Published in final edited form as:
Oncogene. 2008 January 31; 27(6): 794–802. doi:10.1038/sj.onc.1210674.

Activation of estrogen signaling pathways collaborates with loss of Brca1 to promote development of ERα-negative and ERα-positive mammary preneoplasia and cancer

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Abstract

BRCA1 can regulate estrogen receptor-α (ERα) activity. This study tested the hypotheses that Brca1 loss in mammary epithelium alters the estrogenic growth response and that exposure to increased estrogen or ERα collaborates with Brca1 deficiency to accelerate preneoplasia and cancer development. Longer ductal extension was found in mammary glands of $Brca1^{f/f;MMTV\text{-}Cre}$ mice during puberty as compared to wild-type mice. Terminal end bud differentiation was impaired in Brca1 mutant mice with preservation of prolactin-induced alveolar differentiation. Exogenous estrogen stimulated an abnormal sustained increase in mammary epithelial cell proliferation and the appearance of ERα-negative preneoplasia in postpubertal Brca1 mutant mice. Carcinogenesis was investigated using $Brca1^{f/f;MMTV\text{-}Cre}$ mice hemizygous for p53. Exogenous estrogen increased the percentage of mice with multiple hyperplastic alveolar nodules. Targeted conditional ERα overexpression in mammary epithelial cells of mice that were Brca1 mutant and hemizygous for p53 increased the percentage of mice exhibiting multiple hyperplastic nodules, invasive mammary cancers and cancer multiplicity. Significantly more than half of the preneoplasia and cancers were ERα negative even as their initiation was promoted by ERα overexpression.

Keywords

breast cancer; mouse model; brca1; estrogen; ERα

Introduction

Women carrying germ-line mutations of the breast cancer susceptibility gene (*BRCA1*) are predisposed to breast and ovarian cancers (Miki *et al.*, 1994). While most BRCA1 mutation-

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc).

related breast cancers are estrogen receptor-α (ERα) and progesterone receptor (PR) negative (Foulkes *et al*., 2004), there is a role for estrogen in early stages of cancer progression. Ovariectomy decreases breast cancer in women (Rebbeck *et al*., 2002) and mice (Bachelier *et al*., 2005). BRCA1 represses ERα-mediated gene transcription (Fan *et al*., 2002), inhibits estrogen signaling to extracellular signal-regulated protein kinase and reduces cell proliferation (Razandi *et al*., 2004). Tamoxifen-mediated ERα agonist activity promotes cancer development in Brca1-deficient mice (Jones *et al*., 2005).

Mammary gland development is synchronized by hormones and growth factors (Fendrick *et al*., 1998). Estrogen signaling through ERα promotes cellular proliferation and differentiation and stimulates terminal end bud (TEB) formation and ductal development (Bocchinfuso and Korach, 1997). TEBs are the growing ends of mammary ducts that appear during puberty and differentiate into terminal ductal ends when pubertal mammary gland development is completed. Cell proliferation is transient in mammary epithelial cells with downregulation by negatively regulating factors including the transforming growth factor-β (TGF-β) family (Grimm and Rosen, 2006). Placental lactogen and prolactin govern alveolar differentiation during pregnancy (Dembinski and Shiu, 1987).

Like humans, Brca1 mutant mammary cancers that develop in mice are predominantly ERα negative (Xu *et al*., 1999; Brodie *et al*., 2001; Bachelier *et al*., 2005). Human BRCA-1 mutant breast cancers frequently exhibit p53 mutation (Gudmundsdottir and Ashworth, 2006), and p53 haploinsufficiency increases cancer incidence in mouse models. Brca1 loss in mice produces an exaggerated proliferative response to exogenous progesterone (Ma *et al*., 2006) and mifepristone is cancer protective (Poole *et al*., 2006). Finally, Brca1-deficient mammary glands demonstrate increased ANG1 (Furuta *et al*., 2006), Igf-1, Irs-1 and Igf-1r (Shukla *et al*., 2006), indicating that multiple pathways implicated in breast carcinogenesis are altered by Brca1 loss.

This study tested the hypotheses that Brca1 loss in mammary epithelium alters the estrogenic growth response and that increased estrogen signaling collaborates with Brca1 deficiency to accelerate preneoplasia and cancer development. Two experimental approaches were employed: exogenous estrogen exposure and a targeted conditional increase in ERα expression in mammary epithelial cells (Frech *et al*., 2005a, b). Results supported the hypothesis that estrogen signaling plays a role in early stages of Brca1 mutation-related breast cancer development.

Results

Mammary glands of Brca1$^{f/f;MMTV\text{-}Cre}$ mice exhibit longer ductal extension during puberty and impaired terminal end bud differentiation

To test if Brca1 loss leads to an abnormal growth response to endogenous estrogen during puberty, ductal extension was analysed on mammary gland whole mounts from 3- to 6-week-old Brca1$^{f/f;MMTV\text{-}Cre}$ and wild-type mice. At 3 weeks of age, ductal extension was comparable in Brca1-deficientand wild-type mice (Figures 1a and b); however at age 6 weeks ductal extension was significantly longer in Brca1$^{f/f;MMTV\text{-}Cre}$ as compared to wild-type mice (39.8±3.6 vs 17.3±2.9, *t*-test, $P < 0.006$; Figures 1e–g). To test if Brca1 loss compromised the differentiation response of TEBs to estrogen and progesterone, 3-week-old Brca1$^{f/f;MMTV\text{-}Cre}$ and wild-type mice were exposed to continuous 17β-estradiol and progesterone for 2 weeks. Wild-type mice exhibited the expected TEB differentiation into terminal ductal ends (Figure 1d) while TEBs from Brca1$^{f/f;MMTV\text{-}Cre}$ mice did not (Figure 1c). In contrast, both Brca1$^{f/f;MMTV\text{-}Cre}$ and wild-type mice exhibited normal alveolar differentiation (Figures 2a–d) when exposed to prolactin in organ culture. Because increased p27 expression is found in transgenic mice expressing dominant-negative Brca1 (Deans *et*

al., 2004), p27, p21 and estrogen signaling-associated gene products c-myc, cyclin d1 and cyclin e were examined but no significant differences were found with Brca1 loss (Figure 3). In summary, Brca1 loss impaired estrogen-and progesterone-induced differentiation but not the different developmental stage of prolactin-induced differentiation.

