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Promotion of mammary cancer development by tamoxifen in a mouse model of Brca1-mutation-related breast cancer

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Loss of full-length Brca1 in mammary epithelial cells of the mouse mammary tumor virus (MMTV)-Cre Brca1 conditional exon 11 deletion mouse model results in the development of mammary adenocarcinomas with similar genetic changes to those found in human BRCA1-mutation-related breast cancers. We used this experimental model to evaluate the chemopreventive effect of tamoxifen on the development of mammary preneoplasia and adenocarcinoma. No protective effects of tamoxifen administration on mammary cancer development were found. Instead, tamoxifen treatment significantly increased rates of mammary epithelial cell proliferation and the prevalence of mammary hyperplasia at 6 months of age. Tamoxifen-exposed mice developed adenocarcinomas at younger ages than control mice and a higher percentage of mice developed adenocarcinomas by 12 months of age. Both whole mouse and tissue culture cell models were used to test if loss of full-length Brca1 was associated with a relative increase in the agonist activity of tamoxifen. Tamoxifen induced increased ductal growth in MMTV-Cre Brca1 conditional mice compared to wild type. Estrogen receptor alpha (ERα) expression was downregulated in the tamoxifen-induced hyperplasias. Reducing BRCA1 levels in MCF-7 cells using siRNA resulted in a relative increase in the agonist activity of tamoxifen. Results suggest a model of mammary cancer progression in which loss of full-length Brca1 altered the agonist/antagonist activity of tamoxifen, resulting in tamoxifen-induced mammary epithelial cell proliferation with subsequent loss of ERα expression and development of ERα-negative hyperplasias and adenocarcinomas.
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Introduction

Cre-lox-mediated conditional mouse models of Brca1-mutation-related breast cancer were first introduced in 1999 (Xu *et al*., 1999). The mouse mammary tumor virus (MMTV)-Cre transgene utilized to induce tissue-specific loss of full-length Brca1 in mammary epithelial cells first induces recombination and loss of full-length Brca1 during late embryogenesis (Wagner *et al*., 1997, 2001). Haploid loss of p53 promotes mammary cancer development in this model (Brodie *et al*., 2001). The adenocarcinomas developing in this model demonstrate invasive features characteristic of human breast cancer and exhibit estrogen-independent growth similar to the majority of BRCA1-mutation-related human breast cancers (Brodie *et al*., 2001). The adenocarcinomas demonstrate different molecular signatures. Reports to date document overexpression of either ErbB2, cyclin D1 or c-*myc* in addition to loss of functional p53 (Brodie *et al*., 2001).

There are a variety of hormonal manipulations (ovariectomy, selective estrogen response modifier (SERM) treatment, aromatase treatment) that have proven to be highly effective in the treatment of estrogen receptor alpha (ERα)-positive breast cancer and can cause the regression of even relatively large tumors (Chlebowski, 2002; Jordan, 2004). In contrast, these treatments have been considered to be ineffective in the treatment of ERα-negative tumors. In humans, the majority of BRCA1-mutation-related breast cancers are ERα negative (Metcalfe *et al*., 2004), but two lines of evidence point to a possible role for estrogen in the early stages of cancer progression. First, a number of studies have shown that ovariectomy, with follow-ups of 6–10 years, decreases the occurrence of breast cancer almost 50% (Metcalfe *et al*., 2004) and greater degrees of protection are found when it is performed at younger ages (Meijers-Heijboer *et al*., 2001; Rebbeck *et al*., 2002). We initially repeated these studies in the present MMTV-Cre Brca1 conditional exon 11 mouse model and found that ovariectomy decreased the production of mammary tumors beginning roughly 5 months after bilateral oophorectomy (Bachelier *et al*., 2002). The efficacy of ovariectomy in this model was also associated with reduced development of the mammary gland.

Second, a direct repressive function of BRCA1 on ERα transcriptional activity has been described (Fan *et al*., 1999, 2001; Zheng *et al*., 2001). Owing to this, it has been hypothesized that tamoxifen, an SERM, might have a protective role at early stages of cancer progression even if the majority of cancers are ERα negative. Clinical studies published to date demonstrate mixed results (Narod *et al*., 2000; Schrag *et al*., 2000; King *et al*., 2001; Robson, 2002), where one study showed efficacy (Narod *et al*., 2000) and a second study, albeit with low tumor numbers, showed minor enhancement (King *et al*., 2001).

Tamoxifen is a mixed agonist/antagonist (Gallo and Kaufman, 1997). In normal mice, tamoxifen treatment results in regression of mammary ductal structures (Kotoula *et al*., 1993) and, tamoxifen, unlike chronic estrogen treatment, does not downregulate levels of nuclear-localized ERα (Cheng *et al*., 2004). It does, however, downregulate levels of nuclear-localized ERβ (Cheng *et al*., 2004). Tamoxifen can prevent progression of ERα-negative cancers in certain mouse models (Osborne *et al*., 1990; Yoshidome *et al*., 2000; Yang *et al*., 2003). The timing of tamoxifen administration during disease progression may be important as tamoxifen promotes cancer development in MMTV-ErbB2 transgenic mice when delivered later during the course of disease (Menard *et al*., 2000).

In contrast to the relatively clear data demonstrating the efficacy of ovariectomy in human BRCA1 mutation carriers, the data with tamoxifen have been more equivocal. This study was initiated to determine whether or not tamoxifen treatment would prevent progression of mammary cancer in a genetically defined mouse model with loss of full-length Brca1 and haploid insufficiency of p53.

Results

Tamoxifen treatment increased the prevalence of mammary hyperplasia in Brca1$^{Co/Co}$MMTV-Cre/p53+/− female mice

Placebo-treated Brca1$^{Co/Co}$MMTV-Cre/p53+/− female mice demonstrated a pattern of cancer progression characterized by areas of normal-appearing mammary histology, with focal areas of mammary hyperplasia in 50% of the mice by 6 months of age (Figure 1). Hyperplastic alveolar nodules (HANs) developed in 90% of the mice and adenocarcinomas developed in 35% of the mice by 12 months of age. To determine if tamoxifen treatment altered development of hyperplasia, the prevalence of hyperplasia was compared in tamoxifen- and placebo-treated 6-month-old mice. Unexpectedly, tamoxifen treatment significantly increased the prevalence of mammary hyperplasia at 6 months of age from 50% in the placebo-treated mice to 100% in the tamoxifen-treated mice (Figure 2) (χ^2, $P<0.05$). Consistent with previously published results (Kotoula *et al*., 1993), wild-type control female mice treated with the same tamoxifen pellets utilized for the Brca1$^{Co/Co}$MMTV-Cre/p53+/− female mice demonstrated mammary ductal regression (data not shown).

