (*vide infra*). There was a second, smaller peak late in the menstrual cycle on days 20–23.

Menstrual Cycle Distribution: Sex Steroid Receptors

The distribution of ER values for patients segregated by phase of menstrual cycle demonstrated that ER values were significantly different during the various phases of the menstrual cycle ($P = 0.03$, one-way ANOVA). Peak ER values were observed during the proliferative phase, days 3–7. Mean tissue levels of ER (7.5 ± 3.0 fmole/mg protein, mean ± SEM) and the proportion of

TABLE 1. Mean Values (SEM) of Receptors and Cyst Proteins

Menstrual phase*	N	ER† x	ER† SEM	PR‡ x	PR‡ SEM	CDP§ x	CDP§ SEM	PBP§ x	PBP§ SEM
Proliferative	14	7.5	2.9	5.9	4.4	4350	1970	6060	2380
Follicular	15	1.9	0.6	36.0	27.8	8180	2850	17750	6320
Luteal	7	0.6	0.3	0.1	0.1	450	260	1810	380
Secretory	13	1.8	0.8	11.8	8.6	6560	2620	11080	4520
Menstrual	7	0.4	0.2	2.0	1.2	5420	1150	13410	5100

* See text for definition.
† ER fmole/mg protein; p = 0.03, ANOVA.
‡ p = N.S.
§ Cyst protein in ng/mg protein; p < 0.005 Kruskal-Wallis test.

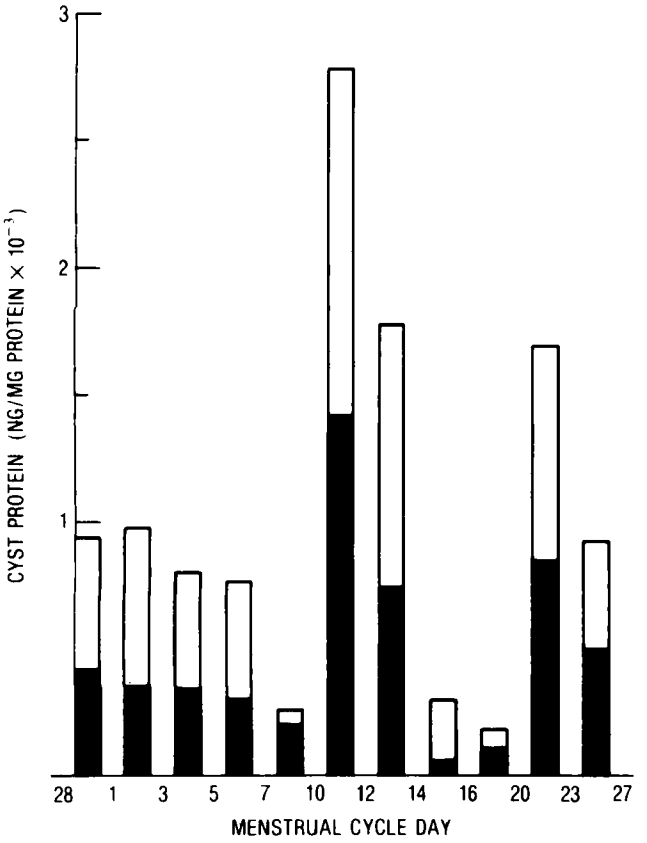


FIG. 3. Menstrual cycle variations of CDP and PBP in the normal human breast. Mean values of CDP (solid bar) and PBP (open bar) are shown for days of the menstrual cycle. Running averages were calculated from adjacent days with approximately five samples per point.

specimens with ER > 3 fmole/mg protein (8/14; 57%) were significantly higher during this phase than at other times during the menstrual cycle (Tables 1 and 2). When running averages were computed for ER levels by day of menstrual cycle, it was apparent that estrogen receptor levels peaked on days 5–8 (Fig. 5A). A small, second peak was observed late in the menstrual cycle on days 25 and 26.

