Oncogenes and Tumor Suppressors

Blockade of AP-1 Potentiates Endocrine Therapy and Overcomes Resistance

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Abstract

The transcription factor AP-1 is downstream of growth factor (GF) receptors (GFRs) and stress-related kinases, both of which are implicated in breast cancer endocrine resistance. Previously, we have suggested that acquired endocrine resistance is associated with increased activity of AP-1 in an in vivo model. In this report, we provide direct evidence for the role of AP-1 in endocrine resistance. First, significant overlap was found between genes modulated in tamoxifen resistance and a gene signature associated with GF-induced estrogen receptor (ER) cistrome. Interestingly, these overlapping genes were enriched for key signaling components of GFRs and stress-related kinases and had AP-1 motifs in their promoters/enhancers. Second, to determine a more definitive role of AP-1 in endocrine resistance, AP-1 was inhibited using an inducible dominant-negative (DN) cJun expressed in MCF7 breast cancer cells in vitro and in vivo. AP-1 blockade enhanced the antiproliferative effect of endocrine treatments in vitro, accelerated xenograft tumor response to tamoxifen and estrogen deprivation in vivo, promoted complete regression of tumors, and delayed the onset of tamoxifen resistance. Induction of DN-cJun after the development of tamoxifen resistance resulted in dramatic tumor shrinkage, accompanied by reduced proliferation and increased apoptosis. These data suggest that AP-1 is a key determinant of endocrine resistance by mediating a global shift in the ER transcriptional program.

Implications: AP-1 represents a viable therapeutic target to overcome endocrine resistance. Mol Cancer Res; 14(5) 470–81. ©2016 AACR.

Introduction

More than 70% to 80% of breast cancers express estrogen receptor α (ER), which has been implicated in the etiology and progression of this disease. Endocrine therapy using different agents that block the estrogen (E2)/ER pathway has proven highly successful in the clinical setting. Tamoxifen (Tam) is a selective modulator of ER that is widely used for the treatment of pre- and postmenopausal patients with all stages of ER+ breast cancer (1). Aromatase inhibitors (AI) in postmenopausal women block the synthesis of E2 and have become the treatment of choice for many of these patients (2, 3). Despite their documented benefits, however, resistance is common in many women treated with both tamoxifen and AIs, either early (de novo or intrinsic resistance) or after prolonged therapeutic intervention (acquired resistance). Understanding the mechanisms of ER signaling and resistance to endocrine therapy remains critical in order to improve outcomes of breast cancer patients.

ER is a member of the greater family of nuclear receptor transcription factors (TF), which upon activation by E2, will then bind to DNA and regulate gene transcription by recruiting a complex of coregulator proteins. Importantly, these coregulators, as well as ER itself, undergo posttranslational modifications in response to diverse cellular signals coming from tumor cells, the microenvironment, growth factor receptors (GFRs), and stress-related kinases, with a potential impact on signaling activity as a consequence (4–6). These modifications can modulate ER transcriptional activity and result in ligand-independent or
tamoxifen-mediated activation of the receptor (7, 8). ER can modulate gene transcription either by directly binding to DNA on sites that contain E2 response elements (ERE) or by tethering to gene promoters/enhancers via interaction with other TFs, such as AP-1 (9). Interestingly, tamoxifen can stimulate the ER/AP-1 complex rather than inhibit it (10). Furthermore, the interaction of ER with AP-1 and other factors can be due to ligand-independent activation and, therefore, may not be susceptible to the estrogen-lowering effects of aromatase inhibition (11).

The AP-1 transcription complex is a collection of dimeric proteins that belong to the Jun (c-Jun, JunB, JunD), Fos (FosB, Fra-1, Fra-2), Maf, and ATF subfamilies. AP-1 complexes, including those of c-Jun, regulate the transcription of genes involved in cancer cell proliferation, survival, and invasiveness (12, 13). Levels and activity of the various members of the AP-1 complex are also regulated by multiple signals, including microenvironment stimuli, mitogenic GRs, and stress-related kinases associated with tumor progression (14). We have previously shown in preclinical models that the development of endocrine resistance is associated with oxidative stress and upregulation of EGFR and HER2 with activation of downstream proliferation and survival pathways (15, 16). Resistance is only partially overcome by targeting EGFR in these tumors, with similar observations in patients (17, 18), suggesting that other survival pathways also contribute to resistance. In addition, we also demonstrated that endocrine resistance is associated with increased levels of phosphorylated JNK, a major regulator of c-Jun activity and phospho-c-Jun itself, resulting in augmented AP-1 transcriptional activity (16). Similarly, increased levels of phospho-JNK and phospho-c-Jun, and enhanced GFR signaling, have been observed in patients with tamoxifen-resistant tumors (19, 20). Additional in vitro data show that tamoxifen-stimulated cells display higher levels of AP-1 DNA binding and transcriptional activity (21, 22) and that high expression of AP-1-dependent genes, such as VEGF, cyclin D1, and uPA, predicts poor tamoxifen response (22).

