RESEARCH

Estrogen receptor subtypes dictate the proliferative nature of the mammary gland

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Abstract

Estrogen induces proliferation of breast epithelial cells and is responsible for breast development at puberty. This tightly regulated control is lost in estrogen-receptor-positive (ER+) breast cancers, which comprise over 70% of all breast cancers. Currently, breast cancer diagnosis and treatment considers only the α isoform of ER; however, there is a second ER, ER β . Whilst ER α mediates estrogen-driven proliferation of the normal breast in puberty and breast cancers, ER β has been shown to exert an anti-proliferative effect on the normal breast. It is not known how the expression of each ER (alone or in combination) correlates with the ability of estrogen to induce proliferation in the breast. We assessed the levels of each ER in normal mouse mammary glands subdivided into proliferative and non-proliferative regions. ER α was most abundant in the proliferative regions of younger mice, with ER β expressed most abundantly in old mice. We correlated this expression profile with function by showing that the ability of estrogen to induce proliferation was reduced in older mice. To show that the ER profile associated with breast cancer risk, we assessed ER expression in parous mice which are known to have a reduced risk of developing ER α breast cancer. ER α expression was significantly decreased yet co-localization analysis revealed ER β expression increased with parity. Parous mice had less unopposed nuclear ER α expression and increased levels of ER β . These changes suggest that the nuclear expression of ERs dictates the proliferative nature of the breast and may explain the decreased breast cancer risk with parity.

Key Words

- ▶ parity
- ▶ estrogen receptor alpha
- ▶ estrogen receptor beta
- ▶ mammary gland
- ▶ breast cancer

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Introduction

Estrogen has long been linked to breast cancer stimulation and underlies the effectiveness of anti-estrogen therapies such as tamoxifen to block the growth and recurrence of hormonally responsive breast cancer. At present, anti-estrogen therapy focuses on blocking the action of ER α ,

one of two ER subtypes present within the breast, due to its overexpression in 70% of breast cancers (Masood 1992). ER α is expressed within a subset of luminal epithelial cells in the mouse mammary epithelium and mediates the proliferative effects of estrogen (Pettersson *et al.*

2000, Ström *et al.* 2004). A series of ER α -deficient mouse models have revealed that it is required for mammary ductal elongation, pregnancy-induced tertiary branching and the proliferation and maintenance of differentiating alveolar cells (Bocchinfuso *et al.* 2000, Dupont *et al.* 2000, Feng *et al.* 2007). The role of ER α in cancer development is evidenced in its overexpression in the majority of breast cancers and increased expression in the breast tissue of populations at high breast cancer risk (Lawson *et al.* 1999). Furthermore, deregulation of ER α expression in mammary epithelial cells is sufficient to increase proliferation and development of ductal carcinoma *in situ* (Frech *et al.* 2005). Likewise, loss of ER α is associated with a reduction in mammary tumorigenesis in transgenic mice (Bocchinfuso & Korach 1997, Hewitt *et al.* 2002).

ER β , the product of a second estrogen receptor gene (*ESR2*) located on a different chromosome (Gosden *et al.* 1986, Enmark *et al.* 1997), has almost complete homology to the original ER α in the DNA-binding domain (97%), and part homology to the ligand-binding domain (55%) (Li *et al.* 2004). In contrast to ER α , high expression of ER β correlates with decreased proliferative markers and longer disease-free survival of breast cancer (Roger *et al.* 2001, Omoto *et al.* 2002, Esslimani-Sahla *et al.* 2004, Myers *et al.* 2004a, Gruvberger-Saal *et al.* 2007, Sugiura *et al.* 2007, Leygue & Murphy 2013, Hieken *et al.* 2015) yet its expression is rarely considered for clinical management of disease.

ER β is expressed in high (near ubiquitous) levels throughout the epithelium of the breast, yet mice deficient in ER β appear to have a normal mammary histology (Krege *et al.* 1998). ER β appears to play a more subtle role in mammary gland development, mediating the terminal differentiation of the gland during pregnancy and lactation as well as an effect on intercellular junctions and proliferation (Forster *et al.* 2002). Over the last decade, evidence for an anti-proliferative role for ER β in numerous organs has also emerged (Risbridger *et al.* 2007, Leygue & Murphy 2013) and work by ourselves and others has demonstrated a role for ER β in negatively regulating the estrogen-induced pro-proliferative actions of ER α . For example, if ER β and ER α are co-expressed in a cancer cell, estradiol and phytoestrogens have been shown to reduce proliferation in breast and prostate cancer cell lines *in vitro* (Ström *et al.* 2004, Powell *et al.* 2012). Coupled with the aforementioned observations of high ER β expression correlating with less aggressive breast cancer cases, the ratio of ER α :ER β in clinical breast cancers specimens has also been shown to increase with increasing stage of breast cancer (Powell *et al.* 2012). However, support for

this anti-proliferative role of ER β has largely come from work in the setting of breast cancer, and there remains a limited understanding of the interaction of the ERs in the normal breast.

Transcript and protein levels of ER α and ER β have been assessed in mammary tissue of rats throughout development (Saji *et al.* 2000, 2001), showing a widespread expression of ER β throughout development except during the proliferative phase of pregnancy when ER β levels were lost. However, the protein expression work was performed at low resolution and does not allow the assessment of subcellular localization of each ER. Here, we have assessed the levels of expression of ER α and ER β in the mouse mammary gland and correlated expression and estradiol sensitivity with proliferation rate of the gland in different regions and at different ages. We show for the first time that mice at low risk of breast cancer (parous mice) have a less proliferative ER signature; low ER α and high nuclear expression of ER β . We propose from this work that the levels of both receptors within the normal breast epithelium are important in determining the proliferative nature of the gland and could be used to predict developmental stages of oncogenic vulnerability.

Materials and methods

Animals

All animal work was completed under Monash University Animal Ethics Committee approval. All mice were housed in microisolated cages and provided with water and chow *ad libitum*. 5- to 6-week-old FVB females were bred with similarly aged FVB males. Pregnant mothers carried their offspring to term and underwent full nursing (lactation for 3 weeks) before pups were removed at the normal weaning age (21 days). Mothers were then left for 10 weeks to return to a resting parous state, avoiding the transient increased triple negative breast cancer risk period that immediately follows pregnancy (Schedin 2006). Age-matched virgin FVB females were used as controls. For estrogen sensitivity studies, mice at 6 to 9 weeks of age were ovariectomized and treated with estrogen or vehicle 7 days later, a time point selected based on assays by Haslam (Haslam 1989).

Tissue collection

Each animal assessed was taken at the estrus stage of the cell cycle as determined by vaginal smear except where estrus cyclicity was not yet established. Fourth mammary glands were excised and divided into 3 portions (proximal

to the nipple, middle region and distal tips) to compare receptor expression and proliferative response between different regions of the mammary gland. We assessed these three regions separately as the distal tips have been shown to be the most proliferative (Russo & Russo 1980). Mammary gland portions were formalin fixed, paraffin embedded and sectioned prior to immunolabeling.