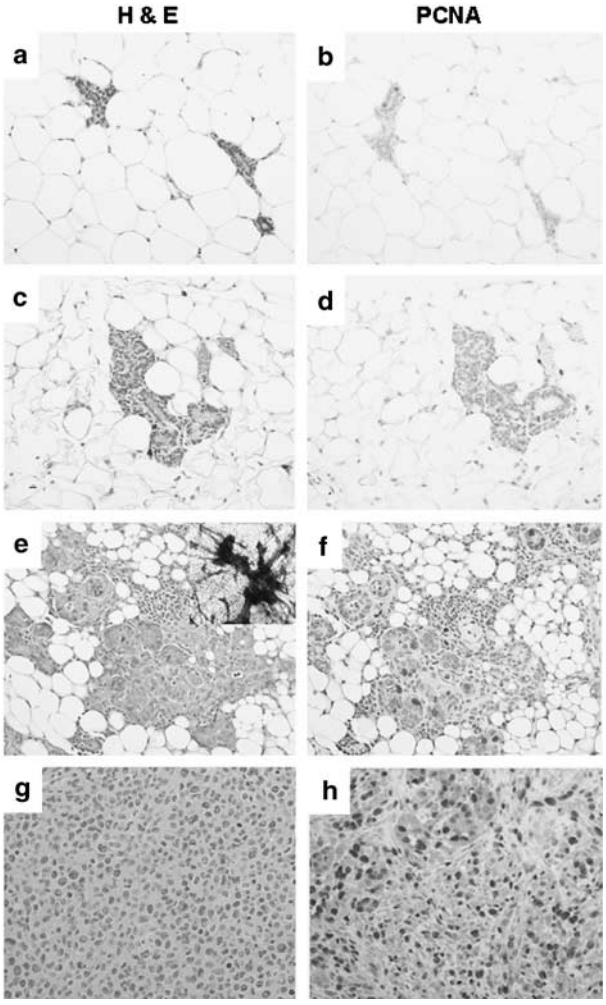

Figure 1 Progression of pathological changes in the inguinal (#4) mammary glands of the Brca1 conditional knockout mouse model monitored by H&E-stained sections (**a**, **c**, **e** and **g**) and whole-mount analyses (inset in **e**). Progression of changes in cell proliferation illustrated using PCNA immunohistochemistry (**b**, **d**, **f** and **h**). (**a**) Normal ductal morphology in a nonpregnant 6-month-old mouse. (**b**) PCNA immunohistochemistry of normal-appearing ductal structure in a nonpregnant 6-month-old mouse. (**c**) Representative mammary epithelial cell hyperplasia from a 6-month-old mouse. (**d**) PCNA immunohistochemistry of hyperplastic foci in a 6-month-old mouse. (**e**) Representative HAN in the mammary gland from a 12-month-old mouse. Inset shows HAN on whole mount. (**f**) PCNA immunohistochemistry of an HAN from a 12-month-old mouse. (**g**) Undifferentiated mammary adenocarcinoma from a 12-month-old mouse. (**h**) PCNA immunohistochemistry of an undifferentiated adenocarcinoma from a 12-month-old mouse. The percentage of proliferating epithelial cells is highest in the adenocarcinomas (50%), lower in the HAN (10%) and hyperplastic foci (4%) and lowest in the normal-appearing ducts (2%). Magnification = × 20. Scale bars = 10 μm

Tamoxifen treatment increased the rate of mammary epithelial cell proliferation

To test if the increased prevalence of mammary hyperplasia was associated with an increase in the rate of mammary epithelial cell proliferation, proliferative indices were compared in placebo- and tamoxifen-treated 6-month-old mice both in normal-appearing

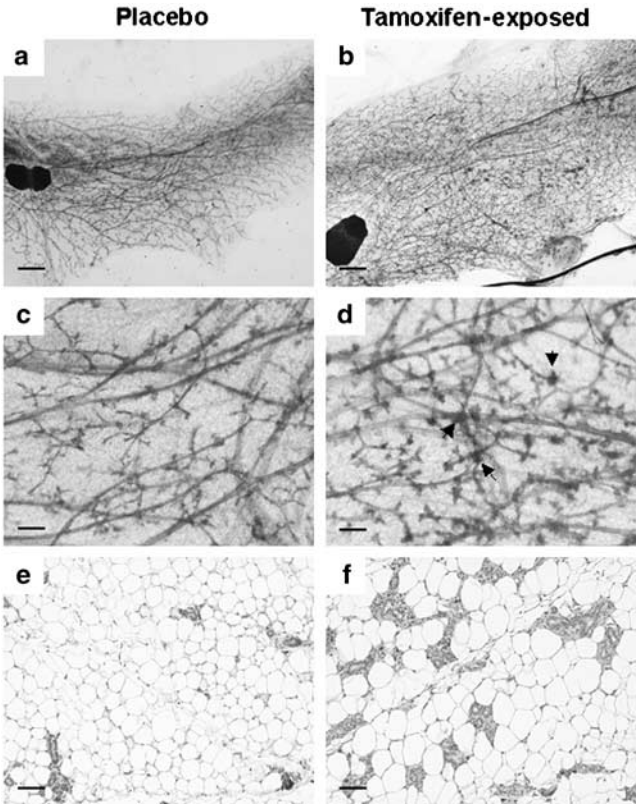

Figure 2 Whole-mount and histology preparation in nontumor-bearing inguinal (#4) mammary glands of 6-month-old placebo (**a**, **c** and **e**) and tamoxifen-treated (**b**, **d** and **f**) Brca1$^{Co/Co}$ MMTV-Cre/p53 + /− female mice. HANs can be identified at branch points in (**d**) (arrow heads) and mammary hyperplasia identified on H&E-stained histological sections (**f**). Magnification in (, **b**, **e** and **f**) = × 10. Scale bars = 100 μm; magnification in (**c** and **d**) = × 4. Scale bar = 200 μm

ductal structures and areas of hyperplasia (Table 1). The mean mammary epithelial cell proliferative indices of tamoxifen-treated mice were significantly higher in both normal-appearing ducts (10 ± 2%) (mean ± standard error (s.e.) and areas of hyperplasia (11 ± 1%) than in placebo-treated mice (normal-appearing ducts: 2 ± 1% and hyperplasias: 4 ± 4%) (Kruskal–Wallis, $P < 0.012$, Table 1, Figure 3a and b). There was no significant differences in the mean apoptotic index between the placebo- and tamoxifen-treated groups in either the normal-appearing ducts or the areas of hyperplasia (< 1% for both) (Figure 3c and d). In wild-type mice, chronic estrogenic stimulation, but not chronic tamoxifen treatment, results in downregulation of nuclear-localized ERα. In contrast, chronic tamoxifen stimulation results in downregulation of nuclear-localized ERβ but not ERα (Saji *et al*., 2000; Cheng *et al*., 2004). We hypothesized that if the relative agonist activity of tamoxifen was increased by loss of full-length Brca1, there would be loss of nuclear-localized ERα with retention of nuclear-localized ERβ in the hyperplasias of the tamoxifen-treated Brca1$^{Co/Co}$MMTV-Cre/p53 + /− mice. In support of this hypothesis, immunohistochemical studies demonstrated a significant loss of nuclear-localized ERα but retention of nuclear-localized ERβ in the hyperplastic lesions of tamoxifen-treated mice (Kruskal–Wallis, $P < 0.002$) (Table 2, Figure 3e and f). There was no change in progesterone receptor (PR) expression following tamoxifen exposure (data not shown).

