

Estrogen receptor beta in breast cancer

C Palmieri, G J Cheng¹, S Saji¹, M Zelada-Hedman¹, A Wärrin¹, Z Weihua¹, S Van Noorden, T Wahlstrom², R C Coombes, M Warner¹ and J-Å Gustafsson¹

Department of Cancer Medicine, Cancer Cell Biology Group, Cancer Research Campaign Laboratories, Imperial College School of Medicine, Hammersmith Campus, Du Cane Road, London W12 ONN, UK

¹Department of Bioscience, Karolinska Institute, Novum, Huddinge University Hospital, SE-14186, Huddinge, Sweden

²Department of Obstetrics and Gynecology, Helsinki University Central Hospital, Haartmaninkatu 2, 00290 Helsinki, Finland

(Requests for offprints should be addressed to J-Å Gustafsson; Email: jan-ake.gustafsson@mednut.ki.se)

(S Saji is now at Tokyo Metropolitan Komagome Hospital, Department of Surgery, Breast Oncology Unit, 3–18–22 Honkomagome, Bunkyo-ku, 113–8677 Tokyo, Japan)

Abstract

Estrogen is essential for normal growth and differentiation in the mammary gland. It also supports growth of approximately 50% of primary breast cancers. For this reason, removal of estrogen or blocking of its action with the anti-estrogen, tamoxifen, is the main treatment for estrogen receptor alpha (ER α)-positive tumors. In 1996, when oncologists became aware of a second ER, ER β , there was some doubt as to whether this receptor would be of importance in breast cancer because the clinical consensus was that responsiveness to tamoxifen is related to the presence of ER α in breast cancer. Today we know that ER α and ER β have distinct cellular distributions, regulate separate sets of genes and can oppose each other's actions on some genes. We also know that ER β is widely expressed in both the normal and malignant breast and that there are proliferating cells in the breast which express ER β . In this review we summarize what is known about ER β in breast cancer and examine the possibility that ER β -selective ligands may well represent a useful class of pharmacological tools with a novel target, namely proliferating cells expressing ER β .

Endocrine-Related Cancer (2002) 9 1–13

Introduction

The major endocrine-related cancers in the human population display appropriate gender-specific hormonal responsiveness, i.e. estrogen for breast and endometrial cancer in females and androgen for prostate cancer in males. Since normal growth of breast, uterus and prostate is dependent on the respective sex steroids, the involvement of these hormones in malignant growth in these organs is not surprising. In normal tissues, hormone-stimulated growth is controlled, and tissues like the prostate and breast do not have unlimited growth even during continuous exposure to their respective hormones. Uncontrolled growth in response to hormones could be due to either overactivity of the signals for growth or underactivity of the signals which normally stop proliferation.

The androgen receptor, the estrogen receptor (ER) alpha and the genes which they regulate, have been extensively studied and are the main targets in clinical approaches to controlling hormonally responsive prostate and breast cancer respectively (Ali & Coombes 2000). In 1996 it became

evident that one major player in the story, the second ER, ER β , had gone unnoticed (Kuiper *et al.* 1996) and that its presence may well have some bearing on the development and treatment of hormone-dependent cancers. ER β is highly expressed in the prostate (Kuiper *et al.* 1996) and is expressed together with ER α in both normal and malignant breasts (Dotzlaw *et al.* 1997, Vladusic *et al.* 1998, Fuqua *et al.* 1999, Speirs *et al.* 1999, Cullen *et al.* 2000, Iwao *et al.* 2000, Jarvinen *et al.* 2000, Speirs & Kerin 2000, Omoto *et al.* 2001, Roger *et al.* 2001). It is, therefore, not surprising that a great deal of interest is focused on the role of ER β in normal and malignant growth of these two organs. In the ventral prostate of mice, ER β is involved in regulation of epithelial growth, and its absence, in ER β -/- mice, results in hyperplasia of the prostatic epithelium (Weihua *et al.* 2001). In the case of breast cancer, some of the most important questions which need to be answered are whether it is of clinical value to measure ER β along with ER α and whether ER β can be a novel target in therapy. In this review we will summarize what has been learnt about ER β in the normal

breast and in breast cancer and present a part of our large, as yet unpublished, study on ER β in archived breast cancer samples.

Estrogen and breast cancer

Estrogen is a modulator of cellular growth and differentiation in the mammary gland. It mediates most of its functions through two members of the nuclear receptor superfamily, ER α and ER β . These receptors are hormone-dependent transcriptional regulators which, in the presence of appropriate ligands, bind to estrogen-response elements (EREs) on DNA (Tsai & O'Malley 1994 for review) or interact with proteins in other pathways (Batistuzzo de Medeiros 1997, Galienand & Garcia 1997, Paech et al. 1997, Porter et al. 1999) and affect transcription of specific genes. Numerous animal studies show that estrogen can induce and promote breast cancer, and removal of the ovaries or administration of anti-estrogens can oppose this (Vignon et al. 1987, Hulka & Stark 1995). However, the mechanisms through which estrogen exerts its proliferative effects are not understood. One of the prevailing concepts is that the proliferative action of estrogen on the epithelium is indirect, i.e. that estrogen stimulates secretion of growth factors from breast stroma and these factors stimulate epithelial cells to proliferate (Wiesen et al. 1999).

One of the main reasons for the uncertainty about the mechanism of estrogen-mediated proliferation in the mammary gland, is that, although there is clearly epithelial growth in mammary glands in response to estrogen, it appears that

the proliferating cells are not the ones which express ER α . In immunohistochemical studies, markers of proliferation, such as Ki67 and cyclin A, are not found in cells which express ER α (Clarke et al. 1997a,b, Zeps et al. 1999). In the rodent mammary gland, ER β -containing cells can proliferate but the majority of cells expressing proliferation markers do not express either ER (Saji et al. 2000). This is in direct contrast to estrogen action in breast cancer cell lines where ER α -positive cells proliferate in response to estradiol (Allegra & Lippman 1978, Chalbos et al. 1982). Furthermore, when ER α is introduced into ER α -negative cell lines, proliferation is often inhibited in response to estrogen and in many cases cell death results (Garcia et al. 1992, Jiang & Jordan 1992, Jeng et al. 1994, Levenson & Jordan 1994). There appears to be a fundamental difference between normal breast epithelium and breast cancer cell lines in their response to estrogen. What has been unclear is whether or not cells within a breast tumor respond to estradiol in a fashion similar to breast cancer cell lines.

