BRCA1 Suppresses Osteopontin-mediated Breast Cancer*

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BRCA1 is a well described breast cancer susceptibility gene thought to be involved primarily in DNA repair. However, mutation within the BRCA1 transcriptional domain is also implicated in neoplastic transformation of mammary epithelium, but responsible mechanisms are unclear. Here we show in a rat mammary model system that wild type (WT) BRCA1 selectively inhibits OPN protein and induces anchorage-independent growth, adhesion to fibronectin, and invasion through Matrigel. A mutant BRCA1 allele (Mut.BRCA1) associated with familial breast cancer lacks OPN suppressor effects, binds to WT.BRCA1, and impedes WT.BRCA1 suppression of OPN. Stable transfection of rat breast tumor cell lines with Mut.BRCA1 dramatically up-regulates OPN protein and induces anchorage-independent growth. In human primary breast cancer, BRCA1 mutation is significantly associated with OPN overexpression. Taken together, these data suggest that BRCA1 mutation may confer increased tissue-specific cancer risk, in part by disruption of BRCA1 suppression of OPN gene transcription.

Osteopontin (OPN) is an extracellular matrix glycosphosphoprotein that binds to α5-containing integrins (1) and has an important role in neoplastic transformation, malignant cell attachment, and migration (2, 3). Overexpression of benign, nonmetastatic rat mammary cells with OPN cDNA endows the transfectants with the ability to metastasize in vivo (4), whereas OPN inhibition by antisense cDNA impedes cell growth and tumor-forming capacity (5). We and others have shown that OPN overexpression in human primary breast cancer is associated with early metastasis and poor outcome (6, 7) and has poor prognosis in gastric cancers (8, 9). BRCA1 encodes a multifunctional nuclear phosphoprotein with tumor suppressor functions that include regulation of the cell cycle (10–12), apoptosis (12, 13), DNA repair (14, 15), and gene transcription through interaction with specific DNA-binding factors (12, 16–18). BRCA1 may repress c-Myc and inhibit both c-Myc and Ras transforming activity in cultured cells (12). Impairment of BRCA1 function by overexpression of a truncated RNA helicase A peptide that binds to the BRCA1 C terminus enhances pleomorphic nuclei, aberrant mitoses, and tetraploidy that typify the malignant phenotype (19).

Although BRCA1 tumor suppressor functions may be impaired by germ line mutation in ubiquitous adult tissues, BRCA1-associated cancers predominantly arise in breast or ovary (20). Substantial evidence implicates estradiol- and growth factor-mediated signaling pathways in the tissue specificity of BRCA1-dependent cancer (21–24), although there is a wide gap in our knowledge of how signaling is orchestrated during neoplastic progression. Estradiol responses in target tissues are mediated by the estrogen receptor α (ERα), often in concert with the activation protein-1 (AP-1) factor, c-Jun (25–27). Epidermal growth factor-dependent activation of erbB2/HER2/Neu up-regulates the transcriptional activity of the Ets factor, polycloma virus enhancer activator 3 (PEA3), that has key regulatory roles in both mammary gland development and oncogenesis (28, 29). These transcriptional regulators ERα, AP-1 and Ets factors can cooperate in stimulating transcription of OPN (30), an adhesive glycosphosphoprotein capable of inducing malignant transformation of rat mammary epithelium in vitro and metastasis in vivo (31). This transcription complex can activate OPN through cognate ERα, AP-1 and Ets-binding sites within its promoter (30). High level OPN expression may have adverse prognostic significance (6) and may be associated with up-regulation of ERα, AP-1, or Ets factors in sporadic human breast cancers (30). Because BRCA1 protein may bind ERα (12, 32), AP-1 (33), and Ets-transcription factors (34), we now investigate the effects of wild type (WT), mutated (Mut.), or truncated (Trunc.) BRCA1 upon these transcription factors on resultant OPN expression and upon OPN-mediated neoplastic transformation of mammary epithelium in vitro. We also assess the effects of germ line BRCA1 mutation upon expression of OPN in human breast cancer.

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The on-line version of this article (available at http://www.jbc.org) contains additional text and supplemental Fig. 1.

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2 The abbreviations used are: OPN, osteopontin; WT, wild type; Mut., mutant; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Trunc., truncated; siRNA, short interfering RNA; IP, immunoprecipitation; mAb, monoclonal antibody; HA, hemagglutinin; ERα, estrogen receptor α; DMEM, Dulbecco’s modified Eagle’s medium; E2, estradiol.

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**MATERIALS AND METHODS**

**Plasmids and Oligonucleotides—**Expression vectors for mouse PEA3 (30), for human c-Jun (30), and for ERα (35) have all been tested previously for producing authentic products (36). Expression vectors for human WT, Mut., and Trunc.BRCA1 Rc/CMV have been described previously (37). The Mut.BRCA1 encoded a point mutation (A178E) in its C-terminal region. This is a germ line mutation associated with very early onset familial breast cancer (38). This mutant is associated with impaired transcriptional activity (39), possibly by improper folding of the C-terminal region, which may either stabilize or destabilize the structure. Trunc.BRCA1 was generated by PCR to produce a premature termination within exon 11 (nucleotide 1259), a common site of breast cancer-associated truncating mutations. It encodes a truncated protein of 50 kDa lacking the C-terminal region. It fails to induce GADD45 expression (37), which is normally triggered by WT.BRCA1 in response to DNA damage. To investigate any interaction between WT.BRCA1 and Mut.BRCA1, a Mut.BRCA1 expression vector and a control Tip60 expression vector (40) were generated with a 5′-HA (hemagglutinin) epitope tag (Clontech) from their original expression vectors. For permanent transfection WT.BRCA1 was also released from WT.BRCA1 Rc/CMV and then subcloned into pBK-CMV (Stratagene, TX) to yield WT.BRCA1-pBK-CMV. The 2.3-kbp rat OPN promoter (41) and a control pGL-3 empty vector were used, as described previously (30). Inducible expression of OPN was achieved using the TRex system (Invitrogen).

**Mutagenesis of the Osteopontin Promoter—**The OPN promoter was amplified using luciferase reporter constructs (OPN-Luc) with mutated SFREs (termed S1M or S2M), AP-1 (termed mAP), and Ets-binding sites (termed ∆EB1, ∆EB1/2, or ∆EB1/2/3) were made as follows. OPNS1M-Luc, OPNS2M-Luc, OPNS1S2M-Luc, and mAP-OPN-Luc were generated with the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), as described previously (36). OPNAEB1, OPNAEB1/2, and OPNAEB1/2/3 were made by PCR using oligonucleotides starting at one of residues −1775, −1250, or −576 (5′-AAA CTG TGG GTG TCG-3′, 5′-TAA AAC CTG CTT AAG T-3′, or 5′-CCC ATG CTG TCC TGG A-3′) and terminating at residues −6 (5′-CCC ATG CTG TCC TGG A-3′). The three fragments were then cloned separately into the pGL-3 vector.

