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An Estrogen Receptor-α/p300 Complex Activates the *BRCA-1* Promoter at an AP-1 Site That Binds Jun/Fos Transcription Factors: Repressive Effects of p53 on *BRCA-1* Transcription[1]

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Abstract

One of the puzzles in cancer predisposition is that women carrying *BRCA-1* mutations preferentially develop tumors in epithelial tissues of the breast and ovary. Moreover, sporadic breast tumors contain lower levels of *BRCA-1* in the absence of mutations in the *BRCA-1* gene. The problem of tissue specificity requires analysis of factors that are unique to tissues of the breast. For example, the expression of estrogen receptor-α (ERα) is inversely correlated with breast cancer risk, and 90% of *BRCA-1* tumors are negative for ERα. Here, we show that estrogen stimulates *BRCA-1* promoter activity in transfected cells and the recruitment of ERα and its cofactor p300 to an AP-1 site that binds Jun/Fos transcription factors. The recruitment of ERα/p300 coincides with accumulation in the S-phase of the cell cycle and is antagonized by the antiestrogen tamoxifen. Conversely, we document that overexpression of wild-type p53 prevents the recruitment of ERα to the AP-1 site and represses *BRCA-1* promoter activity. Taken together, our findings support a model in which an ERα/AP-1 complex modulates *BRCA-1* transcription under conditions of estrogen stimulation. Conversely, the formation of this transcription complex is abrogated in cells overexpressing p53.

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Introduction

The breast and ovarian cancer susceptibility gene *BRCA-1* [1,2] encodes for a transcription factor, which contributes to recombination and DNA repair functions [3–5]. Reduced levels of wild-type BRCA-1 protein have been detected in a large percentage of sporadic breast tumors in the absence of mutations in the *BRCA-1* gene [6], suggesting that disruption of *BRCA-1* expression may be a contributing factor to the onset of mammary carcinogenesis [7]. Exposure to ovarian estrogens has been recognized as one risk factor in breast tumorigenesis based on the evidence that therapies with the estrogen receptor agonist tamoxifen (TMX) reduced the incidence of breast cancer [8]. Effects of estrogen on responsive genes are mediated by two estrogen receptors: estrogen receptor-α (ERα) and estrogen receptor-β (ERβ). In the classic pathway, ERα contacts the DNA at specific estrogen-responsive elements (EREs) comprising target genes and recruits coactivators and cofactors that enhance transcription [9]. Alternatively, ERα may physically interact with p160/p300 proteins bound to an AP-1 (Jun/Fos) complex that contacts DNA [10]. The profile of cofactors and the type of ligand have been shown to influence the transcription activity of ERα [11,12].

The expression of *BRCA-1* peaks in the S-phase of the cell cycle [13–15] and is induced by estrogen in breast cancer cell lines [16,17] and estrogen plus progesterone in the mammary gland of ovariectomized mice [18]. Although estrogen depletion reduces *BRCA-1* expression [19], the stimulatory effects of estrogen on *BRCA-1* expression are believed to be indirect based on the observations that the proximal *BRCA-1* promoter lacks consensus EREs that bind ERα and that *de novo* protein synthesis is required for *BRCA-1* upregulation [20]. To clarify the mechanisms of estrogen stimulation of *BRCA-1* expression, we investigated whether estrogen regulated *BRCA-1* transcription through an alternative pathway involving the recruitment of complexes containing ERα at non-EREs in the *BRCA-1* promoter. We report that, in response to estrogen, an ERα/p300

Abbreviations: AP-1, activator protein-1; ChIP, chromatin immunoprecipitation; DMEM, Dulbecco's modified Eagle's medium; E_2, 17β-estradiol; ERα, estrogen receptor-α; ERE, estrogen-responsive element; FCS, fetal calf serum

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complex is recruited to an AP-1 domain located in the proximal *BRCA-1* promoter and activates *BRCA-1* transcription, whereas the overexpression of p53 prevents the recruitment of ERα and represses *BRCA-1* promoter activity.

Materials and Methods

Cells, Transient Transfections, and Luciferase Assay

HCT116 and HCT116 p53KO cells were a generous gift from B. Vogelstein. MCF-7 and HeLa cells were obtained from the American Type Culture Collection (Rockville, MD). The plasmid pTam67 was originated by cloning the Tam67 cDNA into the *Eco*RI site of pCR3.1. Transient transfections were performed using the Lipofectamine-Plus procedure according to the manufacturer's instructions (Life Technologies, Inc., Carlsbad, CA). Briefly, 24 hours after cells were plated, each well was cotransfected with the appropriate plasmid and an internal control plasmid pRL-TK (renilla luciferase gene). Cells were incubated with the DNA–liposome complex for 3 hours at 37°C in 5% CO_2. Following transfection, cells were maintained in Dulbecco's modified Eagle's medium (DMEM) plus 5% charcoal-stripped fetal bovine serum (FBS) and allowed to recover for 48 hours. Cells were then treated in DMEM containing either control (ethanol vehicle) or 10 nM 17β-estradiol (E_2) for the times indicated.

Western Blot Analysis and Flow Cytometry

Western blot analysis for *BRCA-1* was performed as described previously [21]. Cell extracts were normalized to protein content and separated by 4% to 12% gradient sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Immunoblotting was carried out with antibodies raised against *BRCA-1* (Ab-2; Oncogene Research Products, Cambridge, MA). Normalization of Western blots was confirmed by incubating immunoblots with β-actin antibody-1 (Oncogene Research Products). The immunocomplexes were detected by enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL). Flow cytometry was performed in triplicate as described previously [22]. Briefly, cells were harvested with trypsin and washed in phosphate-buffered saline (PBS). Then cells were treated with RNAse and stained with propidium iodide (70 μM in PBS). Cell cycle distribution profiles were recorded with a FACscan (Becton Dickinson, San Jose, CA), using a CELLQuest program.