Antibodies

Primary antibodies against ER isoforms were mouse monoclonal anti human ER α 1:40 (clone ID5, DAKO) and chicken anti human ER β 1:50 a kind gift from Jan-Ake Gustafsson. The specificity of chicken polyclonal 503 IgY ER β antibody has been tested previously using peptide pre-absorption in rat tissue (Saji *et al.* 2000) and also in ER β knockout mice (Maneix *et al.* 2015). It recognizes mouse ERbeta and mouse ER β 18aa ins but does not pick up human ER β 2 (or ER β cx) (Ogawa *et al.* 1998). We performed our own validation using MDA-MB-231 cells (which do not express ER β) and MDA-MB-231 cells overexpressing ER β 1 (Supplementary Methods and Supplementary Fig. 1, see section on supplementary data given at the end of this article) and show that only MDA-MB-231 cells expressing ER β 1 showed positive staining. We also performed validation studies with the CWK ER β antibody (CWK-F12, DSHB) generated by B Katzenellenbogen that was validated as specific for ER β using rapid immunoprecipitation mass spectrometry of endogenous protein (RIME) (Nelson *et al.* 2017). Both the ER α and ER β primary antibodies also have demonstrated cross-reactivity to mouse tissue in previous studies (Weihua *et al.* 2001, Dall *et al.* 2017). Rat monoclonal anti BrDU antibody (clone BU1/75 (ICR1), AbD Serotec, Kidlington, UK), rabbit polyclonal anti-phosphorylated Ki67 (Leica Biosystems) and purified mouse anti-E-cadherin (clone 36/E-Cadherin, BD Biosciences, Franklin Lakes, NJ, USA) were also used. For immunohistochemistry, biotinylated secondary antibodies were used correlating to the appropriate isotype. For immunofluorescence, anti-mouse IgG1 555 fluorescently labeled secondary antibody was used for ER α , anti-chicken 488 fluorescently labeled secondary antibody was used for ER β and anti-mouse IgG2a 647 fluorescently labeled secondary antibody was used for E-Cadherin (Invitrogen).

Immunolabelling on paraffin sections

Sections were dewaxed and rehydrated through graded alcohols into water. Heat-mediated antigen retrieval was

performed in 0.01 M citrate buffer (pH 6.0) using either a pressure cooker for ER α , ER β and Ki67 or microwave for BrDU. Where appropriate endogenous peroxidase blocking by incubating sections in 3% hydrogen peroxide (Merck Millipore) in methanol. Non-specific immunoreactivity was blocked by incubation with mouse immunoglobulin blocking reagent (M.O.M. Kit, Vector Laboratories, Peterborough, UK) and CAS block (Invitrogen). Primary antibodies as detailed earlier were applied to sections overnight at 4°C. Sections were washed several times in 0.05% TBS/tween and then incubated in appropriate secondary antibodies for 30 min up to 1.5 h at room temperature. Sections were washed using 0.05% TBS/tween. For immunohistochemical labeling, sections were treated with avidin-biotin-HRP complex (ABC-HRP; DAKO A/S) prior to protein localization using diaminobenzidine (liquid DAB; Dako). Sections were then counterstained and coverslipped for imaging. The CWK-F12 ER β antibody was diluted to 1:25 and used on the Ventana Benchmark Ultra Autostainer. Briefly, tumor cell lines were deparaffinized, antigen retrieval done with Cell Conditioner 1 (CC1) for 40 min at 100°C and pre-primary peroxidase inhibitor for 5 min at room temperature. Primary antibody incubations were completed for 60 min at room temperature. Visualization on the autostainer was performed with OptiView DAB IHC Detection Kit (Ventana Medical Systems, Tucson, AZ, USA) and then counterstained with Mayer's Hematoxylin (Amber Scientific, Midvale, Australia) for 60 s. We also tried manual staining with the same CWK-F12 antibody and pressure cooking, but staining was not successful.

For immunofluorescence, sections were stained with DAPI (Roche)/PBS to label nuclei before coverslipping in Vectashield mounting media (Vector Laboratories) for imaging.

Image capture

Immunohistochemical staining was imaged and captured on an Olympus BX-51 microscope (Olympus). All immunofluorescent sections were examined at room temperature using a NIKON CI inverted confocal microscope. PMT levels for all channels were set using a positive control young virgin mammary gland section. All images, including negative controls, were then taken with these settings to allow cross comparison. Images of sections were obtained using 60 $\times$ /1.40–0.60 oil λ lenses and captured via NIS elements image acquisition software. Multicolor images were collected sequentially in three

channels and co-localization overlays were generated using ImageJ software.

Quantification of ER α expression

Tissue blocks from each portion of the mammary gland were completely sectioned and a uniform systematic random sampling scheme was employed to quantitate ER α -positive cells as described in previous literature (Bianco *et al.* 2006), where every 20th section was selected for immunostaining. Tissues from all groups were processed in the same immunohistochemical assay to eliminate inter-assay variation. Once stained, quantification of immunoreactivity was performed using a BioPrecision2 microscope stage (LUDL), 99A400 Focus drive (LUDL), MAC5000 Controller (LUDL) and ND-281 Encoder (Heidenhain) coupled to an Olympus BX-51 microscope (Olympus). Sections were examined at 10 $\times$ magnification, mapped to define tissue boundaries, and sampled at pre-determined intervals along the *x* and *y* axis using a single point grid counting frame to allow sampling of fraction of 10–40% of the selected tissue sections. Using brightfield imaging under 40 $\times$ magnification, positive cells were scored and totaled for each representative section, and then compiled for each region (proximal, middle, distal) for each animal. The results are expressed as the percentage of total cells counted. The images were captured using a PixeLink PL-623C digital camera (PixeLink) coupled to a computer.

Quantification of ER α /ER β co-localization

At least 5 images from each of $n=4$ –5 nulliparous or parous animals were assessed for the subcellular localization of ER α and ER β . DAPI and E-cadherin staining were used to distinguish nuclear and cytoplasmic regions of the cell (Supplementary Fig. 2). The total number of epithelial cells was counted for each image as were the ER α +, ER β + and ER α /ER β double positive cells. The percentages of ER expressing cells were expressed as a fraction of total cells.

Quantification of BrDU

At least 5 images from each of $n=5$ mice at 6 or 9 weeks of age treated with oil or estradiol were taken and the percentage of BrDU-positive epithelial cells as a function of the total epithelial cells was determined in each section.

