

# Frequency and Distribution of Estrogen Receptor-positive Cells in Normal, Nonlactating Human Breast Tissue<sup>1</sup>

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## ABSTRACT

We have analyzed the frequency and distribution of cells expressing estrogen receptor (ER) in cryosections of normal human breast tissue using quantitative microspectrophotometry and the estrogen receptor immunocytochemical assay. We found that the human mammary gland contained a small but distinct population of ER-positive cells, comprising approximately 7% of the total epithelial cell population from all biopsies. Stromal cells were found to be ER negative. The ER-positive cells were distributed as scattered single cells, with the highest frequency and intensity of measured staining in the lobules as compared to the interlobular ducts. Moreover, on the average, 87% of the ER-positive cells were luminal epithelial cells or occupied an intermediate position in the duct wall. The intermediate cells were found not to express basal cell phenotype as determined by combined immunocytochemistry to ER and "common acute lymphoblastic leukemia antigen" selectively decorating myoepithelial cells (B. A. Gusterson *et al.*, *J. Natl. Cancer Inst.*, 77: 343-349, 1986).

## INTRODUCTION

No data on the frequency and distribution of estrogen receptor-positive cells in normal breast tissue are available, although ER<sup>4</sup> obviously play a significant role in the biology of the mammary gland. Thus, the changes observed in the mammary gland during puberty, pregnancy, lactation, menopause, and menstrual cycle are all correlated to physiologically varying amounts of circulating estradiol in the blood stream (1-4). Moreover, more than 50% of malignant neoplasms arising from the breast gland epithelium are estrogen receptor positive (5). Also some risk factors for development of breast cancer, such as early menarche, late menopause, nulliparity, and giving birth to first child after the age of 30 years (6), probably reflect a life span variation in levels of circulating estrogen. These observations all strongly suggest that the normal human breast gland comprises cells with high-affinity receptors to circulating estrogen. However, it is not evident from these observations which cell type (stromal, parenchymal, or both) the ER-positive cell population is affiliated to. The aim of the present study was therefore to evaluate the immunoreactivity of an antibody to estrogen receptors in cryosections of the normal, nonlactating mammary gland using the recently introduced ER-ICA (7).

## MATERIALS AND METHODS

Normal breast tissue consisted of 18 biopsy specimens removed from patients (age range, 15-59 years) undergoing reduction mammoplasty

primarily due to "mammary hypertrophy" or "asymmetry." The tissue received was frozen immediately in *n*-hexane or isopentane cooled to -70°C in solid carbon dioxide. Up to ten sections were cut at different levels of the biopsy in a cryostat with a section thickness setting of 4 µm and a chamber temperature of -18°C.

The sections were picked up on clean uncoated glass slides and after about 30 s at room temperature (rt) transferred to 3.5% formaldehyde in 0.1 M phosphate buffer, pH 7.35, for 15 min, also at rt. Then sections were rinsed for 5 min in PBS at rt, transferred to fresh PBS at rt, and stored as such until they were all ready (maximal time, 30 min). Next, the sections were fixed for 1-3 min in methanol at -20°C followed by fixation for 1-3 min in acetone at -20°C. After a 5-min rinse in PBS at rt, they were incubated for demonstration of estrogen receptors by using the ER-ICA kit purchased from Abbott Laboratories Diagnostics Division (Vedbaek, Denmark), as recommended by the package insert. All incubations were performed at rt. Sections were incubated for 15 min with blocking reagent, 30 min with primary antibody, 30 min with bridging antibody, 30 min with peroxidase-antiperoxidase complex, and 10 min with diaminobenzidine-H<sub>2</sub>O<sub>2</sub>. All incubation steps were separated from each other by a double 5-min rinse in PBS. Sections incubated in a similar manner, except that the primary antibody was replaced by the control antibody (Abbott Diagnostics), served as controls. The frequency of estrogen receptor-positive cells in cryosections was determined after a brief (and very weak) hematoxylin staining of the ER-ICA-incubated sections, using a ×25 objective and a ×12.5 eye piece. Both ER-positive and ER-negative cells were counted. Areas with fibrocystic changes (here restricted to epithelial hyperplasia in duct walls or lobules or the appearance of cysts) were not evaluated.

