Estrogen Receptor Regulates E2F1 Expression to Mediate Tamoxifen Resistance

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Abstract

Antiestrogen resistance often develops with prolonged exposure to hormone therapies, including tamoxifen, and is a major problem in the treatment of breast cancer. Understanding the mechanisms involved in the development of antiestrogen resistance is an important step in the development of new targeted therapies. Two forms of antiestrogen resistance exist: *de novo* resistance and acquired resistance. To mimic acquired resistance, we have established a tamoxifen-resistant breast cancer cell line (MCF-7TamR) by treating parental MCF-7 cells with tamoxifen over a period of 6 months to select for cells with the resistant phenotype. Characterization of the MCF-7TamR cells under normal, hormone-deprived, and tamoxifen-treated conditions suggests that these cells continue to grow in the presence of tamoxifen. Additionally, a greater percentage of resistant cells enter the S phase under tamoxifen conditions, compared with parental MCF-7 cells. Consistent with these growth results, molecular analysis indicates that tamoxifen-resistant cells express higher levels of cyclin E1, cdk2, ACTR, and E2F1. Faslodex or ICI 182, 780 (ICI)-mediated degradation of estrogen receptor (ER) reduced the proliferation of these cells, as well as the level of E2F1 expression in tamoxifen-resistant cells, suggesting that tamoxifen resistance and E2F1 expression are in part dependent on ER. We further showed that tamoxifen enhances the ERα/Sp-1 interaction and promotes the recruitment of ERα and Sp-1 to the proximal promoter of E2F1 in resistant cells. Collectively, our findings suggest that tamoxifen resistance is a result of increased ERα/Sp-1 interaction, which enhances the expression of E2F1 to promote tamoxifen resistance. *Mol Cancer Res; 8(3); 343–52. ©2010 AACR.*

Introduction

Antiestrogens are the most frequently used drugs for hormone therapy of estrogen receptor (ER)–positive breast cancer patients. The efficacy of these drugs depends strongly on the biological characteristics of the tumor tissue. Breast cancer is classified as either ER positive or ER negative, depending on the presence or absence of ER, particularly ERα. Although there are two known isoforms of ER, ERα and ERβ, much of what we know about ER-dependent breast cancer has focused on ERα. The presence of ERα in breast cancer provides a target for therapeutic intervention. However, cells in the more advanced stages of breast cancer no longer respond to hormone withdrawal, irrespective of receptor status. Conventional endocrine therapy, such as tamoxifen-based treatment, which inhibits the ligand-binding domain of the receptor, has shown some success. However, resistance typically develops with prolonged treatment to tamoxifen. Two forms of antiestrogen resistance have been observed: *de novo* resistance and acquired resistance. The absence of ER is the cause of the most common form of *de novo* resistance, whereas unresponsiveness to antiestrogens is responsible for the major form of acquired resistance. In many cases, ER is still present in cells with acquired resistance. However, the role of ERα in the development of tamoxifen-resistant breast cancer remains unclear (1-5). Understanding the role of ERα in the development and progression of hormone-unresponsive and receptor-dependent breast cancer is an important step in the development of future therapeutics.

In an attempt to understand the molecular mechanisms behind tamoxifen resistance, we developed a tamoxifen-resistant cell line (MCF-7TamR) by maintaining ER-positive breast cancer cells, MCF-7, in 10^{-8} mol/L tamoxifen for more than 6 months to select for resistant phenotypes. Molecular analyses indicated that resistant cells expressed higher levels of the cell cycle regulators cyclin E1, cdk2, and E2F1, all of which have been implicated in cancer progression (6-13). We further showed that tamoxifen enhances the ERα/Sp-1 interaction and promotes the recruitment of ERα and Sp-1 to the proximal promoter of E2F1 in MCF-7TamR cells. Taken together, our results suggest that

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acquired tamoxifen resistance is due to overexpression of E2F1 as a result of the elevated ERα/Sp-1 interaction in resistant cells.

Materials and Methods

Cell Culture and Cell Growth Analysis

Human MCF-7 breast cancer cells were obtained from the American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS; Hyclone) and antibiotics. A tamoxifen-resistant MCF-7 cell line (MCF-7TamR) was developed from the parental line by maintaining cells in a medium containing 10^{-8} mol/L tamoxifen over 6 mo. Resistant cells were characterized by using a cell proliferation assay under three conditions: (a) normal or FBS condition, (b) hormone-deprived condition using a medium containing 10% charcoal-dextran stripped FBS (10% CDS-DMEM, Hyclone), and (c) tamoxifen (10^{-7} mol/L, Tocris)–treated condition. Cell proliferation was measured by cell counting at 2, 4, and 6 d after plating of triplicate samples, and the results were compared with those of the parental cells.

For ER depletion experiments, MCF-7TamR cells were plated in six-well plates under normal conditions. Cells were treated with or without 10^{-7} mol/L Faslodex or ICI 182, 780 (ICI), and cell proliferation was measured by cell counting at 2, 4, and 6 d after plating of triplicate samples.

For the RNAi silencing experiments, MCF-7TamR cells were seeded in 24-well plates and transfected with ERα or control siRNAs (scrambled) using an siRNA transfection reagent (Santa Cruz Biotechnology). Five hours after transfection, the medium was changed to a hormone-deprived medium with or without 10^{-7} mol/L tamoxifen. Cells were counted 3 and 4 d after treatment and subsequently collected for Western blot analysis.

Western Blot Analysis

MCF-7 and MCF-7TamR cells were plated in six-well plates under normal (FBS), hormone-deprived, or tamoxifen-treated (10^{-7} mol/L) conditions. Cells were harvested at 24 or 48 h after plating and lysed in 1% SDS-HEPES buffer. Protein expression was evaluated by Western blot analysis with protein-specific antibodies: α-cyclin E1, α-Cdk2, α-E2F1, α-ACTR (BD), α-cyclin D1 (Transduction Laboratories), and α-actin (Sigma).

