The expression of oestrogen receptor (ER)- β and its variants, but not ER α , in adult human mammary fibroblasts

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Abstract

Whilst oestrogen receptor (ER)- α and ER β have been shown to be important in the development of the mammary gland, the cell-specific expression pattern of these two receptors within the human breast is not clear. Although it is well established that in the developing rodent mammary gland stromal ER α mediates the secretion of growth factors which stimulate the proliferation of the ductal epithelium, the expression of ER α in human adult breast stromal fibroblasts is controversial, and the expression of ER β has not been properly defined. In the present study, we have evaluated the expression of ER α and ER β by immunohistochemistry in normal tissue samples, and in purified human breast fibroblasts by Western blotting, RT-PCR analysis and ligand-binding sucrose gradient assay. Our data clearly demonstrated that ER β variants, including ER β 1, ER β 2, ER β 5, ER β δ and ER β ins, but not ER α , are expressed in human adult mammary fibroblasts. These results are supported by the findings that an ER β -selective ligand, BAG, but not the ER α high-affinity ligand oestradiol, can induce fibroblast growth factor-7 release and activate transcription from an oestrogen-responsive element promoter in these adult human mammary fibroblasts. Together, these observations revealed that, in the adult breast and in breast cancer, the proliferative signals derived from the stroma of adult mammary glands in response to oestrogen are not mediated by ER α and provide new insights into the nature of stromal–epithelial interactions in the adult mammary gland. In addition, the expression of these ER β variants in cells where there is no ER α suggested that these ER β splice forms may have functions other than that of modulating ER α activity.

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Introduction

Oestrogen (e.g. oestradiol) plays a crucial role in regulating the growth and differentiation of the normal mammary gland and has an important role in the development and progression of breast cancer (Girdler & Brothrick 2000). The actions of oestrogen are mediated by its interaction with two oestrogen receptors, ER α and ER β , which are ligand-activated nuclear transcription factors and members of the steroid/nuclear receptor superfamily (Kuiper *et al.* 1996, Parker 1998, Nilsson *et al.*

2001). Although ER α and ER β are structurally similar (Brzozowski *et al.* 1997), there are significant differences in their ligand affinities and interaction with the transcriptional machinery, which result in important physiological and pharmacological differences in the signalling of the two receptors. Differential activation of the oestrogen-responsive element (ERE)-regulated and the activator protein-1 (AP-1)-controlled reporter genes by ER α and ER β has been documented (Paech *et al.* 1997).

There are several mechanisms through which oestrogen stimulates growth of mammary

epithelium. One mechanism, which is crucial in the developing mammary gland, is the indirect effect of oestrogen on the mammary epithelium. In this case, oestrogen induces the mammary stroma to secrete growth factors, which in turn stimulate epithelial proliferation (Cunha *et al.* 1997). There is a considerable body of evidence showing that stromal ER α is involved in rodent mammary gland morphogenesis (Haslam & Shyamala 1981, Cunha *et al.* 1997). Recent studies in mice using chimeric tissue recombinations between ER α null and normal mice indicate that oestrogen-mediated proliferation of epithelial cells is dependent upon the presence of ER α in mouse mammary stromal cells, but not in epithelial cells (Yang *et al.* 1995, Bocchinfuso & Korach 1997). However, this is not an absolute requirement since the stroma from ER α null mice can support epithelial growth if oestrogen and progesterone are administered (Yang *et al.* 1995). Consistent with this notion is a recent immunohistochemical study on mouse transplanted human organoids showing that ER α is expressed in luminal epithelium but not the stroma (Parmar *et al.* 2002).

The adult mammary gland undergoes morphogenesis and remodelling throughout reproductive life. Morphogenesis is regulated by circulating steroids and polypeptide hormones, as well as by local epithelial–mesenchymal signals. The production and release of mesenchymal factors are under endocrine control. For example, the hepatocyte growth factor (HGF)/scatter factor (SF) promotes ductal outgrowth and tubule formation in the mammary gland (Niranjan *et al.* 1995), and the secretion of HGF/SF from the stroma has been shown to be increased by oestrogen (Sunil *et al.* 2002). Fibroblast growth factor (FGF7) (also termed keratinocyte growth factor (KGF)) is also another important mesenchymal-derived epithelial growth factor that has been shown to be regulated by oestrogen; however, there are species differences in its function on epithelial cells and its regulation by oestrogen. In humans, FGF7 stimulates both normal and malignant breast cancer epithelial cell proliferation (Palmieri *et al.* 2003). One report has indicated that the expression of FGF7 is not affected by oestrogen in normal breast stromal cells but is stimulated by oestrogen in stromal cells from breast cancers (Zhang *et al.* 1998). Administration of FGF7 to rats causes ductal proliferation, whilst in mice it results in cystic ducts (Yi *et al.* 1995). In

mice, oestrogen was found to cause an increase in FGF7 in the mammary gland *in vivo*, but response was slow, and the dose needed was higher than that needed for growth of the uterus (Pedchenko & Imagawa 2000).

Although the evidence for the presence of ER α in the developing mammary stroma is strong, there is some question about which of the ERs is expressed in the stroma of the adult gland. ER α has been detected in stromal cells derived from tissues of fetuses, infants and young female individuals (Boyd *et al.* 1996, Keeling *et al.* 2000) but not stromal cells from adult samples (Boyd *et al.* 1996). By *in situ* hybridization, Sasano *et al.* (1999) detected ER α mRNA predominantly in ductal epithelial cells and also in some stromal cells. Others have also shown by immunohistochemical staining that there is a lack of ER α in both cancerous and normal human breast stromal cells (Walker *et al.* 1992, Leygue *et al.* 1999). Although ER β is less well studied than ER α , there is evidence that ER β is expressed in both epithelium and stroma (Crandall *et al.* 1998, Palmieri *et al.* 2002).

