Published in final edited form as:
Clin Cancer Res. 2009 June 1; 15(11): 3672–3679. doi:10.1158/1078-0432.CCR-08-2343.

Interaction of TFAP2C with the Estrogen Receptor-α Promoter Is Controlled by Chromatin Structure

George W. Woodfield[1], **Michael J. Hitchler**[2], **Yizhen Chen**[1], **Frederick E. Domann**[2], and **Ronald J. Weigel**[1,3]

[1]Department of Surgery, University of Iowa, Iowa City, Iowa

[2]Department of Radiation Oncology, University of Iowa, Iowa City, Iowa

[3]Department of Biochemistry, University of Iowa, Iowa City, Iowa

Abstract

Purpose—Transcriptional regulation of estrogen receptor-α (ERα) involves both epigenetic mechanisms and *trans*-active factors, such as TFAP2C, which induces ERα transcription through an AP-2 regulatory region in the ERα promoter. Attempts to induce endogenous ERα expression in ERα-negative breast carcinomas by forced overexpression of TFAP2C have not been successful. We hypothesize that epigenetic chromatin structure alters the activity of TFAP2C at the ERα promoter.

Experimental Design—DNA methylation, histone acetylation, and chromatin accessibility were examined at the ERα promoter in a panel of breast carcinoma cell lines. TFAP2C and polymerase II binding were analyzed by chromatin immunoprecipitation. Epigenetic chromatin structure was altered using drug treatment with 5-aza-2′-deoxycytidine (AZA) and trichostatin A (TSA).

Results—The ERα promoter in the ERα-negative lines MDA-MB-231, MCF10A, and MCF7-5C show CpG island methylation, histone 3 lysine 9 deacetylation, and decreased chromatin accessibility compared with ERα-positive cell lines MCF7 and T47-D. Treatment with AZA/TSA increased chromatin accessibility at the ERα promoter and allowed TFAP2C to induce ERα expression in ERα-negative cells. Chromatin immunoprecipitation analysis showed that binding of TFAP2C to the ERα promoter is blocked in ERα- negative cells but that treatment with AZA/TSA enabled TFAP2C and polymerase II binding.

Conclusion—We conclude that the activity of TFAP2C at specific target genes depends upon epigenetic chromatin structure. Furthermore, the combination of increasing chromatin accessibility and inducing TFAP2C provides a more robust activation of the ERα gene in ERα-negative breast cancer cells.

Breast cancer is currently the most common cancer and the second most common cause of cancer deaths among women in the United States (1). Predicting the biological response to hormonal treatment and chemotherapy depends upon the overexpression of certain markers, including estrogen receptor-α (ERα) and ErbB2/HER2/neu (2,3). Approximately 75% of breast cancers overexpress ERα, and overexpression of the receptor predicts an improved prognosis and response to hormonal therapy compared with cancers with low or absent ERα expression (4,5). Given the effectiveness and low complications associated with hormonal therapy, the

Requests for reprints: Ronald J. Weigel, 200 Hawkins Drive, 1509 JCP, University of Iowa, Iowa City, IA 52242. Phone: 319-353-7474; Fax: 319-356-8378; Ronald-Weigel@uiowa.edu.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

possibility of inducing hormone response in ERα-negative cancer remains an attractive treatment strategy.

Recent work on the regulation of ERα expression in breast cancer suggests that both epigenetic chromatin structure and *trans*-active factors play a role in the regulation of receptor expression. Differences in expression of the receptor comparing ERα-positive and ERα-negative cell lines can be explained by differences in transcription of the ERα gene (6). Transcription also seems to be the mechanism of control in approximately one third of ERα-negative primary breast cancers, and tumors with low or absent ERα mRNA have a worse tumor grade (7). Methylation of the CpG island in the ERα promoter has been found in certain ERα-negative breast carcinoma cell lines and tumors (8,9). Treatment of the ERα-negative breast cancer cell line MDA-MB-231 with 5-aza-2′-deoxycytidine (AZA) was reported to reactivate ERα expression, and a synergistic induction of ERα expression was possible with the combination of AZA and trichostatin A (TSA; refs. 10,11). However, other groups examining the MDA-MB-231 cell line failed to confirm that ERα expression was induced with AZA or TSA treatment (12,13). Another line of investigation has focused on the activity of certain transcription factors involved in regulating ERα expression in breast cancer. The forkhead transcription factors FoxM1 and FOXO3a have been implicated in the regulation of the ERα promoter (14,15). TFAP2C has been shown to activate expression of the ERα promoter, and binding of AP-2 factors induces a prominent hypersensitive site in the chromatin near the transcriptional start site (16,17). More recently, it has been reported that elimination of TFAP2C in a variety of ERα-positive cell lines significantly reduces the expression of ERα and eliminates functional hormone response (18).

Translational Relevance

Hormonal therapy offers an effective and well-tolerated treatment for breast cancer, but its efficacy is restricted to a set of breast cancers that express estrogen receptor-α (ERα). The possibility of inducing ERα expression in ERα-negative cancer cells has been an attractive potential for using hormonal therapy in hormone unresponsive breast cancer. However, the complexity of ERα gene regulation has hindered the clinical application of this approach. Herein, we show that binding of TFAP2C to the AP-2 regulatory region of the ERα promoter is blocked in ERα-negative cells by epigenetic chromatin structure. Drugs that increase chromatin accessibility enable TFAP2C to bind to the ERα promoter and induce ERα expression. The combination of altering chromatin structure and overexpression of TFAP2C provides a more robust activation of the ERα gene in ERα-negative breast cancer cells and may have clinical utility in altering the hormone-responsive phenotype of cancer cells.

Finding evidence for the regulation by both epigenetic structure and *trans*-active factors raises an interesting question as to how these two mechanisms might interact in the control of ERα expression. At the present time, there has not been a consistent and reproducible means for inducing ERα expression in ERα-negative breast carcinomas. We hypothesize that epigenetic chromatin structure can influence the functional activity of TFAP2C at the ERα promoter. To address this possibility, we have examined how the combination of epigenetic structure of the ERα promoter and forced overexpression of TFAP2C participate in control of ERα promoter activity.

Materials and Methods

Cell lines

Cell lines were obtained from American Type Culture Collection and maintained as previously described (19). The MCF7-5C cell line was a generous gift from Dr. Craig Jordan (20). AZA and TSA (Sigma Aldrich) were used at the final concentrations of 2.5 μmol/L and 100 ng/mL, respectively (11). For experiments with drug treatment, cells were treated with AZA for 4 to 5 d and with TSA for 12 to 24 h before analysis. The adenoviral vector expressing TFAP2C was previously described (17).

Analysis of CpG island methylation

CpG DNA methylation was determined by cloning and sequencing multiple clones recovered from bisulfite-treated DNA using the MethylSEQr reagent kit (Applied Biosystems), which was used according to the manufacturer's instructions.