Tamoxifen treatment increased the incidence of adenocarcinoma

To establish whether or not the increased prevalence of hyperplasia found in the tamoxifen-treated mice was

Table 1 Effect of treatments on cell proliferation in mammary ductal cells and mammary epithelial cell hyperplastic foci

Treatments	*# of mice*	*# of fields/ mouse*	*Ductal structures*				*Hyperplastic structures*			
			Total # structures counted	*Total # cells counted*	*# PCNA positive*	*Proliferative index mean ± s.e.*	*Total # structures counted*	*Total # cells counted*	*# PCNA positive*	*Proliferative index mean ± s.e.*
Placebo	6	10	103	6189	105	2 ± 1%	63	2321	92	4 ± 4%
Tamoxifen	6	10	69	3296	267	10 ± 2%[a]	131	7783	837	11 ± 1%[a]

[a]Kruskal–Wallis, $P \leqslant 0.012$, compared to placebo treatment

Table 2 Effect of treatments on nuclear ERα positivity in mammary ductal cells and mammary epithelial cell hyperplastic foci

Treatments	*# of mice*	*# of fields/ mouse*	*Ductal structures*				*Hyperplastic structures*			
			Total # structures counted	*Total # cells counted*	*# ERα positive*	*% ER positive mean ± s.e.*	*Total # structures counted*	*Total # cells counted*	*# ERα positive*	*% ER positive mean ± s.e.*
Placebo	6	10	177	5420	310	6 ± 2%	23	2468	141	5 ± 2%
Tamoxifen	6	10	62	3149	52	1 ± 0%	106	6602	0	0[a]

[a]Kruskal–Wallis, $P \leqslant 0.002$, compared to placebo treatment

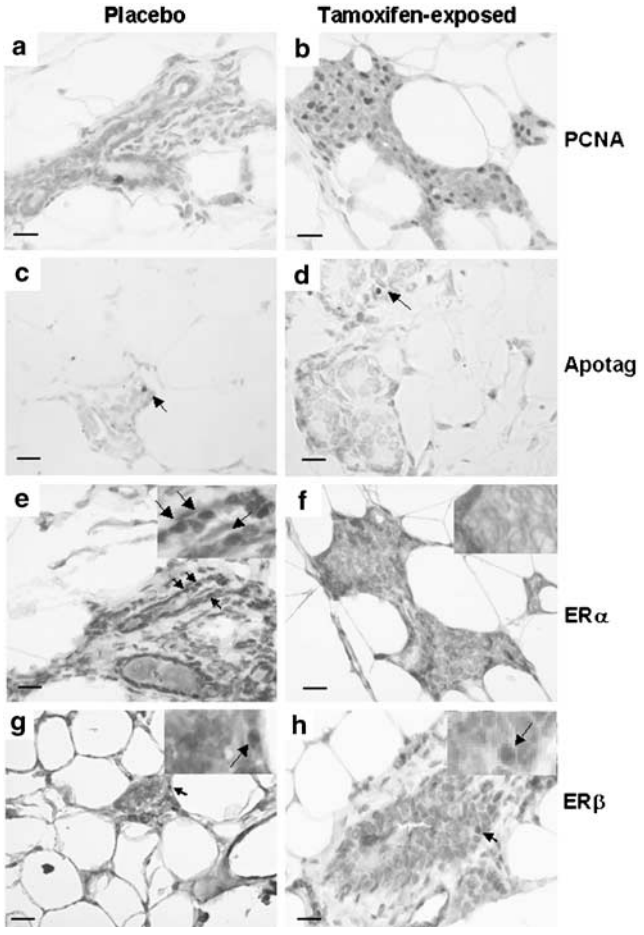

Figure 3 Immunohistochemical detection of PCNA (**a** and **b**) apoptotic cells (**c** and **d**), nuclear-localized ERα (**e** and **f**) and nuclear-localized ERβ (**g** and **h**) in the nontumor-bearing inguinal (#4) mammary glands of 6-month-old placebo (**a**, **c**, **e** and **g**) and tamoxifen-treated (**b**, **d**, **f** and **h**) Brca1$^{Co/Co}$MMTV-Cre/p53+/− female mice. The arrows in (**c** and **d**) point to the one apoptotic cell present in the fields shown. Arrows in (**e**) and (**g** and **h**) indicate representative mammary epithelial cells with nuclear-localized ERα and ERβ, respectively. The same image is shown in the top right inset in (**e**, **f**, **g** and **h**) at higher magnification. Magnification = × 40. Scale bars = 20 μm

