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*Report*

# Pattern of distribution of cells positive for estrogen receptor α and progesterone receptor in relation to proliferating cells in the mammary gland *

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*Key words:* breast epithelium, cell proliferation, differentiation, estrogen receptors, progesterone receptors

**Summary**

Since cell proliferation is indispensable for the growth and development of the breast, and estrogens are considered to play a major role in promoting cell proliferation, while progesterone influences its differentiation, the present work was designed with the purpose of verifying the relationship between cells containing steroid hormone receptors and proliferating cells in the normal human breast. Twelve breast samples were analyzed for their content of lobules type 1 (Lob1), Lob2, Lob3, and Lob4, and the number of cells containing estrogen receptor alpha (ER-α), progesterone receptor (PgR), or expressing Ki67 antibody was determined by double immunocytochemical technique with specific antibodies. The highest percentage of ER-α, PgR, and Ki67 positive cells was found in Lob1, with a progressive reduction in the more differentiated Lob2 and Lob3. ER-α and PgR positive cells were found exclusively in the breast epithelium and were negative for Ki67, while cells positive for Ki67 did not express receptors. These findings were compared with the distribution of ER-α and PgR in the autoradiographs of mammary gland of young virgin rats inoculated with $^{3}$H-thymidine for determination of the DNA labeling index (DNA-LI). Both the DNA-LI and the percentage of ER-α and PgR positive cells were maximal in the epithelium of terminal end buds, and these values were reduced in alveolar buds and lobules. ER-α and PgR positive cells did not proliferate, and those cells that had incorporated $^{3}$H-thymidine were negative for both receptors. Our results led us to conclude that the content of ER-α and PgR in the normal mammary tissue varies with the degree of lobular development, in parallel with cell proliferation. However, the expression of receptors occurs in cells other than the proliferating cells, indicating that they represent at least two separate cell populations. These findings open new avenues towards the understanding of the mechanisms through which estrogens and progesterone affect the proliferative activity of breast epithelial cells, and their role in the initiation of the cascade of events that leads a normal cell to cancer.

## Introduction

Even though the breast is influenced by a myriad of hormones and growth factors [1–9], estrogens are considered to play a major role in promoting the proliferation of both the normal and the neoplastic breast epithelium [10–16]. The influence of estrogens on the proliferative activity of mammary epithelial cells has been traditionally considered to be mediated by at least three different mechanisms: a receptor–mediated [11, 17–24], an autocrine/paracrine loop [15, 16], or a negative feedback, according to which estrogens remove the effect of one or several inhibitor factors present in the serum [13, 25]. Unfortunately, none of these mechanisms has been precisely defined with regard to their role in the normal development and differentiation of the breast, or the initiation and progression of the neoplastic process. This picture was complex enough until recently, when only a single type of estrogen receptor (ER) was known to exist as the mediator of the genomic effects of this hormone in specific target tissues. The recent cloning of a gene encoding a second type of ER, the ER-β, from the mouse, rat, and humans, with an affinity for estradiol similar to that of the classical ER (now identified as ER-α), has prompted a reevaluation of the estrogen signaling system [26–28].

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Cell proliferation is indispensable for the normal growth and development of the breast. The fact that the normal epithelium contains receptors for both estrogen and progesterone lends support to the receptor-mediated mechanism as a major player in the hormonal regulation of breast development. The role of these hormones on the proliferative activity of the breast has been for a long time, and still is, the subject of heated controversies. The breast epithelium of sexually mature normally cycling women does not exhibit maximal proliferation during the follicular phase of the menstrual cycle [5–9, 29–34], when estrogens reach peak levels of 200–300 pg/ml and progesterone is less than 1 ng/ml [35]. Maximal proliferative activity occurs during the luteal phase, when progesterone reaches levels of 10–20 ng/ml and estrogen levels are 2–3-fold lower than during the follicular phase [35]. In breast cells grown *in vitro*, or when breast tissues are implanted in athymic nude mice, however, estrogens stimulate cell proliferation and progesterone has no effect, or even inhibits cell growth [14, 17, 33, 34].

Our studies of the proliferative activity of the mammary epithelium in both rodents and humans have demonstrated cell division varies with the degree of differentiation of the mammary parenchyma [1–4, 10, 36–38]. In humans the highest level of cell proliferation is observed in the undifferentiated lobules type 1 (Lob1) present in the breast of young nulliparous females [1–4, 10]. The progressive differentiation of Lob1 into Lob2 and Lob3, occurring under the hormonal influences of the menstrual cycle, and the full differentiation into Lob4 during pregnancy, result in a concomitant reduction in the proliferative activity of the mammary epithelium [1–4, 37]. The relationship of lobular differentiation and cell proliferation with the hormone responsiveness of the mammary epithelium is just beginning to be unraveled. Of interest is the fact that the content of ER-α and progesterone receptors (PgR) in the lobular structures of the breast is directly proportional to the rate of cell proliferation, being maximal in the undifferentiated Lob1, and decreasing progressively in Lob2, Lob3, and Lob4 [12–16]. The present work was designed with the purpose of testing whether those cells expressing either ER-α or PgR were those responding with proliferation, supporting the postulated receptor-mediated mechanism of cell growth stimulation. For these purposes we analyzed normal human breast tissues utilizing a double staining procedure for identifying simultaneously ER-α or PgR positive cells and proliferating cells, and compared these findings with results obtained from the rat mammary gland in which cells synthesizing DNA were autoradiographically identified and compared with those containing ER-α and PgR.

## Materials and methods

### *Human breast tissue procurement and processing*

Normal breast tissues were obtained from reduction mammoplasty specimens from 12 sexually mature women who were premenopausal, ranging in age from 17 to 39 years, all of whom were regularly cycling. An average of 100 g of tissue was collected from every specimen, fixed in 10% neutral buffered formalin for a minimum of 24 h, embedded in paraffin, sectioned at 5 μm thickness, and processed for light microscopic examination and immunocytochemical detection of cell proliferation, ER-α, and PgR, utilizing the corresponding antibodies described below. All the breast samples were examined histologically for verifying the absence of mammary pathology and for quantitation of ductal and lobular structures.

### *Rat mammary glands*

Mammary tissue was obtained from virgin Sprague Dawley rats obtained from Taconic Farm (New York, NY). The rats were housed three to a cage in an environmentally controlled clean air room with a 12 h light/12 h darkness cycle. They were fed Purina Certified Rodent Chow 5002 pellets and tap water *ad libitum*. When the animals reached the age of 55 days they received an intraperitoneal injection of 5 μCi $^{3}$H-thymidine/g body weight ($^{3}$H-thymidine specific activity 86.0 μCi/μg) (Covance Laboratories, Vienna, VA). The animals were sacrificed 1 h later, and the mammary glands were dissected, fixed in 10% neutral buffered formalin, and processed for light microscopy, immunocytochemistry, and autoradiography as described elsewhere [36].