**Small Interfering RNA to BRCA1—**pRETROSUPER-siRNA BRCA1 or BRCA1-siRNA was prepared as described below. The specific siRNA transcripts targeted against human BRCA1 were obtained from a previously described SUPER RNA interference library (Cancer Research, UK). Briefly, the 19-mer sequences from the BRCA1 gene were converted into pairs of complementary 59-mer hairpin oligonucleotides. The complementary 59-mer oligonucleotides targeting the BRCA1 gene were annealed and ligated into the pRETROSUPER vector and transfected into competent DH5α bacteria. Glycerol stocks of transformants were prepared with the well containing bacteria with the short interfering RNA (siRNA) construct targeting the BRCA1 gene. DNA from the siRNA construct was isolated. The DNA corresponding to the siRNA construct was determined by sequencing. The three siRNA oligonucleotide sequences for BRCA1 (siRNA-BRCA1), designed according to the human mRNA sequence (GenBank® accession numbers U14680, NM_007296), were as follows: 5′-GTACGAGATTATGTCACT-3′, 5′-CATAACAGATGGGTGGA-3′, and 5′-GTT-GCAGAATCTGGCAGA-3′.

**Cell Lines and Cell Culture—**The rat mammary (Rama 37) nonmetastatic benign tumor-derived cell line (42) and derivative stably transfected cell subclones were cultured in Dulbecco’s modified Eagle’s medium (DMEM), 10% (v/v) fetal calf serum, 100 μg/ml penicillin, 100 μg/ml streptomycin (Invitrogen). The MCF-7 human breast cancer cell line was obtained from ECACC, Wiltshire (UK), and propagated in DMEM, 5% (v/v) fetal calf serum, 50 mg ml−1 insulin, 10−8 M estradiol.

**Stable and Transient Transfections—**Permanently expressing OPN cells were produced from the endogenous rat gene by transfection of C9-Met-DNA into Rama 37 cells as described previously (43, 44) yielding C9 cells. Results from single-cell clones and a pool of transformants were substantially the same. Rama 37 and C9 subclones that stably expressed WT.BRCA1, Mut.BRCA1, and Trunc.BRCA1 were raised as described previously (43) and were designated for R37 as WT.BRCA1 R37, Mut.BRCA1 R37, and Trunc.BRCA1 R37 and for C9 as WT.BRCA1 C9, Mut.BRCA1 C9, and Trunc.BRCA1 C9. Single cell clones from each of the above transfectants were pooled and collected in 950 μg/ml hygromycin to provide multicolonial cell lines for each of the above transfectants; six single cell clones were also retained. Results for the pooled transformant cell clones were shown, and those obtained with single cell cloned cell lines were substantially the same (data not shown). Rama 37 and subclones that stably expressed WT.BRCA1, Mut.BRCA1, and Trunc.BRCA1 and that also contained the inducible construct TRex-OPN were also raised and were designated OPN-TRex R37, OPN-TRex/WT.BRCA1 R37, OPN-TRex/Mut.BRCA1 R37, and OPN-TRex/Trunc.BRCA1 R37. These OPN-TRex-transfected cells contained a model tetracycline-inducible promoter that also contained the major AP-1 and Ets-binding elements present in the endogenous OPN promoter (30, 45–47). In detail these stable cell lines expressing OPN in the TRex-inducible system were made by transfection of OPN cDNA in pcDNA4/T0 and pcDNA6TR (both Invitrogen) into one of Rama 37, WT.BRCA1 C9, Mut.BRCA1 C9, and Trunc.BRCA1 C9 and subsequent selection in 400 μg/ml hygromycin (Invitrogen). Results were shown for the pooled transformant cell lines, and those for single cell cloned cell lines were substantially the same (not shown).

All cell lines were cultured in Dulbecco’s modified Eagle’s medium, 10% (v/v) fetal calf serum, 100 μg/ml penicillin, 100 μg/ml streptomycin (Invitrogen) and harvested. To study the effect on OPN production of both WT.BRCA1 and Mut. BRCA1, WT.BRCA1 C9 and OPN-TRex/WT.BRCA1 R37 cells were seeded in multiwell plates at 2.5 × 105 cells/3.5-cm diameter well, in 1 ml of serum-free medium. After 24 h they were transiently cotransfected with a predetermined amount of 400 ng of Mut.BRCA1 in its expression vector.

OPN promoter activity was estimated in transient transfections using the promoter coupled to firefly luciferase cDNA and normalized to Renilla luciferase activity in the dual luciferase assay.
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For the study of the effect on OPN production of BRCA1-siRNA, Rama 37 cell lines were transiently transfected with the siRNA plasmids containing a mixture of the three BRCA1-siRNAs using Lipofectamine and PLUS-C reagent (Invitrogen); these cell lines were termed R37/BRCA1-siRNA.

The human breast cancer cell lines MCF-7-WT.BRCA1, MCF-7-empty vector, and MCF-7-Mut. BRCA1 were generated by stably transfecting the MCF-7 breast cancer cells with either WT. BRCA1, empty vector, or Mut. BRCA1 to generate separate pools of transformant cell clones as described previously (43). Results for the pools of transformants were described, and those for the single cell clones were substantially the same. The MCF-7 cell line contains a single normal allele of BRCA1 (48).

Western Blotting for Proteins—For detection of BRCA1 or OPN proteins in stably or transiently transfected cells, 10 μg of protein of whole cell extracts from each transfectant cell line were electrophoresed through 10% (w/v) polyacrylamide, 1% (w/v) SDS gels and Western-blotted with rabbit polyclonal antibody to BRCA1, which can detect both human and rat (sc-7867, Santa Cruz Biotechnology, Santa Cruz, CA) or mouse monoclonal antibody (mAb) to OPN (Developmental Studies Hybridoma Bank) (6, 49). Western blots for time course and dose range of tetracycline for induction of OPN protein in the OPN-Trex cell lines were conducted as described above and visualized with mAb to OPN, as described previously (30, 43). The normal autoradiographic exposure time was 1 min.
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Northern Blotting—Total RNA was extracted from cells and subjected to Northern blot analysis, as described previously (50). The membranes were hybridized separately and in turn to OPN and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probes and subjected to autoradiography. To OPN and glyceraldehyde-3-phosphate dehydrogenase (50). The membranes were hybridized separately and in turn subjected to Northern blot analysis, as described previously (50).