In cryosections from 5 biopsy specimens, the amount of final reaction product, a polymer of oxidized diaminobenzidine, was quantified using a Vickers M85A scanning and integrating microspectrophotometer. Measurements were performed at 460 nm, which is the absorption maximum of the oxidized diaminobenzidine polymer (8). A ×40 objective, flying spot 1 (diameter, 0.5 µm in the specimen plane), and a field mask size A2 (scanning area of 78.5 µm<sup>2</sup>) were used. The size of the scanning field mask was just sufficient to encompass 1 nucleus. Generally 50 nuclei, both in lobules and interlobular ducts, were measured separately in at least 3 cryosections. To calculate the absorbance due to specific binding of primary antibody, the mean absorbance value obtained from cryosections exposed to control serum was subtracted from that obtained from consecutive sections exposed to the primary antibody of the ER-ICA kit.

In some sections, myoepithelial cells were demonstrated along with ER-positive nuclei by combined immunocytochemistry to the CALLA (9) and the ER-ICA. Sections fixed in 3.5% formaldehyde in 0.1 M phosphate buffer, pH 7.35, were incubated for 5 min at rt with PBS-NGS, followed by incubation for 30 min in PBS-NGS containing DAKO-CALLA antibody (M 727), 1:50 (kindly provided by Dakopatts, Glostrup, Denmark). Then the sections were rinsed for three times for 5 min in PBS-NGS, followed by a 30-min incubation at rt in PBS-NGS containing horseradish peroxidase-conjugated goat anti-mouse IgG, 1:20 (Medac, Code 6450; Gesellschaft für klinische Spezialpräparate, mbH, Hamburg, West Germany; purchased from Pharmavit, Birkerød, Denmark). After three 5-min rinses in PBS-NGS, the sections were incubated for 30 min at rt in PBS containing 0.5 mg/ml diaminobenzidine and 0.5 µl/ml 30% H<sub>2</sub>O<sub>2</sub>. Immediately after this incubation, the sections (not allowed to dry) were further processed for the ER-ICA. No apparent loss of ER expression was recorded using this type of double incubation. To visualize some selected histological configurations for micrographs, cryosections were incubated to detect cyokeratins. A few sections were fixed for 5 min in methanol at -20°C

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The abbreviations used are: ER, estrogen receptor(s); CALLA, common acute lymphoblastic leukemia antigen; ER-ICA, estrogen receptor immunocytochemical assay; rt, room temperature; PBS, phosphate-buffered saline; NGS, 5% normal goat serum.

followed by incubation as described above for CALLA with a rabbit anti-human keratin antiserum (DAKO, Code A575) diluted 1:400 and a horseradish peroxidase-conjugated swine anti-rabbit immunoglobulin at 1:20 (DAKO).

**Statistics.** Since none of the data fulfilled the criteria for normal distribution, data with similar distribution were compared using the Wilcoxon rank sums tests and centered signed rank statistics. Otherwise the median 2-sample test was used. The statistics were performed using the VMS Version of SAS Release 5.03, The SAS Institute, Inc., Cary, NC.

**RESULTS**

By immunocytochemistry, the normal human breast tissue was found to contain estrogen receptor-positive cells in all the biopsy specimens investigated. ER-positive cells were present exclusively in parenchymal tissue, *i.e.*, ducts and lobules (as defined in Fig. 1), whereas no staining was seen in stromal cells (Figs. 2 and 3). Positive staining was distinctly confined to the nucleus of individual cells, either as a weak central staining or as a strong staining of the entire nucleus (Figs. 2 and 3).