Exogenous estrogen stimulated a sustained increase in mammary epithelial cell proliferation and abnormal growth in postpubertal Brca1$^{f/f;MMTV\text{-}Cre}$ mice

To test if Brca1 loss altered estrogen response, postpubertal Brca1$^{f/f;MMTV\text{-}Cre}$ and wild-type mice were implanted with a 17β-estradiol or placebo pellet and mammary glands were analysed 8 weeks later. A sustained increase in cellular proliferation was found in Brca1$^{f/f;MMTV\text{-}Cre}$ mice (1.2±0.4 vs 11.2±1.8, placebo vs 17β-estradiol-treated, Mann–Whitney, P <0.0001; Figures 4a and b) unlike wild-type mice (2.9±1.7 vs 1.6±0.3, placebo vs 17β-estradiol-treated, Mann–Whitney, P = 0.96; Figures 4c and d). Brca1$^{f/f;MMTV\text{-}Cre/p53+/-}$ mice showed the same response as Brca1$^{f/f;MMTV\text{-}Cre}$ mice (data not shown). Whole mount analyses demonstrated dense mammary epithelial cell growth in estrogen- treated intact and ovariectomized Brca1$^{f/f;MMTV\text{-}Cre}$ (Figures 5a, b, e and f) but not in wild-type mice (Figures 5c, d, g and h). Progesterone may contribute to the more uniform growth pattern in intact (Figure 5b) as compared to ovariectomized mice (Figure 5f). Percentages of nuclear-localized ERα were not different between placebo-treated Brca1$^{f/f;MMTV\text{-}Cre}$ and wild-type mice (5.7±0.9 vs 3.5±0.6, Mann–Whitney, P <0.17; Figures 4e and g). ERα was downregulated in Brca1$^{f/f;MMTV\text{-}Cre}$ and wild-type mice following estrogen treatment (Figures 4f and h). No significant differences in the cell percentages demonstrating nuclear-localized ERβ or PR expression were found (Figures 4e–h). The proliferative response did not alter epithelial cell organization (Figure 6). Steady-state RNA expression levels of Tgfβ1, Tgfβ2, Tgfβr-1, Tgfβr-2 (analysis of variance (ANOVA), P <0.0001), Tgfβ3 (ANOVA, P <0.02) and Igfbp-4 (ANOVA, P <0.04) were lower in mammary glands from Brca1$^{f/f;MMTV\text{-}Cre}$ compared to wild-type mice (Supplementary data). Brca1 loss did not alter expression levels of Vegfa, Flt4, Kdr, Figf, Kit, Tgfα, C-met, Hgf, Fgf7 and Fgfr2.

p53 deficiency cooperates with estrogen stimulation in the development of hyperplastic alveolar nodules (HANs)

p53 haploinsufficiency increases the percentage of Brca1$^{f/f;MMTV\text{-}Cre}$ mice that develop invasive mammary adenocarcinomas (Xu *et al*., 1999). HANs are noninvasive preneoplastic lesions identified in mammary gland whole mounts that reflect early events in cancer development(Heppner *et al*., 2000). Estrogen exposure increased the mean number of HANs/gland and the percentage of mice with HANs similar to the effects of p53 haploinsufficiency. p53 haploinsufficiency increased the mean number of HANs from 0.3±0.1 in Brca1$^{f/f;MMTV\text{-}Cre}$ mice to 2.0±0.5 in Brca1$^{f/f;MMTV\text{-}Cre/p53+/-}$ mice (*t*-test, P <0.013) and percentage of mice with HANs from 19 to 45% (χ^2, P <0.011). Estrogen exposure increased the mean number of HANs in Brca1$^{f/f;MMTV\text{-}Cre}$ mice to 2.0±0.8 (*t*-test, P <0.004) and percentage of mice with HANs to 86% (χ^2, P <0.003). The combination of p53 haploinsufficiency and estrogen exposure increased mean number of HANs to 13.0±2.6 (*t*-test, P <0.0001) and the prevalence to 92% (χ^2, P <0.002) as compared to untreated Brca1$^{f/f;MMTV\text{-}Cre/p53+/-}$ mice. Study duration was limited by development of ureteral obstruction (Arbeit *et al*., 1996) 1–5 months after pellet placement in 71% of the Brca1$^{f/f;MMTV\text{-}Cre}$ and 1–3 months after pellet placement in 25% of the Brca1$^{f/f;MMTV\text{-}Cre/p53+/-}$ mice. Fourteen percent of estrogen-exposed Brca1$^{f/f;MMTV\text{-}Cre}$ mice and 25% of the Brca1$^{f/f;MMTV\text{-}Cre/p53+/-}$ mice developed invasive mammary cancer similar to percentages found in untreated mice (Xu *et al*., 1999; Jones *et al*., 2005).

