

Molecular Basis for Estrogen Receptor α Deficiency in BRCA1-Linked Breast Cancer

Alison M. Hosey, Julia J. Gorski, Margaret M. Murray, Jennifer E. Quinn, Wen Y. Chung, Gail E. Stewart, Colin R. James, Susan M. Farragher, Jude M. Mulligan, Alistair N. Scott, Peter A. Dervan, Patrick G. Johnston, Fergus J. Couch, Peter A. Daly, Elaine Kay, Amanda McCann, Paul B. Mullan, D. Paul Harkin

- Background** BRCA1-mutant breast tumors are typically estrogen receptor alpha (ER α) negative, whereas most sporadic tumors express wild-type BRCA1 and are ER α positive. We examined a possible mechanism for the observed ER α -negative phenotype of BRCA1-mutant tumors.
- Methods** We used a breast cancer disease-specific microarray to identify transcripts that were differentially expressed between paraffin-embedded samples of 17 BRCA1-mutant and 14 sporadic breast tumors. We measured the mRNA levels of estrogen receptor 1 (ESR1) (the gene encoding ER α), which was differentially expressed in the tumor samples, by quantitative polymerase chain reaction. Regulation of ESR1 mRNA and ER α protein expression was assessed in human breast cancer HCC1937 cells that were stably reconstituted with wild-type BRCA1 expression construct and in human breast cancer T47D and MCF-7 cells transiently transfected with BRCA1-specific short-interfering RNA (siRNA). Chromatin immunoprecipitation assays were performed to determine if BRCA1 binds the ESR1 promoter and to identify other interacting proteins. Sensitivity to the antiestrogen drug fulvestrant was examined in T47D and MCF-7 cells transfected with BRCA1-specific siRNA. All statistical tests were two-sided.
- Results** Mean ESR1 gene expression was 5.4-fold lower in BRCA1-mutant tumors than in sporadic tumors (95% confidence interval [CI] = 2.6-fold to 40.1-fold, $P = .0019$). The transcription factor Oct-1 recruited BRCA1 to the ESR1 promoter, and both BRCA1 and Oct-1 were required for ER α expression. BRCA1-depleted breast cancer cells expressing exogenous ER α were more sensitive to fulvestrant than BRCA1-depleted cells transfected with empty vector (T47D cells, the mean concentration of fulvestrant that inhibited the growth of 40% of the cells [IC₄₀] for empty vector versus ER α : $>10^{-5}$ versus 8.0×10^{-9} M [95% CI = 3.1×10^{-10} to 3.2×10^{-6} M]; MCF-7 cells, mean IC₄₀ for empty vector versus ER α : $>10^{-5}$ versus 4.9×10^{-8} M [95% CI = 2.0×10^{-9} to 3.9×10^{-6} M]).
- Conclusions** BRCA1 alters the response of breast cancer cells to antiestrogen therapy by directly modulating ER α expression.

J Natl Cancer Inst 2007;99:1683–94

Germline mutations in the BRCA1 tumor suppressor gene confer a genetic predisposition to breast and ovarian cancers (1). A substantial body of evidence suggests that, in addition to its roles in DNA damage response and the ubiquitination pathway, BRCA1 is directly involved in transcriptional regulation. For example, the carboxyl terminus of BRCA1 can act as a transcriptional activator when fused to a heterologous DNA-binding domain (2,3). BRCA1 was later shown to be a component of the RNA polymerase II holoenzyme complex via its interaction with RNA helicase A (4) and to act as either a coactivator or a corepressor of transcription through its interactions with a number of well-characterized transcription factors [reviewed in (5)]. For example, BRCA1 can transcriptionally coactivate the CDKN1A and GADD45 genes through p53 and the 2'5'-oligoadenylate synthetase gene through STAT1 and transcriptionally corepress genes such as hTERT, ANG1, and S100A7 through interactions with C-Myc (5,6). The ability of BRCA1 to act as either a transcriptional coactivator or corepressor may be related to its ability to

Affiliations of authors: Centre for Cancer Research and Cell Biology, Queen's University Belfast, Belfast, Northern Ireland, U.K. (AMH, JJJ, MMM, JEQ, GES, CRJ, PGJ, PBM, DPH); School of Medicine and Medical Science (P.A. Dervan, WYC, AM), and the Conway Institute of Biomolecular and Biomedical Science (WYC, AM), University College Dublin, Dublin, Ireland; Department of Pathology, Mater Misericordiae Hospital, Dublin, Ireland (P. A. Dervan); Department of Laboratory Medicine and Pathology, Mayo Clinic College of Medicine, Rochester, MN (FJC); Department of Haematology and Oncology, Trinity College, St James's Hospital, Dublin, Ireland (P. A. Daly); Department of Histopathology, Education and Research Centre, Beaumont Hospital, Royal College of Surgeons in Ireland, Dublin, Ireland (EK); Almac Diagnostics, Craigavon, U.K. (SMF, JMM, ANS).

Correspondence to: D. Paul Harkin, PhD, Centre for Cancer Research and Cell Biology, Queen's University Belfast, 97 Lisburn Rd, Belfast, Northern Ireland, BT9 7BL, U.K. (e-mail: d.harkin@qub.ac.uk).

See "Funding" and "Notes" following "References."

DOI: 10.1093/jnci/djm207

© The Author 2007. Published by Oxford University Press. All rights reserved. For Permissions, please e-mail: journals.permissions@oxfordjournals.org.

CONTEXT AND CAVEATS

Prior knowledge

BRCA1-mutant breast tumors are typically estrogen receptor alpha (ER α) negative, whereas most sporadic tumors express wild-type BRCA1 and are ER α positive. The molecular basis for the ER α -negative phenotype of BRCA1-mutant tumors has not been defined.

Study design

In vitro studies in human breast cancer cells were used to examine the mechanism for the disparity in ER α expression identified by molecular profiling of 17 BRCA1-mutant and 14 sporadic human breast tumors. The sensitivity of human breast cancer cells to the antiestrogen drug fulvestrant was examined as function of BRCA1 expression.

Contribution

BRCA1-mutant tumors fail to express ER α due to the loss of BRCA1-mediated transcriptional activation of the gene encoding ER α . Expression of exogenous ER α in BRCA1-depleted breast cancer cells increased their sensitivity to fulvestrant.

Implications

BRCA1 alters the response of breast cancer cells to antiestrogen therapy by directly modulating ER α expression.

Limitations

A small number of human breast tumors were used for molecular profiling. The mechanism by which some of the BRCA1-mutant tumors retained ER α positivity is not known.

interact with the basal transcription machinery as well as with proteins that are involved in chromatin remodeling (7,8).

BRCA1-mutant breast tumors exhibit a distinct pathologic phenotype and typically do not express estrogen receptor alpha (ER α) (9,10). Furthermore, gene expression profiling has revealed that BRCA1-mutant breast tumors cluster with the ER α -negative basal subtype of breast cancer (11). The molecular basis for the ER α -negative phenotype of BRCA1-mutant tumors has not been defined. However, evidence exists for a functional link between BRCA1 and estrogen signaling pathways. For example, BRCA1 has been shown to inhibit ER α signaling, which results in the negative regulation of expression of downstream genes [reviewed in (12)]. BRCA1 has also been reported to regulate estrogen synthesis through transcriptional inhibition of the gene encoding aromatase, the rate-limiting enzyme in estrogen biosynthesis (13). In this study, we examined further the relationship between BRCA1 and ER α levels to identify the mechanism of ER α deficiency in BRCA1-mutant breast tumors.

Materials and Methods

Tumor Samples and Cell Lines

We used formalin-fixed, paraffin-embedded samples of 17 BRCA1-mutant breast tumors and 14 sporadic breast tumors. Breast tumor samples used in this study were from surgical resections performed at the Mayo Clinic (Rochester, MN; nine BRCA1 mutant, seven sporadic) or Beaumont Hospital (Dublin, Ireland; eight BRCA1 mutant, seven sporadic) and were obtained following approval by the respective ethical committee of each institution. Written

informed consent was obtained from all patients who provided tissue. All breast tumor samples were confirmed to have a tumor content of at least 50% and were age and stage matched. The optimal matching approach developed by Rosenbaum (14) was used to match three candidate control subjects with sporadic breast cancer to each case subject with BRCA1-mutant breast cancer. Control subjects had the same stage of disease (I–IV) and nodal status (negative versus positive nodes) as each case subject. Control subjects with age at surgery closest to case subjects were then chosen.

The human breast cancer HCC1937 cell line is p53 mutant and ER α negative and expresses a mutated BRCA1 protein (5382insC) that lacks the last 34 amino acids (15). HCC1937 cells were obtained from the European Collection of Cell Cultures (ECACC; Wiltshire, U.K.) and were grown in RPMI-1640 medium (Invitrogen, Paisley, U.K.) supplemented with 20% fetal calf serum (FCS), 1 mM sodium pyruvate, and 50 μ g/mL penicillin–streptomycin (Invitrogen). HCC-EV and HCC-BR cell lines were generated by stable transfection of HCC1937 cells with empty vector (Rc-CMV; Invitrogen, Paisley, U.K.) or a Rc-CMV-BRCA1 plasmid that expresses full-length wild-type BRCA1 under control of the cytomegalovirus promoter. Transfected cells were grown in medium that contained 0.2 mg/mL of geneticin (G418; Sigma, Dorset, U.K.) to select for the neomycin resistance gene present on the Rc-CMV plasmid. Polymerase chain reaction was performed to detect the presence of BRCA1-containing vector in clones of stably transfected cells using a forward primer to BRCA1 (BRCA1-F, 5'-AGGAGCTTTTCATCATTCACCC-3') and a reverse primer to the Rc-CMV 3' untranslated region (RcCMV-R, 5'-AACTAGAAGGCACAGTCGAGG-3'). HCC1937 cells stably transfected with Rc-CMV (HCC-EV cells) or Rc-CMV-BRCA1 (HCC-BR cells) were grown in medium that contained 0.2 mg/mL G418. Human breast cancer T47D and MCF-7 cell lines express wild-type BRCA1 and ER α and were maintained as previously described (16). Briefly, MCF-7 cells (obtained from ECACC) and T47D cells (obtained from Cancer Research UK, London) were grown in Dulbecco's modified Eagle medium (Invitrogen) or RPMI-1640, respectively, supplemented with 10% FCS, 50 μ g/mL penicillin–streptomycin, and 1 mM sodium pyruvate (Invitrogen). All cells were grown in 5% CO₂ in a humidified incubator.