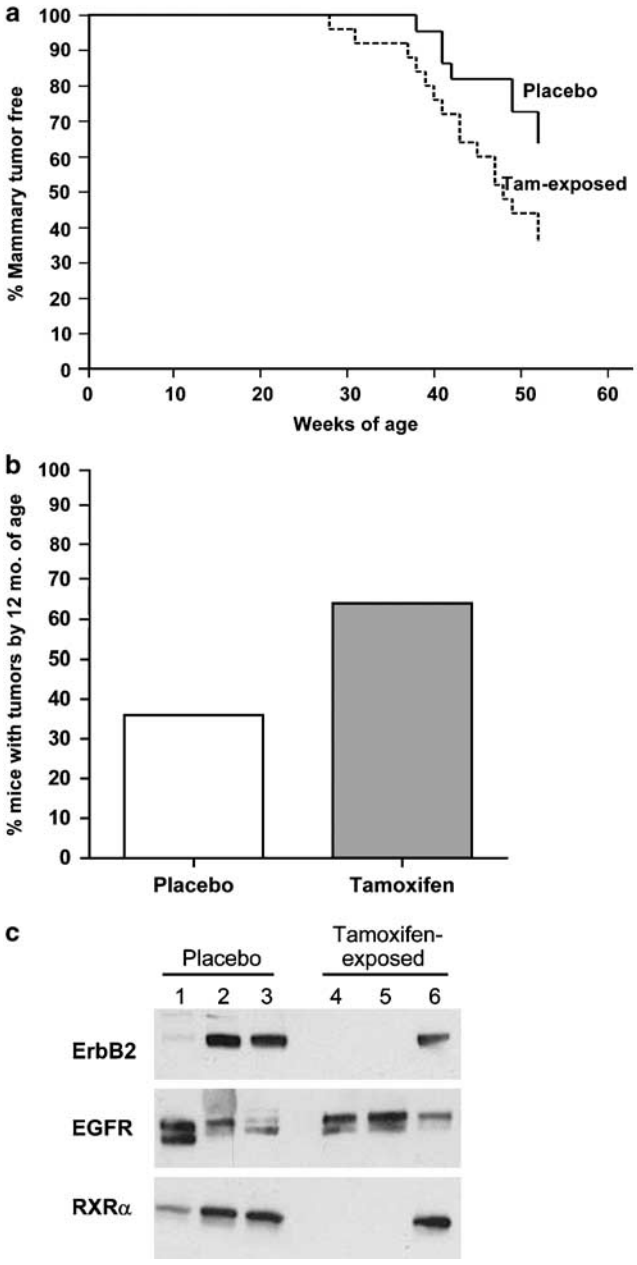

Figure 4 Comparison of time to first palpable tumor in placebo- and tamoxifen-treated Brca1$^{Co/Co}$MMTV-Cre/p53+/− female mice. (**a**) Tumor-free survival of mice treated with tamoxifen (25 mice) was significantly different from the placebo group (22 mice) (Kaplan–Meier, $P = 0.039$). (**b**) Tamoxifen exposure increased the percentage of mice developing adenocarcinomas by 12 months of age (χ^2, $P < 0.05$). (**c**) Immunoblot analysis of ErbB2, EGFR and RXRα protein expression in representative adenocarcinomas from placebo- and tamoxifen-treated Brca1$^{Co/Co}$ MMTV-Cre/p53+/− female mice. Shown are representative immunoblots of total protein (40 μg protein/lane) samples of adenocarcinomas from six individual mice after administration of either placebo (lanes 1–3) or tamoxifen (lanes 4–6). Expression of ErbB2 and EGFR were found in 66 and 33%, respectively, of the adenocarcinomas from tamoxifen-treated mice and 60% and 60, respectively, of the adenocarcinomas from placebo-treated mice. RXRα expression was found in 75% of the adenocarcinomas from tamoxifen-treated mice and 100% of the adenocarcinomas from placebo-treated mice. The differences were not statistically significant (χ^2, $P \geqslant 0.05$)

correlated with earlier or more frequent development of adenocarcinoma, cohorts of placebo- and tamoxifen-treated mice were followed to 12 months of age for development of mammary adenocarcinoma. Tamoxifen treatment significantly shortened the time to development of first palpable tumor (Kaplan–Meier, $P < 0.039$) and significantly increased the percentage of mice developing adenocarcinomas by 12 months of age (65% as compared to 35%; χ^2, $P < 0.05$) (Figure 4a and b). Tamoxifen treatment did not alter significantly the differentiation status, or tumor multiplicity. As overexpression of either ErbB2 or epidermal growth factor receptor (EGFR) has been associated with tamoxifen resistance (Kurokawa and Arteaga, 2003), steady-state levels of ErbB2 and EGFR were evaluated in the adenocarcinomas of tamoxifen-treated mice and compared to the levels found in adenocarcinomas from

placebo-exposed mice. There were no statistically significant differences in the prevalence of either ErbB2 or EGFR overexpression between the two groups (Figure 4c). This indicates that the increased incidence of adenocarcinoma development in the tamoxifen-treated mice was not due to selection for high expression of ErbB2 or EGFR. We evaluated expression levels of the retinoid X receptor alpha (RXRα) in both adenocarcinomas and hyperplastic mammary tissue from placebo- and tamoxifen-treated mice because RXR-selective ligands have chemopreventive activity and have been shown to improve response in combination with tamoxifen (Bischoff *et al*., 1999; Grubbs *et al*., 2003). Both the hyperplastic mammary tissue from 6-month-old mice (data not shown) and a significant proportion of the adenocarcinomas (Figure 4c) demonstrated expression of this receptor, suggesting that this could be an alternative therapeutic approach for mammary cancers that arise from loss of Brca1 function.

Decreased expression levels of Brca1 were associated with a relative increase in the agonist activity of tamoxifen

The MCF-7 mammary epithelial cell tissue culture cell model was used to test if a decrease in BRCA1 expression levels could be linked to an increase in the agonistic activity or a decrease in the antagonistic activity of tamoxifen. Steady-state levels of BRCA1 protein were reduced significantly using siRNA (Figure 5a). Consistent with previously published results (Fan *et al*., 1999, 2001), decreased levels of BRCA1 expression in this model resulted in increased levels of estrogen response element (ERE)-mediated luciferase activity in response to 17-β estradiol (Figure 5b). Similarly, reducing BRCA1 expression levels resulted in a modest but significant increase in luciferase activity in response to tamoxifen consistent with a relative increase in the agonist activity of tamoxifen and a decrease in the relative amount of antagonist activity (Figure 5c). Reducing BRCA1 expression levels using siRNA had no effect on steady-state expression levels of ERα (Figure 6).

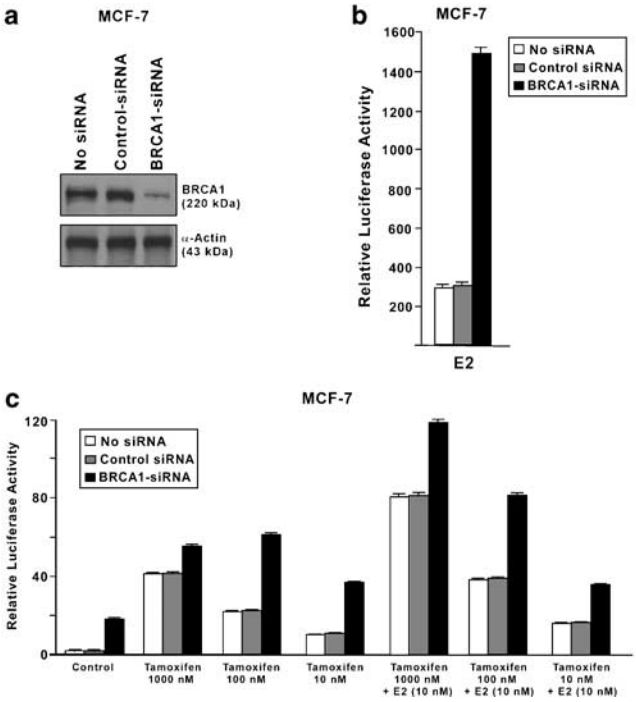

Figure 5 Reducing BRCA1 expression in MCF-7 cells using siRNA resulted in a relative increase in the agonist activity of tamoxifen and a decrease in the relative amount of antagonist activity. Reduction of BRCA1 expression levels by siRNA (**a**). Immunoblot analysis of BRCA1 protein expression in MCF-7 cells treated with no siRNA, control siRNA or BRCA1-siRNA. α-Actin was used as a control for loading and transfer. ER transcriptional activity in MCF-7 cells pretreated with BRCA1-siRNA, control siRNA or no siRNA in the presence or absence of 17-β estradiol or tamoxifen for 24 h shown in (**b**) and (**c**), respectively. Results obtained from the same set of experiments but shown in different panels due to the differences in scales. Luciferase values are expressed relative to the absolute control value (i.e. no siRNA, no E2 or tamoxifen). Values are means $\pm$ s.e.m.'s for $n=4$ replicate wells