Reverse Transcription-PCR Analysis

MCF-7 and MCF-7TamR cells were plated in six-well plates under normal, hormone-deprived, or tamoxifen-treated (10^{-7} mol/L) conditions. Cells were harvested at 24 and 48 h after plating, and total RNA was isolated using Trizol, following the manufacturer's protocol. Three micrograms of total RNA were used for reverse transcription with Moloney murine leukemia virus reverse transcriptase (Invitrogen) and oligo-dT18 primers. Gene expression was monitored using semiquantitative PCR over 28 cycles (Bio-Rad). Primer sequences for reverse transcription-PCR (RT-PCR) are listed below:

cyclin D1F: TCCTGTGCTGCGAAGTGGAAAC; cyclin E1F: ATACAGACCCACAGAGACAG; cdk2F: TTTGCTGAGATGGTGACTCGC; E2F1F: CGCATCTATGACATCACCAACG; c-myc F: TGACACTGTCCAACTTGACCCTCTT; CTDF: GGTGGAATACTTTGCCTGCCTTC; cyclin AF: CCCCCAGAAGTAGCAGAGTTTGTG; ACTRF: GATTAGGAGAAAACTTGGATCC; ActinF: GAGAAAATCTGGCACCACACC; cyclin D1R: AAATCGTGCGGGGTCATTGC; cyclin E1R: TGCCATCCACAGAAATACTT; cdk2R: CACTGGAGGAGGGGTGAGATTAG; E2F1R: GAAAGTTCTCCGAAGAGTCCACG; c-myc R: TCGCAAGACTCCAGCGCCTTCTC; CTDR: CCGAGACGCTCCCAGACATC; cyclin AR: GCTTTGTCCCGTGACTGTGTAGAG; ACTRR: TGTTTCCGTCTCGATTCACCA; ActinR: ATACCCCTCGTAGATGGGCAC

For quantitative RT-PCR analysis, gene sequences were amplified in the presence of the SYBR green fluorophore (Superarray) and detected using an iCycler real-time PCR instrument (Bio-Rad). Fluorescence values were collected after each elongation step, along with a melting curve analysis at the end of the PCR. Fold difference (N) was calculated using the equation $N = E (C_T \text{ treated} - C_T \text{ control})$ between treated cells and mock-treated (control) cells. E is defined as the PCR efficiency of the specific primer pair and was determined by the standard curve [$E = 10$ (1 / slope of standard curve)]. C_T is the threshold cycle, which is defined as the minimum cycle number at which fluorescence of the PCR product is first detected.

Bromodeoxyuridine Assay

MCF-7 and MCF-7TamR cells were plated in six-well plates containing untreated glass slides under hormone (estrogen), hormone-deprived, or 10^{-7} mol/L tamoxifen conditions. Cells were pulse-labeled for 2 h with 10 μmol/L bromodeoxyuridine (BrdUrd, Sigma) and harvested by fixation in 70% ethanol for 30 min at room temperature. Slides containing cells were treated with 2 mol/L HCl and 0.5% Triton X-100 for 30 min and rinsed in PBS (pH 8.5). BrdUrd incorporation was monitored using a BrdUrd antibody conjugated to fluorescein isothiocyanate (FITC), prepared in 0.5% Tween 20/1× PBS. Slides were washed three times with 1× PBS plus 0.5% Tween and counterstained with 4′,6′-diamidino-2-phenylindole (DAPI) for 3 min. Cells were examined using a fluorescence microscope and digital images were captured. The percentage of cells in the S phase was determined by counting the number of BrdUrd-positive cells in five different frames and dividing by the total number of DAPI-positive cells.

Coimmunoprecipitation

MCF-7 and MCF-7TamR cells were plated in hormone-depleted or tamoxifen-containing medium for 48 h. Cells

were harvested, lysed in 0.5% NP40 lysis buffer, and sonicated for 60 s. Cell lysates were immunoprecipitated with a 50:50 ratio of HC-20 and MC-20 ERα antibodies (Santa Cruz Biotechnologies) for 1.5 h and then incubated with protein A beads for 1 h. Protein complexes were washed three times with 1× PBS and then separated with SDS-PAGE before Western blot analysis with α-ERα (Neomarkers), α-Sp-1(Santa Cruz Biotechnology), and α-c-Jun (Santa Cruz Biotechnology) antibodies.

Luciferase Reporter Assay

The reporter gene assay was done by transfecting MCF-7TamR cells with the firefly luciferase reporter plasmid pGL2-E2F1-*Luc* and the pRL-SV40 *Renilla* luciferase plasmid (Promega) using Lipofectamine (Invitrogen), according to the manufacturer's protocol. Five hours after transfection, cells were treated with or without 10^{-7} mol/L ICI 182,780. Cells were harvested 48 h posttransfection and analyzed using a dual luciferase assay kit (Promega). In the second reporter gene assay, ERα was overexpressed by transfecting MCF-7TamR cells with the pCMV-ERα, pGL2-E2F1-*Luc*, and pRL-SV40 *Renilla* luciferase plasmids. All reporter gene assays were done in triplicate, with the entire experiment repeated at least three times. The pGL2-E2F1-*Luc* plasmid was a generous gift from Joseph Nevins (Duke Institute of Genome Sciences and Policy, Duke University, Durham, NC; ref. 14).

Chromatin Immunoprecipitation

MCF-7 and MCF-7TamR cells were plated in 10-cm plates in hormone-deprived medium with and without tamoxifen for 24 h. Cells were harvested by fixation with formaldehyde. Cells were then scraped and collected by centrifugation. Chromatin immunoprecipitation (ChIP) was done as previously described (11). Briefly, equal numbers of cells were lysed and sonicated in lysis buffer to fragment the chromatin into 1-kb fragments. The chromatin mixtures were immunoprecipitated with antibodies

against specific target proteins: α-ERα (50:50 mixture of HC-20/MC-20), α-Sp-1, or α-ACTR (Santa Cruz Biotechnology). Antibody complexes were purified using either protein A or G, and complexes were reverse crosslinked to release the chromatin fragments. DNA fragments were purified using QiaQuick columns, and occupancy at specific promoter sites was detected by PCR using promoter-specific primers. The presence of a PCR product indicates the recruitment of protein to the promoter region. The sequences of the ChIP primers are listed below:

E2F1-ChIPF: GCAAGTTGAGGATGGAAGAGGTG; cdk2-ChIPF: GATGGAACGCAGTATACCTCTC; E2F1-ChIPR: TGGGGACACGGGAACATAGG; cdk2-ChIPR: AAAGCAGGTACTTGGGAAGAGTG

