BRCA1 Represses Amphiregulin Gene Expression

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Abstract

BRCA1, the breast cancer- and ovarian cancer-specific tumor suppressor, can be a transcriptional repressor or a transcriptional activator, depending on the promoter context. To identify the genes activated or repressed by BRCA1, we have analyzed microarray results from cells depleted of BRCA1 and revealed a number of genes regulated by BRCA1 on the level of transcription. Among the genes repressed by BRCA1, we have identified amphiregulin (AREG) and early growth response-1 (EGR1). Results indicate that BRCA1 regulates AREG transcription directly through binding to the AREG promoter, however, we could not detect BRCA1 on the EGR1 promoter, suggesting that EGR1 is indirectly regulated by BRCA1. In an attempt to identify the mechanism of the AREG transcriptional repression by BRCA1, we have mapped two independent BRCA1 response elements on the AREG located at positions −202/−182 and +19/+122. BRCA1 depletion leads to induction of the AREG protein. Taken together, our data build the connection between BRCA1 loss of function and AREG upregulation—a change in gene expression often observed in breast cancer. Cancer Res; 70(3); 996–1005. ©2010 AACR.

Introduction

Loss of function of the tumor suppressor BRCA1 (breast cancer–associated 1) protein is responsible for the high percentage of familial and sporadic breast cancers. BRCA1 inactivating mutations are found in ~50% of patients with familial breast cancer (1, 2); however, in sporadic breast cancer, mutations in BRCA1 are almost never found. Nevertheless, sporadic cases are often characterized by decreased BRCA1 mRNA and protein levels (3). Moreover, in many clinical samples, ~14% of sporadic breast cancers revealed hypermethylated BRCA1 promoter—an evidence of epigenetic downregulation (4).

BRCA1 protein participates in key cellular processes, including transcription, repair of DNA damage, cell cycle checkpoints, and centrosome dynamics (5–7). The question of how BRCA1 mediates its role as a tumor suppressor is unresolved. BRCA1 is primarily a nuclear protein (1), and one of its first identified roles is transcriptional stimulation (8, 9).

BRCA1 copurifies with an RNA polymerase II (RNAPII)–containing complex (9–11). The mechanism by which BRCA1 protein regulates transcription has been elucidated. BRCA1 stimulates transcription at a variety of promoters by stabilizing the preinitiation complex (12). BRCA1 can also act as a transcriptional repressor when in the presence of active ubiquitination cofactors, by conjugating ubiquitin on RNAPII and preventing the initiation of RNA synthesis (13). It is likely that the specificity of whether BRCA1 stimulates or represses the transcription reaction depends on the factors that recruit BRCA1 protein to the promoter (12, 13).

How might the function of BRCA1 as a transcriptional repressor contribute to its function as a tumor suppressor? We hypothesize that the recruitment of BRCA1 to a gene promoter regulates the transcription of a factor that is critical for tumorigenesis. In the current study, we performed gene expression microarray analysis with RNAi-depleted BRCA1 samples and identified genes significantly repressed by BRCA1, including amphiregulin (AREG) and early growth response 1 (EGR1). We found that BRCA1 is present on the AREG promoter. Using reporter assays, we determined that the response to BRCA1 repression mapped to two sites, the −202/−182 and +19/+122 positions of the AREG promoter. By contrast, our results suggest that BRCA1 indirectly regulates EGR1 transcription, possibly through AREG protein induction. Taken together, our data show the connection between BRCA1 tumor suppressor and AREG, a ligand of the epidermal growth factor receptor (EGFR), which is important in breast cancer development and progression.

Materials and Methods

Constructs. Plasmids for the expression of specific shRNA were prepared as described (14). The BRCA1-specific shRNA plasmid was the same one used previously (13). Reporter constructs were based on the AREG promoter–containing
vector described by Plowman and colleagues (15) and were prepared by subcloning of the promoter fragments −643/+122, −202/+122, −182/+122, −159/+122, −136/+122, −110/+122, −44/+122, −202/+19, −182/+19, −159/+19, −136/+19, and −110/+19 into pGL3 basic vector (Promega). The primer sequences used for subcloning are presented in Supplementary Materials (Supplementary Table S1).

