Research article

Involvement of a specificity proteins-binding element in regulation of basal and estrogen-induced transcription activity of the BRCA1 gene

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Abstract

Introduction Increased estrogen level has been regarded to be a risk factor for breast cancer. However, estrogen has also been shown to induce the expression of the tumor suppressor gene, BRCA1. Upregulation of BRCA1 is thought to be a feedback mechanism for controlling DNA repair in proliferating cells. Estrogens enhance transcription of target genes by stimulating the association of the estrogen receptor (ER) and related coactivators to estrogen response elements or to transcription complexes formed at activator protein (AP)-1 or specificity protein (Sp)-binding sites. Interestingly, the BRCA1 gene lacks a consensus estrogen response element. We previously reported that estrogen stimulated BRCA1 transcription through the recruitment of a p300/ER-α complex to an AP-1 site harbored in the proximal BRCA1 promoter. The purpose of the study was to analyze the contribution of cis-acting sites flanking the AP-1 element to basal and estrogen-dependent regulation of BRCA1 transcription.

Methods Using transfection studies with wild-type and mutated BRCA1 promoter constructs, electromobility binding and shift assays, and DNA-protein interaction and chromatin immunoprecipitation assays, we investigated the role of Sp-binding sites and cAMP response element (CRE)-binding sites harbored in the proximal BRCA1 promoter. The purpose of the study was to analyze the contribution of cis-acting sites flanking the AP-1 element to basal and estrogen-dependent regulation of BRCA1 transcription.

Results We report that in the BRCA1 promoter the AP-1 site is flanked upstream by an element (5’-GGGGCGGAA-3’) that recruiting Sp1, Sp3, and Sp4 factors, and downstream by a half CRE-binding motif (5’-CGTAA-3’) that binds CRE-binding protein. In ER-α-positive MCF-7 cells and ER-α-negative Hela cells expressing exogenous ER-α, mutation of the Sp-binding site interfered with basal and estrogen-induced BRCA1 transcription. Conversely, mutation of the CRE-binding element reduced basal BRCA1 promoter activity but did not prevent estrogen activation. In combination with the AP-1/CRE sites, the Sp-binding domain enhanced the recruitment of nuclear proteins to the BRCA1 promoter. Finally, we report that the MEK1 (mitogen-activated protein kinase kinase-1) inhibitor PD98059 attenuated the recruitment of Sp1 and phosphorylated ER-α, respectively, to the Sp and AP-1 binding element.

Conclusion These cumulative findings suggest that the proximal BRCA1 promoter segment comprises cis-acting elements that are targeted by Sp-binding and CRE-binding proteins that contribute to regulation of BRCA1 transcription.

Introduction Gene expression in mammary tissue is under the control of ovarian steroids, including estrogen, which regulates transcription activity of target promoters by stimulating the recruitment of either the estrogen receptor (ER)-α or ER-β [1]. In the classical pathway, the liganded ER-α homodimerizes and
binds directly to DNA at estrogen response elements (EREs) [2]. The physical contact of the ER-α homodimer with DNA facilitates the subsequent recruitment of coregulators [3]. The latter factors contribute to chromatin reorganization and transcriptional coactivation [4]. Alternatively, the ER-α can physically interact with DNA-bound protein complexes containing, among other factors, the activator protein (AP)-1 [5], cAMP response element (CRE [TGACGTCA])-binding protein (CREB) [1], or specificity protein (Sp) family members [6,7]. The Sp factors have been shown to bind directly to the consensus GC-rich region 5’-(G/T)GGGGCGG(G/A)(G/A)-3’ or DNA-bound AP-1 transcription complexes [8].

Members of the Sp family of transcription factors exert differential effects on gene transcription. For example, Sp1 and Sp4 have been shown to act as transcriptional activators, whereas Sp3 was reported to antagonize Sp1 activation functions by competing for promoter occupancy [9-11]. The Sp family members play a significant role in tissue and organ development, because mice lacking at least one of the Sp proteins exhibit various physiological abnormalities, including embryonic lethality [12], growth retardation [13,14], and sterility [15]. Increased expression of Sp1 has been detected in gastric [16,17] pancreatic [18], and breast [19] tumors. In addition to inducing the recruitment of ER to target promoters, estrogen influences gene expression through the activation of mitogen-activated protein kinases (MAPKs), which phosphorylate the ER-α and other transcription factors including Sp1 [20] and AP-1 [2]. Phosphorylation of these factors increases their binding affinity for cognate DNA elements or DNA-bound coregulators [21].

