# Estrogen receptor beta in breast cancer

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## 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 (ERα)-positive tumors. In 1996, when oncologists became aware of a second ER, ERβ, 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 ERα in breast cancer. Today we know that ERα and ERβ have distinct cellular distributions, regulate separate sets of genes and can oppose each other's actions on some genes. We also know that ERβ is widely expressed in both the normal and malignant breast and that there are proliferating cells in the breast which express ERβ. In this review we summarize what is known about ERβ in breast cancer and examine the possibility that ERβ-selective ligands may well represent a useful class of pharmacological tools with a novel target, namely proliferating cells expressing ERβ.

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## 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, ERβ, 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. ERβ is highly expressed in the prostate (Kuiper *et al*. 1996) and is expressed together with ERα 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 ERβ in normal and malignant growth of these two organs. In the ventral prostate of mice, ERβ is involved in regulation of epithelial growth, and its absence, in ERβ-/- 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 ERβ along with ERα and whether ERβ can be a novel target in therapy. In this review we will summarize what has been learnt about ERβ in the normal


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breast and in breast cancer and present a part of our large, as yet unpublished, study on ERβ 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, ERα and ERβ. 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 antiestrogens 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 ERα. In immunohistochemical studies, markers of proliferation, such as Ki67 and cyclin A, are not found in cells which express ERα (Clarke *et al*. 1997*a*,*b*, Zeps *et al*. 1999). In the rodent mammary gland, ERβ-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 ERα-positive cells proliferate in response to estradiol (Allegra & Lippman 1978, Chalbos *et al*. 1982). Furthermore, when ERα is introduced into ERα-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 ERα-containing cells do not proliferate, the biological significance of the well-documented induction of cyclin D by estrogen and the interaction of ERα with cyclin D (Neuman *et al*. 1997, Zwijsen *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 ERα is rapidly down-regulated in response to estrogen in a ubiquitin-mediated

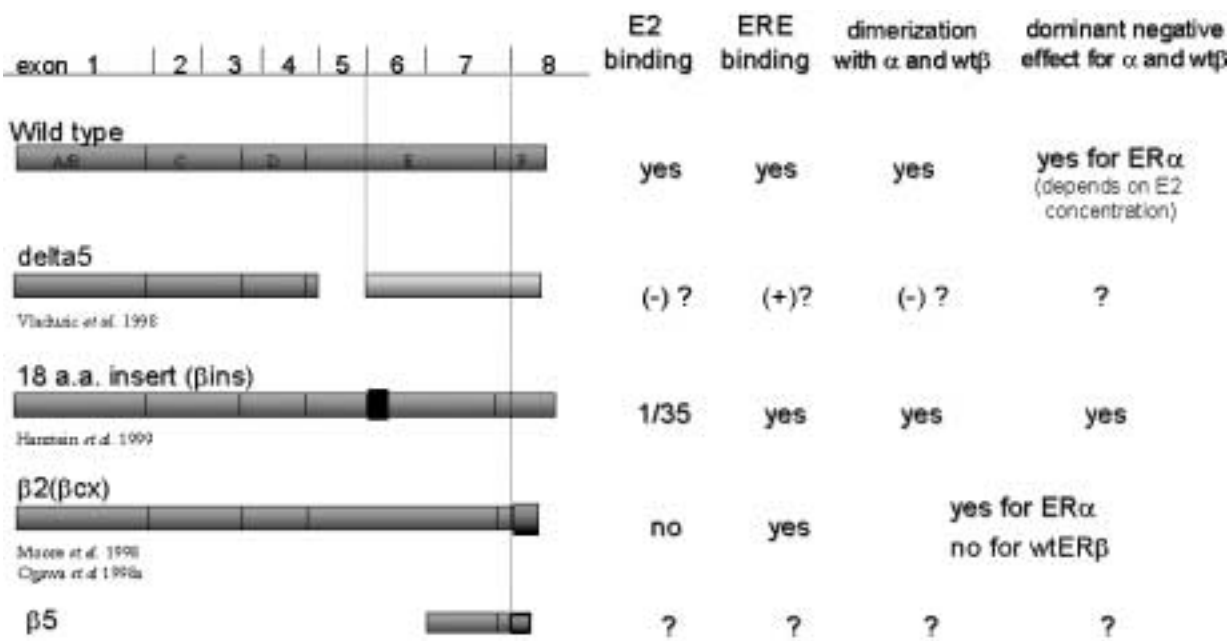


**Figure 1** ERβ splice variants. The reported exon–intron junctions of human ERβ sequences are indicated. Models of each variant protein and the location of primers are shown.