Determining the proliferative response to exogenous estrogen

The responsiveness of the mammary gland to the proliferative effects of estradiol can be assessed using ovariectomized mice treated with oil or estradiol (Haslam 1989). Pubertal or 9-week-old mice were ovariectomized and treated with either vehicle (peanut oil, Sigma), or one of three doses of estradiol: low (0.1 μ g per gram body weight), medium (1 μ g/gram BW) or high (10 μ g/gram BW) 7 days post-surgery. All mice were killed after 48 h but were treated with 50 μ g/g body weight bromodeoxyuridine (BrDU, Sigma) intraperitoneal 2 h prior to collection to allow proliferation to be assessed.

Statistical analysis

Results are presented as mean $\pm$ standard error of the mean (S.E.M.). Stereological assessment of ER α expression was tested for normality using a D'Agostino-Pearson omnibus test and then a non-parametric Mann-Whitney *U* test was used to assess statistical differences (GraphPad Prism software). For co-localization analysis of ER α and ER β , a 2-way ANOVA with Tukey's multiple comparisons test was used to assess statistical differences. Differences between experimental and control groups were considered significant at $P<0.05$.

Results

The mouse mammary gland shows structural differences including duct thickness, side branching and endowment of proliferative subtending branches in the regions located distal from the nipple (Russo & Russo 1980). To explore ER α and ER β expression throughout the mammary gland, the tissue was divided into 3 equal portions (proximal, middle and distal regions) and the expression assessed according to each portion. The expression of ER α was limited to a subset of epithelial cells throughout the gland at all ages. However, at 4–6 weeks of age, the number of ER α -expressing cells was higher than that in the mature adult mammary gland (Fig. 1). The number of ER α -expressing cells in young mice was also higher in the more distal regions than that in the proximal regions (Fig. 1A (ii, iii) and B (ii, iii)).

In contrast to ER α , ER β expression was ubiquitously expressed in all epithelial cells of the mouse mammary gland, at all ages (Fig. 2). Unlike ER α expression, ER β was observed at equal levels across all regions of the ductal

Figure 1

Estrogen receptor (ER) α staining is more abundant in mammary glands from young mice. ER α immunohistochemical staining in 4-week (A), 6-week (B) and 18-week (C) old mice. Expression was assessed in various regions of the gland that were proximal (i), middle (ii) and distal (iii) to the nipple. Scale represents 20 μ m. A full-colour version of this figure is available at <https://doi.org/10.1530/JOE-17-0582>.

tree including the terminal end buds (TEBs) (Fig. 2A (iii) and B (iii)).

To assess whether the expression levels of ER α and ER β were indicative of the proliferative nature of the mammary gland, 4-, 6- and 18-week old mammary glands were stained with Ki67 as a marker of cell division. As shown in Fig. 3, Ki67 expression was largely absent in the 4-week-old mammary gland (Fig. 3B) but increased in abundance in the 6-week-old mammary gland.

By 18 weeks, the mammary gland exhibited modest Ki67 expression (Fig. 3C). Correlating with the increase in ER α expression observed in the middle and distal regions of the 6-week-old mammary gland (Fig. 1B (ii, iii)), Ki67 was also observed to be highest in these regions (Fig. 3B (ii, iii)).

To investigate if ER expression profiles correlated with the ability of estrogen to functionally induce the increase in proliferation observed in Fig. 3, we used an assay of estrogen sensitivity originally used to define when the

Figure 2

Estrogen receptor (ER) β staining is similar across all ages and regions of the mammary gland. ER β immunohistochemical staining in 4-week (A), 6-week (B) and 18-week (C) old mice. Expression was assessed in various regions of the gland that were proximal (i), middle (ii) and distal (iii) to the nipple. Scale represents 20 μ m. A full-colour version of this figure is available at <https://doi.org/10.1530/JOE-17-0582>.

Figure 3

Ki67 staining is more abundant in mammary glands from 6-week old mice. Ki67 immunohistochemical staining in 4-week (A), 6-week (B) and 18-week old mice. Expression was assessed in various regions of the gland that were proximal (i), middle (ii) and distal (iii) to the nipple. Scale represents 20 μ m. A full-colour version of this figure is available at <https://doi.org/10.1530/JOE-17-0582>.

mouse mammary gland first becomes responsive to the estrogen-induced proliferation (Haslam & Shyamala 1980, Haslam 1989). Estrogen treatment (0.1 μ g/g BW) induced proliferation (indicated by detection of BrDU) in the epithelial and stromal cells across the mammary glands of 6-week-old animals that had been ovariectomized (Fig. 4), with greatest effect in the distal tips which had the highest ER α to ER β ratio (Figs 1B (iii) and 2B (iii)).

Pubertal mice are presumed to be ultra-sensitive to estrogen whilst they undergo ductal elongation to fill the mammary fat pad. Consistent with this, the adult mammary tissue showed a less proliferative ER profile compared to the 6-week-old mammary gland (decreased ER α (Fig. 1C) and increased ER β (Fig. 2C)). To confirm that the adult mice were functionally less sensitive to estrogen by nature of their altered ER profile, we used the *in vivo* assay of estrogen sensitivity described earlier. At the same dose of estrogen used to successfully stimulate epithelial cell proliferation in 6-week-old animals (Fig. 4), whilst the stroma did show a proliferative response, the epithelial cells in 9-week-old adult mice were unresponsive. This agrees with previous work showing that the epithelial cells are less sensitive than stromal cells to an estrogen stimulus (Haslam 1989). Instead they required 2 orders of magnitude higher dose to induce epithelial cell proliferation (Fig. 5). Even at this higher dose (10 μ g/g BW), the adult mice showed a reduced response to estrogen-induced epithelial cell proliferation compared to pubertal animals (Fig. 6).

If the proliferative nature of the mammary gland aligns with breast cancer risk (increased proliferation = increased risk), we would expect the ER expression profile to reflect this. To test such a prediction, we assessed a mouse model of decreased ER+ breast cancer risk, parity (Russo *et al.* 1991, Medina & Smith 1999). Parous mice were generated by mating 6-week-old females and allowing them one cycle of pregnancy, lactation and involution before a 10-week period, allowing their mammary glands to return to pre-pregnant ductal architecture. ER α in the parous glands was decreased compared to age- and estrous-cycle-stage-matched nulliparous controls (Fig. 7). We sectioned the entire mammary gland from 5 animals and performed stereological quantification similar to the protocol we have used previously to assess follicle numbers in mouse ovaries (Myers *et al.* 2004b). Our analysis allowed at least 100 cells per section to be assessed for ER α positivity (Fig. 7A, B and C). The number of ER α + cells was significantly reduced in the middle and distal regions ($P=0.03$) (Fig. 7D).