In the present study, we have investigated the presence of ER α and ER β in breast stroma from normal breast tissue and from breast cancer. We purified fibroblasts from normal and malignant breasts and found that ER β 1, and its splice variants ER β cx (Ogawa *et al.* 1998) and ER β 85, but not ER α , are expressed in these human breast fibroblasts. Furthermore, treatment of these fibroblasts with interleukin (IL)-1 β and ER β -specific agonist are more effective than oestrogen in stimulating fibroblasts to secrete the growth factor FGF7.

Materials and methods

Tissue samples

Human breast samples were obtained with the approval of the local ethics committee and consent from women who underwent breast reduction mammoplasty and surgery for carcinoma of the breast at Charing Cross Hospital and Central Middlesex Hospital, London, UK.

Immunohistochemical staining

Paraffin sections of breast tissue were obtained from the histopathology archive at Charing Cross

Hospital, London. In all the immunostaining experiments, paraffin sections (4 μ m) were dewaxed in xylene and rehydrated through graded alcohol. Endogenous peroxidase was blocked by incubation for 15 min with a solution of 0.6% hydrogen peroxide and antigens were retrieved by microwave sections in 1 mM citrate buffer (pH 6.0) for 20 min at 800 W. Tissue sections were incubated for 10 min at room temperature with antibody diluent containing 5% normal rabbit serum, 0.1% bovine serum albumin and 0.1% sodium azide in phosphate-buffered saline (PBS). For ER β immunostaining, a rabbit anti-human ER β polyclonal (06-629) antibody from Upstate Biotechnology (Lake Placid, NY, USA) (1/100 dilution in antibody diluent) was applied to sections and incubated overnight at 4 °C. ER β cx was detected by a specific antibody described recently (Saji *et al.* 2002). For ER α staining, monoclonal mouse anti-ER α (1D5; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was applied at a dilution of 1/500 in antibody diluent and the sections incubated overnight at 4 °C. After incubation with the primary antibodies, the sections were rinsed in PBS with 0.05% Tween 20 prior to addition of the appropriate peroxidase-conjugated secondary antibody in PBS. As secondary antibodies, peroxidase-conjugated rabbit anti-mouse IgG (Dako, Ely, UK) and goat anti-rabbit IgG (Sigma, Poole, Dorset, UK) were used at dilutions of 1:100. After incubation for 1 h at room temperature the secondary antibodies were washed off with PBS with 0.05% Tween 20. Colour staining was developed with hydrogen peroxide and 3,3'-diaminobenzidine tetrahydrochloride (DAB; Dako). After colour development, the sections were also counterstained with Mayer's haematoxylin, dehydrated through graded alcohol to xylene, and mounted with Pertex (Histolab, Havershill, UK).

Breast fibroblast purification and culture

Breast fibroblasts were prepared from reduction mammoplasty and breast carcinoma tissues as described previously (Gomm *et al.* 1995). The tissues were first assessed by histopathologists to ascertain the exact pathological status before being used for breast fibroblast preparations. Briefly, 0.5 cm² breast tissue was digested overnight with 1 mg/ml collagenase (Sigma) and 1 mg/ml hyaluronidase (Sigma) in RPMI 1640 (Gibco-BRL,

Life Technologies Inc., Paisley, UK) plus 5% fetal calf serum (FCS) at 37 °C, followed by centrifugation at 150 *g* for 5 min. After removing the lipid layer, the tissue suspension was subjected to three sedimentations at 1 *g* (or under gravity) to enrich for the ductal and lobular elements. The supernatants containing single cells (including blood cells, fibroblasts and endothelial cells) were removed and cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL, Life Technologies Inc.) supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin to positively select for stromal cells. The blood cells in the cell suspension were eliminated by three rounds of depletion using anti-common acute lymphoblastic leukaemia antigen (CALLA/CD10; Sera-Lab, Loughborough, UK) conjugated to 4.5 μ m Dynabeads (DynaL, Bromborough, UK) according to the manufacturer's protocol. Dynabead-conjugated rat monoclonal anti-epithelial membrane antigen was then used to eliminate epithelial and myoepithelial cells as described (Gomm *et al.* 1995). The purified breast fibroblasts were then expanded and used for RNA and protein preparation. One of the fibroblast lines (line 8) is conditionally immortalized and has been described previously (O'Hare *et al.* 2001).

RNA preparation and cDNA production by RT

Total RNA was isolated using the RNeasy kit (Qiagen, Crawley, UK) and quantified by absorbance at 260 nm. Total RNA (2 μ g) was reverse transcribed in a 12 μ l reaction mixture composed of 10 U MMLV reverse transcriptase (Gibco-BRL, Life Technologies Inc.), 2 mM dNTPs, 6.7 mM MgCl₂ and 5 μ M random hexamer primers. The mixture was incubated at 40 °C for 60 min, followed by heating at 95 °C for 10 min.