The breast cancer susceptibility gene BRCA1 encodes a phosphoprotein that is involved in the DNA damage response [22,23] and regulation of cell cycle checkpoints [24]. About 30% to 40% of sporadic breast cancers, which represent the late normal BRCA1 expression.

In proliferating breast epithelial cells, the cellular levels of BRCA1 mRNA and protein have been shown to increase in response to estrogen [27-29]. The BRCA1 promoter harbors two distinct transcriptional start sites (exon-1A and exon-1B). However, translation of BRCA1 mRNA always starts from the ATG codon located on exon-2 [30]. Upregulation of BRCA1 expression in response to estrogen may represent a feedback mechanism that represses ER-signaling during the early stages of breast tumorigenesis [31]. This interpretation is supported by evidence indicating that the BRCA1 protein represses the transcriptional activity of the liganded ER-α and estrogen-regulated genes [32]. However, the BRCA1 gene appears not to be regulated by estrogen through the classical pathway, because the BRCA1 promoter lacks canonical EREs. Recently, our laboratory reported that estrogen stimulated BRCA1 promoter activity by inducing the recruitment of an ER-α/p300 transcription complex to an AP-1 element in close proximity to the transcriptional start site of exon-1B [33,34]. The objective of the present work was to elucidate the roles played by Sp-binding and CRE-binding elements flanking the AP-1 site in regulation of BRCA1 transcription.

Materials and methods
The experimental research reported in the manuscript has been performed with the approval of the Institutional Biosafety Committee of the University of Arizona (Tucson, AZ, USA), on 20 December 2005.

Cell culture and chemicals
MCF-7 and HeLa cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco’s modified Eagle’s medium (DMEM)/F12 (Sigma-Aldrich Chemical Co., St. Louis, MO, USA), supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT, USA) as described previously [29]. Estrogen, penicillin/streptomycin solution, and DMEM/F12 were purchased from Sigma-Aldrich Chemical Co. Antibodies against ER-α, Sp1, Sp3, and Sp4 were purchased from Santa Cruz Biotechnologies, Inc. (Santa Cruz, CA, USA). The antibody against phosphorylated ER-α (Ser118) was obtained from Cell Signaling (Beverly, MA, USA).

Site-directed mutagenesis
Details concerning the cloning of a 1.7-kilobase BRCA1 promoter fragment into pGL3 Basic (pGL3BRCA1) are described elsewhere [35]. Mutation of the Sp (GGGGCGG to GCTAAG) and half-CRE (CGTAA to CtgAC) core sequence in the BRCA1 promoter was carried out by site-directed mutagenesis (Stratene, La Jolla, CA, USA) using the following primers synthesized by Sigma-Genosys (The Woodlands, TX, USA): Sp-F-Mut: 5’-GGTACGGCCTaGAACCTGAGGCGCTAAGG CG-3’; Sp-R-Mut: 5’-CGCCTTACCGCCTCTCA GGTTCtagCCTACC-3’; CRE-F-Mut: 5’-GGAAACCTAGAGGGCtgACGGGTTGTGAAG-3’; and CRE-R-Mut: 5’-CTTCACACCGCCCTtgcaGCCTTCTCAG GTTCC-3’. The insertion of mutations was confirmed by direct sequencing.

Transfection studies
Experimental conditions for cell culture and transfection of reporter plasmids were those described previously [33]. Briefly, MCF-7 and HeLa cells were cultured for 3 days in phenol red-free DMEM/F12 and supplemented with 5% charcoal-stripped FBS. Then, cells were seeded in six-well plates 24 hours before transfection using the Lipofectamine-Plus (Invitrogen, Carlsbad, CA, USA). Plasmids encoding renilla were also cotransfected to account for variations in transfection.
efficiency and cell density. Luciferase reporter activity was monitored with a Luminometer 20/20 (Turner Biosystems, Sunnyvale, CA, USA) and expressed as relative luciferase units corrected for renilla.