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degradation pathway (Lonard *et al*. 2000). One interpretation of this phenomenon is that ERα must be removed from the cell in order for the cell to progress through the cell cycle. The absence of ERα 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 ERα-containing cells but that ERα 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 ERα and high expression of ERβ, 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 ERα 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, ERα-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 ERα 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 ERα-positive patients respond to tamoxifen and that some patients, classified as ERα-negative, do benefit from tamoxifen therapy (McGuire *et al*. 1982). There are several possible explanations for how tamoxifen might produce its beneficial effects in ERα-positive cancers: (i) ERα-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 ERα-containing cells, which, although they may not be the proliferating cells, represent the bulk of some tumors; (iii) ERα-containing cells may be the malignant cells but when they enter the cell cycle ERα is down-regulated and tamoxifen targets these cells when they are in $G_0$ 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 ERα in estrogen-induced growth of the breast, there is also the question

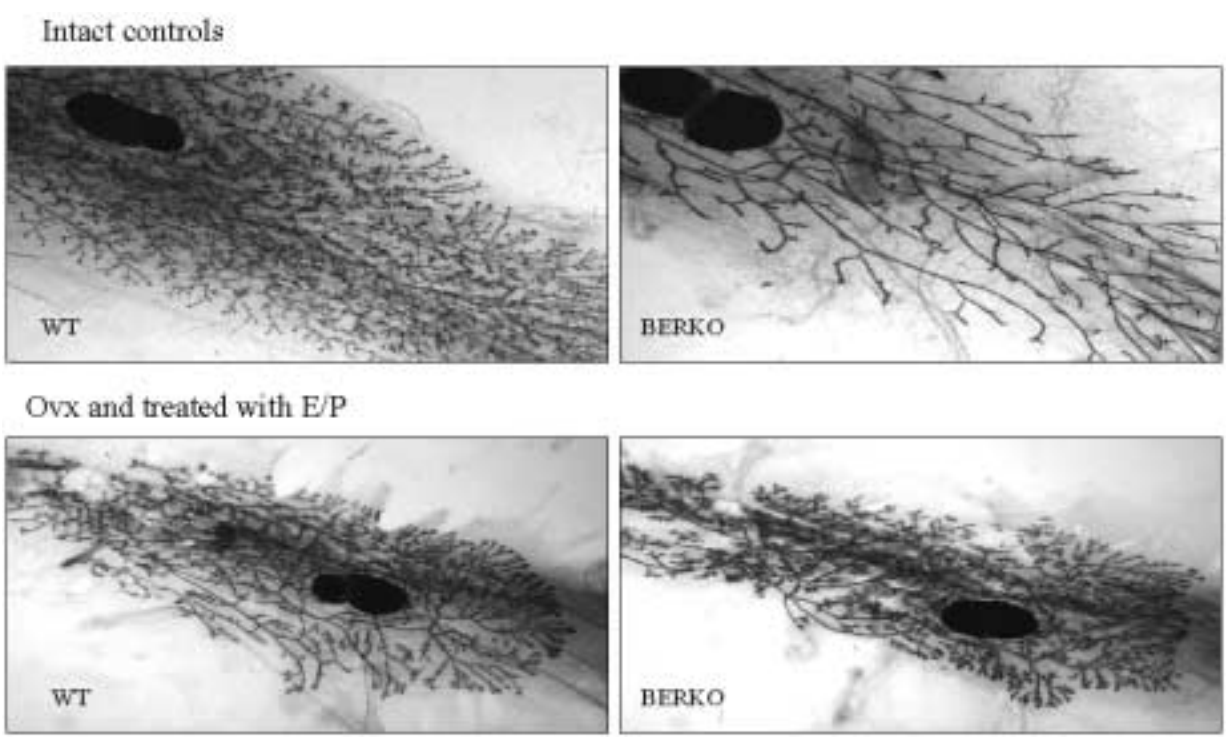


**Figure 2** Whole mounts of WT and BERKO breasts. Whole mount preparations of the fourth abdominal mammary glands of BERKO and WT female mice showing the development of the mammary ductal tree. The upper panels show mammary glands of mature (4-month-old) intact mice. BERKO mice do not have regular estrous 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.


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

## Breast phenotype in ERα-/- and ERβ-/- mice

In the rat mammary gland, ERβ is more abundant than ERα (Saji *et al*. 2001) but the role of ERβ in this organ is still not clear. Even though ERβ-/- mice are available (Krege *et al*. 1998), there are two confounding factors which make it difficult to define the role of ERβ in the mammary gland. One is ovarian dysfunction in ERβ-/- 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 ERβ splice variant ERβ ins (Fig. 1) in the rat mammary gland (Saji *et al*. 2001). In ERβ 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 ERα and may function as a repressor of ERα (Saji *et al*. 2001). If this is the case, some aspects of the mammary gland in ERβ-/- mice may be due to unrestrained ERα activity.