If ER α -containing cells do not proliferate, the biological significance of the well-documented induction of cyclin D by estrogen and the interaction of ER α with cyclin D (Neuman et al. 1997, Zwijzen et al. 1997), is difficult to understand. One possible, but untested, explanation for the absence of ERs in proliferating cells in the mammary gland, is that estrogen triggers proliferation in these cells but that ERs must be down-regulated for progress through the cell cycle to occur. Recent data suggest that ER α is rapidly down-regulated in response to estrogen in a ubiquitin-mediated

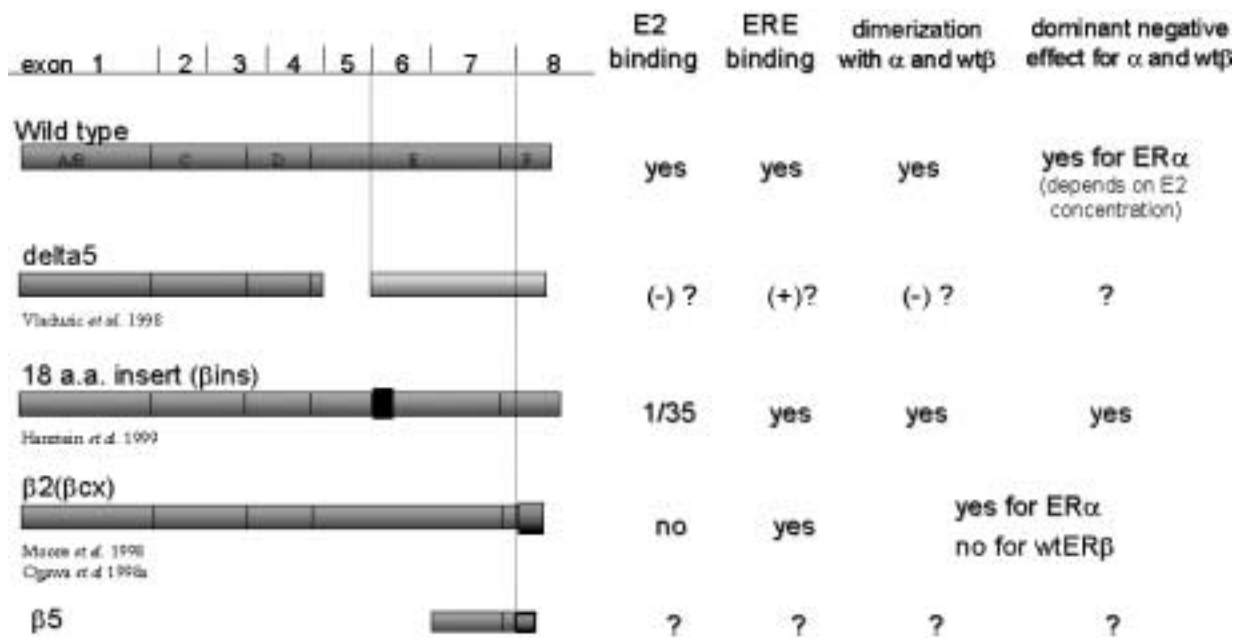


Figure 1 ER β splice variants. The reported exon-intron junctions of human ER β sequences are indicated. Models of each variant protein and the location of primers are shown.

degradation pathway (Lonard *et al.* 2000). One interpretation of this phenomenon is that ER α must be removed from the cell in order for the cell to progress through the cell cycle. The absence of ER α in cells expressing proliferation markers such as cyclin A and Ki67 would then have a different meaning. It would mean that proliferation is initiated in ER α -containing cells but that ER α is down-regulated early in the cell cycle. Such a mechanism could explain why, during the highest proliferative phase of the breast, i.e. pregnancy, there is very little expression of ER α and high expression of ER β , but most of the cells which express the proliferating cell antigen contain neither receptor (Saji *et al.* 2000), and why there is lower expression of ER α in the breasts of women during the luteal phase of the menstrual cycle when proliferative activity in the breast is highest (Ricketts *et al.* 1991). If this interpretation of the data is correct, the difference between normal and cancer cell lines would then be that progress through the cell cycle can occur in the presence of ERs in cancer cells.

Surprisingly, we have found (Jensen *et al.* 2001) that unlike breast cancer cell lines, ER α -expressing cells in breast cancers do not express proliferation markers. This observation has raised questions about what exactly is the mechanism of anti-estrogen therapy in breast cancer. The presence

of ER α in breast at the time of diagnosis of breast cancer is taken as an indication of hormone responsiveness (Allegra & Lippman 1980) and on this basis treatment with anti-estrogen therapy, such as tamoxifen, is commenced. It is well known that about two-thirds of ER α -positive patients respond to tamoxifen and that some patients, classified as ER α -negative, do benefit from tamoxifen therapy (McGuire *et al.* 1982). There are several possible explanations for how tamoxifen might produce its beneficial effects in ER α -positive cancers: (i) ER α -containing cells may produce growth factors which stimulate proliferation of surrounding cancer cells and tamoxifen may inhibit production of these growth factors and hence proliferation of the tumor; (ii) tamoxifen may be lethal to ER α -containing cells, which, although they may not be the proliferating cells, represent the bulk of some tumors; (iii) ER α -containing cells may be the malignant cells but when they enter the cell cycle ER α is down-regulated and tamoxifen targets these cells when they are in G₀ or early in the cell cycle; and (iv) tamoxifen may prevent metastatic spread by reducing production of estrogen-regulated proteases required for invasion.

In addition to questions about the role of ER α in estrogen-induced growth of the breast, there is also the question

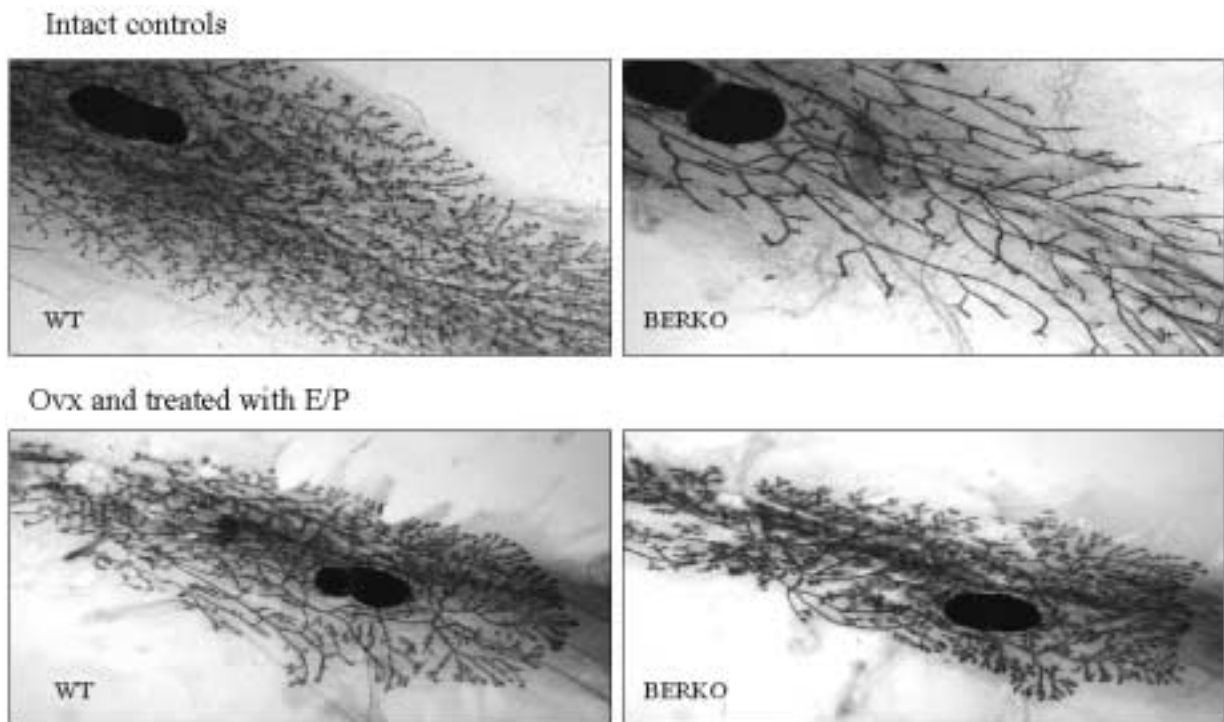


Figure 2 Whole mounts of WT and BERKO breasts. Whole mount preparations of the fourth abdominal mammary glands of BERKO and WT female mice showing the development of the mammary ductal tree. The upper panels show mammary glands of mature (4-month-old) intact mice. BERKO mice do not have regular estrous cycles and never show the branching seen in the WT mammary glands. Lower panels show mammary glands of ovariectomized (Ovx) pubertal mice, treated with estrogen and progesterone (E/P) (1:1000) for 15 days, starting at the age of 30 days. This hormone treatment produced extensive branching in both WT and BERKO mouse mammary glands.

of whether ER β plays a role in the breast. Some answers to this question have come from mice in which the ER β gene is inactivated.