**DNA-protein pull-down assay**

Eighty to ninety per cent confluent breast MCF-7 cells were cultured for 3 days in phenol red-free DMEM/F12 and supplemented with 5% charcoal-stripped FBS. Cells were then maintained in phenol red-free DMEM/F12 plus vehicle with 5% charcoal-stripped FBS with or without 10 nmol/l estrogen before nuclear extracts were obtained using the Nuclear/Cytoplasmic Extract Kit (Pierce Biotechnology, Rockford, IL, USA). The biotin-labeled double stranded oligonucleotides were based on the human BRCA1 promoter sequence. The DNA-protein pull-down assay was performed as described previously [36]. Briefly, MCF-7 nuclear extracts were mixed with biotin-labeled oligonucleotides and streptavidin agarose beads with 70% slurry. After 2 hours of incubation at room temperature, beads were centrifuged and washed with cold phosphate buffer saline. Bound proteins were then separated on 4% to 12% PAGE and Western blot analysis was performed with antibodies against Sp1, Sp3, total ER-α, or phosphorylated ER-α. Oligonucleotides used for the pull-down assay were as follows: Sp/AP-1, forward: 5’-GGTACGGCCCTAGCTGAGGCCTAA-3’; Sp/AP-1, reverse: 5’-TTACGGCTCTCAGGTTACCCCTACC-3’. Samples were electrophoresed through a 5% nondenaturing polyacrylamide gel at 200 V for 90 minutes. Finally, the gel was dried and exposed to a phosphor screen, and digital phosphorimages were retrieved using the Storm system (Molecular Dynamics, Sunnyvale, CA, USA).

**Electrophoretic mobility binding and shift assays**

The procedure used for electromobility binding assay was described previously [33]. Briefly, MCF-7 nuclear extracts were mixed with biotin-labeled oligonucleotides and streptavidin agarose beads with 70% slurry. After 2 hours of incubation at room temperature, beads were centrifuged and washed with cold phosphate buffer saline. Bound proteins were then separated on 4% to 12% PAGE and Western blot analysis was performed with antibodies against Sp1, Sp3, total ER-α, or phosphorylated ER-α. Oligonucleotides used for the pull-down assay were as follows: Sp/AP-1, forward: 5’-GGTACGGCCCTAGCTGAGGCCTAA-3’; Sp/AP-1, reverse: 5’-TTACGGCTCTCAGGTTACCCCTACC-3’. Samples were electrophoresed through a 5% nondenaturing polyacrylamide gel at 200 V for 90 minutes. Finally, the gel was dried and exposed to a phosphor screen, and digital phosphorimages were retrieved using the Storm system (Molecular Dynamics, Sunnyvale, CA, USA).

**Chromatin immunoprecipitation assay and real-time PCR**

Chromatin immunoprecipitation assay and real-time PCR were carried out as described previously [34,37]. Briefly, MCF-7 cells were prepared in phenol red-free DMEM/F12 supplemented with 5% charcoal-stripped FBS for 3 days. After treatment with control DMEM plus vehicle or 10 nmol/l estrogen, chromatin was crosslinked with formaldehyde (1% final concentration) for 10 minutes at room temperature and subsequently glycine was added to quench the formaldehyde crosslinking. Cells were then harvested and resuspended in lysis buffer (1% SDS, 10 mmol/l EDTA, 50 mmol/l Tris-HCl, and protease inhibitor cocktail). After sonication (10 × 15 seconds), samples were diluted in chromatin immunoprecipitation (ChIP) buffer (1% Triton X-100, 2 mmol/l EDTA, 150 mmol/l NaCl, 20 mmol/l Tris-HCl, and protease inhibitor cocktail). Dilutions of chromatin preparations were reserved as either input (no antibody) or used for immunoprecipitation with the desired antibody or IgG as a negative control. The chromatin immunoprecipitation was conducted utilizing the Magna ChIP kit (17–611) obtained from Millipore (Billerica, MA, USA), in accordance with the protocol provided. Briefly, the sonicated samples were immunoprecipitated overnight at 4°C with the antibodies and G magnetic beads. The magnet beads were pelleted and sequentially washed with a low salt immune complex wash buffer, high salt immune complex wash buffer, LiCl immune complex wash buffer, and TE buffer. The protein-DNA complexes were eluted and crosslinking was reversed by incubation with ChiP elution buffer and proteinase K for 2 h at
62°C, followed by incubation for 10 minutes at 95°C. The free DNA was then purified using the provided spin columns.

PCR primers used to amplify the BRCA1 promoter region flanking the Sp/AP-1/CRE binding sites were as follows: forward: 5'-CTGACAGATGGGTATTCTTTGACG-3'; and reverse: 5'-GCATATTCCAGTTCCCTGAACTGAGGCGTGTAAGGCGT-3'; followed by incubation for 10 minutes at 95°C. The free DNA was then purified using the provided spin columns.