Electrophoretic Mobility Shift Assay (EMSA)

Cells were plated in DMEM plus 5% charcoal-stripped FBS. After 24 hours, cells were treated for 24 hours with 10 nM E_2 then subsequently harvested. Briefly, cells were trypsinized then washed with ice-cold DPBS. Cells were resuspended in ice-cold 25 mM Hepes buffer containing 1.5 mM EDTA, 1 mM DTT, 0.5 mM PMSF, and 5 μg/ml aprotinin, and placed on ice for 10 minutes. Cells were pelleted and resuspended in 1 ml of ice-cold 25 mM Hepes buffer containing 1.5 mM EDTA, 10% (vol/vol) glycerol, 1 mM DTT, 0.5 mM PMSF, and 5 μg/ml aprotinin. The cell suspension was transferred to a mortar for drilling with a Teflon pestle until more than 90% of the cells in a 2-μl aliquot were unable to exclude trypan blue. After centrifugation, cell pellets were resuspended in 150 μl of ice-cold 25 mM Hepes buffer containing 1.5 mM EDTA, 10% (vol/vol) glycerol, 0.5 M KCl, 1 mM DTT, 0.5 mM PMSF, and 5 μg/ml aprotinin, and placed on ice with intermittent vortexing. Cell debris was removed by centrifugation. Supernatants containing nuclear protein were stored at −70°C. Nuclear protein concentration was determined using the BCA protein assay (Pierce Chemical Company, Rockford, IL). Oligonucleotides used for binding and gel retardation assay were: *BRCA-1*, 5′-AACCTGAGAGGCGTAAGGCGTT-3′ (sense) and 5′-AACGCCTTACGCCTCTCAGGTT-3′ (antisense); and consensus TRE, 5′-CAAACACATGAGTAATGTGTT-3′ (sense) and 5′-AACACATTACTCATGTGTTTG-3′ from the human collagenase promoter. The complementary oligonucleotides were annealed then phosphorylated at the 5′-end with [γ-^{32}P]ATP and T4 polynucleotide kinase. Unincorporated nucleotides were removed using the TE-10 spin columns (Clontech, Mountain View, CA). Binding assays were performed by incubating 5 μg of nuclear protein in the binding buffer then incubated with the labeled oligonucleotides for 20 minutes. For supershift assays, antibodies (Affinity Bioreagents, Boulder, CO) were incubated with 1-μg nuclear extracts for 2 hours prior to addition of labeled oligonucleotides. For cold competition, a 100-fold excess of the respective unlabeled oligonucleotides was added to the binding reaction 10 minutes prior to addition of the labeled oligonucleotides. 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, Piscataway, NJ).

Chromatin Immunoprecipitation (ChIP) Assay

Cells were collected after fixation of protein and DNA through the addition of formaldehyde for a final concentration of 1% to the cell culture medium and incubated at 25°C for 10 minutes. Prior to collection, cells were washed twice in cold DPBS. The cells were resuspended in a lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris–HCl, and protease inhibitor cocktail). After sonication, the dilution of chromatin preparations was either reserved as input (no antibody) material or utilized for immunoprecipitation with the desired antibody. After immunoprecipitation, DNA was recovered and subjected to polymerase chain reaction (PCR) analysis using the following primers: *BRCA-1*, forward: ATCGGTACCAAGTGATGC TCTGGGGTACTG, reverse: ACTAGATCTACCTCATGAC-CAGCCGACGTT (237 bp), flanking the AP1-binding site. As a positive control for estrogen treatment and ChIP assay for ERα, we tested for the recruitment of ERα at the ERE region of the estrogen-inducible *pS2* gene using the primers: forward: TATGAATCACTTCTGGAGTGA; reverse: GAGCGT-TAGATAACATTTGCC (289 bp). As negative controls, we tested for the recruitment of ERα at exon 7 of the *BRCA-1* gene using forward: 5′-ATGCAAACAGCTATAATTTTG-3′; reverse, 5′-CAAGGAAGGATTTTCGGGTTC-3′ (140 bp) and through coincubation with IgG.

Results

Estrogen Induces BRCA-1 Promoter Activity

In transient transfection assays with ERα-positive MCF-7 breast cancer cells, we found that the treatment with E_2 for 24 hours, but not in earlier time points (data not shown), stimulated by a factor of 2.0 the transcriptional activity of a 1.69-kb *BRCA-1* promoter–reporter construct (pGL3BRCA-1) [21] containing both transcription start sites for exons 1A and 1B (Figure 1*A*). The E_2 treatment stimulated luciferase reporter activity from a positive control promoter–reporter construct (p3XERE) containing an array of three consensus EREs (Figure 1*B*) and the accumulation of BRCA-1 protein (Figure 1*C*). The requirement for ERα in the E_2-dependent regulation of *BRCA-1* transcription was confirmed in cervical HeLa and colon HCT116 cancer cells transiently transfected with pGL3BRCA-1 plus a vector encoding for ERα (pERα). For these experiments, we adopted transfection conditions similar to those of previous studies, which examined the role of *BRCA-1* on ERα signaling [23]. In detail, we transfected ~70% confluent 24-well dishes with 0.5 μg of pERα. Our results indicated that in ERα-negative HeLa (Figure 1*D*) and HCT116 (Figure 1*E*) cells transfected with either pGL3BRCA-1 or an empty vector (pCR3.1), E_2 treatment was not sufficient to induce *BRCA-1* promoter activity. However, *BRCA-1* transcription became responsive to E_2 following cotransfection with various amounts of pERα as documented by stimulation of luciferase activity from pGL3BRCA-1. Similarly, transcription from the positive control p3XERE reporter construct was induced by E_2 on cotransfection with pERα. The cotransfection of ERα into HeLa and HCT116 cells produced a fold induction in *BRCA-1* transcription, similar to that observed in transfected MCF-7 cells expressing endogenous ERα and treated with E_2.

In search of non-ERE sites located in the proximal *BRCA-1* promoter that may recruit transcription complexes containing ERα, we mapped a sequence (5′-CTGAG-3′) with significant homology to consensus sequences for AP-1 transcription factors [24] at positions −27/−31 upstream of the transcription start site on exon 1B (Figure 2*A*). Site-directed mutagenesis of the candidate AP-1–like (CTGAG to CACTA) site abrogated basal and E_2-dependent activity of the *BRCA-1* promoter, as evidenced by the significant reduction in luciferase activity observed in MCF-7 cells transfected with a mutated *BRCA-1* promoter–luciferase reporter construct (pAP1mut) (Figure 2*B*). Moreover, cotransfection with an expression vector encoding for a dominant-negative variant (pTam67) of c-Jun abrogated basal and E_2-induced *BRCA-1* promoter activity (Figure 2*C*), confirming the requirement for AP-1 in basal and estrogen-dependent regulation of *BRCA-1* transcription.