Breast phenotype in ER α ^{-/-} and ER β ^{-/-} mice

In the rat mammary gland, ER β is more abundant than ER α (Saji et al. 2001) but the role of ER β in this organ is still not clear. Even though ER β ^{-/-} mice are available (Krege et al. 1998), there are two confounding factors which make it difficult to define the role of ER β in the mammary gland. One is ovarian dysfunction in ER β ^{-/-} mice (Cheng et al. 2002), which manifests as absence of corpora lutea, lack of progesterone and lack of cyclical growth in the mammary gland. The other confounding factor is the presence of substantial quantities of the ER β splice variant ER β ins (Fig. 1) in the rat mammary gland (Saji et al. 2001). In ER β ins, there is an insertion in the ligand-binding domain of an 18 amino acid sequence which reduces the affinity of the receptor for estradiol by 40-fold (Chu & Fuller 1997, Maruyama et al. 1998). This variant is co-expressed in cells with ER α and may function as a repressor of ER α (Saji et al. 2001). If this is the

case, some aspects of the mammary gland in ER β ^{-/-} mice may be due to unrestrained ER α activity.

In prepubertal female ER β ^{-/-} mice, there is normal ductal development. This confirms what has already been observed i.e. that mammary ductal elongation, which is known to be estrogen-driven, is ER α -mediated (Couse & Korach 1999). Normal terminal end buds were also seen in the growing mammary ducts of both ER β ^{-/-} and age-matched wild-type (WT) glands. Sexually mature ER β ^{-/-} female mammary glands are less developed (fewer side branches and alveoli) than those of WT littermates (Fig. 2). In less than half of the ER β ^{-/-} mice was there any evidence of estrous cycles and this might explain the poor development of the ER β ^{-/-} mammary gland. When ovariectomized mice were implanted s.c. with pellets composed of estrogen and progesterone (1:1000) for 15 days, mammary glands in the ER β ^{-/-} mice were indistinguishable from those of similarly treated WT mice (Fig. 2). This indicates that ER β is not necessary for estrogen-induced proliferation of the mammary gland. Interestingly, in ER α ^{-/-} mice, when pituitary hormones are neutralized, estrogen elicits normal growth of the mammary gland (Couse et al. 2000, Korach 2000). Thus the results from the ER knockout mice reveal that neither ER α ^{-/-} nor ER β ^{-/-},

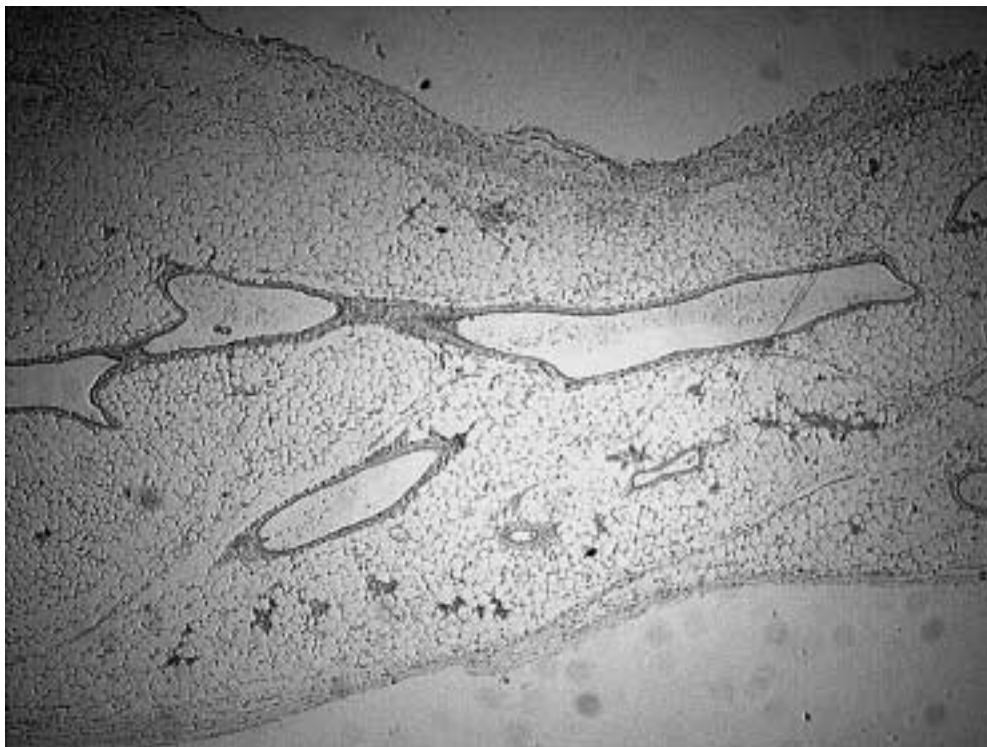


Figure 3 Histological appearance of the mammary gland of ER β ^{-/-} mouse at 2 years of age. Mammary glands were excised and fixed in 4% paraformaldehyde. The section illustrates multiple cysts. These are found throughout the BERKO mammary gland.