The staining pattern of the mammary ducts and lobules was far from homogeneous. Thus, the ER-ICA distinctly outlined two different subpopulations of mammary epithelial cells, those with, and those without nuclear estrogen receptors. The estrogen receptor-positive subpopulation comprised (the biopsies taken all together) approximately 7% of the total population of epithelial cells in the mammary ducts and lobules. These 7%

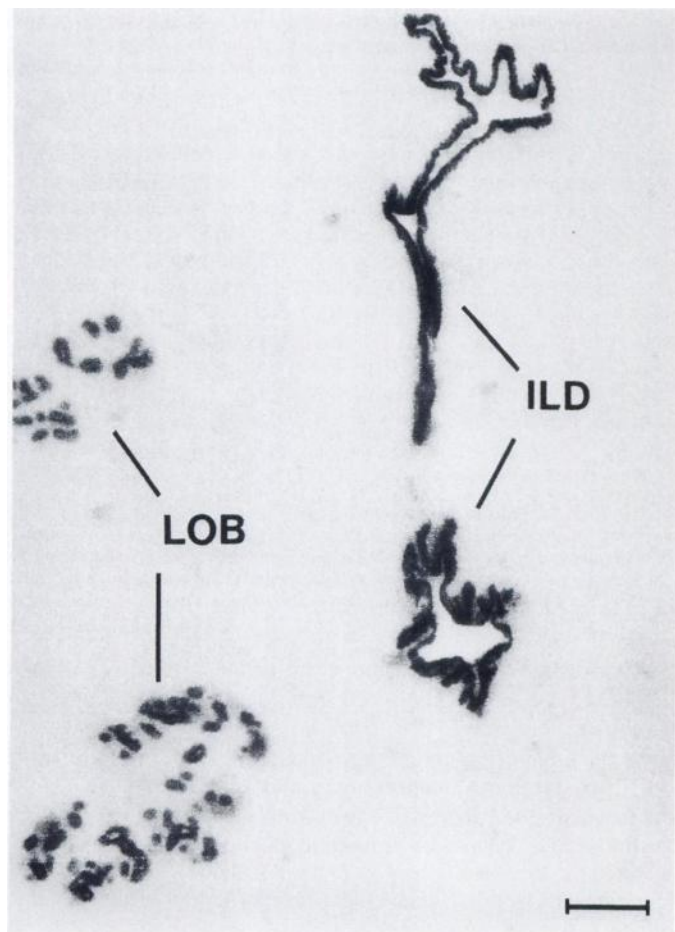


Fig. 1. Immunocytochemical detection of epithelial specific cytokeratins in a cryosection of "normal" human breast tissue to demarcate the epithelial components. The picture shows what is defined as typical lobules (LOB) and interlobular ducts (ILD) in the present study.  $\times 55$ ; bar, 200  $\mu\text{m}$ .