To assess whether the subcellular locations of ER α and ER β aligned with proliferation, we performed immunofluorescence staining on parous and nulliparous mammary glands (Fig. 8). The ER α expression was again observed to be decreased in parous mammary glands compared to age-matched controls (Fig. 8B and E). ER β expression was observed to be ubiquitously expressed throughout both parous and nulliparous glands (Fig. 8C and F). Due to the ubiquitous expression of

Figure 4

High estrogen sensitivity in young (6 week old) mice. BrDU staining in the mammary glands of mice treated with either peanut oil control (A and B) or 0.1 µg/g BW 17β estradiol (C and D). Epithelial (A and C) and stromal staining (B and D) is shown within regions of the gland proximal (i), middle (ii) and distal (iii) to the nipple. Scale represents 20 µm. A full-colour version of this figure is available at <https://doi.org/10.1530/JOE-17-0582>.

ERβ in mammary epithelial cells, we performed all immunofluorescence on the same day and took all images using the same laser strength and detector settings, allowing quantification of ERα and ERβ expression in parous and age-matched nulliparous glands. As shown in Fig. 8F, the intensity of ERβ expression was higher in parous mammary glands compared to nulliparous.

When we assess co-localization of the ERs in parous and nulliparous mammary glands (Fig. 9 and Supplementary Fig. 2), ERα expression was restricted to the nucleus; however, ERβ showed either cytoplasmic or nuclear expression. In nulliparous mice, the percentage of cells expressing ERα alone was 9, 20 and 15% in proximal, middle and distal regions, respectively (Fig. 9C (i)). In accordance with our stereology findings, the parous glands had significant reductions in ERα-expressing cells in the middle and distal regions (28- and 13- fold reduction, respectively). In contrast, the percentage of cells with unopposed nuclear ERβ expression was significantly higher in parous mammary glands compared to nulliparous across all regions (increase of 3-, 2- and 4-fold for proximal, middle and distal, respectively)

(Fig. 9C (ii)). The percentage of cells with nuclear ERα and ERβ co-expression was unchanged between parous and nulliparous mammary glands (Fig. 9C (iii)). Cells with nuclear ERα and cytoplasmic ERβ were significantly reduced in parous mammary glands compared to nulliparous in the proximal region only (9-fold decrease) (Fig. 9C (iv)). The middle and distal regions also showed a trend for a decrease in the parous mammary glands but this did not reach significance. The level of cytoplasmic ERβ alone expression was variable across the gland and was not significantly altered with parity (Fig. 9C (v)). We did not observe in any of our normal mammary sections co-localization of the ERs in the same sub-nuclear location. Even when both receptors were expressed in the nucleus, ERα was restricted to the euchromatin, and ERβ was present in the heterochromatin (Fig. 9A inset).

We assessed whether the changes in ERα and ERβ due to parity lead to a decrease in proliferation in the mammary gland using Ki67. The parous animals showed significantly lower levels of Ki67 staining compared to nulliparous mammary glands, indicating low levels of proliferation induced by parity (Fig. 10).

Figure 5

Low estrogen sensitivity in the epithelial mammary cells of adult (9 week old) mice. BrDU staining in the mammary glands of mice treated with either peanut oil control (A and B) or 0.1 $\mu\text{g/g}$ BW 17 β estradiol (C and D) or 10 $\mu\text{g/g/BW}$ (E and F). Epithelial (A, C and E) and stromal staining (B, D and F) is shown within regions of the gland proximal (i), middle (ii) and distal (iii) to the nipple. Scale represents 20 μm . A full-colour version of this figure is available at <https://doi.org/10.1530/JOE-17-0582>.

Discussion

The normal breast is under the tight control of estrogen during growth and development. Both estrogen receptors, ER α and ER β , are expressed in the normal mammary gland (Saji *et al.* 2000) and normal human breast (Petersen *et al.* 1987, Clarke *et al.* 1997, Speirs *et al.* 2000, 2002, Roger *et al.* 2001, Shaw *et al.* 2002) where it is believed they work in concert to maintain control of estrogen actions. While ER α is critical for mediating estrogen-dependent proliferation during mammary development, ER β inhibits cell proliferation and promotes differentiation (Thomas & Gustafsson 2011). Here, we show that during active

ductal development, ER α expression is high and ER β is low correlating with a high proliferative index and high sensitivity to estrogen-induced proliferation. In contrast, at times of maintenance, ER α expression is low and ER β is high which is an expression profile associated with low proliferation and lowered estrogen sensitivity. The proliferative ER expression profile is further decreased (as evidenced by decreased ER α and Ki67 expression and increased ER β) in a parous mammary gland which is known to have a decreased risk of ER+ breast cancer.

Our studies found ER α expression to be most abundant in the 4-week-old mouse mammary gland, consistent with previous reports on protein and RNA levels (Saji

Figure 6 Quantitative assessment of BrDU staining. Mean percentage of BrDU+ epithelial cells in mice following oil or estradiol (E2) administration at (A) 6 weeks of age or (B) 9 weeks of age. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Data are expressed as mean $\pm$ s.e.m. according to the region of the gland assessed.

et al. 2000, Dall *et al.* 2017). ER α expression remained high in the 6-week-old mammary gland, particularly in the TEBS that predominate in young mice and are known

to be enriched for proliferating cells (Russo *et al.* 1982, Dall *et al.* 2017). By 18 weeks of age (by this stage a well-established adult mammary gland), ER α expression was reduced even in the distal tips that are derived from the TEBS. In contrast, ER β expression was highly abundant throughout all developmental stages, similar to the staining patterns observed in the normal human breast previously (Speirs *et al.* 2000, 2002, Shaw *et al.* 2002). The expression pattern of ER α and ER β we report agrees with existing work showing the normal mammary gland contains a high level of ER β - (~85%) positive epithelial cells across all ages and whilst ER α expression is high in young mammary glands, on average only 10% of cells in adult ducts were ER α positive (Petersen *et al.* 1987, Clarke *et al.* 1997, Roger *et al.* 2001, Saji *et al.* 2001).

Previous protein expression studies collectively reported a lack of co-localization between ER α and proliferative markers in normal mammary gland cells (Clarke *et al.* 1997, Russo *et al.* 1999, Shoker *et al.* 1999, Saji *et al.* 2000) which was unexpected given that estrogen was believed to be driving proliferation. Later studies showing the control of proliferation by ER α through paracrine signaling (Wiesen *et al.* 1999, Mallepell *et al.* 2006) may explain the dissociation between ER α and proliferation marker expression. Further, additional studies have reported the immediate degradation of the ER α protein following transmission of the estrogen stimulus to proliferate (Reid *et al.* 2003, Cheng *et al.* 2004, Valley *et al.* 2005). Our data are in support of this in that estradiol-induced proliferation seemed to be controlled

Figure 7

Stereological assessment reveals decreased estrogen receptor (ER) α expression in parous mammary glands. Parous or age-matched nulliparous mammary gland tissue blocks ($n = 5$) were sectioned at 4- μ m sections and every 20th section was used for stereological assessment (A). Each slide had a series of sampling grids applied to it at low power (B) that were assessed at high power (C) to determine the number of positive cells. Only cells within the square or touching green (inclusion boundaries) were included. The compiled results were expressed as percentage of the number of ER α -positive cells of all cells counted for each region of the mammary gland. Error bars represent mean $\pm$ s.e.m. *Statistical significance $P < 0.05$. A full-colour version of this figure is available at <https://doi.org/10.1530/JOE-17-0582>.