PCR

PCR was carried out using the Gene Amp PCR System 2400 or 7700 gene amplifier (Perkin-Elmer, Beaconsfield, UK). The PCR of 50 μ l consisted of 3 μ l cDNA (corresponding to 500 ng RNA), 1 $\times$ PCR buffer (Perkin-Elmer), 2.5 mM MgCl₂, 0.2 mM dNTPs, 0.5 units Taq polymerase (Perkin-Elmer) and 1 μ M of each of the sequence specific primers. At each cycle, the PCR was denatured at 94 °C for 30s, annealed at specific

temperature/time and extended at 72 °C for 60 s. The final extension was at 72 °C for 3 min at the completion of the last cycle. The nucleotide sequences, annealing temperatures, reaction cycles and predicted sizes of the cDNA products of the primers used and names were as follows.

β -actin (1) PCR: forward 5'-GTGCACCTC GCGCGATGCTG-3'; reverse 5'-CCTCACCGA GCGCGGCTACAG-3' (58 °C, 35 cycles, 500 bp).

ER α PCR: EX2U forward 5'-GGAGACATGA GAGCTGCCAC-3'; EX4L reverse 5'-CCAGC AGCATGTCTGAAGATC-3' (58 °C, 40 cycles, 292 bp).

ER β PCR: forward 5'-TGTTACTGGTCCAG GTTCAAAGAGG-3'; reverse 5'-AGCCACAC TTCACCATTCAC-3' (60 °C, 40 cycles, 200 bp).

ER β WT (wild type)/ β ins PCR: forward (LBD U) 5'-GAGCTCAGCCTGTTTCGACC-3'; reverse (LBD L) 5'-GGCCTTGACACAGAGATATTC-3' (57 °C, 38 cycles, 247 bp for WT and 300 bp for β ins).

ER β WT/ δ 5 PCR: forward δ 55'-ATGA TGATGTCCCTGACCAAG-3'; reverse LBD L 5'-GGCCTTGACACAGAGATATTC-3' (57 °C, 38 cycles, 327 bp for WT and 188 bp for δ 5).

ER β WT/ β cx/ β 5 PCR: forward (1U) 5'-CGATGCTTTTGGTTTGGGTGAT-3'; reverse (1 L) 5'-GCCCTCTTTGCTTTTACTGTC-3'; reverse (2 L) 5'-CTTTAGGCCACCGAGTTG ATT-3' (57 °C, 38 cycles, 268 bp for WT, 214 bp for β cx and 295 bp for β 5).

Cytokeratin19 PCR: forward 5'-TCCGCCC GCTTTGTGTCCCTCGT-3'; reverse 5'-AG CATCCTTCCGGTTCTGCTCG-3' (69 °C, 30 cycles, 488 bp).

β -actin (2) PCR: forward 5'-CTGGCACCA CACCTTCTAC-3'; reverse 5'-GGGCACAGT GTGGGTGAC-3' (58 °C, 35 cycles, 237 bp).

All PCR products were resolved on 2% agarose gel by electrophoresis in Tris-acetate-EDTA buffer in the presence of 0.5 μ g/ml ethidium bromide. The DNA bands were visualised in the presence of u.v. illumination.

Western blot analysis and antibodies

Whole cell extracts were prepared by lysing cells with four times packed cell volume of lysis buffer (1% Nonidet P-40, 100 mM NaCl, 20 mM Tris, pH 7.4, 10 mM NaF, 1 mM sodium orthovanadate

and protease inhibitors ('Complete' purchased from Roche, Lewes, UK) on ice for 15 min. Protein yield was quantified by Bio-Rad Dc protein assay kit (Bio-Rad, Hemel Hempstead, UK). One hundred micrograms of lysate were separated by SDS-PAGE, transferred to nitrocellulose membranes and recognised by specific antibodies. The ER β (06-629) and ER α (ID5) antibodies used for immunostaining did not give the best results for Western blotting, and the ER α (F-10) antibody and the polyclonal ER β (LBD) were used instead for Western blotting. The mouse monoclonal ER α (F-10) antibody and the anti-actin polyclonal antibody were purchased from Santa Cruz Biotechnology. The rabbit polyclonal ER β (LBD) and ER β cx antibodies have been described previously (Saji *et al.* 2000, 2002) and were raised against the ligand-binding domain of ER β 1 protein and the C-terminus of ER β cx respectively. The antibodies were detected using horseradish peroxidase-linked goat anti-mouse or anti-rabbit IgG (Dako) and visualised by the enhanced chemiluminescent detection system (Amersham Pharmacia Biotech, Amersham, Bucks, UK.)

Sucrose density gradient assay

Sucrose density gradient assay was performed essentially as described previously (Jensen *et al.* 1968, Palmieri *et al.* 2002). Cell pellets frozen on liquid nitrogen were pulverised in a dismembrator (Braun Melsungen, Melsungen, Germany) for 45 s at 1800 r.p.m. Pulverised cells (100 μ g/ml) were added to a buffer consisting of 10 mM Tris-HCl, pH 7.5, 1.5 mM EDTA and 5 mM sodium molybdate. Cytosol was obtained by centrifugation of the homogenate at 75 000 *g* for 1 h in a 70Ti rotor (Beckman Instruments, High Wycombe, UK) at 4 °C. Cell extracts were incubated for 2 h at 0 °C with 10 nM [³H]oestradiol (Amersham International, Amersham, Bucks, UK) in the absence or presence of 50-fold excess of unlabelled oestradiol (Sigma) as described previously (Saji *et al.* 2000), and the unbound oestradiol was removed with dextran-coated charcoal. Samples (100 μ l) were layered on 4 ml sucrose density gradients (10–30% (w/v) sucrose in 10 mM Tris-HCl, 1.5 mM EDTA, 1 mM -monothioglycerol (Sigma) and 10 mM KCl) and centrifuged for 16 h at 300 000 *g* in an SW-60Ti rotor (Beckman Instruments) at 4 °C. Successive 100 μ l fractions were collected from the

bottom by paraffin oil displacement and assayed for radioactivity by liquid scintillation counting. For Western blotting, fractions were first precipitated with trichloroacetic acid, then resuspended in methanol and incubated on dry ice for 30 min. The protein was recovered by centrifugation. Pellets were dissolved in SDS sample buffer, resolved by SDS-PAGE and subjected to Western blot analysis.