Gene Expression Profiling of Tumor Samples

Total RNA was extracted from one 10- μ m section from each of the 31 formalin-fixed, paraffin-embedded breast tumors with the use of an RNeasy FFPE kit (Qiagen, Crawley, U.K.), according to the manufacturer's instructions. GeneChip Two-Cycle Target Labeling and Control Reagents (Affymetrix, Santa Clara, CA) were used to convert total RNA to complementary DNA (cDNA) and to transcribe the cDNA in vitro to generate biotinylated complementary RNA (cRNA) targets. We used a GeneChip Instrument System (Affymetrix) to fragment the biotinylated cRNA targets and to hybridize the targets to a Breast Cancer DSA microarray (Almac Diagnostics, Craigavon, U.K.), which contains 60856 probe sets that correspond to approximately 60 000 transcripts that have been shown to be expressed in both breast cancer and normal tissue. The hybridized cRNA was stained, washed, and scanned using the GeneChip Scanner 7G (Affymetrix) according to the manufacturer's instructions.

Microarray Data Analysis and Identification of Differentially Expressed Genes

Microarray experiments were performed once for each tumor sample. The scanned intensity for each probe set was saved to Affymetrix CEL files (available at <http://www.ebi.ac.uk/arrayexpress/experiments/E-TABM-366>). Gene expression indices were computed from these files with the use of dChip software (17) by using invariant set normalization and a perfect match-only model.

To detect differential gene expression between BRCA1-mutant and sporadic breast tumor samples, the following criteria were applied: 1) Student's unpaired *t* test with statistical significance defined as a *P* value less than .05; 2) at least a twofold difference in gene expression between BRCA1-mutant and sporadic samples; and 3) any differentially expressed genes had to be classified "present" by the MAS5 algorithm (18) in at least 20% of the samples. The last criterion was implemented to lessen the incidence of spurious (unreliable) discovery of differentially expressed genes. Affymetrix control probe sets were discarded from the analysis. Combined application of these criteria to the gene expression data resulted in detection of 636 differentially expressed genes.

Reverse Transcription–Polymerase Chain Reaction Amplification of Estrogen Receptor 1 mRNA From Tumor Samples

Total RNA was extracted from each of the 31 breast tumor samples as described above and reverse transcribed into cDNA with the use of random primers and Moloney murine leukemia virus reverse transcriptase (Invitrogen), according to the manufacturer's instructions. To validate the high level of differential expression observed with estrogen receptor 1 (ESR1) mRNA between sporadic and BRCA1-mutant tumors in the microarray data, cDNAs were subjected to quantitative real-time polymerase chain reaction (qRT-PCR) amplification using primers that were designed to amplify the 3' end of the ESR1 transcript and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript in independent experiments. ESR1 mRNA levels were quantified by normalization to GAPDH mRNA. The ESR1 primers were 5'-GGTGCCTGAGACACAGACC-3' (forward) and 5'-GTGAGAGAACAGAACTGGC-3' (reverse); the GAPDH primers were 5'-AGGTGGTCTCCTCTGACTTCAA-3' (forward) and 5'-CACCCTGTTGCTGTAGCCAAATTC-3' (reverse). qRT-PCR assays were performed with the use of a QuantiTect SYBR Green PCR kit (Qiagen), according to the manufacturer's instructions, in a DNA Engine Opticon thermal cycler (Bio-Rad, Hertfordshire, U.K.) and were analyzed with the use of Opticon Monitor 3.1 software (Bio-Rad).

Northern Blot Analysis

Total RNA was extracted from HCC1937, T47D, and MCF-7 cells with the use of RNA STAT60 reagent (Tel-Test Inc, Friendswood, TX) and analyzed for ESR1 mRNA expression by northern blotting, as previously described (16). Briefly, 20 µg of RNA was subjected to electrophoresis on a 1% formaldehyde agarose denaturing gel for 3 hours at 70 V. The 18S and 28S ribosomal bands were visualized on a transilluminator (Stratagene, Amsterdam, The Netherlands) under UV light (260 nm). The gel was then washed for 20 minutes in 0.05 M NaOH, rinsed in diethylpyrocarbonate-treated water, and equilibrated in 20× SSC (sodium chloride and trisodium citrate solution;

1× SSC is 0.15 M sodium chloride and 0.015 M sodium citrate) for 30 minutes, and the RNA was transferred to nitrocellulose Hybond-N membrane (Amersham, Buckinghamshire, U.K.) by capillary action in 20× SSC overnight at room temperature. The RNA was cross-linked to the membrane using UV light (Stratalinker 2400, Stratagene), and the membrane was incubated overnight at 65 °C in northern hybridization buffer (0.25 M Na₂HPO₄ [pH 7.2], 7% sodium dodecyl sulfate [SDS]) with ESR1, and GAPDH ³²P-dCTP-labeled radioactive probes, sequentially. The probe for ESR1 RNA was generated by *Xba*I restriction digestion of an ERα pcDNA3.1/Hygro expression construct (a gift from Professor D. McDonnell, Duke University Medical Center, Durham, NC). The probe for GAPDH RNA was a 250-bp oligonucleotide that was generated by PCR amplification using a fetal brain cDNA library (Invitrogen, Paisley, U.K.) as template and the following primers: GAPDH S 5'-ACCACAGTCCATGCCATCAC-3' and GAPDH AS 5'-TCCACCACCCTGTTGCTGTA-3'. Probes were radiolabeled with the use of a Megaprime DNA Labeling System (Amersham) and ³²P-αdCTP (Amersham). The probes were purified with the use of Microcon filters (Millipore, Billerica, MA), denatured by heating to 95 °C for 5 minutes, and added to the membrane for overnight hybridization as described above. The hybridized membranes were washed twice for 20 minutes at 65 °C with low-stringency wash buffer (20 mM Na₂HPO₄ [pH 7.2], 5% SDS) and once with high-stringency wash buffer (20 mM Na₂HPO₄ [pH 7.2], 1% SDS) and then exposed to film at -80 °C for 24 hours for autoradiography.

Immunoblot Analysis

Immunoblot analysis was performed as previously described (19). Briefly, total cellular protein extracts were prepared by adding 200 µL lysis buffer (0.25 M NaCl, 0.1% Igepal, 0.25 M HEPES, 5 mM EDTA, 0.5 mM dithiothreitol [DTT]) to a 90-mm plate containing HCC1937, T47D, or MCF-7 cells, and the lysed cells were harvested with a cell scraper. Each cell lysate was passed through a 21-gauge needle five times, followed by incubation on ice for 10 minutes to ensure adequate lysis. The lysates were centrifuged at 15 000*g* for 15 minutes at 4 °C to pellet the cell debris. The supernatant was stored at -20 °C. Protein concentration in the supernatant was quantified with the use of a Bio-Rad protein assay kit. Equal amounts of protein (typically 60 µg per lane) were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE). Following electrophoresis, the proteins were transferred onto Hybond-P polyvinylidene difluoride membrane (Amersham). Transfer was carried out at 100 V for 2 hours. Following transfer, the Hybond-P membrane was incubated in blocking solution (5% milk [Marvel] in 1× TBST (5 M NaCl, 1 M Tris [pH 7.4], 1 M KCl, 0.1% Tween 20) for 1 hour. Primary antibody to the protein of interest was added to 10 mL of blocking solution. The primary mouse monoclonal antibodies BRCA1 (D-9; Santa Cruz Biotechnology) and GAPDH (6G5; Sigma, Dorset, U.K.) and the rabbit polyclonal antibodies ERα (HC-20) and Oct-1 (C-21) (both from Santa Cruz Biotechnology) were used. BRCA1 and Oct-1 antibodies were used at a dilution of 1:100; ERα was used at a dilution of 1:1000; and GAPDH was used at a dilution of 1:5000. Membranes were washed, and horseradish peroxidase-conjugated secondary antibodies (Amersham) were added (at 1:2000 dilution). The membrane was washed extensively, then subjected to a chemiluminescence

detection agent (Amersham) for 5 minutes. The membrane was then analyzed by autoradiography.

Coimmunoprecipitation

Protein cell lysates were collected as described above. Equal amounts of protein (500 µg) were preincubated with 80 µL of 50% protein G-sepharose slurry (Amersham) pre-equilibrated in IP buffer (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 10% [vol/vol] glycerol, 0.1% [vol/vol] Tween 20, 1 mM DTT, and protease inhibitor cocktail) for 30 minutes at 4 °C with gentle rocking to pre-clear the samples. The samples were centrifuged briefly at 1500g, and the supernatants were collected and incubated with 1 µg of the appropriate antibody for 3 hours or overnight at 4 °C with gentle rocking. The sample volume was increased to 500 µL with IP buffer, 80 µL of pre-equilibrated protein G slurry was added, and the samples were rocked gently for 1 hour. The samples were washed five times for 3 minutes each with 500 µL IP buffer. After the final wash, the protein G beads were resuspended in 20 µL of SDS sample buffer and boiled for 3 minutes. The samples were resolved by SDS-PAGE as described above and subjected to immunoblot analysis to assess complex status. The antibodies used for immunoprecipitation or immunoblotting were described above.