Results

To understand how tamoxifen resistance develops in ER-positive breast cancer cells, our laboratory has developed a tamoxifen-resistant cell line by growing MCF-7 cells in a low concentration of tamoxifen (10^{-8} mol/L) for 6 months, selecting tamoxifen-resistant cells over time. The growth properties of the tamoxifen-resistant (MCF-7TamR) and parental MCF-7 cells were characterized under three conditions: (*a*) normal condition (10% FBS), (*b*) hormone-deprived condition (10% double charcoal-dextran stripped FBS), and (*c*) tamoxifen condition (10^{-7} mol/L tamoxifen). The results in Fig. 1 show that the tamoxifen-resistant and parental cell lines proliferate at the same rate under normal conditions. However, the MCF-7 parental cells grew significantly slower than the MCF-7TamR cells under hormone-deprived conditions. The observed difference increased significantly when the cells were grown in a tamoxifen-containing medium (Fig. 1). The results of this experiment confirm that the MCF-7TamR cell line displays the resistant phenotype.
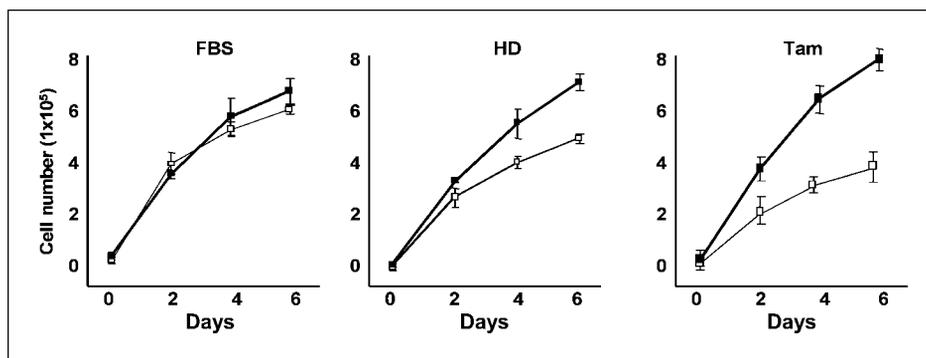

FIGURE 1. Characterization of a tamoxifen-resistant cell line (MCF-7TamR). MCF-7TamR (■) and parental MCF-7 (□) cells were plated in six-well plates under normal conditions (FBS), hormone-deprived conditions (HD), or hormone-deprived conditions with 10^{-7} mol/L tamoxifen (Tam). Cells were counted in triplicate every 2 d after treatment using a hemocytometer. Results are representative of three independent experiments.
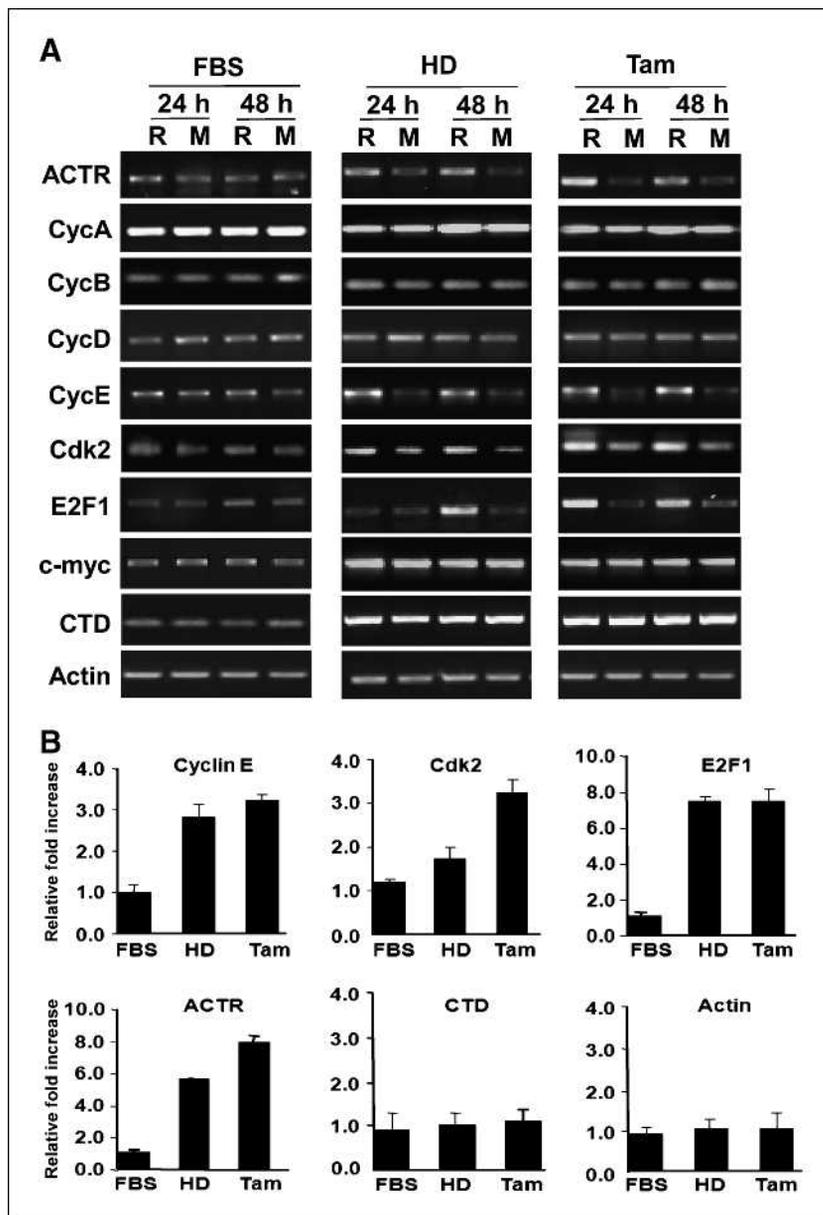
FIGURE 2. Gene expression analysis of tamoxifen-resistant (R) and parental (M) cells. Resistant (MCF-7TamR) and parental (MCF-7) cells were plated in six-well plates under normal conditions (FBS), hormone-deprived conditions (HD), or hormone-deprived conditions with 10^{-7} mol/L tamoxifen (Tam) for 24 or 48 h. Total RNA was isolated, and gene expression was analyzed by (A) semiquantitative RT-PCR and (B) quantitative RT-PCR for the resistant cells at 48 h. Statistics for the quantitative RT-PCR results were derived from triplicate samples.