Chromatin immunoprecipitation analysis

Chromatin immunoprecipitation (ChIP) experiments were carried out as previously described with minor modifications (18,21,22). Twenty million cells were cross-linked for 10 min at 37°C using 1% formaldehyde. Cells were washed twice with PBS, pelleted by centrifugation, and resuspended in 500 μL sonication buffer [50 mmol/L Tris-Cl (pH 8.1), 10 mmol/L EDTA, and 1% SDS + protease inhibitors] and sonicated using conditions determined empirically for each cell line to achieve an optimal fragment length between 1,000 to 300 bp. After sonication, samples were centrifuged at 20,000 × *g* for 10 min at 4°C. The supernatant containing cross-linked DNA/histones was diluted 1:10 using IP dilution buffer [0.01% SDS, 1.1% Triton-X 100, 1.2 mmol/L EDTA, 16.7 mmol/L Tris-Cl (pH 8.1), 167 mmol/L NaCl] plus protease inhibitors. Samples were precleared using 75 μL of protein A agarose (Upstate Biotech) for 1 h at 4°C. For histone H3 analysis, about one tenth of the sample was removed as input control, whereas the remainder was split into two reactions for immunoprecipitation with acetylated histone 3 lysine 9 (H3K9; Upstate Biotech) or control nonspecific IgG (Sigma). For TFAP2C analysis, anti-TFAP2C antibody SC-12762× (Santa Cruz Biotechnology) was used; for RNA polymerase II (Pol II), anti–Pol II antibody CTD4H6 (Upstate/Millipore) was used. Antibodies were allowed to recognize their antigens overnight at 4°C with agitation. Protein/antibody/DNA complexes were collected using 70 μL protein A agarose (Upstate Biotech) followed by washing and elution according to the manufacturer's suggested protocol. Protein/DNA cross-links were reversed using 200 mmol/L NaCl at 65°C for 4 h. DNA was recovered from input ChIP reactions using the Qiagen DNeasy kit (Qiagen) according to manufacturer's suggested protocol. Purified DNA was quantified by using a NanoDrop ND-1000 (NanoDrop). Samples were analyzed by SYBR green quantitative real-time PCR with primers designed to *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*; GAPDHf 5-CGTCCTTGACTCCCTAGTGTCCT-3, GAPDHr 5-CCTACTTTCTCCCCGCTTTTTT-3) and *ERα* (ERαPf 5-TAA-CCTCGGGCTGTGCTCTT-3, ERαPr 5-TTCCCTTGGATCTGAT-GCAGTAG-3). For PCR reactions analyzed by gel electrophoresis, PCR amplification of the ERα gene promoter was done with the primer pair: ER CHIP 2 5′ (5′-gctgtgctctttttccaggt-3′), ER CHIP 2 3′ (5′-ttgctgctgtccaggtacac-3′). For quantitative ChIP, amplification of target amplicons was monitored as a function of increased SYBR green fluorescence. An analysis threshold was set, and the cycle threshold (Ct) was computed for each sample. Fold enrichment of target sequence was calculated using the following formula (fold enrichment = $2^{[(\text{Ct Ip'ed}) - (\text{Ct Input})]}$.

Chromatin accessibility

The accessibility of *cis*-regulatory elements within GAPDH and ERα were carried out as previously described with minor changes (23). Two million cells were washed using PBS, collected by centrifugation, and resuspended, and nuclei were collected using RBS buffer [10 mmol/L Tris (pH 7.5), 10 mmol/L NaCl, 3 mmol/L $MgCl_2$, and 0.05% NP40]. Nuclei preparations were collected by centrifugation and washed with the digestion buffer [50 mmol/L Tris (pH 7.5), 100 mmol/L NaCl, 10 mmol/L $MgCl_2$, and 1 mmol/L DTT] to remove any residual RBS buffer. Isolated nuclei were resuspended into 100 μL of digestion buffer. Half of the nuclei were left untreated to serve as uncut control nuclei, whereas the remaining nuclei were treated with 20 units of RQ1 DNase (Promega) and digested at 37°C for 5 min. After digestion was completed, the reactions were stopped by adding 15 mAU proteinase K and digesting reactions at 65°C for 15 min. DNA was purified using Qiagen DNeasy blood and tissue kit (Qiagen) and quantified by spectrophotometry. The accessibility indices of *cis*-regulatory regions were determined using quantitative real-time PCR, in conjunction with the same primer sets used for quantitative ChIP experiments. Triplicate reactions were setup (5 pmol of each primer, 10 ng of DNA, and 2× SYBR Green PCR master mix) and run using a ABI 7000 sequence detection system (Applied Biosystems) using the following conditions: 10-min denaturation at 95°C, followed by 40 cycles of 94°C for 30 s and 60°C for 30 s. Amplification of both target amplicons was monitored as a function of increased SYBR green fluorescence. The accessibility index for each amplicon was determined by the following formula: $AI = 2^{[(Ct\ Dnase\ treated) - (Ct\ Uncut)]}$).

Reverse transcription-PCR

Real-time quantitative reverse transcription-PCR (RT-PCR) was done by isolating RNA from the using the RNeasy Mini protocol (Qiagen). cDNA was synthesized using SuperScript III first-strand synthesis for RT-PCR (Invitrogen), Assay-on-Demand Gene Expression FAM/MGB Probe, nonprimer limited probes for human ERα (Hs01046-818_m1) or human β-actin endogenous control (4333762F), and TaqMan Universal PCR Master Mix. No AmpErase UNG (Applied Biosystems) were used to perform quantitative PCR using ABI Prism Model 7700 Sequence Detector. In each qPCR assay, samples were run in triplicate, along with three no-template controls and a standard curve. Data were analyzed using ABI Prism 7700 Sequence Detection System software (Applied Biosystems).

Statistical analysis

Statistical analysis of ChIP and chromatin accessibility was determined using a one-way ANOVA analysis with Tukey's multiple comparison posttest. All statistical analysis was done using GraphPad Prism Version 4.0 (Graph-Pad Software, Inc.).

Results

The pattern of expression for TFAP2A, TFAP2C, and ERα

Previous studies established that TFAP2C controls hormone response in breast carcinoma cells through the regulation of multiple pathways of estrogen signaling. One mechanism of estrogen response involves the ability of TFAP2C to activate ERα expression by binding to AP-2 response elements in the ERα promoter. We hypothesized that overexpression of TFAP2C might be able to activate ERα expression in ERα-negative breast carcinoma cell lines. A number of breast carcinoma cell lines were examined for their pattern of expression of TFAP2A, TFAP2C, and ERα. As seen in Fig. 1, the ERα-negative breast carcinoma cell lines MCF7-5C and MDA-MB-231 do not express significant amounts of TFAP2C protein compared with the ERα-positive cell lines MCF7 and T47-D. Of particular interest was the pattern of AP-2 expression in the MCF7-5C cell line, which was derived from the MCF7

parental line by long-term culture in the absence of estrogen (20). The pattern of AP-2 expression in MCF7-5C was reevaluated compared with the MCF10A cell line, which is a commonly used model for normal mammary epithelial cells. The data confirm that hormone-independent MCF7-5C cells have lost TFAP2C expression in concert with their loss of ERα expression compared with the parental MCF7 cell line. Interestingly MCF7-5C was also found to overexpress TFAP2A compared with MCF7, whereas MDA-MB-231 was noted to lack expression of either TFAP2A or TFAP2C. The findings in the ERα-negative cell lines suggested the possibility that overexpression of TFAP2C might induce expression of ERα in MCF7-5C and MDA-MB-231. However, forced overexpression of TFAP2C delivered by adenoviral vector failed to induce expression of ERα in these ERα-negative cell lines (see below and additional data not shown).