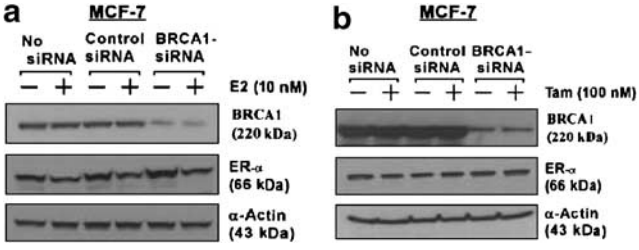

Figure 6 BRCA1-siRNA has no effect on ERα expression. Subconfluent proliferating MCF-7 cells were treated with BRCA1-siRNA, control siRNA (50 nM $\times$ 72 h) or no siRNA (vehicle only), and then incubated $\pm$17-β estradiol (E2, 10 nM) (**a**) or $\pm$tamoxifen (Tam, 100 nM) (**b**) for $T=24$ h. The cells were then harvested to detect BRCA1, ERα and α-actin (control for loading and transfer)

Discussion

This paper describes the effect of tamoxifen on mammary cancer development in the MMTV-Cre Brca1 conditional exon 11 deletion mouse model. Significantly, tamoxifen was not protective in this model when administered at 4 months of age following a first pregnancy. In contrast, tamoxifen stimulated mammary epithelial cell proliferation, ductal growth and promoted the development of mammary adenocarcinomas, acting more like a growth agonist than an antagonist. This increase in mammary epithelial cell proliferation and ductal growth following tamoxifen treatment in Brca1-altered mice was the opposite of the effects in tamoxifen-treated wild-type mice. Consistent with these *in vivo* observations, a relative increase in the agonist activity of tamoxifen was found when BRCA1 expression levels were decreased in tissue culture cells.

Tamoxifen routinely acts as a growth antagonist for the mammary gland by interrupting ERα signal transduction (Kotoula *et al*., 1993). However, in the

uterus and bone, the agonist activity of tamoxifen dominate (Dutertre and Smith, 2000). Additionally, the use of tamoxifen in breast cancer patients has been associated with 'tamoxifen flare', a condition in which a transient increase in tumor growth follows the initial administration of tamoxifen (Plotkin *et al*., 1978; Veldhuis and Santen, 1979; Reddel and Sutherland, 1984). Previous studies attribute this transitory growth of the cancer to a relative enhancement of the agonist activity of tamoxifen before steady-state therapeutic levels are achieved. Several mechanisms have been proposed to explain why tamoxifen may act as an antagonist in some tissues and act as an agonist in others, including cell-specific tamoxifen-induced changes in ERα conformation or modifications that alter the ability of ERα to interact with coactivators or corepressors, expression levels of coactivators or corepressors, or activity of other intracellular signaling pathways (Dutertre and Smith, 2000; Graham *et al*., 2000; McDonnell *et al*., 2002; Osborne and Schiff, 2003; Heldring *et al*., 2004; Jordan, 2004; Smith and O'Malley, 2004). The agonist activity of tamoxifen is increased when the coactivator AIB1/SRC-3 or its isoform AIB1-Δ3 is overexpressed (Reiter *et al*., 2004) and tamoxifen increases steady-state protein levels of SRC-1 and AIB1/SRC-3 (Lonard *et al*., 2004). Higher levels of AIB1 expression in human breast cancer have been associated with poorer outcome following tamoxifen therapy (Osborne *et al*., 2003).

Brca1-mediated changes in coactivator or corepressor expression levels or activity could be a possible explanation for the shift to tamoxifen agonism. Loss of BRCA1 has been reported to increase ERα signaling by reducing expression levels of p300 (Fan *et al*., 2002). Longer polyglutamine repeat length in the *AIB1* gene has been associated with a higher risk of breast cancer development in carriers of BRCA1 mutation, raising the possibility that the *AIB1* gene may collaborate with BRCA1 loss in the development of breast cancer (Rebbeck *et al*., 2001; Kadouri *et al*., 2004).

In humans, loss of function of one *BRCA1* allele has been associated with a relative increase in PR A levels, alterations in ERα-regulated gene expression, but a normal ERα expression pattern (Mote *et al*., 2004). Similarly, in the study reported here, a normal pattern of nuclear-localized ERα expression was found in the normal-appearing mammary ductal cells. However, the abnormal foci of proliferating mammary epithelial cells that developed in the tamoxifen-exposed mice did not demonstrate nuclear-localized ERα. The adenocarcinomas that develop in this model also are predominantly ERα negative (Brodie *et al*., 2001). Taken together, these observations suggest that while estrogenic pathways may act as an initial stimulus for abnormal proliferation, cancer progression may be maintained by ERα-independent mechanisms. This is consistent with observations made in BRCA1-related human breast cancer in which ovariectomy is protective, but the majority of cancers that develop are ERα negative (Kauff *et al*., 2002; Moller *et al*., 2002; Rebbeck *et al*., 2002; Foulkes *et al*., 2004).

Effective chemoprevention for BRCA1-mutation-related cancer in women is an important clinical goal. Data to date from clinical trials are equivocal about the value of tamoxifen for chemoprevention in this population (Narod *et al*., 2000; Schrag *et al*., 2000; King *et al*., 2001; Robson, 2002). An important difference between these trials and the experiments performed here is that in the mouse model full-length Brca1 expression is lost, while in the human studies, the women have loss or mutation of just one allele in the majority of their normal-appearing mammary epithelial cells and loss or mutation of all BRCA1 function only in a subset of these cells or in the carcinomas (Cavalli *et al*., 2004). Additional experiments will need to be performed to determine if shifts in the agonist/antagonist activity of tamoxifen are associated with any of the BRCA1 mutations found in humans or with reduced but not absent BRCA1 expression. The results do point towards the need to develop alternative chemopreventive approaches. One possibility based on the experiments here would be to exploit RXRα as a chemopreventive target (Brown and Lippman, 2000; Sporn and Suh, 2000). Other targets and approaches for combination or intermittent therapy also need to be investigated further (Rendi *et al*., 2004).