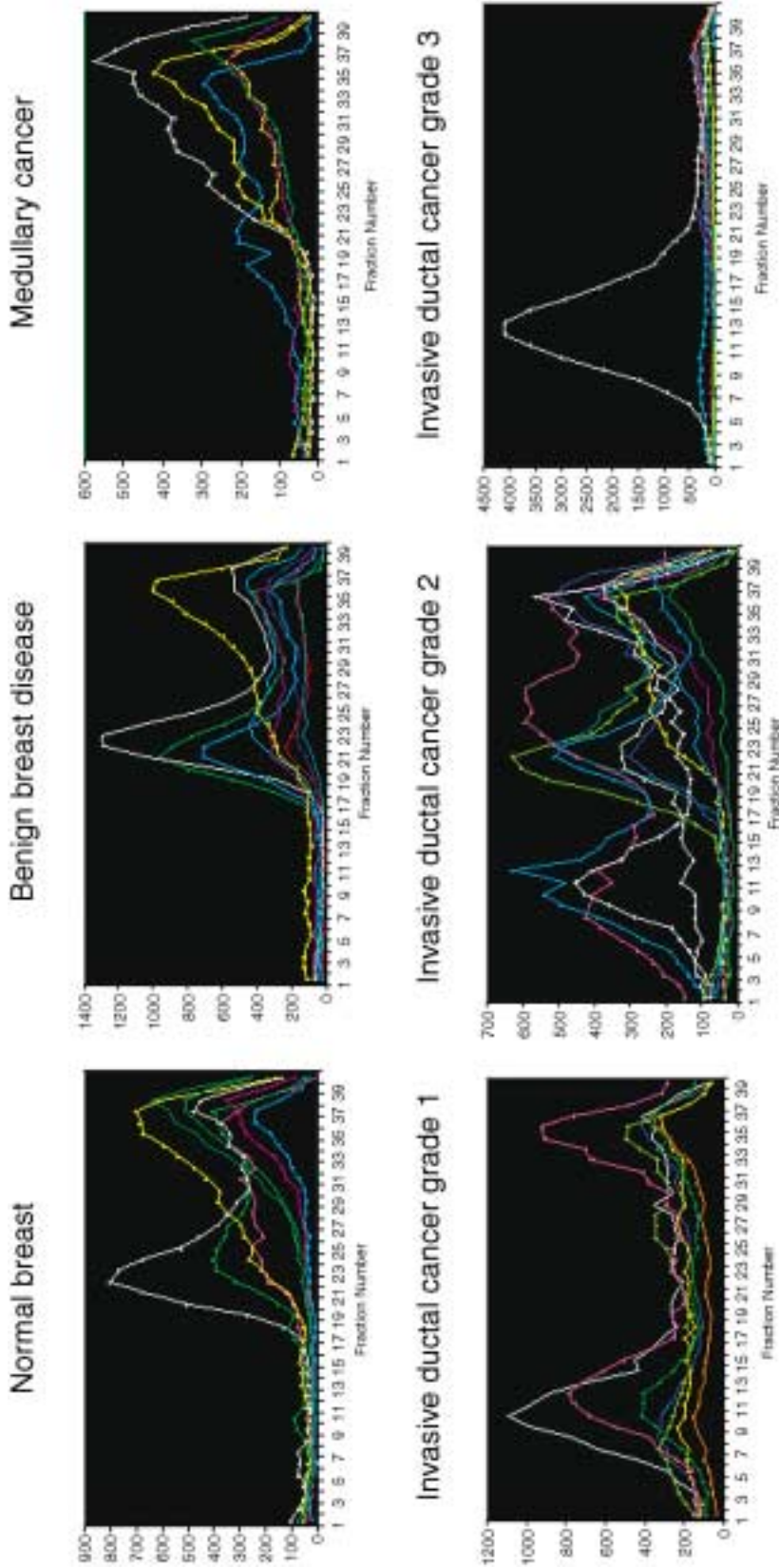


Figure 4 Estradiol binding in 8S (ER α) and 4S (ER β) peaks in normal and breast tumors by sucrose density gradient assay. Tissue was frozen in liquid nitrogen and pulverized in a dismembrator (Braun, Melsungen, Germany) for 45 s at 1800 r.p.m. Pulverized tissue was added to a buffer composed of 10 mM Tris–chloride, pH 7.5, 1.5 mM EDTA and 5 mM sodium molybdate, using 1 ml/100 μ g tissue. Cytosol was obtained by centrifugation of the homogenate at 204 000 g for 1 h in a 70Ti rotor at 4 $^{\circ}$ C. MCF-7 cell cytosol (for calibration of the gradient) was a generous gift from Abbott Laboratories. Breast tissue extracts were incubated for 3 h at 0 $^{\circ}$ C with 10 nM [3 H]estradiol-17 β in either the presence or absence of a 50-fold excess of radio-inert 17 β -estradiol, and the bound and unbound steroids were separated with dextran-coated charcoal. Sucrose density gradients (10–30% (w/v) sucrose) were prepared in a buffer containing 10 mM Tris–HCl, 1.5 mM EDTA, 1 mM α -monothio glycerol, 10 mM KCl. Samples of 200 μ l were layered on 3.5 ml of gradients and centrifuged at 4 $^{\circ}$ C for 16 h at 300 000 g in a Beckman (Palo Alto, CA, USA) L-79K ultracentrifuge with an SW-60Ti rotor. The figure shows high expression of ER β in normal breasts, in benign breast disease and in medullary cancer. ER α expression in these samples is very low. In invasive ductal cancer grade 1, ER α expression is high and ER β low. In invasive ductal cancer type 2, both ER α and ER β are highly expressed. In invasive ductal cancer grade 3, the level of both ERs tends to be reduced, except for one sample which had extremely high levels of ER α .

individually, is necessary for mammary gland growth. It is still not clear whether there can be estrogen-induced growth in the absence of both receptors. Such information will come from studies on the double knockout mice. Even if ERs are not necessary for proliferation in the mammary gland, they can still control both normal and malignant growth through their influences on growth factor-mediated growth and apoptosis. As ER β ^{-/-} mice age, they develop severe cystic breast disease, which is not found in their WT littermates. At 2 years of age, all ER β ^{-/-} mice mammary glands are cystic. A typical gland is shown in Fig. 3.

ER α and ER β in the normal and malignant human breast

In the human breast the role of ER β is even less well understood than it is in rodents. One major confounding factor is

the expression of another ER β splice variant, ER β cx (Ogawa *et al.* 1998a), a dominant negative repressor of ER α . In this variant, an alternative exon 8 is utilized (Fig. 1). This receptor has no measurable affinity for estradiol and, if expressed together with ER α in cells, it silences ER α function (Ogawa *et al.* 1998b). It is not known whether the physiological function of ER β cx is to repress ER α or whether there are, so far unidentified, ligands which will confer novel functions on this receptor. Whatever its functions, its expression in breast cancer (see below) shows that it is one additional factor, in the already complex estrogen story, which cannot be ignored.

Many functions have been suggested for ER β in the breast (Gustafsson & Warner 2000, Knowlden *et al.* 2000, Speirs & Kerin 2000, Warner *et al.* 2000) but no clear picture has emerged about its role in breast cancer. One group suggested that it contributes to the initiation and progression of chemical carcinogen-induced neoplastic transformation in