In prepubertal female ERβ-/- 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 ERα-mediated (Couse & Korach 1999). Normal terminal end buds were also seen in the growing mammary ducts of both ERβ-/- and age-matched wild-type (WT) glands. Sexually mature ERβ-/- female mammary glands are less developed (fewer side branches and alveoli) than those of WT littermates (Fig. 2). In less than half of the ERβ-/- mice was there any evidence of estrous cycles and this might explain the poor development of the ERβ-/- 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 ERβ-/- mice were indistinguishable from those of similarly treated WT mice (Fig. 2). This indicates that ERβ is not necessary for estrogen-induced proliferation of the mammary gland. Interestingly, in ERα-/- 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 ERα-/- nor ERβ-/-,

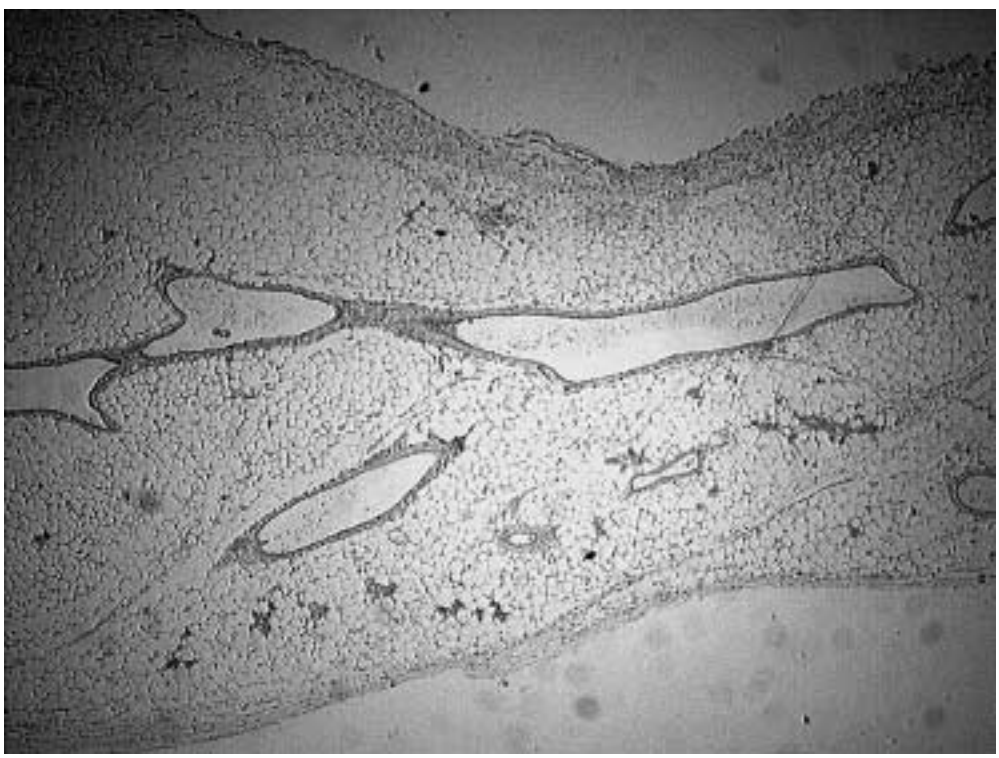

**Figure 3** Histological appearance of the mammary gland of ERβ-/- mouse at 2 years of age. Mammary glands were excised and fixed in 4% paraformaldehyde. The section illustrates multiple cysts. These are found throughout the BERKO mammary gland.


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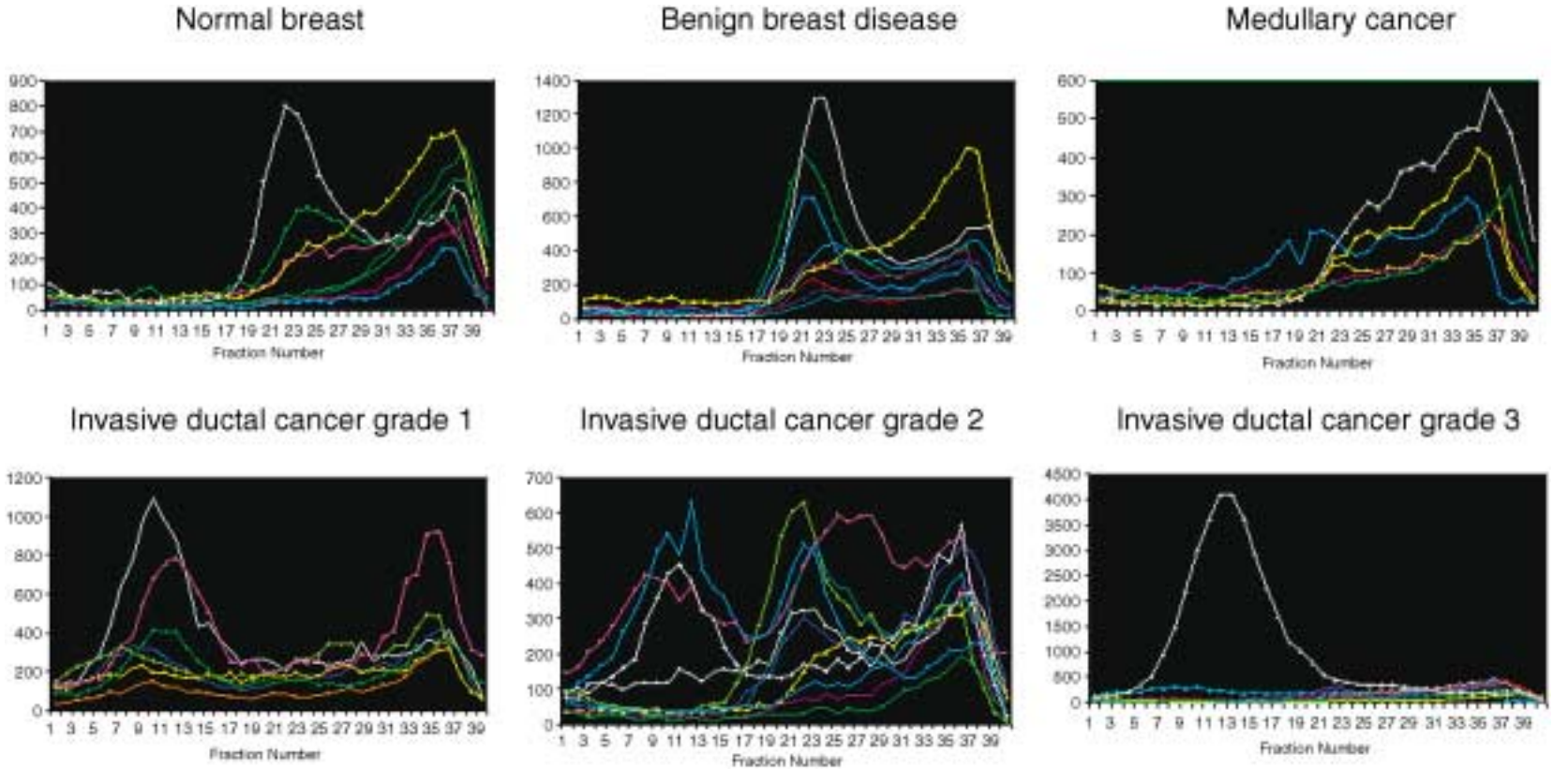