Interestingly, recent genome-wide profiling studies have demonstrated that hyperactive GFR signaling under E2-independent conditions can induce a global shift in the ER DNA-binding sites (cisomere), and in the ER transcriptional program from sites containing the ERE motif, toward those enriched for binding sites (cistrome), and in the ER transcriptional program that could be responsible for endocrine resistance. These observations strengthened our hypothesis that the inhibition of AP-1 may overcome endocrine resistance. To test this hypothesis, we used an inducible dominant-negative (DN)-cJun to inhibit AP-1 activity in vitro and in an in vivo model of endocrine resistance to both tamoxifen and estrogen deprivation mimicking aromatase inhibition. We demonstrate that AP-1 blockade increases tumor sensitivity to endocrine therapy, delays the onset of resistance, and causes dramatic tumor shrinkage, even after the full development of endocrine resistance, through the inhibition of both proliferative and survival signals.

Materials and Methods

Reagents, hormones, and antibodies

17β-Estradiol (E2) pellets (0.36-mg 60-day release) for in vivo studies were purchased from Innovative Research. Tamoxifen citrate for in vivo studies and 4-hydroxy tamoxifen and 17-beta estradiol (E2) for in vitro studies were purchased from Sigma. Doxycycline (Dox) was purchased from Sigma. Antibodies against phosphorylated (p)H3, Ki67, and cleaved caspase 3–7 were obtained from Millipore, Dako, and Cell Signaling Technology, respectively. Antibody against c-Jun was from Oncogene Research Products. Anti-Flag Tag antibody was from Sigma.

Cells and cell culture conditions

Stable MCF7 clones expressing the inducible TAM 67 DN-cJun (MCF7 Tet-off-TAM 67 clones 62 and 67) or the control vector alone (EV 1 and 3) were obtained from the laboratory of P.H. Brown in March 2007. MCF7 Tet-off-TAM 67 clones 62 and 67 were phenotypically validated for inducible DN-cJun expression as described previously (23) and reported below. Similarly, MCF7 control vector alone (EV 1 and 3) clones were phenotypically validated for lack of inducible expression of DN-cJun. These cells were maintained in high-glucose DMEM (Gibco) supplemented with 10% FBS (HyClone), penicillin (100 IU/mL), streptomycin (100 µg/mL), genetecin (G418; Gibco; 400 µg/mL), hygromycin (500 µg/mL), and doxycycline (2 µg/mL) (−Dox) to inhibit the expression of the DN-cJun under a humidified atmosphere of 5% CO2/95% air and at 37°C, as described previously (24, 25). For full induction of the DN-cJun, doxycycline was removed (−Dox) and cells were cultured in the presence of Tet System Approved FBS (Clontech). Low expression of the DN-cJun was obtained by culturing cells in low doxycycline-containing media (+0.1 ng/mL). For experiments employing endocrine treatment, cells were cultured in phenol red-free medium containing 5% charcoal-stripped FBS (starvation medium) for 12 hours, followed by endocrine therapy for 6 days.

Proliferation assays

To induce low or high levels of the DN-cJun, cells were first cultured for 4 days in medium containing Tet System Approved FBS ± Dox. Next, 3,000 cells/well were seeded in 96-well plates in starvation media with continuous exposure to doxycycline as before. Endocrine treatment was started 12 hours later (day 0) using 10−9 M E2, 10−7 M tamoxifen, or 0.01% ethanol to mimic estrogen deprivation (ED). Cell growth was assessed at days 0 and 6 postendocrine treatment as described previously (26). Briefly, for each treatment, cells were grown in 4% glutaraldehyde and stained with 0.05% methylene blue. The dye was subsequently extracted with 3% HCl and absorbance measured at 655 nm. Growth fold change was determined by [optical density (OD) 655 nm at day 6/OD 655 nm at day 0] for each treatment. Experiments were executed in quadruplicate and were repeated at least twice. Results from a representative experiment are shown.