Epigenetic marks of the ERα promoter in ERα-negative cell lines

The inability for TFAP2C to induce ERα expression in ERα-negative cells suggested the possibility that epigenetic changes in the chromatin structure of the ERα gene might play a role in blocking the transcriptional effects of TFAP2C. First, we examined the DNA methylation pattern of the CpG island in the proximal region of the ERα gene promoter. The promoter region analyzed encompassed 28 potential CpG methylation sites and included CpGs incorporated into the AP-2 binding sites, which are required for TFAP2C-mediated activation of the ERα promoter. As seen in Fig. 2, the CpG island in the ERα-positive cell lines MCF7 and T47-D was completely un-methylated. By comparison the region in MCF7-5C cells was found to have complete DNA methylation except for one site in each of two clones. Various degrees of CpG methylation was found in MCF10A and MDA-MB-231 with all clones from these two cell lines showing some CpG site methylation, save for one clone derived from MDA-MB-231. Hence, it seemed plausible that DNA methylation might be one factor inhibiting TFAP2C from inducing ERα expression in ERα-negative cell lines.

Another chromatin mark associated with differences in transcriptional activity is histone acetylation. We examined H3K9 acetylation in the proximal ERα gene promoter using quantitative ChIP analysis. As shown in Fig. 3, there were significant differences in Ac-H3K9 marks comparing ERα-positive and ERα-negative cell lines. Chromatin from MCF7 and T47-D showed Ac-H3K9 marks, whereas the ERα-negative cell lines MCF10A, MDA-MB-231, and MCF7-5C had no evidence for acetylated histones in the ERα promoter. As a positive control, all cell lines showed Ac-H3K9 marks in the GAPDH promoter region, and there were no significant differences comparing the ERα-positive and ERα-negative cell lines.

The finding that the chromatin at the ERα CpG island in ERα-negative cells was more highly DNA methylated and associated with deacetylated histones suggests that the ERα promoter might not be accessible to factors such as TFAP2C. To address this possibility, we compared the ERα promoter region in the different breast cancer cell lines for DNA accessibility. As seen in Fig. 4A, the relative DNA accessibility of the ERα promoter region was significantly lower in the ERα-negative cell lines compared with the ERα-positive lines. However, DNA accessibility for the GAPDH gene was equivalent for all the cell lines examined. AZA can be used to inhibit CpG methylation, and TSA is a his-tone deacetylase inhibitor that can induce histone acetylation.

We examined whether treatment with AZA and TSA could increase the DNA accessibility at the ERα promoter in the ERα-negative cell lines. As seen in Fig. 4B, treatment with AZA and TSA successfully increased the DNA accessibility at the ERα promoter in all the ERα-negative cell lines. Interestingly, there was a trend for AZA/TSA treatment to decrease the accessibility index at the GAPDH locus, which did not reach statistical significance.

AZA/TSA alters ERα inducibility

Based on the finding that treatment with AZA and TSA increased DNA accessibility, we examined whether treatment with these drugs altered the ability for TFAP2C to activate the ERα promoter. In the absence of AZA/TSA, TFAP2C had a modest effect on ERα expression in MDA-MB-231 cells (see Fig. 5A). Drug treatment with AZA alone induced a moderate increase in ERα expression of ~10-fold and the combined treatment with AZA/TSA increased expression of ERα by ~15-fold. These results are consistent with other published works showing that combined treatment with AZA/TSA had a synergistic effect on ERα induction (11). Similar results were obtained with TFAP2C overexpression in the presence of each drug individually. However, in the presence of both AZA/TSA, TFAP2C induced an 80-fold increase in ERα expression in MDA-MB-231 cells. The level of ERα expression achieved by the combination of AZA/TSA and TFAP2C overexpression was ~1% to 5% the level of ERα mRNA in MCF7 cells. Because the level of ERα expression was still relatively low compared with MCF7, the expression of the ERα-responsive gene RERG was also examined (Fig. 5B). The expression pattern for RERG mirrored the increase in ERα expression. TFAP2C alone had no significant effect on RERG expression compared with untreated MDA-MB-231 cells. Overexpression of TFAP2C in the presence of either TSA or AZA alone had a modest effect, whereas overexpression of TFAP2C in the presence of both AZA/TSA resulted in a significant induction of RERG expression. These data indicate that induction of ERα in this system has functional effects on the expression of hormone responsive genes. The effect of TSA and AZA treatment on TFAP2C induction was similarly examined in MCF7-5C cells (Fig. 5C). In the absence of drug, TFAP2C had no significant effect on ERα expression and AZA/TSA alone had a modest effect. However, in the presence of AZA/TSA, overexpression of TFAP2C induced a 16-fold increase in ERα expression.

The ability for AZA/TSA to increase chromatin accessibility and to allow functional activity of TFAP2C at the ERα promoter suggested that drug treatment induced alterations of chromatin structure, allowing TFAP2C to bind to the AP-2 regulatory region of the ERα promoter. We formally investigated this possibility by examining TFAP2C binding to the ERα promoter by ChIP analysis. Consistent with earlier results, binding of endogenous TFAP2C to the ERα promoter can be detected in ERα-positive cells, but not ERα-negative cells (see Fig. 6A). Because MCF7-5C do not express endogenous TFAP2C (see Fig. 1), ChIP analysis with anti-TFAP2C failed to enrich for the ERα promoter and treatment with AZA/TSA did not alter this finding. Similarly, in the absence of AZA/TSA, ChIP analysis failed to detect an interaction between TFAP2C and the ERα promoter even with overexpression of TFAP2C. However, ChIP analysis in cells with overexpression of TFAP2C in the presence of AZA/TSA treatment showed binding of TFAP2C to the AP-2 regulatory region of the ERα promoter. Quantitative ChIP analysis was used to estimate the relative fold enrichment of pull-down of the ERα promoter with anti-TFAP2C antibody (Fig. 6B). Compared with MCF7-5C with overexpression of TFAP2C, AZA/TSA induced a 6-fold increase in binding of TFAP2C detected by ChIP analysis. For comparison, ChIP analysis in MCF7 using endogenous TFAP2C showed a 35-fold increased enrichment. The induction of ERα expression with binding of TFAP2C was associated with a 25-fold increase in loading of RNA Pol II, as determined by quantitative ChIP analysis. We conclude that the chromatin in the region of the ERα locus in ERα-negative cells is inaccessible to TFAP2C binding but that treatment with AZA/TSA increased chromatin accessibility allowing TFAP2C to bind to the AP-2 regulatory region resulting in TFAP2C-mediated loading of Pol II and induction of the ERα promoter. These findings establish a functional relationship between epigenetic chromatin marks and the activity of *trans*-active factors at the ERα gene promoter.