**Figure 4** Estradiol binding in 8S (ERα) and 4S (ERβ) peaks in normal and breast tumors by sucrose density gradient assay. Tissue was frozen in liquid nitrogen and pulverized in a dismembrator (Braun, Melsungen, Germany) for 45 s at 1800 r.p.m. Pulverized tissue was added to a buffer composed of 10 mM Tris–chloride, pH 7.5, 1.5 mM EDTA and 5 mM sodium molybdate, using 1 ml/100 μg tissue. Cytosol was obtained by centrifugation of the homogenate at 204 000 *g* for 1 h in a 70Ti rotor at 4 °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 °C with 10 nM [$^3$H]estradiol-17β in either the presence or absence of a 50-fold excess of radio-inert 17β-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-monothioglycerol, 10 mM KCl. Samples of 200 μl were layered on 3.5 ml of gradients and centrifuged at 4 °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 ERβ in normal breasts, in benign breast disease and in medullary cancer. ERα expression in these samples is very low. In invasive ductal cancer grade 1, ERα expression is high and ERβ low. In invasive ductal cancer type 2, both ERα and ERβ 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 ERα.



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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 ERβ-/- mice age, they develop severe cystic breast disease, which is not found in their WT littermates. At 2 years of age, all ERβ-/- mice mammary glands are cystic. A typical gland is shown in Fig. 3.

## ERα and ERβ in the normal and malignant human breast

In the human breast the role of ERβ is even less well understood than it is in rodents. One major confounding factor is the expression of another ERβ splice variant, ERβcx (Ogawa *et al*. 1998*a*), a dominant negative repressor of ERα. In this variant, an alternative exon 8 is utilized (Fig. 1). This receptor has no measurable affinity for estradiol and, if expressed together with ERα in cells, it silences ERα function (Ogawa *et al*. 1998*b*). It is not known whether the physiological function of ERβcx is to repress ERα 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 ERβ 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