### *Antibodies*

The ER-α was detected in human breast tissues utilizing a mouse monoclonal antibody (clone ER1D5) (Amac Lab, Westbrook, ME) at 1:400 dilution. For the rat mammary gland a rabbit antibody raised against a synthetic peptide coupled to KLH, that recognized both human and rodent ER (Zymed Laboratories, San Francisco, CA), was utilized. Since the ER antibodies utilized detected only ER-α, and only these were tested throughout the experiments, the term ER utilized in the remainder of the manuscript refers exclusively

to ER-α. PgR was detected in both human and rat mammary tissue with the mouse monoclonal antibody clone PR10A9 (Immunotech, Westbrook, ME) at a 1:100 dilution. Cell proliferation was determined utilizing Ki67, a mouse monoclonal antibody raised against a human recombinant peptide corresponding to a 1002 bp Ki-67 cDNA fragment [39] (Oncogene Science, Cambridge, MA).

*Immunocytochemical procedures*

Sections of paraffin-embedded tissues were mounted on aminoalkylsilane-coated slides, deparaffinized, rehydrated, and incubated with 2% hydrogen peroxide at room temperature for 15 min for quenching endogenous peroxidase activity. The sections were sequentially incubated in two changes of Antigen Retrieval Solution *Citra* (Biogenex, San Ramon, CA) at 98°C for 5 min each, diluted normal blocking serum for 20 min, and then with the corresponding primary antibody. For PgR immunocytochemistry, slides were incubated in 0.02% trypsin for 10 min prior to the incubation with the primary antibody. Incubation with primary antibodies was carried out in a humidity chamber at 4°C overnight, followed by a buffer wash. Human breast sections immunoreacted for ER, PgR, and Ki67, and rat PgR were incubated with horse anti-mouse biotinylated secondary antibody. For ER, rat tissues were incubated with goat anti-rabbit biotinylated secondary antibody (Vector Laboratories, Burlingame, CA). The reaction proceeded at room temperature for 30 min. After a buffer rinse the slides were incubated for 30 min with Vectastain Elite ABC kit for mouse or rabbit, as indicated (Vector Laboratories, Burlingame, CA), washed in PBS buffer, and incubated in peroxidase substrate solution containing hydrogen peroxide and 3,3′-diaminobenzidine-HCl for 2 min. Sections incubated with non-immune serum were used as negative controls. All sections were lightly counterstained with hematoxylin. Immunostaining was evaluated by examination of slides under a bright field microscope, and graded according to the intensity of the brown reaction as negative (−), weakly (+), moderately (++), or strongly (+ + +) positive. In sections incubated with Ki67 antibody, cells exhibiting a positive reaction in the outer part of the nucleolus and in the granular component of the nucleus were interpreted to be in the S−, $G_1$−, $G_2$−, or M-phases of the cell cycle. The numbers of cells expressing the nuclear antigen Ki67 were counted and tabulated according to their location in Lob1, Lob2, Lob3, or Lob4. Results were expressed as the percentage of positive cells over the total number of cells counted in each lobular type.

*Double labeling for Ki67 and ER or PgR*

For the simultaneous identification of proliferating and steroid receptor positive cells, the tissue sections were initially processed as described above for single antibody detection, and then incubated first with Ki67 antibody at a dilution of 1:50. After overnight incubation at 4°C the sections were washed in PBS for 10 min, followed by incubation at room temperature with biotinylated horse anti-mouse secondary antibody for 30 min. The sections were then rinsed twice in buffer, incubated with Vectastain Elite ABC-kit for 30 min, and washed in PBS for 10 min, followed by DAB staining for 2 min. After washing twice in buffer the staining for the second antibody was initiated. It was preceded by treatment for antigen retrieval, followed by incubation with either monoclonal mouse anti human ER and PgR antibody overnight at 4°C. For PgR determination the slides were pretreated with 0.02% trypsin for 10 min, washed with PBS for 10 min, and then incubated with biotinylated horse anti-mouse antibody for 30 min at room temperature, washed twice in PBS, and incubated with Vectastain ABC alkaline phosphatase (AP) kit for 30 min at room temperature. The slides were then washed twice in PBS, incubated with AP substrate kit-vector red for 1 h, followed with sequential washes in PBS and tap water, and counterstained with hematoxylin. The level of specificity of these reactions was assessed by comparing the reactions observed in these double-labeled slides with serially obtained tissue sections individually reacted for Ki67, ER, or PgR. An additional control was made by reversing the order of the antibodies, incubating the sections first with the antibody against ER or PgR, followed by Ki67 antibody.

*Double labeling for ER and PgR*

In order to verify whether the cells containing ER were also positive for PgR, a double labeling procedure was applied to breast tissue section. The procedure was identical to that described above, but instead of incubating the slides with Ki67 first they were reacted with ER antibody, followed by PgR antibody.

*Autoradiography-immunocytochemistry procedure*

Paraffin sections of rat mammary tissues were deparaffinized in xylene, rehydrated in decreasing concentrations of ethanol, and coated with NTB2 nuclear track emulsion (Eastman Kodak, Rochester, NY). The sections were drained by gravity, allowed to dry, and then stored in dark boxes for two weeks. Autoradiography

was developed in the dark with Microdol X, fixed and then incubated with the ER or PgR antibodies, completing the immunocytochemical reaction as described above. Epithelial cells synthesizing DNA contained silver grains in the nucleus, and those ER or PgR positive exhibited a brown nuclear reaction. Their number, as well as the number of cells lacking both types of reactions, were counted in ducts, TEB, and lobules. Results were expressed as DNA Labeling Index (DNA-LI), or percentage of cells containing nuclear silver grains over the total number of cells counted in each type of structure, and the percentage of cells positive for each one of the steroid receptors, either singly or in combination. The relationship between cells positive by autoradiography and those positive for ER or PgR was photographically recorded utilizing an Olympus BH-2 bright field microscope with camera attachment.