**Cell culture.** The HeLa cell line (ATCC cell line CCL-2) was maintained in DMEM supplemented with 10% bovine serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin. The SUM149PT cell line (Asterand) was maintained in Ham’s F-12 medium supplemented with 5% fetal bovine serum, 5 μg/mL insulin, 2 μg/mL hydrocortisone, and 5 μg/mL gentamicin. All the cell lines were maintained in a humidified incubator at 37°C and 5% CO2.

**Preparation of cDNA for microarray and TaqMan analysis.** HeLa cells were cotransfected (Lipofectamine; Invitrogen) with 5 μg of shRNA expression plasmid (14) and 20 ng of pBabe-puromycin plasmid. BRCA1 shRNA (gaggacagagccacgaagag) was targeted to the 3’-untranslated region of the BRCA1 mRNA. The control shRNA was targeted against a mutant green fluorescent protein (GFP) construct (ggggcaagacccacgaagag). Puromycin selection (2 μg/mL) was applied 24 h after transfection, and cells were harvested at 72 h posttransfection. BRCA1 siRNA was used to eliminate the possibility of an off-target effect was targeted to position 2616 of the mRNA (GGU UUC AAA GGC CCA GUC AdTdT). The control siRNA was targeted against luciferase (UCG AAG UAU GCC UGC GdTdT). RNA was prepared with either Tri Reagent (Molecular Research), which, with RNA was further purified over RNeasy columns (Qiagen), or Dynabeads mRNA direct kit (Invitrogen). Microarray analysis was performed on separate samples at the Harvard Biopolymers Facility (Boston, MA) and at the Microarray Shared Resource in Comprehensive Cancer Center (Ohio State University, Columbus, OH) on the Affymetrix HG-U133_plus_2 chip. The use of different RNA purification strategies and of different microarray facilities resulted in identification of the most robust changes in gene expression secondary to BRCA1 depletion. For TaqMan assays, cDNA was reverse transcribed from mRNA using the Superscript II kit (Invitrogen). Primers for AREG, EGR1, Jun, BRE, XRC4, DDX58, KRT17, GDF15, and CCL5 were purchased from Applied Biosystems. Real-time PCR reactions were done in nine replicates. GAPDH and TFR2 were used as controls. Calculations of ΔΔCt value were done as described previously (16).

**Chromatin immunoprecipitation.** The cells were treated with 1% formaldehyde for 20 min at 37°C in the incubator, washed three times with 1× PBS buffer, and lysed in a warm buffer (30°C) containing 50 mmol/L Tris-HCl (pH 8.1), 10 mmol/L EDTA, 1% SDS, protease inhibitor cocktail, and 1 mmol/L phenylmethylsulfonylfluoride. The lysate was incubated on ice for 20 min and sonicated to obtain ~500 bp DNA fragments. The samples were diluted 10-fold into dilution buffer [part of chromatin immunoprecipitation (ChIP) Assay Kit (17-295), Upstate (Millipore)], precleared with ssDNA/protein A beads [part of ChIP Assay Kit (17-295), Upstate (Millipore)], and incubated with the antibodies at 4°C overnight. The BRCA1-specific antibody and matched preimmune serum have been described before (17). Spi (SC-59, Santa Cruz Biotechnology) and histone H3 antibodies [06-755, Upstate (Millipore)] were used. The beads were added and incubated for 1 h; washed once with the high-salt solution [part of ChIP Assay Kit (17-295), Upstate (Millipore)], low-salt solution [part of ChIP Assay Kit (17-295), Upstate (Millipore)], and LiCl solution [part of ChIP Assay Kit (17-295), Upstate (Millipore)]; and washed three times with 1× TE buffer (pH 8.0). The samples were eluted from the beads in 1% SDS and 0.1 mol/L NaHCO3 solution and treated with 0.2 mol/L NaCl for 6 h at 65°C, then with 0.1 mg/mL proteinase K [in buffer containing 10 mmol/L EDTA and 70 mmol/L Tris-HCl (pH 6.3)] for 1 h at 45°C and extracted using PCR clean-up kit (Qiagen). Alternatively, the samples were prepared using ChIP-IT Express Enzymatic kit (Active Motif). The samples were analyzed by real-time PCR using SYBR green dye (Bio-Rad), and the primers were described in Supplementary Materials Table S2. Real-time PCR reactions were run on the equipment provided by Plant-Microbe Genomics Facility (Ohio State University, Columbus, OH). Results were analyzed using the methods described previously (Bio-Rad, Bulletin 2916).