Gene Silencing by Short-Interfering RNA

Short-interfering RNA (siRNA) transfection of T47D and MCF-7 cells was performed using Oligofectamine reagent (Invitrogen) as outlined in the manufacturer's instructions. Each siRNA oligonucleotide was used at a concentration of 100 nM, and protein or RNA was extracted from the transfected cells 48 hours later, as described above, and used for immunoblot or northern blot analysis, respectively. The respective siRNA sequences are as follows: BRCA1 mRNA (5'-GCGUGCAGCUGAGAGGCAU-3'), Oct-1 mRNA (5'-CCAGCAGCUCACCUAUUAA-3'), ESR1 mRNA (5'-UCAUCGCAUUCUUGCAAA-3'), scrambled-sequence control siRNA (5'-CCUGGUAGCAGCGAGUCAG-3').

Luciferase Reporter Assay

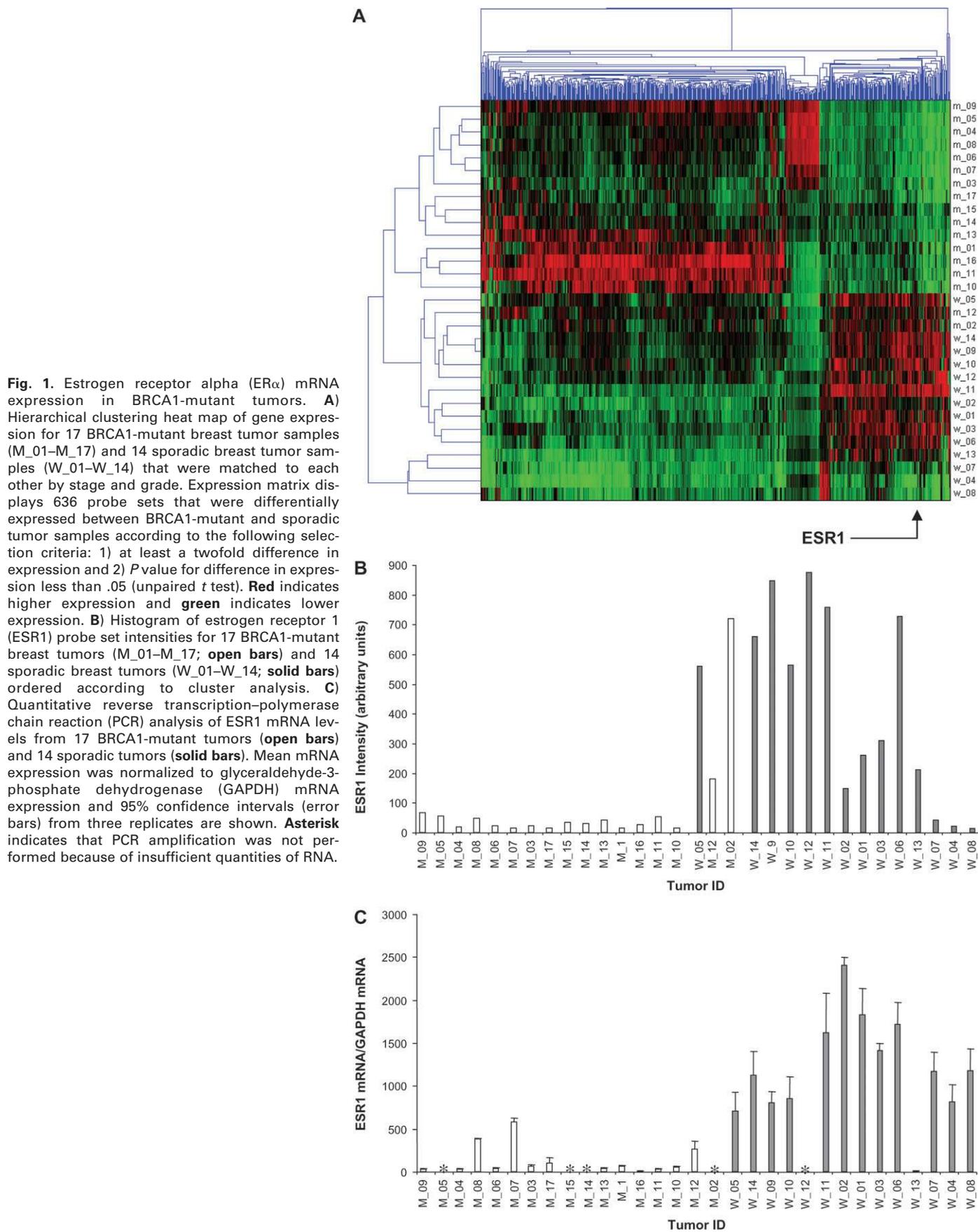
The luciferase reporter assay was performed with the use of a Dual-Luciferase Assay Kit (Promega, Southampton, U.K.), according to manufacturer's instructions. T47D and MCF-7 cells were transfected with scrambled control or BRCA1 siRNA. After 24 hours, cells were trypsinized and seeded into 6-well plates. The following day, cells were transfected with either control pGL3-basic vector or ERα ProAB promoter-firefly plasmid construct (a gift from Dr R. Kiyama, AIST Central 6, Tsukuba, Ibaraki, Japan) with the use of GeneJuice transfection reagent (Novagen, Nottingham, U.K.) in complete medium as recommended. In each case, a control plasmid expressing *Renilla* luciferase (at a ratio of 1:10) was cotransfected. The cells were harvested 24 hours after transfection, and luciferase reporter activity was expressed as the fold change following normalization to *Renilla* luciferase activity. Each transfection was performed in triplicate.

Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation assays were performed as previously described (19). Briefly, HCC-EV, HCC-BR, T47D, and MCF-7 cells were fixed in chromatin-fixing buffer (1.5% formalde-

hyde in phosphate-buffered saline [PBS]) to cross-link protein bound to DNA. Cells were collected following two washings in PBS in 300 µL of cold cell collection buffer (100 mM Tris-HCl [pH 9.4] and 10 mM DTT) and incubated on ice for 15 minutes. Cells were then washed with PBS and lysed sequentially by resuspension and 5-minute centrifugation at 2000g at 4 °C with 1 mL NCP buffer 1 (10 mM EDTA, 0.5 mM EGTA, 10 mM HEPES [pH 6.5], 0.25% Triton X-100) and 1 mL NCP buffer 2 (1 mM EDTA, 0.5 mM EGTA, 10 mM HEPES [pH 6.5], 200 mM NaCl). Following centrifugation, NCP buffer 2 was removed and 1 mL of lysis buffer (10 mM EDTA, 20 mM Tris-HCl [pH 8.1], 0.5% Empigen BB, 1% SDS) was added to each pellet. The samples were sonicated three times for 10 minutes at high settings using a Diagenode Biorupter on 30-second on/off settings. The sonicated samples were then centrifuged for 10 minutes at 15000g. One percent of the supernatant was taken as input DNA, 10% was loaded onto a 0.6% agarose gel to determine the efficiency of the sonication, and the remainder of the sample was diluted 1:1.5 in IP buffer (2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl [pH 8.1], 1% Triton X-100).

The input DNA was incubated overnight at 65 °C to reverse the protein-DNA cross-links. The remainder of the nuclei-chromatin extract was pre-cleared by addition of rabbit preimmune serum (5 µL), goat preimmune serum (5 µL), and protein A/G sepharose bead slurry (80 µL of a 1:1 ratio of protein A and protein G beads; Amersham), with an equal volume of beads buffer (20 mM Tris-HCl [pH 8.1] and 1 mM EDTA) for 2 hours followed by centrifugation at 800g for 1 minute. The resultant supernatants were split into equal aliquots and each aliquot was immunoprecipitated overnight at 4 °C in IP buffer with 1 µg of one of the following antibodies: mouse monoclonal anti-RNA polymerase II (CTD4H8; Upstate, Hampshire, U.K.), mouse monoclonal anti-BRCA1 (Ab-1; Calbiochem, Nottingham, U.K.), rabbit polyclonal anti-Oct-1 (C-21), and rabbit polyclonal anti-ERα (HC-20) (the latter two from Santa Cruz Biotechnology, Santa Cruz, CA), followed by incubation with 80 µL of a 50% protein A/G slurry for 2 hours. Precipitated complexes were serially washed with 300 µL wash buffer 1 (2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl [pH 8.1], 0.1% SDS, 0.1% Triton X-100), 300 µL wash buffer 2 (2 mM EDTA, 250 mM NaCl, 20 mM Tris-HCl [pH 8.1], 0.1% SDS, 0.1% Triton X-100), 300 µL wash buffer 3 (1 mM EDTA, 20 mM Tris-HCl [pH 8.1], 1% deoxycholate, 1% NP40, 250 mM LiCl), and 1 ml wash buffer 4 (20 mM Tris-HCl [pH 8.1], 1 mM EDTA). Complexes were removed from the beads through three subsequent 15-minute incubations, standing vortexing (VXR-VIBRAX, Milian Labware, Gahanna, OH), and 5-minute centrifugations with 50 µL of extraction buffer (1% SDS, 0.1 M NaHCO₃) then incubated at 65 °C overnight to reverse the protein-DNA cross-links. The extracted DNA was purified with the use of a QIAquick PCR purification kit (Qiagen). PCR was performed using specific primers that were designed to amplify a 217-bp region of the ESR1 promoter that is located directly upstream of the transcription start site from the purified immunoprecipitated DNA. Primer sequences were as follows: ER PROM1 5'-AGGAGGGGGAATCAAACAGA-3' and ER PROM2 5'-TTTACTTGTCTCGCTGCTG-3'. PCR products from the purified DNA were visualized by electrophoresis on 2% agarose gels.