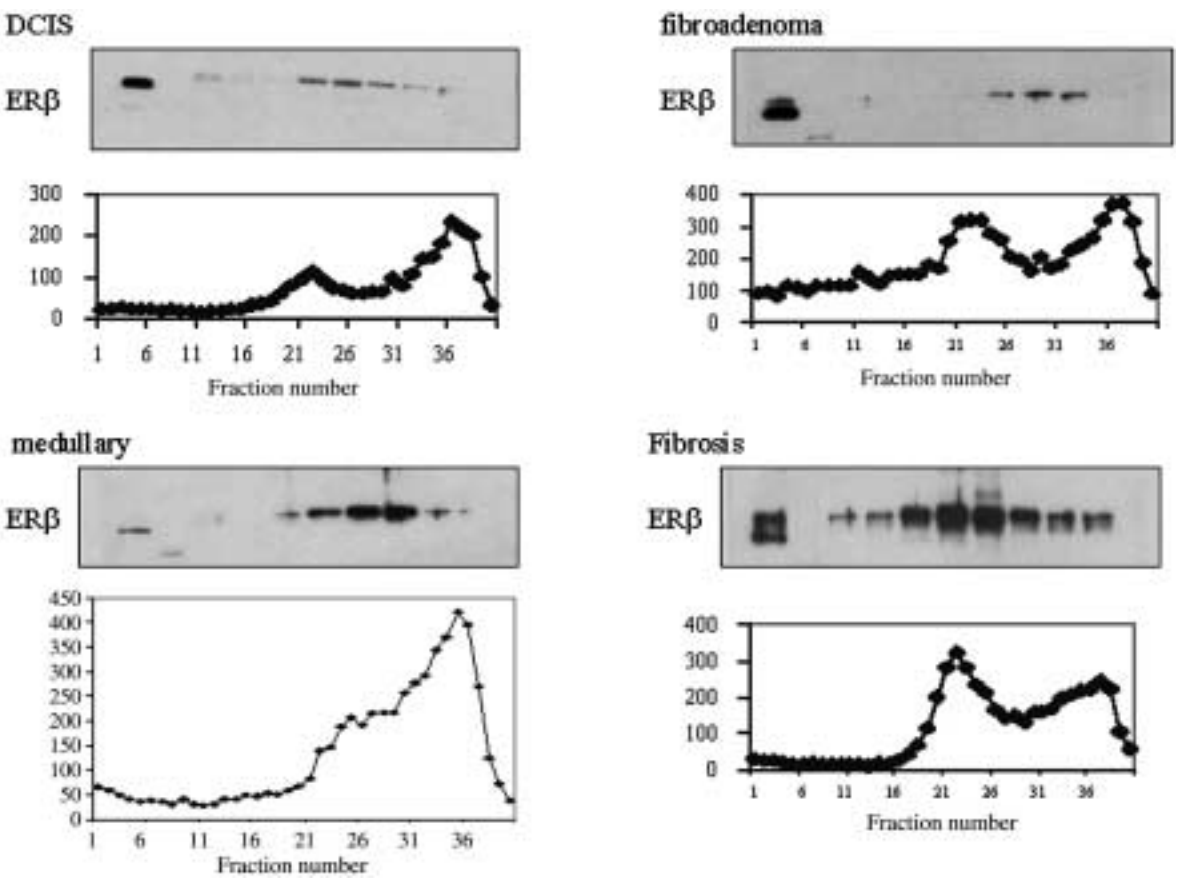


**Figure 5** Detection of ERβ by Western blotting analysis. For Western blotting, sucrose gradient fractions were first precipitated with TCA, and the precipitate resuspended in methanol. Samples were placed on dry ice for 30 min and the protein recovered by centrifugation. Protein pellets were dissolved 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 ERβ antibody was raised in rabbits against the ligand-binding domain of ERβ. The ERα antibody was H222 from Abbott Laboratories. The panel illustrates the high expression of ERβ in fibrocystic disease and medullary cancer.


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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 ERα and ERβ were node-positive and tended to be of higher grade; another (Jarvinen *et al*. 2000), found that ERβ was often co-expressed with ERα and progesterone receptor in breast cancer and that ERβ was significantly associated with negative axillary node status and low tumor grade; while the third study (Mann *et al*. 2001) found that expression of ERβ in more than 10% of cancer cells was associated with better survival. In one RT-PCR study there was increased expression of ERβ mRNA in tamoxifen-resistant breast cancer patients (Speirs & Kerin 2000) and in another study (Roger *et al*. 2001), there was decreased expression of ERβ protein in proliferative preinvasive 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 ERβ 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 ERβ can be used as a routine prognostic indicator either independently or alongside ERα. Regarding the use of ERβ 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 ERβ splice variants, to act as natural antiestrogens and repress ERα function, needs further investigation and evaluation, as this could affect the way we currently interpret ERα immunostaining to guide treatment with antiestrogens.

## Unpublished results from our breast cancer study

In an attempt to resolve some of the issues concerning the role of ERβ 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 ERα 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 differentiaton grade was recorded. We are using multiple techniques to measure