Estrogen Stimulates the Recruitment of ERα to an AP-1 Site in the BRCA-1 Promoter Region

We used ChIP assays to investigate whether or not estrogen stimulated the recruitment of ERα to the *BRCA-1* promoter region containing the AP-1 domain. Based on the information that *BRCA-1* levels peak in S-phase [25], we enriched the fraction of MCF-7 cells positioned in the G_1 phase of the cell cycle (Table 1). Time course experiments showed that E_2 stimulated the recruitment of ERα to a *BRCA-1* promoter fragment comprising the AP-1–like motif (Figure 3*A*). This was accompanied by recruitment of p300 (Figure 3*B*) and accumulation of cells in S-phase (71.5%) (Table 1). Conversely, the E_2-induced accumulation in S-phase and the recruitment of ERα and p300 were antagonized by cotreatment with TMX (Figure 3*C*). Even though the treatment with TMX was stimulated by ~50% *BRCA-1* promoter activity, in combination with E_2, it antagonized the activation of *BRCA-1* transcription. These agonist/antagonist effects of TMX were similar to those exerted by this compound on the expression of other estrogen-responsive genes [10].

The E_2 treatment stimulated the recruitment of c-Jun and FosB (Figure 3*D*), confirming that AP-1 contributed to the formation of a transcription complex at this region. Control experiments indicated that the coincubation of cross-linked chromatin with preimmune IgG did not generate a corresponding *BRCA-1* amplification product (Figure 3*E*). Neither did E_2 stimulate the recruitment of ERα to the coding region of exon 7 in the *BRCA-1* gene (Figure 3*F*). However, the treatment with E_2 triggered the recruitment of ERα to an ERE in the *pS2* gene (Figure 3*G*), thus confirming the efficacy of the E_2 treatment and the experimental conditions for the ChIP assay.

To obtain additional evidence that the *BRCA-1* promoter region containing the AP-1 site was targeted for binding by ERα, we incubated nuclear extracts obtained from E_2-treated MCF-7 cells with a *BRCA-1* oligonucleotide spanning 22 bp (−40/−19) upstream of the exon 1B transcription start site. The incubation of nuclear extracts with the *BRCA-1* oligonucleotide produced two distinct complexes (bands A and B). Band A was supershifted in a dose-dependent fashion following coincubation of the *BRCA-1* oligonucleotide with increasing amounts of an ERα antibody (ERαAb) (Figure 3*H*), suggesting that this complex contained ERα. These results mapped the binding region for ERα to the *BRCA-1* promoter segment comprised between −40 and −19 bp, which included the AP-1–like domain. Conversely, the coincubation of nuclear extracts with control preimmune IgG did not produce a supershifted band. The formation of complex A was competed by coincubation with excess cold oligonucleotide containing the consensus AP-1 sequence 5′-TGACTCA-3′ from the human collagenase promoter (data not shown), confirming that the *BRCA-1* oligonucleotide was a target for AP-1. Taken together, these results suggested that induction of *BRCA-1* transcription by estrogen required the concomitant expression of ERα and its occupancy along with p300 at an AP-1 site in the proximal *BRCA-1* promoter.

Overexpression of p53 Prevents the Recruitment of ERα to AP-1 and Represses BRCA-1 Transcription

In previous studies, p53 has been shown to repress *BRCA-1* expression [21,26], through yet unknown mechanisms. Therefore, we examined whether or not p53 interfered with E_2 stimulation of *BRCA-1* transcription. In transient transfection experiments with MCF-7 cells, we found that basal and

E_2-induced *BRCA-1* promoter activities were repressed following cotransfection with various amounts of a vector encoding for wild-type p53 (p53WT) (Figure 4*A*). In parallel transfection experiments with HCT116 cells lacking p53 (HCTKO, p53−/−), the treatment with E_2 did not influence *BRCA-1* transcription in cells cotransfected with pGL3BRCA-1 plus the empty vector pCMV (Figure 4*B*). The cotransfection with pGL3BRCA-1 plus pERα did not alter basal promoter activity, but resulted in a modest increase (~20%) in *BRCA-1* transcription following E_2 treatment. Conversely, the cotransfection of HCTKO cells with various amounts of p53WT repressed *BRCA-1* promoter activity in a dose-dependent fashion. However, the relative induction by E_2 was greater in cells transfected with higher amounts (0.25–1.0 μg) of p53WT, suggesting that E_2 regulation of *BRCA-1* transcription was influenced by the relative expression levels of p53 and ERα.

Based on the information that p53 physically interacts with ERα [27], we asked whether or not overexpression of p53 modulated the recruitment of ERα to the AP-1 site. ChIP experiments with MCF-7 cells indicated that E_2-induced recruitment of ERα to this site was repressed following overexpression of exogenous p53WT (Figure 5, *A* and *B*). These findings suggested that p53 represses E_2-induced *BRCA-1* transcription by preventing the recruitment of ERα to the *BRCA-1* promoter. Western blot analysis confirmed that in MCF-7 cells overexpressing p53, the induction of *BRCA-1* protein by E_2 was greatly reduced compared with MCF-7 cells transfected with the empty plasmid pCMV. These data suggested that the interaction of ERα at the AP-1 was critical for the E_2-dependent increase in *BRCA-1* protein observed in Western blots.

Discussion

The objective of the present study was to investigate whether or not E_2 activated *BRCA-1* promoter activity and the potential involvement of ERα. Previous studies have documented that E_2 stimulates *BRCA-1* expression [16,17]. However, the lack of EREs in the promoter of *BRCA-1* has led to the suggestion that E_2 activation of *BRCA-1* expression is indirect [20]. In this study, we used a 1.7-kb *BRCA-1* promoter fragment containing both transcriptional start sites of exons 1A and 1B [21] to investigate the E_2 regulation of the *BRCA-1* promoter. The results of transfection studies indicated that E_2 stimulated *BRCA-1* promoter–reporter activity in breast MCF-7 cells expressing endogenous ERα, and in colon (HCT116) and cervical (HeLa) cells cotransfected with a plasmid encoding for ERα. Our transfection conditions were similar to those used in previous studies, which examined the interplay between *BRCA-1* and ERα [23]. In the current study, the efficacy of transfection conditions and E_2 treatment was confirmed by induction in transfected cells of transcription activity from a control expression construct containing an array of three consensus EREs (p3XERE), as well as accumulation of BRCA-1 protein in MCF-7 cells. Conversely, cotreatment with the antiestrogen TMX abrogated E_2-induced *BRCA-1* promoter activity in MCF-7 cells, confirming the involvement of ERα in the regulation of *BRCA-1* transcription.