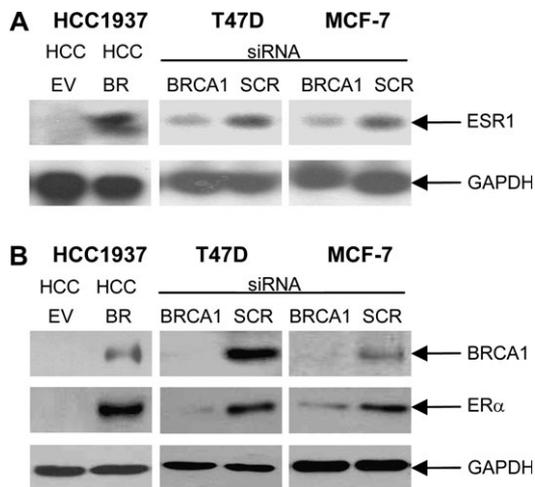


Fig. 2. Effect of BRCA1 expression on expression of estrogen receptor alpha (ER α). **A**) Northern blot analysis of estrogen receptor 1 (ESR1) mRNA expression in breast cancer HCC1937 cells transfected with empty vector (HCC-EV) or BRCA1 expression construct (HCC-BR) and in breast cancer T47D and MCF-7 cells following transfection with a short-interfering RNA (siRNA) oligonucleotide designed to inhibit expression of endogenous BRCA1 (BRCA1) or a scrambled-sequence control oligonucleotide (SCR). The blot was reprobed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression to confirm equal RNA loading and transfer. **B**) Immunoblot analysis of BRCA1, ER α , and GAPDH protein levels in HCC-EV and HCC-BR cells and in T47D and MCF-7 cells transfected with the BRCA1-targeting siRNA or a scrambled-sequence control siRNA.

For chromatin reimmunoprecipitation assays, after the primary immunoprecipitation, antibody–protein complexes were eluted for 30 minutes at 37 °C in 10 mM DTT with agitation, and then subjected to chromatin immunoprecipitation again with a second antibody of interest. For assays carried out with monoclonal antibodies, the appropriate secondary antibody was added for 1 hour before the addition of protein G sepharose. Antibodies and ESR1 primers used were those described above.

Antiestrogen Sensitivity Studies

T47D and MCF-7 cells were seeded at 60%–70% confluency in 90-mm culture plates. The following day, the cells were transfected with scrambled-sequence control or BRCA1-specific siRNA as described above. Twenty-four hours later, the siRNA-transfected cells were seeded at 1.5×10^4 cells per well in 24-well culture dishes for dose inhibition assays. Twenty-four hours later, the cells were treated with fulvestrant (ICI 182,780; Tocris Bioscience, Ballwin, MO) at concentrations that ranged from 10^{-5} M to 10^{-12} M. After 72 hours, the cells were harvested by treatment with trypsin and counted with the use of a Coulter Counter (Beckman-Coulter, Miami, FL) and the concentration of fulvestrant that inhibited the growth of 40% of the cells (IC $_{40}$) was calculated with the use of Prism software. All experiments were performed in triplicate. For all dose inhibition assays, cells were seeded in parallel in 60-mm plates (1.5×10^5 cells per plate) and incubated in medium lacking fulvestrant for protein extraction and immunoblotting to confirm that the expression of BRCA1 was reduced.

We also carried out dose inhibition assays in T47D and MCF-7 cells that transiently expressed exogenous ER α . T47D and MCF-7 cells were transfected with BRCA1-specific siRNA and 24 hours

later were seeded as described above. Six hours after seeding, the cells were transfected with empty pcDNA3.1 expression vector (Invitrogen) or ER α pcDNA3.1 expression vector (a kind gift from Professor D. McDonnell, Duke University Medical Center, Durham, NC) with the use of GeneJuice transfection reagent (Novagen), according to the manufacturer's instructions. Dose inhibition assays and protein extractions were performed 24 hours after transfection, as described above.

Results

Estrogen Receptor 1 mRNA Expression Levels in Breast Tumor Samples

We used a gene expression microarray that is based on the transcriptome of breast cancer to identify genes that were differentially expressed between 17 BRCA1-mutant breast tumors and 14 sporadic breast tumors. The BRCA1-mutant tumors were matched to the sporadic tumors by stage and grade. Of the 636 genes that displayed at least a twofold change in gene expression between BRCA1-mutant tumors and sporadic tumors, we focused on ESR1 because of the reported link between BRCA1 mutations and the loss of ER α expression. The mean ESR1 gene expression was 5.4-fold lower in BRCA1-mutant tumors than in sporadic tumors (95% confidence interval [CI] = 2.6-fold to 40.1-fold, $P = .0019$) (Fig. 1, A).

ESR1 probe set signal intensities derived from the array data for each tumor sample were represented on a histogram to better visualize the difference in ESR1 expression between the BRCA1-mutant tumors and the sporadic tumors (Fig. 1, B). We used qRT-PCR to confirm the microarray data for ESR1 and observed that in general, the BRCA1-mutant tumors had lower expression of ESR1 mRNA relative to GAPDH than the sporadic tumors (mean relative ESR1 expression [arbitrary units]: BRCA1-mutant tumors = 127.4 [95% CI = 23.0 to 231.8], sporadic tumors = 1200.5 [95% CI = 833.7 to 1567.0]). (Fig. 1, C). It is interesting to note that two of the BRCA1-mutant tumors, M_02 and M_12, displayed high levels of ESR1 mRNA expression and segregated with the sporadic tumors by unsupervised cluster analysis (Fig. 1, A), indicating that BRCA1-mutant tumors constitute a heterogeneous group with a minority showing positive expression of ESR1 and a gene expression profile similar to that of sporadic tumors.

Effect of Exogenous BRCA1 Expression on Estrogen Receptor α Levels in HCC1937 Cells

To further investigate whether the presence of a BRCA1 mutation is associated with reduced ER α expression, we examined the effect of reconstituting BRCA1 expression on ER α expression levels in BRCA1-mutant (and low ER α -expressing) HCC1937 breast cancer cells (15). Northern blot analysis confirmed that HCC1937 cells transfected with Rc-CMV BRCA1 (HCC-BR cells) had substantially higher ESR1 mRNA expression than cells transfected with empty vector (HCC-EV cells) (Fig. 2, A). Immunoblot analysis also confirmed that HCC-BR cells expressed higher levels of ER α protein than HCC-EV cells. Therefore, it seems likely that the low expression of ER α protein in the HCC1937 cells was not due to an irreversible genetic effect (Fig. 2, B). To confirm this conclusion, we examined the effect of siRNA-mediated silencing of endogenous BRCA1 gene expression on ESR1 mRNA expression

in T47D and MCF-7 cells. Consistent with our observations in HCC1937 cells, siRNA-mediated inhibition of endogenous BRCA1 expression resulted in a marked reduction in expression of endogenous ESR1 mRNA and ER α protein levels compared with that in control cells transfected with scrambled-sequence siRNA (Fig. 2).

Role of Oct-1 in BRCA1-Associated Estrogen Receptor α Expression

We next examined whether BRCA1 has a direct role in ESR1 transactivation. ESR1 is transcribed into multiple transcripts from at least seven promoters in noncoding exons located upstream of the translation start site (20). A previous study showed that the predominant ESR1 transcripts in breast cancer cell lines are generated from promoter A and, to a lesser extent, promoter B (21). To examine whether BRCA1 regulates ESR1 expression from these promoters, we transfected T47D and MCF-7 cells first with BRCA1-specific or scrambled-sequence siRNAs and then with a luciferase reporter construct that contained ESR1 promoters A and B upstream of the luciferase reporter gene (ProAB) or control luciferase reporter construct. Luciferase reporter assays revealed that siRNA-mediated inhibition of endogenous BRCA1 expression reduced luciferase activity by 39.8% (95% CI = 24.0% to 56.0%) in T47D cells and by 40.2% (95% CI = 26.0% to 54.0%) in MCF-7 cells relative to scrambled siRNA controls (Fig. 3, A). These data suggest that BRCA1 mediates transcriptional activation of ESR1 at least in part through ESR1 promoters A and B.

Having shown that BRCA1 can transactivate and regulate the expression of ESR1, we investigated the mechanism of this regulation at the ESR1 promoter. To examine whether BRCA1 binds to the ESR1 promoter region, we performed chromatin immunoprecipitation assays in HCC1937 cells and T47D cells (Fig 3, B). In HCC1937 cells that were stably transfected with a BRCA1 expression vector to reconstitute BRCA1 expression, BRCA1 bound to the ESR1 promoter (Fig. 3, B, lane 6), as did the positive control protein, RNA polymerase II (Fig. 3, B, lane 4). By contrast, in HCC1937 cells transfected with empty vector, RNA polymerase II bound to the ESR1 promoter (lane 3) but BRCA1 did not (lane 5). Chromatin immunoprecipitation experiments also demonstrated that BRCA1 and RNA polymerase II bound the ESR1 promoter in T47D cells (Fig. 3, B). These data confirmed that BRCA1 physically associates with the ESR1 promoter.

ER α is known to autoregulate its expression and activity (22). We therefore hypothesized that the interaction of BRCA1 with ER α may involve a positive feedback mechanism that regulates the expression of ESR1. To examine this hypothesis, we transfected T47D and MCF-7 cells with an ESR1-specific siRNA to inhibit endogenous ER α expression or with a scrambled-sequence control siRNA (Fig. 4, A). We then performed a series of chromatin immunoprecipitation assays to examine whether ER α interacts directly with its own promoter region (Fig. 4, B). ER α bound to its own promoter (Fig. 4, B, lane 7). BRCA1 interacted with the ESR1 promoter regardless of whether the cells were transfected with ESR1-specific siRNA or scrambled control siRNA (Fig. 4, B, lanes 5 and 6), suggesting that BRCA1-mediated regulation of ESR1 expression occurs through a mechanism that is independent of ER α itself.