Discussion

Recent work has shown that epigenetic chromatin alterations play a critical role in the regulation of gene expression particularly in processes related to development, differentiation, and oncogenesis (24–28). Methylation of CpG islands near the transcriptional start sites of genes is associated with gene silencing (24). Various histone modifications, including acetylation and methylation, are associated with differential transcriptional states and have lead to the concept of a "histone code," which establishes heritable alterations of chromatin structure with functional effects on gene expression (25,29). Particular attention has been given to methylation and acetylation of histone H3. Acetylation and methylation of lysine 4 (H3K4ac and H3K4me) and acetylation of lysine 9 (H3K9ac) tends to be associated with activation, whereas methylation of lysines 9 and 27 (H3K9me and H3K27me) tend to be repressive (25, 26,29). In addition, there can be associations between CpG island methylation and specific histone marks. For example, functional differences have been described between dimethylated and tri-methylated lysine 4 of H3 (H3K4me2+ versus H3K4me3+), and patterns of H3K4 methylation have been found in ES cells to be restricted to genes with CpG islands (27).

Mechanisms through which chromatin modifications alter patterns of gene expression are currently an intensive area of investigation, and one important question is how gene specificity is established. The ability of sequence specific DNA binding factors to interact with enzymes capable of altering chromatin structure is clearly one mechanism that allows for gene-specific epigenetic chromatin modifications (30). A model system that we have extensively studied is the role of TFAP2C in the regulation of genes involved in hormone response including the ERα gene. Herein, we have showed that the chromatin of the ERα gene promoter is inaccessible to TFAP2C in ERα-negative breast cell lines due at least in part to alterations in epigenetic structure, which includes both CpG island methylation and histone H3 deacetylation. Furthermore, treatment of ERα-negative cells with AZA and TSA increased chromatin accessibility and allowed TFAP2C to bind to the regulatory region of the ERα promoter resulting in induction of ERα mRNA expression. Thus, a strategy that induces chromatin accessibility and overexpression of TFAP2C may be a more robust approach for altering the phenotype of ERα-negative breast carcinomas.

One potential limitation of the finding is the apparent low level of ERα induction compared with MCF7 cells. However, it is not known if the level of induction (1-5% of MCF7 ERα mRNA levels) represents relatively low level of expression in all cells or if this represents high ERα expression in a small fraction of the cells. The experiments were conducted over a several-day period, and it is possible that the epigenetic alterations required to allow ERα activation may not have had time to occur in the majority of cells. On the other hand, the levels of expression of ERα were sufficient to allow for induction of hormone-responsive genes showing functional effects of ERα expression. If the effects are limited to a subset of cells, it may be possible to delineate mechanisms that render some cells resistant to induction and thereby expand the effects to all cells in a tumor. Alternatively, the relatively low level of ERα expression may indicate that additional *trans*-active factors are necessary for full activation.

AP2 family members typically bind to G + C-rich *cis*-elements, and their binding *in vitro* is inhibited by cytosine methylation within their cognate elements (31–33). Our current findings extend those results by showing that TFAP2C DNA binding *in vivo* is inhibited in association with DNA methylation and histone deacetylation of its binding site in the ERα promoter and that TFAP2C DNA binding *in vivo* can be activated by pretreating breast carcinoma cells with the epigenetic modifying drugs AZA and TSA. These findings indicate that TFAP2C, and likely other AP-2 family members, requires an unmethylated, open, and accessible chromatin structure for optimal DNA binding and transactivation *in vivo*. Our results also indicate that

the functional consequence of TFAP2C interaction with a promoter, such as ERα is to increase Pol II loading and subsequent activation of the basal transcriptional complex.

One particularly interesting finding resulted from an examination of the hormone-independent MCF7-5C cell line, which was derived from the parental hormone-responsive MCF7 cell line after a long-term culture under conditions of estrogen deprivation (20). The MCF7-5C cells have lost ERα expression, and we have identified extensive epigenetic chromatin modifications in the AP-2 regulatory region of the ERα promoter, including a nearly complete methylation in the CpG island and significant increases in the presence of deacetylated histone H3. One intriguing finding is that MCF7-5C cells have also lost expression of TFAP2C and show overexpression of TFAP2A compared with MCF7 parental cells. Our previous work identified a role for TFPA2C in regulating expression of a set of genes involved in control of hormone response (18). If epigenetic alterations of certain TFAP2C target genes (such as ERα) were to occur first, then TFAP2C would be functionally inhibited and one might expect there to be no further selective pressure to alter TFAP2C expression. However, the loss of TFAP2C expression implies that inhibition of TFAP2C may have preceded the epigenetic chromatin changes and may be functionally linked to the development of the epigenetic marks in the ERα promoter. We are examining additional TFAP2C target genes to determine if the findings presented for the ERα gene can be found in other AP-2 regulatory regions. The data presented suggest that TFAP2C may be involved in maintaining an epigenetic structure associated with active transcription of AP-2 regulated genes.

The alteration in the pattern of TFAP2C expression has implications for breast cancer progression. TFAP2C is known to control a set of genes associated with the hormone responsive breast cancer phenotype. Finding that the expression of TFAP2C is lost with progression from a hormone responsive to a hormone unresponsive phenotype in a breast cancer cell line model raises the possibility that regulation of TFAP2C expression may be a mechanism related to breast cancer progression and development of hormone independence. Recognizing the limitation of cell line models, an examination of primary cancer specimens will need to be done to determine if loss of TFAP2C is a common mechanism involved in progression to hormone independence. A recent study examined TFAP2C expression in a panel of breast cancer cell lines using immunohistochemistry (34). TFAP2C was found to be an independent predictor of poor survival and a decreased duration of response to antihormonal therapy. Interestingly, in ERα-positive patients, the association between TFAP2C expression and time to progression was lost. These findings support a role for TFAP2C in the regulation of hormone response in primary breast cancers. However, in light of our findings, attempting to make a direct correlation between TFAP2C expression levels and function of TFAP2C does not take into account that epigenetic modification can alter the pattern of functional activity of AP-2 factors. Additional studies will need to be done to understand the role of TFAP2C in ERα-negative breast carcinomas.

Acknowledgments

Grant support: NIH grants R01CA109294 (R.J. Weigel) and R01CA73612 (F.E. Domann) and the Kristen Olewine Milke Breast Cancer Research Fund. M.J. Hitchler received partial salary support from T32CA078586.