FGF7 release and transfection assays

Purified human breast fibroblasts were normally cultured in DMEM with 10% FCS, 2 mM glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin. To assess the effects of different growth factors and ligands on the production of FGF7, the purified breast fibroblasts were incubated with different cytokines and ER ligands in breast culture medium (BCM) consisting of 1:1 mix of DMEM (without phenol red) and Ham's F12 supplemented with 15 mM HEPES, 2 mM glutamine, 5 μ g/ml insulin, 10 μ g/ml apo-transferrin, 100 μ M ethanolamine, 1 μ g/ml hydrocortisone, 10 ng/ml epidermal growth factor, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 50 U/ml polymyxin B and 2.5 μ g/ml amphotericin (all from Sigma) (Gomm *et al.* 1995, 1997). The amount of FGF7 produced by the fibroblasts in the culture medium was measured using the human KGF Quantikine Kit (R&D Systems, Abingdon, UK) according to the manufacturer's protocol. The human KGF Quantikine Kit was an ELISA-based quantification assay, and the absorbance was measured at a wavelength of 450 nm within 30 min using a 550 plate reader (Bio-Rad). IL-1 β was purchased from R&D Systems. Oestradiol, tamoxifen and kaempferol (3,4',5,7-tetrahydroxyflavone) were obtained from Sigma and ICI 182,780 from Trocris Cookson Inc. (St Louis, MO, USA). The ER β -selective agonist BAG (L-851 057) (Cheng *et al.* 2004) was supplied by Merck, West Drayton, UK. All the ER ligands were prepared in ethanol and added at the concentrations indicated.

For transfection assays, an ERE-driven chloramphenicol acetyltransferase (CAT) gene reporter plasmid was co-transfected with a pSG5- β -galactosidase transfection control into the human mammary fibroblasts cultured in BCM using the calcium phosphate precipitation method (Lam *et al.* 1995). These plasmids have all been described

previously (Ali *et al.* 1993, Chen *et al.* 1999). The transfected human mammary fibroblasts were then treated as described for FGF7 release assay and harvested for CAT and β -galactosidase after 3 days. The CAT expression was quantified using a CAT ELISA kit (Roche Diagnostics GmbH) as described by the manufacturer.

Results

Immunohistochemical localisation of ER α and ER β in human breast tissues

Immunohistochemical staining of formalin-fixed paraffin-embedded sections of normal human breast tissue are shown in Fig. 1. As previously reported (Jensen *et al.* 2001), ER α was expressed primarily in the epithelium, but not in the stroma (Fig. 1A), while staining for ER β was observed in both the epithelium and stroma (Fig. 1B and C). This staining pattern was not detected with a non-specific control antibody (Fig. 1D and E) and the staining was blocked by 100-fold excess of the peptide used as antigen (Fig. 1F). The ER β antibody used in Fig. 1A–F was raised against an N-terminal peptide common to both ER β 1 and ER β cx. In order to investigate the cellular localization of ER β cx, a specific antibody raised against the unique amino acid sequence coded for by the ER β cx-specific exon was used. The result showed that as shown in Fig. 1G and H ER β cx is also present in both epithelial cells and fibroblasts of breast (Fig. 1I–L).

Expression of ER α and ER β mRNA in human breast fibroblasts

The expression of ER α and ER β mRNA was investigated by RT-PCR analysis. RNA was extracted from a panel of human breast fibroblasts purified from normal breast tissue derived from reduction mammoplasty as well as fibroblasts from women who had undergone surgery for malignant breast tumours. The panel consisted of seven normal (lines 1–7) and six malignant (lines 9–14) fibroblasts, as well as an immortalised human fibroblast cell line (line 8) which was derived from a reduction mammoplasty. For comparison, we also included the malignant breast epithelial cell lines MCF-7, MDA-MB-231 and T47D for ER α -specific RT-PCR, and MCF-7 and MDA-MB-231 for ER β RT-PCR (Fig. 2). β -actin was used as a