Figure 8
ER β staining intensity stronger in parous mammary glands compared to nulliparous glands. Immunofluorescence staining of DAPI (A and D), ER α (B and E) and ER β (C and F) in nulliparous (A, B and C) and parous (D, E and F) mammary glands. Expression was assessed in various regions of the gland that were proximal (i), middle (ii) and distal (iii) to the nipple. Scale represents 20 μ m. A full-colour version of this figure is available at <https://doi.org/10.1530/JOE-17-0582>.

by ER α expression. Ki67 expression was higher in 6-week-old mammary glands compared to 18-week-old mammary glands and likewise lower levels of estradiol was able to stimulate mammary epithelial cell proliferation in ovariectomized 6-week-old mice compared to 10-week-old mice. This proliferative expression profile aligned closely with the expression of ER α and not ER β . ER α is a major prognostic factor for breast cancer. It is expressed in the majority of breast cancers and also in the breast tissue of populations at high risk of breast cancer (Lawson *et al.* 1999). Deregulation of ER α expression increases proliferation and leads to the development of ductal

carcinoma *in situ* (Frech *et al.* 2005). Thus, an increased ER α expression in the developing mammary gland, whilst important for ductal tree outgrowth, may signify a time in the mammary gland that is vulnerable to the oncogenic events that accompany increased proliferation. Early work by Russo *et al.* supports such a hypothesis by reporting higher incidence rates of mammary tumors in young mice treated with a chemical carcinogen compared to their older counterparts (Russo *et al.* 1979). Similarly, women exposed to environmental carcinogens such as pesticides and radiation are at an increased risk of developing breast cancer later in life if they were young at the time

Figure 9

Parous mammary glands have a less proliferative ER profile. Representative images from nulliparous (A) and parous (B) glands stained with DAPI (blue), ERβ (green), ERα (red). Overlay is shown in the left hand upper corner. Scale represents 10 μm. (C) Quantification across the gland (proximal, middle and distal) assessing the subcellular localization of ERs including nuclear ERα alone (i), nuclear ERβ alone (ii), nuclear ERα and ERβ (iii), nuclear ERα with cytoplasmic ERβ (iv) and cytoplasmic ERβ alone (v). Data represent mean ± s.e.m. for $n=4-5$ animals. * $P<0.05$, *** $P<0.001$, **** $P<0.0001$. A full-colour version of this figure is available at <https://doi.org/10.1530/JOE-17-0582>.

of exposure compared to those exposed at older ages (McGregor *et al.* 1977, Hoffman *et al.* 1989, Boice *et al.* 1991, Land *et al.* 2003, Cohn *et al.* 2007).

Providing further support for high ERα expression, correlating with an increased proliferative signature and higher risk of breast cancer, is the significant decrease in ERα we observed in our parous mice, which are known to be at a decreased risk of developing ER+ tumors (Russo *et al.* 1991, Medina & Smith 1999). This agrees

with flow-cytometry data in mice showing a decrease in luminal Sca-1+ (luminal ER+) cells with parity (Meier-Abt *et al.* 2013) and human studies assessing the downstream target progesterone receptor (Muenst *et al.* 2017). Thus, the decreased ERα expression in parous mice may explain in part their decreased risk of cancer.

In estrogen-responsive breast cancer subtypes, ERα expression increases whilst ERβ expression decreases with increasing grade of breast cancer (Lawson *et al.* 1999,

Figure 10

Proliferation levels are decreased in parous mammary glands. Ki67 staining in nulliparous (A) and parous (B) mice. Expression was assessed in various regions of the gland that were proximal (i), middle (ii) and distal (iii) to the nipple. Scale represents 20 μm. A full-colour version of this figure is available at <https://doi.org/10.1530/JOE-17-0582>.

Roger *et al.* 2001, Powell *et al.* 2012). Within invasive breast cancers patients with ER β -positive tumors had increased disease-free survival (Sugiura *et al.* 2007), which has been shown to also be independent of ER α status (Omoto *et al.* 2001, 2002). In line with this *in vitro* studies, we have revealed that ER β overexpression in ER α + breast cancers inhibits estrogen-stimulated proliferation and tumor growth (Omoto *et al.* 2003, Paruthiyil *et al.* 2004, Ström *et al.* 2004, Behrens *et al.* 2007). We assessed the level of ER β co-expression and found that in addition to the loss of nuclear ER α -expressing cells, parous mice had a significant increase in nuclear ER β -expressing cells compared to age-matched nulliparous controls. This suggests that the lower levels of proliferation in parous individuals shown here and by others (Russo & Russo 1996) may be due to the collaborative effects of lower levels of the pro-proliferative ER α and higher levels of the anti-proliferative ER β in the gland.

The work herein shows an increase in nuclear ER β expressing cells in parous individuals which correlates with a decrease in proliferation. This finding is somewhat supported by early *in vitro* work showing that overexpression, or even expression of ER β at the same levels of ER α in breast cancer cell lines, resulted in a decrease in proliferation following estradiol treatment (Omoto *et al.* 2003, Paruthiyil *et al.* 2004, Ström *et al.* 2004, Behrens *et al.* 2007). Whilst we did observe a decrease in ER α expression and increase in ER β in the parous mammary glands, there was no difference in the percentage of cells co-expressing ER α and ER β in the nucleus. However, we have measured ER expression and co-localization in the normal setting, which is very different to the aforementioned forced co-expression studies using breast cancer cell lines. It was interesting that we did not observe direct co-localization of ER α and ER β in the same nuclear location; however, we realize that a more sensitive method such as the proximity ligation assay would be required to confirm this. This would appear to contradict theories surrounding how ER β regulates the proliferative actions of ER α , in that they state that they are required to be expressed in the same subcellular compartment (Pettersson *et al.* 2000, Saji *et al.* 2001, Powell *et al.* 2012). However, we did find proliferation (as measured by Ki67) was lower in parous mammary glands (that have low ER α alone and high nuclear ER β alone expression) compared to nulliparous mammary glands. Therefore, our work suggests that ER β may control ER α transcriptional activity without the need for direct physical interaction, but this requires further testing.