References

1. Jemal A, Siegel R, Ward E, Murray T, Xu J, Thun MJ. Cancer statistics, 2007. CA Cancer J Clin 2007;57:43–66. [PubMed: 17237035]
2. Smith IE, Dowsett M. Aromatase inhibitors in breast cancer. N Engl J Med 2003;348:2431–42. [PubMed: 12802030]
3. Hudis CA. Trastuzumab-mechanism of action and use in clinical practice. N Engl J Med 2007;357:39–51. [PubMed: 17611206]

4. Fisher ER, Redmond CK, Liu H, Rockette H, Fisher B. Correlation of estrogen receptor and pathologic characteristics of invasive breast cancer. Cancer 1980;45:349–53. [PubMed: 7351016]
5. Jordan C, Wolf MF, Mirecki DM, Whiford DA, Welshons WV. Hormone receptor assays: clinical usefulness in the management of carcinoma of the breast. CRC Critical Reviews in Clin Lab Sci 1988:97–152.
6. Weigel RJ, deConinck EC. Transcriptional control of estrogen receptor in estrogen receptor-negative breast carcinoma. Cancer Res 1993;53:3472–4. [PubMed: 8339250]
7. Carmeci C, deConinck EC, Lawton T, Bloch DA, Weigel RJ. Analysis of estrogen receptor messenger RNA in breast carcinomas from archival specimens is predictive of tumor biology. Am J Pathol 1997;150:1563–70. [PubMed: 9137083]
8. Ottaviano YL, Issa JP, Parl FF, Smith HS, Baylin SB, Davidson NE. Methylation of the estrogen receptor gene CpG island marks loss of estrogen receptor expression in human breast cancer cells. Cancer Res 1994;54:2552–5. [PubMed: 8168078]
9. Lapidus RG, Ferguson AT, Ottaviano YL, et al. Methylation of estrogen and progesterone receptor gene 5′ CpG islands correlates with lack of estrogen and progesterone receptor gene expression in breast tumors. Clin Cancer Res 1996;2:805–10. [PubMed: 9816234]
10. Ferguson AT, Lapidus RG, Baylin SB, Davidson NE. Demethylation of the estrogen receptor gene in estrogen receptor-negative breast cancer cells can reactivate estrogen receptor gene expression. Cancer Res 1995;55:2279–83. [PubMed: 7538900]
11. Yang X, Phillips DL, Ferguson AT, Nelson WG, Herman JG, Davidson NE. Synergistic activation of functional estrogen receptor (ER)-α by DNA methyltransferase and histone deacetylase inhibition in human ER-α-negative breast cancer cells. Cancer Res 2001;61:7025–9. [PubMed: 11585728]
12. Jang ER, Lim SJ, Lee ES, et al. The histone deacetylase inhibitor trichostatin A sensitizes estrogen receptor α-negative breast cancer cells to tamoxifen. Oncogene 2004;23:1724–36. [PubMed: 14676837]
13. Alao JP, Lam EW, Ali S, et al. Histone deacetylase inhibitor trichostatin A represses estrogen receptor α-dependent transcription and promotes proteasomal degradation of cyclin D1 in human breast carcinoma cell lines. Clin Cancer Res 2004;10:8094–104. [PubMed: 15585645]
14. Guo S, Sonenshein GE. Forkhead box transcription factor FOXO3a regulates estrogen receptor α expression and is repressed by the Her-2/neu/phosphatidylinositol 3-kinase/Akt signaling pathway. Mol Cell Biol 2004;24:8681–90. [PubMed: 15367686]
15. Madureira PA, Varshochi R, Constantinidou D, et al. The Forkhead box M1 protein regulates the transcription of the estrogen receptor α in breast cancer cells. J Biol Chem 2006;281:25167–76. [PubMed: 16809346]
16. McPherson LA, Baichwal VR, Weigel RJ. Identification of ERF-1 as a member of the AP2 transcription factor family. Proc Natl Acad Sci U S A 1997;94:4342–7. [PubMed: 9113991]
17. Schuur ER, McPherson LA, Yang GP, Weigel RJ. Genomic structure of the promoters of the human estrogen receptor-α gene show changes in chromatin structure induced by ap2γ. J Biol Chem 2001;276:15519–26. [PubMed: 11278455]
18. Woodfield GW, Horan AD, Chen Y, Weigel RJ. TFAP2C controls hormone response in breast cancer cells through multiple pathways of estrogen signaling. Cancer Res 2007;67:8439–43. [PubMed: 17875680]
19. Ghosh MG, Thompson DA, Weigel RJ. PDZK1 and GREB1 are estrogen-regulated genes expressed in hormone-responsive breast cancer. Cancer Res 2000;60:6367–75. [PubMed: 11103799]
20. Jiang SY, Wolf DM, Yingling JM, Chang C, Jordan VC. An estrogen receptor positive MCF-7 clone that is resistant to antiestrogens and estradiol. Mol Cell Endocrinol 1992;90:77–86. [PubMed: 1301400]
21. McPherson LA, Loktev AV, Weigel RJ. Tumor Suppressor Activity of AP2α Mediated through a Direct Interaction with p53. J Biol Chem 2002;277:45028–33. [PubMed: 12226108]
22. Hitchler MJ, Wikainapakul K, Yu L, Powers K, Attatippaholkun W, Domann FE. Epigenetic regulation of manganese superoxide dismutase expression in human breast cancer cells. Epigenetics 2006;1:163–71. [PubMed: 17965603]
23. Rose SL, Fitzgerald MP, White NO, et al. Epigenetic regulation of maspin expression in human ovarian carcinoma cells. Gynecol Oncol 2006;102:319–24. [PubMed: 16457875]

24. Jones PA, Baylin SB. The epigenomics of cancer. Cell 2007;128:683–92. [PubMed: 17320506]
25. Santos-Rosa H, Caldas C. Chromatin modifier enzymes, the histone code and cancer. Eur J Cancer 2005;41:2381–402. [PubMed: 16226460]
26. Bernstein BE, Mikkelsen TS, Xie X, et al. A bivalent chromatin structure marks key developmental genes in embryonic stem cells. Cell 2006;125:315–26. [PubMed: 16630819]
27. Orford K, Kharchenko P, Lai W, et al. Differential H3K4 methylation identifies developmentally poised hematopoietic genes. Dev Cell 2008;14:798–809. [PubMed: 18477461]
28. Pasini D, Hansen KH, Christensen J, Agger K, Cloos PA, Helin K. Coordinated regulation of transcriptional repression by the RBP2 H3K4 demethylase and polycomb-repressive complex 2. Genes Dev 2008;22:1345–55. [PubMed: 18483221]
29. Nightingale KP, O'Neill LP, Turner BM. Histone modifications: signalling receptors and potential elements of a heritable epigenetic code. Curr Opin Genet 2006;16:125–36.
30. Gazin C, Wajapeyee N, Gobeil S, Virbasius CM, Green MR. An elaborate pathway required for Ras-mediated epigenetic silencing. Nature 2007;449:1073–7. [PubMed: 17960246]
31. Comb M, Goodman HM. CpG methylation inhibits proenkephalin gene expression and binding of the transcription factor AP-2. Nucleic Acids Res 1990;18:3975–82. [PubMed: 1695733]
32. Hermann R, Doerfler W. Interference with protein binding at AP2 sites by sequence-specific methylation in the late E2A promoter of adenovirus type 2 DNA. FEBS Lett 1991;281:191–5. [PubMed: 1826660]
33. Huang Y, Peng J, Oberley LW, Domann FE. Transcriptional inhibition of manganese superoxide dismutase (SOD2) gene expression by DNA methylation of the 5′ CpG island. Free Radic Biol Med 1997;23:314–20. [PubMed: 9199894]
34. Gee JM, Eloranta JJ, Ibbitt JC, et al. verexpression of TFAP2C in invasive breast cancer correlates with a poorer response to anti-hormone therapy and reduced patient survival. J Pathol 2009;217:32–41. [PubMed: 18825690]