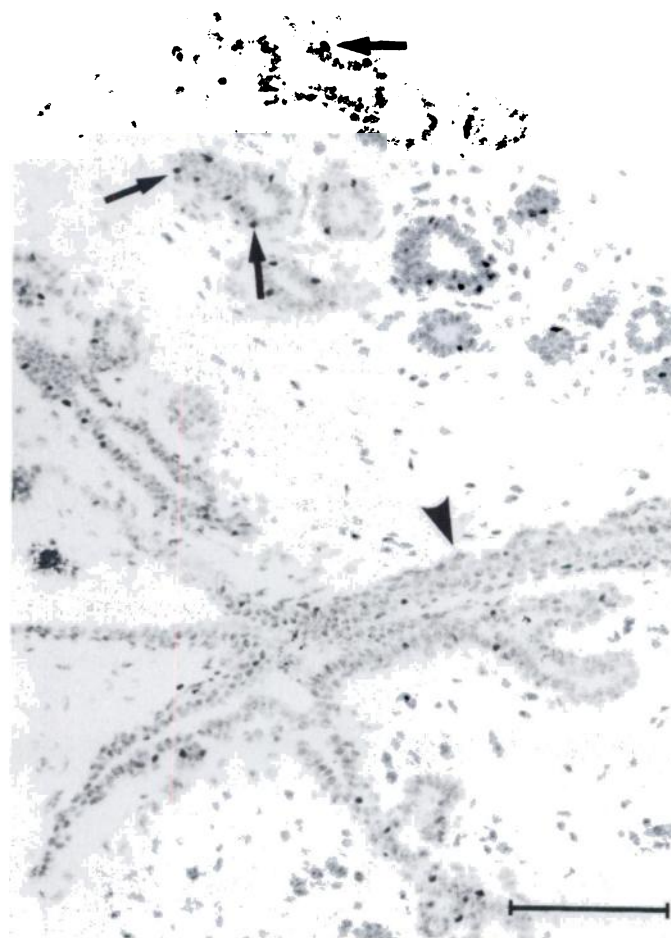


Fig. 2. Low magnification of a portion of a lobule including its terminal ductule (arrowhead). Arrows, ER-positive nuclei with predominant basal position.  $\times 210$ ; bar, 100  $\mu\text{m}$ .

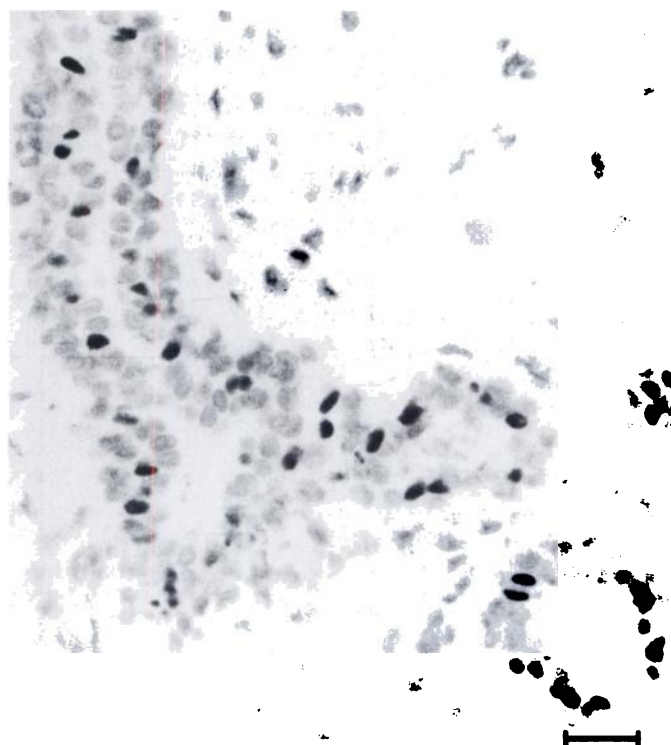


Fig. 3. Higher magnification of elements of a lobule with ER-positive nuclei predominantly located in a luminal position of the duct wall.  $\times 500$ ; bar, 20  $\mu\text{m}$ .

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were distributed as scattered single cells or small clusters throughout mammary gland, with the highest frequency in the lobules (Table 1). Moreover, a stronger intensity of staining was generally measured in the lobules as compared to the interlobular ducts (Table 2). Both pronounced inter- and intra-individual variations in frequency and staining intensity were recorded.

The ER-positive cells of the lobules were localized in an intermediate position between luminal epithelial cells and basal epithelial cells or in a typical luminal position (Table 3; Figs. 3 and 4). Less frequently the cells were localized in a typical basal position (Table 3; Fig. 2). To determine whether the intermediate cell type belonged to the basal cell lineage, an immunocytochemical detection of CALLA [an antigen present at the lateral membrane of basal cells in the human breast gland (9)]

Table 1 Frequency of ER-positive epithelial cells in lobules and interlobular ducts, in cryosections from 15 mammary glands

Each frequency is based on counts of 1000 randomly selected nuclei in sections of at least two distant biopsies per mammary gland.