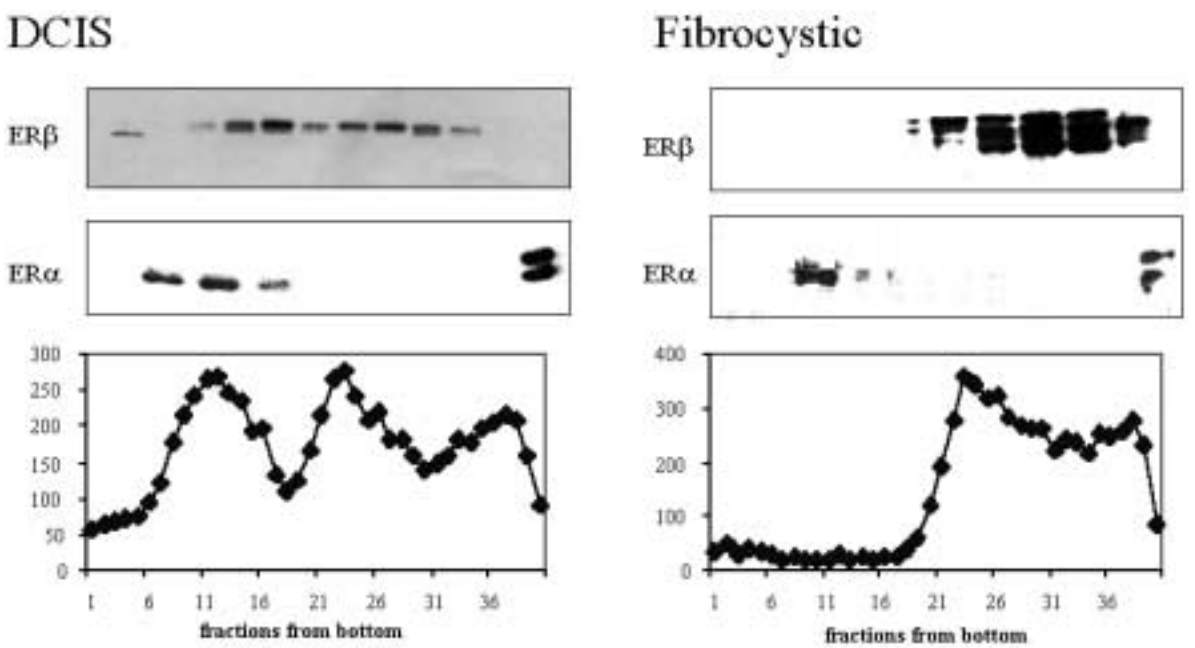


**Figure 6** Breast sample with non-estrogen-binding form of ERα. In the DCIS sample, there was estrogen binding in the 8S peak and a corresponding ERα band on Western blots in the 8S fractions. In the fibrocystic sample there was no estrogen binding in the 8S peak but there was a band of the correct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


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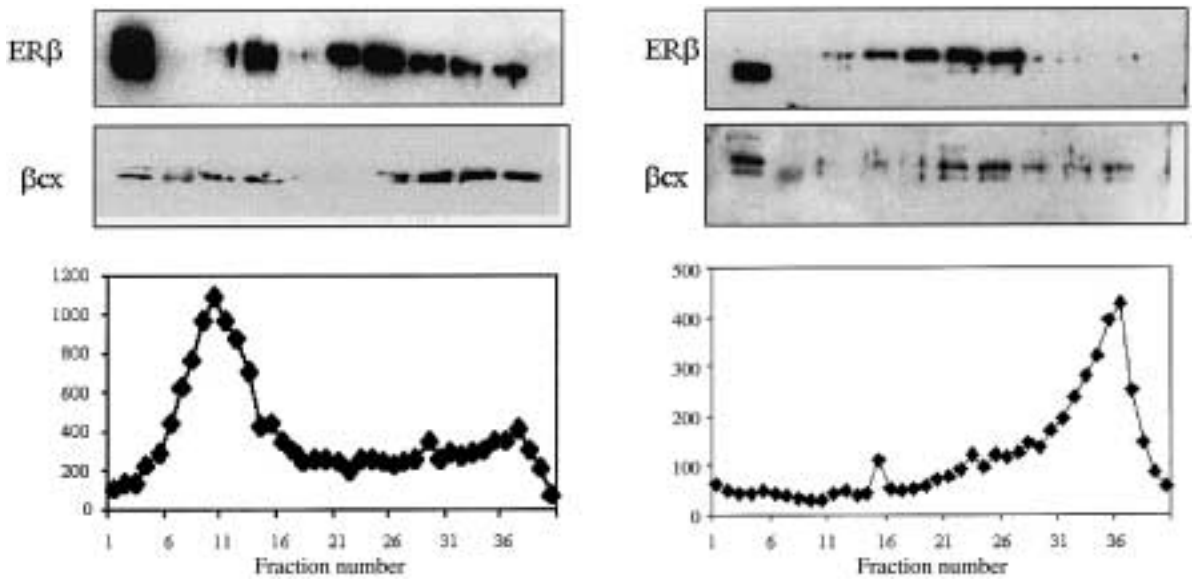


**Figure 7** Breast cancers expressing ERβcx. The two ductal cancers (LH panel, grade 1; RH panel, grade 3) shown in this figure had no estrogen-binding 4S peak. ERβ, as detected by the LBD antibody, was found throughout the gradient. With an ERβcx-specific antibody, ERβcx distribution in the gradient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

ERα and ERβ 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 ERβ 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 ERα and ERβ was revealed by sucrose density gradient 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 ERα. The unexpected finding was that the 4S estradiol-binding peak contained ERβ, 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 μm) 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. ERα monoclonal antibody (6F11) was from Novocastra (Newcastle, UK); ERβ 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 °C with normal goat serum diluted 1:10 in PBS. Antibodies were diluted individually in PBS containing 3% BSA. Dilution for ERβ was 1:100 and for ERα antibody 1:500. Sections were incubated with antibodies overnight at 4 °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 ERα and ERβ expression in invasive ductal cancer. In the fibrocystic tumor, ERα is expressed in the epithelial cells while ERβ is expressed in both epithelial and stromal cells.