To elucidate the molecular mechanism of tamoxifen resistance in breast cancer, we examined the differential gene expression of the two cell lines. MCF-7TamR and parental MCF-7 cells were plated in normal (FBS), hormone-depleted (HD), or tamoxifen-containing medium (TAM). Cells were harvested 24 and 48 hours after plating for gene expression analysis using semiquantitative and quantitative RT-PCR (Fig. 2A and B). Consistent with our proliferation data, no significant differences in gene expression were observed between the resistant and parental cells under normal (FBS) conditions (Fig. 2). However, the expression levels of cyclin E1, cdk2, E2F1, and ACTR (AIB1, SRC-3, TRAM, RAC3) were markedly increased in the resistant cells at 24 hours after

exposure to tamoxifen conditions. Furthermore, a less dramatic but reproducible increase in expression of these genes was also observed under hormone-deprived conditions. These results suggest that tamoxifen-resistant (MCF-7TamR) cells express elevated levels of key cell cycle genes, leading to increased proliferation in these cells.

To determine whether the difference observed at the level of gene expression was reflected at the protein level, we used Western blot analysis to examine the protein expression profiles of resistant and parental cells under the same conditions (Fig. 3). Consistent with the gene expression analysis, the increased levels of cyclin E1, cdk2, E2F1, and ACTR mRNAs in tamoxifen-resistant cells correlated with an increase in the expression levels of these proteins

under tamoxifen treatment (Fig. 3). In contrast, the expression levels of these proteins were not significantly different between the normal and resistant cells grown under normal (FBS) conditions (Fig. 3). A decrease in cyclin D1 expression was also detected in the parental cells (MCF-7) after 48 hours of tamoxifen treatment. This finding was expected, as tamoxifen is an ER antagonist known to decrease the expression of ER target proteins, such as cyclin D1 (15, 16).

Given that cyclin E1, cdk2, and E2F1 are important regulators of the G_1-S transition, we questioned whether there was a difference between the tamoxifen-resistant and parental cells in the progression of cells into the S phase. S-phase progression is monitored by incorporation of BrdUrd, a thymidine analogue that is incorporated into newly synthesized DNA. MCF-7 and MCF-7TamR cells were plated under hormone (estrogen), hormone-deprived, or tamoxifen conditions. Cells were pulse-labeled with BrdUrd for 2 hours and harvested for S-phase analysis using a BrdUrd-specific antibody conjugated to FITC. Cells were also counterstained with DAPI to detect total nuclei. Cells positive for BrdUrd staining represent cells that actively synthesized DNA during the 2-hour pulse period. Consistent with our growth and gene expression analyses, no significant differences ($P = 0.12$) were observed between parental (MCF-7) and resistant (MCF-7TamR) cells in the percentage of cells in the S phase when maintained under hormone (estrogen) conditions (the percentages of S-phase cells were 26.4% and 27.8%, respectively; Fig. 4A). Under hormone-deprived conditions, a significant difference ($P = 0.05$) in the number of BrdUrd-positive cells was observed between the parental and resistant cell lines (18.3% in MCF-7 versus 24.4% in MCF-7TamR; Fig. 4B). More significantly, the difference in the percentage of BrdUrd-positive cells was even greater in cells grown in the presence of tamoxifen (14.7% in MCF-7 cells versus 30.2% in MCF-7TamR cells; $P = 0.05$; Fig. 4C). These results also suggest that tamoxifen may act as an agonist, rather than an antagonist, in the resistant cells, as 30.2% of cells were in the S phase under tamoxifen conditions versus 24.4% in hormone-deprived conditions (Fig. 4B and C). As expected, the parental MCF-7 cells sensitive to tamoxifen displayed a lower percentage (14.7%) of cells entering the S phase in the presence of tamoxifen (Fig. 4C). These results further validate that the MCF-7TamR cells exhibit a resistant phenotype.

To determine whether the ER plays a role in tamoxifen resistance, we examined the effects of decreased levels of ER on the growth of tamoxifen-resistant cells. To reduce the endogenous levels of ER, we treated cells with ICI 182,780, a pure antiestrogen that binds to ER and promotes receptor degradation. We then analyzed the effects of ICI-mediated receptor degradation on the proliferation of the parental and resistant cells. As expected, ICI diminished the proliferation of parental MCF-7 cells. To our surprise, treatment with ICI also reduced the growth of cells resistant to tamoxifen (MCF-7TamR; Fig. 5A). These data suggest that tamoxifen-resistant cell growth is, in part, dependent on the presence of an intact ER. However, no sig-

nificant differences were detected in the expression levels of the ER target genes cyclin D1, c-myc, and CTD (Fig. 2), suggesting that ER may act through an alternative mechanism to mediate antiestrogen-resistant growth.

To further investigate the molecular mechanism of antiestrogen-resistant breast cancer cell growth, we examined gene expression in resistant cells treated with ICI. We found that the levels of E2F1 decreased significantly as early as 24 hours after ICI treatment, closely correlating with the decreased expression of ERα. No significant changes were observed in the expression levels of cyclin E1 or cdk2 until at later time points following ICI treatment (Fig. 5B). We also examined cyclin D1, a known ER target gene. As expected, the expression of cyclin D1 decreased with the decrease in expression of ERα (Fig. 5B). These results suggest that the expression of E2F1 in tamoxifen-resistant cells is dependent on the presence of ER.

To determine whether ERα is necessary for the transcription of E2F1, we performed a luciferase reporter assay. MCF-7TamR cells were cotransfected with E2F1-*Luc*, renilla luciferase, and an ERα expression plasmid (pCMV-ERα) for 48 hours. Luciferase activity was monitored using a dual luciferase assay with renilla luciferase as the internal control. Our results suggest that ERα can transactivate the E2F1 promoter 8-fold more than the control (empty p-CMV vector; Fig. 5C). To further establish the role of ERα in the transcription of E2F1, we also transfected MCF-7TamR cells with E2F1-*Luc* and subsequently treated the cells with ICI to downregulate the expression of endogenous ERα in MCF-7TamR cells (Fig. 5D). The decreased levels of ERα reduce transactivation significantly from 38-fold to 10-fold. Taken together, our results suggest that ERα may regulate E2F1 expression in tamoxifen-resistant cells.