Targeted ERα overexpression in mammary epithelial cells of Brca1$^{f/f;MMTV\text{-}Cre/p53+/-CERM}$ mice increased the percentage of mice exhibiting multiple hyperplastic nodules and invasive multiple mammary cancers

To increase estrogen signaling pathway activity specifically in mammary epithelial cells and avoid toxicity from systemic estrogen exposure, the conditional estrogen receptor α in mammory tissue (CERM) system (Frech *et al*., 2005a, b) was introduced into *Brca1*$^{f/f;MMTV\text{-}Cre}$ mice to generate *Brca1*$^{f/f;MMTV\text{-}Cre/CERM}$ and *Brca1*$^{f/f;MMTV\text{-}Cre/p53+/-/CERM}$ mice. *Brca1*$^{f/f;MMTV\text{-}Cre/CERM}$ mice demonstrated a modest nonsignificant increase in the mean number of HANs/gland (0.6±0.4) and HAN prevalence (40%) as compared to *Brca1*$^{f/f;MMTV\text{-}Cre}$ mice. ERα overexpression in *Brca1*$^{f/f;MMTV\text{-}Cre/p53+/-/CERM}$ mice increased mean number of HANs/gland (14.3±3.7, *t*-test, P <0.0001) and HAN prevalence (100%, χ^2, P <0.023) compared to *Brca1*$^{f/f;MMTV\text{-}Cre/p53+/-}$ mice. Levels were comparable to estrogen-treated *Brca1*$^{f/f;MMTV\text{-}Cre/p53+/-}$ mice. The percentage of *Brca1*$^{f/f;MMTV\text{-}Cre/p53+/-/CERM}$ mice developing invasive cancer was increased (100%, χ^2, P <0.023) as compared to control *Brca1*$^{f/f;MMTV\text{-}Cre/p53+/-}$ mice (53%) and mean number of palpable invasive cancers per mouse was increased to 2.6±0.4 in mice from 0.6±0.6 in (*t*-test, P <0.0001). The percentage of mice with multiple invasive cancers was higher in *Brca1*$^{f/f;MMTV\text{-}Cre/p53+/-/CERM}$ mice (86%) as compared to *Brca1*$^{f/f;MMTV\text{-}Cre/p53+/-}$ mice (10%). In summary increasing the activity of the estrogen signaling pathway through upregulation of ERα expression promoted the development of both HANs and invasive mammary cancer in the setting of p53 haploinsufficiency.

ERα-negative preneoplasia and cancers developed in both Brca1$^{f/f;MMTV\text{-}Cre/p53+/-}$ and Brca1$^{f/f;MMTV\text{-}Cre\ p53+/-/CERM}$ mice

ERα-negative preneoplasia and cancers developed in Brca1$^{f/f;MMTV\text{-}Cre/p53+/-}$ (Figures 7q and r) and Brca1$^{f/f;MMTV\text{-}Cre/p53+/-/CERM}$ mice (Figures 7f and n). The fact that increased ERα expression facilitated the appearance of HANs (Figure 7a, inset), preneoplasia (Figures 7a and e) and invasive cancers (Figures 7i and m) raised the possibility that preneoplasia and cancers would be ERα positive. ERα immunohistochemistry was performed on both preneoplasia (Figures 7b and f) and invasive cancers (Figures 7j, n and r) fromBrca1$^{f/f;MMTV\text{-}Cre/p53+/-}$ and Brca1$^{f/f;MMTV\text{-}Cre/p53+/-/CERM}$ mice. Preneoplasia and cancers from Brca1$^{f/f;MMTV\text{-}Cre/p53+/-}$ mice were ERα negative (Figures 7r). In contrast, some (Figures 7b and j), but not all (Figures 7f and n), preneoplasia and invasive cancers from Brca1$^{f/f;MMTV\text{-}Cre/p53+/-/CERM}$ mice demonstrated nuclear-localized ERα. Expression of the ERα target gene PR followed the ERα staining pattern (Figures 7d, h, l, p and t). Cre expression was found in Brca1$^{f/f;MMTV\text{-}Cre/p53+/-/CERM}$ (Figures 7c, g, k and o) and Brca1$^{f/f;MMTV\text{-}Cre/p53+/-}$ (Figure 7s) mice with parallel expression patterns of ERα and Cre in ERα-positive Brca1$^{f/f;MMTV\text{-}Cre/p53+/-/CERM}$ mice (Figures 7c and k), suggesting that some cells simultaneously lost full-length Brca1 and expressed ERα. However, even when ERα overexpression promoted development of mammary cancer, ERα-negative preneoplasia and cancer still emerged.

Discussion

Loss of full-length Brca1 in mammary epithelial cells in mice was associated with an abnormal response to estrogen stimulation that correlated with enhanced development of preneoplasia and invasive mammary cancer. Impaired differentiation responses during puberty and after exogenous estrogen and progesterone exposure were identified. Reduction of BRCA1 by RNA interference in a 3D culture system also results in failure of differentiation into mammary acini and increased cell proliferation (Furuta *et al*., 2005). This impaired differentiation response could be one reason why pregnancy does not appear

to be as protective against the development of breast cancer in women who carry a *BRCA*1 mutation as other populations (Cullinane *et al*., 2005). Anomalous but not absent grow th and differentiation responses are consistent with the fact that either loss of full-length Brca1 or *BRCA*1 mutation does not abort mammary gland development; instead it produces a gland that functions normally in many ways but is predisposed toward cancer development.