**Reporter gene assay.** HeLa cells on a six-well plate were cotransfected (Lipofectamine; Invitrogen) with 1.6 μg of shRNA expression plasmid targeting either BRCA1 or, as a control, mutant GFP (14), 1.6 μg promoter-reporter construct, and 66 ng of pBabe-puromycin plasmid. Puromycin selection (2 μg/mL) was applied 24 h after transfection; 72 h after transfection, cells were lysed using the lysis buffer from the luciferase assay system kit (Promega). The fluorescent units were normalized to the sample protein content measured using BCA protein assay kit (Pierce). The transfection efficiency was checked by cotransfection of the GFP expression plasmid.

**Results**

**Inhibition of BRCA1 reveals a number of repressed and activated genes in cancer-associated pathways.** In an effort to determine which genes might be contributing to cancer development and progression when BRCA1 function is lost, we performed an Affymetrix array analysis by comparing the gene expression pattern of HeLa cells in which BRCA1 protein was depleted using RNAi (Fig. 1A).

Our study revealed the large number of genes that were either upregulated or downregulated following depletion of BRCA1, and we chose 2.3-fold change as a reasonable cutoff for further investigation. The list of these genes is shown in Table S3 of Supplementary Materials. The Ingenuity Pathway Analysis (IPA) software was used to analyze the lists of genes and reveal their contribution to disease, and to identify genetic networks and pathways that include the affected genes. Analysis of the gene list (253 genes), using IPA software, revealed that 85 genes are expressed in the mammary gland and 153 genes are expressed in breast cancer cell lines (Table S4 of Supplementary Materials). Moreover, IPA revealed the involved biological functions and diseases. The distribution
of the genes according to their function and their role in the pathologic processes is presented in Table 1. The pathologic processes that involve cancer (cell cycle, cell growth, cell movement, and gene expression) are all consistent with known functions of BRCA1 in controlling cell cycle progression after DNA damage (18), DNA repair (19), and control of centrosomes (18, 20).

The distribution of the genes according to the diseases and disorders to which they might be related is also shown in Table 1. One hundred of these genes are involved in cancer development and progression. The functional analysis of the genes reveals that they are involved in cell growth and proliferation (69 molecules), cell death (67 molecules), cellular development, gene expression, and cell cycle regulation (Table 1). The results shown in Table 1 illustrate how BRCA1 loss can contribute to cancer development and progression. Because the changes in gene expression due to BRCA1 depletion are consistent with known processes, functions, and diseases associated with BRCA1, an investigation of specific genes from this dataset is supported.

**Inhibition of BRCA1 leads to increased AREG mRNA expression.** Loss of BRCA1 protein, as may occur in a breast cancer tumor cell, would be expected to result in a derepression of genes that the tumor suppressor normally inhibits.