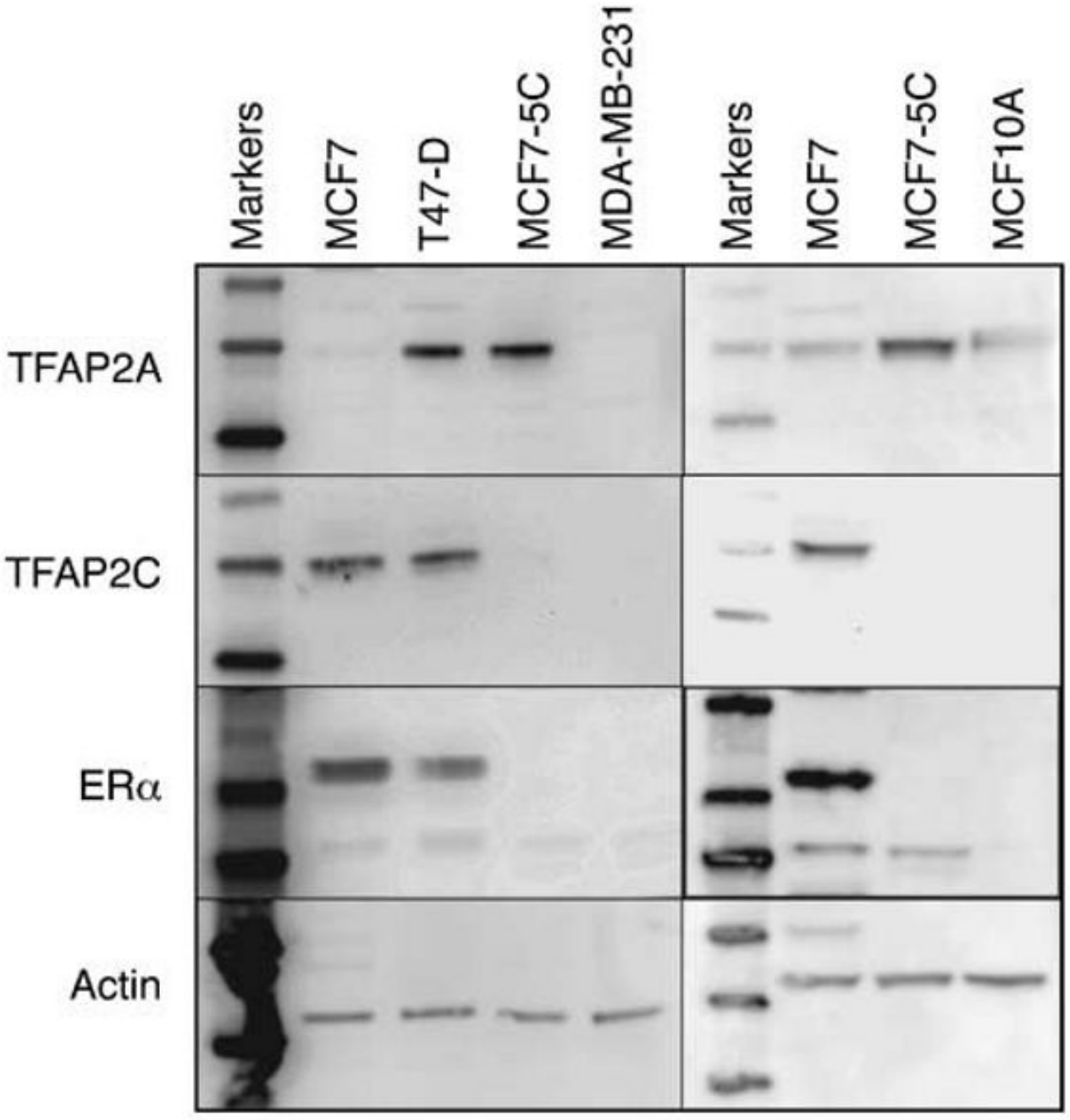

Fig. 1.
Expression of ERα and AP-2 factors in breast cancer cell lines. Western blots of proteins from breast cell lines as indicated shows expression pattern for TFAP2A, TFAP2C, ERα, and actin.

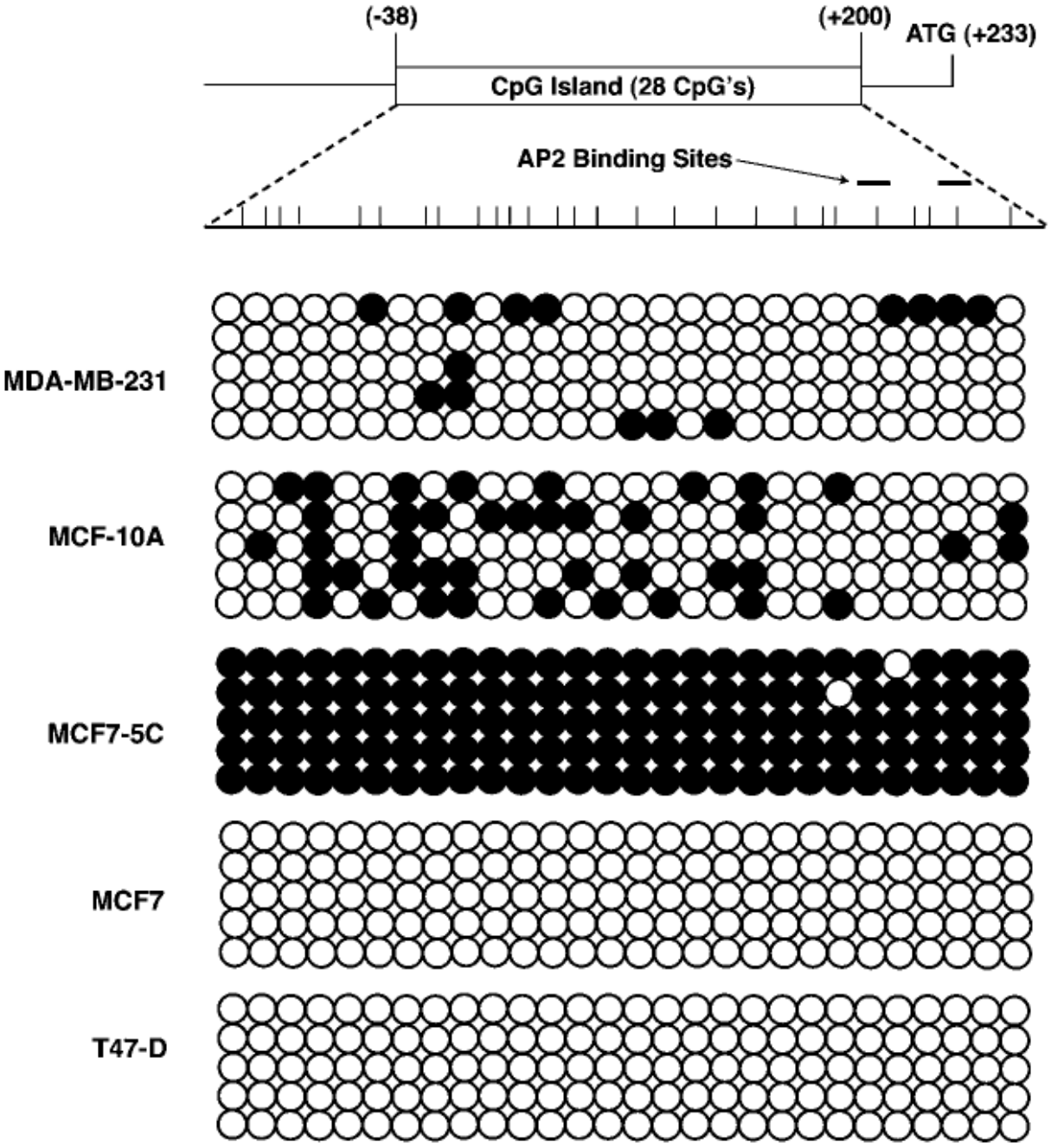

Fig. 2.

