Abstract

Breast cancers expressing estrogen receptor-α (ERα) are associated with a favorable biology and are more likely to respond to hormonal therapy. In addition to ERα, other pathways of estrogen response have been identified including ERβ and GPR30, a membrane receptor for estrogen, and the key mechanisms regulating expression of ERs and hormone response remain controversial. Herein, we show that TFAP2C is the key regulator of hormone responsiveness in breast carcinoma cells through the control of multiple pathways of estrogen signaling. TFAP2C regulates the expression of ERα directly by binding to the ERα promoter and indirectly via regulation of FoxM1. In so doing, TFAP2C controls the expression of ERα target genes, including pS2, MYB, and RERG. Furthermore, TFAP2C controlled the expression of GPR30. In distinct contrast, TFAP2A, a related factor expressed in breast cancer, was not involved in estrogen-mediated pathways but regulated expression of genes controlling cell cycle arrest and apoptosis including p21(CDPI) and IGFBP-3. Knockdown of TFAP2C abrogated the mitogenic response to estrogen exposure and decreased hormone-responsive tumor growth of breast cancer xenografts. We conclude that TFAP2C is a central control gene of hormone response and is a novel therapeutic target in the design of new drug treatments for breast cancer. [Cancer Res 2007;67(18):8439–43]

Introduction

The prediction of whether a breast cancer will respond to hormonal therapy is largely dependent on the expression of estrogen receptor-α (ERα; ref. 1). Whereas normal mammary epithelial cells express minimal amounts of the receptor (4 fmol/mg protein), ERα-positive breast cancers commonly overexpress the receptor by one to three orders of magnitude (2). In addition to ERα, other signaling pathways for estrogen have been identified in breast carcinoma cells including ERβ (3) and GPR30 (4, 5), a membrane receptor for estrogen. Controversy exists about the contribution of each estrogen signaling pathway to hormone response and mechanisms controlling expression of ERα and other signaling pathways are yet to be defined. Previous studies identified the TFAP2 family as one set of factors involved in human ERα promoter expression in breast cancer cells (6, 7). The TFAP2A and TFAP2C proteins are commonly expressed in breast cancer and overexpression of these factors can up-regulate the cloned ERα promoter (7). Furthermore, overexpression of TFAP2A and TFAP2C can induce a hypersensitive site at the AP2 regulatory region of the ERα promoter in human mammary epithelial cells (8). However, several critical questions remain to be addressed. First, it has not been shown whether endogenous TFAP2 factors regulate expression of ERα in breast cancer cells. Second, it remains a question as to whether TFAP2 factors are key regulators of ERα expression and whether the activity of TFAP2 on ERα expression is sufficient to have functional effects on hormone responsiveness. Finally, it is important to determine whether the various TFAP2 family members differ in their activity with respect to ERα regulation. To address these questions, we manipulated endogenous TFAP2 expression in breast carcinoma cell lines and examined the effects on ERα expression, alternate gene expression profiles, and physiologic responses to estrogen treatment.

Materials and Methods

Cell lines and culture. All cells were obtained from American Type Culture Collection. Hormone manipulation was done as described previously (9) with 10 nmol/L β-estradiol and 10 μmol/L 4-OH-tamoxifen (Sigma-Aldrich).

Small interfering RNA transfections. Small interfering RNAs (siRNA) for TFAP2A and TFAP2C were obtained from Dharmacon and used according to the manufacturer. SMARTpools for TFAP2A and TFAP2C and single siRNA (TFAP2C1, D-005238-01-0200; TFAP2A5, D-006348-03) were used with comparable results as the SMARTpools.

Gene expression array analysis. Total RNA was isolated using the RNeasy Mini kit (Qiagen) and subjected to analysis on the Human Genome U133 Plus 2.0 Arrays (Affymetrix) in conjunction with the DNA Core Facility at the University of Iowa following the GeneChip Expression Analysis Technical Manual. ArrayAssist software (Stratagene) implementing the robust microarray analysis algorithm was used to generate gene expression values.

Quantitative and semiquantitative reverse transcription-PCR. Gene expression array results were validated using quantitative real-time PCR using Taqman gene expression assays, supplied by ABI (Applied Biosystems). Semiquantitative reverse transcription-PCR (RT-PCR) for ERα and pS2 was done as described previously (9).

Western blots. Western blots were done using the following antibodies: TFAP2C 6E4/4, TFAP2A 3B5, actin 1-19, ERα HC-20, and pS2 FL-84 (Santa Cruz Biototechnology) and TFAP2C 6E4/4 05-409, TFAP2A 8G8/5 05-820, and ERα 60C 05-634 (Upstate/Chemicon).