Biopsy	Age of patients <sup>a</sup> (yr)	No. of ER-positive cells	
		Lobules	Interlobular ducts <sup>a</sup>
W416	19	27	3
W426	45	67	18
W428	17	Not done	
W430/1	17	34	13
W430/2	59	51	0
W430/3	15	49	39
W431/2	50	Not done	
W432/1	18	44	40
W434	18	79	31
W438	46	52	0
W444	52	100	1
W445	20	20	3
W447	21	16	5
W450/1	28	278	88
W458/1	22	260	79
W464/1	54	223	158
W466/1	20	182	32
W470	18	Not done	
Mean ± SD		98.8 ± 90.3	
		(P < 0.0058) <sup>c</sup>	
Mean ± SD			34.0 ± 44.1

<sup>a</sup> By occurrence no biopsies from patients in the age group between 30 and 40 years were obtained. Patients below the age of 30 years (n = 12) were taken to be in a premenopausal status without further verification. Patients in the age range from 40 to 50 years (n = 2) were positively identified as being in a premenopausal status based on file information. Patients in the age group above 50 years (n = 4) could not be classified due to lack of specific file information. However, only one biopsy from patients in this category (W431/2) showed histological signs indicating a state of involution.

<sup>b</sup> The ER-positive cells in interlobular ducts often accumulated as clusters of cells in one or two isolated ducts.

<sup>c</sup> A significant difference is seen between the frequency of ER-positive cells in lobules and interlobular ducts by the Wilcoxon rank sums test.

Table 2 Intensity of staining (absorbance) in nuclei of ER-positive epithelial cells present in lobules and interlobular ducts of some selected biopsy specimens

Biopsy	Absorbance ± SE	
	Lobules	Interlobular ducts
W426	0.066 ± 0.005	0.017 ± 0.002 <sup>a</sup>
W450/1	0.181 ± 0.010	0.088 ± 0.005
W458/1	0.083 ± 0.005	0.057 ± 0.002
W466/1	0.050 ± 0.004	0.012 ± 0.004
W470	0.044 ± 0.003	0.061 ± 0.004 <sup>b</sup>

<sup>a</sup> The mean absorbance values are based on measurements of 50 randomly selected nuclei, except in this interlobular duct, where only 20 nuclei were positively stained.

<sup>b</sup> In some biopsy specimens, a single interlobular duct, throughout many consecutive sections, contained clusters of ER-positive cells with a relatively strong staining intensity, while the intercellular duct profiles in sections from other parts of the same mammary gland showed the usual weak staining intensity (see Table 1, Footnote b). In such a case, the mean absorption value exclusively represents the focal high staining intensity.

Table 3 Distribution of ER-positive epithelial cells in luminal, intermediate, and basal position in the normal mammary duct wall in cryosections of 17 normal breast specimens

Nuclei were encountered only if they formed part of a bi- or multilayer of epithelial cells in the duct wall.

Biopsy	No. of cells counted	% of ER-positive cells located in		
		Luminal position	Intermediate position <sup>a</sup>	Basal position
W416	85	68	26	6
W426	243	52	36	12
W428	83	4	54	42
W430/1	94	32	65	3
W430/2	237	60	34	6
W430/3	177	57	37	6
W431/2	81	40	49	10 <sup>b</sup>
W432/1	185	42	34	24
W434	215	15	67	18
W438	189	41	44	15
W444	100	55	29	16
W445	99	11	35	53 <sup>b</sup>
W447	24	84	16	0
W450/1	98	52	43	5
W458/1	166	69	31	0
W464/1	146	64	32	4
W466/1	97	74	24	2
Mean ± SD		48.2 ± 22.6	(P < 0.0002) <sup>d</sup>	
		NS <sup>d</sup>		
Mean ± SD			38.6 ± 13.8	(P < 0.0001) <sup>e</sup>
Mean ± SD				13.1 ± 14.7

<sup>a</sup> The frequency of ER-positive cells in intermediate position may not represent the relative amount of ER-positive cells in the intermediate epithelial cell population, since the size of this population is unknown.