![Figure 1. Affymetrix data identify a number of genes repressed by BRCA1, among them AREG and EGR1, and the pathway analysis reveals EGR1 as a downstream target for AREG. A, shRNA-mediated BRCA1 depletion in HeLa cells. HeLa cells were transfected with a plasmid expressing shRNA specific for GFP (Control, lane 1) or for BRCA1 (lane 2). Cells were harvested 72 h after transfection, and Western blots were probed with antibodies against BRCA1 (top) and RNA polymerase II large subunit (Rpb1, loading control, bottom). B, validation of the data obtained from the Affymetrix microarray analysis by real-time PCR. The controls in Affymetrix microarray data and in real-time PCR were normalized to 1 and shown in white and light gray. The Affymetrix microarray data and the real-time PCR data for the samples where BRCA1 was depleted are shown in dark gray and black, respectively. X axis, genes used in the study; Y axis, fold change relative to the control. C, pathway analysis of the genes identified by Affymetrix microarray and validated by quantitative real-time PCR. A biological circuit with AREG taken from IPA software of microarray results is shown. AREG, GDF15, EGR1, and KRT17 all had significant changes in expression following depletion of BRCA1; EGFR did not significantly change expression but was included in the regulatory module due to the pathway analysis. The position of each gene in the figure corresponds to its cellular localization. The dashed line illustrates the indirect connections between the molecules; the solid lines indicate direct connection between molecules. The line without arrows shows interaction; the lines with arrows indicate activation.
Genes that were significantly upregulated in our dataset and identified using IPA to affect cancer development and progression were selected from the microarray data and further validated by real-time PCR (Fig. 1B). *AREG, EGR1, DDX58, KRT17, GDF15*, and *CCL5* were confirmed by real-time PCR (Fig. 1B). The genes *BRE, Jun, and XRCC4* were not confirmed by real-time PCR and therefore excluded from the study. *AREG, EGR1, DDX58, KRT17, GDF15*, and *CCL5* were all expressed in breast cancer cell lines according to IPA (Supplementary Table S4).

Among the genes upregulated in HeLa cells with depleted BRCA1, we have identified *AREG* and *EGR1* to be of particular interest because both genes have been reported to have high levels of expression in breast cancer (21, 22); moreover, both genes were upregulated in Affymetrix microarray experiment and validated by quantitative real-time PCR. Affymetrix data revealed 3.7-fold increase in *AREG* mRNA, and quantitative reverse transcriptase-PCR (RT-PCR) revealed a 6.3-fold increase in *AREG* expression after depletion of BRCA1 by RNAi (Fig. 1B). Therefore, we conclude that the BRCA1 silencing leads to the increased *AREG* mRNA expression in HeLa cells. For *EGR1*, we have observed 2.5-fold increase in expression from Affymetrix data and 11.3-fold increase in quantitative real-time PCR (Fig. 1B). To eliminate the possibility of off-target effects, we depleted BRCA1 in HeLa cells using a siRNA targeting a different BRCA1 sequence than that specified by the shRNA used in the preceding experiments. The quantitative real-time PCR data revealed a 2.3-fold increase in *AREG* expression and 2.9-fold increase in *EGR1* expression (Supplementary Fig. S1); therefore, we have concluded that *AREG* and *EGR1* mRNA levels increase in response to BRCA1 depletion. Moreover, IPA analysis revealed that *EGR1* is a downstream target for *AREG* (Fig. 1C; Supplementary Fig. S2) and indicated how genes identified by microarray and confirmed by quantitative real-time PCR can all be linked: *AREG* overexpression indirectly leads to *EGR1* upregulation, which, in turn, stimulates growth differentiation factor 15 (GDF15). *AREG* has been reported to bind to EGFR and to activate it

### Table 1. The distribution of genes upregulated or downregulated >2.3-fold in response to BRCA1 silencing according the top networks, diseases and disorders to which they might be involved, and their function in the cell