Estrogen stimulated sustained proliferation of ERα-negative mammary epithelial cells when Brca1 function was impaired. Estrogen-induced cell proliferation is normally mediated through paracrine signaling pathways including epidermal growth factor (EGF)- and insulin growth factor (IGF)-associated pathways (Fendrick *et al*., 1998; Silberstein, 2001). BRCA1 can inhibit EGF and IGF signaling (Razandi *et al*., 2004) and loss of Brca1 in mouse mammary glands is associated with increased expression levels of Igf-1, Irs-1 and Igf-1r (Shukla *et al*., 2006). This study showed that loss of Brca1 was associated with increased expression levels of Igfbp4 and decreased expression levels of both receptors and ligands from the inhibitory TGF-β signaling pathway. This suggests a model in which loss of Brca1 leads to increased activity of IGF signaling pathways and decreased activity of the TGF-β pathways resulting in an abnormal and sustained proliferative response to estrogen. The target cell for this proliferative response was not identified; however, it is known that over half of the adenocarcinomas that develop in this model demonstrate gene expression patterns reminiscent of human basal cell cancers, a type of human cancer that is overrepresented in BRCA1 mutation carriers (Herschkowitz *et al*., 2007).

Retention of retinoid X receptor-α (RXRα) expression in the proliferating cells suggests that RXR ligands, perhaps in combination with PPARγ, could be a chemopreventive approach for Brca1 mutation-related cancer through differentiation and/or reduced IGF levels (Decensi *et al*., 2003).

Expression of PR is regulated by the estrogen signaling pathway in mammary tissue and mediates some estrogen-induced actions (Fendrick *et al*., 1998). The PR inhibitor mifepristone is cancer protective in a different but related p53 null mouse model of *Brca1* mutation-related breast cancer (Poole *et al*., 2006). Mifepristone increases TGF-β1 expression, decreases cell proliferation and promotes apoptosis in ERα- and PR-positive and -negative breast cancer cell lines (El Etreby *et al*., 1998; Liang *et al*., 2003). In tumor explants, progesterone increases IGF availability (Krzysiek *et al*., 2003) and mifepristone reduces this by stimulating secretion of IGFBP-3 (Milewicz *et al*., 2005). It is possible that mifepristone may reduce cancer development by upregulation of inhibitory factors such as TGF-β1 and/or downregulation of stimulatory pathways including IGF. Progesterone and IGF increase ductal elongation in the mammary gland (Ruan *et al*., 2005) and progesterone and the PR-B isoform has been shown to increase insulin receptor substrate-2 expression (Vassen *et al*., 1999; Cui *et al*., 2003). One can speculate that the enhanced ductal elongation observed in the Brca1-deficient mice was secondary to increased PR and IGF signaling in the pubertal mice.

Estrogen pathway stimulation was as effective as p53 haploinsufficiency in promoting the development of preneoplasia in the $Brca1^{f/f;MMTV\text{-}Cre}$ mice butt he combination of estrogen pathway activation with p53 haploinsufficiency in $Brca1^{f/f;MMTV\text{-}Cre/p53+/-}$ and $Brca1^{f/f;MMTV\text{-}Cre/p53+/-/CERM}$ mice was more potent than either intervention alone. Increasing ERα expression levels in the $BRCA1^{f/f;MMTV\text{-}Cre/p53+/-/CERM}$ mice promoted development of both ERα-positive and -negative invasive mammary cancer. This stands in contrast to another CERM-related model in which TAg and ERα overexpression are combined to produce uniformly ERα-positive mammary cancers (Tilli *et al*., 2003). The results in the Brca1 mouse model are compatible with data from human *BRCA1* mutation

carriers where approximately two-thirds of the breast cancers are ERα negative (Foulkes *et al*., 2004).

The study suggests a model in which loss of Brca1 function results in an aberrant proliferative response to estrogen signaling leading to development of preneoplasia and cancer. In this model, p53 insufficiency is a collaborating factor. It is possible that decreased expression levels of inhibitory factors such as TGF-β family members and increased expression of stimulatory factors including the IGF signaling pathway contribute to the abnormal estrogen-induced growth response.

Materials and methods

Mice

Female C57Bl/6 *Brca1*$^{f/f;MMTV\text{-}Cre}$, *Brca1*$^{f/f;MMTV\text{-}Cre/p53+/-}$, *Brca1*$^{f/f;MMTV\text{-}Cre/CERM}$, *Brca1*$^{f/f;MMTV\text{-}Cre/p53+/-/CERM}$ and wild-type mice were identified using PCR. Mice were maintained under guidelines approved by the Georgetown University Animal Care and Use Committee. MMTV-Cre transgene expression initiates during embryogenesis (Wagner *et al*., 2001). Subcutaneous pellet placement and ovariectomy (Raafat *et al*., 1999) was performed under isofluorane anesthesia. Mammary gland organ culture was carried out as described (Ginsburg and Vonderhaar, 2000). Mice were scored as having multiple invasive cancers when more than one palpable cancer was present. Supplementary data provides experimental groups and numbers of mice.

Mammary gland whole mounts, histology and immunohistochemistry

Mounted #4 mammary glands of 3- to 6-week-old mice were measured for ductal extension. In older mice, HANs were counted and prevalence calculated as the percentage of mice/group exhibiting one or more HANs/#4 mammary gland. The other #4 mammary gland from each mouse was fixed in 10% buffered formalin, embedded in paraffin using standard techniques and 5 μm sections stained using hematoxylin and eosin (H&E) or immunohistochemical detection performed: proliferating cell nuclear antigen (PCNA) (1:100; U7032; DAKO, Carpinteria, CA, USA), ERα (1:50; IM2133; Beckman Coulter Immunotech, Miami, FL, USA), ERβ (1:100; GTX70174; GeneTex Inc., San Antonio, TX, USA), PR (1:100; NCL-L-PG-AB; Novacastra, UK), RXRα (1:100; sc-553; Santa Cruz, Santa Cruz, CA, USA), smooth-muscle actin (SMA, 1:100; 1A4, MS-113-P; NeoMarkers, Fremont, CA, USA), c-Myc (1:50; sc-764; Santa Cruz), P21 (1:50; ms-387; NeoMarkers), P27 (1:50; sc-1641; Santa Cruz), cyclin D1 (1:50; sc-8396; Santa Cruz) and cyclin E (1:300; sc-481; Santa Cruz) Cre (1:400; 69050; Novagen, San Diego, CA, USA). Proliferative index and percent ERα-, ERβ- and PR-positive cells were calculated. Digital photographs were taken using a Nikon Eclipse E800M microscope with DMX1200 camera (Nikon Instruments Inc., Melville, NY, USA).