IHC

Tumor tissue was processed and immunohistochemical staining was performed as described previously (27). Briefly, antigen retrieval was performed with 10 mM sodium citrate, pH 6 (for Flag antibody) or with Tris-HCl, pH 9 (for Ki67, cleaved caspase-3, and phospho-H3) in a pressure cooker at full pressure for 10 minutes. Mouse IgGs were blocked using the M.O.M Kit (Vector...
Laboratories) according to the manufacturer’s instructions. Anti-Flag antibody (1:100 dilution) was incubated overnight in a humidified chamber at 4°C, while K667 (1:200), cleaved caspase-3 (1:50), and phospho-H3 (1:400) were incubated for 1 hour at room temperature. Slides were then incubated with the secondary, biotinylated antibody for 30 minutes. Sections were then incubated with streptavidin–peroxidase for 30 minutes, and the enzyme was visualized after 15 minutes of incubation with diaminobenzidine. Nuclei were counterstained with hematoxylin before mounting. Markers were scored by counting positive and negative cells in four randomly selected high power fields, and results were expressed as the percentage of positive cells.

Protein extracts and immunoblots

Protein extracts and immunoblots were performed as described previously (26). Briefly, cell cultures were harvested in lysis buffer (Cell Signaling Technology) supplemented with 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 1 × protease inhibitor mixture (Roche Molecular Biochemicals). Twenty-five micrograms of protein from each sample was separated under denaturing conditions by electrophoresis on SDS-PAGE and transferred onto nitrocellulose membranes (Invitrogen). Blots were blocked with appropriate blocking buffer and then reacted at 4°C with primary antibodies at dilutions as per the manufacturer’s instructions overnight. Immunoblots were run in duplicate at a minimum, to confirm results.

Xenograft studies

Xenografts were established by injecting 15–20 × 10⁶ cells subcutaneously into ovariectomized 5- to 6-week-old athymic female mice (Harlan Sprague Dawley) that had been supplemented with 0.36-mg 60-day release E2 pellets (Innovative Research) subcutaneously into ovariectomized 5- to 6-week-old athymic female mice (Harlan Sprague Dawley) that had been supplemented with 0.36-mg 60-day release E2 pellets (Innovative Research) before mounting. Markers were scored by counting positive and negative cells in four randomly selected high power fields, and results were expressed as the percentage of positive cells.

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Computing GF-induced ER/AP-1–dependent gene signature associated with tamoxifenresistance

Our previously described gene signature of MCF7 xenografts that acquired tamoxifen resistance (15) was intersected with a list of genes putatively associated with growth factor (EGF)-induced ER-binding sites (EGF-induced ER cistrome) recently identified with genome-wide chromatin immunoprecipitation using ER antibody, followed by DNA microarray analysis (ChIP-on-chip; ref. 11). Genes putatively associated with sites of EGF-induced ER binding were defined as genes from the ReFSq database that have a functional ER-binding site within 20 kb upstream or downstream of their transcription start site. ER-binding sites were defined as high-stringency EGF-induced sites (FDR 1%) that fail to recruit ER following E2 treatment based on the low-stringency E2-induced ER cistrome as described previously (FDR 20%; ref. 11). To identify the list of genes putatively associated with EGF-induced ER-binding sites that harbour an AP-1 motif, we restricted the previous analysis to sites containing the AP-1 motif (TRANSFAC: M00926.AP-1; PSSM score > 5). Enrichment of the genes in our MCF7 xenograft tamoxifen-resistant signature within the list of genes putatively associated with EGF-induced ER-binding sites was tested using a one-sided Fisher exact test. Ingenuity Pathway Analysis software (Ingenuity Systems Inc.) was further used to identify the top biologic networks represented by the tamoxifen-resistant upregulated ER/AP-1 gene signature. In brief, the list of the upregulated tamoxifen-resistant genes overlapping with the list of genes putatively associated with the EGF-induced ER cistrome (n = 93 genes) was uploaded into the web application www.ingenuity.com and an analysis was run. The three top-scoring networks were merged into a single network, and the visualization tool was used to display graphically both the relationship among the genes in the list and also the relationship between the same genes and other molecules in the Ingenuity database.

Statistical analysis

On the basis of our previous studies in similar xenograft models (28), time to tumor doubling (TTD) was defined a priori as the time when tumor volume had increased 2 times from the value measured at the time of randomization for each mouse; time to response (TTR) was defined as the time when tumor volume had decreased to half from randomization. Complete tumor regression was defined as complete tumor disappearance (no palpable nodule or no measurable disease) for at least 2 consecutive measurements and time to complete response (TCR) was defined as the time when complete tumor regression was observed. The Kaplan–Meier method was used to determine the median TTD, median TTR, and median TCR. All P values for the xenograft studies were based on comparisons of variables among groups by use of the generalized Wilcoxon test. Complete tumor regression rates at day 100 and 95% confidence intervals were calculated for each group of animals. The two-sample t test or Wilcoxon rank sum test was used for 2-group comparisons of tumor proliferation and other immunohistochemically assessed biomarkers. All statistical tests were two sided.