The distribution of PR values was different from the distribution of ER values in that levels of PR were highest during the follicular phase of differentiation (III). Levels of PR were significantly higher during this phase compared to luteal ($P = 0.008$) or proliferative ($P = 0.07$) phases but not to the other phases (Wilcoxon rank sum). The proportion of specimens with PR > 3 fmole/mg protein was also higher during the follicular phase, although several specimens were positive during the secretory phase as well (Table 2). When running averages were calculated for PR values by day of menstrual cycle, there were two peaks of PR observed (Fig. 5B). The first peak, days 13–14, coincided with peak CDP and PBP. The second peak occurred late in the cycle on days 21–23.

Discussion

Progesterone receptor[4,5] and estradiol 17-beta dehydrogenase[5,17] have been shown to be menstrual cycle dependent in normal human endometrium. Such proteins appear to be products of intact estrogen–estrogen receptor–chromatin interactions and may serve as markers for the integrity of estrogen receptor-mediated events. There is considerable evidence to suggest that progesterone receptor is a similar marker in human breast carcinoma.[24] Utilizing "normal" tissue obtained from breasts containing carcinoma, Pollow *et al.*[16] observed menstrual cycle variations in progesterone receptor with peak levels noted at mid-cycle. The menstrual cycle dependent variations of PR, CDP, and PBP in normal breasts demonstrated in this study support the concept that PR and these breast cyst fluid proteins are related to estrogen-induced transcriptional events. The correlation between morphologic features of breast menstrual cycling and levels of CDP and PBP during the phases of the menstrual cycle indicate that these cytosol proteins may be useful markers of ER–PR associated differentiation in the normal breast. These data extend our previous observations which found that well-differentiated tumors contained significantly higher levels of CDP and PBP than poorly-differentiated breast carcinomas.[19]

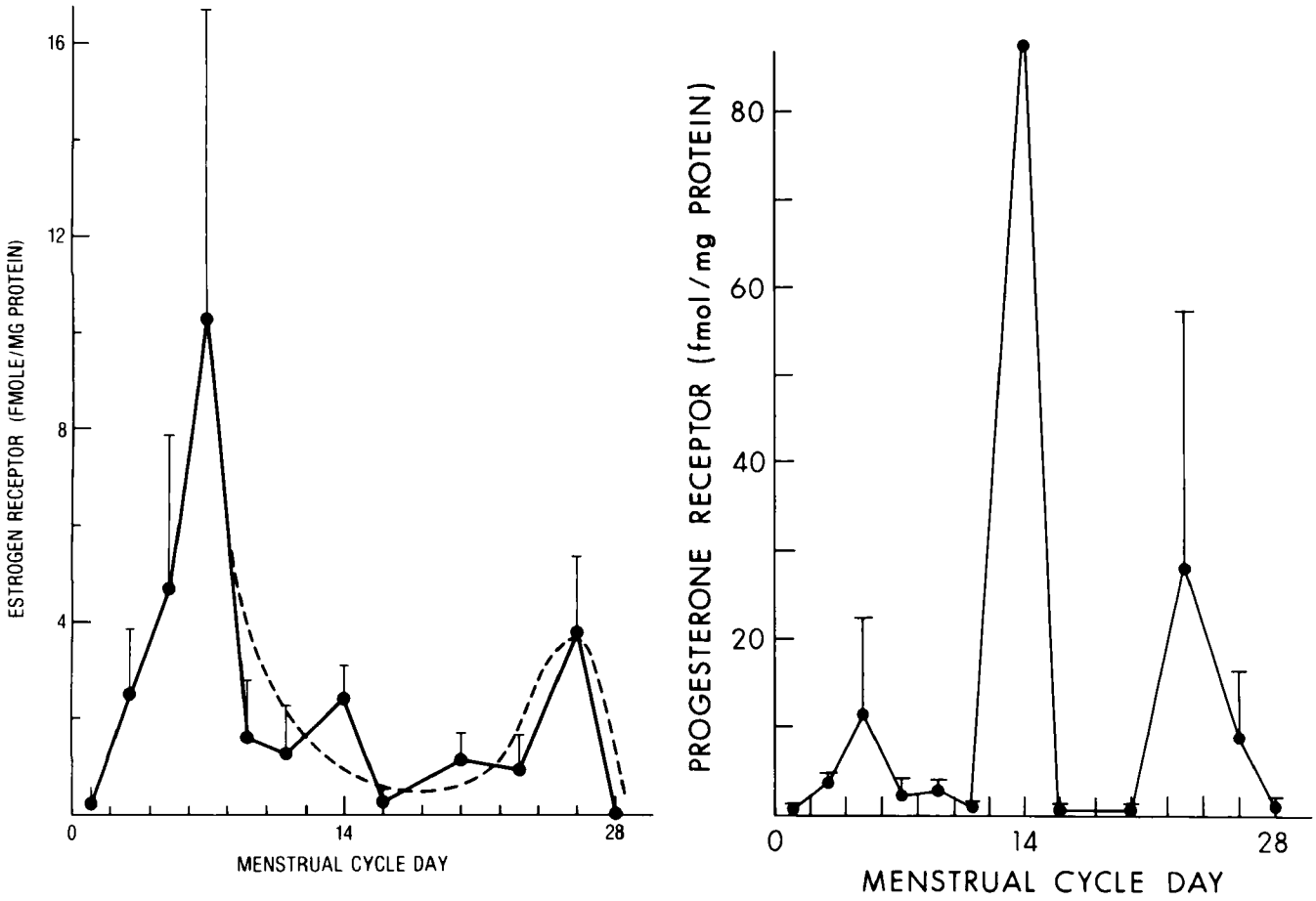
Normal human endometrium has also been shown to have menstrual cycle-dependent variations in ER levels.[4,5] Other investigators, using tissue not rigidly defined to be normal and without consideration to possible menstrual cycle variation, have reported that estrogen receptor was present in less than 20% of non-neoplastic specimens.[10–15]