Methylation of CpG island of ERα promoter. The CpG island of the main ERα promoter (top) was analyzed for methylation by bisulfite sequencing. The locations of known AP-2 binding sites within the CpG island. Results of sequence from five separate clones derived from chromatin from the cell lines indicated. Twenty-eight CpGs with open circles for unmethylated base and closed circle for methylated base.

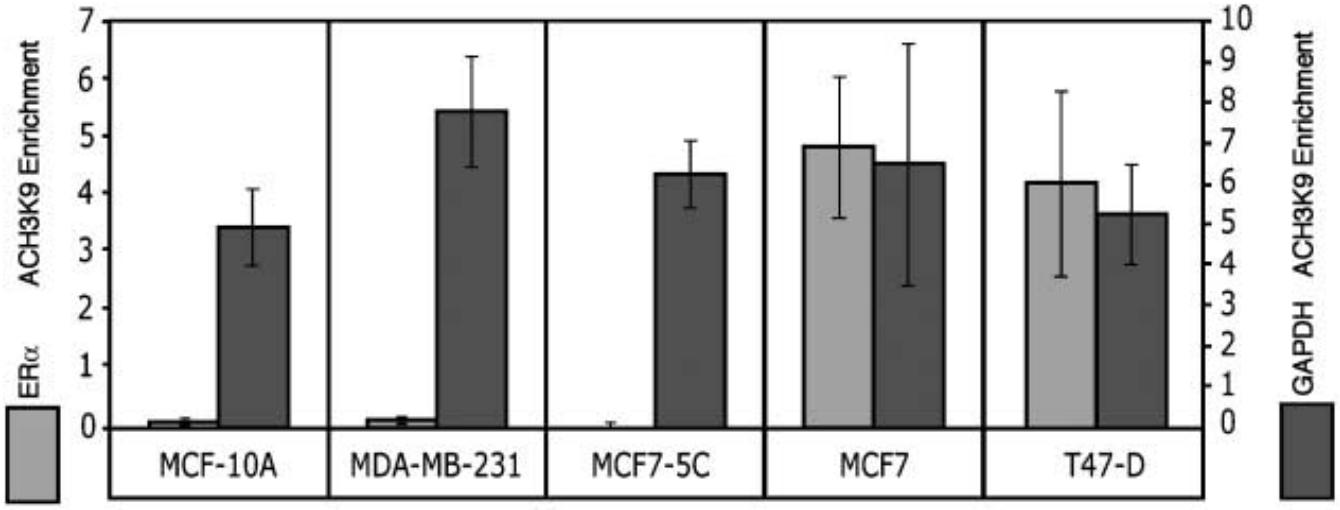

Fig. 3.

H3K9 Acetylation at the ERα promoter. ChIP analysis for H3K9ac for the ERα and GAPDH genes for the various cell lines as indicated. Numbers on *y* axis indicate relative fold AcH3K9 enrichment (see Materials and Methods). There were significant differences in H3K9ac of the ERα promoter comparing the ERα-negative cell lines (MCF-10A, MDA-MB-231, and MCF7-5C) to the ERα-positive cell lines (MCF7 and T47-D) with $P < 0.05$. Results for H3K9ac of ERα gene were not significantly different comparing the three ERα-negative cell lines to each other or comparing the two ERα-positive lines. There were no significant differences in H3K9ac of the GAPDH gene promoter for any of the cell lines. Results are for three separate experiments.

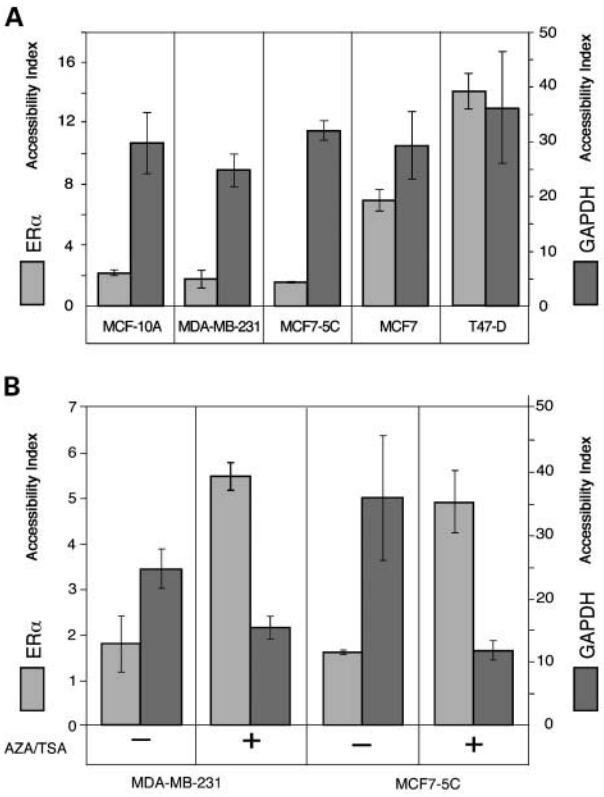

Fig. 4.

Chromatin accessibility of ERα gene promoter. *A*, chromatin accessibility was analyzed for the ERα and GAPDH gene promoters for the cell lines indicated. Numbers on *y* axis indicate accessibility index (see Materials and Methods). There was significantly greater chromatin accessibility at the ERα promoter comparing the ERα-positive cell lines to the ERα-negative lines ($P < 0.05$). No significant differences in chromatin accessibility for the GAPDH gene promoter were detected comparing the various cell lines. Results are for three separate experiments. *B*, chromatin accessibility for the ERα and GAPDH gene promoters for MDA-MB-231 and MCF7-5C cell lines either without (−) or with (+) treatment with AZA/TSA. Numbers on *y* axis indicate accessibility index. In both cell lines, AZA/TSA treatment significantly increased chromatin accessibility of the ERα gene promoter ($P < 0.05$) whereas no significant changes were observed for the GAPDH gene, although there was a trend for a relative decrease in accessibility index with AZA/TSA. Results are for three separate experiments.