Results

A gene signature of ER/AP-1 cooperation is enriched in signaling pathways known to be involved in endocrine resistance

We have previously shown in our MCF7 xenograft model that acquired tamoxifen resistance is driven in part by GFR signaling with repression of classic ER genomic activity (15). Indeed, GFRs, including EGFR, and their downstream kinases are implicated in
the development of endocrine resistance (15, 29, 30). In addition, our work as well that of others has provided evidence that AP-1 transcriptional activity is augmented in endocrine-resistant breast cancer (16, 19, 21). Recent studies mapping ER cistromes using the ChIP-on-chip technology showed that the ER DNA-binding sites in the presence of EGF and in the absence of E2 were completely distinct from those induced by E2. This altered genomic activity of ER depended on AP-1 DNA-binding sites and on AP-1 transcriptional activity (11). We therefore hypothesized that the observed role of AP-1 in endocrine-resistance may be related to the reprogramming of the ER cistrome under GFR activation. To this end, we intersected our previously developed tamoxifen-resistant MCF7 xenograft gene signature (15) and the list of genes putatively associated with the EGF-induced ER cistrome (Fig. 1A). We found a significant enrichment of the genes associated with the EGF-unique ER DNA-binding sites within our tamoxifen-resistant signature \[P = 2E^{-16}\] (upregulated) and \(P = 7E^{-7}\) (downregulated), one-sided Fisher exact test; Fig. 1A and B). Remarkably, 92% of these DNA-binding sites harbored an AP-1 motif (Fig. 1C; gene lists are provided in Supplementary Tables S1 and S2). To explore the potential biology underlying this gene list, we next analyzed the list of the 93 tamoxifen-resistant upregulated overlapping genes by network analysis, which enables visualizing relationships among molecules. Interestingly, merging of the three most represented networks in this analysis showed a network comprising signaling molecules, GFRs, ligands, microenvironment, and stress-related kinase pathways thought to be important in breast cancer endocrine resistance, including TGFβ, VEGF, PDGF, ERBB2, IL1R, PI3K, AKT, PKC, ERK1/2, JNK, p38 MAPK, FOS, and NFKB, among others (Fig. 1D).

**DN-cln expression inhibits MCF7 cell proliferation and potentiates the effect of endocrine treatment in vitro**

To more directly study the functional role of AP-1 in endocrine-resistant breast cancer, we adopted a genetic approach using an inducible DN-cln. The DN-cln mutant is a deletion form of the human cln lacking the transactivation domain (named trans activation mutant- TAM-67; ref. 23). The DN-cln is expressed in this cellular system upon doxycycline removal, leading to the inhibition of AP-1 activity by dimerizing with wild-type AP-1 protein to produce low-activity dimers containing only one transactivation domain. Two clones stably expressing the inducible Flag-tagged DN-cln (MCF7 Tet-off TAM-67, clone 67 and 62) were described previously and used for these studies (23). The effect of AP-1 blockade on endocrine treatment was assessed under the induction of low and high levels of DN-cln using various doxycycline concentrations in the cell growth media. Figure 1E demonstrates a gradual induction of DN-cln in cell lysates of clone 62 upon titrating down the doxycycline concentration for 4 days. Decreasing doxycycline concentrations in the culture medium to 1 ng/mL were still able to fully inhibit the expression of DN-cln. Low levels of the DN-cln were first detected in the presence of 0.1 ng/mL doxycycline and were fully induced upon complete doxycycline withdrawal (−Dox).

Clone 67 and 62 grown in the presence of 1 ng/mL of doxycycline (normal AP-1), 0.1 ng/mL doxycycline (partially impaired AP-1), or absence of doxycycline (maximally impaired AP-1) were treated for 6 days with E2, estrogen deprivation (ED) with ethanol, or ED plus tamoxifen, and cell proliferation was then assayed. Two vector-alone stably transfected clones (EV) were used as controls. In the presence of normal AP-1, all clones showed significant and substantial growth inhibition by both ED and tamoxifen treatments, although the degree of sensitivity, as expected, varied somewhat among the clones (Fig. 1F). Doxycycline withdrawal resulted in no significant changes in EV control clones. In contrast, in the DN-cln clones, a gradual decrease in proliferation was observed upon increasing expression of DN-cln in cells treated with E2 or with endocrine therapy (Fig. 1F). Most importantly, maximal impairment of AP-1 (−Dox) resulted in the induction of cell death only in conjunction with endocrine treatment (ED and tamoxifen), as shown by the substantial decrease in cell numbers at the completion of the treatment (day 6) compared with baseline (day 0). These data show that AP-1 inhibition significantly adds to the inhibitory effects of endocrine therapy in vitro and that this effect on tumor inhibition was most pronounced in the presence of antiestrogen treatment.