<table>
<thead>
<tr>
<th>Top networks</th>
<th>Score</th>
<th>Molecules</th>
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<tbody>
<tr>
<td>Immune response, viral function, nucleic acid metabolism</td>
<td>46</td>
<td>26</td>
</tr>
<tr>
<td>Cardiac hypertrophy, cardiovascular disease, developmental disorder</td>
<td>39</td>
<td>23</td>
</tr>
<tr>
<td>Cancer, cell cycle, gastrointestinal disease</td>
<td>34</td>
<td>21</td>
</tr>
<tr>
<td>Cancer, cellular growth and proliferation, hematologic disease</td>
<td>32</td>
<td>20</td>
</tr>
<tr>
<td>Cardiovascular system development and function, cell morphology, cellular development</td>
<td>30</td>
<td>19</td>
</tr>
<tr>
<td>Cancer, cellular movement, neurologic disease</td>
<td>21</td>
<td>15</td>
</tr>
<tr>
<td>Amino acid metabolism, cancer, small-molecule biochemistry</td>
<td>21</td>
<td>15</td>
</tr>
<tr>
<td>Cardiovascular system development and function, molecular transport, small-molecule biochemistry</td>
<td>20</td>
<td>14</td>
</tr>
<tr>
<td>Cell signaling, nucleic acid metabolism, small-molecule biochemistry</td>
<td>18</td>
<td>13</td>
</tr>
<tr>
<td>Organ morphology, reproductive system development and function, amino acid metabolism</td>
<td>18</td>
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<td>Cancer, cell death, gene expression</td>
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<td>13</td>
</tr>
<tr>
<td>Drug metabolism, molecular transport, nucleic acid metabolism</td>
<td>16</td>
<td>12</td>
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<th>Diseases and disorders</th>
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<tr>
<td>Viral function</td>
<td>2.66E−06 to 1.57E−03</td>
<td>9</td>
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<tr>
<td>Organism injuries and abnormalities</td>
<td>2.84E−06 to 2.47E−03</td>
<td>29</td>
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<tr>
<td>Dermatologic diseases and conditions</td>
<td>6.25E−06 to 3.91E−04</td>
<td>22</td>
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<tr>
<td>Genetic disorder</td>
<td>6.25E−06 to 2.70E−03</td>
<td>12</td>
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<table>
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<th>Molecular and cellular functions</th>
<th>Score</th>
<th>Molecules</th>
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<td>Cell death</td>
<td>2.27E−08 to 2.79E−03</td>
<td>67</td>
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<tr>
<td>Cellular growth and proliferation</td>
<td>2.86E−07 to 2.79E−03</td>
<td>69</td>
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<td>Cellular development</td>
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<td>Gene expression</td>
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<tr>
<td>Cell cycle</td>
<td>1.50E−05 to 2.79E−03</td>
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(23). Pathway analysis reveals that EGFR interacts with KRT17 and stimulates EGR1 expression, which, in turn, stimulates GDF15 (Fig. 1C). The stimulation of this module could be initiated by increases in AREG concentration. Our subsequent experiments are focused on AREG and EGR1 because the increases in mRNA levels for these genes ranked among the highest in the Affymetrix data, and both of these genes are linked to breast cancer.

**BRCA1 binds to the AREG promoter.** To test whether BRCA1 regulation of AREG is direct, we assayed, using the ChIP assay, for the presence of BRCA1 protein on the AREG promoter. Using either antibodies specific to BRCA1 or a matched preimmune serum, the quantitative real-time PCR of the immunoprecipitated DNA used primers covering two regions, −490/−184 and −201/+13, of the AREG promoter. We observed a 3- and 5-fold increase relative to the matched preimmune serum for the promoter regions at −490 to −184 and −201 to +13, respectively, relative to the mRNA start site (Fig. 2A and B). As a positive control, ChIP assay using antibody specific for histone H3 showed an 8.8-fold increase in PCR signal when compared with the negative control preimmune serum for the region −490/−184, and 37.3-fold increase when compared with the negative control preimmune serum for the region −201/+13 (data not shown). We conclude that BRCA1 is present on the AREG promoter and directly represses AREG transcription. Because histone H3 is certainly abundant in multiple sites on the AREG promoter, it is not surprising that the ChIP assay revealed a higher-fold increase relative to BRCA1. As an additional positive control, we assayed by ChIP for the presence of the Sp1 transcription factor on the AREG promoter using antibodies specific for Sp1 because its binding site is located at positions −3/−8 of the AREG promoter (ref. 15; Fig. 2A). As negative controls, we assayed by ChIP...
for AREG exon 4 (∼5.5 kb away from transcription start site) and exon 6 (∼9.8 kb away from the transcription start site) regions using antibodies specific for BRCA1 and Sp1. As shown in Fig. 2C, Sp1 and BRCA1 samples had an increase in relative promoter binding compared with the preimmune matched serum on the AREG regulatory region −490/−184. By contrast, neither Sp1 nor BRCA1 bound to the AREG exon 4 and AREG exon 6 regions. Taken together, these results indicate that BRCA1 indeed binds directly to the proximal promoter region of the AREG gene.