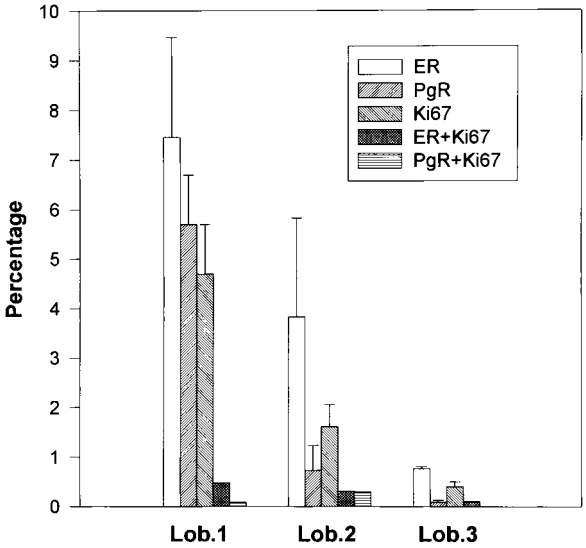


*Figure 1.* Percentage of cells positive for estrogen receptor (ER), progesterone receptor (PgR), Ki67, and of cells positive for both ER and Ki67 (ER + Ki67), or PgR and Ki67 (PgR + Ki67) (ordinate). Cells were quantitated in lobules type 1 (Lob1), type 2 (Lob2), and type 3 (Lob3) of the breast (abscissa).

## Results

The architectural pattern of all the breast samples was evaluated in tissue sections stained with hematoxylin and eosin. Normal tissues were analyzed for their content of Lob1, Lob2, Lob3, and Lob4, which were classified according to previously established criteria [2, 37]. Briefly, the size, morphological characteristics, and number of ductules comprising each lobular unit were the criteria applied for classifying the lobules present in each breast sample. The breast tissues of the 12 donors contained Lob1 (Figures 1, 2a–f), in which a total of 19,339 cells were counted (Table 1). Four of the breast samples studied were composed exclusively of Lob1. Five of the samples contained Lob2, and three of the samples contained Lob3 in addition to Lob1. In these samples a total of 8,490 and 17,750 cells were counted, respectively (Table 1). None of the breast tissues studied contained Lob4.

### *Distribution of proliferating cells in relation to steroid receptor positive cells in breast tissues*

Cell proliferation was detected in sections incubated with Ki67 antibody. The quantification of cells exhibiting a brown nuclear reaction characteristic of DAB stain, or cycling cells, revealed that the highest percentage of positive cells was found in Lob1 (Figure 1, Table 1). Cell proliferation was reduced by three-fold in Lob2, and by more than ten-fold in Lob3 (Figures 1, 2a,b; Table 1). The proliferating cells were almost exclusively found in the epithelium lining ducts and lobules, while only occasional positive cells were found in the myoepithelium, or in the intralobular and interlobular stroma. The same pattern of reactivity was observed in tissue sections incubated with the ER and PgR antibodies. Positive cells were found exclusively in the epithelium, with Lob1 containing the highest number of positive cells. Their number decreased progressively in Lob2 and Lob3 (Figure 1, Table 1).

The use of the double staining procedure for Ki67 and ER or PgR allowed us to quantitate and to deter-

*Figure 2.* (a–f) Lob1 ductules of the human breast: (a) the single-layered epithelium lining the ductule contains Ki67 positive cells (brown nuclei), and ER positive cells (red-purple nuclei) (x40); (b) the single-layered epithelium lining the ductule contains brown Ki67 positive cells, and red-purple PgR positive cells. The specificity of the reaction was verified by inverting the order of the stains; (c) and (d) ER positive cells, brown, Ki67 positive cells, purple red; (e) brown nuclei of PgR positive cells, and a Ki67 positive cell in mitosis appears stained purple red; (f) sections doubly stained for ER (brown) and PgR (red-purple). Most of the cells exhibit a simultaneous positive reaction for both antibodies, the overlap of the colors results in a reddish-brown nuclear discoloration in cells positive for both ER and PgR. Sections were stained with DAB/alkaline phosphatase-vector red, with light hematoxylin counterstain, and photographed at x40. (g) and (h) Rat mammary gland autoradiography, immunoreacted with ER (g) and PgR (h) antibodies: (g) Lob1 ductules. The black stippling of silver grains indicates $^{3}$H-thymidine incorporation (S phase), the brown nuclear reaction ER positive cells; (h) multilayered epithelium of a terminal end bud (TEB) containing cells in the S phase of the cycle (stippled nuclei) and the brown reaction of PgR positive cells (DAB-Hematoxylin) (x40).