Statistical analyses

Means and s.e. were analysed using Student's *t*-test and two-way ANOVA tests (Graphpad Software, San Diego, CA, USA). Nonparametric data were analysed using Mann–Whitney two-tailed and χ^2-tests (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was supported by NIH NCI MAO/RFP NO1-CN-05024 (PAF and LPJ), RO1CA08000 (EMR, PAF and KT), 2T32CA09686-08 (LPJ) and the Susan G Komen Breast Cancer Foundation PDF040244 (LPJ and SA) and PDF0503642 (MTT).

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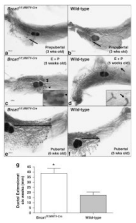

Figure 1.

Mammary glands of Brca1$^{f/f;MMTV\text{-}Cre}$ mice exhibit longer ductal extension during puberty and impaired TEB differentiation. Mammary gland whole mounts of 3-week (**a** and **b**), 17β-estradiol (E) + progesterone (P)-treated 5-week (**c** and **d**) and 6-week-old (**e** and **f**) Brca1$^{f/f;MMTV\text{-}Cre}$ and wild-type female mice. Long thin arrows indicate extent of ductal elongation (**a** and **b**). Black arrowheads indicate retained TEBs (**c** and inset). Thin white arrows indicate differentiated terminal ductal ends (**d** and inset). Bar graph comparing ductal elongation (**g**). Magnification, × 0.5; scale bar, 1000 μm.

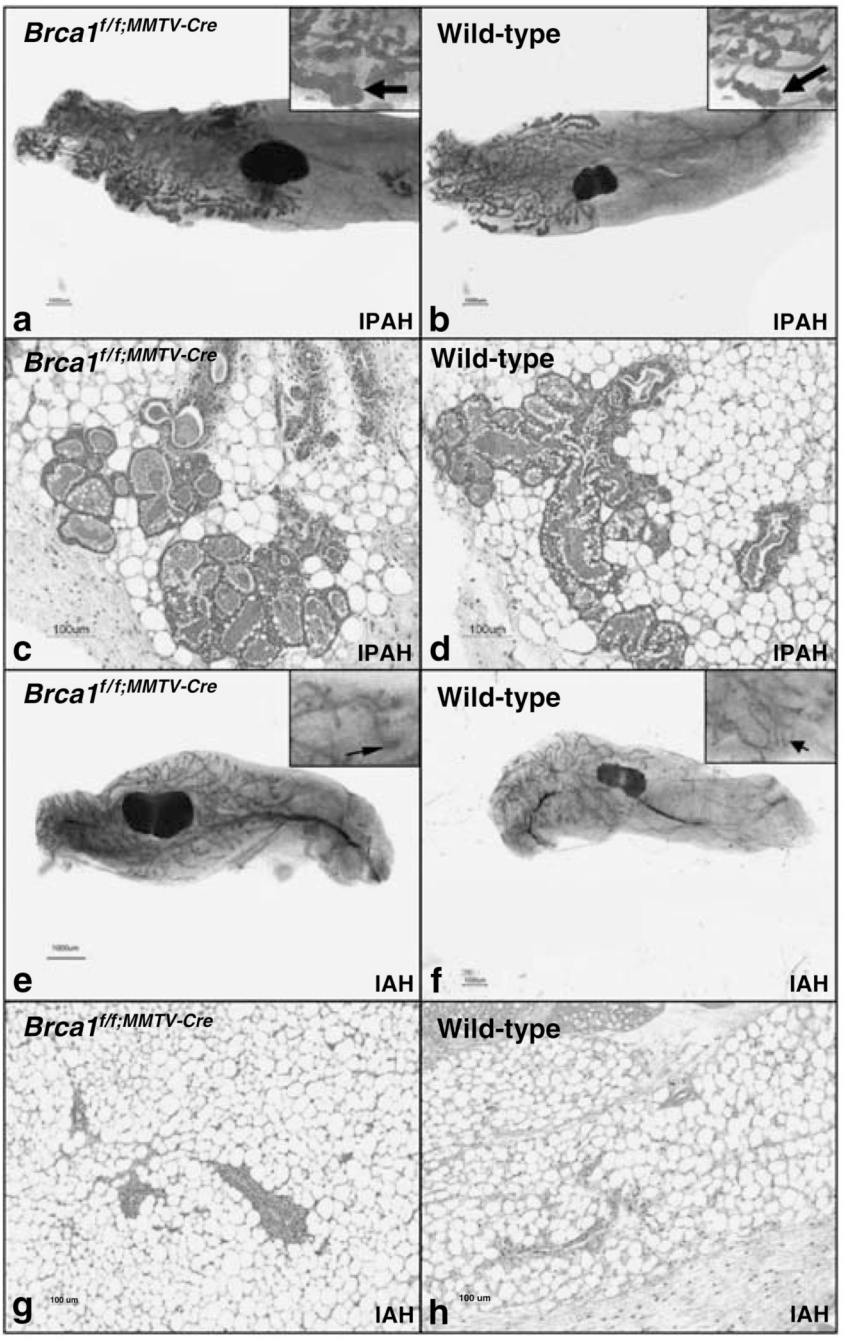

Figure 2.