Cell cycle analysis. Twelve hours after siRNA transfection, cells were transferred into phenol-free DMEM (Life Technologies, Invitrogen) medium plus 10% charcoal-stripped fetal bovine serum (Gemini Bio-Products), with the addition of 5 μmol/L bovine insulin (Sigma-Aldrich) for 48 h, after which the cells were treated with medium alone or medium containing β-estradiol or β-estradiol and 4-OH-tamoxifen. After 12 h, cells were harvested, stained with 0.05 mg/mL propidium iodide (Sigma-Aldrich), 0.1% Triton X-100, and 10 mg/mL sodium citrate, and subjected to fluorescence-activated cell sorting analysis.

Chromatin immunoprecipitation. Chromatin immunoprecipitation (ChIP) analysis was done as described previously (10, 11).

Tumor xenografts. Female BALB/c nude mice, 4 to 6 weeks old (Charles River), were implanted with a 1.7-mg estrogen pellet (Innovative Research)
and $5 \times 10^6$ MCF7 cells transfected with TFAP2C, TFAP2A, or TFAP2C scrambled siRNA were injected into the mammary fat pad. For visible tumors, surface area was estimated by measuring with calipers and reported as $3.14 \times \text{length} / 2 \times \text{width} / 2$. For microscopic tumors, volume was estimated from histologic sections assuming ellipsoid formula ($\text{length} \times \text{width} \times \text{depth} \times 0.5233$). Immunohistochemical stains for ERα were done in the University of Iowa Pathology Core.

**Results and Discussion**

TFAP2C regulates expression of ERα. To test the hypothesis that endogenous TFAP2 factors are involved in the normal regulation of ERα expression, ERα-positive MCF7 cells were transfected with siRNA directed against TFAP2C or TFAP2A and mRNA was measured by RT-PCR. By 96 h after transfection, elimination of TFAP2C reduced the level of ERα mRNA to 17% the level found in mock-transfected cells (Fig. 1A). By comparison, ERα expression in cells transfected with a siRNA directed against TFAP2A was not significantly different from mock-transfected cells. Western blot analysis of ERα and TFAP2C protein showed a dose-dependent effect of TFAP2C siRNA transfection with the loss of ERα expression paralleling the effect on TFAP2C expression (Fig. 1B). Western blot analysis confirmed that the expression of ERα protein was reduced to 16% after transfection with TFAP2C siRNA compared with mock-transfected cells (Fig. 1B).

Because MCF7 express relatively low levels of TFAP2A (Fig. 1D), the lack of an effect of TFAP2A knockdown on ERα expression might have been due to low endogenous levels of TFAP2A in this cell line. We examined two other ERα-positive cell lines, T47-D and ZR75-1, which expressed higher levels of TFAP2A. As shown in Fig. 1D, knockdown of TFAP2C expression in T47-D and ZR75-1 resulted in a consistent reduction in ERα expression that was comparable with the reduction seen in MCF7 cells. By contrast to TFAP2C, knockdown of TFAP2A resulted in a slight increase in ERα expression. These findings showed that positive regulation of ERα expression is specific for TFAP2C, which had not been evident in systems using overexpression of AP2 factors (7).

TFAP2C targets the ERα promoter and induces genes in the ERα pathway. We propose that TFAP2C regulates expression of ERα by directly targeting the ERα promoter at the AP2-regulatory region (8). ChIP was used to investigate the interaction between TFAP2C and the ERα promoter. As seen in Fig. 2A, ChIP analysis was done with anti-TFAP2C antibody, which pulled down the regulatory Figure 1. TFAP2C regulates ERα expression in breast cancer cell lines. A, quantitative RT-PCR was used to examine mRNA expression for ERα, TFAP2C, and TFAP2A in MCF7 cells (average of three transfections) after mock transfection or transfection with siRNA directed against TFAP2C or TFAP2A as indicated. Transfection of a TFAP2C scrambled siRNA showed results that were identical to mock transfection (see Supplementary Data). For expression of ERα, mock transfection was significantly different from transfection with TFAP2C siRNA at 96 h (mock versus TFAP2C, 1 versus 0.17; $P < 0.002$; see Supplementary Data for table of all data). B, Western blots show a dose-response for TFAP2C and ERα expression with increasing amount (nanomole per liter) of single TFAP2C siRNA in MCF7 cells. Histogram shows normalized protein for TFAP2C and ERα from dose-response data above. C, Western blot analysis of TFAP2C and ERα protein was done in MCF7 cells mock transfected or transfected with TFAP2C siRNA. Histogram shows relative amount of ERα protein in mock- and TFAP2C siRNA-treated cells in average of three transfections. D, Western blots are shown for TFAP2A, TFAP2C, ERα, and actin protein in MCF7, T47-D, or ZR75-1 cells after mock transfection or after transfection with siRNA specific for TFAP2A or TFAP2C (see Supplementary Data for relative amount of TFAP2 mRNA expression and for antibody specificity).
region of the human ERα promoter containing the AP2-binding site in the ERα-positive cell lines MCF7, ZR75-1, and T47-D cells but not in ERα-negative cell lines HCT116, HBL-100, and MDA-MB-231. These results are consistent with a model in which TFAP2C directly targets the AP2 sites in the ERα promoter and is consistent with earlier studies that identified an interaction between TFAP2C and the chromatin of the ERα promoter in the AP2 regulatory region (8).