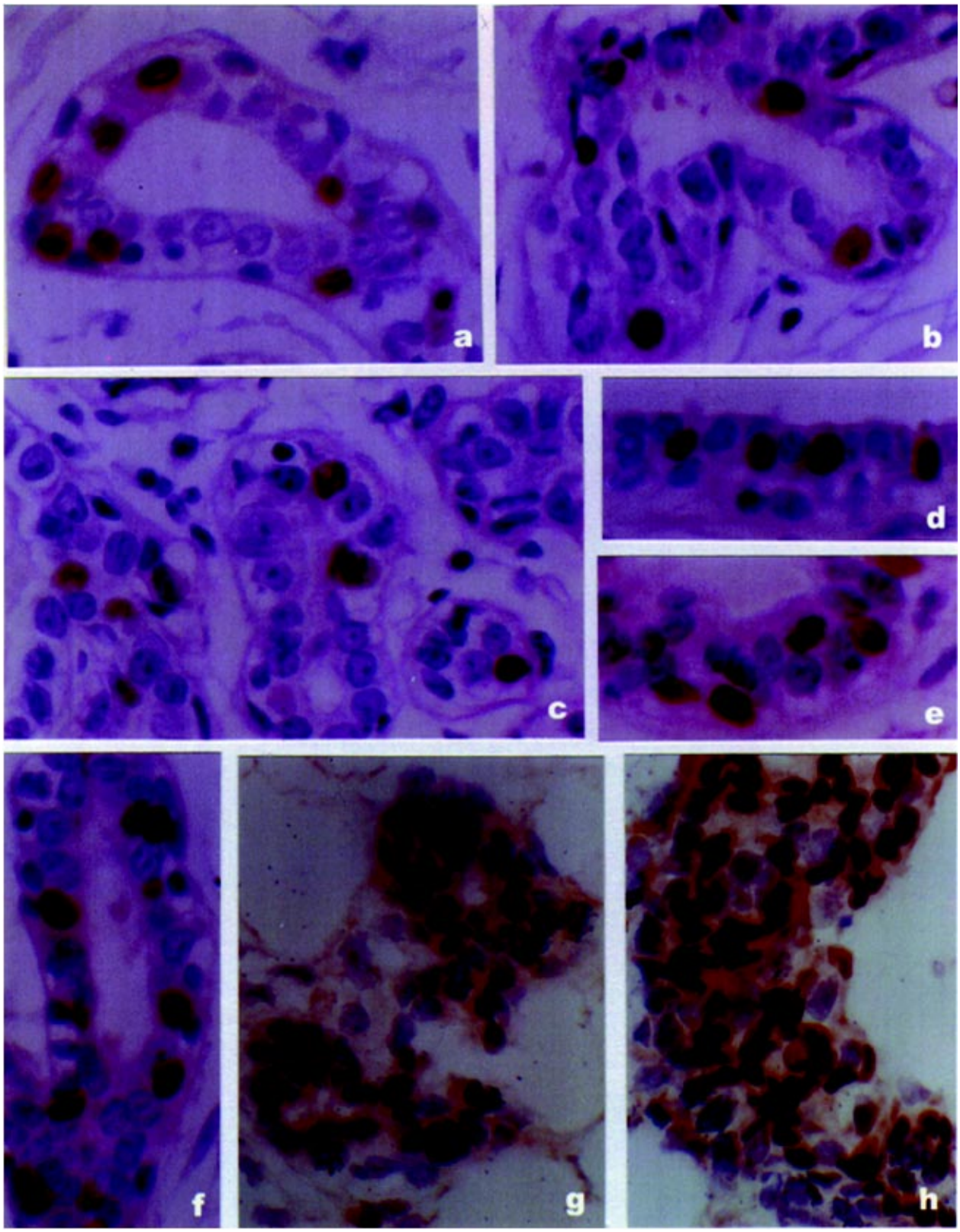


*Figure 2.*

*Table 1.* Distribution of Ki67, ER, and PgR positive cells in the lobular structures of the human breast

| Lobule type | No. cells | Ki67 | ER | PgR | Ki67 + ER | Ki67 + PgR |
|---|---|---|---|---|---|---|
| Lob1 | 19,339[a] | $4.72 \pm 1.00$[d,e] | $7.46 \pm 2.88$[h] | $5.70 \pm 1.36$[k] | $0.48 \pm 0.28$[n] | $0.09 \pm 0.01$[o] |
| Lob2 | 8,490[b] | $1.58 \pm 0.45$[f] | $3.83 \pm 2.44$[i] | $0.73 \pm 0.57$[l] | $0.31 \pm 0.21$ | $0.28 \pm 0.27$ |
| Lob3 | 17,750[c] | $0.40 \pm 0.18$[g] | $0.76 \pm 0.04$[j] | $0.09 \pm 0.04$[m] | $0.01 \pm 0.01$ | $0.01 \pm 0.01$ |

[a]Total number of cells counted in Lob1 in breast tissue samples of 12 donors.
[b]Total number of cells counted in Lob2 in breast tissue samples of five donors.
[c]Total number of cells counted in Lob3 in breast tissue samples of three donors.
[d]Proliferative activity determined by the percentage cells Ki67 positive, expressed as the mean ± SD. Differences were significative in [e]Lob1 vs. [f]Lob2 ($t = 1.98$, $p < 0.05$), [f]Lob2 vs. [g]Lob3 ($t = 2.27$, $p < 0.04$), and [e]Lob1 vs. [g]Lob3 ($t = 2.56$, $p < 0.01$). ER positive cells were significantly different in [h]Lob1 vs. [j]Lob3 ($t = 2.04$, $p < 0.05$). PgR positive cells were significantly different in [k]Lob1 vs. [l]Lob2 ($t = 2.27$, $p < 0.05$), and [k]Lob1 vs. [m]Lob3 ($t = 2.60$, $p < 0.03$).
[n]Percentage of cells positive for both Ki67 and ER, expressed as the mean ± SD.
[o]Percentage of cells positive for both Ki67 and PgR, expressed as the mean ± SD.

*Table 2.* Distribution of cells synthesizing DNA (DNA-LI) and of cells positive for ER and PgR in the rat mammary gland

| Type of structure | No. cells | DNA-LI | ER | PgR | DNA-LI + ER | DNA-LI + Pg |
|---|---|---|---|---|---|---|
| TEB | 7,092[a] | $20.31 \pm 1.02$[c,d] | $17.21 \pm 7.35$[f] | $43.35 \pm 9.61$[h] | $1.01 \pm 0.23$[j] | $3.31 \pm 0.55$[l] |
| AB + lobules | 5,861[b] | $3.26 \pm 0.68$[e] | $12.40 \pm 5.52$[g] | $39.81 \pm 7.15$[i] | $0.43 \pm 0.13$[k] | $0.14 \pm 0.05$[m] |

[a]Total number of cells counted in terminal end buds (TEB) in the mammary gland of six virgin rats.
[b]Total number of cells counted in alveolar buds (AB) and lobules in the mammary gland of six virgin rats.
[c]Proliferative activity, expressed as the percentage cells incorporating $^3$H-thymidine, or DNA labeling index (DNA-LI), expressed as the mean ± SD. The DNA-LI was significantly higher in [d]TEB vs. [e]AB + lobules ($t = 13.64$, $p < 0.000001$). The percentage of ER positive cells was significantly lower than the percentage of PgR positive cells in TEBs (f vs. h) ($t = 11.77$, $p < 0.00000$), and in AB + lobules (g vs. i), ($t = 13.27$, $p < 0.000000$).
ER positive cells were significantly higher in TEB than in AB + lobules (f vs. g) ($t = 2.26$, $p < 0.02$). The percentage of PgR positive cells in TEB ([h]) was not significantly different from that in AB + lobules ([i]). The percentage of cells doubly positive for $^3$H-thymidine incorporation and ER did not differ significantly in TEB ([j]) and AB + lobules ([k]). The percentage of cells doubly positive for $^3$H-thymidine incorporation and PgR was significantly higher in TEB than in AB + lobules (l vs. m) ($t = 5.07$, $p < 0.000003$).