In summary, this study demonstrates that loss of Brca1 can alter the agonist/antagonist activity of tamoxifen in mammary epithelial cells and illustrates that cancer progression in a mouse model of Brca1-mutation-related mammary adenocarcinoma proceeds through sequential steps from ERα-negative hyperplastic proliferative foci through HANs to adenocarcinoma.

Materials and methods

Mice and genotyping

Brca1 conditional knockout mice carrying the MMTV-Cre recombinase gene in a p53 heterozygous background (Xu *et al*., 1999) were maintained on a C57Bl/6 genetic background. Nontransgenic C57Bl/6 mice were used as controls. All mice were maintained in accordance with institutional guidelines approved by the Georgetown University Animal Care and Use Committee. The presence of the floxed Brca1 alleles, the absence of wild-type Brca1 alleles and the presence of the MMTV-Cre transgene were identified using polymerase chain reaction (PCR) on tail DNA as described previously (Wagner *et al*., 1997; Xu *et al*., 1999). The p53 heterozygote allele was identified using a modification of previously published techniques (5′ primer: p53 wild-type allele – forward primer (5′-CTGTCTTCCAGATACTCGGGATAC-3′); reverse primer (5′-CCAATGGTGCTTGGACAATGTG-3′). p53 knockout allele – forward primer (5′-CTGTCTTCCAGA TACTCGGGATAC-3′); reverse primer: (5′-ATCGCCTTC TATCGCCTTCTTGACGAGTTC-3′)). In all, 35 PCR cycles were run (45 s at 94°C; 1 min 20 s at 57°C; 3 min at 72°C) (Donehower *et al*., 1992; Xu *et al*., 1999).

Tamoxifen treatment studies

Mice were bred at 2 months of age. After parturition, female mice were anesthetized and implanted subcutaneously with a

25 mg 60-day constant release tamoxifen pellet ($n=31$) or a placebo pellet ($n=28$) (Innovative Research of America, Sarasota, FL, USA). For studies of mammary hyperplasia, mice were euthanized at 6 months of age ($n=6$ tamoxifen treated and $n=6$ placebo treated) and necropsied. For mammary adenocarcinoma studies ($n=25$ tamoxifen treated and $n=22$ placebo treated), mice were monitored weekly for the development of primary palpable tumors and then euthanized when a palpable tumor reached 1 cm^3 or by 12 months of age, whichever came first. Tumor development was recorded as the animal age when a tumor was first detected by palpation. Complete necropsies were performed to examine for the presence or absence of nonpalpable tumors, more than one tumor and metastases. Mammary tissue, mammary cancer tissue and tissue containing metastases were removed and processed for whole mount, formalin fixed for histology or immediately frozen in liquid nitrogen and stored at $-80^{\circ}C$ for gene expression analysis as appropriate.

Whole-mount analysis, histological studies, in situ *detection of apoptosis and immunohistochemistry*

Mammary tissue and adenocarcinoma specimens were fixed in 10% buffered formalin overnight at 4°C and embedded in paraffin using standard techniques. Sections (5 μm) were cut for hematoxylin and eosin (H&E) staining and immunohistochemistry. Eight nonadjacent sections from each mammary gland of each mouse were examined for the presence or absence of normal ductal structures, hyperplasia and adenocarcinoma. *In situ* detection of apoptotic cell nuclei was performed using the ApopTag Apoptosis Detection Kit (Intergen Company, Purchase, NY, USA) and the apoptotic index determined (Li *et al*., 1996). Immunohistochemical detection of proliferating cell nuclear antigen (PCNA) expression was performed using the EPOS PCNA Immunostaining system (U7032, DAKO, Carpinteria, CA, USA) and the proliferative index (Tilli *et al*., 2003) determined separately for the normal-appearing ducts and the areas of hyperplasia in the $Brca1^{Co/Co}$MMTV-Cre/p53 +/− female mice. ERα, ERβ and PR immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue sections using the mouse on mouse (M.O.M) peroxidase kit (PK-2200, Vector Laboratories Inc., Burlingame, CA, USA). Tissues sections were deparaffinized and rehydrated. For ERα immunohistochemistry, antigens were exposed to preheated 1 × High pH Target Retrieval Solution (S3307, Dako) for 25 min. For ERβ and PR immunohistochemistry, slides were pressure cooked for 4 min and immersed in preheated 1 × High pH Target Retrieval Solution (Dako). Slides were allowed to cool for an additional 20 min in the retrieval solution, quenched with 3% hydrogen peroxide, blocked with mouse IgG-blocking reagent, incubated with M.O.M diluent and a 1 : 25 dilution of the ERα antibody (IM2133, Beckman Coulter Immunotech, Miami, FL, USA) for 1 h at room temperature, or a 1 : 100 dilution of the ERβ antibody (GTX70174, GeneTex Inc., San Antonio, TX, USA) for overnight at 4°C, or a 1 : 100 dilution of the PR antibody (NCL-L-PGR-AB, Novacastra, UK) for 1 h at room temperature. The tissues were exposed to M.O.M biotinylated anti-mouse IgG reagent for 10 min at room temperature, M.O.M elite stain for 30 min, then stained with diaminobenzidine peroxidase substrate kit (SK-4100, Vector Laboratories Inc.) for 5 min and counterstained with hematoxylin, and then mounted with GVA-mount (Vector Laboratories Inc.). Digital photographs were taken using the Nikon Eclipse E800M microscope setup with Nikon DMX1200 software (Nikon Instruments Inc., Melville, NY, USA).

Immunoblot analysis and semiquantitative RT–PCR of mammary tissue

Frozen mammary adenocarcinoma was homogenized in protein lysis buffer to extract whole proteins ($n=17$). Protein concentration was quantified by bicinchoninic protein assay (Pierce, Rockford, IL, USA). Protein (40 μg) from each sample was solubilized in sample dilution buffer, separated by denaturing 8% Tris-glycine gels (Invitrogen Life Technologies, Carlsbad, CA, USA) and electrophoretically transferred to polyvinylidene difluoride membranes for immunoblot analysis. Membranes were blocked in 5% nonfat dry milk in Tris-buffered saline and 1% Tween (TBS-T) overnight at 4°C. Membranes were incubated with primary antibodies against ErbB2 (sc-284, Santa Cruz, Santa Cruz, CA, USA), EGFR (sc-03, Santa Cruz) or RXRα (sc-553, Santa Cruz) for 1 h at room temperature and washed with TBS-T. Bound antibody was detected by incubation with a 1 : 5000 dilution of a horseradish peroxidase-linked anti-rabbit secondary antibody (sc-2004; Santa Cruz) for 1 h at room temperature. Immunoreactive protein was detected with the enhanced chemiluminescence (ECL) plus Western blotting detection kit (RPN2133, Amersham Biosciences, Piscataway, NJ, USA). Total RNA was isolated by Trizol extraction (Invitrogen, Carlsbad, CA, USA) from mammary gland tissue snap-frozen at the time of necropsy, quantified on a spectrophotometer and cDNA synthesis performed. PR mRNA was detected using forward primer (5′-CATGTCAGTGGACAGATGCT-3′) and reverse primer (5′-ACTTCAGACATCATTTCCGG-3′).