**Expression of DN-cln augments the response to endocrine treatment in vivo**

To determine whether the effects of AP-1 blockade on endocrine sensitivity observed in vitro were also observed in the in vivo setting, we next studied xenografts of the MCF7 Tet-off DN-cln clone 67 and 62 cells in mice treated with endocrine therapy. Xenograft tumors of clone 67 and clone 62 cells were established in the presence of E2 and doxycycline supplementation. When tumors reached 150 to 200 mm3 in size, mice were randomized to control (continued E2 supplementation) or endocrine treatment with ED alone or ED plus tamoxifen, either in the presence (normal AP-1) or in the absence (impair ed AP-1) of doxycycline. Inhibition of AP-1 improved response to endocrine therapy by significantly shortening time to response (TTR), defined as the time to tumor size halving from randomization, in the tamoxifen treatment arms for both clones (Fig. 2A and B) and the ED arm for clone 62, with a trend toward significance for clone 67 (Fig. 3A and B; Table 1; clone 67: tamoxifen \(P = 0.006\) and ED \(P = 0.068\); clone 62: tamoxifen \(P = 0.014\) and ED \(P = 0.0006\)).

More importantly, AP-1 inhibition, together with endocrine therapy, caused complete disappearance of many tumors and also significantly shortened time to complete response (TCR), defined as the time from randomization to complete regression of the tumors in the tamoxifen-treated mice for both clones (Fig. 2C and D) and the ED-treated mice for clone 62, but not clone 67 (Fig. 3C and D; Table 1; clone 67: tamoxifen \(P = 0.003\) and ED \(P = 0.7\); clone 62: tamoxifen \(P = 0.001\) and ED \(P < 0.0001\)). About 33% of clone 67 and 73% of clone 62 tumors in mice treated with tamoxifen plus DN-cln were undetectable after 100 days of treatment compared with 0% and 20%, respectively, of tumors in mice treated with tamoxifen alone. Similar data were observed in clone 62 mice treated with ED [percentage of complete response (%CR) at day 100: 85.7% vs. 0% in ED+ DN-cln vs. ED alone, respectively; Table 1]. Finally, in clone 67 in which acquired resistance to tamoxifen and tumor progression were detected within 200 days, DN-cln (−Dox) in combination with tamoxifen significantly delayed the onset of tamoxifen resistance by prolonging the time to tumor doubling (TTD) \(P = 0.003\); Fig. 2E; Table 2). At day 200, no tumors had developed resistance and progressed with tamoxifen plus the DN-cln, compared with half of those treated with tamoxifen alone.
Genes upregulated in TamR \( n = 423 \)

Genes downregulated in TamR \( n = 217 \)

**A**

Genes putatively associated with EGF-induced ER-binding sites \( n = 1489 \)

\[ P = 2 \times 10^{-16} \]

**B**

\[ P = 7 \times 10^{-7} \]

**C**

**D**

**E**

**F**

**Vector control 1**

**Vector control 3**

**DN-cJun Clone 62**

**DN-cJun Clone 67**

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In contrast to the in vitro data, inhibition of AP-1 in E2-treated mice had no effect on tumor growth as measured by TTD (Fig. 3E and F; clone 67 $P = 0.3$ and clone 62 $P = 0.6$; Table 2), suggesting that increased AP-1 activity becomes relevant only in tumors from mice treated with endocrine therapy, where AP-1 may function as an escape pathway to circumvent ER blockade.

To ensure that the observed growth delay was not due to doxycycline treatment itself, we inoculated mice with an MCF7 subclone that is stably transfected with vector alone (EV3) and then randomized them to E2-treated control or endocrine treatment either in the presence or the absence of doxycycline. As expected, no significant differences in growth were observed with or without doxycycline (Supplementary Fig. S1A–S1C; Supplementary Tables S3 and S4; Tam + Dox vs. Tam – Dox: TTR $P = 0.144$; TTR $P = 0.136$; TTD $P = 0.5$; ED + Dox vs. ED – Dox: TTR $P = 0.771$; TCR $P = 0.597$; E2 + Dox vs. E2 – Dox – Dox: TTD $P = 0.871$). Dn-cJun expression overcomes the growth of endocrine resistant tumors in vivo by eliciting a cytotoxic effect