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The primary objective of this study was to examine whether cis-acting elements flanking an AP-1 site located in the proximal BRCA1 promoter played a role in the basal and estrogen-dependent regulation of BRCA1 transcription. We mapped immediately upstream from the AP-1 site a GC-rich region located at positions -41/-36 (GGGGCGGAA) that shared perfect homology to the consensus Sp-binding sequence (5'-[G/T]GGGGCGG[G/A] [G/A]-3') [8] (Figure 1). We also mapped a half-CRE-binding site (5'-CGTAA-3') immediately downstream (-23/-27) from the AP-1 element. Previous investigations from our laboratory have documented that estrogen-dependent stimulation of BRCA1 transcription required the assembly of an ERα/p300 complex at the AP-1 site [33]. Because Sp-binding factors and CRE-binding factors have been shown to cooperate with AP-1 in regulation of transcription, we examined the role played by these sites in controlling basal and estrogen-induced BRCA1 promoter activity. Breast cancer MCF-7 cells were transiently transfected with either a reporter-luciferase construct containing 1.7 kilobase of the wild-type BRCA1 promoter (pGL3BRCA1, -1552 to +140 means following a significant (P < 0.05) analysis of variance test was performed by Fisher’s protected least significant difference test.

**Results**

The primary objective of this study was to examine whether cis-acting elements flanking an AP-1 site located in the proximal BRCA1 promoter played a role in the basal and estrogen-dependent regulation of BRCA1 transcription. We mapped immediately upstream from the AP-1 site a GC-rich region located at positions -41/-36 (GGGGCGGAA) that shared perfect homology to the consensus Sp-binding sequence (5'-[G/T]GGGGCGG[G/A] [G/A]-3') [8] (Figure 1). We also mapped a half-CRE-binding site (5'-CGTAA-3') immediately downstream (-23/-27) from the AP-1 element. Previous investigations from our laboratory have documented that estrogen-dependent stimulation of BRCA1 transcription required the assembly of an ERα/p300 complex at the AP-1 site [33]. Because Sp-binding factors and CRE-binding factors have been shown to cooperate with AP-1 in regulation of transcription, we examined the role played by these sites in controlling basal and estrogen-induced BRCA1 promoter activity. Breast cancer MCF-7 cells were transiently transfected with either a reporter-luciferase construct containing 1.7 kilobase of the wild-type BRCA1 promoter (pGL3BRCA1, -1552 to +140

| Promoter element | Oligonucleotides                                                                 |
|------------------|----------------------------------------------------------------------------------|
| AP-1/CRE         | Sense: 5'-AACCTGAGAGGCGTAAAGGCGT-3', Antisense: 5'-ACGCCCTTACCGCTAGTTCTAGT-3' |
| Sp/AP-1/CRE      | Sense: 5'-GGGTAGGGCGGAACTGAGAGGCGT-3', Antisense: 5'-ACGCCCTTACCGCTAGTTCTAGT-3' |
| Sp-Mut/AP-1/CRE  | Sense: 5'-GGGTAGGGCGGAACTGAGAGGCGT-3', Antisense: 5'-ACGCCCTTACCGCTAGTTCTAGT-3' |
| Intervening AP-1/CRE sequences: | Sense: 5'-GGGTAGGGCGGAACTGAGAGGCGT-3', Antisense: 5'-ACGCCCTTACCGCTAGTTCTAGT-3' |
| Sp/ Ap-1/CRE-Mut | Sense: 5'-GGGTAGGCGGAACTGAGAGGCGT-3', Antisense: 5'-ACGCCCTTACCGCTAGTTCTAGT-3' |
| Intervening AP-1/CRE sequences: | Sense: 5'-GGGTAGGGCGGAACTGAGAGGCGT-3', Antisense: 5'-ACGCCCTTACCGCTAGTTCTAGT-3' |

AP, activator protein; CRE, cAMP response element; Sp, specificity protein.
In MCF-7 cells transfected with pGL3-BRCA1, the treatment with estrogen induced (about 2.3-fold) BRCA1 promoter activity (Figure 2a) as compared with the control vehicle (DMEM), thus confirming the results of previous findings from our laboratory [34]. However, basal reporter activity was reduced by about 80% in MCF-7 cells transfected with pSpmut. We observed a small increase (1.2-fold; \( P < 0.10 \)) in BRCA1 promoter activity upon treatment with estrogen, but this stimulation was considerably lower than that observed in cells transfected with the wild-type pGL3 BRCA1 promoter construct. Conversely, the mutation of the half CRE-binding site reduced by about 35% basal BRCA1 promoter activity but did not interfere with estrogen-dependent activation (about 2.0-fold) of BRCA1 transcription. These results clearly highlighted the differential role of the Sp site compared with the half-CRE in the estrogen-dependent regulation of BRCA1. The efficacy of the transfection conditions and estrogen treatment were confirmed by the activation of transcription from a positive control promoter-luciferase construct harboring three consensus EREs (p3XERE; Figure 2b).