Our work shows that high ER α expression levels dictate the estrogen sensitivity and in turn proliferative nature of the mammary gland. We also show a lowered ER α and higher intensity ER β expression profile in parous mice correlating with a lower level of proliferation which may explain their reduced risk of ER+ breast cancer.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/JOE-17-0582>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References

- Behrens D, Gill JH & Fichtner I 2007 Loss of tumorigenicity of stably ERbeta-transfected MCF-7 breast cancer cells. *Molecular and Cellular Endocrinology* **274** 19–29. (<https://doi.org/10.1016/j.mce.2007.05.012>)
- Bianco JJ, McPherson SJ, Wang H, Prins GS & Risbridger GP 2006 Transient neonatal estrogen exposure to estrogen-deficient mice (aromatase knockout) reduces prostate weight and induces inflammation in late life. *American Journal of Pathology* **168** 1869–1878. (<https://doi.org/10.2353/ajpath.2006.050623>)
- Bocchinfuso WP & Korach KS 1997 Mammary gland development and tumorigenesis in estrogen receptor knockout mice. *Journal of Mammary Gland Biology and Neoplasia* **2** 323–334. (<https://doi.org/10.1023/A:1026339111278>)
- Bocchinfuso WP, Lindzey JK, Hewitt SC, Clark JA, Myers PH, Cooper R & Korach KS 2000 Induction of mammary gland development in estrogen receptor-alpha knockout mice. *Endocrinology* **141** 2982–2994. (<https://doi.org/10.1210/endo.141.8.7609>)
- Boice JD, Preston D, Davis FG & Monson RR 1991 Frequent chest X-ray fluoroscopy and breast cancer incidence among tuberculosis patients

- in Massachusetts. *Radiation Research* **125** 214–222. (<https://doi.org/10.2307/3577890>)
- Cheng G, Weihua Z, Warner M & Gustafsson J-Å 2004 Estrogen receptors ER α and ER β in proliferation in the rodent mammary gland. *PNAS* **101** 3739–3746. (<https://doi.org/10.1073/pnas.0307864100>)
- Clarke RB, Howell A, Potten CS & Anderson E 1997 Dissociation between steroid receptor expression and cell proliferation in the human breast. *Cancer Research* **57** 4987–4991.
- Cohn BA, Wolff MS, Cirillo PM & Sholtz RI 2007 DDT and breast cancer in young women: new data on the significance of age at exposure. *Environmental Health Perspectives* **115** 1406–1414. (<https://doi.org/10.1289/ehp.10260>)
- Dall GV, Vieusseux JL, Korach KS, Arao Y, Hewitt SC, Hamilton KJ, Dzierzak E, Boon WC, Simpson ER, Ramsay RG, *et al.* 2017 SCA-1 labels a subset of estrogen-responsive bipotential repopulating cells within the CD24⁺ CD49fhi mammary stem cell-enriched compartment. *Stem Cell Reports* **8** 417–431. (<https://doi.org/10.1016/j.stemcr.2016.12.022>)
- Dupont S, Krust A, Gansmuller A, Dierich A, Chambon P & Mark M 2000 Effect of single and compound knockouts of estrogen receptors alpha (ERalpha) and beta (ERbeta) on mouse reproductive phenotypes. *Development* **127** 4277–4291.
- Enmark E, Peltö-Huikko M, Grandien K, Lagercrantz S, Lagercrantz J, Fried G, Nordenskjöld M & Gustafsson JA 1997 Human estrogen receptor beta-gene structure, chromosomal localization, and expression pattern. *Journal of Clinical Endocrinology and Metabolism* **82** 4258–4265. (<https://doi.org/10.1210/jcem.82.12.4470>)
- Esslimani-Sahla M, Simony-Lafontaine J, Kramar A, Lavaill R, Mollevi C, Warner M, Gustafsson JA & Rochefort H 2004 Estrogen receptor beta (ER beta) level but not its ER beta cx variant helps to predict tamoxifen resistance in breast cancer. *Clinical Cancer Research* **10** 5769–5776. (<https://doi.org/10.1158/1078-0432.CCR-04-0389>)
- Feng Y, Manka D, Wagner KU & Khan SA 2007 Estrogen receptor-alpha expression in the mammary epithelium is required for ductal and alveolar morphogenesis in mice. *PNAS* **104** 14718–14723. (<https://doi.org/10.1073/pnas.0706933104>)
- Forster C, Makela S, Warri A, Kietz S, Becker D, Hultenby K, Warner M & Gustafsson JA 2002 Involvement of estrogen receptor beta in terminal differentiation of mammary gland epithelium. *PNAS* **99** 15578–15583. (<https://doi.org/10.1073/pnas.192561299>)
- Frech MS, Halama ED, Tilli MT, Singh B, Gunther EJ, Chodosh LA, Flaws JA & Furth PA 2005 Deregulated estrogen receptor alpha expression in mammary epithelial cells of transgenic mice results in the development of ductal carcinoma in situ. *Cancer Research* **65** 681–685.
- Gosden JR, Middleton PG & Rout D 1986 Localization of the human oestrogen receptor gene to chromosome 6q24 – q27 by in situ hybridization. *Cytogenetics and Cell Genetics* **43** 218–220. (<https://doi.org/10.1159/000132325>)
- Gruvberger-Saal SK, Bendahl PO, Saal LH, Laakso M, Hegardt C, Eden P, Peterson C, Malmstrom P, Isola J, Borg A, *et al.* 2007 Estrogen receptor beta expression is associated with tamoxifen response in ERalpha-negative breast carcinoma. *Clinical Cancer Research* **13** 1987–1994. (<https://doi.org/10.1158/1078-0432.CCR-06-1823>)