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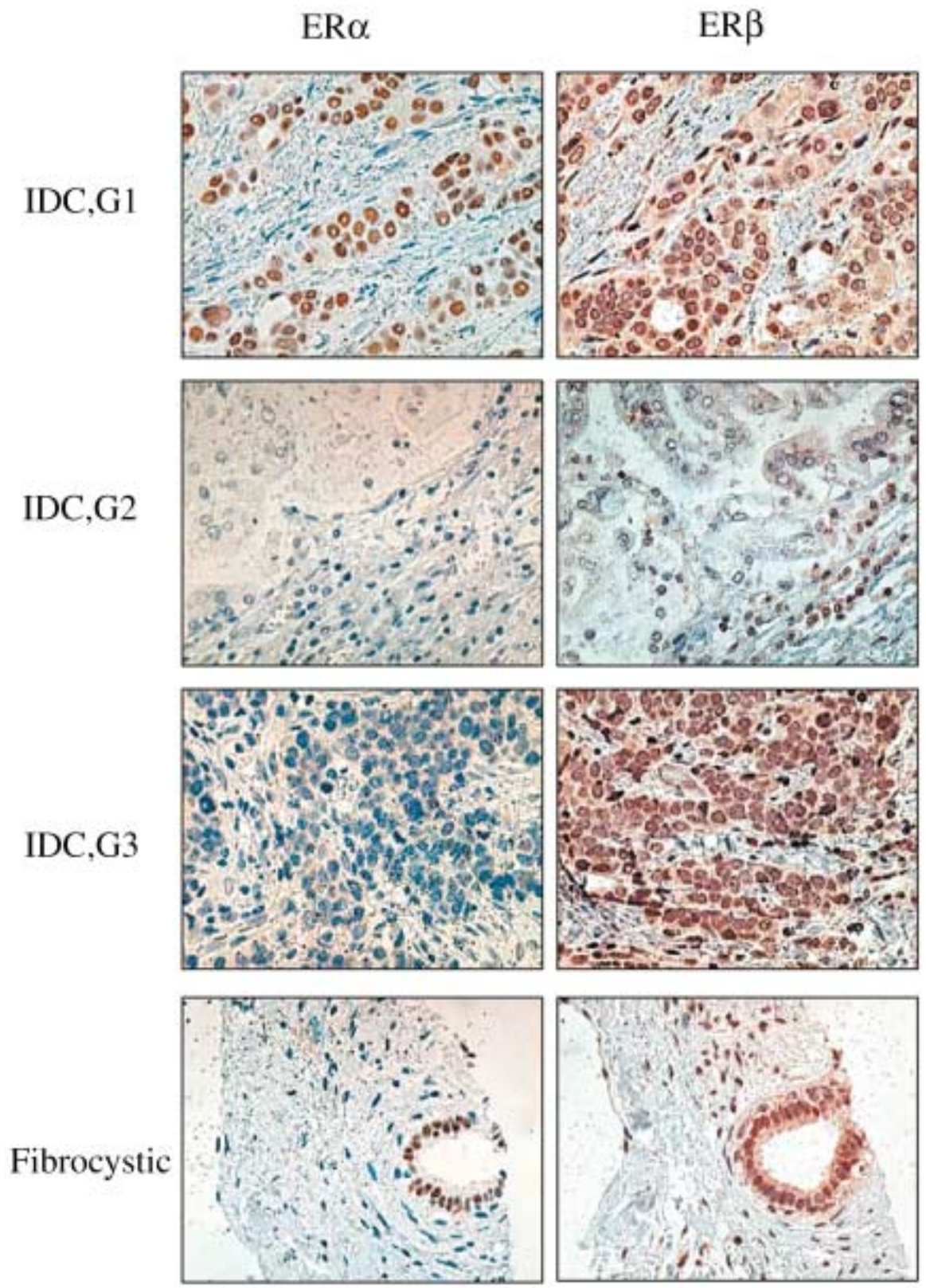


**Figure 8** See opposite page for legend.



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higher sedimentation rate of ERα suggests that it is in a larger multi-protein complex than is ERβ and careful examination of the ERβ 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, ERα, 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, ERβ, (range 20–475 fmol/mg protein) was present in both normal and malignant breasts (80% of these samples were positive for ERβ). All grade 1 invasive ductal carcinomas were devoid of ERβ but had high levels of ERα. As discussed above, the presence of ERα 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 ERα-positive. Interestingly, although ERα 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 ERα protein such as point mutations, post-transcriptional modifications of the receptor or the presence of ERβ splice variants which prevent estrogen binding by forming heterodimers with ERα (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. ERα 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 ERβ with a molecular mass of 60–63 kDa (Figs 5–7).

Immunohistochemistry (Fig. 8) confirmed that ERα 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 ERβ 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 ERβ protein. In order to understand this result, we developed another antibody, one specific for the cx exon. Western blotting with this antibody showed that ERβcx was abundantly expressed in fractions which had positive signals with the antibody raised against the ligand-binding domain of ERβ (Fig. 7). Clearly, ERβcx 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 ERβ1 is present. It is not clear whether there is no ERβ1 or if it is present but its binding to estradiol is inhibited by ERβcx. The presence of ERβcx was confirmed by RT-PCR (Fig. 9). The ratio of ERβ1 to ERβcx mRNA varied considerably between samples and in some cases ERβcx was the only ERβ isoform detected.