The current study demonstrates that the normal mammary gland has menstrual cycle dependent variations of estrogen and progesterone receptor. As a result, the proportion of specimens with ER greater than 3 fmole/mg was highly dependent on the menstrual phase. Most

TABLE 2. Proportion of Positive Estrogen and Progesterone Receptors

Menstrual phase	N	ER*		PR†	
		No. >3 fm	(%)	No. >3 fm	(%)
Proliferative	14	8	(57)	2	(15)
Follicular	15	5	(33)	5	(33)
Luteal	7	0	(0)	0	(0)
Secretory	13	3	(25)	2	(16)
Menstrual	7	0	(0)	2	(28)

* ER 8S peak in SDGA, positive > 3 fmole/mg protein.
† PR total binding in DCCA, positive > 3 fmole/mg protein.



FIGS. 5A AND 5B. Menstrual cycle variations of ER and PR in the normal human breast. (A, left) Mean values of ER are shown for days of the menstrual cycle. (B, right) Mean values for PR are shown for days of the menstrual cycle. Running averages calculated as described. Vertical line: SEM.

positive samples (57%) occurred during the proliferative (follicular) phase, while a significantly lower incidence was observed during other phases. This menstrual cycle dependence may account for some of the discrepancies among reported levels of ER found in normal breast tissue.[10-15] The overall incidence of ER positive specimens was 16/56 (28%) in this series. This is somewhat higher than reported values and may relate to the fact that receptor analyses were performed on epithelial-enriched tissue obtained by subgross dissection.

Sex steroid receptor control of breast carcinomas is usually assessed by measurement of cytosolic estrogen and progesterone receptors.[25] Since receptor analysis has important prognostic[26] and therapeutic[27] implications and is used to stratify patients in adjuvant chemotherapy trials, accurate evaluation of the integrity of receptor control is crucial in premenopausal patients with breast carcinoma. In view of the low levels of ER normally observed during the luteal phase, it would be difficult to determine whether a low ER in a breast carcinoma sampled during this phase reflects a lack of receptor control or whether it reflects a functioning receptor mechanism. Although Saez *et al.*[29] reported that breast carcinoma specimens obtained from premenopausal women did not have menstrual cycle variations in levels of ER and PR, they did not consider relative tumor differentiation,[25] nor did they evaluate the influence of menstrual cycle irregularities or exogenous hormone use. Furthermore, the patients in that study did not demonstrate the expected menstrual cycle variation of serum estradiol and had low luteal phase progesterone levels. It would therefore seem premature to conclude that menstrual cycling of ER and PR does not occur in some breast carcinomas as it does in normal breast tissue.

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