To study the effects of AP-1 inhibition in tumors that had already developed resistance to endocrine therapy, we injected the expression of the DN-cJun at a later time point when tumors were growing on endocrine therapy. To this end, we identified tumor pairs of either clone 67 or clone 62 with similar size and/or growth characteristics that were either slowly growing or more rapidly progressing after long-term (>10 months) tamoxifen treatment in the presence of doxycycline (normal AP-1). Mice from different experiments bearing these “paired tumors” were allocated to either continued doxycycline or doxycycline withdrawal to induce the expression of the DN-cJun, all in the presence of continued tamoxifen treatment. Although these tumors displayed diverse and heterogeneous growth characteristics under tamoxifen, all the tumors in the doxycycline-withdrawal group showed shrinkage within 4–6 weeks after DN-cJun induction (Fig. 4). In contrast, tumors in mice in the control group (+Dox) remained stable in size or continued to grow over the same time frame, confirming that the effect observed in the doxycycline-withdrawal group was due to AP-1 inhibition.

To understand the mechanisms by which AP-1 blockade inhibits tamoxifen-resistant growth, tumor samples from mice in the Tam ± Dox groups (7–10 tumors per treatment group) were assessed by IHC for apoptosis (cleaved caspase 3–7; CC3–7) and proliferation (Ki67 and phospho-histone 3: pH3).

Expression of the DN-cJun was assessed using IHC with an anti-Flag antibody. Importantly, control tumors from the E2-supplemented mice ± Dox were also assayed in parallel. As expected, expression of the DN-cJun was detected only in –Dox conditions as shown by IHC staining with an anti-Flag antibody [Fig. 4C; and further confirmed by Western blotting using a cJun antibody (Supplementary Fig. S1D)]. AP-1 inhibition reduced proliferation and increased apoptosis in association with tamoxifen compared with tamoxifen alone (average Ki67 expression: 51% vs. 34.7%, $P = 0.04$; average pH3 expression: 2.9% vs. 1.6% $P = 0.006$; average CC3–7 expression: 2.4% vs. 7.4% in Tam + Dox vs. Tam – Dox, $P = 0.01$). No significant differences in apoptosis or proliferation rates were detected in E2-treated mice ± Dox (Fig. 4B and C).

Taken together, these findings strongly suggest that AP-1 activity functions as a compensatory pathway when ER signaling is blocked and can thereby mediate resistance to tamoxifen and ED in this model. Furthermore, AP-1 inhibition can reverse the endocrine-resistant phenotype in vivo both by inhibiting tumor cell proliferation and by eliciting a cytotoxic effect.

Discussion

Multiple pathways, including GF, stress, and those originating from microenvironmental stimuli, have been proposed to mediate intrinsic or acquired resistance to endocrine therapy. Previous work from our group and others has provided evidence for increased INK/AP-1 pathway activity and GFR signaling in endocrine resistance (16, 19–22). Our data support the concept that AP-1 may integrate signals from multiple pathways that cause endocrine resistance and that inhibition of AP-1 function is a very effective strategy for improving sensitivity and overcoming resistance to endocrine therapies. We show that there is a significant overlap between the gene signature of tamoxifen-resistant MCF7 xenografts (15) and the gene signature associated with the EGF-induced ER cistrome (11). Most of these overlapping genes have an AP-1 motif in their promoter/enhancer regions. Interestingly, the upregulated overlapping genes belong to a network of GFRs, stress-related kinases, and signaling molecules activated by the microenvironment, all of which have been implicated in endocrine resistance (31). These data suggest that AP-1, by engaging altered ER-dependent genomic networks, may act as a major node integrating diverse signaling pathways mediating endocrine resistance.

Indeed, in our in vivo breast cancer model of endocrine resistance, blockade of AP-1 function by DN-cJun leads to accelerated response to endocrine treatment, a higher response rate, a
remarkably higher complete tumor disappearance rate, and a marked increase in the time to treatment resistance in endocrine-sensitive tumors. Furthermore, blocking this pathway after the development of endocrine resistance restores tamoxifen sensitivity by inducing apoptosis and reducing cell proliferation. Our in vivo data from the DN-cJun model suggest that AP-1 is indeed crucial for the development of endocrine resistance, as AP-1 blockade does not allow tumors to progress while undergoing endocrine treatment. Moreover, our study suggests that AP-1 is fundamental for sustaining the resistant phenotype, as AP-1 blockade completely reverses the progression and growth of long-term tamoxifen-treated tumors, without any significant effects on E2-stimulated growth.

Other evidence suggests that AP-1 plays an important role in breast cancer biology and ER function. cJun overexpression in breast cancer cells induces changes correlating with an aggressive and invasive phenotype, promotes tumor formation in mice, and leads to reduced sensitivity to tamoxifen (32). AP-1 DNA-binding activity is significantly elevated in sublines of hormone-independent MCF7 tumors stimulated in vivo by c-Jun.