Complex Detection by Immunoprecipitation—For immunoprecipitation in vitro, products were generated in a coupled transcription-translation cell-free protein-synthesizing reticulocyte lysate with [35S]methionine, and the expression vector for BRCA1 or nonradioactive amino acids and expression vectors for ERα, c-Jun, PEA3, Mut. BRCA1-HA, Tip60-HA, unprogrammed lysate for the empty vector pCMV-HA, and immunoprecipitated with mAbs to the different transcription factors as well as HA antibody as described previously (36). Characterization of the mAbs to ERα, c-Jun, and PEA3 has been undertaken previously (30, 36). A rabbit polyclonal antibody to BRCA1, raised against the N-terminal epitope, recognizes human, mouse, and rat BRCA1 in Western blots (sc-7867; Santa Cruz Biotechnology). The rabbit polyclonal antibody to HA tag raised against an internal region of the influenza HA protein (sc-805) also produced the correct sized proteins on Western blots (Santa Cruz Biotechnology). In detail, cDNA templates for ERα, c-Jun, PEA3, and WT.BRCA1 were prepared using a Hybaid kit (Hybaid, Middlesex, UK) and resuspended in RNase-free distilled water. An in vitro coupled transcription and translation kit (T7/T3-TnT; Promega, Madison, WI) was used according to the manufacturer’s instructions. After completion of the 90-min reaction, samples were kept on ice. One ml of immunoprecipitation buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.2 mM Na3VO4, 0.5% (w/v) Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 25 μg/ml leupeptin, 25 μg/ml aprotonin, and 25 μg/ml pepstatin) was added to each sample, mixed, and incubated on ice for 30 min. Twenty μl of protein A/G-agarose, prewashed three times in immunoprecipitation buffer, were added to each sample and incubated for an additional 4 h at 4 °C with rotation to remove any proteins that had interacted nonspecifically with protein A/G-agarose. Protein A/G-agarose was removed by cen-
trifugation at 14,000 rpm for 3 min in a benchtop centrifuge. The supernatant containing the £^{35}S$-labeled BRCA1 was mixed in turn with supernatants containing one of nonradioactive ER$\alpha$, c-Jun, and PEA3, and the combined supernatants were then incubated with 2.5 µg of the requisite antibody (ER$\alpha$, c-Jun, or PEA3 antibody) overnight at 4 °C with rotation. Twenty µl of protein A/G-agarose were added to each sample and incubated at 4 °C for an additional 60 min. Protein A/G-agarose antibody conjugates were recovered by centrifugation at 14,000 rpm for 3 min, resuspended in 1 ml of Buffer A (phosphate-buffered saline, 0.2% (w/v) Triton X-100, and 350 mM NaCl), and recentrifuged. Samples were resuspended in 1 ml of Buffer B (phosphate-buffered saline, 0.2% (w/v) Triton X-100), centrifuged, and resuspended in SDS sample buffer. Samples were resolved by electrophoresis on 10% (w/v) polyacrylamide gels at 200 V for 45 min with equal amounts of protein being loaded per lane. The gel was fixed for 30 min in 10% (v/v) propanol, 10% (v/v) acetic acid, dried under vacuum, and exposed to Kodak X-Omat AR x-ray film for 6–24 h before developing the film.

**IP-Western for the Interaction of PEA3 and BRCA1**—Rama 37 stably expressing WT.BRCA1 and/or PEA3 were lysed in buffer (250 mM NaCl, 20 mM sodium phosphate (pH 7.0), 30 mM sodium pyrophosphate (pH 7.0), 5 mM EDTA, 0.1 mM Na$_3$VO$_4$, 10 mM NaF, 0.1% Nonidet P-40) supplemented with protease inhibitors. The lysates were sonicated for 30 s and incubated on ice for 1 h. Cell debris was removed by centrifugation, and the supernatants were incubated overnight with protein A-Sepharose and with 1 µg of the antibody against BRCA1 for the supernatants containing PEA3 and BRCA1 or for the supernatants containing PEA3 alone. Immuno-precipitates were subjected to SDS-10% PAGE using Tris-glycine buffers, followed by transfer to a
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polyvinylidene difluoride membrane. The filters were incubated with PEA3 antibody and developed as described previously (36).

Endogenous IP and Western Blotting—Rama 37 cells were harvested and lysed in IP buffer (0.5% (v/v) Nonidet P-40 solution: 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.5% (v/v) Nonidet P-40, 10% (v/v) glycerol, and protease inhibitors). Total cell lysates were incubated with protein G beads with normal IgG as a pretreatment. The supernatants were then treated with BRCA1 antibody and immunoprecipitated with protein G beads for 3 h. After immunoprecipitation, beads were washed four times. Laemml buffer was added, and the samples were loaded onto 6–10% SDS-polyacrylamide gel, and transferred to nitrocellulose membrane (Millipore). Immunoblot analysis was performed using primary antibodies. The primary antibodies were as follows: BRCA1 (sc-7867, rabbit polyclonal; Santa Cruz Biotechnology), c-Jun (sc-1694, rabbit polyclonal; Santa Cruz Biotechnology), PEA3 (sc-113, mouse monoclonal; Santa Cruz Biotechnology). Immunodetection was performed using the enhanced chemiluminescence system (Amersham Biosciences) (32).

Soft Agar Assays—Cells were removed by trypsinization and resuspended at 1.5 × 10^5 cells/ml in normal medium. 10 ml of 1× DMEM with 10% (v/v) fetal calf serum, 1% (w/v) L-glutamine, 20 µg/ml gentamycin, 2 µg/ml penicillin were plated in 0.3% agar layered on top of 0.6% agar in 100-mm plates, and colonies were counted after 5–7 days of incubation at 37 °C in 5% (v/v) CO₂ in air (51).

Cell-Substrate Adhesion Assay—Multwell tissue culture plates (96 wells, Nunc, Naperville) were coated with 100 µl of serial dilutions (0–20 µg/ml, 100 µl/well) of human fibronectin by overnight adsorption at 4 °C. Sufficient wells were left blank to determine background crystal violet staining. Adherent cells were stained with crystal violet, and the dye in each well was solubilized. Background crystal violet staining was subtracted from all the values, and results were determined as an absorbance at 550 nm (52).

Matrigel Invasion Assay—Biocoat 250 µg/ml Matrigel invasion chambers 6.4 mm in diameter (Falcon-Ulster Anesthetics, Maneyrea, UK) were used to assess the invasiveness of suitably transfected Rama 37 cells. The migratory cells were stained by Gurr’s eosin and methylene blue, and the stain was released in acetic acid, and its absorbance was measured at 650 nm as described previously (51).