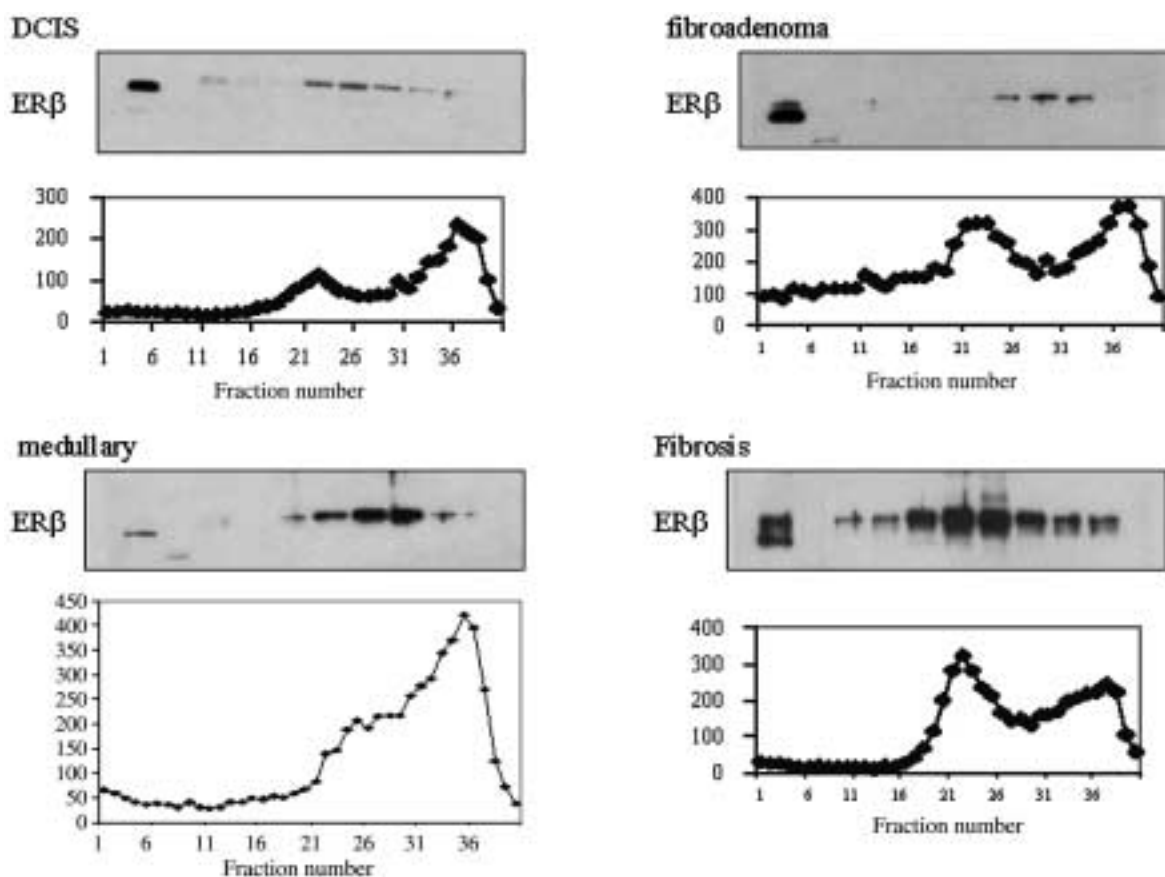


Figure 5 Detection of ER β by Western blotting analysis. For Western blotting, sucrose gradient fractions were first precipitated with TCA, and the precipitate resuspended in methanol. Samples were placed on dry ice for 30 min and the protein recovered by centrifugation. Protein pellets were dissolved in SDS sample buffer and resolved by SDS-PAGE in 4–20% gradient gels with a Tris–glycine buffer system. Transfer to ProBlot (Applied Biosystems, Foster City, CA, USA) membranes was done by electroblotting. Signals were developed using SuperSignal West Femto Maximum Sensitivity Substrate from Pierce (Rockford, IL, USA). The ER β antibody was raised in rabbits against the ligand-binding domain of ER β . The ER α antibody was H222 from Abbott Laboratories. The panel illustrates the high expression of ER β in fibrocystic disease and medullary cancer.

breast because expression was induced in chemical carcinogen-transformed human breast epithelial cells (Hu *et al.* 1998). Other groups have shown the expression mRNAs of both ERs in normal and malignant human breast tissue by RT-PCR (Leygue *et al.* 1997, Vladusic *et al.* 1998, 2000, Spiers *et al.* 2000). Of three published immunohistochemical studies, one (Leygue *et al.* 1997) demonstrated that those tumors that co-expressed ER α and ER β were node-positive and tended to be of higher grade; another (Jarvinen *et al.* 2000), found that ER β was often co-expressed with ER α and progesterone receptor in breast cancer and that ER β was significantly associated with negative axillary node status and low tumor grade; while the third study (Mann *et al.* 2001) found that expression of ER β in more than 10% of cancer cells was associated with better survival. In one RT-PCR study there was increased expression of ER β mRNA in tamoxifen-resistant breast cancer patients (Speirs & Kerin 2000) and in another study (Roger *et al.* 2001), there was decreased expression of ER β protein in proliferative pre-invasive mammary tumors.

Because of technical difficulties with antibodies and the need for fresh or frozen samples, there are very few studies where ERs are measured by Western blotting. However, one such study has been published and showed that full-length ER β protein could be detected in three human breast tumors of unspecified histopathology (Fuqua *et al.* 1999). From all of the data published so far, it is not yet clear how ER β can be used as a routine prognostic indicator either independently

or alongside ER α . Regarding the use of ER β as a potential novel target in the treatment of breast cancer, it is currently unclear whether agonists or antagonists will be useful. The potential role of ER β splice variants, to act as natural anti-estrogens and repress ER α function, needs further investigation and evaluation, as this could affect the way we currently interpret ER α immunostaining to guide treatment with anti-estrogens.

Unpublished results from our breast cancer study

In an attempt to resolve some of the issues concerning the role of ER β in breast cancer, we have initiated a large study of ER profiles in primary and recurrent breast cancer. So far, 63 frozen samples obtained from the histopathology archive at Charing Cross Hospital, London, UK have been processed. They were composed of 33 invasive ductal carcinomas, five medullary cancers, 14 samples from fibrocystic disease, six samples of normal tissue adjacent to cancer and two *in situ* ductal cancers. All samples were previously typed for ER α by ligand-binding assays and/or immunohistochemistry. Another twenty-four paraffin-embedded samples were from the Helsinki University Central Hospital, Finland. They were composed of five benign tumors, 25 invasive ductal cancers and four lobular cancers. Information on patient's age, menopausal status, pathological diagnosis and differentiation grade was recorded. We are using multiple techniques to measure

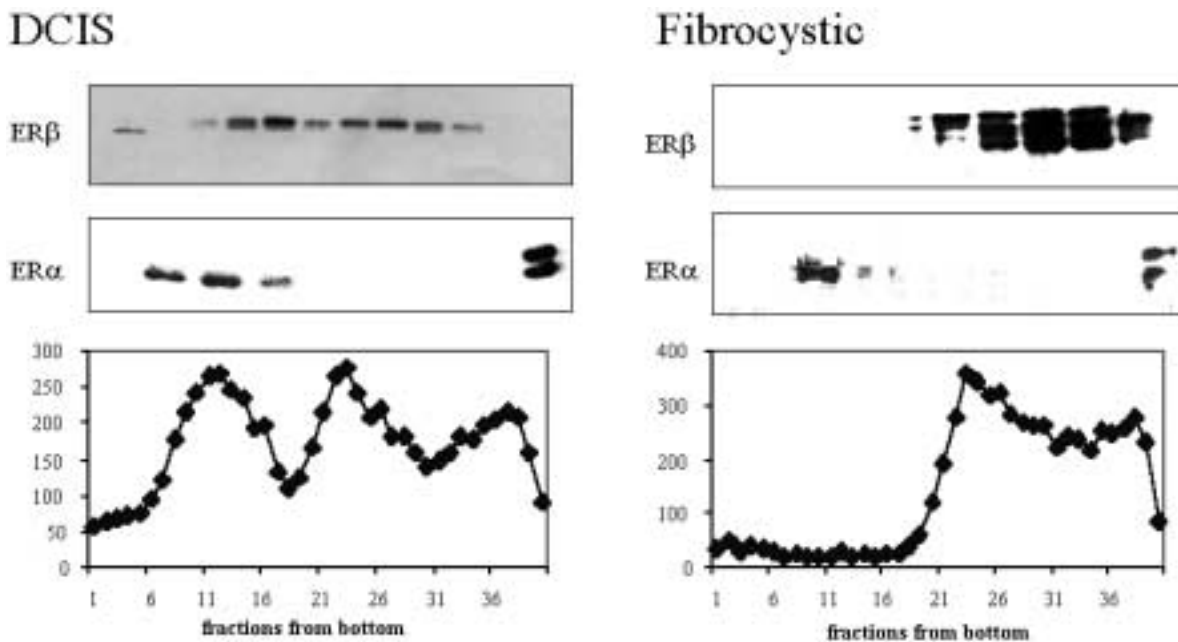


Figure 6 Breast sample with non-estrogen-binding form of ER α . In the DCIS sample, there was estrogen binding in the 8S peak and a corresponding ER α band on Western blots in the 8S fractions. In the fibrocystic sample there was no estrogen binding in the 8S peak but there was a band of the correct size on Western blots.