The E2F1 promoter lacks the classic estrogen response element 5′-GGTCAnnnTGACC-3′; however, ERα is
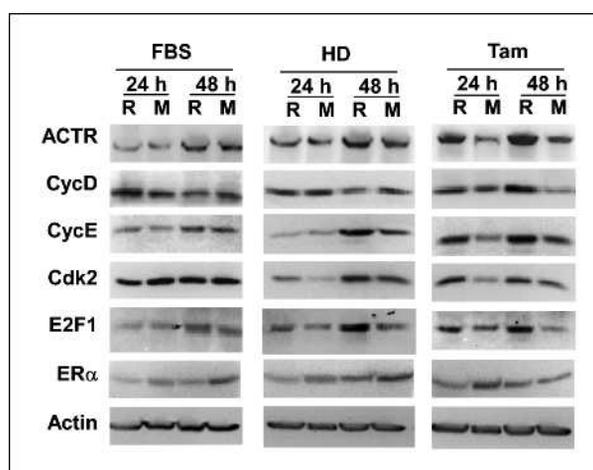

FIGURE 3. Western blot analysis of tamoxifen-resistant (R) and parental (M) cells. Resistant and parental cells were plated in six-well plates under normal conditions (FBS), hormone-deprived conditions (HD), or hormone-deprived conditions with 10^{-7} mol/L tamoxifen (Tam) for 24 or 48 h. Total cell lysates were analyzed by Western blot.
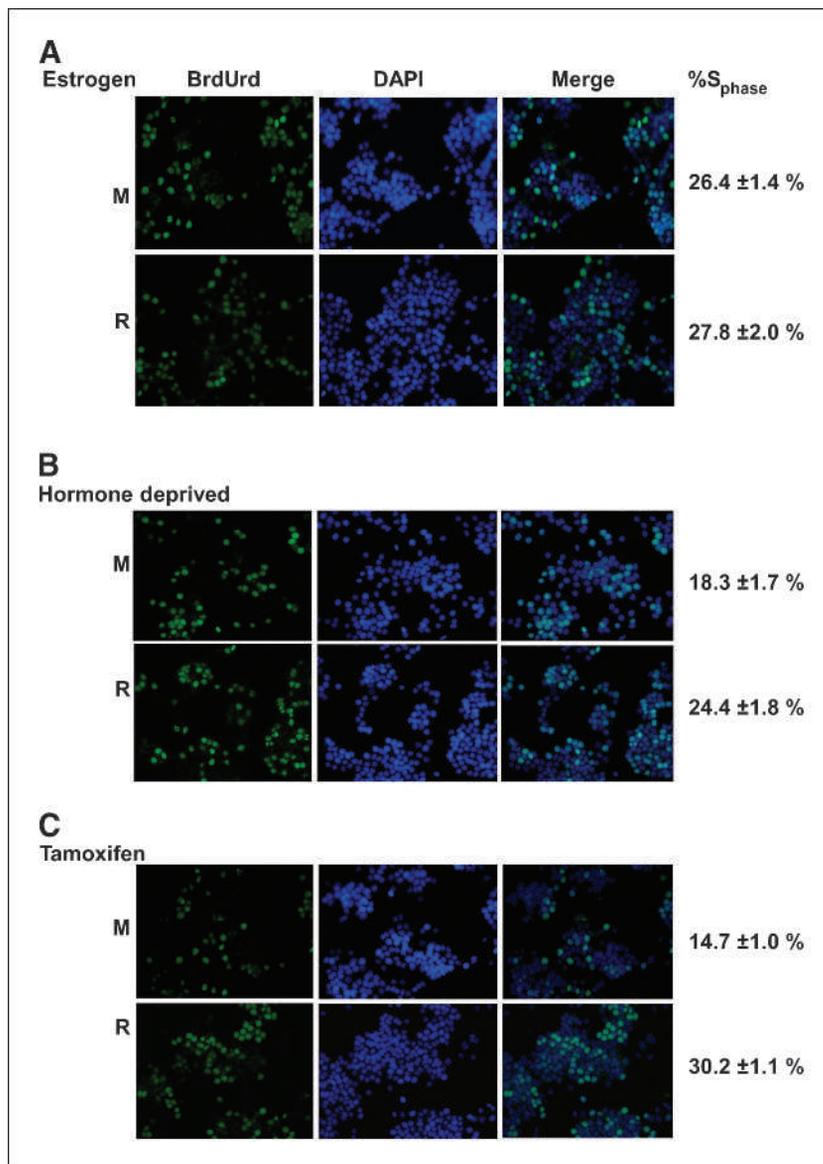
FIGURE 4. MCF-7TamR cells displayed a greater percentage of cells in the S phase. MCF-7 (M) and MCF-7TamR (R) cells were plated in hormone-depleted medium and treated with (A) 10^{-7} mol/L β-estradiol (Estrogen), (B) mock-treated with PBS (Hormone deprived), or (C) 10^{-7} mol/L tamoxifen (Tamoxifen). Cells were pulse-labeled for 2 h with 10 μmol/L BrdUrd and harvested via fixation in 70% ethanol for 30 min at room temperature. DNA synthesis was determined by an immunofluorescence assay with an α-BrdUrd antibody conjugated to FITC. Total DNA was visualized using DAPI and the percentage of cells in the S phase was determined by dividing the number of BrdUrd-positive cells (FITC) by the number of DAPI-positive cells. Results are representative of three independent experiments.

known to interact with several other transcription factors, including Sp-1 (17-20). Several studies have previously shown that tamoxifen increases ERα/Sp-1 interactions (17, 19, 20). To determine whether ERα interacts with Sp-1 in tamoxifen-resistant cells upon tamoxifen treatment, we performed a coimmunoprecipitation experiment. MCF-7 and MCF-7TamR cells were hormone deprived for 24 hours and treated with or without 10^{-7} mol/L tamoxifen. Cell lysates were immunoprecipitated with α-ERα antibodies, and the presence of Sp-1 in the immunoprecipitates was analyzed by Western blot. The results in Fig. 6A show that although Sp-1 is found in complex with ERα in parental cells under tamoxifen treatment, no significant interaction is found in the absence of tamoxifen. Strikingly, a greater fraction of Sp-1 coprecipitated

with ERα in the resistant cells when compared with the parental cells under both conditions. The ERα-/Sp-1 interaction was confirmed by reciprocal coimmunoprecipitation with antibody against Sp-1 (data not shown).