Figure 1 Immunostaining of normal human breast tissues for ER α and ER β expression. Representative photomicrographs of immunohistochemical staining of breast sections using DAB as substrate. All micrographs were at 200 $\times$ magnification unless indicated. (A) Normal human breast tissue was stained with anti-ER α (1D5) antibody. Positive staining was detected in epithelial cells only. (B) Normal human breast tissue was stained with an anti-ER β (06-629) antibody. Staining is observed in the ductal epithelial cells and stromal fibroblasts. (C) Normal human breast stroma was stained with the anti-ER β antibody. (D) Normal human breast tissue was stained with an unrelated antibody, demonstrating no ductal epithelial and stromal staining. (E) Normal human breast tissue was stained with the anti-ER β antibody, showing positive staining for epithelial cells and some stromal cells. (F) Normal breast tissue (as in E) was stained with the anti-ER β antibody in the presence of antigenic peptides. (G) Control normal prostate tissue was stained with the anti-ER β antibody, showing positive staining for epithelial cells only. (H) Normal prostate tissue was stained with the anti-ER β antibody in the presence of the antigenic (blocking) peptides, showing no sign of staining. (I) Normal human breast tissue was stained the anti-ER β antibody (LBD) (100 $\times$ magnification). (J) Normal human breast tissue was stained with an anti-ER β cx-specific antibody (Saji *et al.* 2002) (100 $\times$ magnification). (K) Normal human breast tissue was stained with the anti-ER β cx-specific antibody. (L) Normal human breast tissue was stained with the anti-ER β cx-specific antibody in the presence of the antigenic peptides, showing little sign of staining. Nuclear localisation is observed in all the positive staining.

Figure 2 Expression of ER α , ER β , cytokeratin 19 and β -actin mRNA in purified breast fibroblasts and breast epithelial cancer cell lines. Total RNA extracted from 14 normal and malignant breast fibroblasts, and the epithelial cancer cell lines MCF-7 and MDA-MB-231 was reverse transcribed and analysed for the expression of ER α , ER β , cytokeratin 19 and β -actin mRNA by PCR using the primers shown. The ER-positive cell line T47D was also included as an extra positive control for ER α PCR. Schematic diagrams of the exons and functional domains of ER α and ER β are shown in the upper panel.

control for cDNA synthesis, PCR efficiency and gel loading.

The ER α status of the human fibroblasts was investigated using RT-PCR with PCR primers recognising sequences within regions A/B and D of human ER α mRNA respectively. These primers produced a product of 292 bp from the cDNA derived from MCF-7 and T47D cells. This band indicated the presence of ER α mRNA (Fig. 2). The negative control was the MDA-MB-231 cell line which is known not to express ER α and with which no PCR signal was found. With cDNA from fibroblasts from either normal or malignant human breasts, no ER α -specific band was amplified (Fig.

2). With ER β -specific primers, a PCR product of the expected size, 200 bp, was produced in all the samples from both the normal and malignant human breast fibroblasts and the two breast epithelial cell lines, MCF-7 and MDA-MB-231. It is notable that a minor but higher molecular weight PCR product was also obtained using the ER β primers F and B. This represents a splice variant with an insertion into the C and D regions, which are important for DNA binding and dimerisation.

Human breast fibroblasts were prepared from an established technique in our laboratory and their morphological appearance was examined and subsequently confirmed to be that of fibroblasts and

Figure 3 Expression of ERβ variants in purified breast fibroblasts and breast epithelial cancer cell lines. The expression of ERβ variants ERβ1, ERβcx, ERβ5 and ERβins mRNA transcripts in 14 normal and malignant breast fibroblasts, and the epithelial cancer cell lines MCF-7 and MDA-MB-231 was analysed by RT-PCR using splice variant-specific primers. Schematic diagrams of the structures and functional domains of common ERβ splice variants are shown in the upper panel. β-actin cDNA was amplified in parallel. a.a., amino acids; wt, wild type.

not epithelial cells. Cytokeratin 19 was used as a marker for epithelial cells to assess the degree of contamination of fibroblasts with epithelial cells. RT-PCR analysis with cytokeratin 19 primers produced a predicted product of 500 bp from the MCF-7 and MDA-MB-231 cell lines, but not from any of the purified human breast fibroblasts, indicating that the human fibroblasts isolated were not contaminated with epithelial cells (Fig. 2).

Expression of ERβ variant mRNAs in human breast fibroblasts

The expression of ERβ splice variants in populations of purified breast fibroblasts was investigated

by PCR analysis (Fig. 3). Three categories of variants were investigated: ERβins-, a splice variant with an insert of 54 bp in the ligand-binding domain (Chu *et al.* 1997, Petersen *et al.* 1998) commonly found in rodents but not in human tissues; ERβδ5, which lacks exon 5; ERβcx, in which the C-terminal 61 amino acids are replaced by a unique 26 amino acid sequence; and ERβ5 which is a truncated receptor consisting only of amino acids 409–472. The 14 fibroblast samples were processed separately in two subgroups. The expression levels of various ERβ isoforms were judged by the relative intensity of the bands between different samples within the same subgroup.

The presence of ER β ins was examined using the primer pair LBD U and LBD L, complementary to sequences in exon 5 and exon 6 of the *ER β* gene respectively. In only one cDNA sample was a band corresponding to ER β ins amplified. The one positive signal seen in lane 7 could represent a novel splice variant arising from a rare human mutation.

For detection of ER β δ 5 variants, primers recognising sequences in exon 4 and exon 6 of the gene were used. The PCR result showed that in all the normal and malignant breast fibroblasts there was a normal ER β mRNA with an intact 5th exon. However, as revealed by the presence of the shorter 188 bp (δ 5-specific) PCR product, the normal fibroblasts in lanes 3 and 7 as well as the malignant line 12 also expressed the ER β splice variant which lacks the 5th exon. This ER β δ 5 splice form was also present in the MDA-MB-231, but not the MCF 7 and T47D epithelial cell lines. It is notable that these δ 5-specific primers also span the region of the ligand-binding domain and thus include the region where ER β ins would be detected. The presence in the normal fibroblast in lane 7 of a PCR product about 50 bp longer than expected confirmed that there was a novel mRNA in this sample.