The action of ERα at an ERE (5′-GGTCAnnnTGACC-3′) is well understood and involves the direct binding of ER homodimer to DNA and the recruitment of coactivators including CBP/p300 and p160 [9,28]. Alternatively, ERα has been shown to activate the transcription of several E_2-inducible genes including IGF-1 [29], collagenase [30], and cyclin D [31] at AP-1 sequences that bind members of the Jun and Fos families. Inspection of the proximal *BRCA-1* promoter revealed its presence in close proximity to the exon 1B transcription start site of an element (CTGAG) homologous to a sequence that binds AP-1 factors [24]. Several observations presented in this report support the notion that this site is important for basal and E_2-induced regulation of *BRCA-1* transcription. First, mutation of the candidate AP-1 site, as well as overexpression of a dominant-negative variant of c-Jun (pTam67), led to repression of *BRCA-1* promoter activity in transfected MCF-7 cells. These results were in agreement with those of previous investigations documenting that mutation of sites binding Jun/Fos proteins and expression of Tam67 reduced basal and E_2-inducible AP-1 transactivation [32–34]. Second, the results of ChIP assays provided direct evidence that the *BRCA-1* promoter region flanking the AP-1 site was targeted by c-Jun and FosB in the treatment of MCF-7 cells with E_2. Compared to other members of the Jun and Fos families, c-Jun and FosB are considered strong transactivators [35], and their recruitment to the *BRCA-1* gene highlights the role of AP-1 in E_2-dependent activation of *BRCA-1* transcription. Third, treatment with E_2 induced the recruitment of ERα and its cofactor p300 to the *BRCA-1* promoter region comprising the AP-1, whereas the antiestrogen TMX antagonized these effects. Results of EMSAs mapped the recruitment of ERα to a 21-bp promoter region (−40 and −19 bp) spanning the AP-1 site. These cumulative data are consistent with a model that attributes to ERα and its coactivator p300 a key role in the activation of *BRCA-1* transcription through an AP-1. The physiological relevance of these findings is that ERα/AP-1 interactions may lead to activation of *BRCA-1* promoter activity, which is paralleled by an increase in BRCA-1 protein. Unlike gel

Figure 1. *Estrogen induces BRCA-1 promoter activity in transiently transfected MCF-7 cells. (A) MCF-7 cells were precultured for 4 days in phenol red–free DMEM containing 5% charcoal dextran–stripped FBS (Hyclone Laboratories, Logan, UT). Then, a 1.69-kb fragment of the BRCA-1 5′ flanking region driving the expression of a luciferase cassette (pGL3BRCA-1) was transiently transfected, using Lipofectamine Plus (Life Technologies, Inc.), into MCF-7 cells, which were cultured in DMEM or DMEM plus 10 nM E_2 (Sigma, St. Louis, MO) for various periods of time. (B) The treatment with E_2 induces promoter activity from a positive control vector (p3XERE) transfected into MCF-7 cells. (C) Western blot analysis with antibodies for BRCA-1 (Ab-2) and β-actin (Ab-1) (Oncogene Research Products) documents that BRCA-1 protein levels are induced in MCF-7 cells cultured for 24 hours in DMEM plus 10 nM E_2. (D) HeLa and (E) HCT116 cancer cells were cotransfected with pGL3BRCA-1 and either a plasmid encoding for ERα (pERα) or an empty vector (pCR3.1). Transfected cells were cultured in DMEM or DMEM plus 10 nM E_2 for 24 hours. Control plates were transfected with p3XERE. Bars represent mean luciferase units corrected for the internal control renilla ±SE from two independent experiments performed in quadruplicate.*