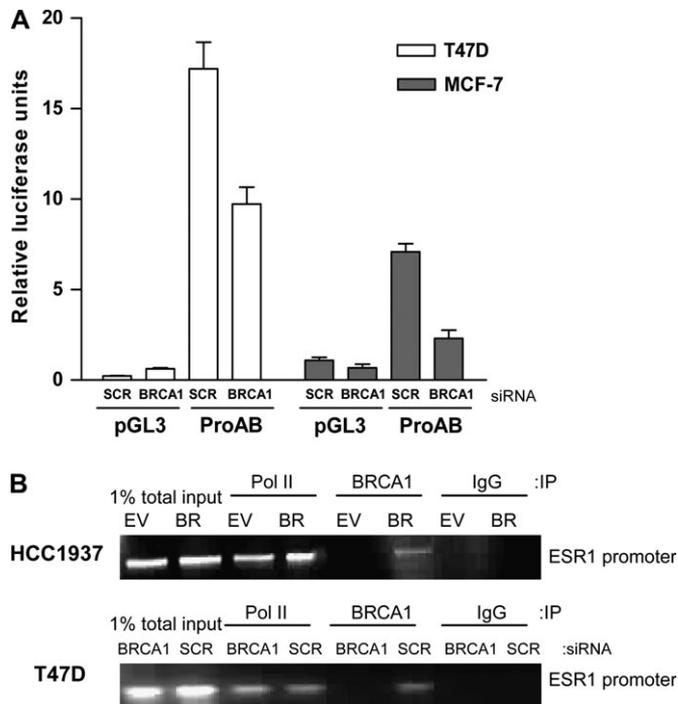


Fig. 3. BRCA1 directly regulates estrogen receptor 1 (ESR1) promoter activity. **A**) Luciferase reporter assay performed on T47D cells and MCF-7 cells that were transfected first with BRCA1-specific short-interfering RNA (siRNA) or a scrambled-sequence control oligonucleotide (SCR) and, 48 hours later, with a reporter construct bearing the firefly luciferase gene driven by the ESR1 promoters A and B (ProAB) or empty luciferase vector (pGL3). A cotransfected *Renilla* luciferase expression plasmid served as an internal control. After incubation for 24 hours, firefly luciferase activity was assayed and normalized to *Renilla* luciferase activity. Mean relative firefly luciferase unit (normalized to *Renilla* luciferase activity) and 95% confidence intervals (error bars) from three replicates are shown. **B**) Chromatin immunoprecipitation assay of HCC1937 cells transfected with empty vector (EV) or BRCA1 expression construct (BR) and T47D cells transfected with a scrambled-sequence control oligonucleotide (SCR) or BRCA1-specific siRNA (BRCA1). Recruitment of RNA polymerase II (Pol II) and BRCA1 to the ESR1 promoter was analyzed using primers specific to the ESR1 promoter. One percent of total input DNA was used as a loading control, and isotype-matched IgG was used as an internal control for the immunoprecipitation.

Evaluation of putative transcription factor-binding sites in the upstream promoter region of ESR1 through Alibaba 2.1 Transcription Factor Binding Prediction Software (<http://www.gene-regulation.com/pub/programs.html>) revealed a number of potential octamer elements. These are the specific sequences that are recognized by the ubiquitous transcription factor Oct-1, which is known to interact with BRCA1 (23,24). To investigate whether Oct-1 plays a functional role in the transcriptional regulation of ESR1, we examined ESR1 mRNA expression and ER α protein expression in T47D and MCF-7 cells that were transfected with a siRNA designed to inhibit endogenous Oct-1 expression. Compared with scrambled-sequence siRNA-transfected control cells, T47D and MCF-7 cells transfected with Oct-1 siRNA had reduced levels of ESR1 mRNA and of Oct-1 and ER α protein, as demonstrated by northern and immunoblot analyses, respectively (Fig. 4, C). These data indicate that Oct-1 plays a role in the transactivation of ESR1 in these cell lines.

BRCA1 has previously been shown to bind Oct-1 and thereby regulate the expression of the genomic stability protein Gadd45 and

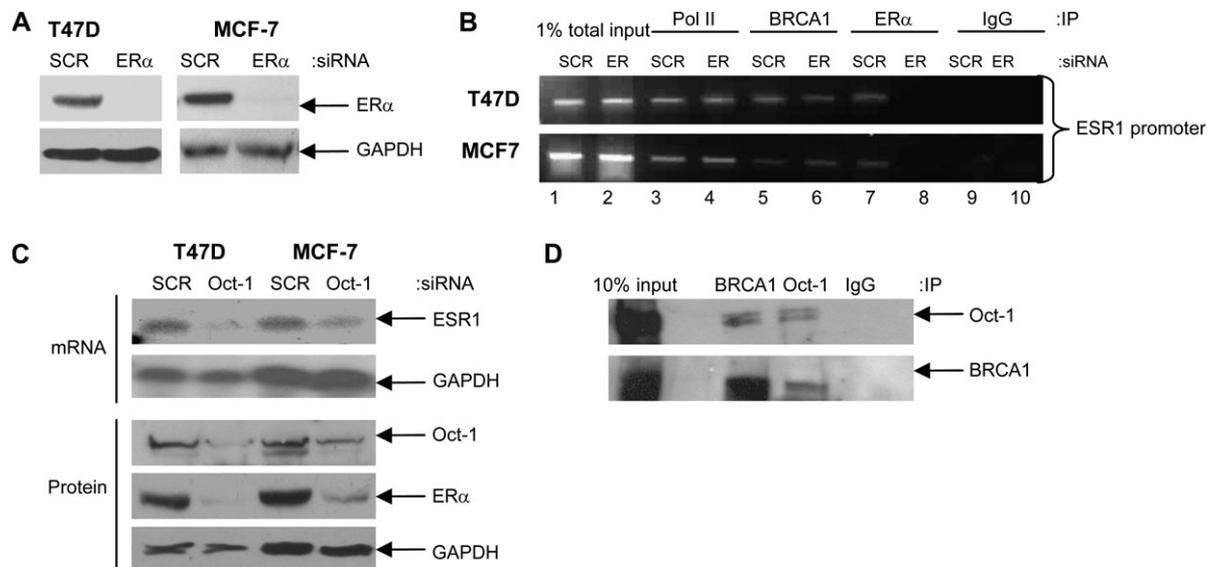


Fig. 4. BRCA1-mediated transactivation of estrogen receptor 1 (ESR1) requires Oct-1 but not estrogen receptor alpha (ER α). **A**) Immunoblot analysis of ER α expression in T47D and MCF-7 cells transfected with ESR1-specific siRNA (ESR1) or scrambled-sequence control oligonucleotide (SCR). Equal loading was confirmed by reprobings the blot with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody. **B**) Chromatin immunoprecipitation assay using primers specific to the ESR1 promoter shows recruitment of RNA polymerase II (Pol II), BRCA1, and ER α to the ESR1 promoter in T47D and MCF-7 cells. ER α is bound to the ESR1 promoter in control cells transfected with scrambled-sequence siRNA (SCR) (lane 7) but not in cells with siRNA-mediated inhibition of endogenous ER α (lane 8). BRCA1 is present on the ESR1 promoter in both control cells transfected with scrambled-sequence siRNA (lane 5) and cells with siRNA-mediated depletion of

endogenous ER α (lane 6). One percent of total input DNA was used as a loading control (lanes 1 and 2). RNA Pol II and isotype-matched IgG were used as positive and negative controls, respectively (lanes 3, 4, 9, and 10). **C**) Northern blot analysis of ESR1 and GAPDH mRNA expression and immunoblot analysis of ER α , Oct-1, and GAPDH protein expression. T47D and MCF-7 cells were transfected with either a scrambled control oligo (SCR) or Oct-1-specific siRNA oligo. Reprobing for GAPDH mRNA and protein confirmed equal loading. **D**) Coimmunoprecipitation analysis demonstrating association of endogenous BRCA1 and Oct-1 in T47D cells. Protein lysate (500 μ g) was immunoprecipitated with BRCA1 antibody or Oct-1 antibody and immunoblotted for Oct-1 (top panel) or BRCA1 (bottom panel). Ten percent input lysate was loaded as a positive control. IgG was used as a negative control for immunoprecipitation.

the spindle checkpoint protein Mad2 (23,24). Consistent with these observations, we found that BRCA1 and Oct-1 coimmunoprecipitate from T47D cell protein extracts (Fig. 4, D). Furthermore, chromatin immunoprecipitation assays revealed that Oct-1 also binds the ESR1 promoter in both T47D and MCF-7 cells (Fig. 5, A, lane 7). Oct-1 still bound the ESR1 promoter following siRNA-mediated inhibition of endogenous BRCA1 expression, suggesting that Oct-1 binding to the ESR1 promoter was independent of BRCA1 (Fig. 5, B, lanes 7 and 8). However, BRCA1 binding to the ESR1 promoter was abrogated following siRNA-mediated depletion of endogenous Oct-1, suggesting that recruitment of BRCA1 to the ESR1 promoter is dependent on Oct-1 (Fig. 5, A, lanes 5 and 6).

To investigate whether both BRCA1 and Oct-1 simultaneously occupy the promoter of ESR1, the antibody-protein complexes resulting from chromatin immunoprecipitation with an antibody to one protein (e.g., anti-BRCA1) were subjected to chromatin immunoprecipitation with an antibody to the other protein (e.g., anti-Oct-1) (Fig. 5, C). Reciprocal chromatin immunoprecipitation revealed that BRCA1 and Oct-1 were found to be simultaneously associated with the ESR1 promoter (Fig 5, C, lanes 3 and 6). By contrast, reciprocal chromatin immunoprecipitation revealed no such association between ER α and BRCA1 or Oct-1 on the ESR1 promoter (Fig. 5, C, lanes 10 and 11). Since BRCA1 and Oct-1 have been shown to interact by coimmunoprecipitation and to colocalize on the ESR1 promoter, these observations suggest that BRCA1 and Oct-1 may act in concert to coactivate ESR1 transcription.