Immunohistochemistry of Human Breast Cancers—Specimens from primary tumors of 11 patients with familial breast cancer associated with mutant BRCA1 and of 11 age-matched control patients with sporadic breast cancer not associated with mutant BRCA1 were preserved in buffered formalin, embedded in paraffin wax, and sectioned (53, 54). The specific BRCA1 mutations in the patient cohort of the present study have been described previously (53, 54). The presence or absence of mutated BRCA1s was identified by heteroduplex analysis on lymphocyte DNA followed by cycle sequencing to confirm variants. Mutations were spread quite evenly along the length of the gene. The majority of mutations (88%) were either frameshift or nonsense mutations that would be predicted to result in premature truncation of the BRCA1 protein as described previously (53, 54). It was not determined whether the specific tumors were deleted in the wild type allele (53). The sections were stained immunocytochemically for OPN (6) or for BRCA1, the latter using mAb MS110 from Oncogene Research Products (Cambridge, MA) (55, 56) as described previously (30). All carcinoma sections were analyzed independently by two observers. The percentage of carcinoma cells or cell nuclei expressing OPN or BRCA1, respectively, from 10 fields per section at ×200 magnification, from 2 sections per specimen was recorded. Staining was categorized as positive with a threshold of ≥10% OPN or BRCA1-positive carcinoma cells or nuclei, respectively. The association of positive immuno-
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RESULTS

Effect of BRCA1 on Osteopontin Gene Expression—From parental Rama 37 (R37) and C9-Met-DNA-transfected R37 cells (C9), subclones were raised that stably expressed wild type, a human mutant, or truncated BRCA1 (WT.BRCA1, Mut.BRCA1, or Trunc.BRCA1). Parental Rama 37 subclones were designated WT.BRCA1 R37, Mut.BRCA1 R37, or Trunc.BRCA1 R37 and C9 subclones designated WT.BRCA1 C9, Mut.BRCA1 C9, or Trunc.BRCA1 C9. Similar cell lines were generated from Rama 37 cells containing the model tetracycline-inducible OPN (OPN-TRex R37 cells), and these were designated OPN-TRex/WT.BRCA1 R37, OPN-TRex/Mut.BRCA1 R37, or OPN-TRex/Trunc.BRCA1 R37, respectively, as described under “Materials and Methods.” Immunoblot analysis using a polyclonal antibody to the BRCA1 N-terminal region indicated similar elevated levels of BRCA1 expression in WT.BRCA1 R37, Mut.BRCA1 R37, and Trunc.BRCA1 R37 cells (5.5-, 5.2-, and 5.3-fold, respectively) over that in control untransfected Rama 37 cells (set at 1-fold) (Fig. 1A). Similar BRCA1 expression levels were observed in WT/Mut- (Fig. 1B) or Trunc.BRCA1 C9 cells (Fig. 1B) and in OPN-TRex R37/WT/ Mut- or Trunc.BRCA1 transformed subclones (data not shown).

Using the above cell systems OPN protein levels were reduced in WT.BRCA1 R37 cells but increased in Mut.BRCA1 R37 cells, whereas Mut.BRCA1 and Trunc.BRCA1 enhanced OPN mRNA and protein levels by 1.5–1.6-fold (Fig. 1, G and H). In all cases BRCA1 and OPN mRNA levels were normalized against constitutively expressed GAPDH mRNA (Fig. 1, D and G), and protein levels were normalized against constitutively expressed β-actin (Fig. 1, A–C, E, F, and H) in parental Rama 37 cells. These data suggest that WT.BRCA1 inhibits and mutant or truncated BRCA1 enhances OPN expression in rat mammary cells. Transfection of Rama 37 cells with expression vectors for BRCA1-siRNA reduced BRCA1 protein expression by 3.3-fold and increased OPN protein expression by 4-fold, respectively (Fig. 1E). These data suggest that endogenous OPN protein expression in Rama 37 cells is inversely dependent on expression of endogenous BRCA1.

Effect of BRCA1 on Transactivation of the OPN Promoter—Interactive effects of WT/Mut/Trunc.BRCA1 variants with OPN-activating transcription factors ERα, c-Jun, and PEA3 were investigated, using a 2.3-kbp wild type OPN promoter-luciferase construct (OPN-Luc). Transfection of Rama 37 cells with OPN-Luc, an ERα expression vector, and treatment with 10−8 m estradiol stimulated OPN promoter luciferase reporter activity by 6.9-fold (Student’s t test, p = 0.015) (Fig. 2A). In cotransfection experiments over a range of increasing concentrations, WT.BRCA1 in an expression vector progressively inhibited ERα-stimulated OPN-reporter activity to near base-line levels, with optimal inhibition at 75 ng of WT.BRCA1 vector/reaction (Fig. 2A). In controls BRCA1 protein expression corresponded approximately to the level of transfected WT.BRCA1 expression vector (Fig. 2B). This histochemical staining for OPN or BRCA1 and mutant BRCA1-containing carcinomas was assessed for each specimen using Fisher’s two-sided exact test (6).
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A

FIGURE 5. Effects of BRCA1 and mutants on properties associated with the malignant state of C9-Met-DNA-transformed R37 cells. A, effects on proliferation. Abbreviations used are as described under "Materials and Methods." Increase in cell number over 3 days is shown; values are the mean ± S.D. of triplicate experiments. B, effects on anchorage-independent growth. Effect of permanent transfection of expression vectors for BRCA1 and mutants on soft agar colony formation of C9 or on Rama 37 cells is shown (10^6 cells per dish). Cells were grown for 6 days in DMEM as overlays on 0.8% (w/v) agarose beds (see "Materials and Methods"). Results shown represent the mean colony number ± S.D. of triplicate experiments. Abbreviations are as follows: C9/Mut.BRCA1, Mut.BRCA1 cDNA in an expression vector transfected C9 cells; C9/Trunc.BRCA1, Trunc.BRCA1 cDNA transfected C9 cells; C9/WT.BRCA1, WT.BRCA1 cDNA transfected C9 cells, and C9/Mut/WT.BRCA1, Mut.BRCA1 cDNA transiently transfected WT.BRCA1 C9 cells (see "Materials and Methods"). C, effects on adhesion to fibronectin. Rama 37 (R37) and C9-Met-DNA-transformed R37 subclones (C9 subclones) were permanently transfected with expression vectors for BRCA1 or mutants and designated as in B above. The WT.BRCA1 C9 cells were also transiently transfected with an expression vector for Mut.BRCA1 to yield WT.BRCA1/Mut.BRCA1 C9 cells (C9/Mut/WT.BRCA1). The cells were grown on 20 µg/ml fibronectin-coated multiwell plates saturated with 1% (w/v) BSA. Cells were seeded and allowed to attach for 6 h. The number of adherent cells was measured by the A_550 of a colorimetric reaction, as described under "Materials and Methods." Results shown represent the mean ± S.D. of experiments in triplicate. D, effects on cell invasion. Rama 37 (R37) and C9-Met-DNA transformed subclones (C9); C9, WT.BRCA1 C9, Mut.BRCA1 C9, and Trunc.BRCA1 C9 as well as WT.BRCA1 C9 cells transiently transfected by an expression vector for Mut.BRCA1 (WT.BRCA1/Mut.BRCA1 C9) were tested; the abbreviations used are those in B above. Cells were cultured in Boyden chambers using fibronectin in the lower chamber as a chemoattractant. Cells that invaded through the dividing membrane were fixed, stained, the stain released with 10% (v/v) acetic acid, and the resultant absorbance was measured at 650 nm (see "Materials and Methods"). Results shown represent the mean ± S.D. of four experiments.