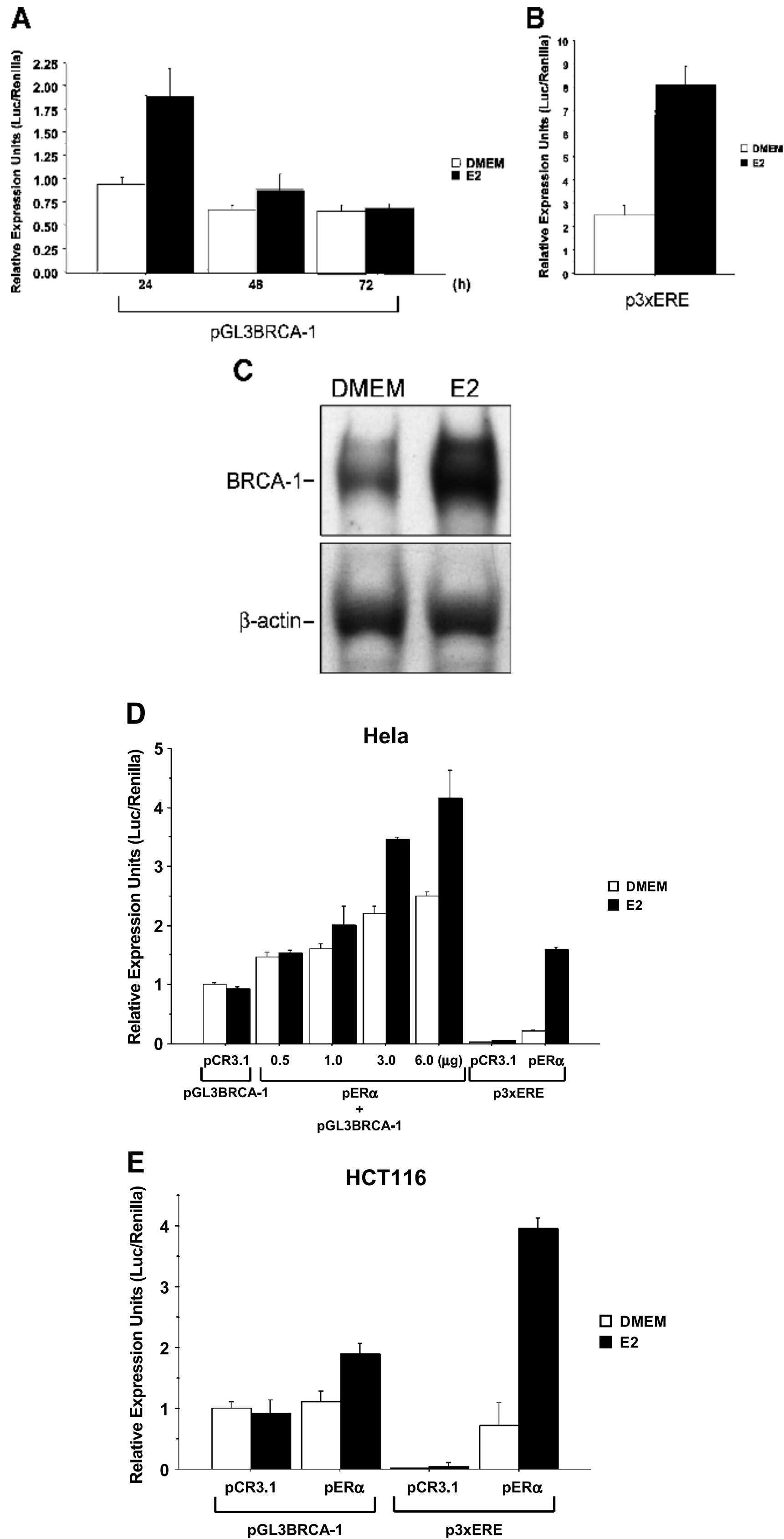

Figure 1.

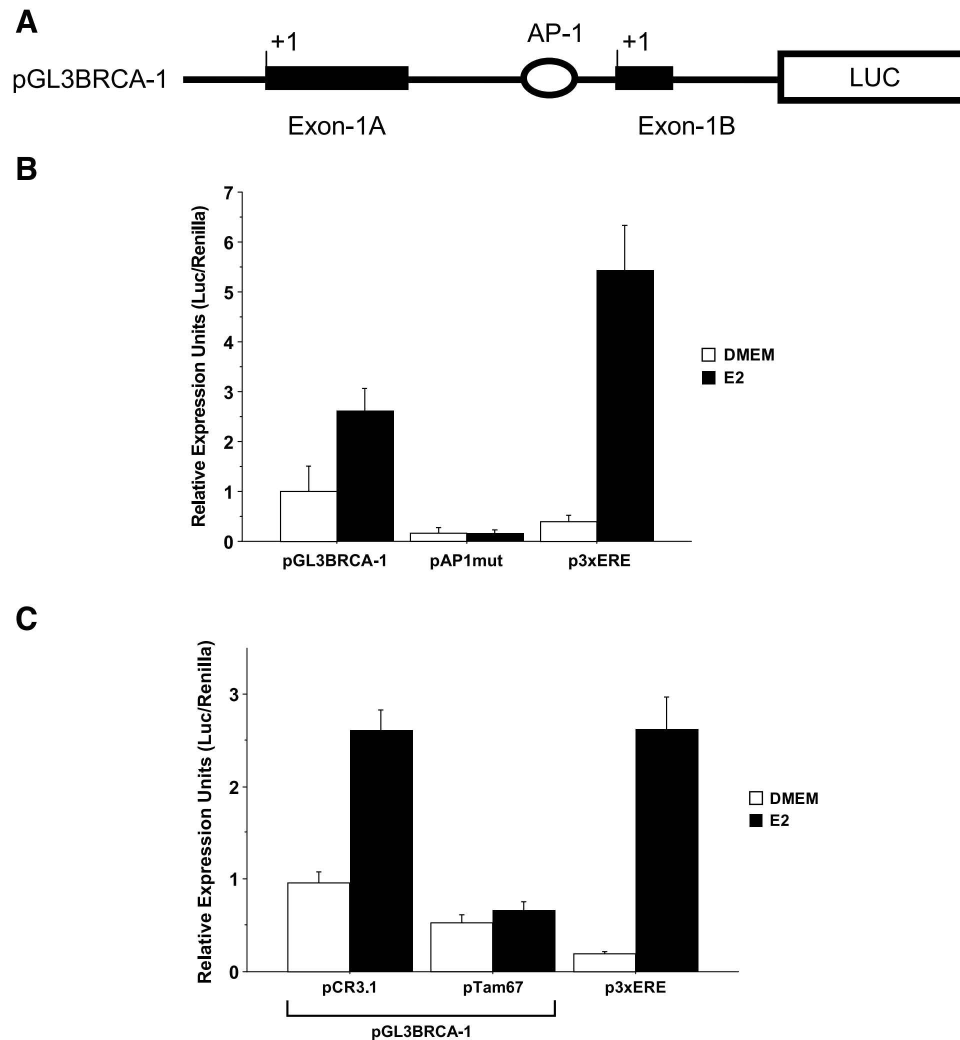

Figure 2. *An AP-1–like site contributes to estrogen-induced BRCA-1 promoter activity. (A) Position of the candidate AP-1 element in the BRCA-1 promoter. (B) MCF-7 cells were precultured for 4 days in DMEM with 5% FBS and then transiently transfected with pGL3BRCA-1 or pGL3BRCA-1 mutated at the AP-1 site (pAP1mut). p3XERE is a positive control for treatment with 10 nM E_2. (C) Transient transfection of MCF-7 cells with a dominant-negative c-Jun plasmid (pTam67) represses basal and estrogen-regulated BRCA-1 transcription. (B and C) Data represent mean luciferase units corrected for the internal control renilla ±SE from two independent experiments performed in quadruplicate.*

mobility studies with segments of the *BRCA*-1 promoter, the ChIP assay offered the opportunity to examine the recruitment of these transcription factors to the *BRCA-1* promoter in the context of the native chromatin.