Normal prolactin-induced alveolar differentiation in *Brca1*$^{f/f;MMTV\text{-}Cre}$ and wild-type mice. Mammary gland whole mounts (**a, b, e** and **f**) and H&E (**c, d, g** and **h**) sections from *Brca1*$^{f/f;MMTV\text{-}Cre}$ and wild-type female mice after 7 days in organ culture medium containing insulin (I) + aldosterone (A) + hydrocortisone (H) with (**a–d**) or without (**e–h**) prolactin (P). Arrows indicate alveolar differentiation in higher magnification insets (thick arrows) (**a** and **b**), TEBs (thin arrows) (**e**) or terminal ductal ends (arrowhead) (**f**). Whole mount: magnification, × 0.5; scale bar, 1000 μm. H&E: magnification, × 10; scale bar, 100 μm.

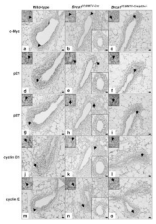

Figure 3.

Expression patterns of c-myc, p27, p21, cyclin d1 and cyclin e are similar in TEBs of wild-type, *Brca1*$^{f/f;MMTV\text{-}Cre}$ and *Brca1*$^{f/f;MMTV\text{-}Cr/p53+/-}$ mice. Immunohistochemical staining of c-myc (**a–c**), p21 (**d–f**), p27 (**g–i**), cyclin d1 (**j–l**) and cyclin e (**m–o**) in TEBs of 6-week-old wild-type (**a, d, g, j** and **m**), Brca1$^{f/f;MMTV\text{-}Cre}$ (**b, e, h, k** and **n**) and Brca1$^{f/f;MMTV\text{-}Cre\ P53+/-}$ (**c, f, i, l** and **o**) mice. Arrows indicate nuclear-localized c-myc, p21, p27, cyclin d1 or cyclin e expression. Large images, × 20; small insets, × 40. Medium insets are cross sections, × 20; scale bars, 20 μm.

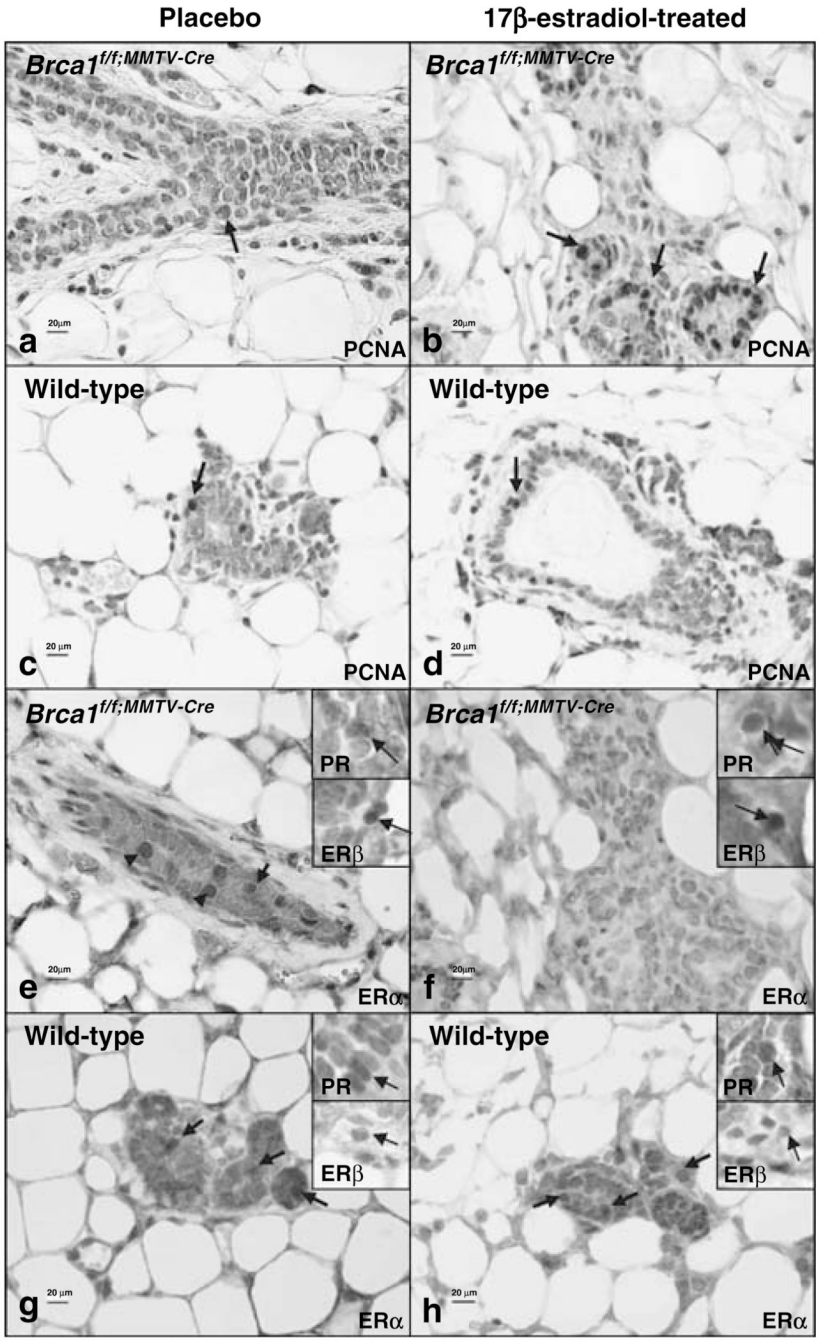

Figure 4.