A critical question is whether the loss of ERα caused by TFAP2C knockdown is significant enough to have functional consequences for ERα-regulated genes, such as pS2 (12). As shown in Fig. 2B, the TFAP2C siRNA significantly reduced the level of mRNA for TFAP2C and ERα. When pS2 mRNA was analyzed in parallel, we found that knockdown of TFAP2C significantly reduced the expression of pS2 mRNA. We estimate that the expression of pS2 mRNA was reduced to <5% of mock-transfected cells, which agrees with earlier studies showing that estrogen treatment resulted in a 20-fold induction of pS2 mRNA in MCF7 cells (9, 12). Western blot analysis confirmed that knockdown of TFAP2C expression eliminated pS2 protein expression (Fig. 2C). By comparison, MCF7 cells treated with TFAP2A siRNA retained expression of pS2, which showed the expected repression with 4-OH-tamoxifen.

TFAP2C regulates multiple pathways of estrogen signaling. We were interested in determining what other genes might be controlled by TFAP2C, either directly or indirectly, through regulation of ERα and potentially other signaling pathways. Furthermore, it was important to determine if TFAP2C controlled the expression of genes outside of the ERα pathway or if the effect of TFAP2C was limited to estrogen-responsive genes. We were also interested in determining what genes might be responsive to TFAP2A by comparison to knockdown of TFAP2C. To this end, we did an analysis of the changes in global patterns of gene expression in MCF7 cells with elimination of either TFAP2C or TFAP2A. The patterns of gene expression with alteration of TFAP2 activity were compared with changes in expression induced by estrogen exposure. A summary of the array data is presented in Fig. 3A, which was confirmed using quantitative RT-PCR for selected genes of interest as shown in Fig. 3B. In Fig. 3B, expression of the genes of interest was also examined with the combination of TFAP2C knockdown and estrogen elimination. Knockdown of TFAP2C altered the pattern of expression of known ERα-regulated genes, including MYB (13) and BERG (14), both of which were found to be appropriately regulated by estrogen exposure. Interestingly, TFAP2C controlled the expression of several genes outside the estrogen-regulated pathways; most prominent among these being GPR30, FoxM1, several of the cyclin family members, and RADS1. Of particular significance, the expression of GPR30 was originally identified in ERα-positive breast cancers (4) and was subsequently found to be a membrane receptor for estrogen (5, 15). Furthermore, FoxM1 has been shown recently to contribute to the regulation of ERα expression in breast cancer (16). In distinct contrast, TFAP2A controlled expression of genes, such as p21(cip1), which induces G1 and G2-S cell cycle arrest (17). Regulation of p21(cip1) by TFAP2 is consistent with previous reports (17) and these data indicate that control of this cyclin-dependent kinase inhibitor was most notable with knockdown of TFAP2A. TFAP2A was found to regulate expression of IGFBP-3, which is inversely associated with progression of breast (18) and prostate cancer (19). Furthermore, the elimination of estrogen induced expression of IGFBP-3 and the combination of estrogen elimination and TFAP2A knockdown resulted in an intermediate effect on expression. Another noteworthy finding is the identification of genes, such as PDZK1, which was regulated by estrogen but was not altered significantly by TFAP2C. PDZK1 was reported previously to be an estrogen-regulated gene; however, it was also shown not to be controlled directly by ERα (9).

The lack of an effect on PDZK1 by TFAP2C suggests that PDZK1 either can be regulated by relatively low levels of ERα or may be regulated by an alternate mechanism of estrogen responsiveness.

TFAP2C controls mitogenic response to estrogen and estrogen-induced tumor growth. The physiologic effect of estrogen exposure in hormone-responsive breast cancer is to induce mitosis and tumor growth. The finding that ERα and GPR30 are controlled by TFAP2C suggests that mitogenic response to estrogen would also be affected by TFAP2C knockdown. However, other genes induced by estrogen (e.g., PDZK1) seemed to be outside the regulation of TFAP2C and could indicate that elimination of TFAP2C will fail to alter the physiologic responses to β-estradiol. Hence, we examined cell cycle response to β-estradiol stimulation in MCF7 cells transfected with siRNA directed against either TFAP2C or TFAP2A. As seen in Fig. 4A, MCF7 cells transfected with siRNA specific for TFAP2A maintained the expected robust mitogenic response to β-estradiol treatment, which was appropriately inhibited by 4-OH-tamoxifen. By comparison, the mitogenic response to treatment with β-estradiol was dramatically blunted in MCF7 cells transfected with siRNA directed against TFAP2C. This result shows that knockdown of TFAP2C in MCF7 cells significantly reduced the ability of the cells to enter S phase in response to estrogen exposure.