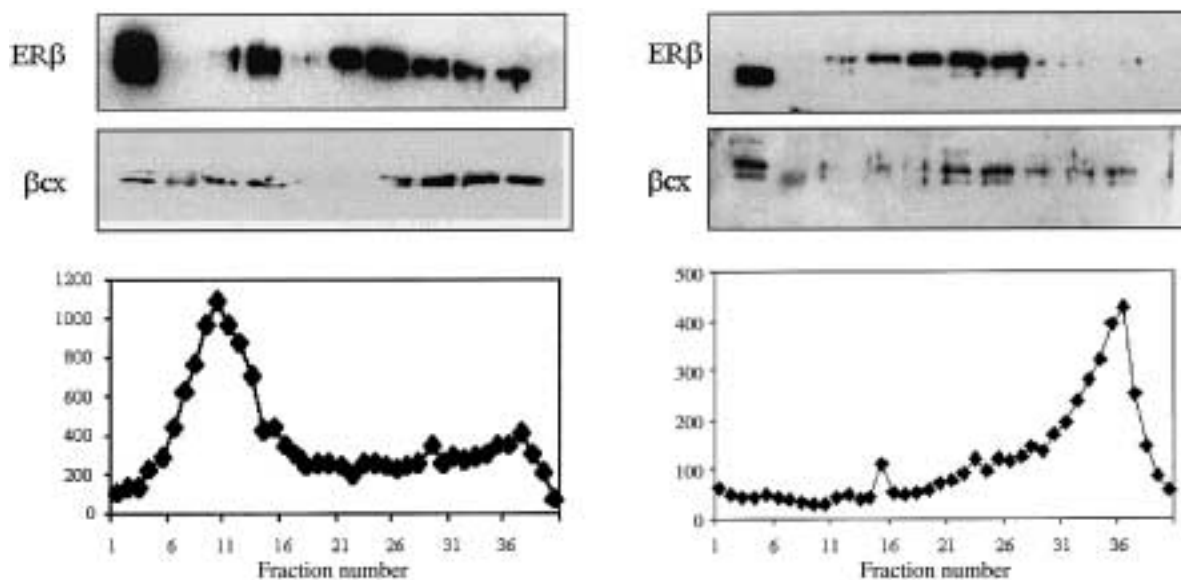


Figure 7 Breast cancers expressing ER β cx. The two ductal cancers (LH panel, grade 1; RH panel, grade 3) shown in this figure had no estrogen-binding 4S peak. ER β , as detected by the LBD antibody, was found throughout the gradient. With an ER β cx-specific antibody, ER β cx distribution in the gradient coincided with that found with the LBD antibody.

ER α and ER β proteins in the frozen samples. Expression levels and binding capacity of the two ERs were measured by sucrose density gradient centrifugation of low-salt tissue extracts with estradiol as ligand. The proteins were analyzed by Western blotting and localized in the tissues by immunohistochemistry. The various ER β isoforms were measured by RT-PCR. The fixed samples were examined by immunohistochemistry and this study has been published (Jensen *et al.* 2001).

One of the most unexpected and useful differences between ER α and ER β was revealed by sucrose density gra-

dient centrifugation. There is a clear difference in sedimentation profiles of the two receptors. As depicted in Fig. 4, in the presence of 10 nM [3 H]estradiol there are two distinct peaks of estradiol binding detectable in low-salt extracts of human breast samples. As expected (Greene *et al.* 1977), the 8S peak contained ER α . The unexpected finding was that the 4S estradiol-binding peak contained ER β , exclusively (Fig. 5). Since there were clear differences between various breast samples in the quantity of estradiol bound and the ratio of the two peaks, this method appears to be a simple and efficient method to assess the presence of the two ERs. The

Figure 8 Immunohistochemistry. Paraffin sections (4 μ m) were dewaxed in xylene and rehydrated through graded alcohol to water. Endogenous peroxidase was blocked by incubation for 30 min with a solution of 1% hydrogen peroxide and antigen retrieval was performed by microwaving sections in 0.01 M citrate buffer, pH 6.0, for 20 min at 800 W. ER α monoclonal antibody (6F11) was from Novocastra (Newcastle, UK); ER β rabbit polyclonal (06-629) was from Upstate Biotechnology (Lake Placid, NY, USA). Biotinylated secondary antibodies, goat anti-mouse IgG, and goat anti-rabbit IgG and avidin-biotin (ABC) kits were obtained from Vector Laboratories, Inc., Burlingame, CA, USA. Tissue sections were incubated for 1 h at 4 $^{\circ}$ C with normal goat serum diluted 1:10 in PBS. Antibodies were diluted individually in PBS containing 3% BSA. Dilution for ER β was 1:100 and for ER α antibody 1:500. Sections were incubated with antibodies overnight at 4 $^{\circ}$ C. For negative controls, the primary antibody was replaced with PBS or with primary antibodies after absorption with the corresponding antigens. Prior to addition of the secondary antibody, sections were rinsed in PBS containing 0.05% Tween 20. The ABC method was used to visualize the signal according to the manual provided by the manufacturer (Vector). Sections were incubated in biotinylated goat anti-rabbit or goat anti-mouse immunoglobulin (1:200 dilution) for 2 h at room temperature, followed by washing with PBS and incubation in avidin-biotin-horseradish peroxidase for 1 h. After thorough washing in PBS, sections were developed with 3,3'-diaminobenzidine tetra-hydrochloride substrate (Dako, Glostrup, Denmark), slightly counterstained with Mayer's hematoxylin, followed by dehydration through an ethanol series, xylene and mounting. The percentage of positively stained cells was averaged after counting the positively and negatively stained cells from four high-magnification fields with the software Image-Pro Plus (version 4.1), Media Cybernetics, Silver Spring, MD, USA. The pictures show that there are different patterns of ER α and ER β expression in invasive ductal cancer. In the fibrocystic tumor, ER α is expressed in the epithelial cells while ER β is expressed in both epithelial and stromal cells.