We conclude from these studies that ERβ is usually expressed in normal breasts and in benign and malignant breast disease. The important remaining question is whether the presence of ERβ 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 ERα and ERβ 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 ERβ isoforms co-localize with ERα in cells. If ERβcx is expressed in cells with ERα, this may represent one mechanism by which ERα-positve 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 ERα, the question of whether ERβcx functions as an 'anti-estrogen' *in vivo* must be examined. Of course, before this question can be answered, we need to know whether ERβ splice variants have alternative ligands or alternative routes of activation and whether they silence ERα action at sites of interaction other than on EREs.

## Unanswered questions

There are important questions which need to be addressed with regard to ERβ and its splice variants in breast cancer. (i) Can their measurement be used to improve the prognostic value of ERα or could it even be used as an independent prognostic indicator? (ii) What is the functional significance of ERβ splice variants such as ERβcx and can their presence be used to predict the likely response to hormonal therapy? (iii) Does ERβ 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 ERβ 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



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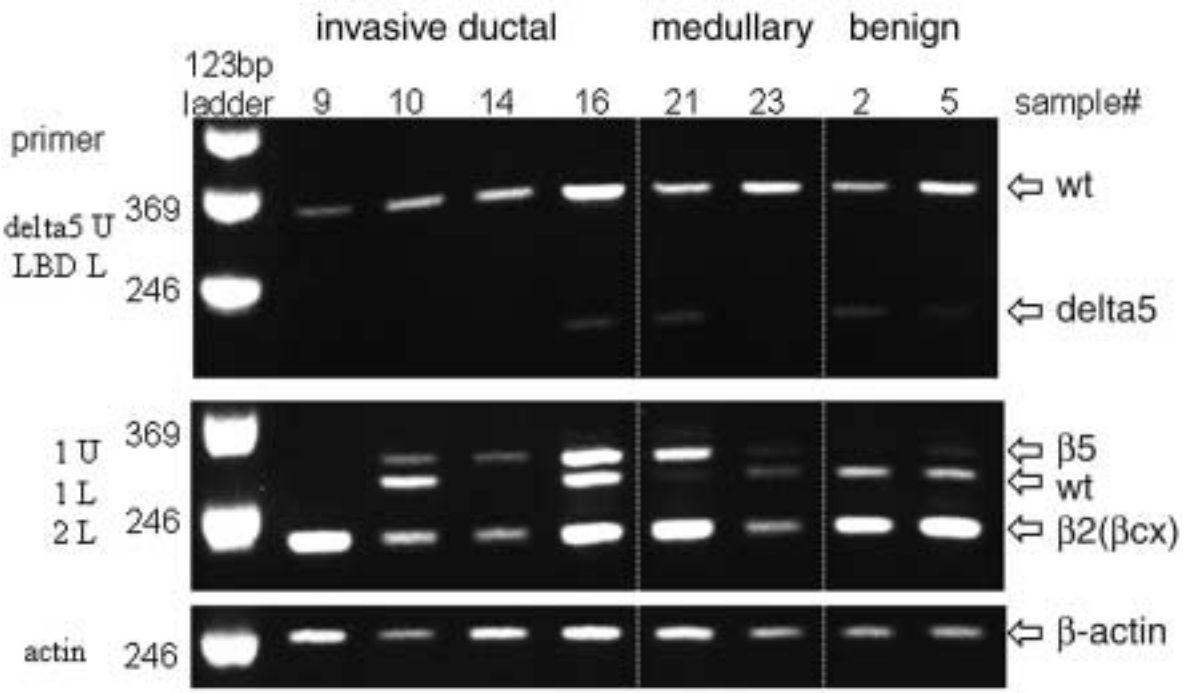


**Figure 9** Identification of ERβ splice variants by RT-PCR in breast cancer. Total RNA was extracted from frozen breast tissue sections using RNAwiz (Ambion, Austin, TX, USA) according to the manufacturer's instructions, and quantified spectrophotometrically. Five micrograms of total RNA were treated 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 μl according to the manufacturer's instructions. Six primer pairs were designed to detect the following ERβ isoforms: (1) the exon 5 deletion isoform (δ5); (2) ERβ ins; (3) ERβcx and β5. The primer sets for ERβ variants were as follows: ERβ LBD U, 5′-GAGCTCAGCCTGTTCGACC; ERβ LBD L, 5′-GGCCTTGACACAGAGATATTC; ERβ δ5 U, 5′-ATGATGATGTCCCTGACCAAG; ERβ 1U, 5′-CGATGCTTTGGTTTGGGTGAT; E ERβ 1L, 5′-GCCCTCTTTGCTTTTACTGTC and ERβ 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'-CTGGCACCACACCTTCTAC 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 × 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 δ5 variant of ERβ 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 ERβcx 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 ERα, ERβ and ERβ 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 ERβ, but before this can happen the above questions need to be answered.

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