mine in the same tissue sections the spatial relationship between those cells that were proliferating and those that reacted with the ER or PgR antibody, appearing purple-red in color due to the alkaline phosphatase-vector red staining (Figures 2a–e). The number of cells that expressed ER and/or PgR was similar to that of cells positive for Ki67, and the highest percentage of positive cells was also observed in Lob1 for both steroid hormones. The percentage of ER and PgR positive cells in Lob1 did not differ significantly, 7.5% and 5.7%, respectively (Figure 2f, Table 1). In Lob2 the percentage of ER and PgR positive cells was reduced to 3.8% and 0.7%, respectively, and in Lob3 their number became negligible (Table 1). Of interest was the observation that even though there were similarities in the relative percentages of Ki67, ER, and PgR positive cells, and in the progressive reduction in the percentage of positive cells as the lobular differentiation progressed, those cells positive for Ki67 were not the same that reacted positively for ER or PgR (Figures 2a,b; Table 1). Very few cells, less than 0.5% in Lob1, and even fewer in Lob2 and Lob3, appeared positive for both Ki67 and ER (Ki67 + ER) (Table 1). This double reactivity was identified by the darker staining of the nuclei, which appeared dark purple-brown. The percentage of cells exhibiting double labeling with Ki67 and PgR antibodies (Ki67 + PgR) (Table 1) in Lob1 was lower than the percentage of double labeled ER positive cells. There was a slight increase in Lob2, decreasing in Lob3, the percentages being quite similar to those observed in Ki67 + ER labeled cells (Table 1).

When the sequence in which the antibodies were incubated was reversed, and ER or PgR incubation preceded the Ki67 reaction, the color of the reactions

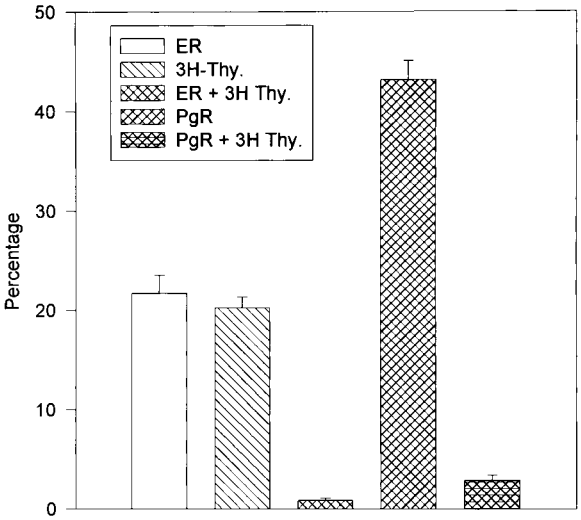


*Figure 3.* Histogram showing the percentage of cells positive for ER, $^3$H-thymidine incorporation during DNA synthesis ($^3$H-thy.), Double-labeled (ER $+^3$ H-thy.), PgR, and double labeled (PgR $+^3$ H-thy.) (ordinate). Cells were counted in TEB and AB + lobules (abscissa).

changed, with ER (Figures 2c,d) and PgR (Figure 2e) appearing brown, and Ki67 purple-red. Nevertheless, the percentages of cells positive for each one of the antibodies and their distribution did not change, confirming the results previously reported.

The double staining reaction incubating tissue sections with ER first, followed by PgR antibody, revealed that the nuclei of the epithelial cells positive for these receptors exhibited a dark brown-purple discoloration due to an overlap of the brown color of DAB and the purple-red of vector red, indicative of a simultaneous reaction with both steroid hormone receptor antibodies (Figure 2f). The number of positive cells was similar to the values obtained when quantifying a single receptor (Table 1). Occasional cells stained a lighter brown or a brighter purple-red, indicating that there were some cells containing only ER or PgR. Their number, however, was negligible.

### *Cell proliferation and ER and PgR content in the rat mammary gland*

The mammary gland of the young virgin rats was composed of a ductal system ending in club-shaped terminal end buds (TEB) or primitive ductular, alveolar buds (AB) and lobular structures (Figures 2g,h; Table 2). The epithelium lining the TEB was multilayered, exhibiting an active incorporation of $^3$H-thymidine in more than 20% of the cells (Figure 2h). The DNA-LI was markedly lower in AB and lobules (Table 2, Figure 2g). Proliferating cells were also detected in the stroma, but they were fewer in number, and they were not included in this analysis.

ER and PgR positive cells were detected only in the epithelium lining TEB, AB, ducts or lobules, but no positive cells were found in the stroma. The detection of ER and PgR in the epithelium of TEB, AB, and lobules (Figures 2g, h) revealed that the number of cells positive for both receptors varied with the type of structure considered. TEB contained the highest percentage of both ER and PgR positive cells, and both the values were reduced in AB and lobules. In contrast to what was observed in the human breast, however, the number of PgR positive cells was significantly higher than the number of ER positive cells in the corresponding structures (Table 2; Figure 3). It was clear that the immunocytochemical reaction with each one of the hormone receptor antibodies was positive in cells that lacked the stippling of the silver grains, whereas those cells that had incorporated $^3$H-thymidine had a sharp black stippling over the pale blue counterstain of hematoxylin, but were negative for both ER and PgR (Figures 2g,h and 3). In TEB the ratio of ER positive and $^3$H-thymidine labeled cells was roughly 1:1, while in AB and lobules it was 4:1. In all types of structures, however, the labeled cells were juxtaposed to the ER positive cells (Figure 2g). The ratio of PgR positive and $^3$H-thymidine labeled cells was 2:1 and 10:1 in TEB and AB + lobules, respectively (Table 2, Figure 2h). PgR positive cells formed clusters surrounding isolated $^3$H-thymidine labeled cells (Figure 2h).

## Discussion

Results reported here indicate that the content of ER and PgR in the normal breast tissue, as detected immunocytochemically, varies with the degree of lobular development, in a linear relationship with the rate of cell proliferation of the same structures. The utilization of a double labeling immunocytochemical technique for staining in the same tissue section of steroid hormone receptors and proliferating cells, i.e. Ki67 positive, allowed us to determine that the expression of the receptors occurs in cells other than the proliferating cells, confirming results reported by other authors [32]. Results obtained by performing immunocytochemical stains for ER, PgR, and Ki67 in human breast tissues were compared vis-à-vis with the *in vivo* incorporation of $^3$H-thymidine into cells that were synthesizing DNA in the mammary glands of virgin rats. The analysis of the mammary glands of young virgin Sprague-Dawley rats confirmed previous findings on