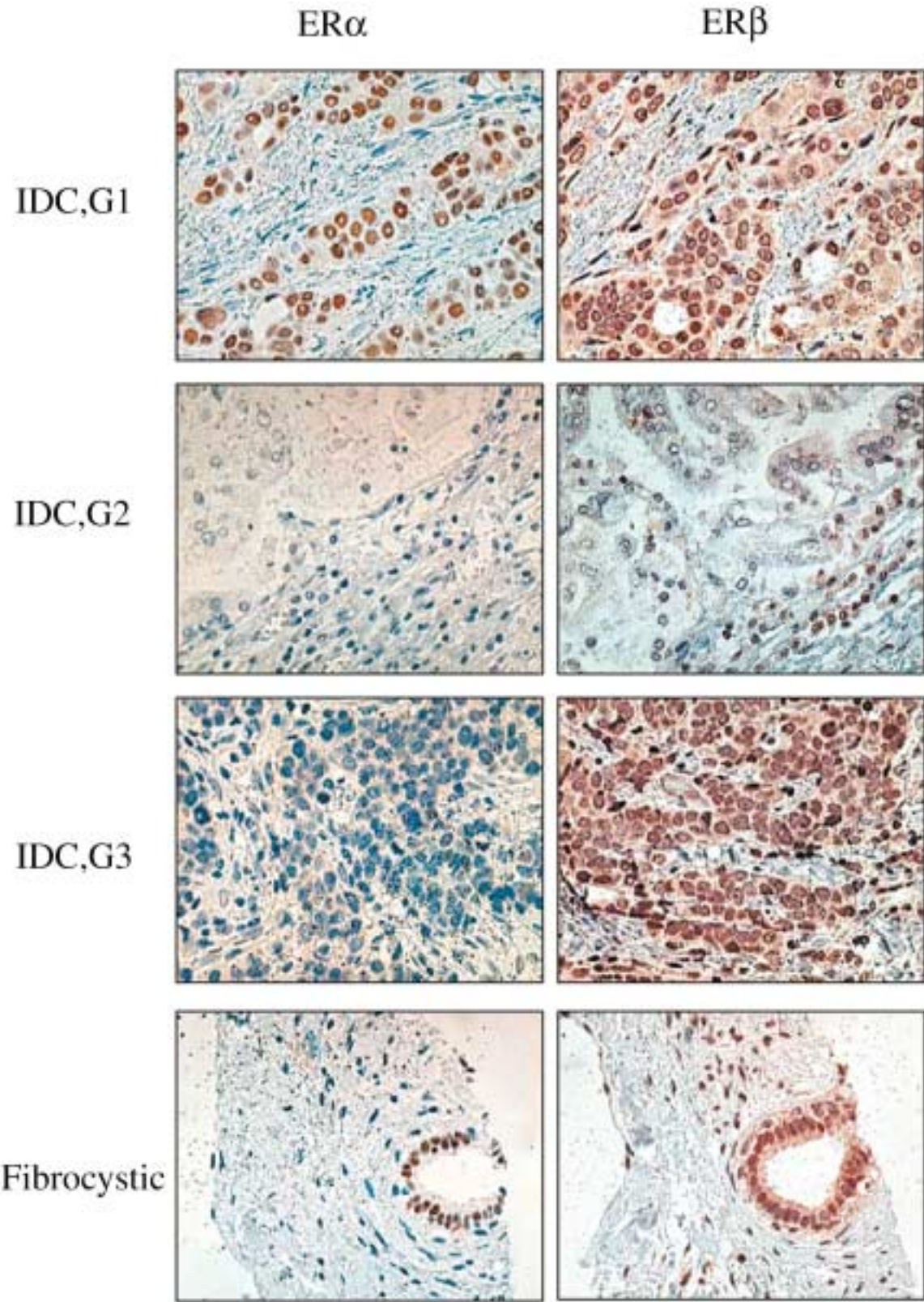


Figure 8 See opposite page for legend.

higher sedimentation rate of ER α suggests that it is in a larger multi-protein complex than is ER β and careful examination of the ER β complex could provide useful information about the proteins which associate with it in the cell.

Of the breast cancer samples, 13% had an 8S peak only, 42% had a 4S peak only and 31% had both peaks. In 11% of the samples no specific estradiol binding was observed. The 8S peak, ER α , was prominent (range 13–3700 fmol/mg protein) in invasive ductal cancer, but was not detected in normal tissue or in benign breast disease. The 4S peak, ER β , (range 20–475 fmol/mg protein) was present in both normal and malignant breasts (80% of these samples were positive for ER β). All grade 1 invasive ductal carcinomas were devoid of ER β but had high levels of ER α . As discussed above, the presence of ER α in a cell might be an indication of a non-proliferative state, and this might explain why the grade 1 invasive ductal cancers are less aggressive. Grade 2 and 3 invasive ductal cancers had various ratios of the two receptors and in some samples no estrogen binding could be detected.

From our estrogen binding data, 44% of the breast cancer samples would be classified as ER α -positive. Interestingly, although ER α was present only in the 8S peak, never in the 4S peak, there were some samples in which no estradiol binding could be detected, but which had distinct and specific bands of the correct size on Western blots with H222 antibody (Abbott Laboratories, North Chicago, IL, USA) (Fig. 6). There could be several reasons for lack of estrogen binding in samples which do express ER α protein such as point mutations, post-transcriptional modifications of the receptor or the presence of ER β splice variants which prevent estrogen binding by forming heterodimers with ER α (Palmieri et al. 2000). The reason for the lack of estradiol binding has not been investigated but it does suggest that discrepancies between the ligand-binding assay and the immunodetection assay can occur. This therefore requires further attention.

Western blotting confirmed what was found with the binding data. ER α was highly expressed in invasive ductal cancer but its expression was low in normal breasts and in fibrocystic disease (Fig. 5). The 4S estradiol-binding peak always contained ER β with a molecular mass of 60–63 kDa (Figs 5–7).

Immunohistochemistry (Fig. 8) confirmed that ER α was highly expressed in grade 1 invasive ductal cancer. Its expression varied in grades 2 and 3 invasive ductal cancer and was low in fibrocystic disease. Despite the lack of estrogen binding in the 4S peak in grade 1 invasive ductal cancer, there was abundant, specific ER β staining in sections of breasts from patients with this disease. Western blotting with these samples confirmed that although there was no 4S estradiol-binding peak, there was abundant expression of ER β protein. In order to understand this result, we developed another antibody, one specific for the cx exon. Western blotting with this antibody showed that ER β cx was abundantly ex-

pressed in fractions which had positive signals with the antibody raised against the ligand-binding domain of ER β (Fig. 7). Clearly, ER β cx is expressed as a protein in breast cancer. Furthermore, since there was no estradiol binding in the 4S peak of the sucrose gradient, it appears that no ligand-binding form of ER β 1 is present. It is not clear whether there is no ER β 1 or if it is present but its binding to estradiol is inhibited by ER β cx. The presence of ER β cx was confirmed by RT-PCR (Fig. 9). The ratio of ER β 1 to ER β cx mRNA varied considerably between samples and in some cases ER β cx was the only ER β isoform detected.

We conclude from these studies that ER β is usually expressed in normal breasts and in benign and malignant breast disease. The important remaining question is whether the presence of ER β in breast cancer could influence treatment of the disease. The answer to this question awaits identification of the array of genes which are regulated by this receptor. Identification of such genes is important if we are to understand the actions of this nuclear receptor and the effect of its dysregulation in the human breast. Currently there is not enough information available for decisions on whether ER α and ER β complement or oppose each other's actions on certain target genes within the breast. It is not known whether these receptor proteins form heterodimers *in vivo* or which ER β isoforms co-localize with ER α in cells. If ER β cx is expressed in cells with ER α , this may represent one mechanism by which ER α -positive cancers are resistant to the actions of tamoxifen. On the other hand, if tamoxifen exerts its beneficial effects in the treatment of breast cancer by opposing the action of ER α , the question of whether ER β cx functions as an 'anti-estrogen' *in vivo* must be examined. Of course, before this question can be answered, we need to know whether ER β splice variants have alternative ligands or alternative routes of activation and whether they silence ER α action at sites of interaction other than on EREs.