Based on this information, we analyzed the cooperativity between the Sp site and ER-\( \alpha \) in estrogen-activation of the BRCA1 promoter. For these experiments, we selected the Hela cell line because it does not express ER-\( \alpha \) and in previous studies [34], we successfully used this system to examine estrogen-regulated BRCA1 expression. In preliminary experiments, we ascertained that the cotransfection of Hela cells with p3XERE plus an empty vector (pCR3.1) did not yield measurable luciferase activity (Figure 3a). Compared with control vehicle (DMEM), the treatment with estrogen stimulated luciferase reporter activity in Hela cells cotransfected with a vector containing the cDNA cassette for ER-\( \alpha \) (pER\( \alpha \)).
plus p3XERE. These results confirmed the validity of the Hela cell line as a model system to study the regulation of BRCA1 by estrogen. In Hela cells transfected with pGL3BRCA1 plus the empty vector pCR3.1, the treatment with estrogen did not induce BRCA1 promoter activity, although it was stimulated about 2.0-fold upon cotransfection with pERα (Figure 3b). In Hela cells cotransfected with the pSpmut construct alone, the basal promoter activity was reduced by 80%, and estrogen activation was reduced in magnitude from 2.0-fold to 1.4-fold ($P < 0.05$). These results confirmed the important role of the crosstalk between the Sp site and ER-α in basal and estrogen-dependent regulation of BRCA1 transcription.

Next, we examined whether the Sp sequence influenced the recruitment of nuclear factors to the adjacent region of the BRCA1 promoter harboring the AP-1 and CRE sites. BRCA1 oligonucleotides were coincubated with nuclear extract obtained from MCF-7 cells cultured in control vehicle (DMEM) or in the presence of estrogen. The results of electromobility binding assays indicated that estrogen stimulated the binding of nuclear factors to the BRCA1 oligonucleotide harboring the AP-1 and CRE sites as evidenced by the increase in intensity of a DNA:protein complex (complex A; Figure 4a). However, we observed a striking increase in the intensity of this complex when nuclear extracts were coincubated with the BRCA1 oligonucleotide spanning the Sp-1, AP-1, and CRE domains. Moreover, a second complex (complex B) of lower molecular weight was visualized by gel electrophoresis after incubation of nuclear extracts with BRCA1 oligonucleotide. These results suggested that the complex B probably comprised Sp proteins.

The role played by the Sp, AP-1, and CRE sites in the recruitment of nuclear factors was further investigated in competition studies with oligonucleotides harboring mutations for each site (Figure 4b). Compared with the wild-type labeled BRCA1 oligonucleotide, the incubation of nuclear extracts with cold wild-type Sp/AP-1/CRE oligonucleotide reduced the binding by nuclear factors (Figure 4c; lane 3). Conversely, the coincubation with cold oligonucleotides harboring mutated Sp, AP-1, or CRE differentially restored binding as compared to the wild-type labeled control (lanes 4 to 10). The most significant recovery of binding was observed with cold oligonucleotides mutated for Sp1 (lane 4), AP-1 (lane 5), and CRE (lanes 9 and 10). These cumulative data confirmed that the Sp, AP-1, and CRE sites played an important role in the formation of transcription complexes at this segment of the BRCA1 promoter.

To test whether the GC-box was a binding site for Sp factors and the ER-α, we performed DNA-protein pull-down assays. Nuclear extracts harvested from control (DMEM) and estrogen-treated MCF-7 cells were incubated with BRCA1 oligonucleotides comprising the GC-box. Western blots revealed the constitutive occupancy of Sp-1, Sp3, and ER-α in control vehicle (DMEM) cells (Figure 5a). For the Sp3 protein, we visualized two doublets of approximately 60 and 100 kDa, which represent the two short and two long forms from different translational initiation [38]. A similar pattern for Sp3 has been reported following Western blotting of cellular extracts obtained from MCF-7 cells [39,40]. The treatment with estrogen increased the binding levels of Sp1 and ER-α protein, but not the association of Sp3. The incubation of nuclear extracts with BRCA1 oligonucleotides mutated for the Sp-binding site (Spmut) reduced significantly the binding by Sp1 and Sp3, but minimally reduced the association of ER-α. These results suggested that Sp binding was not required for the recruitment of the ER-α to the adjacent AP-1 site.