The dynamics of cofactor recruitment to the AP-1 site is likely complex and involves an orderly recruitment of transcription factors and alterations in chromatin state [9]. For example, the participation of p300, which possesses intrinsic histone acetyl transferase (HAT) activity [36], in the formation of the transcription complex recruited at the AP-1 may be a key event that may lead to chromatin remodeling and activation of *BRCA-1* transcription. This notion is supported by the current observations that cotreatment with TMX antagonized the recruitment of p300 to the AP-1 site and antagonized the stimulatory effects of E_2 on *BRCA-1* promoter activity. In this study, although we did not assess the presence of p160s, members of this family of transcription factors have been shown to physically interact with ERα at AP-1 sites [28]. One possibility is that Jun/Fos heterodimers bound to the *BRCA-1* DNA may recruit p300–p160s, whereas ERα may be recruited to the complex through contacts with the coactivator p160 [37]. Furthermore, it is plausible that ERα may form bridges with accessible transcription

Table 1. Estrogen Stimulates G_1-Phase to S-Phase Transition of MCF-7 Cells.

Treatment	G_1	S	G_2/M
4 days in DMEM	65.0	34.5	0.5
After 24 hr in DMEM	40.5	59.0	0.5
E_2	28.0	71.5	0.5
TMX	54.6	42.0	3.4
TMX + E_2	35.2	61.2	3.6

MCF-7 cells were precultured for 4 days in phenol red–free DMEM containing 5% charcoal dextran–stripped FBS. Then cells were cultured for 24 hours in basal DMEM or DMEM supplemented with 10 nM E_2, 1 μM TMX, or their combination. Cell were stained with propidium iodide and used for flow cytometry. Cell cycle distribution profiles were recorded with a FACscan (Becton Dickinson) using a CELLQuest program.