- Haslam SZ 1989 The ontogeny of mouse mammary gland responsiveness to ovarian steroid hormones. *Endocrinology* **125** 2766–2772. (<https://doi.org/10.1210/endo-125-5-2766>)
- Haslam SZ & Shyamala G 1980 Progesterone receptors in normal mammary gland: receptor modulations in relation to differentiation. *Journal of Cell Biology* **86** 730–737. (<https://doi.org/10.1083/jcb.86.3.730>)
- Hewitt SC, Bocchinfuso WP, Zhai J, Harrell C, Koonce L, Clark J, Myers P & Korach KS 2002 Lack of ductal development in the absence of functional estrogen receptor alpha delays mammary tumor formation induced by transgenic expression of ErbB2/neu. *Cancer Research* **62** 2798–2805.
- Hieken TJ, Carter JM, Hawse JR, Hoskin TL, Bois M, Frost M, Hartmann LC, Radisky DC, Visscher DW & Degnim AC 2015 ERbeta expression and breast cancer risk prediction for women with atypias. *Cancer Prevention Research* **8** 1084–1092. (<https://doi.org/10.1158/1940-6207.CAPR-15-0198>)
- Hoffman DA, Lonstein JE, Morin MM, Visscher W, Harris BSH & Boice JD 1989 Breast cancer in women with scoliosis exposed to multiple diagnostic X rays. *Journal of the National Cancer Institute* **81** 1307–1312. (<https://doi.org/10.1093/jnci/81.17.1307>)
- Krege JH, Hodgin JB, Couse JF, Enmark E, Warner M, Mahler JF, Sar M, Korach KS, Gustafsson JA & Smithies O 1998 Generation and reproductive phenotypes of mice lacking estrogen receptor beta. *PNAS* **95** 15677–15682. (<https://doi.org/10.1073/pnas.95.26.15677>)
- Land CE, Tokunaga M, Koyama K, Soda M, Preston DL, Nishimori I & Tokuoka S 2003 Incidence of female breast cancer among atomic bomb survivors, Hiroshima and Nagasaki, 1950–1990. *Radiation Research* **160** 707–717. (<https://doi.org/10.1667/RR3082>)
- Lawson JS, Field AS, Champion S, Tran D, Ishikura H & Trichopoulos D 1999 Low oestrogen receptor alpha expression in normal breast tissue underlies low breast cancer incidence in Japan. *Lancet* **354** 1787–1788. ([https://doi.org/10.1016/S0140-6736\(99\)04936-3](https://doi.org/10.1016/S0140-6736(99)04936-3))
- Leygue E & Murphy LC 2013 A bi-faceted role of estrogen receptor beta in breast cancer. *Endocrine-Related Cancer* **20** R127–R139. (<https://doi.org/10.1530/ERC-12-0389>)
- Li X, Huang J, Yi P, Bambara RA, Hilf R & Muyan M 2004 Single-chain estrogen receptors (ERs) reveal that the ER α / β heterodimer emulates functions of the ER α dimer in genomic estrogen signaling pathways. *Molecular and Cellular Biology* **24** 7681–7694. (<https://doi.org/10.1128/MCB.24.17.7681-7694.2004>)
- Mallepell S, Krust A, Chambon P & Briskin C 2006 Paracrine signaling through the epithelial estrogen receptor α is required for proliferation and morphogenesis in the mammary gland. *PNAS* **103** 2196–2201. (<https://doi.org/10.1073/pnas.0510974103>)
- Maneix L, Antonson P, Humire P, Rochel-Maia S, Castaneda J, Omoto Y, Kim HJ, Warner M & Gustafsson JA 2015 Estrogen receptor β exon 3-deleted mouse: the importance of non-ERE pathways in ER β signaling. *PNAS* **112** 5135–5140. (<https://doi.org/10.1073/pnas.1504944112>)
- Masood S 1992 Estrogen and progesterone receptors in cytology: a comprehensive review. *Diagnostic Cytopathology* **8** 475–491. (<https://doi.org/10.1002/dc.2840080508>)
- McGregor DH, Land CE, Choi K, Tokuoka S, Liu PI, Wakabayashi T & Beebe GW 1977 Breast cancer incidence among atomic bomb survivors, Hiroshima and Nagasaki, 1950–69. *Journal of the National Cancer Institute* **59** 799–811. (<https://doi.org/10.1093/jnci/59.3.799>)
- Medina D & Smith GH 1999 Chemical carcinogen-induced tumorigenesis in parous, involuted mouse mammary glands. *Journal of the National Cancer Institute* **91** 967–969. (<https://doi.org/10.1093/jnci/91.11.967>)
- Meier-Abt F, Milani E, Roloff T, Brinkhaus H, Duss S, Meyer DS, Klebba I, Balwierz PJ, van Nimwegen E & Bentes-Alj M 2013 Parity induces differentiation and reduces Wnt/Notch signaling ratio and proliferation potential of basal stem/progenitor cells isolated from mouse mammary epithelium. *Breast Cancer Research* **15** R36. (<https://doi.org/10.1186/bcr3419>)
- Muenst S, Mechera R, Daster S, Piscuoglio S, Ng CKY, Meier-Abt F, Weber WP & Soysal SD 2017 Pregnancy at early age is associated with a reduction of progesterone-responsive cells and epithelial Wnt signaling in human breast tissue. *Oncotarget* **8** 22353–22360. (<https://doi.org/10.18632/oncotarget.16023>)
- Myers E, Fleming FJ, Crotty TB, Kelly G, McDermott EW, O'Higgins NJ, Hill AD & Young LS 2004a Inverse relationship between ER-beta and SRC-1 predicts outcome in endocrine-resistant breast cancer. *British Journal of Cancer* **91** 1687–1693. (<https://doi.org/10.1038/sj.bjc.6602156>)
- Myers M, Britt KL, Wreford NG, Ebling FJ & Kerr JB 2004b Methods for quantifying follicular numbers within the mouse ovary. *Reproduction* **127** 569–580. (<https://doi.org/10.1530/rep.1.00095>)