Because ERα coprecipitates with Sp-1, we questioned whether Sp-1 is directly involved in the regulation of genes that are upregulated during the development of tamoxifen resistance. To address this question, we performed a ChIP assay to determine whether ERα and Sp-1 are recruited to the E2F1 promoter. MCF-7 and MCF-7TamR cells were both plated in either hormone-deprived or tamoxifen-containing medium for 24 hours. Cells were harvested for ChIP analysis, and recruitment of ERα and Sp-1 was analyzed by PCR. The results shown in Fig. 6B indicate that under tamoxifen treatment, higher levels of ERα are

recruited to the E2F1 promoter in the resistant cells than in the parental MCF-7 cells (3.5-fold, compare lanes 3 and 4). This difference was less significant under hormone-deprived conditions (1.6-fold, compare lanes 1 and 2), which is consistent with the gene expression data. Similarly, the occupancy of Sp-1 on the E2F1 promoter was elevated in the resistant cells treated with tamoxifen compared with the parental cells (4-fold), whereas little difference was seen between the two cell lines under hormone-deprived conditions (1.8-fold). We also included ACTR and CARM1 in our ChIP analysis. ACTR/AIB1 was initially identified as a nuclear receptor coactivator that mediates transcriptional activation (21-24) and recent studies have shown that it is involved in the transcription of the E2F1 gene (11, 13, 25). CARM1 is a

protein arginine methyltransferase that has been shown to regulate estrogen-stimulated breast cancer cell growth through upregulation of E2F1 (1, 2, 42). As expected, the occupancy of ACTR and CARM1 on the E2F1 promoter mimics the occupancy patterns of ERα and Sp-1, suggesting that ACTR and CARM1 are important components of the transcriptional complex that regulates the expression of E2F1. We also examined the occupancies of ERα, Sp-1, ACTR, and CARM1 at the cdk2 promoter. As shown in Fig. 5B, ICI-mediated degradation of ERα did not alter the expression levels of cdk2 until at later time points, suggesting that ERα may not be directly involved in cdk2 expression. Consistent with this observation, the results in Fig. 6C show no significant ERα occupancy on the proximal promoter of cdk2 in either cell
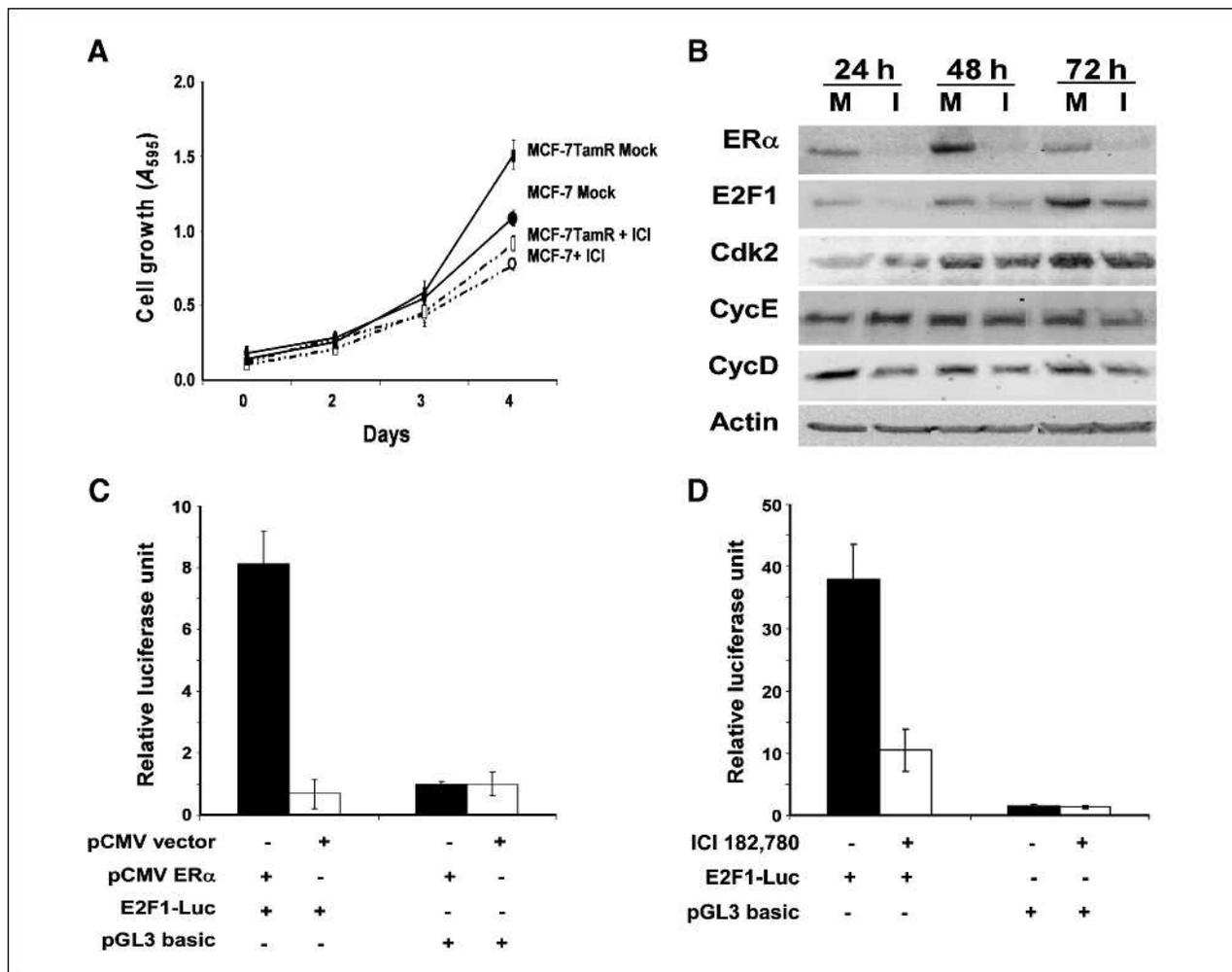