Detection of the ER β cx and ER β 5 variants by PCR involved the use of three primers in the same reaction, namely 1U, 1L and 2L, corresponding to sequences in exon 7 and in exon 8 present only in ER β cx and ER β 5 mRNA respectively. The PCR results showed that all but two of the fibroblast cell lines possessed the ER β 1 C-terminal sequence. Only the normal fibroblast (lane 3) and the malignant (lane 9) lacked the sequence at the 3' end of ER β 1. Interestingly, all of the human breast fibroblast lines, both normal and malignant, contained the ER β cx variant, as did the epithelial cell lines. ER β 5 transcript was only expressed in two of the six fibroblast cell lines (11 and 12) derived from malignant breast tumours and in two of the eight normal fibroblast cell lines (5 and 8) derived from normal fibroblasts.

Expression of ER β but not ER α protein in human breast fibroblasts

Cell lysates were prepared from the majority of the samples and subjected to Western blot analysis for ER expression (Fig. 4). The expression of ER α and

the two major ER β variants (ER β 1 and ER β cx) in breast fibroblasts was investigated with specific antibodies. Western blots for ER α revealed that only the cell line MCF-7 expressed ER α of the expected molecular weight of 66 kDa. MDA-MB-231 (a known ER α -negative breast epithelial cell line) and all of the normal and malignant human breast fibroblasts were negative for ER α . Western blot analysis with an ER β antibody (LBD) raised against the ligand-binding domain of ER β 1 (Saji *et al.* 2000, 2002) identified a predominant ER β product of 57 kDa, corresponding to ER β 1, at significant levels in all but two of the fibroblasts. It is notable that the ER β expression levels in these two fibroblast lines 3 and 9 were extremely low when compared with the other fibroblast lines. This was consistent with our previous RT-PCR results showing that both lines 3 and 9 expressed very low levels of ER β 1. Unlike ER β 1, ER β cx appeared to be expressed in all breast fibroblasts studied, again consistent with the RT-PCR result. This result also demonstrated that the epithelial cell lines MCF-7 and MDA-MB-231 expressed both ER β 1 and ER β cx. These data confirmed that, at the protein level, human breast fibroblasts express only ER β and not ER α .

Expression of ER β with little or no oestrogen-binding activity in breast fibroblasts

We next examined the expression levels and binding capacity of ER α and ER β in the human breast fibroblasts and the MCF-7 and MDA-MB-231 cell lines using sucrose density gradient centrifugation assay with [3 H]oestradiol as ligand (Palmieri *et al.* 2002). Consistent with previous data, the ER α -containing radiolabelled 8S peak was only detected in the extract from the MCF-7 cells, and not in extracts from the human breast fibroblasts or the ER α -negative MDA-MB-231 cell line (Fig. 5). This expression pattern was confirmed by Western blotting of sucrose density gradient fractions corresponding to a sedimentation rate of 8S. Although it has been shown that ER β sediments on sucrose gradients as a 4S peak (Weihua *et al.* 2001, Palmieri *et al.* 2002), no specific 4S oestradiol-binding peak was observed for the human breast fibroblasts or the MCF-7 and MDA-MB-231 cell lines. Western blotting of the fractions corresponding to the 4S region of the gradient

Figure 4 Western blot analysis of human breast fibroblasts using different ER α and ER β antibodies. Cell lysates (100 μ g) extracted from malignant breast fibroblasts and the epithelial cancer cell lines MCF-7 and MDA-MB-231 were resolved by SDS-PAGE, transferred to nitrocellulose membranes and Western blotted for ER α , ER β 1 and ER β cx expression. Equal loading was confirmed by ponceau (Sigma) staining of the transferred nitrocellulose membrane. ER α was detected using the mouse monoclonal ER α (F-10) antibody, ER β 1 was recognised by the rabbit polyclonal ER β (LBD) antibody and ER β cx by the ER β cx-specific rabbit polyclonal antibody (Saji *et al.* 2002). The expression level of actin was used as a control for gel loading.

showed that ER β was present in these fractions. One interpretation of these results is that ER β cx which does not bind oestradiol is the dominant ER in the human breast fibroblasts as well as the MCF-7 and MDA-MB-231 cell lines. When the 4S sucrose gradient fractions were probed for ER β cx expression, it was found that the human breast fibroblasts and the MCF-7 and MDA-MB-231 cell lines all expressed the ER β cx protein.

Role of ER and oestrogen in adult human mammary fibroblasts

FGF7 is a breast stromal fibroblast-derived mitogen that stimulates mammary epithelial cell growth. Previous reports have indicated that the expression of FGF7 mRNA and protein in mammary gland is oestrogen regulated (Zhang *et al.* 1998, Pedchenko & Imagawa 2000). To obtain further information on the functional role of the ER species present in the human breast fibroblasts, the effects of various ER α and β ligands on FGF7 release from these fibroblasts were analysed by ELISA (Fig. 6.). Consistent with previous reports (Palmieri *et al.*