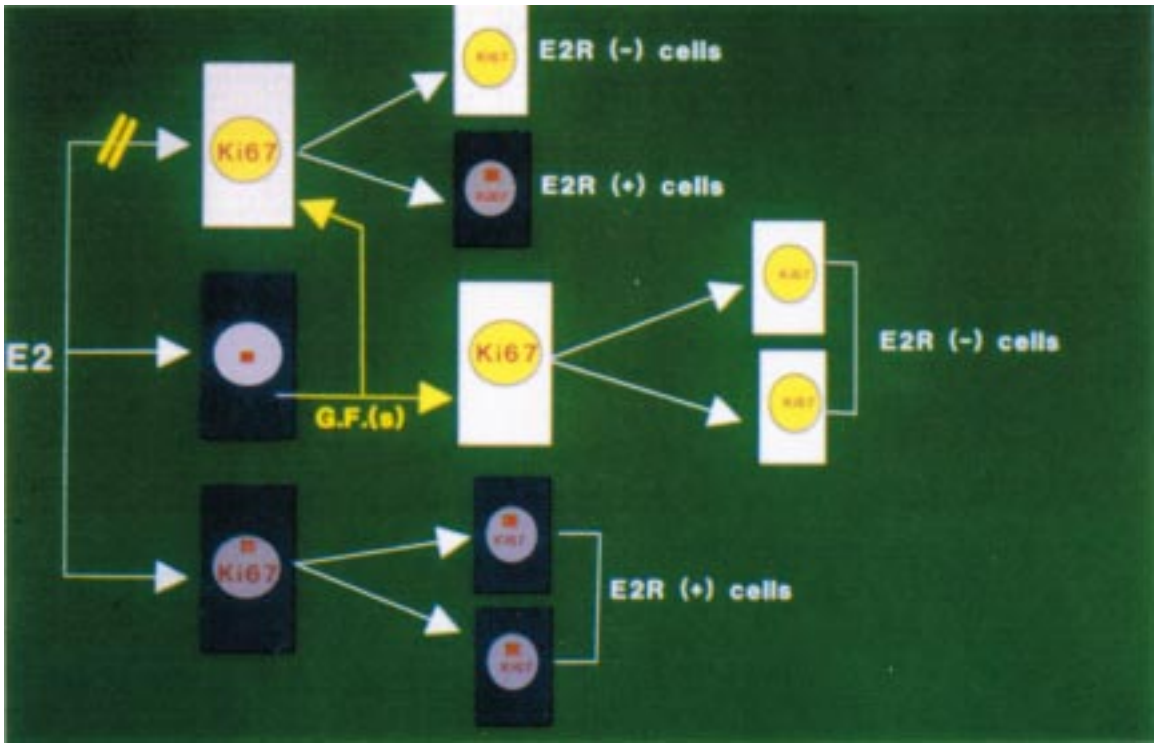


*Figure 4.* Schematic representation of the postulated pathways of estrogen actions on breast epithelial cells. Cells expressing three different phenotypes might be present in the epithelium: estrogen receptor (ER) negative Ki67 positive cells that are capable of proliferating, ER positive cells that do not proliferate, and a small proportion of ER and Ki67 positive cells. Estrogen might stimulate ER positive cells to produce a growth factor that in turn stimulates neighboring ER negative cells capable of proliferating. ER + Ki67 positive cells can proliferate and could be stimulated by estrogen to originate ER positive daughter cells or probably tumors. ER negative cells may convert to ER positive cells during neoplastic transformation.

the greater proliferative activity of TEBs [36]. It also revealed that the TEBs, ABs, and lobules of the virgin rat mammary gland contain receptors for both estrogen and progesterone, and that the number of cells positive for both receptors was higher in the epithelium of TEB, progressively declining in the more differentiated AB and lobules. The higher concentration of ER and PgR in the immature mammary gland of rodents and other species has been reported by other authors [40]. Similar to what has been observed in humans, the rat mammary gland contains steroid hormone receptor positive cells only in the ductal and lobular epithelium, but no positive cells were found in the stroma. These findings contrast with results obtained by cytosolic determination that reported that a high percentage of receptors were located in the mammary stroma [40].

The findings that proliferating cells are different from those that are ER and PgR positive support data that indicate that estrogen controls cell proliferation by an indirect mechanism. This phenomenon has been demonstrated using supernatants of estrogen-treated ER positive cells that stimulate the growth of ER negative cell lines in culture. The same phenomenon has been shown *in vivo* in nude mice bearing ER negative breast tumor xenografts [41, 42]. ER positive cells treated with antiestrogens secrete TGF β that inhibits the proliferation of ER negative cells [21].

Our studies have shown that the proliferative activity and the percentage of ER and PgR positive cells are highest in Lob1 in comparison with the various lobular structures composing the normal breast. These findings provide a mechanistic explanation for the higher susceptibility of these structures to be transformed by chemical carcinogens *in vitro* [43, 44], supporting as well the observations that Lob1 are the site of origin of ductal carcinomas [45]. However, the relationship between ER positive and ER negative breast cancers is not clear [46, 47]. It has been suggested either that ER negative breast cancers result from the loss of the ability of the cells to synthesize ER during clinical evolution of ER positive cancers, or that are ER positive and ER negative cancers are different entities [46,

48]. Our data allowed us to postulate that Lob1 contain at least three cell types, ER positive cells that do not proliferate, ER negative cells that are capable of proliferating, and a small proportion of ER positive cells that can proliferate as well (Figure 4). Therefore, estrogen might stimulate ER positive cells to produce a growth factor that in turn stimulates neighboring ER negative cells capable of proliferating (Figure 4). In the same fashion, the small proportion of cells that are ER positive and can proliferate could be the source of ER positive tumors. The possibility exists, as well, that the ER negative cells convert to ER positive cells. The conversion of ER negative to ER positive cells has been reported [49, 50]. The newly discovered ERβ opens the possibility that those cells traditionally considered to be ER-α negative might be ERβ positive [26–28]. We have recently found that ER-β is expressed during the immortalization and transformation of ER negative human breast epithelial cells, supporting this hypothesis [51].

The findings that proliferating cells in the human breast are different from those that contain steroid hormone receptors explain much of the *in vitro* data [52–55]. Of interest are the observations that while the ER positive MCF-7 cells respond to estrogen treatment with increased cell proliferation, and that the enhanced expression of the receptor by transfection also increased the proliferative response to estrogen [52, 56], ER negative cells, such as MDA-MB 468 and others, when transfected with ER, exhibit inhibition of cell growth under the same type of treatment [53–57]. Although the negative effect of estrogen on those ER negative cells transfected with the receptor has been interpreted as an interference with the transcription factor used to maintain estrogen independent growth [56], there is no definitive explanation for their lack of survival. These data can be explained in the light of the present work, in which proliferating and ER positive cells are two separate populations. Furthermore, we have observed that when Lob1 of normal breast tissue are placed in culture they lose the ER positive cells, indicating that only proliferating cells, that are also ER negative, can survive, and constitute the stem cells (unpublished observation). These observations are supported by the fact that MCF-10F, a spontaneously immortalized human breast epithelial cell line derived from breast tissues containing Lob1 and Lob2, is ER negative [57, 58].