Figure 3

(a) Hela cells were cotransfected with pGL3BRCA1 or pSpmut containing a mutated specificity protein (Sp) site plus the empty vector pCR3.1 or pERα. Transfected cells were cultured in Dulbecco’s modified Eagle’s medium containing 5% charcoal dextran-stripped fetal bovine serum plus vehicle (DMEM) or DMEM plus 10 nM 17β-estradiol (E2) for 24 hours. (b) Hela cells were cotransfected with an empty plasmid (pCR3.1) plus p3xERE or p3XERE plus a vector containing the cDNA for the estrogen receptor (ER)-α (pERα). Bars represent mean luciferase units corrected for the internal control renilla ± standard error from four independent experiments performed in quadruplicate. Asterisks indicate significant E2 activation ($*P < 0.05$) of BRCA1 promoter activity as compared with vehicle control (DMEM).
Interactions of CREB proteins with the BRCA1 promoter region containing the Sp/AP-1/CRE sequences were investigated by gel electromobility shift assay. Nuclear proteins were incubated with radiolabeled BRCA1 promoter oligonucleotides containing the activator protein (AP)-1/cAMP response element (CRE) or the Sp plus AP-1/CRE sites. MCF-7 cells were cultured for 3 hours in control vehicle (DMEM) or DMEM plus 10 nmol/l 17β-estradiol (E2). Free probe (lane 1). In competition studies, nuclear extracts from MCF-7 cells cultured in the presence of E2 were incubated with radiolabeled BRCA1 oligonucleotides containing the wild-type specificity protein (Sp)/AP-1/CRE sequences in the absence (lane 2) or presence (100-fold) of competing cold wild-type Sp/AP-1/CRE oligonucleotide (lane 3), or 100-fold of competing cold oligonucleotides mutated for Sp (lane 4), AP-1 (lanes 5 and 6), intervening sequences between AP-1 and CRE (lanes 7 and 8), and CRE (lanes 9 and 10). The arrows indicate the DNA:protein complexes visualized by electromobility binding assay.

Sp site enhances the binding of nuclear proteins to the BRCA1 promoter. (a) Nuclear extracts normalized to 5 μg of nuclear protein obtained from MCF-7 cells were incubated with radiolabeled BRCA1 promoter oligonucleotides containing the activator protein (AP)-1/cAMP response element (CRE) or the Sp plus AP-1/CRE sites. MCF-7 cells were cultured for 3 hours in control vehicle (DMEM) or DMEM plus 10 nmol/l 17β-estradiol (E2). FP, free probe (lane 1). (b, c) In competition studies, nuclear extracts from MCF-7 cells cultured in the presence of E2 were incubated with radiolabeled BRCA1 oligonucleotides containing the wild-type specificity protein (Sp)/AP-1/CRE sequences in the absence (lane 2) or presence (100-fold) of competing cold wild-type Sp/AP-1/CRE oligonucleotide (lane 3), or 100-fold of competing cold oligonucleotides mutated for Sp (lane 4), AP-1 (lanes 5 and 6), intervening sequences between AP-1 and CRE (lanes 7 and 8), and CRE (lanes 9 and 10). The arrows indicate the DNA:protein complexes visualized by electromobility binding assay.

Interactions of CREB proteins with the BRCA1 promoter region containing the Sp/AP-1/CRE sequences were investigated by gel electromobility shift assay. Nuclear proteins were incubated with the Sp1/CRE or Sp/AP1/CRE oligonucleotides and, upon gel separation, we observed (Figure 5b) a pattern of retarded bands (A and B) that was similar to that depicted in Figure 4a. Upon coincubation with an antibody for CREB, we visualized a slower migrating band suggesting this protein was associated with the Sp/CRE or Sp/AP-1/CRE oligonucleotide. These findings suggested that the Sp site or estrogen treatment did not influence the constitutive recruitment of CREB to the AP-1/CRE region.
Further confirmation of the interactions of Sp1, Sp3, Sp4, ER-α, and CREB with the BRCA1 promoter was obtained by ChIP assay followed by real-time PCR analysis. The treatment with estrogen increased the occupancy of Sp1, Sp4, and ER-α. The association of Sp3 and CREB was slightly increased by estrogen but not to a significant degree as compared with control DMEM (Figure 5c). These in vivo results complemented those obtained with DNA-pull down and electro-
The MEK-1 inhibitor reduces the E2-induced binding of Sp1 and ER-α to the BRCA1 promoter. Nuclear protein extracts (200 μg) were obtained from MCF-7 cells cultured for 1.5 hours in Dulbecco’s modified Eagle’s medium containing 5% charcoal dextran-stripped fetal bovine serum containing vehicle (DMEM), or DMEM plus 10 nmol/l 17β-estradiol (E2), the MEK-1 (mitogen-activated protein kinase) inhibitor PD98059, or E2 plus PD98059. DNA:protein complexes were generated by coincubation of nuclear extracts with the wild-type specificity protein (Sp1/activator protein (AP)-1 oligonucleotide and separated by SDS-PAGE. The recruitment of Sp1, total ER-α, or phosphorylated ER-α was visualized by Western blotting with specific antibodies. Panels are representative of two independent experiments. Numbers indicate molecular weight markers (kDa).

