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

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Background
BRCA1-mutant breast tumors are typically estrogen receptor alpha (ERα) negative, whereas most sporadic tumors express wild-type BRCA1 and are ERα positive. We examined a possible mechanism for the observed ERα-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 ERα), which was differentially expressed in the tumor samples, by quantitative polymerase chain reaction. Regulation of ESR1 mRNA and ERα 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 ERα expression. BRCA1-depleted breast cancer cells expressing exogenous ERα 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 ERα: >10⁻⁵ versus 8.0 × 10⁻⁸ M [95% CI = 3.1 × 10⁻¹⁰ to 3.2 × 10⁻⁸ M]; MCF-7 cells, mean IC40 for empty vector versus ERα: >10⁻⁵ versus 4.9 × 10⁻⁸ M [95% CI = 2.0 × 10⁻⁹ to 3.9 × 10⁻⁸ M]).

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

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 (ERα) (9,10). Furthermore, gene expression profiling has revealed that BRCA1-mutant breast tumors cluster with the ERα-negative basal subtype of breast cancer (11). The molecular basis for the ERα-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 ERα 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 ERα levels to identify the mechanism of ERα 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 ERα 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 µg/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′-AGGAGCTTTCTATCATTTCCCCC-3′) and a reverse primer to the Rc-CMV 3′ untranslated region (RcCMV-R, 5′-AATCTAGAGGCAACAGTGAGG-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 ERα 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 µg/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-µm section from each of the 31 formalin-fixed, paraffin-embedded breast tumors with the use of an RNaseasy 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 60,856 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 washed, stained, 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’-GGTGCGCTGAGACACAGACC-3’ (forward) and 5’-GTGAGAGAGACGAAAACCTGGC-3’ (reverse); the GAPDH primers were 5’-AGGTGGTCTCCTCTGACTTCA-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 XbaI restriction digestion of an Eρα pdDNA3.1/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’-ACCACAGTCCATGCCCATC3’ and GAPDH AS 5’-TCCACACCCCTGTTGCTGTA-3’. Probes were radiolabeled with the use of a Megaprime DNA Labeling System (Amersham) and 32P-dCTP (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 000g 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...
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) preequilibrated 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 preclar 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 preequilibrated 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′-GGGUGCGACUGAGGAGCUA-3′), Oct-1 mRNA (5′-CCAGACGCUCCACCUAUUA-3′), ESR1 mRNA (5′-UCAUGCUUCCCCUGAAA-3′), scrambled-sequence control siRNA (5′-CCUGGUGACGCGGAGUCAG-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 EREs 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% formaldehyde 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 DT T) 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 Bioruptor 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 precleared 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 (CTD-H8; 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 ERα 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′-TTTACCTGTCTCGTCGG-3′. PCR products from the purified DNA were visualized by electrophoresis on 2% agarose gels.
Fig. 1. Estrogen receptor alpha (ERα) mRNA expression in BRCA1-mutant tumors. A) Hierarchical clustering heat map of gene expression for 17 BRCA1-mutant breast tumor samples (M_01–M_17) and 14 sporadic breast tumor samples (W_01–W_14) that were matched to each other by stage and grade. Expression matrix displays 636 probe sets that were differentially expressed between BRCA1-mutant and sporadic tumor samples according to the following selection criteria: 1) at least a twofold difference in expression and 2) P value for difference in expression less than .05 (unpaired t test). Red indicates higher expression and green indicates lower expression. B) Histogram of estrogen receptor 1 (ESR1) probe set intensities for 17 BRCA1-mutant breast tumors (M_01–M_17; open bars) and 14 sporadic breast tumors (W_01–W_14; solid bars) ordered according to cluster analysis. C) Quantitative reverse transcription–polymerase chain reaction (PCR) analysis of ESR1 mRNA levels from 17 BRCA1-mutant tumors (open bars) and 14 sporadic tumors (solid bars). Mean mRNA expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression and 95% confidence intervals (error bars) from three replicates are shown. Asterisk indicates that PCR amplification was not performed because of insufficient quantities of RNA.
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% confluence 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 x 10⁴ 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 (IC₄₀) 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 x 10⁵ cells per plate) and incubated in medium lacking fulvestrant for protein extraction and immunoblotting to confirm the expression of BRCA1.