In contrast to the results with the AREG promoter, ChIP experiments aiming to investigate whether BRCA1 binds the EGR1 promoter revealed the absence of BRCA1 on the EGR1 promoter (Fig. 2B), suggesting that the effect of BRCA1 on EGR1 transcription upregulation is indirect. This result is consistent with the outcome of the analysis using the IPA software, which showed that EGR1 stimulation is a downstream target of increasing AREG protein concentration (Fig. 1C; Supplementary Fig. S2). AREG is an extracellular ligand that interacts with the EGFR present on the surface of HeLa cells (24), leading to activation of a number of downstream genes, including EGR1, which was significantly increased by BRCA1 depletion.

**BRCA1 repression of AREG transcription maps to two elements of the AREG promoter.** We mapped the sequences of the AREG promoter DNA that are BRCA1 responsive, by using a gene expression reporter assay. The reporter construct contained the AREG regulatory sequence −643/+122 relative to the mRNA 5′ end previously described by Plowman and colleagues (15). The reporter gene, luciferase, was placed downstream of the AREG promoter. HeLa cells were transfected with the reporter plasmid, the plasmid that expresses the shRNA that specifies BRCA1 mRNA, and the plasmid carrying puromycin resistance. Cells that received these plasmids were then selected by growth in the presence of puromycin, and BRCA1 protein was effectively depleted (Fig. 3A; Supplementary Fig. S3).

Depletion of BRCA1 led to 2.5-fold derepression of the reporter containing the AREG regulatory sequence −643/+122 (Fig. 3B). The deletion of region −643/−203 from the promoter-reporter construct revealed that this sequence was not important for the BRCA1 repression and therefore mapped the BRCA1-responsive region to −202/+122. These results for the BRCA1 repression of the reporter containing −202/+122 of the AREG promoter were validated using a siRNA specific for a different BRCA1 sequence, ruling out off-target effects as causing this repression (Supplementary Fig. S4). The comparison of the promoter-reporter constructs containing −202/+122 and −202/+19 fragments of the AREG promoter with the further progressive deletion
constructs corresponding to −182/+122 and −182/+19 fragments revealed that BRCA1 depletion can lead to derepression of the reporters containing −202/+122, −202/+19, and −182/+122 fragments of the promoter but not −182/+19, leading to the conclusion that BRCA1 binds to two regions on the EREG promoter located at −202/−182 and +19/+122 positions. Moreover, these data illustrate that −202/−182 and +19/+122 BRCA1-responsive sites are independent.

BRCA1 regulates transcription on the variety of promoters; however, on its own, it does not bind DNA with sequence specificity (25, 26). Therefore, we hypothesize that BRCA1 protein binds indirectly to the −202/−182 and +19/+122 DNA elements through other factors specifically bound at these sites, and the identification of these other factors is the subject of continuing investigations.

**BRCA1 represses AREG protein levels.** The previous experiments have shown that BRCA1 is a transcriptional repressor of the EREG promoter. Depletion of BRCA1 by RNAi resulted in the increased AREG transcript level. According to the pathway analysis (Fig. 1C), the function of AREG protein can cause the upregulation of EGR1 gene that we observed (Fig. 1B). Thus, we tested whether BRCA1 control of AREG transcription could affect protein levels. Western blot analysis of BRCA1-depleted HeLa cell lysates revealed that RNAi targeting BRCA1 leads to the increase of AREG protein level (Fig. 4A). AREG has been reported to be present in cells as 16, 21, 25, and 28 kDa proteins (21). Our data reveal that a protein with migration consistent with a 25 kDa polypeptide and reactive with the AREG-specific antibody is induced following depletion of BRCA1 (Fig. 4A). This result supports the concept that BRCA1 protein represses AREG protein expression.