**Figure 6**

Discussion

Understanding how estrogen regulates BRCA1 expression may contribute to clarifying the role played BRCA1 under normal physiological conditions and pathophysiological perturbations that predispose to breast cancer. Previous studies from our laboratory documented that estrogen stimulated BRCA1 expression by inducing the recruitment of an ER-α/p300 complex at an AP-1 site located close to the transcription start site of exon-1b [33]. The objective of this study was to define the role of Sp and CRE binding sites flanking the AP-1 element. The Sp-binding domain (5'-GGGGCGGAA-3') shared homology with the Sp1 consensus sequence (5'-[G/T]GGGCGG[G/A][G/A]-3'). We found that mutation of the Sp site led to a striking reduction in basal activity and reduced the estrogen activation. Cumulative data from DNA:protein binding and ChIP assays with BRCA1 oligonucleotides spanning the GC-rich and AP-1 domain suggested that estrogen stimulated the recruitment of Sp-1, Sp4, and ER-α but did not affect the constitutive occupancy by Sp-3. Therefore, the estrogen-dependent activation of the proximal BRCA1 promoter requires the intact Sp-binding site and appears to be mediated by increased occupancy of Sp1/Sp4 and ER-α as compared with Sp3.

Previous studies have documented that Sp3 can both enhance and inhibit transactivation. For example, the estrogen-dependent regulation of the VEGFR2 (vascular endothelial growth factor receptor 2) promoter was primarily regulated by ER-α/Sp3 and ER-α/Sp4, but not Sp1 [42]. Conversely, recent findings by the same group [43] documented a role for Sp1, Sp3, and Sp4 in regulating the VEGFR1 gene. Similarly, other reports corroborated a role for ER-α/Sp1 in estrogen-dependent activation of the interleukin-1α [44], insulin-like growth factor-1 [45], and cdc25A [46] gene promoter. Our results indicate that Sp1 and Sp4 play a specific role in estrogen-dependent regulation of BRCA1 transcription. Interestingly, mutation of the Sp site did not prevent the recruitment of the ER-α to BRCA1 oligonucleotides harboring the AP-1 domain, but it reduced transcriptional activity. These findings suggest that the estrogen-induced association of Sp1 and Sp4 at the GC-box may be functionally required for estrogen-mediated transactivation of the BRCA1 promoter. The observation that the Sp-binding site enhanced the recruitment of nuclear proteins to BRCA1 oligonucleotides comprising the AP-1 and CRE elements suggests the Sp site may increase the stability of the ER-α/p300 complex recruited at the adjacent AP-1 site. In electromobility shift assays, we observed constitutive recruitment of CREB to a half-CRE (5'-CGTAA-3') located just downstream from the AP-1 motif. However, the constitutive occupancy by CREB was not altered by the estrogen treatment or the presence of the upstream Sp-binding site.

Exposure to estrogen has been shown to activate signaling cascades, including the MAPK kinase (MEK)/MAPK phosphorylation pathway that leads to phosphorylation of ER-α and related coactivators [41]. Therefore, we examined whether the MEK/MAPK pathway modulated the binding of Sp1 and ER-α to the BRCA1 promoter regions comprising the Sp and AP-1 sequences. Compared with MCF-7 cells cultured in control vehicle (DMEM), the treatment with estrogen stimulated the binding of Sp1 and ER-α. This effect was counteracted by the treatment with the MEK1 kinase inhibitor PD98059 (Figure 6). The constitutive binding of Sp1 and ER-α to the BRCA1 oligonucleotide was not influenced by the treatment with the PD98059 compound alone. As a positive control, we measured the association of phosphorylated ER-α, whose binding to the BRCA1 oligonucleotide was reduced upon cotreatment with estrogen plus PD98059. These data suggested that the recruitment of Sp1 and ER-α to the region of the BRCA1 promoter comprising respectively the Sp and AP-1 binding elements was mediated through MEK-dependent activation of phosphorylation.