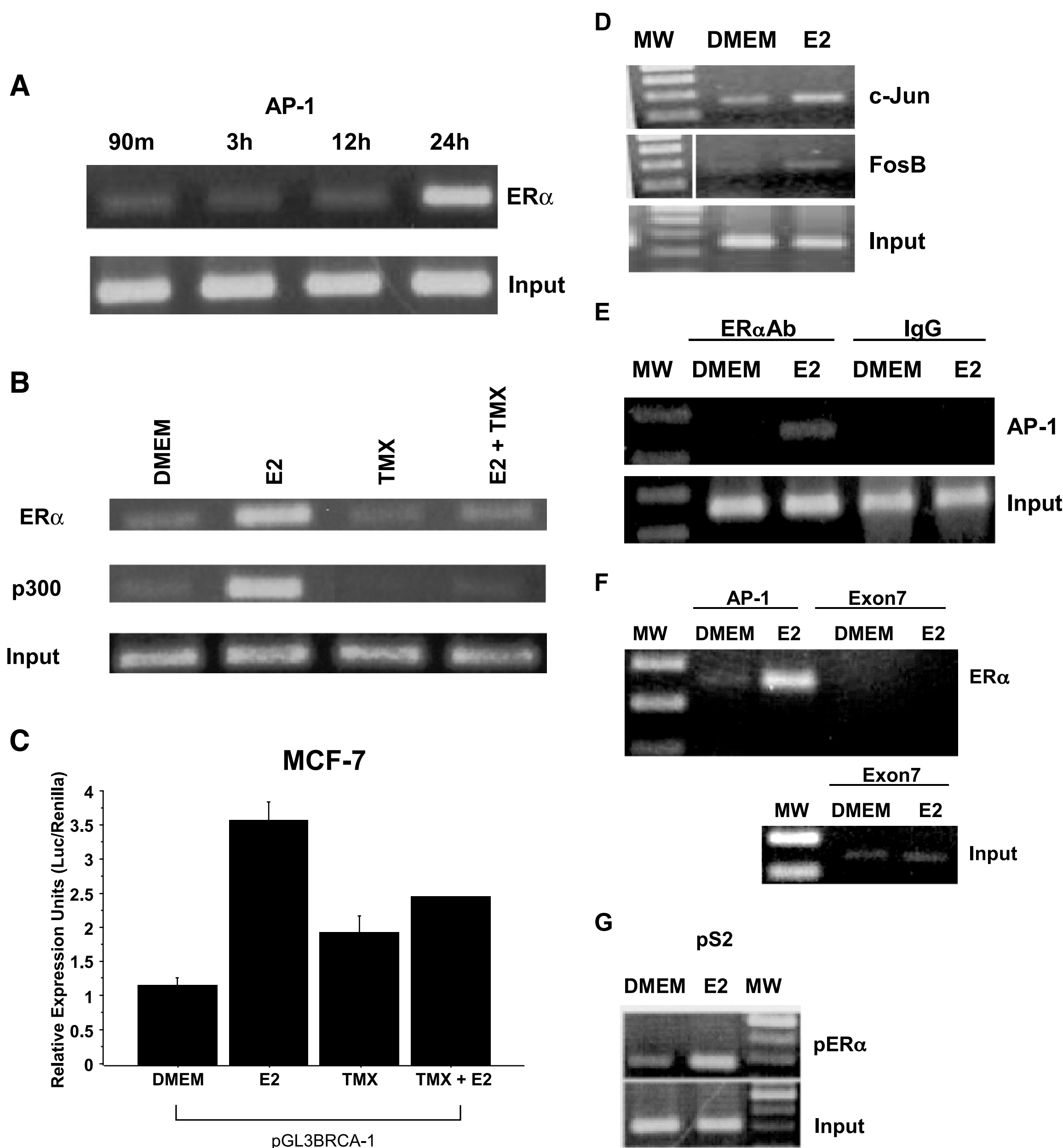

Figure 3. *Treatment of MCF-7 cells with estrogen stimulates BRCA-1 promoter occupancy by ERα and p300 to an AP-1 site. (A) MCF-7 cells were precultured for 4 days in DMEM with 5% FBS and then treated with 10 nM E_2 for various periods of time. Cells were processed for ChIP assay using an antibody against ERα (Neomarkers, Fremont, CA). Inputs are control bands generated by PCR from cross-linked chromatin. (B) At 24 hours, estrogen stimulates the recruitment of ERα and p300 (antibody from Affinity Bioreagents), whereas 1 μM TMX (Sigma) antagonizes E_2-dependent recruitment of ERα and p300. (C) E_2-induced BRCA-1 transcription in transiently transfected MCF-7 cells is antagonized by cotreatment with TMX. (D) E_2 stimulates the recruitment of c-Jun and FosB. (E) Coincubation with IgG followed by PCR amplification does not produce a band comprising the AP-1 segment. (F) The recruitment of ERα to a region of exon 7 in the BRCA-1 gene (negative control) is not stimulated by E_2, which stimulates (G) the recruitment of ERα to an ERE in the pS2 gene (positive control). The size of the amplicon was 237 bp for BRCA-1 (−98 to +139 bp from +1 on exon 1B), 289 bp for the pS2 ERE, and 140 bp for exon 7 of the BRCA-1 gene. (H) EMSA for ERα at the BRCA-1 promoter. MCF-7 cells were precultured for 4 days in DMEM with 5% FBS and then treated for 24 hours with E_2. Nuclear extracts were coincubated with a ^{32}P-labeled BRCA-1 oligonucleotide (−40/−19 bp) plus various amounts of mouse IgG or an antibody for ERα. The ERα antibody supershifted a complex (band A) in a dose-dependent manner, thus confirming the presence of ERα at this region; FP, free probe.*

factors recruited at adjacent sites in the *BRCA-1* promoter. For example, we identified just upstream of the AP-1 site a consensus sequence (5′-GGGCGG-3′) for the transcription factor Sp1, which has been shown to interact with ERα [38]. This potential interaction may stabilize the ERα/p300 transcription complex formed at the AP-1. Finally, another factor that may influence the degree and temporal activation of the *BRCA-1* gene by E_2 is the relative abundance and profile of AP-1 proteins recruited to the *BRCA-1* promoter. Regardless of the precise complement of cofactors and coactivators recruited at the AP-1 site, our studies showed that ERα and p300 play an important role in E_2-dependent activation of the *BRCA-1* promoter. The recruitment of an ERα/p300 complex to an AP-1 rather than an ERE site may integrate the role of AP-1 in the regulation of cell proliferation [37] with that of *BRCA-1* in cell growth response. The fact that the

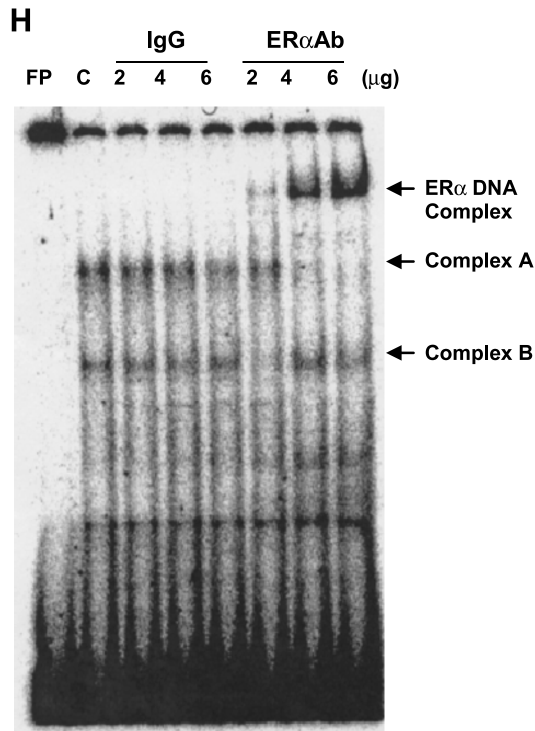

Figure 3. *(continued)*

kinetics and magnitude of *BRCA-1* induction by E_2 are different from those of inducible genes containing EREs [20] may be due to the type of transcriptional response that is required for *BRCA-1*. Elevation of *BRCA-1* expression as cells enter S-phase may offer a control mechanism that activates DNA repair and cell cycle checkpoints before DNA replication occurs [14].

Previous studies by other investigators [39,40] and our laboratory [21] documented that *BRCA-1* expression levels were downregulated in response to p53 induction and that these effects were due to transcriptional repression by p53. However, no consensus p53-binding sites have been found in the *BRCA-1* promoter [39], suggesting that the repressive effects of p53 on *BRCA-1* transcription are not mediated through direct binding to target DNA sequences. In this study, we found that overexpression of p53 counteracted the E_2-dependent upregulation of *BRCA-1* promoter activity. Results of ChIP assays documented that the recruitment of ERα to the AP-1 site in the *BRCA-1* promoter was abrogated in MCF-7 cells overexpressing exogenous p53. One potential explanation for these results is that p53 may physically interact with ERα, thus hampering the transcriptional activity of the liganded ERα [27]. The interaction between p53 and ERα occurs at multiple sites on the ERα protein and interferes with the ability of ERα to bind to EREs or other proteins in an ERα-mediated transcription complex [41]. In addition, p53 may interfere with the recruitment of p300 and factors associated with AP-1, while stimulating the recruitment of corepressors and histone deacetylases. For example, the physical interaction of p53 with histone deacetylase-1 and mSin3a has been reported to mediate transcriptional repression of the *Map4* gene [42]. Other reports documented that p53 binds to p300 in a region that is required for its intrinsic HAT activity [36]. The formation of p53–p300 complexes has been shown to reduce the amount of p300 available and repress transcription from an AP-1 site, whose activation is p300-dependent [43].