We also carried out dose inhibition assays in T47D and MCF-7 cells that transiently expressed exogenous ERα. 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 ERα 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 ERα 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, M₉₂ and M₁₂, 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 ERα expression, we examined the effect of reconstituting BRCA1 expression on ERα expression levels in BRCA1-mutant (and low ERα–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 ERα protein than HCC-EV cells. Therefore, it seems likely that the low expression of ERα 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 ERe 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.

ERα is known to autoregulate its expression and activity (22). We therefore hypothesized that the interaction of BRCA1 with ERα 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 ERα expression or with a scrambled-sequence control siRNA (Fig. 4, A). We then performed a series of chromatin immunoprecipitation assays to examine whether ERα interacts directly with its own promoter region (Fig. 4, B). ERα 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 ERα itself.

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 ERα protein expression in T47D and MCF-7 cells that were transfected with an 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 ERα 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
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, A, lanes 5 and 6). 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 ERs 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 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 ERα (29), which results in the decreased expression of ERα. 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 all 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 IC50 = 1.3 × 10−9 M [95% CI = 1.0 × 10−10 to 5.8 × 10−9 M]; MCF-7 cells: mean IC50 = 3.2 × 10−8 M [95% CI = 1.0 × 10−8 to 1.4 × 10−7 M]) but not of cells transfected with BRCA1-specific siRNA (T47D cells: mean IC50 > 10−4 M; MCF-7 cells: mean IC50 > 10−3 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.
Finally, to examine whether the resistance of BRCA1-depleted cells to fulvestrant treatment was directly related to reduced expression of ERα, we carried out growth inhibition assays in T47D and MCF-7 cells that were sequentially transfected with BRCA1-specific siRNA and then with an ERα expression construct or empty vector. BRCA1-depleted cells expressing exogenous ERα were more sensitive to fulvestrant than BRCA1-depleted cells transfected with empty vector (T47D cells, mean IC50 for empty vector versus ERα: >10−5 versus 8.0 × 10−9 M [95% CI = 3.1 × 10−10 to 3.2 × 10−9 M]; MCF-7 cells, mean IC50 for empty vector versus ERα: >10−5 versus 4.9 × 10−9 M [95% CI = 2.0 × 10−9 to 3.9 × 10−9 M]) (Fig. 6, B).

Collectively, these data indicate that loss of BRCA1-mediated transcriptional activation of ERα expression results in increased resistance to an ERα 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 ERα; up to 90% of BRCA1-mutant tumors exhibit loss of ERα expression (9,10). In this study, we identified a novel transcriptional mechanism that explains the link between BRCA1 mutation and the ERα negativity frequently observed in breast cancers that develop in these carriers. Our results suggest that BRCA1-mutant tumors fail to express ERα 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 ERα 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 ERα and ID4, a transcription factor that is a negative regulator of BRCA1 expression (35).

We propose a model for how the observed loss of ERα 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 ERα 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 ERα (Fig. 7, B).

Gene expression profiling has revealed that breast cancers can be classified into at least three distinct subtypes: luminal, basal, and...
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 fulvestrant (Fig. 6).

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 with fulvestrant with the number of untreated control cells. B) Sigmoidal dose-response curves (bottom 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.
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 ERα 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 ERα expression and response to fulvestrant therapy. Hence, ERα deficiency in BRCA1-mutant breast tumors may result in a reduced response to an antiestrogen drug that functions as an ERα antagonist (e.g., a pure ERα 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 ERα expression, several studies have shown that a small number of BRCA1-mutant tumors retain ERα 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 ERα 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 ERα positivity.

Previous studies have demonstrated that BRCA1 can inhibit ERα signaling [reviewed in (12)]. In addition, BRCA1 has been implicated in the negative regulation of production of estradiol (the biologic ligand for ERα) 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 ERα and provides a simple mechanism to explain the high frequency of ERα negativity observed in BRCA1-deficient tumors.

References