- Nelson AW, Groen AJ, Miller JL, Warren AY, Holmes KA, Tarulli GA, Tilley WD, Katzenellenbogen BS, Hawse JR, Gnanapragasam VJ, *et al.* 2017 Comprehensive assessment of estrogen receptor beta antibodies in cancer cell line models and tissue reveals critical limitations in reagent specificity. *Molecular and Cellular Endocrinology* **440** 138–150. (<https://doi.org/10.1016/j.mce.2016.11.016>)
- Ogawa S, Inoue S, Watanabe T, Orimo A, Hosoi T, Ouchi Y & Muramatsu M 1998 Molecular cloning and characterization of human estrogen receptor betacx: a potential inhibitor of estrogen action in human. *Nucleic Acids Research* **26** 3505–3512. (<https://doi.org/10.1093/nar/26.15.3505>)
- Omoto Y, Inoue S, Ogawa S, Toyama T, Yamashita H, Muramatsu M, Kobayashi S & Iwase H 2001 Clinical value of the wild-type estrogen receptor beta expression in breast cancer. *Cancer Letters* **163** 207–212. ([https://doi.org/10.1016/S0304-3835\(00\)00680-7](https://doi.org/10.1016/S0304-3835(00)00680-7))
- Omoto Y, Kobayashi S, Inoue S, Ogawa S, Toyama T, Yamashita H, Muramatsu M, Gustafsson JA & Iwase H 2002 Evaluation of oestrogen receptor beta wild-type and variant protein expression, and relationship with clinicopathological factors in breast cancers. *European Journal of Cancer* **38** 380–386. ([https://doi.org/10.1016/S0959-8049\(01\)00383-5](https://doi.org/10.1016/S0959-8049(01)00383-5))
- Omoto Y, Eguchi H, Yamamoto-Yamaguchi Y & Hayashi S 2003 Estrogen receptor (ER) beta1 and ERbetacx/beta2 inhibit ERalpha function differently in breast cancer cell line MCF7. *Oncogene* **22** 5011–5020. (<https://doi.org/10.1038/sj.onc.1206787>)
- Paruthiyil S, Parmar H, Kerekatte V, Cunha GR, Firestone GL & Leitman DC 2004 Estrogen receptor beta inhibits human breast cancer cell proliferation and tumor formation by causing a G2 cell cycle arrest. *Cancer Research* **64** 423–428. (<https://doi.org/10.1158/0008-5472.CAN-03-2446>)
- Petersen OW, Hoyer PE & van Deurs B 1987 Frequency and distribution of estrogen receptor-positive cells in normal, nonlactating human breast tissue. *Cancer Research* **47** 5748–5751.
- Pettersson K, Delaunay F & Gustafsson JA 2000 Estrogen receptor beta acts as a dominant regulator of estrogen signaling. *Oncogene* **19** 4970–4978. (<https://doi.org/10.1038/sj.onc.1203828>)
- Powell E, Shanle E, Brinkman A, Li J, Keles S, Wisinski KB, Huang W & Xu W 2012 Identification of estrogen receptor dimer selective ligands reveals growth-inhibitory effects on cells that co-express ERalpha and ERbeta. *PLoS ONE* **7** e30993. (<https://doi.org/10.1371/journal.pone.0030993>)
- Reid G, Hübner MR, Métivier R, Brand H, Denger S, Manu D, Beaudouin J, Ellenberg J & Gannon F 2003 Cyclic, proteasome-mediated turnover of unliganded and liganded ERα on responsive promoters is an integral feature of estrogen signaling. *Molecular Cell* **11** 695–707. ([https://doi.org/10.1016/S1097-2765\(03\)00090-X](https://doi.org/10.1016/S1097-2765(03)00090-X))
- Risbridger GP, Ellem SJ & McPherson SJ 2007 Estrogen action on the prostate gland: a critical mix of endocrine and paracrine signaling. *Journal of Molecular Endocrinology* **39** 183–188. (<https://doi.org/10.1677/JME-07-0053>)
- Roger P, Sahla ME, Makela S, Gustafsson JA, Baldet P & Rochefort H 2001 Decreased expression of estrogen receptor beta protein in proliferative preinvasive mammary tumors. *Cancer Research* **61** 2537–2541.
- Russo J & Russo IH 1980 Influence of differentiation and cell kinetics on the susceptibility of the rat mammary gland to carcinogenesis. *Cancer Research* **40** 2677–2687.
- Russo IH & Russo J 1996 Mammary gland neoplasia in long-term rodent studies. *Environmental Health Perspectives* **104** 938–967. (<https://doi.org/10.1289/ehp.96104938>)
- Russo J, Wilgus G & Russo IH 1979 Susceptibility of the mammary gland to carcinogenesis: I differentiation of the mammary gland as determinant of tumor incidence and type of lesion. *American Journal of Pathology* **96** 721–736.
- Russo J, Tay LK & Russo IH 1982 Differentiation of the mammary gland and susceptibility to carcinogenesis. *Breast Cancer Research and Treatment* **2** 5–73.
- Russo IH, Koszalka M & Russo J 1991 Comparative study of the influence of pregnancy and hormonal treatment on mammary carcinogenesis. *British Journal of Cancer* **64** 481–484. (<https://doi.org/10.1038/bjc.1991.335>)
- Russo J, Ao X, Grill C & Russo IH 1999 Pattern of distribution of cells positive for estrogen receptor alpha and progesterone receptor in relation to proliferating cells in the mammary gland. *Breast Cancer Research and Treatment* **53** 217–227. (<https://doi.org/10.1023/A:1006186719322>)
- Saji S, Jensen EV, Nilsson S, Rylander T, Warner M & Gustafsson J-Å 2000 Estrogen receptors α and β in the rodent mammary gland. *PNAS* **97** 337–342. (<https://doi.org/10.1073/pnas.97.1.337>)
- Saji S, Sakaguchi H, Andersson S, Warner M & Gustafsson J-AK 2001 Quantitative analysis of estrogen receptor proteins in rat mammary gland. *Endocrinology* **142** 3177–3186. (<https://doi.org/10.1210/endo.142.7.8260>)
- Schedin P 2006 Pregnancy-associated breast cancer and metastasis. *Nature Reviews Cancer* **6** 281–291. (<https://doi.org/10.1038/nrc1839>)
- Shaw JA, Udokang K, Mosquera JM, Chauhan H, Jones JL & Walker RA 2002 Oestrogen receptors alpha and beta differ in normal human breast and breast carcinomas. *Journal of Pathology* **198** 450–457. (<https://doi.org/10.1002/path.1230>)
- Shoker BS, Jarvis C, Clarke RB, Anderson E, Hewlett J, Davies MPA, Sibson DR & Sloane JP 1999 Estrogen receptor-positive proliferating cells in the normal and precancerous breast. *American Journal of Pathology* **155** 1811–1815. ([https://doi.org/10.1016/S0002-9440\(10\)65498-3](https://doi.org/10.1016/S0002-9440(10)65498-3))
- Speirs V, Adams IP, Walton DS & Atkin SL 2000 Identification of wild-type and exon 5 deletion variants of estrogen receptor β in normal human mammary gland. *Journal of Clinical Endocrinology and Metabolism* **85** 1601–1605. (<https://doi.org/10.1210/jcem.85.4.6493>)
- Speirs V, Skliris GP, Burdall SE & Carder PJ 2002 Distinct expression patterns of ERα and ERβ in normal human mammary gland. *Journal of Clinical Pathology* **55** 371–374. (<https://doi.org/10.1136/jcp.55.5.371>)
- Ström A, Hartman J, Foster JS, Kietz S, Wimalasena J & Gustafsson J-Å 2004 Estrogen receptor β inhibits 17β-estradiol-stimulated proliferation of the breast cancer cell line T47D. *PNAS* **101** 1566–1571. (<https://doi.org/10.1073/pnas.0308319100>)
- Sugiura H, Toyama T, Hara Y, Zhang Z, Kobayashi S, Fujii Y, Iwase H & Yamashita H 2007 Expression of estrogen receptor β wild-type and its variant ERβcx/β2 is correlated with better prognosis in breast cancer. *Japanese Journal of Clinical Oncology* **37** 820–828. (<https://doi.org/10.1093/jjco/hym114>)
- Thomas C & Gustafsson JA 2011 The different roles of ER subtypes in cancer biology and therapy. *Nature Reviews Cancer* **11** 597–608. (<https://doi.org/10.1038/nrc3093>)
- Valley CC, Métivier R, Solodin NM, Fowler AM, Mashek MT, Hill L & Alarid ET 2005 Differential regulation of estrogen-inducible proteolysis and transcription by the estrogen receptor α N terminus. *Molecular and Cellular Biology* **25** 5417–5428. (<https://doi.org/10.1128/MCB.25.13.5417-5428.2005>)
- Weihsua Z, Mäkelä S, Andersson LC, Salmi S, Saji S, Webster JI, Jensen EV, Nilsson S, Warner M & Gustafsson J-A 2001 A role for estrogen receptor β in the regulation of growth of the ventral prostate. *PNAS* **98** 6330–6335. (<https://doi.org/10.1073/pnas.111150898>)
- Wiesen JF, Young P, Werb Z & Cunha GR 1999 Signaling through the stromal epidermal growth factor receptor is necessary for mammary ductal development. *Development* **126** 335–344.

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