Figure 2.
Mice bearing xenograft tumors of either clone 67 (A and C) or clone 62 (B and D) cells were randomly allocated to continued E2 supplementation, ED, or ED in combination with tamoxifen (Tam), either in the presence or in the absence of doxycycline to induce DN-cJun expression causing AP-1 blockade. In the tamoxifen-treated group, inhibition of AP-1 significantly reduced time to response (TTR) (A and B) and time to complete response (TCR) (C and D). Tamoxifen-treated clone 67 tumors also showed significant prolongation in time to tumor doubling (TTD) (E) when AP-1 function was impaired.
tamoxifen (16, 21), as well as in tamoxifen-resistant patient samples (19). In ER\textsuperscript{+} cells, continuous exposure to tamoxifen can induce ER agonistic activity at AP-1–regulated promoter sites (33). Indeed, it is well known that ER has nonclassical genomic functions, where it does not make direct contact with DNA on ERE sites but rather is tethered to gene promoters/enhancers via interaction with other TFs, such as AP-1, NF\textkappa B, and others (9). This ER function regulates the expression of genes that are critical for proliferation, survival, and growth. In this form of gene regulation, ER is known to modulate the expression of distinct sets of AP-1–dependent genes (34). Indeed, AP-1 is a TF downstream of diverse mitogenic, survival, and stress stimuli, including GFR and stress-related kinase signaling, implicated in breast cancer endocrine resistance. Interestingly, recent systematic analysis of ER cistromes under GF stimulus (EGF) in the absence of E2 showed that EGF induces the modulation of a distinct set of ER-dependent genes, which are also dependent on AP-1 (11). In our analysis, a large fraction of genes modulated in our \textit{in vivo} tamoxifen-resistant signature have an AP-1–binding site in their regulatory promoter/enhancer regions, thus suggesting that reprogramming of ER nuclear genomic function through its binding to AP-1

![Figure 3](image-url)

\textit{Figure 3.} Inhibition of AP-1 in ED-treated mice significantly reduced TTR (A and B) and TCR (C and D) for clone 62, but not clone 67. However, in E2-supplemented mice, AP-1 inhibition did not delay tumor growth, as measured by TTD, in either clone 67 (E) or clone 62 (F) xenografts.
sites might be a feature of endocrine therapy resistance. Of note, gene network analysis of the 93 ER/AP-1–dependent genes that are upregulated in our tamoxifen-resistant signature implicated several different signaling pathways, which have been previously shown to be important in endocrine resistance. The multiplicity and diversity of these pathways may explain why inhibition of just EGFR has only a partial effect in overcoming tamoxifen resistance (15), whereas inhibition of AP-1, which in this study, results in a more dramatic effect in this model, including complete tumor regression in mice.

It has previously been shown that DN-cJun induces growth arrest in E2–treated tumors in vitro and in vivo (35). Although we confirm these results in vitro, we did not observe the same results in vivo. The most likely explanation for the discrepancy is different experimental conditions under which the in vivo studies were conducted. In our experiments, AP-1 blockade was induced when tumors were larger in size. Therefore, established xenograft tumors in the presence of E2 may be less dependent on AP-1. In contrast, tamoxifen-resistant tumors responded well to AP-1 blockade, suggesting that AP-1 may play a role that is relatively specific to endocrine-resistant growth, consistent with our prior observation that AP-1 transcriptional activity is much greater after tamoxifen resistance.

On the basis of the data presented here, we propose the following working model (Fig. 5). In endocrine-sensitive cells, E2 regulation of gene transcription is mediated predominantly by ER interaction with ERE DNA-binding sites (classic nuclear genomic activity). In response to endocrine therapy, AP-1 becomes a major determinant of transcription for diverse adaptive pathways responsible for resistance. These multiple pathways, including ER itself, converge on AP-1 to enhance its activity, which in turn activates a molecular shift in the ER genomic network to facilitate an AP-1/ER

### Table 1. TTR, TCR, and rate of CRs to endocrine treatment ± AP-1 inhibition in xenografts of MCF7 Tet-off DN-c-Jun clones 62 and 67