**Table 1**

| kDa | Sp1 | Sp1 | Phospho-ERα (Ser 118) | ERα |
|-----|-----|-----|----------------------|-----|
| 118 |     |     |                      |     |
| 85  |     |     |                      |     |
| 47  |     |     |                      |     |
| 47  |     |     |                      |     |
One possible model that integrates the current results is depicted in Figure 7. Activation of BRCA1 transcription by estrogen may be dependent on the increased interaction between Sp1, Sp4, and ER-α bound respectively to the Sp and AP-1 elements located in the proximal region of the BRCA1 promoter. The occupancy by CREB at the half-CRE may serve as an ancillary factor that regulates constitutive activity. Interactions between ER-α, Sp1, and Sp4 may trigger the subsequent recruitment of cofactors such as p300, which can function as a histone acetyltransferase [4], thus leading to estrogen-dependent activation of the BRCA1 gene. This model finds support in the experimental evidence that mutation of the Sp or AP-1 [33] element prevented the estrogen-dependent activation of the BRCA1 promoter.

The role of Sp proteins has been proposed to be promoter selective and dependent on the presence of flanking sequences [42]. The Sp1 factor has been demonstrated to interact physically with the ER-α and ER-β [7], but only the ER-α/Sp1 interaction led to transcriptional activation of estrogen-responsive genes, such as Hsp27 [6], cad [47], and cathepsin-D [48]. Conversely, ER-α/Sp1 interactions played a minimal role in activation of VEGFR2, and ER-α/Sp3 and ER-α/Sp4 were critical factors [42]. In this study, we found that the treatment with estrogen increased the binding of the BRCA1 promoter by Sp1 and Sp4, but it did not alter the levels of bound Sp3. Similar to other genes involved in the oncogenic process [49,50], the relative occupancy ratio between Sp proteins may play a critical role in transcriptional regulation of the BRCA1 gene. For example, elevated Sp3 levels have been shown to promote pancreatic and breast cancer cell growth by inhibiting transcription of growth inhibitory genes including those encoding the cyclin-dependent kinase inhibitor p27 and the type II receptor for transforming growth factor-β [8,51]. Moreover, pancreatic cells transfected with small inhibitory RNA targeting Sp3 exhibited increased cell cycle distribution in G0/G1, and decreased distribution in S phase [8]. Conversely, Sp1 sites were required for activation of the p21 promoter [52]. Based on the observation that Sp1-mediated transcriptional activation can be repressed by Sp3 [53], a shift in the Sp1/Sp4 versus Sp3 binding ratio may alter normal estrogen regulation of the BRCA1 gene. The model depicted in Figure 7 of cooperative interactions between DNA-bound Sp proteins and ER-α recruited at an AP-1 site is similar to that reported for the E2F1 promoter, whose transactivation by the ER-α was found to be independent of direct interactions with promoter elements and was regulated by a multiprotein complex comprising ER/Sp1/NF-Y [54].

Regulation of BRCA1 by estrogen may require activation of the MAPK cascade. Estrogen binds to the ER-α and stimulates MAPK-mediated phosphorylation of MEK1/2, which in turn phosphorylates ERK1/2 (extracellular signal regulated kinase1/2) [55]. The activated ERK1/2 may add phosphate groups to ER-α and Sp and modulate protein-DNA or protein-protein interactions of these factors at target promoters. Our results document that activation of the MEK pathway plays a key role in the estrogen-regulated recruitment of Sp1 and phosphorylated ER-α to the BRCA1 promoter.

**Conclusion**

We report that estrogen-dependent activation of BRCA1 transcription is mediated by a GC-rich region, which is a binding target for Sp proteins. The Sp-binding motif is located in close proximity to an AP-1 site, which is a target for an ER-α/p300 complex. Estrogen stimulates the recruitment of Sp1 and Sp4, but not Sp3, to the GC-rich region. We also provide evidence that the AP-1 site is flanked by a CRE-binding domain that regulates constitutive expression. The close proximity of the Sp, AP-1, and CRE-binding sites may be important for the recruitment of transcriptional complexes and regulation of basal and estrogen-dependent BRCA1 transcription. Finally, we provide evidence that the MAPK pathway regulates the association of Sp1 and ER-α with the BRCA1 promoter. Overall, these results offer new evidence for both genomic and nongenomic regulation of BRCA1 transcription activity by estrogen.
Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
JKH, SCD, and DFR conceived the study. JKH conducted the
mutational studies. SCD conducted the DNA-pull down assays, Western blot analyses, and ChIP studies. SSM con-
ducted the binding and electromobility shift assays. MQK and
DFR performed transcription studies. JKH, SCD, SSM, and
DFR contributed to the drafting of the manuscript, which was
approved by all authors.

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