Exogenous estrogen stimulated an abnormal sustained increase in mammary epithelial cell proliferation in postpubertal Brca1$^{f/f;MMTV-Cre}$ mice. Immunohistochemical detection of proliferating cell nuclear antigen (PCNA), estrogen receptor-α (ERα), progesterone receptor (PR, insets) and estrogen receptor-β (ERβ, insets) in mammary gland sections from 6-month-old placebo (**a, c, e** and **g**) and 17β-estradiol-treated (**b, d, f** and **h**) *Brca1$^{f/f;MMTV-Cre}$* and wild-type female mice. Arrows indicate cells demonstrating nuclear-localized PCNA, ERα, PR or ERβ expression. Magnification, × 40; scale bars, 20 μm.

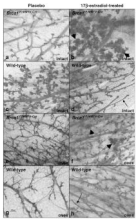

Figure 5.

Exogenous estrogen stimulated abnormal growth in postpubertal Brca1$^{f/f;MMTV\text{-}Cre}$ mice. Mammary gland whole mounts of 6-month-old placebo (**a, c, e** and **g**) and 17β-estradiol-treated (**b, d, f** and **h**) intact (**a–d**) and ovariectomized (ovex) (**e–h**) *Brca1$^{f/f;MMTV\text{-}Cre}$* and wild-type female mice. Thick arrowheads indicate areas of abnormal ductal growth found in the 17βestradiol-treated *Brca1$^{f/f;MMTV\text{-}Cre}$* mice. Thin arrows indicate normal-appearing ductal structures in the placebo-treated *Brca1$^{f/f;MMTV\text{-}Cre}$* and placebo and 17β-estradiol-treated wild-type mice. Magnification, × 4; scale bar, 200 μm.

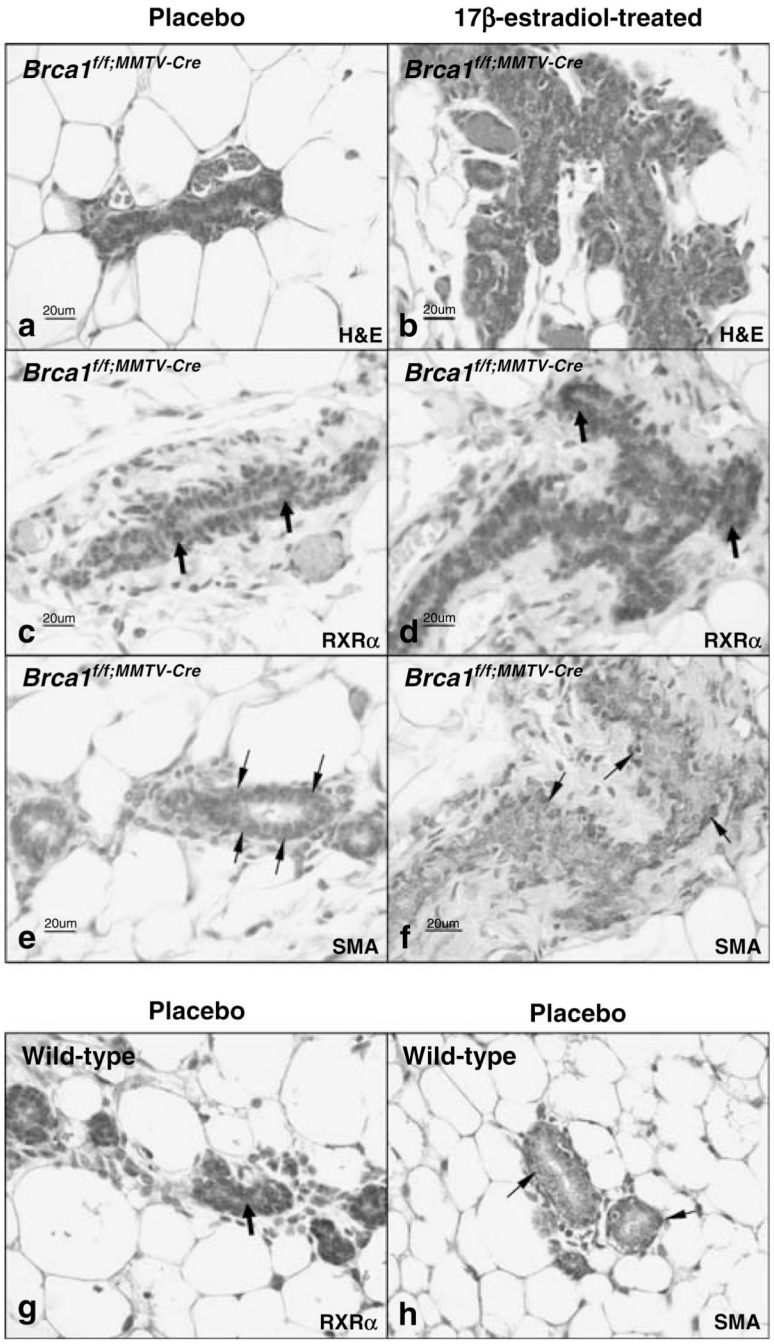

Figure 6.