Until recently, it was believed that estrogens acted through a single nuclear estrogen receptor that transcriptionally activated specific target genes, but there is mounting evidence that a membrane receptor coupled to alternative second messenger signaling mechanisms [59, 60] is also operational, and may stimulate the cascade of events leading to cell proliferation. This knowledge suggests that ER(α) negative cells found in the human breast may respond to estrogens through this or other pathways. Although more studies need to be done in this direction, it is clear that the findings that in the normal breast the proliferating and steroid hormone receptor positive cells are different open new possibilities for clarifying the mechanisms through which estrogens might act on the proliferating cells to initiate the cascade of events leading to cancer.

## References


1. Russo J, Russo IH: Role of hormones in human breast development: The menopausal breast. In: Progress in the Management of Menopause. Parthenon Publishing, London, 1997, pp 184–193
2. Russo IH, Russo J: Role of hormones in cancer initiation and progression. J Mammary Gland Biol Neoplasia 3: 49–61, 1998
3. Russo J, Russo IH: Role of differentiation in the pathogenesis and prevention of breast cancer. Endocrine Related Cancer 4: 7–21, 1997
4. Calaf G, Alvarado ME, Bonney GE, Amfoh KK, Russo J: Influence of lobular development on breast epithelial cell proliferation and steroid hormone receptor content. Int J Oncol 7: 1285–1288, 1995
5. Lippman ME, Dickson RB, Gelmann EP, Rosen N, Knabbe C, Bates S, Bronzert D, Huff K, Kasid A: Growth regulation of human breast carcinoma occurs through regulated growth factor secretion. J Cell Biochem 35: 1–16, 1987
6. Meyer JS: Cell proliferation in normal human breast ducts, fibroadenomas, and other duct hyperplasias, measured by nuclear labeling with tritiated thymidine. Hum Path 8: 67–81, 1977
7. Masters JRW, Drife JO, Scarisbrick JJ: Cyclic variations of DNA synthesis in human breast epithelium. J Natl Cancer Inst 58: 1263–1265, 1977
8. Ferguson DJP, Anderson TJ: Morphologic evaluation of cell turnover in relation to the menstrual cycle in the 'resting' human breast. Br J Cancer 44: 177–181, 1981
9. Anderson TJ, Ferguson DJP, Raab GM: Cell turnover in the 'resting' human breast: influence of parity, contraceptive pill, age and laterality. Br J Cancer 46: 376–382, 1982
10. Russo J, Russo IH: Estrogens and cell proliferation in the human breast. J Cardiovasc Pharmacol 28: 19–23, 1996
11. Kumar V, Stack GS, Berry M, Jin JR, Chambon P: Functional domains of the human estrogen receptor. Cell 51: 941–951, 1987
12. King RJB: Effects of steroid hormones and related compounds on gene transcription. Clin Endocrinol 36: 1–14, 1992
13. Soto AM, Sonnenschein C: Cell proliferation of estrogen-sensitive cells: the case for negative control. Endocr Rev 48: 52–58, 1987
14. Huseby RA, Maloney TM, McGrath CM: Evidence for a direct growth-stimulating effect of estradiol on human MCF-7 cells *in vitro*. Cancer Res 144: 2654–2659, 1987