Unanswered questions

There are important questions which need to be addressed with regard to ER β and its splice variants in breast cancer. (i) Can their measurement be used to improve the prognostic value of ER α or could it even be used as an independent prognostic indicator? (ii) What is the functional significance of ER β splice variants such as ER β cx and can their presence be used to predict the likely response to hormonal therapy? (iii) Does ER β represent a novel target in breast cancer and thus potentially allow the development of additional hormonal therapies that can add to effectiveness of existing agents? The answer to these questions will depend on studies that look at the expression of ER β and its variants by Western blotting, PCR and immunohistochemistry. The response to hormonal therapy and subsequent outcome will then need to be analyzed in such patients. Such studies should, where possible, examine samples from patients being treated in the

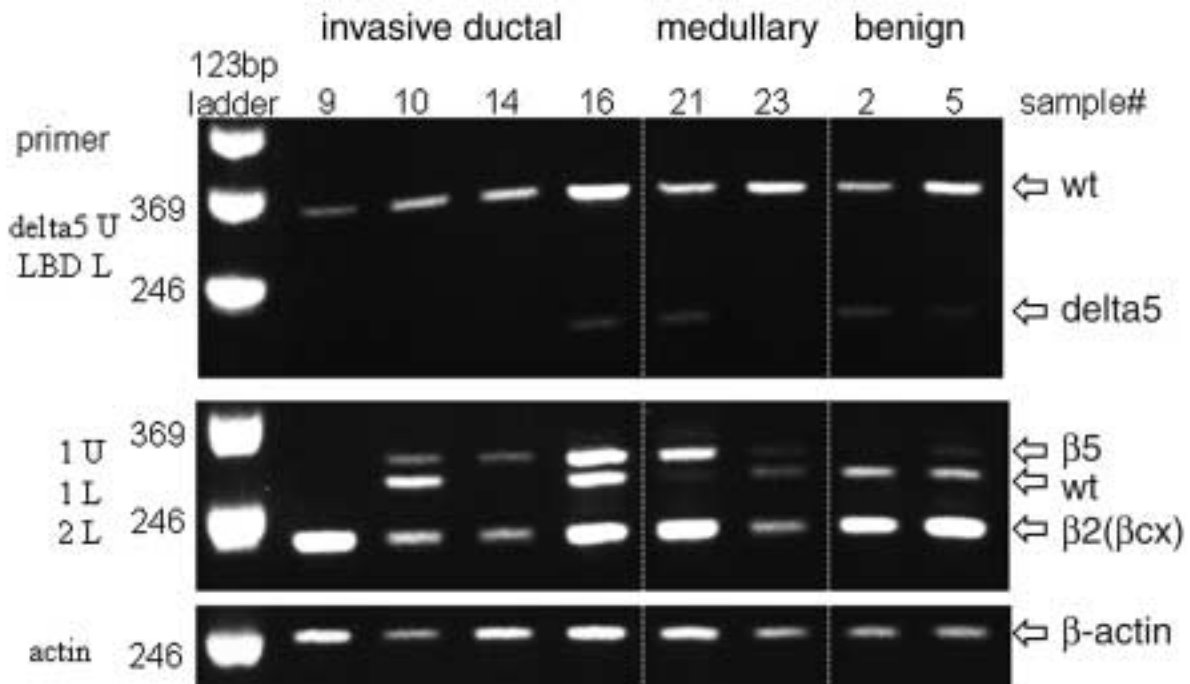


Figure 9 Identification of ER β splice variants by RT-PCR in breast cancer. Total RNA was extracted from frozen breast tissue sections using RNAwiz (Ambion, Austin, TX, USA) according to the manufacturer's instructions, and quantified spectrophotometrically. Five micrograms of total RNA were treated with 5 units RNase-free DNase (Promega, Madison, WI, USA) for 60 min at 37 °C to remove genomic DNA from the RNA samples. After inactivation of DNase for 10 min at 65 °C, samples were reverse-transcribed using SuperScript preamplification system (Life Technologies, Inc., Gaithersburg, MD, USA) in a final volume of 20 μ l according to the manufacturer's instructions. Six primer pairs were designed to detect the following ER β isoforms: (1) the exon 5 deletion isoform ($\delta 5$); (2) ER β ins; (3) ER β cx and $\beta 5$. The primer sets for ER β variants were as follows: ER β LBD U, 5'-GAGCTCAGCCTGTTCGACC; ER β LBD L, 5'-GGCCTTGACACAGAGATATTC; ER β $\delta 5$ U, 5'-ATGATGATGCCCTGACCAAG; ER β 1U, 5'-CGATGCTTTGGTTGGGTGAT; E ER β 1L, 5'-GCCCTTTGCTTTTACTGTC and ER β 2L, 5'-CTTTAGGCCACCGAGTTGATT. The location of each primer and the size of respective products are shown on the left-hand side of the figure. β -Actin primers were 5'-CTGGCACACACCTTCTAC for sense and 5'-GGGCACAGTGTGGGTGAC for antisense. The conditions for the PCR were as follows: 95 °C for 5 min, then 36 cycles (or 25 cycles for the β -actin primer set) at 95 °C for 30 s, 60 °C for 30 s and 72 °C for 60 s, finally 72 °C for 5 min. PCR products were resolved on a 2% agarose gel containing ethidium bromide in 0.5 \times TBE running buffer. In the negative controls the cDNA template was replaced with DNase-treated RNA, and the identity of the positive band was confirmed by direct sequencing of the PCR product or by cloning the PCR product into a TA cloning vector, selection of clones and sequencing of the insert from the vector. None of the tested samples had LBD insertion isoform. The $\delta 5$ variant of ER β was detected in samples 5, 16 and 21, which had no estradiol binding on sucrose gradients, as well as in 2 which had no estradiol binding in the 8S peak. Triple primer PCR, used for evaluating the ratio of the various isoforms (middle panel), revealed that the expression of C-terminally truncated isoforms is common. Patient 9 (invasive ductal carcinoma, grade 3) had exclusively ER β cx and had very high binding of estradiol in the 8S peak. Sample 14, which had estradiol binding in the 4S peak on sucrose gradients, had no WT C-terminal sequence.

neoadjuvant, adjuvant and metastatic setting, as well as patients who develop recurrent breast cancer during or after completing adjuvant hormonal treatment. With results from such detailed studies, it may be possible in the future that, based on ER α , ER β and ER β splice variant status, patients can be stratified for their likely response to hormonal therapy. In addition, there is the potential for such information to assist in identification of patients who may develop hormone-independent breast cancer. The future also holds the possibility of a new era in the therapy of hormonal treatment

based on targeting ER β , but before this can happen the above questions need to be answered.

Acknowledgements

The patient and very skilful work of Christina Thulin-Andersson is gratefully acknowledged. This research was supported by grants from the Swedish Cancer Fund, the UK Cancer Research Campaign, and by KaroBio AB, Sweden.

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