2003), treatment with IL-1 β induced a significant increase in the release of FGF7 (*t*-test, $P < 0.001$), indicating that these human breast fibroblasts were capable of producing FGF7. When fibroblasts in culture were treated with various physiological concentrations of oestradiol (10^{-10} , 10^{-9} , 10^{-8}), there was no significant increase in release of FGF7 into the culture medium. Similarly, the anti-oestrogens tamoxifen and ICI 182,780 had little effect on the release of FGF7. This is in agreement with the earlier sucrose gradient oestradiol-affinity assay showing that the ER β in human breast fibroblasts has little oestrogen-binding activity. Interestingly, when the breast fibroblasts were incubated with the ER β -selective ligand, BAG (Cheng *et al.* 2004), which has $100 \times$ higher affinity for ER β compared with ER α , significant levels of FGF7 release were observed (*t*-test, $P < 0.001$). Similarly, another ligand, kaempferol (Kuiper *et al.* 1998), which has been demonstrated to have high affinity for ER β , also increased FGF7 production in human breast fibroblasts (*t*-test, $P < 0.001$). We next transfected into these breast fibroblasts an ERE-responsive reporter construct to assay for ERE-dependent transcriptional activity in the

Figure 5 Total ligand-binding sucrose density gradient and Western blot analysis of human breast fibroblast, MCF-7 and MDA-MB-231 cell lysates. Human breast fibroblast, MCF-7 and MDA-MB-231 cell lysates were subjected to sucrose density gradient assay using [3 H]oestradiol as ligand. Fractions (100 μ l) were collected from the bottom of the gradients and assayed for radioactivity by liquid scintillation counting. A representative typical human breast fibroblast reactivity profile is shown (A). Parallel control gradients for human breast fibroblast, MCF-7 and MDA-MB-231 were performed in the presence of 50-fold excess unlabelled oestradiol. These profiles overlapped with those of MDA-MB-231 and human breast fibroblasts (data not shown). Fractions corresponding to sedimentation rates of 8S (fractions 6–20) and 4S (fractions 24–30) were pooled and subjected to Western blot analysis for ER α , ER β 1 (using LBD antibody) and ER β cx expression (B).

presence of oestradiol and BAG. The results showed that while oestrogen failed to significantly transactivate the ERE-dependent reporter (*t*-test, $P=0.064$), BAG could activate transcription from this construct (*t*-test, $P=0.006$). These findings

supported the notion that human breast fibroblasts express ER β but not ER α , and that ER β species present in the human breast fibroblasts have low oestradiol-binding activity and little function in oestrogen action.

Discussion

Our previous immunohistochemical studies have suggested that ER β but not ER α is expressed in human breast stroma (Jensen *et al.* 2001, Palmieri *et al.* 2002). In the present study, we further confirmed this observation with more detailed immunohistochemical staining, using additional breast tissue samples and ER β -specific antibodies. More importantly, using purified populations of adult female human breast fibroblasts, we went on to study the expression of ER α and ER β using RT-PCR, Western blot analysis and sucrose density gradient assay, and again found that ER β but not ER α was expressed in both normal and malignant human breast fibroblasts. This is the first time that purified populations of breast fibroblasts have been analysed for their ER α and ER β content and unique information is provided regarding the expression of ER β variants in human breast fibroblasts. We have previously found a predominant expression of ER β cx, a splice variant which does not bind oestrogen, and which is a dominant repressor of ER α with less effect on ER β 1 (Ogawa *et al.* 1998).

At first glance, the absence of ER α from breast stroma might seem to be in conflict with some published data (Boyd *et al.* 1996, Keeling *et al.* 2000, Koerner *et al.* 2001), but it appears that several studies showing the presence of ER α in human breast stroma were done on developing gland, fetal, newborn or young female individuals (Keeling *et al.* 2000) and this prompts us to speculate that ER α is expressed in the stroma of the developing gland and that there is a switch to ER β as the gland matures. Consistent with this explanation, Boyd *et al.* (1996) noted that the number of fibroblasts positive for ER α decreased as

the glandular tissue became more differentiated/mature, and Koerner *et al.* (2001) found that ER α -positive fibroblasts were more readily detectable in young teenage females compared with older subjects. In the rat mammary gland, administration of growth hormone has been shown to result in expression of ER α in mammary stromal cells (Feldman *et al.* 1999). Although at present there are no data on the regulation of the human stromal ER α , it is possible that stimulation by growth hormone, or another hormone expressed during periods of growth of the organism, may explain these age-related phenomena, and aberrant expression of these stochastic factors could account for some of the instances when ER α has been found in the stroma of adult human breast.

Previous studies using mouse models have shown that epithelial–stromal cell interactions are important for the normal development and function of the mammary gland. Cuhna *et al.* (1997) have found that the growth of mammary ducts in mice requires the presence of ER in the stroma. In addition, it has been shown that the effects of oestrogen on proliferation in the mammary epithelium of mice are modulated by the mammary stroma (Woodward *et al.* 1998), and a similar observation has been documented in the uterus (Cooke *et al.* 1997). A possible mechanism for the effect of oestrogen on the mammary epithelium which is mediated by the stroma has been put forward recently by Zhang *et al.* (2002). These authors found that treatment of murine fibroblasts with oestrogen caused an increase in the production of HGF, which caused proliferation of epithelial cells. In light of the present finding that ER β but not ER α is expressed in adult human breast fibroblasts, the mesenchymal–epithelial relationship as well as the possible effects of oestrogen acting indirectly on