B

Inhibitory effect was not observed when cells were transiently transfected with 75 ng/reaction of expression vectors for Mut.BRCA1 or Trunc.BRCA1 (Fig. 2C). Indeed, Mut. BRCA1 and Trunc.BRCA1 each cooperated with ERα to increase the overall amount of OPN promoter reporter activity by 8.7- and 8.9-fold above control, respectively, and significantly above that with ERα/E2 alone (p = 0.0004 and p = 0.0003) (Fig. 2C).

Transfection of Rama 37 cells with OPN-Luc and previously determined optimal concentrations of expression vectors for c-Jun and/or PEA3 (200 ng/reaction for each) (30), alone and in combination, increased OPN promoter luciferase reporter activity by 10-, 8-, 9-, and 38–44-fold, respectively, over the unstimulated OPN promoter reporter construct (Fig. 2D). Transient cotransfection of cells with 75 ng/reaction WT.BRCA1 but not Mut.BRCA1 or Trunc.BRCA1 in an expression vector significantly inhibited the stimulation of OPN promoter luciferase-reporter activity induced by expression vectors for c-Jun or PEA3 alone or c-Jun/PEA3 in combination by 6.7-, 5.3-, and 14.6-fold respectively (p ≤ 0.02) (Fig. 2D). Stimulation of the OPN promoter reporter construct by expression vectors for ERα, c-Jun, and PEA3 was selectively blocked by mutations in their recognition sequences on the promoter. Transfection of the expression vector for BRCA1 inhibited the enhancing activities of only those transcription factors with intact recognition sequences on the promoter (supplemental Fig. 1, A–C). Because there is no known DNA recognition sequence for BRCA1 present in the 2.3-kbp OPN promoter, our results suggest that BRCA1 may interact directly with transcriptional factors ERα, c-Jun, and PEA3 to inhibit activation of the OPN promoter.

Coimmunoprecipitation Experiments of BRCA1 and Transcription Factors—To investigate whether WT.BRCA1 and ERα WT.BRCA1
and c-Jun, and WT.BRCA1 and PEA3 could interact directly, coimmunoprecipitation experiments were performed on transcription factors generated in coupled transcription/translation cell-free protein-synthesizing reticulocyte lysates. 35S-Labeled WT.BRCA1-containing lysates were incubated with nonradioactive ERα-, c-Jun-, PEA3-containing or unprogrammed lysates; any complexes so produced were immunoprecipitated with the relevant anti-ERα, c-Jun, or PEA3 antibodies (30) and analyzed on polyacrylamide gels (Fig. 3, A and B). An antibody to ERα coimmunoprecipitated a 35S-labeled protein of 220 kDa from WT.BRCA1-producing lysates (Fig. 3A, lane 1). Similarly, antibodies to c-Jun and PEA3, respectively, precipitated a 35S-labeled WT.BRCA1 protein of 220 kDa, with c-Jun-producing lysates (Fig. 3A, lane 3) and PEA3-producing lysates (Fig. 3A, lane 5). These 35S-labeled 220-kDa proteins corresponded to the major radioactive protein in the lysates and possessed the same molecular weight as reported for human WT.BRCA1 (11, 37). The specificity of the immunoprecipitated complexes formed between WT.BRCA1, ERα, c-Jun, and PEA3 was tested as follows. When 35S-labeled WT.BRCA1-containing lysates were incubated with unprogrammed nonradioactive lysates and then immunoprecipitated with anti-ERα, c-Jun, or PEA3, no radioactive WT.BRCA1 protein of 220 kDa was observed on the resultant polyacrylamide gels (Fig. 3A, lanes 2, 4, and 6). When 35S-labeled Mut.BRCA1 or Trunc.BRCA1-containing lysates were incubated with nonradioactive ERα-, c-Jun-, or PEA3-containing lysates and then immunoprecipitated with anti-ERα, anti-c-Jun, or anti-PEA3, no radioactive Mut.BRCA1 or Trunc.BRCA1 proteins of 220 or 50 kDa, respectively, were observed on the resultant polyacrylamide gels (data not shown). However, when lysates containing 35S-labeled WT.BRCA1 and nonradioactive Mut.BRCA1 containing the HA tag (Mut.BRCA1-HA) were incubated and then immunoprecipitated with a mAb to the HA tag, a radioactive protein of the same size (220 kDa) as BRCA1 was observed on the polyacrylamide gels (Fig. 3B, lane 1). This radioactive band was not observed in controls in which an unrelated transcription factor Tip 60-HA (40) (Fig. 3B, lane 2), no transcription factor (lane 3), or unprogrammed HA-containing lysates (lane 4) replaced those containing Mut.BRCA1-HA. Finally the interaction between WT.BRCA1 and PEA3 has been confirmed by IP-Western blotting (Fig. 3C). In controls expression vectors for either BRCA1 (Fig. 3C, lane 1) or PEA3 (Fig. 3C, lane 2), when transfected into Rama 37 cells, produced proteins of the correct size. When Rama 37 cell extracts containing both expressed proteins were immunoprecipitated with antibody to BRCA1, a band corresponding to PEA3 was observed on subsequent Western blots for PEA3 of the solubilized precipitate (Fig. 3C, lane 3). This band was absent when the cell extracts contained only recombinant PEA3 (Fig. 3C, lane 4). These results show that WT.BRCA1 can interact specifically with transcriptional cofactors of the OPN promoter ERα, c-Jun, and PEA3 and with mutated BRCA1, which, in turn, fails to interact with the same transcriptional cofactors.