AREG was reported to be upregulated in breast cancer (21); moreover, it is known that loss of BRCA1 function can lead to breast cancer development. We asked whether loss of BRCA1 can be connected to breast cancer pathology through AREG upregulation. As a model to study how BRCA1 silencing can affect AREG protein levels in breast tissue, we have chosen SUM149PT invasive breast carcinoma cell line that was reported to have low BRCA1 transcript level due to an inactivating mutation (27). Interestingly, published reports have shown that the SUM149PT cell line has increased AREG protein levels compared with the MCF10A cell line (21), indicating that BRCA1 loss and AREG upregulation could be related.

To test whether BRCA1 regulates AREG protein levels, we performed an experiment wherein BRCA1 was reexpressed in SUM149PT cells and compared the AREG level to untreated samples. The data shown in Fig. 4B illustrates that BRCA1 expression leads to the moderate yet significant decrease of the AREG protein level (25 kDa band). The fact that the effect was moderate was not surprising and could be explained by the fact that the transfection efficiency in SUM149PT cell line was ∼40% (data not shown).

Taken together, our data from two different cell lines show that BRCA1 regulates AREG expression. From this finding, we suggest a model in which loss of BRCA1 often observed in breast cancer patients results in AREG upregulation, which was shown to stimulate proliferation and migration (21).

**Discussion**

The BRCA1 tumor suppressor has been shown to have various cellular roles, including transcription regulation, DNA damage repair, and cell cycle regulation (28). Loss of BRCA1 due to inactivation or mutation leads to cancer. Despite intensive study for several years, the question of how the loss of function of BRCA1 leads to cancer development and progression remains unresolved. In this study, we have found that (a) depletion of BRCA1 protein results in derepression of a number of genes, including AREG and EGR1; (b) BRCA1 binds to the AREG promoter but not the EGR1 promoter; (c) the AREG promoter contains two independent DNA sites that are responsive to BRCA1; and (d) AREG protein levels are controlled by BRCA1 protein. Taken together, these results suggest that a part of the tumorigenic phenotype of breast cancer might be explained through loss of BRCA1 repression of AREG gene expression.

![Figure 4](cancerres.aacrjournals.org)
To address the role of BRCA1 repression of transcription in tumor suppression, we first identified the genes that significantly change their expression levels in response to BRCA1 depletion in HeLa cells. We have chosen the HeLa cell line for our experiments because it is a well-characterized cell line that has endogenously expressed BRCA1; moreover, in HeLa, BRCA1 can be efficiently depleted. These data are in agreement with previously published results showing that BRCA1 can act both as a transcriptional repressor and as a transcriptional activator depending on the promoter context (12, 13). Among the genes that showed high fold change, we have selected those that were shown to play a role in cancer, among them **AREG** and **EGR1**. After confirming our Affymetrix results by real-time PCR, we have focused our further study on these two genes.

The comparison of our data in HeLa cells with published results from MCF10A mammary epithelial cells in three-dimensional culture following depletion of the BRCA1/CtIP/ZBRK1 repressor complex (29) revealed similar effect for **AREG** and **EGR1**. In that study, depletion of BRCA1 resulted in a 50% increase in expression of **AREG** and **EGR1**. Although the effect on these genes after BRCA1 depletion had a lower magnitude, the trend is in the same direction, suggesting that BRCA1 control of **AREG** is not limited to HeLa cells.