Figure 6 Effect of various cytokines and ER ligands and agonists on the release of FGF7 and ERE-dependent transactivation activity by breast fibroblasts. (A) Purified breast fibroblasts were incubated with different concentrations of IL-1 β , oestrogen, ICI 182,780, tamoxifen, BAG and kaempferol for 3 days, and the level of FGF7 release into the culture medium assayed using ELISA. Since most compounds were dissolved in ethanol, ethanol was also included as a control. Each point represents the means $\pm$ S.E.M. with $n=6$. Further statistical analysis was performed using *t*-tests between the control and each individual treatment, and *P* values are shown above each histogram. (B) Transcription activity of an ERE-responsive reporter plasmid was analysed in human breast fibroblasts in response to oestradiol and BAG treatment. An ERE-responsive promoter CAT reporter construct was transfected into human breast fibroblasts and then analysed for CAT activity 3 days later. The CAT activity was normalised with β -galactosidase activity from a co-transfected control plasmid, and the relative activity calculated, as was the fold induction. The data shown are representative of three independent experiments, and error bars represent the S.E.M. of the data. Statistical analysis was performed using *t*-tests between the ethanol control and each individual treatment, and the *P* values are shown.

the epithelium via stromal ER also need to be re-evaluated. Moreover, the present data indicate that some of the previous studies on mouse stroma–epithelium interactions may not be applicable to the adult human breast situation.

Besides demonstrating that ER β is the sole ER isotype expressed in breast fibroblasts, our RT-PCR and Western blotting results also showed that ER β 1 and ER β cx are the predominant variants in breast fibroblasts, with ER β cx expressed in all fibroblasts examined and ER β 1 in all but two of the fibroblasts. It is interesting to note that ER β cx was expressed at a higher frequency in these human breast fibroblasts than ER β 1, suggesting that ER β cx could be the major form of ER β in breast fibroblasts. The other variants, including ER β 5 and ER β 55, were also detected in the fibroblasts, but they were present at low levels in human breast fibroblasts. Our result also identified a potential rare insertion variant present in one of the fibroblast cell lines (line 7); however, its functional significance requires further investigation. No significant difference in ER β variant expression was observed between normal and malignant fibroblasts. Although ER β ins is frequently expressed in rat mammary glands (Saji *et al.* 2001), our data showed that it is not normally detected in either normal or malignant human breast fibroblasts. It has been proposed that human ER β cx has a role similar to that suggested for ER β ins in rat mammary glands, which is to antagonise ER α activity (Saji *et al.* 2001).

Previous studies have shown that ER β cx is expressed in breast cancer (Chu *et al.* 1997, Petersen *et al.* 1998, Omoto *et al.* 2002, Palmieri *et al.* 2002). However, there have been diverse reports regarding the potential role of ER β cx. Some evidence has shown that high levels of ER β cx mRNA expression are associated with tamoxifen-resistant and more aggressive breast cancers (Leygue *et al.* 1999, Speirs *et al.* 1999, Saji *et al.* 2002). In contrast, a reduced expression of ER β cx has been reported to be associated with metastatic breast tumours and those that have a high number of tumour-positive lymph nodes (Ahr *et al.* 2001, 2002). Moreover, vascular invasion has also been found to correlate significantly with an ER β cx-negative phenotype (Saji *et al.* 2002) and, recently, ER β cx expression has been associated with favourable response to endocrine therapy and prognostic outcome (Palmieri *et al.* 2004). The

selective expression of ER β cx strongly suggests that splicing of the ER β gene is regulated in a cell-specific manner. This is not the first report in which ER β cx and/or ER β 1 are expressed in populations of cells where there is no ER α . In the endometrium, ER β cx protein and RNA were expressed in decidual glands and stroma cells in the absence of ER α (Henderson *et al.* 2003) and in Sertoli cells of the testis there was expression of ER β cx but not ER β 1 (Saunders *et al.* 2002).

In the present study, ER β cx appears to be the predominant form of ER β in fibroblasts from both normal and malignant adult mammary glands. Previous *in vitro* studies have shown that it is a very good repressor of ER α function at EREs (Ogawa *et al.* 1998) but the activity of ER β cx alone or in combination with either ER α or ER β at 'non-classical' oestrogen-responsive sites, such as AP-1 and Sp1, has not been investigated (Paech *et al.* 1997). Since ERs mediate proliferation as well as differentiation, a more thorough look at which functions of ER α are influenced by ER β cx is needed in order to define the physiological role of ER β cx. In the present study, there was no detectable ER α in breast stroma. If ER β cx has a physiological function in the stroma, that function is not to inhibit ER α activity. The oestrogen-binding assay revealed that the forms of ER β present in the 4S region of the sucrose gradient do not bind efficiently to radiolabelled oestradiol. This lack of oestrogen binding supports the PCR data that ER β cx, which has little or no oestrogen-binding activity, is more abundant than ER β 1 and indicates that ER β cx may antagonise ER β 1 activity. Consistent with this, we obtained data from transient transfection assays showing that oestrogen is inefficient in activating gene transcription from an ERE-containing reporter in the human breast fibroblasts, while the same promoter can be induced by BAG, an ER β -specific ligand. Similarly, our data also showed that BAG, but not oestrogen, can induce the production of FGF7 from these fibroblasts.

The findings by Omoto *et al.* (1998) and Ogawa *et al.* (1998) that ER α function can be quenched by ER β cx raised the question as to whether measurement of ER β cx should be done as part of the diagnosis in breast cancer since it could identify some ER α -positive cancers which are resistant to tamoxifen. The present finding that ER β cx is also expressed in breast stroma means that for such

measurements to be of value, not only the expression level but also the cellular distribution of ER β and variants would have to be considered in future diagnosis and studies of the human breast. Further studies on the regulation of ER β splice variant expression and function in the stromal compartment will help in our understanding of mammary gland development and malignant transformation, and assist in the identification of novel targets for therapeutic intervention.

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