To determine whether interaction occurred between endogenous BRCA1 and endogenous c-Jun or PEA3 in untransfected Rama 37 cells, their cell lysates were immunoprecipitated with control or BRCA1 antibody, followed by Western blotting of the precipitates with antibodies to BRCA1, c-Jun, and to PEA3 as indicated. A strong interaction of endogenous BRCA1 with endogenous c-Jun or PEA3 was observed in Rama 37 cells (Fig. 3D, lane 2). This interaction was dependent on the presence of BRCA1 in the complex, and without it no interaction occurred (Fig. 3D, lane 1). These results indicate that complexes occurred between BRCA1 and c-Jun or PEA3 in untransfected Rama 37 cells.

Reversal of BRCA1 Inhibition of OPN Expression by a Cancer-related Mutant—Effects of mutant BRCA1 (Mut.BRCA1) upon WT.BRCA1 transcriptional repression of the OPN promoter luciferase-reporter construct (Fig. 4) and of enhanced OPN.
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mRNA and protein (Fig. 1, D and E) were investigated in transiently transfected Rama 37 and WT.BRCA1 C9 cells, respectively. Mut.BRCA1 impaired WT.BRCA1 suppression of the OPN promoter in a dose-dependent manner. At a transfection dose of 100 ng/reaction, the Mut.BRCA1 plasmid impeded WT.BRCA1 plasmid (75 ng/reaction) suppression of OPN promoter luciferase activity, whereas at 400 ng/reaction, the Mut-BRCA1 plasmid effectively blocked WT.BRCA1 plasmid suppression of the OPN promoter reporter (Fig. 4). Moreover, transient transfection of the expression plasmid for Mut.BRCA1 (400 ng/reaction) in the WT.BRCA1 C9 cells, completely inhibited the suppressive effect of WT.BRCA1 plasmid on the levels of OPN mRNA (Fig. 1D, lane 6) and OPN protein (Fig. 1E, lane 6), causing them to be raised by 6- and 4.5-fold respectively, to levels observed in parental C9 cells (Fig. 1, D and E). In contrast the levels of the constitutively expressed GAPDH mRNA and β-actin protein were unchanged (Fig. 1, D and E). Similar effects were observed on the expression of OPN mRNA (Fig. 1G, lane 6) and OPN protein (Fig. 1H, lane 6) in OPN-TRex/WT.BRCA1 R37 cells transiently transfected with the expression plasmid for Mut.BRCA1 (400 ng/reaction). These data suggest that Mut.BRCA1 impedes WT.BRCA1 suppression of the OPN promoter, OPN transcript, and protein.

Effect of BRCA1 on the Morphology, Proliferation, and Anchorage-independent Growth of OPN-transformed Cells—Low OPN-producing cells (parental Rama 37 or OPN-TRex R37 cells without tetracycline) were flattened with round nuclei, whereas high OPN producers (C9 or OPN-TRex R37 cells with tetracycline) became elongated, spindel-shaped with a pleomorphic outline. Transfection of parental low OPN-producing Rama 37 cells with expression vectors for WT.BRCA1, Mut.BRCA1, or Trunc.BRCA1 had little effect on cellular morphology (data not shown). Transfection of high OPN-producing C9 or tetracycline-treated OPN-TRex R37 cells with an expression vector for WT.BRCA1 but not Mut.BRCA1 or Trunc.BRCA1 changed cell morphology to a flattened phenotype with rounded nuclei (data not shown). These results suggest that the OPN-related cell phenotypic changes can be suppressed by WT.BRCA1.

In growth studies, proliferation of WT.BRCA1 C9 cells was over 2.5-fold less than that of C9, Mut.BRCA1 C9, or even Rama 37 cells after 3 days (Fig. 5A). In soft agar assays C9 cells, Mut.BRCA1 C9 and Trunc.BRCA1 C9 cells produced a 10-fold increase in colony number per plate compared with control Rama 37 cells (Fig. 5B). The 10-fold increase in colony number per plate for C9 cells was specifically abolished by supertransfection with an expression vector for WT.BRCA1 (Fig. 5B). Transient transfection of WT.BRCA1 C9 cells with 400 ng/reaction Mut.BRCA1 in an expression vector increased colony numbers by 8-fold over that of cells without Mut.BRCA1 cDNA transfection, restoring them to that of C9 cells alone (Fig. 5B). In the absence of tetracycline, there were no significant effects of wild type, mutated, or truncated BRCA1 upon cell growth of OPN-TRex R37 cells (Fig. 6A). After tetracycline treatment (1.2 µg/ml), however, growth of OPN-TRex/WT.BRCA1 R37 cells was over 3-fold less than that of OPN-TRex R37, OPN-TRex/Mut. BRCA1 R37, or OPN-TRex/Trunc.BRCA1 R37 cells (Fig. 6B). Tetracycline treatment of OPN-TRex R37 cells, OPN-TRex/Mut. BRCA1 R37, and OPN-TRex/Trunc.BRCA1 R37 cells induced a 6-7-fold increase in colony number per soft agar plate compared with Rama 37 or OPN-TRex R37 cells without tetracycline controls. This effect was not observed in tetracycline-treated OPN-TRex/WT. BRCA1 R37 cells (Fig. 6C). Hence, inhibition of anchorage-dependent and -independent growth by WT.BRCA1 appears to be dependent upon OPN overexpression in the Rama 37 rat mammary epithelial subclones.

Effect of BRCA1 on Adhesion and Invasion—There was a 2.5-fold increase in adhesion for C9 cells over Rama 37 cells themselves. This increase was specifically abolished by stable supertransfection with an expression vector for WT.BRCA1, but not with those for Mut.BRCA1 or Trunc.BRCA1 (Fig. 5C). Cell invasion through Matrigel was assayed by a dye-based system (51, 57). C9, Mut.BRCA1 C9, and Trunc.BRCA1 C9 cells induced an 8-9-fold increased in cell invasion, in comparison with R37 cells. Invasion of WT.BRCA1 C9 cells was reduced to 2.9 that of C9 cells (Fig. 5D). Transient transfection of WT.BRCA1 C9 cells with 400 ng/reaction Mut.BRCA1 in an expression vector increased the level of cell adhesion by 2.9-fold (p = 0.005) (Fig. 5C) and that of invasion by 2.8-fold (p = 0.005) (Fig. 5D) over that of cells without Mut.BRCA1 cDNA transfection, restoring them to those of C9 cells alone.