siRNA treatment and immunoblot analysis of MCF-7 cells

Subconfluent proliferating MCF-7 cells were treated with BRCA1-siRNA, control-siRNA (50 nM × 72 h) or no siRNA (vehicle only) before being harvested and subjected to Western blotting to detect BRCA1, ERα or α-actin. The sequences of the siRNAs used were: BRCA1-siRNA-3 (5′-AATGC-CAAAGTAGCTAATGTA-3′) and scrambled-sequence control siRNA (5′-GTCACGATAAGACAATGATAT-3′) (Xiong *et al*., 2003). All siRNAs were chemically synthesized by Dharmacon Inc. For siRNA treatments, subconfluent proliferating cells were treated with each siRNA (50 nM), using siPORT Amine reagent (Ambion). The cells were incubated with siRNA for 72 h (to reduce BRCA1 protein levels to <25% of control) prior to the start of the experiment. The control siRNA has no effect on BRCA1 levels (Xiong *et al*., 2003); neither siRNA is toxic to the cells under these experimental conditions, as determined using MTT assays. Equal aliquots of total protein (50 μg/lane) were electrophoresed on a 4–13% SDS–polyacrylamide gradient gel, transferred to nitrocellulose membranes (Millipore) and blotted using primary antibodies directed against human BRCA1 (sc-642, rabbit polyclonal, Santa Cruz, 1 : 200), ERα (sc-8002, mouse monoclonal, Santa Cruz, 1 : 400) and α-actin (sc-1616, goat polyclonal, Santa Cruz, 1 : 500). After incubation with the appropriate horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences), immune complexes were visualized by using the ECL detection system (Amersham Biosciences), with colored markers (BioRad, Hercules, CA, USA) as molecular size standards.

ER transcriptional assays

Subconfluent proliferating MCF-7 cells in 24-well dishes were pretreated with BRCA1-siRNA, control (scrambled-sequence) siRNA (50 nM × 72 h) or no siRNA (vehicle only); the cells were then transfected overnight (using Lipofectamine™, Invitrogen Life Technologies) with the estrogen-responsive

luciferase reporter ERE-thymidine kinase (TK)-luciferase gene (Luc), which consists of the vitellogenin A2 ERE upstream of a minimal TK promoter and Luc (0.25 μg plasmid DNA per well) as described previously (Fan *et al*., 2002). The cells were then washed, allowed to recover for a few hours and then treated $\pm$17-β estradiol (E2) (10 nM) and $\pm$tamoxifen (10, 100 and 1000 nM) for 24 h in serum-free phenolphthalein-free DMEM. The cells were then harvested for luciferase assays. Luciferase values expressed relative to the absolute control value (i.e. no siRNA, no E2 or tamoxifen). Values are means $\pm$ s.e.m. for $n=4$ replicate wells. A second independent experiment gave similar results.

Statistical analyses

Nonparametric data were analysed using Kruskal–Wallis, Mann–Whitney and χ^2 tests. The time to first palpable tumor in the different groups was compared using a Kaplan–Meier test. $P<0.05$ was considered statistically significant.