**AREG** and **EGR1** upregulation of transcription as a response to BRCA1 depletion illustrates BRCA1 involvement in the regulation of these genes; however, the microarray results alone do not answer the question of whether BRCA1 directly regulates **AREG** and **EGR1** transcription. ChIP data showed the presence of BRCA1 on **AREG** promoter but not on the **EGR1** promoter. Moreover, **EGR1** is a downstream effector in a gene activation cascade initiated by **AREG** (30). Taken together, our data suggest that BRCA1 is present on the **AREG** promoter and acts as a transcriptional repressor, and that **EGR1** upregulation is secondary to **AREG** derepression when BRCA1 is depleted. Moreover, **EGR1** leads to direct activation of GDF15, which is upregulated in breast cancer (refs. 31, 32; Fig. 1C); therefore, our data suggest how BRCA1, **AREG**, **EGR1**, and GDF15 can be connected together.

Because our data revealed that BRCA1 silencing is indirectly involved in the upregulation of **EGR1** transcription, we have focused our further study on **AREG**. **AREG** is a member of the epidermal growth factor family and is a ligand of EGFR. It is expressed as a transmembrane precursor that is cleaved by ADAM17 protease and released to activate its receptor (23). Analysis of **AREG**-depleted mice revealed an essential role of **AREG** in mammary epithelial development (23, 33). **AREG** overexpression has been frequently observed in colon, breast, prostate, pancreas, lung, and ovarian cancer, as well as in squamous cell carcinomas and myeloma cells (34–40). **AREG** can promote its own expression through the activation of EGFR in a positive-feedback **AREG** autocrine loop (21). Overexpression of **AREG** in MCF10A cell line leads to increased cell motility and invasion (21).

Although **AREG** function has been well described (21, 23, 41) and the **AREG** promoter has been characterized (15), a full understanding of **AREG** transcriptional control remains undetermined. Only a few transcription factors have been shown to regulate **AREG** transcription, among them Sp1 binding at positions −37/−8 (15), cAMP-responsive element binding protein binding at positions −57/−64 (42), and WT1 zinc finger transcription factor binding at positions −68/−84 of the **AREG** promoter (43). 17β-Estradiol has been shown to induce **AREG** mRNA expression through activation of estrogen receptor (44), although no estrogen response element is characterized on this promoter. None of these known enhancers correlates with the BRCA1 elements that we mapped, using the reporter assay, on the **AREG** promoter.
BRCA1 has been reported to regulate transcription on a variety of promoters; however, on its own, it binds DNA only in a non-sequence-specific manner (25, 26). BRCA1 can regulate transcription by interacting with other transcription factors that can bind to specific sites on DNA, and, in this way, BRCA1 alters the function of these transcription factors (28, 45). We have identified two independent BRCA1 response elements on the AREG promoter located at the −202/−182 and +19/+122 positions. We hypothesize that because BRCA1 is a transcriptional repressor of the AREG promoter, BRCA1 protein, in a complex with BARD1, binds to a sequence-specific transcription factor/activator bound to either of the identified DNA elements, and represses AREG expression (Fig. 5). Identification of the BRCA1 binding sequence-specific transcription factor(s) is the subject of continuing investigation.

AREG is often overexpressed in breast cancer (46); moreover, AREG expression in breast cancer samples does not correlate with estrogen receptor α (ERα) (46, 47), and AREG and EGFRI expression in invasive carcinomas is correlated with the absence of estrogen receptor (48). Tumors resulting from BRCA1 mutations are frequently ER independent. The ER-independent invasive ductal carcinoma cell line SUM149PT has been characterized by low BRCA1 transcript due to the 2288delT BRCA1 inactivating mutation (27) and has an increased AREG mRNA and protein levels (21). Our results suggest that there is a direct link to explain this prior observation. Our experiments show for the first time that BRCA1 depletion leads to AREG upregulation in the HeLa cell line, and BRCA1 expression in SUM149PT cell line leads to reduction in the amount of AREG protein, supporting the idea that BRCA1 controls AREG expression. Moreover, AREG overexpression in MCF10A leads to activation of the genes involved in cell mobility and invasion (21), which allows us to suggest that BRCA1 loss of function can contribute to cancer development and progression through AREG upregulation (Fig. 5). Taken together, our data suggest that a part of the tumorigenic phenotype of breast cancer might be explained through loss of BRCA1 repression of AREG gene expression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

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