15. Huff KK, Knabbe C, Lindsey R, Kaufman D, Bronzert D, Lippman ME, Dickson RB: Multihormonal regulation of insulin-like growth factor-1-related protein in MCF-7 human breast cancer cells. Mol Endocrinol 2: 200–208, 1988
16. Dickson RB, Huff KK, Spencer EM, Lippman ME: Introduction of epidermal growth factor related polypeptides by 17β-estradiol in MCF-7 human breast cancer cells. Endocrinol 118: 138–142, 1986
17. Katzenellenbogen BS, Kendra KL, Norman MJ, Berthois Y: Proliferation, hormonal responsiveness and estrogen receptor content of MCF-7 human breast cancer cell growth in the short-term and long-term absence of estrogens. Cancer Res 47: 4355–4360, 1987
18. Petersen OW, Hoyer PE, van Deurs B: Frequency and distribution of estrogen receptor positive cells in normal, nonlactating human breast tissue. Cancer Res 47: 5748–5751, 1987
19. Jacquemier JD, Hassouin J, Torente M, Martin PM: Distribution of estrogen and progesterone receptors in healthy tissue adjacent to breast lesions at various stages: immunohistochemical study of 107 cases. Breast Cancer Res Treat 15: 109–117, 1990
20. McGuire W, Carbone P, Vollmer R (eds): Estrogen Receptors in Human Breast Cancer. Raven Press, New York, 1975
21. Dickson R, Lippman M: Control of human breast cancer by estrogen, growth factors and oncogenes. In: Lipman ME, Dickson RB (eds) Estrogen Receptors in Human Breast Cancer. Raven Press, New York, 1975
22. Wittliff JL: Steroid-hormone receptors in breast cancer. Cancer 53: 630–643, 1984
23. Watts CKW, Handel ML, King RJB, Sutherland RL: Oestrogen receptor gene structure and function in breast cancer. J Steroid Biochem Molec Biol 41: 5293–5536, 1992
24. Aakvaag A, Utaacker E, Thorsen T, Lea OA, Lahooti H: Growth control of human mammary cancer cells (MCF-7 cells) in culture: effect of estradiol and growth factors in serum containing medium. Cancer Res 50: 7806–7810, 1990
25. Dell'aquilla ML, Pigott DA, Bonaquist DL, Gaffney EV: A factor from plasma derived human serum that inhibits the growth of the mammary cell line MCF-7: characterization and purification. J Natl Cancer Inst 72: 291–298, 1984
26. Kuiper GGJM, Enmark E, Pelto-Huikko M, Nilsson S, Gustaffson J-Å: Cloning of a novel estrogen receptor expressed in rat prostate and ovary. Proc Natl Acad Sci USA 93: 5925–5930, 1996
27. Byers M, Kuiper GGJM, Gustaffson J-Å, Park-Sarge OK: Estrogen receptor-β mRNA expression in rat ovary: downregulation by gonadotropins. Mol Endocrinol 11: 172–182, 1997
28. Vladusic EA, Hornby AE, Guerra-Vladusic FK, Lupu R: Expression of estrogen receptor β messenger RNA variant in human breast cancer. Cancer Res 58: 210–214, 1998
29. Longacre TA, Bartow SA: A correlative morphologic study of human breast and endometrium in the menstrual cycle. Am J Surg Pathol 10: 382–393, 1986
30. Going JJ, Anderson TJ, Battersby S: Proliferative and secretory activity in human breast during natural and artificial menstrual cycles. Am J Pathol 130: 193–204, 1988
31. Potten CS, Watson RJ, Williams GT: The effect of age and menstrual cycle upon proliferative activity of the normal human breast. Br J Cancer 58: 163–170, 1988
32. Clark RB, Howell A, Potter CS, Anderson E: Dissociation between steroid receptor expression and cell proliferation in the human breast. Cancer Res 57: 4987–4991, 1997
33. Laidlaw IJ, Clark RB, Howell A, Owen AWMC, Potten CS, Anderson E: Estrogen and progesterone stimulate proliferation of normal human breast tissue implanted in athymic nude mice. Endocrinol 136: 164–171, 1995
34. Clarke RB, Howell A, Anderson E: Estrogen sensitivity of normal human breast tissue *in vivo* and implanted into athymic nude mice; analysis of the relationship between estrogen-induced proliferation and progesterone receptor expression. Breast Cancer Res Treat 45: 121–183, 1997
35. Goodman HM: Basic Medical Endocrinology. Raven Press, New York, 1994, pp 288–290
36. Russo J, Russo IH: Influence of differentiation and cell kinetics on the susceptibility of the rat mammary gland to carcinogenesis. Cancer Res 40: 2677–2687, 1980
37. Russo J, Rivera R, Russo IH: Influence of age and parity on the development of the human breast. Breast Cancer Res Treat 23: 211–218, 1992
38. Russo J, Russo IH: Biology of disease. Biological and molecular bases of mammary carcinogenesis. Lab Invest 57: 112–137, 1987
39. Key G, Becker MH, Baron B, Duchrow M, Schluter C, Flad HD, Gerdes J: New Ki-67-equivalent murine monoclonal antibodies (MIB1-3) generated against bacterially expressed parts of the Ki-67 cDNA containing three 62 base pair repetitive elements encoding for the Ki-67 epitope. Lab Invest 68: 629–636, 1993
40. Haslam S: Role of sex steroid hormones in normal mammary gland function. In: Neville MC, Daniel CW (eds) The Mammary Gland: Development, Regulation and Function. Plenum Press, New York, 1987, pp 499–533
41. Clarke R, Dickson RB, Lippman ME: Hormonal aspects of breast cancer. Growth factors, drugs and stromal interactions. Crit Rev Oncol Hematol 12: 1–23, 1992
42. Knabbe C, Lippman ME, Wakefield LM, et al.: Evidence that transforming growth factor β is a hormonally regulated negative growth factor in human breast cancer cells. Cell 48: 417–428, 1987
43. Russo J, Reina D, Frederick J, Russo IH: Expression of phenotypical changes by human breast epithelial cells treated with carcinogens *in vitro*. Cancer Res 48: 2837–2857, 1998
44. Russo J, Calaf G, Russo IH: A critical approach to the malignant transformation of human breast epithelial cells. Crit Rev Oncogen 4: 403–417, 1993
45. Russo J, Gusterson BA, Rogers A, Russo IH, Wellings SR, van Zwieten MJ: Biology of breast disease. Comparative study of human and rat mammary tumorigenesis. Lab Invest 62: 244–278, 1990
46. Habel LA, Stamford JL: Hormone receptors and breast cancer. Epidemiol Rev 15: 209–219, 1993
47. Harlan LC, Coates RJ, Block G: Estrogen receptor status and dieting intakes in breast cancer patients. Epidemiology 4: 25–31, 1993
48. Moolgavkar SH, Day NE, Stevens RG: Two-stage model for carcinogenesis: epidemiology of breast cancer in females. J Natl Cancer Inst 65: 559–569, 1980
49. Kodama F, Green GL, Salmon SE: Relation of estrogen receptor expression to clonal growth and antiestrogen effects on human breast cancer cells. Cancer Res 45: 2720–2724, 1985
50. Podhajcer OL, Bravo AL, Sorin I, Guman N, Cerdeiro R, Mordoh J: Determination of DNA synthesis, estrogen

receptors, and carcinoembryonic antigen in isolated cellular subpopulations of human breast cancer. Cancer 58: 720–729, 1986
51. Hu, YF, Lau KM, Ho SM, Russo J: Increased expression of estrogen receptor β in chemically transformed human breast epithelial cells. Int J Oncol 12: 1225–1228, 1998
52. Foster JS, Wimalasena J: Estrogen regulates activity of cyclin-dependent kinases and retinoblastoma protein phosphorylation in breast cancer cells. Mol. Endocrinol 10: 488–498, 1996
53. Wang W, Smith R, Burghardt R, Safe SH: 17β estradiol-mediated growth inhibition of MDA-MB 468 cells stably transfected with the estrogen receptor: cell cycle effects. Mol. Cell Endocrinol 133: 49–62, 1997
54. Levenson AS, Jordan VC: Transfection of human estrogen receptor (ER) cDNA into ER negative mammalian cell lines. J Steroid Biochem Mol Biol 51: 229–239, 1994
55. Weisz A, Bresciani F: Estrogen regulation of proto-oncogenes coding for nuclear proteins. Crit Rev Oncogen 4: 361–388, 1993
56. Zajchowski DA, Sager R, Webster L: Estrogen inhibits the growth of estrogen receptor negative, but not estrogen receptor positive, human mammary epithelial cells expressing a recombinant estrogen receptor. Cancer Res 53: 5004–5011, 1993
57. Pilat MJ, Christman JK, Brooks SC: Characterization of the estrogen receptor transfected MCF-10A breast cell line 139B6. Breast Cancer Res Treat 37: 253–266, 1996
58. Calaf G, Tahin Q, Alvarado ME, Estrada S, Cox T, Russo J: Hormone receptors and cathepsin D levels in human breast epithelial cells transformed by chemical carcinogens. Breast Cancer Res Treat 29: 169–177, 1993
59. Aronica SM, Kraus WL, Katzenellenbogen BS: Estrogen action via the cAMP signaling pathway. Stimulation of adenylate cyclase and cAMP regulated gene transcription. Proc Natl Acad Sci USA 91: 8517–8521, 1994
60. Pappos TC, Gametahu B, Watson CS: Membrane estrogen receptors identified by multiple antibody labeling and impeded-ligand binding. FASEB J 9: 404–410, 1994

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