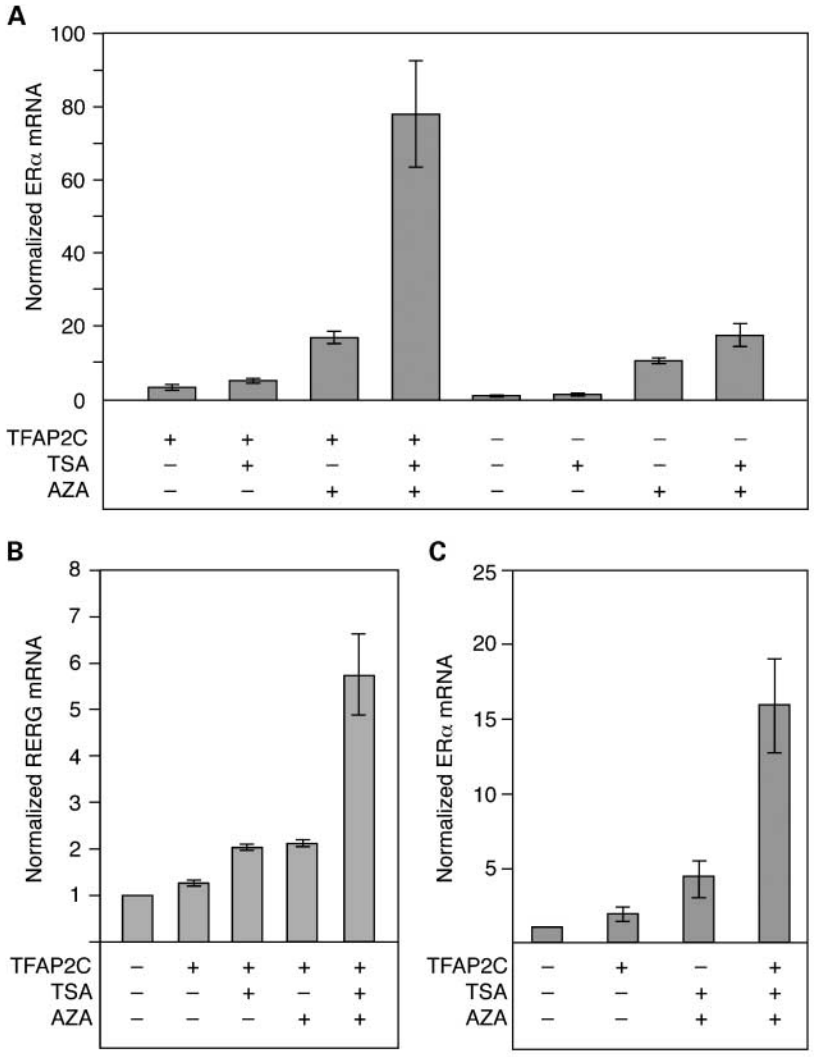

Fig. 5.

TFAP2C induces ERα mRNA in the presence of AZA/TSA. *A*, MDA-MB-231 cells were analyzed for ERα mRNA by quantitative RT-PCR. Overexpression of TFAP2C was induced by infection at multiplicity of infection of 100 with Ad-TFAP2C (+) or mock infected (−). Cells were either untreated with drugs (−) or exposed to AZA (+) or TSA (+) or both drugs as indicated. Results of normalized ERα mRNA are shown as determined by quantitative RT-PCR. Differences between ERα expression with TFAP2C overexpression only (lane 8) compared with TFAP2C expression in the presence of AZA/TSA (lane 4) were significant ($P < 0.005$). The relative expression of ERα induced in MDA-MB-231 is ~1% to 5% of ERα mRNA in MCF7 cells. *B*, MDA-MB-231 cells with or without overexpression of TFAP2C and treated with TSA, AZA, or both drugs as indicated were analyzed for expression of the ERα-responsive gene RERG. Results of normalized RERG mRNA are shown as determined by quantitative RT-PCR. Differences between RERG expression comparing TFAP2C and TSA/AZA with no drugs or TSA or AZA alone was significant ($P < 0.01$). *C*, MCF7-5C cells were analyzed for ERα mRNA after overexpression of TFAP2C was induced by infection at multiplicity of infection of 100 with Ad-TFAP2C (+) or mock infected (−). Cells were either untreated with drugs (−) or exposed to a combination of AZA/TSA (+) as indicated. Expression

of ERα was significantly different comparing overexpression of TFAP2C without drugs (lane 2) compared with overexpression of TFAP2C in the presence of AZA/TSA (lane 4); $P < 0.02$.

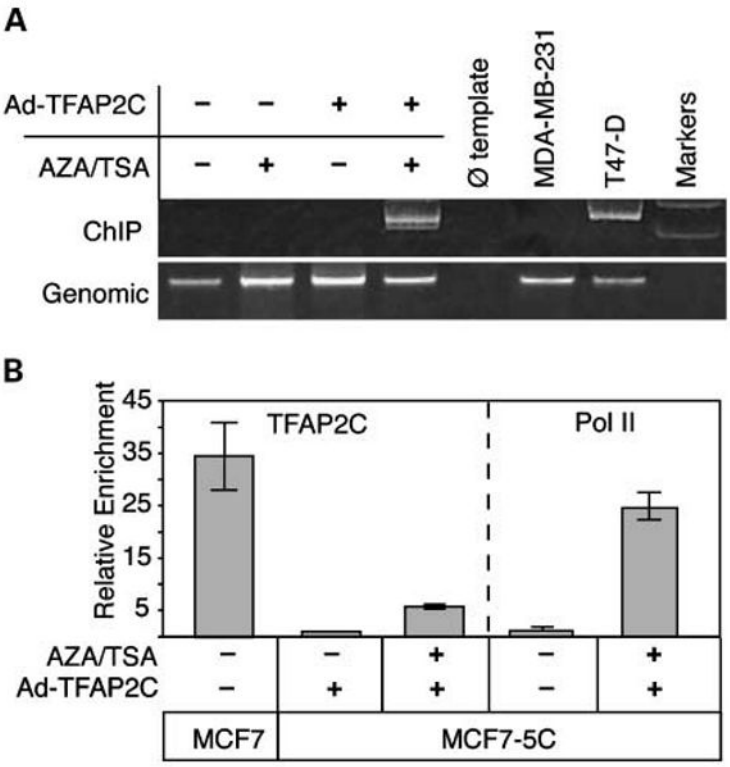

Fig. 6.

ChIP analysis of TFAP2C and RNA Pol II interaction with ERα promoter. *A*, MCF7-5C cells were mock infected (−) or infected with Ad-TFAP2C (+) in either the absence of drugs (−) or with treatment with the combination of AZA and TSA (+). Analysis was done in parallel with MDA-MB-231 cells as negative control, T47-D as a positive control, or in the absence of template as indicated. Amplification of input genomic DNA before immunoprecipitation (bottom). *B*, quantitative ChIP analysis done with TFAP2C or Pol II antibody for relative fold enrichment of ERα promoter in MCF7-5C cells after overexpression of TFAP2C without or with drug treatment with AZA and TSA. Comparison with ChIP done in MCF7 cells is shown as a positive control. Data are normalized to enrichment in MCF7-5C cells with overexpression of TFAP2C. Difference of fold enrichment comparing MCF7-5C overexpressing TFAP2C with AZA/TSA is 6-fold greater than without drug treatment ($P < 0.005$). Binding of Pol II by quantitative ChIP shows a 25-fold increase with overexpression of TFAP2C in presence of AZA/TSA compared with untreated MCF7-5C cells ($P < 0.0001$).