<sup>b</sup> In some cases, the sum of the frequencies did not reach 100 due to truncation of decimals.

<sup>c</sup> The frequency of ER-positive cells with luminal position was significantly higher than that of cells with basal position, using a median 2-sample test.

<sup>d</sup> NS, no significant difference in frequency was observed between cells in luminal and cells in intermediate position.

<sup>e</sup> The frequency of ER-positive cells in intermediate position was significantly higher than that of cells in basal position, using a median 2-sample test.

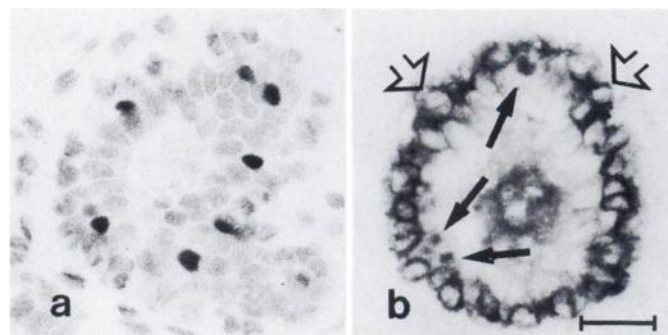


Fig. 4. a, ER-positive nuclei with predominant intermediate position in the duct wall; b, section incubated to detect both ER and CALLA. It is evident that the intermediate epithelial cells with ER-positive nuclei (small arrows) are different from the basal cells characterized by a marked CALLA staining. Open arrows, nuclei of the CALLA-positive basal cells. × 500; bar, 20 μm.

was performed in combination with the ER-ICA assay. As seen in Fig. 4, the intermediate cell type lacked this differentiated myoepithelial feature.

In the involuting mammary gland (W431/2), ER-positive cells persisted in rudimentary mammary ductules. At this stage the frequency of ER-positive cells in these ductules was as high as that seen in lobules of premenopausal women.

DISCUSSION

The major result of the present study is the unequivocal demonstration of a small but distinct population of estrogen receptor-positive epithelial cells in the normal human mammary gland. However, some inherent limitations in the experi-

mental approach should be considered. Thus, a part of the quantification protocol is based on simple counting of ER-positive cells by eye. The threshold of detection is therefore not well defined, and weak positive reactions may not be registered, implying that the frequency of ER-positive cells (approximately 7%) could be an underestimate. Also, the 7% ER-positive cells have not been further subclassified according to the patterns of the menstrual cycle, since this was not the object of the present study. Previous studies have shown an influence of the menstrual cycle on ER expression in the human breast gland (2). Another important problem in studies of ER expression is the material taken to represent so-called normal human breast tissue. This material is obtained from a selected group of individuals operated on primarily due to mammary hypertrophy, and it is not known to what extent this group reflects the general ("normal") female population in terms of mammary ER expression. However, reduction mammoplasty specimens represent the most widely used source of normal, nonlactating human breast tissue and, as such, should be well characterized. In doing so, it is particularly important to recognize that "normal" breast tissue may contain scattered elements of fibrocystic changes which, in their less obvious form (mild adenosis or fibrosis in the absence of cysts and epithelial hyperplasia), are difficult to discriminate from pure normality. Although in the present study several sections from distant places in the mammary glands were analyzed and compared, in order to exclude focal fibrocystic changes, it cannot be ruled out that some of the variability seen in ER expression may be accounted for by inclusion of some incidents of minor abnormal histology. This should, however, not interfere with the major result of the present study.

Application of the immunocytochemical detection of ER-positive cell populations may be of importance, for instance, in relation to short-term cultivation of human breast epithelial cells with some defined phenotypic traits (10, 11). Such work

is now in progress in our laboratory.

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