FIGURE 5. The growth of tamoxifen-resistant cells is dependent on ERα. A, MCF-7TamR (square) and MCF7 (circle) cells were plated in six-well plates in triplicate. Cells were treated with (open) and without (solid) 10^{-7} mol/L ICI. Cell growth was monitored every 2 d by direct cell counting in triplicate. B, MCF-7TamR cells were treated with 10^{-8} mol/L ICI (I) or mock-treated (M) for 24, 48, or 72 h. Protein expression was analyzed by Western blot. C, MCF-7TamR cells were transfected with pCMV ERα and E2F1-*Luc*; the corresponding control vectors pCMV and pGL3 basic were used as controls. D, MCF-7TamR cells were transfected with E2F1-*Luc* or with pGL3 basic (control). Following transfection, cells were treated with or without 10^{-7} mol/L ICI. Results represent multiple experiments done in triplicate.
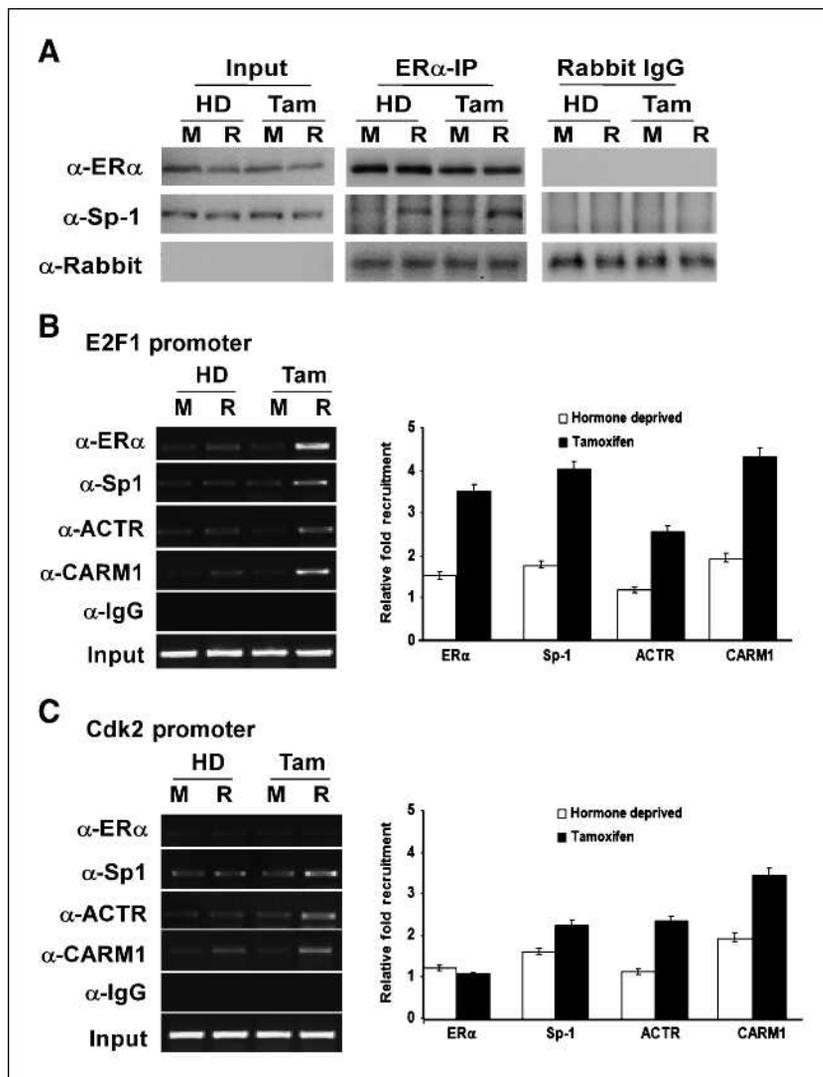
FIGURE 6. ERα interacts with Sp-1 in tamoxifen-resistant cells. A, MCF-7TamR cells were plated in hormone-depleted medium and treated with 10^{-7} mol/L tamoxifen (Tam) or mock-treated with PBS (HD). Cells were harvested for coimmunoprecipitation at 24 h after treatment. Cell lysates immunoprecipitated with α-ERα antibodies or normal rabbit IgG and immunoprecipitated complexes were separated by SDS-PAGE, and protein levels were analyzed by Western blot with antibodies against ERα and Sp-1. The input represents 10% of the total protein submitted to immunoprecipitation. MCF-7 (M) and MCF-7TamR (R) cells were hormone deprived (HD) and treated with 10^{-7} mol/L tamoxifen (Tam). ChIP analysis was done with α-ERα, α-Sp-1, α-ACTR, and α-CARM1, and recruitment of proteins to the (B) E2F1 and (C) Cdk2 promoters was determined using promoter-specific primers and semiquantitative RT-PCR. The band intensities of the PCR products were quantitated using Quantity One (Bio-Rad).

type under hormone-deprived or tamoxifen conditions. On the other hand, Sp-1, ACTR, and CARM1 were recruited to the cdk2 promoter under both conditions, with higher levels observed in resistant cells grown in the presence of tamoxifen.

To understand the role of E2F1 in tamoxifen-resistant breast cancer cell proliferation, we analyzed the growth of resistant cells following siRNA-mediated silencing of E2F1 (Fig. 7A). MCF-7TamR cells were transfected with either Ri-E2F1 (RiE) or a control siRNA (RiC) and maintained in a hormone-deprived medium in the presence (black) and absence (white) of tamoxifen (Fig. 7A). The results presented in Fig. 7A show that depletion of E2F1 reduced cell growth under both conditions (compare RiE to RiC). More importantly, cells transfected with Ri-E2F1 regain responsiveness to tamoxifen, suggesting that E2F1 plays a significant role in the development of tamoxifen resistance. Western blot analysis was used to monitor E2F1 expression in cells transfected with Ri-E2F1 and Ri-control

(Fig. 7B). Collectively, our results suggest that ERα cooperates with Sp-1 to regulate the expression of E2F1 and promote tamoxifen resistance.