Treatment of OPN-TRex R37 cells, OPN-TRex/Mut. BRCA1 R37 cells, and OPN-TRex/Trunc.BRCA1 R37 cells with 1.2 µg/ml tetracycline produced about a 9-fold increase in adhesion to fibronectin-coated dishes, in comparison with cells not treated with tetracycline. This tetracycline-induced increase in adhesion was not observed in tetracycline-treated OPN-TRex/WT.BRCA1 R37 cells (p = 0.015) (Fig. 6D). Tetra-
cycloheximide treatment (1.2 μg/ml) of OPN-TreX R37 cells, OPN-TreX/Mut.BRCA1 R37 cells, and OPN-TreX/Trunc.BRCA1 R37 cells induced 2–3-fold increases in cell invasion, in comparison with untreated OPN-TreX R37 cells. Invasion of tetra-cycloheximide-treated OPN-TreX/WT.BRCA1 R37 cells, however, was ∼40% less than that of the other tetracycline-treated cell subclones (Fig. 6E). Transient transfection of OPN-TreX/WT.BRCA1 R37 cells with 400 ng/reaction Mut.BRCA1 in an expression vector and tetracycline treatment increased the level of cell invasion by 1.8-fold (p = 0.007) over cells without Mut.BRCA1 transfection (Fig. 6E).

Osteopontin Expression and Function in Breast Tumor Cell Lines Stably Transfected with Mut.BRCA1—OPN protein levels were assayed in total cell lysates from MCF-7-WT. BRCA1, MCF-7-empty vector, and MCF-7-Mut.BRCA1 human breast cancer cell lines by Western blotting. OPN was weakly expressed in MCF-7-empty vector (1-fold induction) and MCF-7-WT.BRCA1 cells (0.9-fold induction). Conversely, high expression of OPN was observed in MCF-7-Mut.BRCA1 cells (8-fold induction) (data not shown). Moreover, the Rama 37 cells transfected with Mut.BRCA1, which overexpressed OPN (Fig. 1C), produced about a 14-fold increase in colonies per plate over that with Rama 37 alone. Transfection with WT.BRCA1 was without appreciable effect (Fig. 6C).

BRCA1 Status and Osteopontin/BRCA1 Expression in Familial and Sporadic Human Primary Breast Cancers—Histologic sections of familial BRCA1 mutant or sporadic BRCA1 nonmutant human primary breast cancers were assessed for OPN and BRCA1 expression by immunohistochemistry. Seven of eleven familial mutant BRCA1 cancers expressed OPN in ≥10% carcinoma cells in comparison with only 1/10 BRCA1 nonmutant sporadic breast cancers (Fisher’s exact test; p = 0.024; Fig. 7) (Table 1). In contrast the same fraction of 2/11 familial mutant breast or nonmutant sporadic breast cancers expressed BRCA1 in ≥10% carcinoma cell nuclei (p = 1.0; see Table 1). The fraction of cell nuclei stained for BRCA1 in normal lobules of the breast was 100% (not shown). These results suggested that mutant BRCA1 and not the level of BRCA1 is significantly associated with OPN overexpression in a human breast cancer cell line and in human primary breast cancers.

DISCUSSION

In our model rat mammary system, transfection of C9-Met-DNA into Rama 37 cells increased OPN expression by 4–5-fold and caused enhanced anchorage-independent growth in soft agar, enhanced cellular adhesion to fibronectin-coated surfaces, and enhanced cellular invasion through Matrigel, in agreement with previous reports (31, 44). The C9-Met-DNA R37 cells use integrated copies of C9-Met-DNA to activate the endogenous rat OPN gene by sequestration of an inhibitory transcription factor Tcf-4 (43). Moreover, subsequent transfection of C9-Met-DNA transfected cells with expression vectors for WT.BRCA1, Mut.BRCA1, or Trunc. BRCA1 separately all yield similar protein levels of BRCA1-related products. These results establish suitable model systems in which to compare the effect on OPN expression and function of WT.BRCA1 with that of two of its mutants found in familial breast cancer (38, 39). In this study we show that wild type BRCA1 has a largely suppressive effect on OPN production and function in C9-Met-DNA transfected R37 cells. Conversely, neither Mut.BRCA1 nor Trunc.BRCA1 has such effects. These results are not an artifact of a single clone of transformant cells, because they have been obtained with cell lines produced from pools of single cell clones of transformants and substantially confirmed by analysis of cell lines obtained from single cell clones (see “Materials and Methods”). We have also independently confirmed these results in Rama 37 cell lines transfected either with a relevant inducible model promoter for OPN (Fig. 1, G and H, and Fig. 6, C–E) or with a similar model promoter in a transfected pBK-CMV expression vector for OPN.3 Overexpression of WT.BRCA1 in the parental Rama 37 cells is also associated with a small but significant reduction in basal levels of OPN protein, suggesting that BRCA1 can also suppress the level of expression of OPN from a relatively inactive endogenous rat gene. The inhibitory effect of BRCA1 on mammalian promoters is not universal, however, because it fails to inhibit the exogenously transfected promoter reporter construct 4XVDRE-DR3-Tk-Luc for the vitamin D3-induced protein (58) in Rama 37 cells.3

Because overexpression of WT.BRCA1 in C9-Met-DNA-transfected R37 cells produces similar reductions in the levels of OPN protein and mRNA, it probably acts at the level of OPN transcription. The above conclusion has been confirmed in Rama 37 cells by transient cotransfection of the WT.BRCA1 expression vector, the promoter reporter construct for the rat OPN gene, and expression vectors for the transcription factors ERα, c-Jun, and the Ets factor PEA3. These transcription factors recognize specific enhancer sequences in the endogenous rat OPN promoter (supplemental Fig. 1), thereby stimulating its activity (Fig. 2), and their overexpression is associated with that of OPN in specimens from human breast cancers (30, 36). The inducible and noninducible transfected expression vectors used for OPN place its expression under the control of the cytomegalovirus promoter which, although different from that of the endogenous rat promoter, contains a major core of 11 and 3 recognition sequences for c-Jun (45, 46) and Ets transcription factors (47), respectively, the same transcription factor recognition sequences and transcription factors encountered in the OPN promoter (30). The OPN transgenes are therefore relevant model systems for the endogenous promoter, at least for these two transcription factors and the effect of BRCA1 and ERα on modulating their activity. This study has shown that wild type but not mutated or truncated BRCA1 suppresses ERα, c-Jun, or PEA3 stimulation of the OPN promoter reporter in Rama 37 cells. These findings suggest that the inhibitory effect of WT.BRCA1 on transactivation of the OPN promoter reporter may arise from a specific interaction between BRCA1 and components of the OPN transcription activation complex in both the endogenous and transfected OPN genes. However, BRCA1 does not typically bind to specific DNA sequences (59), and hence our findings suggest that BRCA1 may inhibit OPN expression by effects upon OPN transcription factors, at the protein level. This conclusion is supported by direct coimmu-

3 M. K. El-Tanani, F. C. Campbell, P. Crowe, P. Erwin, D. P. Harkin, P. Pharoah, B. Ponder, and P. S. Rudland, unpublished results.
noprecipitation and by exogenous and endogenous IP-Western blot experiments. Previous reports have also shown that WT.BRCA1 can bind and inhibit transcriptional activity induced by ERα (32, 60), c-Jun (33), and Ets transcription factors (34) at promoters other than that for the OPN gene. The fact that the cancer-related mutants of BRCA1, viz. Mut.BRCA1 and Trunc.BRCA1, fail to bind to and repress the activity of any of the three transcription factors suggests that the mutated/deleted region contains elements of the binding site(s) for all three transcription factors (32–34, 60).