Association Between BRCA1 Expression and Response to Antiestrogen Treatment

Given the importance of antiestrogen therapy for breast cancer in both the preventative and adjuvant settings (25,26), we next investigated the effect of BRCA1 expression on the response of breast cancer cells to antiestrogen treatment. We selected the antiestrogen drug fulvestrant for use in this study because it is a pure steroidal ER α antagonist and because it does not exhibit the partial agonist properties that are frequently associated with selective estrogen receptor modulators (27,28). Fulvestrant also acts as an antiestrogen by reducing the half-life of ER α (29), which results in the decreased expression of ER α . We transfected T47D and MCF-7 cells with scrambled control siRNA or BRCA1-specific siRNA oligonucleotides. The following day, the cells were seeded into 24-well plates and, 24 hours later, treated with a range of concentrations of fulvestrant. In parallel, 48 hours after siRNA transfection, protein was harvested from the cells and subjected to immunoblot analysis to determine the efficiency of the siRNA-mediated depletion of BRCA1 expression levels. In both cell lines, fulvestrant treatment resulted in the dose-dependent growth inhibition of cells that were transfected with the scrambled-sequence control siRNA (T47D cells: mean IC₄₀ = 1.3 \times 10⁻⁹ M [95% CI = 1.0 \times 10⁻¹¹ to 5.8 \times 10⁻⁸ M]; MCF-7 cells: mean IC₄₀ = 3.2 \times 10⁻⁸ M [95% CI = 1.0 \times 10⁻⁹ to 1.4 \times 10⁻⁷ M]) but not of cells transfected with BRCA1-specific siRNA (T47D cells: mean IC₄₀ > 10⁻⁵ M; MCF-7 cells: mean IC₄₀ > 10⁻⁵ M) (Fig. 6, A). These data indicate that wild-type BRCA1 is required for the growth inhibition of breast tumor cells in response to fulvestrant treatment.

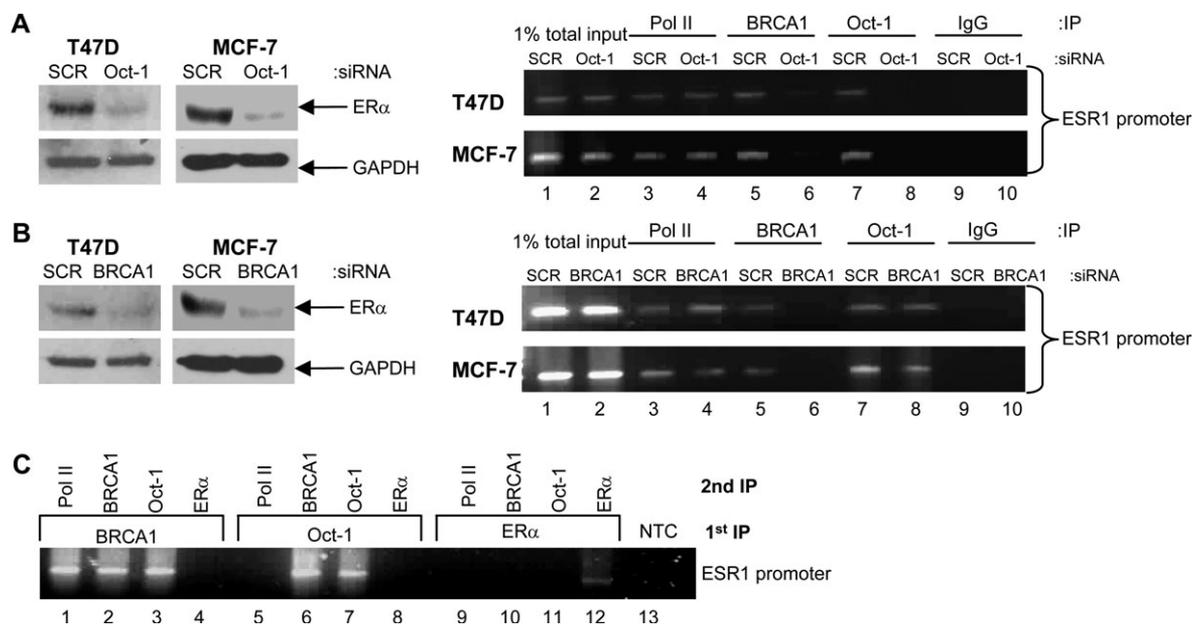


Fig. 5. Oct-1 recruits BRCA1 to the estrogen receptor 1 (ESR1) promoter. **A**) Immunoblot analysis of estrogen receptor alpha (ER α) protein expression with short-interfering RNA (siRNA)-mediated inhibition of endogenous Oct-1 in T47D and MCF-7 cells. From the identical experiment, chromatin immunoprecipitation (ChIP) analysis shows recruitment of RNA polymerase II (Pol II), BRCA1, and Oct-1 to the ESR1 promoter in T47D and MCF-7 cells. BRCA1 binding to the ESR1 promoter was impaired following siRNA-mediated depletion of endogenous Oct-1 (lane 6). One percent of total input DNA was used as a loading control (lanes 1 and 2). RNA Pol II and isotype-matched IgG were used as positive and negative controls, respectively (lanes 3, 4, 9, and 10). **B**) Immunoblot analysis of the effect of siRNA-mediated inhibition of endogenous BRCA1 on ER α protein expression in T47D and

MCF-7 cells. From the identical experiment, ChIP analysis shows recruitment of RNA Pol II, BRCA1, and Oct-1 to the ESR1 promoter. Oct-1 binding to the ESR1 promoter was not impaired following siRNA-mediated inhibition of endogenous BRCA1 (lane 8). One percent of total input DNA was used as a loading control (lanes 1 and 2). RNA Pol II and isotype-matched IgG were used as positive and negative controls, respectively (lanes 3, 4, 9, and 10). **C**) Chromatin reimmunoprecipitation screening for interactions between factors recruited to the ESR1 promoter. Chromatin prepared from T47D cells was subjected to chromatin immunoprecipitation, using antibodies for BRCA1 (lanes 1–4), Oct-1 (lanes 5–8), and ER α (lanes 9–12). Samples were then reimmunoprecipitated with antibodies for RNA Pol II, BRCA1, Oct-1, and ER α , respectively. NTC = no template control.

Finally, to examine whether the resistance of BRCA1-depleted cells to fulvestrant treatment was directly related to reduced expression of ER α , we carried out growth inhibition assays in T47D and MCF-7 cells that were sequentially transfected with BRCA1-specific siRNA and then with an ER α expression construct or empty vector. BRCA1-depleted cells expressing exogenous ER α were more sensitive to fulvestrant than BRCA1-depleted cells transfected with empty vector (T47D cells, mean IC₄₀ for empty vector versus ER α : $>10^{-5}$ versus 8.0×10^{-9} M [95% CI = 3.1×10^{-10} to 3.2×10^{-6} M]; MCF-7 cells, mean IC₄₀ for empty vector versus ER α : $>10^{-5}$ versus 4.9×10^{-8} M [95% CI = 2.0×10^{-9} to 3.9×10^{-6} M]) (Fig. 6, B).

Collectively, these data indicate that loss of BRCA1-mediated transcriptional activation of ER α expression results in increased resistance to an ER α antagonist, which suggests that ESR1 is a functionally important BRCA1 target gene.

Discussion

One of the unexplained features of BRCA1-mutant breast tumors is that they frequently do not express ER α ; up to 90% of BRCA1-mutant tumors exhibit loss of ER α expression (9,10). In this study, we identified a novel transcriptional mechanism that explains the link between BRCA1 mutation and the ER α negativity frequently observed in breast cancers that develop in these carriers. Our results suggest that BRCA1-mutant tumors fail to express ER α due to the

loss of BRCA1-mediated transcriptional activation of ESR1. In sporadic breast cancers, BRCA1 expression is frequently reduced during the transition from carcinoma in situ to invasive cancer (30). This reduction and/or the absence of BRCA1 in sporadic cancers occurs through several mechanisms, including loss of heterozygosity, hypermethylation of the BRCA1 promoter, and loss of transcriptional regulation of BRCA1 (31–33). The reduced BRCA1 expression in sporadic cancers is also associated with ER α negativity (32,33). In agreement with our findings, a recent study demonstrated a positive association between BRCA1 mRNA and ESR1 mRNA expression levels in sporadic breast cancers (34). In further support of a role for BRCA1 as a positive regulator of ESR1 expression, this study (34) also revealed an inverse association between tumor levels of ER α and ID4, a transcription factor that is a negative regulator of BRCA1 expression (35).

We propose a model for how the observed loss of ER α expression in BRCA1-mutant tumors is likely to occur after loss of the wild-type BRCA1 allele (Fig. 7). We suggest that the loss of the wild-type BRCA1 allele, which occurs during neoplastic development in BRCA1 mutation carriers, has a direct effect on ESR1 transactivation, resulting in the loss of ER α mRNA and protein expression (Fig. 7, A). Our model also implies that the reduction in BRCA1 expression levels that frequently occurs in sporadic breast cancers may directly impact on levels of ER α (Fig. 7, B).

Gene expression profiling has revealed that breast cancers can be classified into at least three distinct subtypes: luminal, basal, and