<table>
<thead>
<tr>
<th></th>
<th>Median TTR* (days)</th>
<th>95% CI</th>
<th>P</th>
<th>Median TCR* (days)</th>
<th>95% CI</th>
<th>P</th>
<th>% CR at day 100c</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tam Clone 62 Control (–DOX)</td>
<td>15</td>
<td>56</td>
<td>35–140</td>
<td>0.014</td>
<td>NA</td>
<td>91-NA</td>
<td>0.001</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>DN-c-Jun (–DOX)</td>
<td>11</td>
<td>35</td>
<td>21–42</td>
<td>91</td>
<td>49–112</td>
<td>72.7</td>
<td>46.1–93.7</td>
</tr>
<tr>
<td>Clone 67 Control (–DOX)</td>
<td>17</td>
<td>NA</td>
<td>56–NA</td>
<td>0.006</td>
<td>NA</td>
<td>NA</td>
<td>0.003</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>DN-c-Jun (–DOX)</td>
<td>17</td>
<td>49</td>
<td>35–63</td>
<td>112</td>
<td>91-NA</td>
<td>33.3</td>
<td>15.4–62.5</td>
</tr>
<tr>
<td>ED Clone 62 Control (–DOX)</td>
<td>14</td>
<td>94</td>
<td>56–140</td>
<td>0.0006</td>
<td>NA</td>
<td>182-NA</td>
<td>&lt;0.0001</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>DN-c-Jun (–DOX)</td>
<td>14</td>
<td>35</td>
<td>28–35</td>
<td>66.5</td>
<td>42–84</td>
<td>85.7</td>
<td>63.4–97.7</td>
</tr>
<tr>
<td>Clone 67 Control (–DOX)</td>
<td>17</td>
<td>42</td>
<td>28–63</td>
<td>0.068</td>
<td>77</td>
<td>56–189</td>
<td>0.704</td>
<td>64.7</td>
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<tr>
<td></td>
<td>DN-c-Jun (–DOX)</td>
<td>18</td>
<td>28</td>
<td>28–35</td>
<td>84</td>
<td>63–133</td>
<td>61.1</td>
<td>40–82.5</td>
</tr>
</tbody>
</table>

Abbreviations: CI, confidence interval; DOX, doxycycline; ED, estrogen deprivation; Tam, tamoxifen.
*TTD: time to tumor doubling (time elapsed from randomization to tumor size doubling).
**TCR: time to complete response (time elapsed from randomization to tumor disappearance).
*c% CR: percent of mice with complete response at day 100 (no palpable tumor for two consecutive measurements).

### Table 2. TTD with endocrine treatment ± AP-1 inhibition in xenografts of MCF7 Tet-off DN-c-Jun clones 62 and 67

<table>
<thead>
<tr>
<th></th>
<th>Median TTD* (days)</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2 Clone 62 Control (–DOX)</td>
<td>14</td>
<td>19.5</td>
<td>13.8–27.7</td>
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<tr>
<td></td>
<td>DN-c-Jun (–DOX)</td>
<td>15</td>
<td>16.8</td>
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<td>Clone 67 Control (–DOX)</td>
<td>14</td>
<td>4.5</td>
<td>2.4–9.3</td>
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<td></td>
<td>DN-c-Jun (–DOX)</td>
<td>17</td>
<td>6.1</td>
</tr>
<tr>
<td>Tam Clone 67 Control (–DOX)</td>
<td>17</td>
<td>NA</td>
<td>131-NA</td>
</tr>
<tr>
<td></td>
<td>DN-c-Jun (–DOX)</td>
<td>17</td>
<td>NA</td>
</tr>
</tbody>
</table>

Abbreviations: CI, confidence interval; DOX, doxycycline; Tam, tamoxifen.
*TTD: time to tumor doubling (time elapsed from randomization to tumor size doubling).
Figure 4.
A, mice bearing clone 67 or clone 62 tumors, which were either stable or progressing under long-term tamoxifen (Tam) treatment and normal AP-1 conditions, were allocated to either keep (left) or withdraw doxycycline (Dox; right) to induce DN-cJun expression, causing AP-1 blockade in the presence of continued tamoxifen, and were observed for 4 to 6 weeks. B, box and whisker plots of quantification of IHC analysis. C, representative IHC images of proliferation (Ki67 and pH3) and apoptosis (CC3/7) markers in tamoxifen-treated and E2 control tumors in the presence or absence of DN-cJun expression. H&E, hematoxylin and eosin; CC3/7, cleaved caspase 3-7.
models are warranted to gain a more mechanistic insight into AP-1’s role in endocrine-resistant breast cancer.

Disclosure of Potential Conflicts of Interest
S. De Placido has received speakers bureau honoraria from AstraZeneca, Novartis, and Roche and is a consultant/advisory board member for AstraZeneca, Novartis, Pfizer, and Roche. P.H. Brown is a consultant advisory board member for Susan G. Komen Foundation. C.K. Osborne is a consultant/advisory board member for AstraZeneca and PerkinElmer. R. Schiff reports receiving a commercial research grant from AstraZeneca and is a consultant/advisory board member for Eli Lilly and Company. No potential conflicts of interest were disclosed by the other authors.

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References


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