Overall, these findings provided evidence for a direct role of ERα in the regulation of *BRCA-1* promoter activity by E_2. The increased expression of *BRCA-1* may lead to activation of S-phase checkpoints, including p53 and p21 [40,44–46] and DNA damage-responsive genes [47] (Figure 5). This signaling may be of particular significance in cells of the breast, which undergo cyclic proliferative pressure by

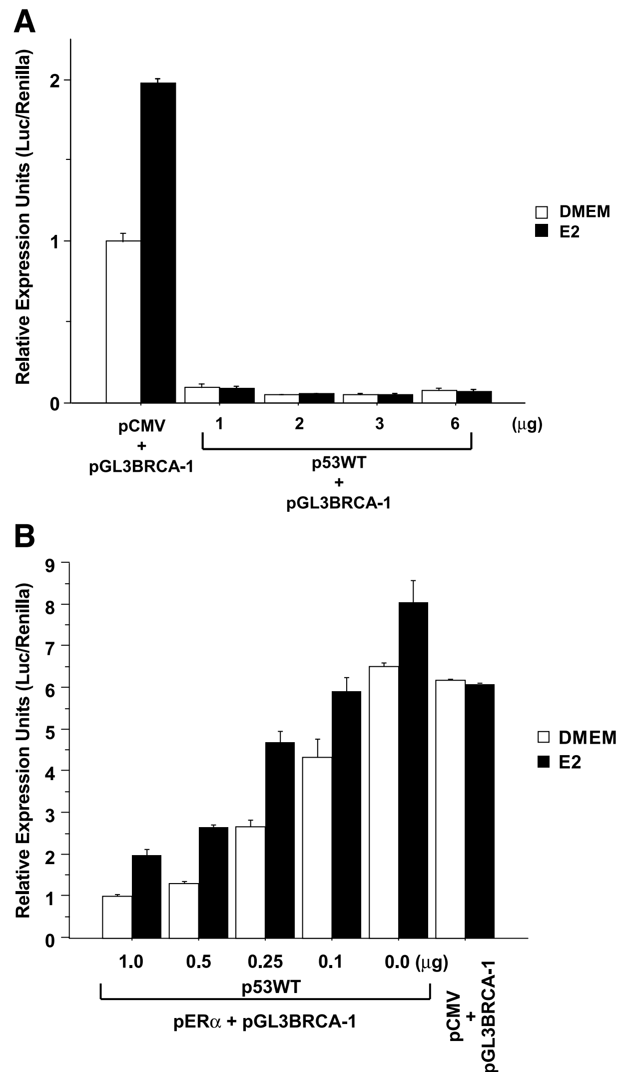

Figure 4. *Overexpression of wild-type p53 represses basal and estrogen-induced BRCA-1 transcription. (A) MCF-7 and (B) HCTKO (p53−/−) cells were precultured for 4 days in DMEM with 5% FBS and then transiently transfected with pGL3BRCA-1 or pGL3BRCA-1 plus various amounts of a plasmid encoding for wild-type p53 (p53WT) (gift from Bert Vogelstein, John Hopkins University, Baltimore, MD) under the control of the cytomegalovirus (CMV) promoter or an empty plasmid (pCMV). Where indicated, the HCTKO cells were cotransfected with 3.0 μg of pERα. Transfected cells were cultured in DMEM or DMEM supplemented with 10 nM E_2 for 24 hours. Bars represent mean luciferase units corrected for the internal control renilla ±SE from two independent experiments performed in quadruplicate.*

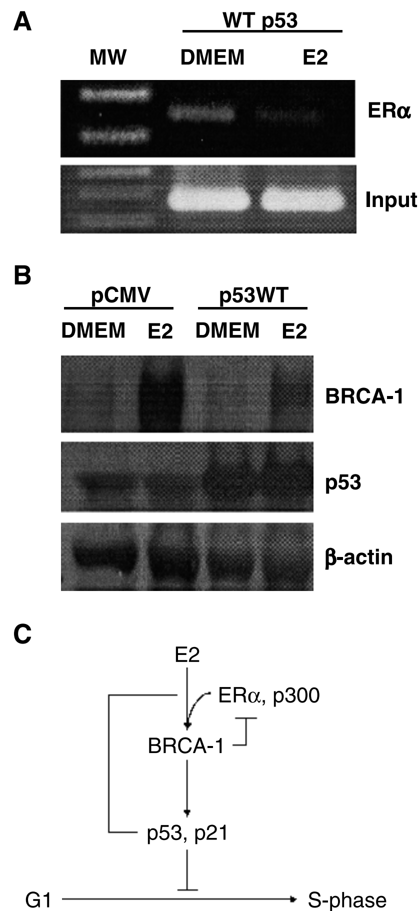

Figure 5. *Overexpression of p53 in MCF-7 cells prevents estrogen-induced accumulation of ERα at the AP-1 site and BRCA-1 protein. (A) Bands are PCR products following ChIP assay for ERα. Inputs are control bands generated by PCR from cross-linked chromatin. (B) Western blot analysis with antibodies for p53 and BRCA-1 documents that p53 protein levels are overexpressed in MCF-7 cells transfected with p53WT, whereas E_2-induced BRCA-1 protein is repressed. (A and B) MCF-7 cells were cultured for 24 hours in DMEM or DMEM plus 10 nM E_2. (C) Proposed model that integrates E_2 regulation of BRCA-1 expression with G_1-phase to S-phase transition. Our data suggest that E_2 stimulates the recruitment of an ERα/p300 transcription complex to an AP-1 site in the BRCA-1 promoter. In turn, increased BRCA-1 levels may activate G_1/S-phase checkpoints to allow time for DNA repair [40] and to block ERα transcriptional activity [23]. However, accumulation of p53 interferes with the recruitment of ERα, leading to transcriptional repression of BRCA-1.*

ovarian estrogens. Without the positive regulation of *BRCA-1* expression by ERα, it is conceivable that E_2 may stimulate progression through S-phase without proper control, and increase the risk for cancer growth in E_2-responsive tissues. Studies have shown that 90% of *BRCA-1* tumors are likely to be ER-negative [48] and may explain the lower levels of *BRCA-1* observed in sporadic breast tumors [6]. In turn, increased expression of *BRCA-1* may inhibit the transcriptional activity of ERα as suggested by earlier studies [23].

Our results also indicated that endogenous p53 levels appeared to have no effects of ERα recruitment on the *BRCA-1* promoter. Conversely, overexpression of p53 antagonized the recruitment of ERα to the AP-1 site. These data may explain, at least in part, how accumulation of p53 leads to repression of *BRCA-1* transcription [39]. Interestingly, upregulation of *BRCA-1* has been shown to stabilize wild-type p53, providing a feedback loop in which these tumor-suppressor genes regulate each other [26]. Overall, the interplay between the positive regulation and the negative regulation by ERα and p53, respectively, on *BRCA-1* expression may be part of an integral signaling pathway that is mediated by AP-1 and determines whether the cell undergoes checkpoints/DNA repairs or apoptosis should the damage be irreparable. Disruption of this trafficking may predispose to the onset of breast cancer.

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