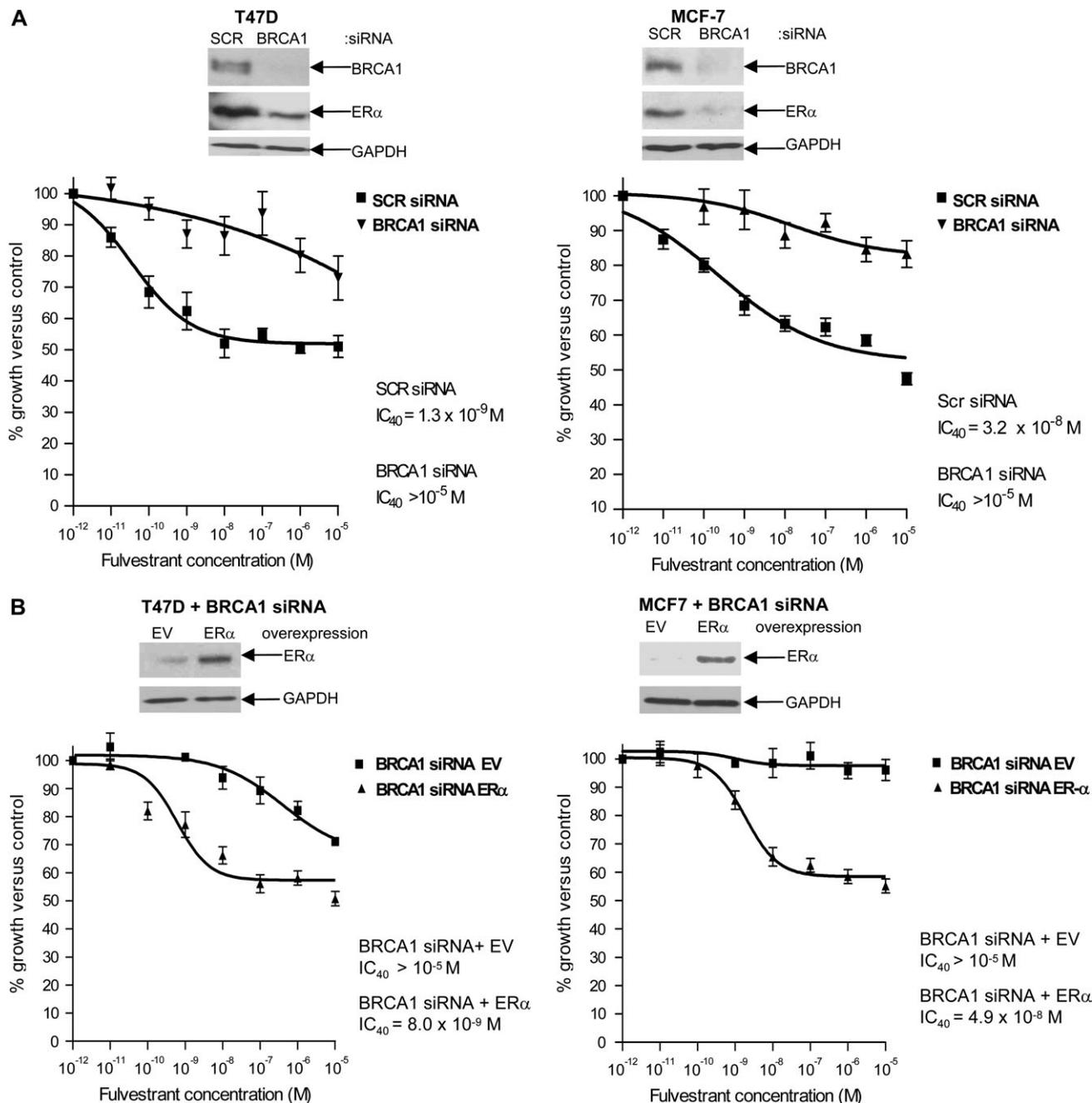


Fig. 6. Association between BRCA1 expression and response to fulvestrant. **A)** Sigmoidal dose–inhibition curves (**bottom panels**) for fulvestrant sensitivity of T47D cells (**left**) and MCF-7 cells (**right**) transfected with BRCA1-specific short-interfering RNA (siRNA) (BRCA1 siRNA) or scrambled-sequence control oligonucleotide (SCR siRNA), with immunoblot analysis (**upper panels**) of BRCA1 and estrogen receptor alpha (ERα) expression. Percentage change in growth was determined by counting cells and comparing the numbers of siBRCA1-transfected cells and siSCR-transfected cells treated with fulvestrant with the number of untreated control cells. **B)** Sigmoidal dose–response curves (**bot-**

tom panels) for fulvestrant sensitivity of T47D cells (**left**) and MCF-7 cells (**right**) transfected with BRCA1-specific siRNA followed by transient transfection with pcDNA3.1 (EV) or pcDNA3.1 ERα expression vectors, with immunoblot analysis (**upper panels**) of ERα expression. The immunoblots were reprobed with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody to confirm equal protein loading. The concentrations of fulvestrant that caused 40% inhibition of cell growth (IC_{40}) were calculated from the sigmoidal dose–inhibition curves. In all cases, the mean values of triplicate experiments are shown and error bars correspond to 95% confidence intervals.

HER2 positive (11). Gene expression profiling has shown that BRCA1-mutant tumors cluster with the basal subtype of breast cancer (11). Other studies have found that BRCA1-mutant tumors have characteristics consistent with this distinct subtype of breast cancer (9,36). Breast cancers of the basal subtype tend to lack expression of luminal epithelial cell markers such as ERα while concomitantly over-

expressing basal epithelial cell markers such as keratin-5 and keratin-17 (37). In future studies, it would be intriguing to investigate if the loss of BRCA1-mediated transcriptional activation of these basal markers plays a direct role in the phenotype of basal-like breast cancer.

We also demonstrated that BRCA1 expression levels affect the response of human breast cancer cells to the ERα antagonist

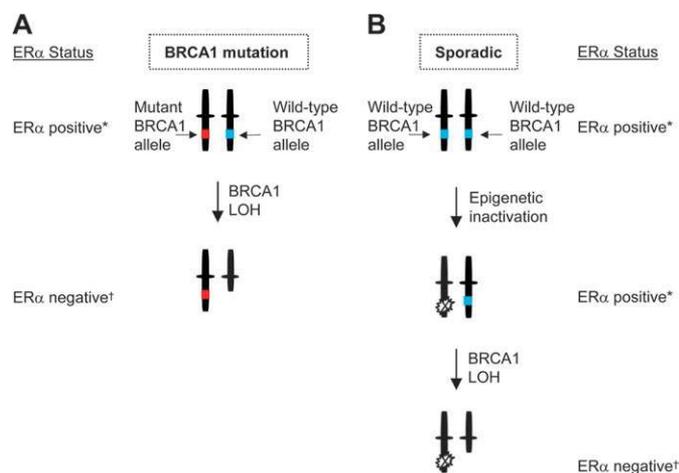


Fig. 7. A model of the sequence of events leading to estrogen receptor alpha (ER α) deficiency in BRCA1 mutated tumors and sporadic tumors. In BRCA1-mutant breast cells (**A**), loss of the wild-type BRCA1 allele by a mechanism involving loss of heterozygosity (LOH) leads to the loss of estrogen receptor 1 transactivation, causing the cells to revert to an ER α -negative phenotype. In sporadic tumors (**B**), reduction of BRCA1 expression by mechanisms involving LOH and epigenetic inactivation may result in a decrease in ER α expression. A complete loss of BRCA1 function in sporadic breast cancers would result in tumors that are ER α negative. **Asterisk** = therapeutic window for antiestrogen-based therapy; **Dagger** = antiestrogen therapy no longer effective.

fulvestrant. Specifically, growth inhibition of both T47D and MCF-7 cells in response to fulvestrant was dependent on wild-type BRCA1, with BRCA1 siRNA-transfected cells exhibiting increased resistance to the antiproliferative effects of this drug. In addition, transient overexpression of ER α in both T47D and MCF-7 cells depleted of BRCA1 expression using siRNA enhanced their sensitivity to fulvestrant.

Taken together, these findings suggest that there is a functionally relevant link between BRCA1-mediated activation of ER α expression and response to fulvestrant therapy. Hence, ER α deficiency in BRCA1-mutant breast tumors may result in a reduced response to an antiestrogen drug that functions as an ER α antagonist (e.g., either a pure ER α antagonist such as fulvestrant or a partial antagonist selective estrogen receptor modulator such as tamoxifen). Our dose-response data therefore add further support to the suggestion that adjuvant antiestrogen therapy may be ineffective for the treatment of BRCA1-mutant tumors (38). However, it is plausible that antiestrogen therapy could still play a role in the prevention of breast cancer in women with BRCA1-mutant breast tumors if such therapy were given before the wild-type BRCA1 allele was lost. For example, one study has shown that tamoxifen treatment for 2–4 years reduces the risk of contralateral breast cancers in BRCA1 mutation carriers by 75% (39). It has also been reported that bilateral prophylactic oophorectomy is associated with a statistically significant reduced risk of breast cancer in BRCA1 mutation carriers (40), suggesting that the development of breast cancer in these carriers has some dependency on ovarian hormone exposure.

Our study is limited by the small number of breast tumors that were analyzed. Although the majority of BRCA1-mutant tumors lack ER α expression, several studies have shown that a small number of BRCA1-mutant tumors retain ER α positivity (41,42).

In this study, two (12%) of the 17 BRCA1-mutant samples (i.e., M_02 and M_12) expressed high levels of ESR1 mRNA and clustered with the sporadic tumors (Fig. 1, A). It is possible that these tumors had increased expression of a positive regulator of ER α that is independent of BRCA1. Alternatively, it is also possible that certain BRCA1 mutations that may affect the other known functions of BRCA1, such as in DNA damage repair or ubiquitination, and segregate with cancer do not abrogate BRCA1-mediated transcription. Further research is required to investigate the mechanism by which certain BRCA1-mutant tumors retain ER α positivity.

Previous studies have demonstrated that BRCA1 can inhibit ER α signaling [reviewed in (12)]. In addition, BRCA1 has been implicated in the negative regulation of production of estradiol (the biologic ligand for ER α) in ovarian granulosa cells (13), whereas treatment of breast cancer cells with estradiol is known to increase BRCA1 expression (43). Further studies are required to investigate the interactions between BRCA1 and the estrogen signaling pathway. Specifically, research is warranted to uncover the role these interactions may play in the development of breast cancers in BRCA1 mutation carriers before the loss of the wild-type allele and to elucidate whether these interactions are important for preventive hormonal therapy in these mutation carriers. This study adds further insight into the complex interaction between BRCA1 and ER α and provides a simple mechanism to explain the high frequency of ER α negativity observed in BRCA1-deficient tumors.