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References

Bachelier R, Li C, Xu X, Lubet R and Deng CX. (2002). *Proc. Am. Assoc. Cancer Res.*, **43,** 512.
Bischoff ED, Heyman RA and Lamph WW. (1999). *J. Natl. Cancer Inst.*, **91,** 2118–2123.
Brodie SG, Xu X, Qiao W, Li WM, Cao L and Deng CX. (2001). *Oncogene*, **20,** 7514–7523.
Brown PH and Lippman SM. (2000). *Breast Cancer Res. Treat.*, **62,** 1–17.
Cavalli LR, Singh B, Isaacs C, Dickson RB and Haddad BR. (2004). *Cancer Genet. Cytogenet.*, **149,** 38–43.
Cheng G, Weihua Z, Warner M and Gustafsson JA. (2004). *Proc. Natl. Acad. Sci. USA*, **101,** 3739–3746.
Chlebowski RT. (2002). *Annu. Rev. Med.*, **53,** 519–540.
Donehower LA, Harvey M, Slagle BL, McArthur MJ, Montgomery Jr CA, Butel JS and Bradley A. (1992). *Nature*, **356,** 215–221.
Dutertre M and Smith CL. (2000). *J. Pharmacol. Exp. Ther.*, **295,** 431–437 ; 6.
Fan S, Ma YX, Wang C, Yuan RQ, Meng Q, Wang JA, Erdos M, Goldberg ID, Webb P, Kushner PJ, Pestell RG and Rosen EM. (2001). *Oncogene*, **20,** 77–87.
Fan S, Ma YX, Wang C, Yuan RQ, Meng Q, Wang JA, Erdos M, Goldberg ID, Webb P, Kushner PJ, Pestell RG and Rosen EM. (2002). *Cancer Res.*, **62,** 141–151.
Fan S, Wang J, Yuan R, Ma Y, Meng Q, Erdos MR, Pestell RG, Yuan F, Auborn KJ, Goldberg ID and Rosen EM. (1999). *Science*, **284,** 1354–1356.
Foulkes WD, Metcalfe K, Sun P, Hanna WM, Lynch HT, Ghadirian P, Tung N, Olopade OI, Weber BL, McLennan J, Olivotto IA, Begin LR and Narod SA. (2004). *Clin. Cancer Res.*, **10,** 2029–2034.
Gallo MA and Kaufman D. (1997). *Semin. Oncol.*, **24,** S1.
Graham JD, Bain DL, Richer JK, Jackson TA, Tung L and Horwitz KB. (2000). *J. Steroid Biochem. Mol. Biol.*, **74,** 255–259.
Grubbs CJ, Hill DL, Bland KI, Beenken SW, Lin TH, Eto I, Atigadda VR, Vines KK, Brouillette WJ and Muccio DD. (2003). *Cancer Lett.*, **201,** 17–24.
Heldring N, Nilsson M, Buehrer B, Treuter E and Gustafsson JA. (2004). *Mol. Cell. Biol.*, **24,** 3445–3459.
Jordan VC. (2004). *Cancer Cell*, **5,** 207–213.
Kadouri L, Kote-Jarai Z, Easton DF, Hubert A, Hamoudi R, Glaser B, Abeliovich D, Peretz T and Eeles RA. (2004). *Int. J. Cancer*, **108,** 399–403.
Kauff ND, Satagopan JM, Robson ME, Scheuer L, Hensley M, Hudis CA, Ellis NA, Boyd J, Borgen PI, Barakat RR, Norton L, Castiel M, Nafa K and Offit K. (2002). *N. Engl. J. Med.*, **346,** 1609–1615.
King MC, Wieand S, Hale K, Lee M, Walsh T, Owens K, Tait J, Ford L, Dunn BK, Costantino J, Wickerham L, Wolmark N and Fisher B. (2001). *JAMA*, **286,** 2251–2256.
Kotoula V, Karkavelas G, Economou L, Sionga A, Boutis L and Kerameos-Foroglou C. (1993). *Histol. Histopathol.*, **8,** 627–636.
Kurokawa H and Arteaga CL. (2003). *Clin. Cancer Res.*, **9** (Part 2)**,** 511S–515S.
Li M, Hu J, Heermeier K, Hennighausen L and Furth PA. (1996). *Cell Growth Differ.*, **7,** 13–20.
Lonard DM, Tsai SY and O'Malley BW. (2004). *Mol. Cell. Biol.*, **24,** 14–24.
McDonnell DP, Wijayaratne A, Chang CY and Norris JD. (2002). *Am. J. Cardiol.*, **90,** 35F–43F.
Meijers-Heijboer H, van Geel B, van Putten WL, Henzen-Logmans SC, Seynaeve C, Menke-Pluymers MB, Bartels CC, Verhoog LC, van den Ouweland AM, Niermeijer MF, Brekelmans CT and Klijn JG. (2001). *N. Engl. J. Med.*, **345,** 159–164.
Menard S, Aiello P, Tagliabue E, Rumio C, Lollini PL, Colnaghi MI and Balsari A. (2000). *Cancer Res.*, **60,** 273–275.
Metcalfe K, Lynch HT, Ghadirian P, Tung N, Olivotto I, Warner E, Olopade OI, Eisen A, Weber B, McLennan J, Sun P, Foulkes WD and Narod SA. (2004). *J. Clin. Oncol.*, **22,** 2328–2335.
Moller P, Borg A, Evans DG, Haites N, Reis MM, Vasen H, Anderson E, Steel CM, Apold J, Goudie D, Howell A, Lalloo F, Maehle L, Gregory H and Heimdal K. (2002). *Int. J. Cancer*, **101,** 555–559.
Mote PA, Leary JA, Avery KA, Sandelin K, Chenevix-Trench G, Kirk JA and Clarke CL. (2004). *Genes Chromosomes Cancer*, **39,** 236–248.
Narod SA, Brunet JS, Ghadirian P, Robson M, Heimdal K, Neuhausen SL, Stoppa-Lyonnet D, Lerman C, Pasini B, de los RP, Weber B and Lynch H. (2000). *Lancet*, **356,** 1876–1881.
Osborne CK and Schiff R. (2003). *Breast*, **12,** 362–367.
Osborne CK, Bardou V, Hopp TA, Chamness GC, Hilsenbeck SG, Fuqua SA, Wong J, Allred DC, Clark GM and Schiff R. (2003). *J. Natl. Cancer Inst.*, **95,** 353–361.
Osborne MP, Telang NT, Kaur S and Bradlow HL. (1990). *Steroids*, **55,** 114–119.
Plotkin D, Lechner JJ, Jung WE and Rosen PJ. (1978). *JAMA*, **240,** 2644–2646.
Rebbeck TR, Lynch HT, Neuhausen SL, Narod SA, Van't Veer L, Garber JE, Evans G, Isaacs C, Daly MB, Matloff E, Olopade OI and Weber BL. (2002). *N. Engl. J. Med.*, **346,** 1616–1622.
Rebbeck TR, Wang Y, Kantoff PW, Krithivas K, Neuhausen SL, Godwin AK, Daly MB, Narod SA, Brunet JS, Vesprini D, Garber JE, Lynch HT, Weber BL and Brown M. (2001). *Cancer Res.*, **61,** 5420–5424.
Reddel RR and Sutherland RL. (1984). *Eur. J. Cancer Clin. Oncol.*, **20,** 1419–1424.

Reiter R, Oh AS, Wellstein A and Riegel AT. (2004). *Oncogene*, **23,** 403–409.
Rendi MH, Suh N, Lamph WW, Krajewski S, Reed JC, Heyman RA, Berchuck A, Liby K, Risingsong R, Royce DB, Williams CR and Sporn MB. (2004). *Cancer Res.*, **64,** 3566–3571.
Robson M. (2002). *Eur. J. Cancer*, **38** (Suppl 6)**,** S18–S19.
Saji S, Jensen EV, Nilsson S, Rylander T, Warner M and Gustafsson JA. (2000). *Proc. Natl. Acad. Sci. USA*, **97,** 337–342.
Schrag D, Kuntz KM, Garber JE and Weeks JC. (2000). *JAMA*, **283,** 617–624.
Smith CL and O'Malley BW. (2004). *Endocr. Rev.*, **25,** 45–71.
Sporn MB and Suh N. (2000). *Carcinogenesis*, **21,** 525–530.
Tilli MT, Frech MS, Steed ME, Hruska KS, Johnson MD, Flaws JA and Furth PA. (2003). *Am. J. Pathol.*, **163,** 1713–1719.
Veldhuis JD and Santen RJ. (1979). *JAMA*, **241,** 2506–2507.
Wagner KU, McAllister K, Ward T, Davis B, Wiseman R and Hennighausen L. (2001). *Transgenic Res.*, **10,** 545–553.
Wagner KU, Wall RJ, St Onge L, Gruss P, Wynshaw-Boris A, Garrett L, Li M, Furth PA and Hennighausen L. (1997). *Nucleic Acids Res.*, **25,** 4323–4330.
Xiong J, Fan S, Meng Q, Schramm L, Wang C, Bouzahza B, Zhou J, Zafonte B, Goldberg ID, Haddad BR, Pestell RG and Rosen EM. (2003). *Mol. Cell. Biol.*, **23,** 8668–8690.
Xu X, Wagner KU, Larson D, Weaver Z, Li C, Ried T, Hennighausen L, Wynshaw-Boris A and Deng CX. (1999). *Nat. Genet.*, **22,** 37–43.
Yang X, Edgerton SM, Kosanke SD, Mason TL, Alvarez KM, Liu N, Chatterton RT, Liu B, Wang Q, Kim A, Murthy S and Thor AD. (2003). *Cancer Res.*, **63,** 2425–2433.
Yoshidome K, Shibata MA, Couldrey C, Korach KS and Green JE. (2000). *Cancer Res.*, **60,** 6901–6910.
Zheng L, Annab LA, Afshari CA, Lee WH and Boyer TG. (2001). *Proc. Natl. Acad. Sci. USA*, **98,** 9587–9592.