Organization of the mammary epithelium was not altered by the abnormal proliferative response. Hematoxylin and eosin (H&E)-stained sections (**a** and **b**) and immunohistochemical detection of retinoid X receptor-α (RXR) (thick arrows) (**c, d** and **g**) and smooth-muscle actin (SMA, thin arrows) (**e, f** and **h**) in mammary gland sections from 6-month-old placebo (**a, c, e, g** and **h**) and 17β-estradiol-treated (**b, d** and **f**) *Brca1*$^{f/f;MMTV\text{-}Cre}$ and wild-type female mice. Magnification, × 40; scale bars, 20 μm.

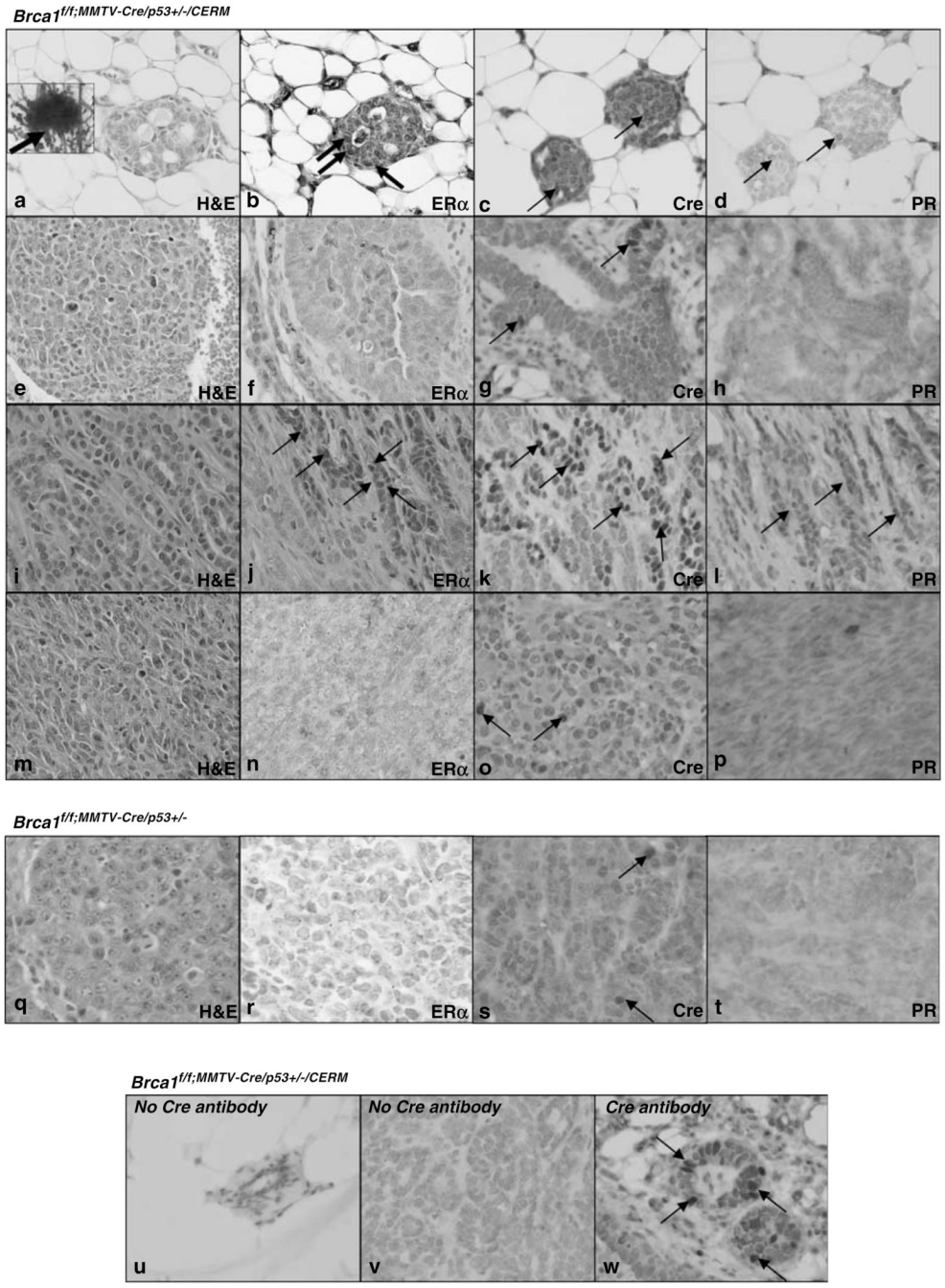

Figure 7.

ERα-negative preneoplasia and cancer in Brca1$^{f/f;MMTV\text{-}Cre/p53+/-}$ and ERα-negative and -positive preneoplasia and cancer in Brca1$^{f/f;MMTV\text{-}Cre/p53+/-/CERM}$ mice. Hematoxylin and eosin (H&E)-stained sections (**a, e, i, m** and **q**), representative HAN from a mammary gland whole mount(**a**, inset) and immunohistochemical detection of nuclear-localized estrogen receptor-α (ERα; **b** and **j**), Cre (**c, g, k, o, s** and **w**), nuclear-localized progesterone receptor (PR) (**d** and **l**) in preneoplasia (**a–h, w**) and invasive cancer (**i–t** and **v**) from Brca1$^{f/f;MMTV\text{-}Cre/p53+/-/CERM}$ and Brca1$^{f/f;MMTV\text{-}Cre/p53+/-}$ mice. Representative ERα-and PR-negative preneoplasia (**f** and **h**) and invasive cancer (**n** and **p**) from Brca1$^{f/f;MMTV\text{-}Cre/p53+/-/CERM}$ mice and invasive cancer (**r** and **t**) from

Brca1$^{f/f;MMTV\text{-}Cre/p53+/-}$ mice. Negative controls: no Cre antibody (**u** and **v**). Magnification, × 40.