References

- (1) Miki Y, Swensen J, Shattuck-Eidens D, Futreal PA, Harshman K, Tavtigian S, et al. A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. *Science* 1994;266:66–71.
- (2) Chapman MS, Verma IM. Transcriptional activation by BRCA1. *Nature* 1996;382:678–9.
- (3) Monteiro AN, August A, Hanafusa H. Evidence for a transcriptional activation function of BRCA1 C-terminal region. *Proc Natl Acad Sci U S A* 1996;93:13595–9.
- (4) Anderson SF, Schlegel BP, Nakajima T, Wolpin ES, Parvin JD. BRCA1 protein is linked to the RNA polymerase II holoenzyme complex via RNA helicase A. *Nat Genet* 1998;19:254–6.
- (5) Mullan PB, Quinn JE, Harkin DP. The role of BRCA1 in transcriptional regulation and cell cycle control. *Oncogene* 2006;25:5854–63.
- (6) Furuta S, Wang JM, Wei S, Jeng YM, Jiang X, Gu B, et al. Removal of BRCA1/CtIP/ZBRK1 repressor complex on ANG1 promoter leads to accelerated mammary tumor growth contributed by prominent vasculature. *Cancer Cell* 2006;10:13–24.
- (7) Bochar DA, Wang L, Beniya H, Kinev A, Xue Y, Lane WS, et al. BRCA1 is associated with a human SWI/SNF-related complex: linking chromatin remodeling to breast cancer. *Cell* 2000;102:257–65.
- (8) Yarden RI, Brody LC. BRCA1 interacts with components of the histone deacetylase complex. *Proc Natl Acad Sci U S A* 1999;96:4983–8.
- (9) Lakhani SR, Reis-Filho JS, Fulford L, Penault-Llorca F, van der Vijver M, Parry S, et al. Prediction of BRCA1 status in patients with breast cancer using estrogen receptor and basal phenotype. *Clin Cancer Res* 2005;11:5175–80.
- (10) Foulkes WD, Metcalfe K, Sun P, Hanna WM, Lynch HT, Ghadirian P, et al. Estrogen receptor status in BRCA1- and BRCA2-related breast cancer: the influence of age, grade, and histological type. *Clin Cancer Res* 2004;10:2029–34.
- (11) Sorlie T, Tibshirani R, Parker J, Hastie T, Marron JS, Nobel A, et al. Repeated observation of breast tumor subtypes in independent gene expression data sets. *Proc Natl Acad Sci U S A* 2003;100:8418–23.
- (12) Rosen EM, Fan S, Isaacs C. BRCA1 in hormonal carcinogenesis: basic and clinical research. *Endocr Relat Cancer* 2005;12:533–48.

- (13) Hu Y, Ghosh S, Amleh A, Yue W, Lu Y, Katz A, et al. Modulation of aromatase expression by BRCA1: a possible link to tissue-specific tumor suppression. *Oncogene* 2005;24:8343–8.
- (14) Rosenbaum P. Optimal matching for observational studies. *J Am Stat Assoc* 1989;84:1024–32.
- (15) Tomlinson GE, Chen TT, Stastny VA, Virmani AK, Spillman MA, Tonk V, et al. Characterization of a breast cancer cell line derived from a germline BRCA1 mutation carrier. *Cancer Res* 1998;58:3237–42.
- (16) Andrews HN, Mullan PB, McWilliams S, Sebelova S, Quinn JE, Gilmore PM, et al. BRCA1 regulates the interferon gamma-mediated apoptotic response. *J Biol Chem* 2002;277:26225–32.
- (17) Li C, Wong WH. Model-based analysis of oligonucleotide arrays: expression index computation and outlier detection. *Proc Natl Acad Sci U S A* 2001;98:31–6.
- (18) Affymetrix. Statistical Algorithms Reference Guide. Affymetrix Technical Note 2002.
- (19) Kennedy RD, Gorski JJ, Quinn JE, Stewart GE, James CR, Moore S, et al. BRCA1 and c-Myc associate to transcriptionally repress psoriasis, a DNA damage-inducible gene. *Cancer Res* 2005;65:10265–72.
- (20) Kos M, Reid G, Denger S, Gannon F. Minireview: genomic organization of the human ERalpha gene promoter region. *Mol Endocrinol* 2001;15:2057–63.
- (21) Flouriot G, Griffin C, Kenealy M, Sonntag-Buck V, Gannon F. Differentially expressed messenger RNA isoforms of the human estrogen receptor-alpha gene are generated by alternative splicing and promoter usage. *Mol Endocrinol* 1998;12:1939–54.
- (22) Castles CG, Oesterreich S, Hansen R, Fuqua SA. Auto-regulation of the estrogen receptor promoter. *J Steroid Biochem Mol Biol* 1997;62:155–63.
- (23) Fan W, Jin S, Tong T, Zhao H, Fan F, Antinore MJ, et al. BRCA1 regulates GADD45 through its interactions with the OCT-1 and CAAT motifs. *J Biol Chem* 2002;277:8061–7.
- (24) Wang RH, Yu H, Deng CX. A requirement for breast-cancer-associated gene 1 (BRCA1) in the spindle checkpoint. *Proc Natl Acad Sci U S A* 2004;101:17108–13.
- (25) EBCTCG. Tamoxifen for early breast cancer: an overview of the randomised trials. Early Breast Cancer Trialists' Collaborative Group. *Lancet* 1998;351:1451–67.
- (26) Fisher B, Costantino JP, Wickerham DL, Redmond CK, Kavanah M, Cronin WM, et al. Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. *J Natl Cancer Inst* 1998;90:1371–88.
- (27) Vendrell JA, Magnino F, Danis E, Duchesne MJ, Pinloche S, Pons M, et al. Estrogen regulation in human breast cancer cells of new downstream gene targets involved in estrogen metabolism, cell proliferation and cell transformation. *J Mol Endocrinol* 2004;32:397–414.
- (28) Howell A, Osborne CK, Morris C, Wakeling AE. ICI 182,780 (Faslodex): development of a novel, "pure" antiestrogen. *Cancer* 2000;89:817–25.
- (29) Wijayarathne AL, McDonnell DP. The human estrogen receptor-alpha is a ubiquitinated protein whose stability is affected differentially by agonists, antagonists, and selective estrogen receptor modulators. *J Biol Chem* 2001;276:35684–92.
- (30) Thompson ME, Jensen RA, Obermiller PS, Page DL, Holt JT. Decreased expression of BRCA1 accelerates growth and is often present during sporadic breast cancer progression. *Nat Genet* 1995;9:444–50.
- (31) Baldassarre G, Battista S, Belletti B, Thakur S, Pentimalli F, Trapasso F, et al. Negative regulation of BRCA1 gene expression by HMGAI proteins accounts for the reduced BRCA1 protein levels in sporadic breast carcinoma. *Mol Cell Biol* 2003;23:2225–38.
- (32) Catteau A, Harris WH, Xu CF, Solomon E. Methylation of the BRCA1 promoter region in sporadic breast and ovarian cancer: correlation with disease characteristics. *Oncogene* 1999;18:1957–65.
- (33) Schmutzler RK, Bierhoff E, Werkhausen T, Fimmers R, Speiser P, Kubista E, et al. Genomic deletions in the BRCA1, BRCA2 and TP53 regions associate with low expression of the estrogen receptor in sporadic breast carcinoma. *Int J Cancer* 1997;74:322–5.
- (34) Roldan G, Delgado L, Muse IM. Tumoral expression of BRCA1, estrogen receptor alpha and ID4 protein in patients with sporadic breast cancer. *Cancer Biol Ther* 2006;5:505–10.
- (35) Beger C, Pierce LN, Marcusson EG, Robbins JM, Welsh P, et al. Identification of Id4 as a regulator of BRCA1 expression by using a ribozyme-library-based inverse genomics approach. *Proc Natl Acad Sci U S A* 2001;98:130–5.
- (36) Foulkes WD, Stefansson IM, Chappuis PO, Begin LR, Goffin JR, Wong N, et al. Germline BRCA1 mutations and a basal epithelial phenotype in breast cancer. *J Natl Cancer Inst* 2003;95:1482–5.
- (37) Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, et al. Molecular portraits of human breast tumours. *Nature* 2000;406:747–52.
- (38) King MC, Wieand S, Hale K, Lee M, Walsh T, Owens K, et al. Tamoxifen and breast cancer incidence among women with inherited mutations in BRCA1 and BRCA2: National Surgical Adjuvant Breast and Bowel Project (NSABP-P1) Breast Cancer Prevention Trial. *JAMA* 2001;286:2251–6.
- (39) Narod SA, Brunet JS, Ghadirian P, Robson M, Heimdal K, Neuhausen SL, et al. Tamoxifen and risk of contralateral breast cancer in BRCA1 and BRCA2 mutation carriers: a case-control study. *Hereditary Breast Cancer Clinical Study Group. Lancet* 2000;356:1876–81.
- (40) Rebbeck TR, Levin AM, Eisen A, Snyder C, Watson P, Cannon-Albright L, et al. Breast cancer risk after bilateral prophylactic oophorectomy in BRCA1 mutation carriers. *J Natl Cancer Inst* 1999;91:1475–9.
- (41) Karp SE, Tonin PN, Begin LR, Martinez JJ, Zhang JC, Pollak MN, et al. Influence of BRCA1 mutations on nuclear grade and estrogen receptor status of breast carcinoma in Ashkenazi Jewish women. *Cancer* 1997;80:435–41.
- (42) Verhoog LC, Brekelmans CT, Seynaeve C, van den Bosch LM, Dahmen G, van Geel AN, et al. Survival and tumour characteristics of breast-cancer patients with germline mutations of BRCA1. *Lancet* 1998;351:316–21.
- (43) Spillman MA, Bowcock AM. BRCA1 and BRCA2 mRNA levels are coordinately elevated in human breast cancer cells in response to estrogen. *Oncogene* 1996;13:1639–45.

Funding

Cancer Research U.K. (C538/A4362; C538/A2556), Breast Cancer Campaign (2003/596), Action Cancer (AC/2003), The R&D Office Northern Ireland (RSG/1374/00), Medical Research Council U.K. (G0200103/61534).

Notes

We thank Linda Wadum, study coordinator, for collection of BRCA1 tumor specimens, Professor Donald McDonnell for the gift of the ER α expression construct, and Dr R. Kiyama for the gift of the ER α luciferase construct. The study sponsors had no role in the design of the study; the collection, analysis, or interpretation of the data; the writing of the manuscript; or the decision to submit the manuscript for publication. D. P. Harkin and P. G. Johnston hold stock in, and are directors of, Almac Diagnostics, the manufacturer of the breast cancer disease-specific microarray.

Manuscript received May 7, 2007; revised September 12, 2007; accepted October 1, 2007.