Discussion

Tamoxifen is an antiestrogen that has been widely used for treatment of ER-positive breast cancer. However, prolonged treatment with tamoxifen often results in the development of antiestrogen resistance. The molecular mechanism of acquired resistance and the role of ERα in this process are poorly understood. In this study, we developed a tamoxifen-resistant cell line to study the mechanism of tamoxifen resistance. The growth pattern of the tamoxifen-resistant (MCF-7TamR) cell line was characterized under three conditions: regular medium with FBS, hormone-depleted medium, and tamoxifen-containing medium. Results from the growth assay confirmed the resistant phenotype of the cells (Fig. 1). The BrdUrd assay showed that the percentage of
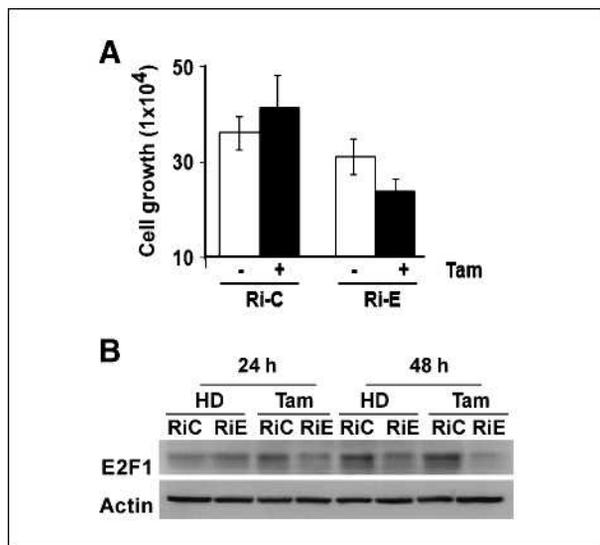
FIGURE 7. Tamoxifen-resistant breast cancer cell growth is dependent on E2F1. MCF-7TamR cells were transfected with Ri-E2F1 (Ri-E) or Ri-Control (Ri-C) and treated with (black) or without (white) 10^{-7} mol/L tamoxifen. A, cell growth was monitored 3 d after transfection, and statistical values represent data from four replicate samples. B, cells were also collected for Western blot analysis to monitor protein expression.

resistant cells entering the S phase was significantly higher than in the parental, nonresistant cell line in both hormone-deprived and tamoxifen conditions. These findings are consistent with the notion that resistant cells more frequently enter the cell cycle than the parental cells (Fig. 4). Gene expression analysis showed that specific cell cycle genes responsible for the regulation of the G_1-S phase transition are upregulated in the resistant cell line. These regulatory genes include E2F1, a transcription factor known to regulate the expression of cyclins and cdks (Figs. 2 and 3; refs. 26-30). In addition to E2F1, levels of cyclin E1, cdk2, and ACTR were also found to be elevated in cells with the tamoxifen-resistant phenotype. All of these genes have been implicated in breast carcinogenesis (6-13).

The role of ERα in the regulation of tamoxifen resistance is unclear. To fully establish a link between ERα and the proliferation of tamoxifen-resistant cells, we used ICI 182,780 to reduce the intracellular levels of ERα (Fig. 5A). Our results strongly suggest that the growth of tamoxifen-resistant cells is partly dependent on ERα. However, the function of ERα in tamoxifen-resistant cells remains unclear. Further molecular-level analysis showed that a decrease in levels of E2F1 accompanied the decrease in the levels of ERα mediated by ICI, whereas levels of cyclin E1 and cdk2 were not affected until later time points (Fig. 5B). This finding suggests that ERα must directly regulate the expression of E2F1, but not the expression of cyclin E1 or cdk2. Overexpression of E2F1 has been shown to be sufficient to promote breast cancer cell proliferation (11, 20, 31, 32) by regulating the expression of cyclins and cdks to mediate the G_1-S tran-

sition (33-35). The observed increase in expression of E2F1 in tamoxifen-resistant cells is consistent with the observation that resistant cells display a greater percentage of cells entering the S phase at any given time (Fig. 4). Further, multiple studies have shown that increased expression of E2F1 is sufficient to promote G_1-S progression in arrested cells (36, 37).

In accordance with previous studies that have shown that ERα cooperates with Sp-1 to regulate the expression of E2F1 (20, 38), our data suggest that ERα interacts with Sp-1 in the presence of tamoxifen in resistant cells (Fig. 6A). Using ChIP analysis, we showed that tamoxifen-resistant cells display increased occupancy of ERα and Sp-1 at the E2F1 proximal promoter. These results provide evidence of a direct link between E2F1 expression and the transcriptional activity of ERα and Sp-1. Consistent with previous studies that have suggested that tamoxifen resistance is mediated by the ACTR-E2F1 pathway (11, 13, 25), our results suggest that ACTR/AIB1 is also part of the transcription complex that regulates the expression of E2F1 (Fig. 6B). Taken together, these data suggest that ERα plays a crucial role in regulating cell proliferation in tamoxifen-resistant cells by interacting with Sp-1 to mediate the expression of E2F1. The work presented here lends support to the current hypothesis that antiestrogens such as tamoxifen, when bound to ER, may facilitate interactions between ERα and other transcription factors (i.e., Sp-1) and thereby regulate the expression of genes necessary for the development of resistance (1-5).

Understanding the role of ERα in tamoxifen resistance is a necessary step in the development of new targeted therapies. The binding of tamoxifen to ERα is known to disrupt the helix 12 conformation of ERα and promote the recruitment of corepressors rather than coactivators (39-41). However, antiestrogen binding does not interrupt protein interactions mediated by the A/B hypervariable domain. Consistent with the reports of others, our data suggest that protein-protein interactions between ERα and other transcription factors, such as Sp-1, may provide an alternative mechanism by which ERα regulates the expression of genes associated with growth, and subsequently leads to antiestrogen resistance. Additionally, the results from this study strongly validate the importance of targeting the ability of ERα to interact with other transcription factors, as this may be the key to treating antiestrogen-resistant breast cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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