In this study, Mut.BRCA1 can actively reverse WT.BRCA1 suppressive effects upon transcription and function of OPN, including suppression of (i) ERα stimulation of the activity of the transiently transfected OPN promoter reporter (Fig. 4), (ii) enhanced OPN mRNA and protein in C9 and OPN-TRex R37 cells (Fig. 1, D, E, G, and H, respectively), and (iii) enhanced cell adhesion (Fig. 5C and Fig. 6D), invasion through Matrigel (Fig. 5D and Fig. 6E) in the same cells, and enhanced growth in soft agar in C9 cells (Fig. 5B). This dominant negative effect of Mut.BRCA1 on the suppressive effect of WT.BRCA1 cannot be due to competitive binding of Mut.BRCA1 with WT.BRCA1 to the three transcription factors tested, because no such binding of the factors to Mut.BRCA1 has been detected. The more likely explanation is that, as shown in Fig. 3B, Mut.BRCA1 binds directly to and thereby probably inactivates WT.BRCA1. One possible explanation for this dominant negative effect is that WT.BRCA1 has to dimerize (61, 62) to perform its suppressive role in transcription. Hence, binding of WT.BRCA1 to Mut.BRCA1 may prevent this. Our findings that cancer-related mutants of BRCA1 enhance considerably OPN expression in parental rat Rama 37 cells (3.2–3.5-fold) (Fig. 1C) and in human MCF-7 cells (12-fold) and induce anchorage-independent growth in Rama 37 cells (Fig. 6C) are consistent with the concept that they exert a dominant negative effect upon the function of an endogenous WT.BRCA1. The more limited increase in expression of OPN from an activated promoter caused by

| Tumor no. | % OPN carcinoma cells staining | OPN staining >10% cells | BRCA1 staining >10% cells | BRCA1 mutant |
|-----------|-------------------------------|-------------------------|---------------------------|-------------|
| 7/11      |                               |                         |                           |             |
| 1         | 0                             | –                       | +                         | –           |
| 2         | 8                             | –                       | –                         | –           |
| 3         | 60                            | +                       | –                         | –           |
| 4         | 1–2                           | –                       | +                         | +           |
| 5         | 3–4                           | –                       | –                         | –           |
| 6         | 70                            | +                       | –                         | +           |
| 7         | <1                            | –                       | –                         | +           |
| 8         | 35                            | +                       | –                         | +           |
| 9         | 35                            | +                       | +                         | –           |
| 10        | 15                            | +                       | –                         | +           |
| 11        | 30                            | +                       | +                         | –           |
| 12        | 3                             | –                       | –                         | +           |
| 13        | 15                            | +                       | +                         | –           |
| 14        | 30                            | +                       | –                         | +           |
| 15        | 3–4                           | –                       | –                         | +           |
| 16        | 2–3                           | –                       | –                         | +           |
| 17        | <1                            | –                       | –                         | +           |
| 18        | <1                            | –                       | –                         | –           |
| 19        | 4                             | –                       | –                         | –           |
| 20        | ND                            | ND                      | +                         | –           |
| 21        | <1                            | –                       | –                         | +           |
| 22        | 4                             | –                       | –                         | –           |

FIGURE 7. Osteopontin immunohistochemistry in familial BRCA1 mutant or sporadic BRCA1 wild type breast cancers. Low (A) and high power (B) views of OPN-positive BRCA1 mutant primary breast cancer. C, high power view of a sporadic BRCA1 wild type, OPN negative primary breast cancer. Magnification: A, ×90, bar = 50 μm; B and C, ×225; bar = 20 μm.
mutants of BRCA1 (Fig. 1, D, E, G, and H) may be due to the reduced effectiveness of WT.BRCA1 in suppressing it.

OPN is usually absent or expressed at a low level in normal tissues but is up-regulated in certain neoplastic and neoplastic epithelia (63–65), including that of the breast (6). Indeed, OPN overexpression has adverse prognostic significance in human breast cancer (6, 7). Significant correlations are observed between levels of OPN and its transcriptional factors ERα, c-Jun, and PEA3 in 29 primary sporadic human breast cancers (30). By comparison lower levels of BRCA1 transcript are associated with the occurrence of distant metastases in one group of sporadic breast cancer patients (66). Here we show that transfection of the expression vector for the cancer-related Mut.BRCA1 into a human breast cancer cell line leads to the overproduction of OPN. Moreover, the incidence of immunohistochemically detectable OPN is increased in a small group of familial primary breast cancers with mutant BRCA1, in comparison with that found in sporadic breast carcinomas containing only wild type BRCA1. For this difference to become statistically significant, the cut-off value for the percentage of immunocytochemically stained carcinoma cells has to be raised from the usual 5% (6) to 10%. This difference in OPN expression in familial breast cancer is not because of a further reduction in level of immunohistochemically detectable BRCA1 in the nucleus of the carcinoma cells over that obtained in sporadic breast cancer (Table 1), in agreement with a previous report (56). The enhancing effect of mutant BRCA1 on other transcription factors active at the OPN promoter such as ERα, c-Jun, and PEA3 (30, 36) may cause enhanced production of OPN in mutant BRCA1 tumors and the development of a more aggressive form of invasive breast cancer. This project shows a suppressor function for BRCA1 upon the OPN transcriptional activation complex and OPN-mediated neoplastic transformation of mammary epithelium. Moreover, one naturally occurring mutant of BRCA1 that occurs in familial breast cancer has lost this suppressive effect, and it inhibits the suppressive effect of WT.BRCA1 in a dominant manner. Our work suggests that loss of transcriptional suppressor function of WT.BRCA1 on the estrogen receptor may make those tissues with high levels of this receptor more susceptible to the induction of osteopontin by mutant BRCA1 and consequent neoplasia.

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