The role of TFAP2C in hormone-responsive tumor growth was examined using a xenograft model in nude mice. Tumor growth of...
MCF7 cells transfected with TFAP2C siRNA was significantly delayed compared with cells transfected with TFAP2A siRNA (Fig. 4B). However, by day 56, differences in tumor size was less significant between the groups and there was noted to be central necrosis in the tumors derived from TFAP2A siRNA-transfected cells that was not found in the TFAP2C siRNA-transfected cells (Fig. 4D). At day 56, all tumors were found to be ERα positive by immunohistochemistry (Fig. 4D). The results suggest that loss of TFAP2C delayed early tumor engraftment with subsequent outgrowth of ERα-positive cells. To assess early tumor engraftment, mice were euthanized 2 weeks after injection and tumor growth was assessed by serial sections of the injection sites before significant central necrosis was observed (Fig. 4D). As seen in Fig. 4C, there were significant differences in tumor size comparing MCF7 cells transfected with TFAP2C siRNA versus TFAP2C scrambled siRNA (P < 0.02). No significant differences were observed comparing cells transfected with scrambled siRNA versus TFAP2A siRNA (P = 0.42).

**Conclusion.** Herein, we show that TFAP2C controls estrogen responsiveness in MCF7 breast carcinoma cells through the regulation of multiple pathways regulating hormone response (see Supplementary Data for schematic of pathways). In distinct contrast, TFAP2A had no effects on estrogen-induced S phase and tumor growth. Interestingly, recent clinical studies of hormonal treatment of breast cancer patients have shown that aromatase inhibitors are superior to tamoxifen in objective response, time to progression, and incidence of contralateral breast cancer (20). The biological basis for increased effectiveness of aromatase inhibitors is thought to be due to suppression of local estrogen production or a potential partial agonist effect of tamoxifen. However, the superior clinical effectiveness of aromatase inhibitors might also be explained by alternate mechanisms of estrogen signaling that are outside the ERα pathway and might not be responsive to the effects of tamoxifen. The results presented herein show that TFAP2C mediates estrogenic responses through several pathways including ERα and GPR30. Hence, TFAP2C may provide a novel therapeutic target for the development of more effective hormonal therapy.

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**Figure 3.** Pattern of gene expression controlled by TFAP2C and TFAP2A compared with estrogen response. A, Venn diagram summarizes number of genes altered (by > factor 2) by β-estradiol (Mock E2 versus Mock No E2), elimination of TFAP2C (siRNA TFAP2C E2 versus Mock E2), or elimination of TFAP2A (siRNA TFAP2A E2 versus Mock E2). Complete array data are available at Gene Expression Omnibus at the National Center for Biotechnology Information under accession no. GSE8640. B, genes of particular interest are displayed in which quantitative RT-PCR was used to confirm changes in gene expression by elimination of β-estradiol, TFAP2C, or TFAP2A for GPR30, FoxM1, MYB, RERG, p21, IGFBP-3, and PDZK1. All data are normalized to mock siRNA transfection in the presence of estrogen. Effect of elimination of both TFAP2 expression and estrogen is shown and shows intermediate effects of each manipulation alone.
Figure 4. TFAP2C controls mitogenic response to estrogen and hormone-responsive tumor growth. A, hormonal effect on cell cycle was examined in MCF7 cells transfected with TFAP2A or TFAP2C siRNA and grown under conditions without estrogen (No E2), 10 nmol/L β-estradiol and 10 μmol/L 4-OH-tamoxifen (+E2/4-OH-T). S phase response to estrogen was not significantly different comparing transfection of TFAP2A siRNA and mock transfection (data not shown). B, growth of tumor xenografts was measured after injection of cells into the mammary fat pad of female nude mice (see Supplementary data for tumor image). Mice were first implanted with an estrogen pellet and MCF7 cells transfected with TFAP2A or TFAP2C siRNA were injected. Tumor size was estimated over time (days). C, early engraftment of tumor xenografts determined by viable tumor cell volume 2 wks after injection of MCF7 cells transfected with either scrambled (SC), TFAP2C, or TFAP2A siRNA. D, histologic sections of tumor xenografts at S2 d from cells transfected with TFAP2A (left and middle left) or TFAP2C (middle and middle right) examined by H&E stain (left and middle) or immunohistochemistry for ERα (middle left and middle right). Histologic section of representative tumor xenograft at 14 d after injection of MCF7 cells transfected with TFAP2A siRNA showing lack of central necrosis (right).

References

TFAP2C Controls Hormone Response in Breast Cancer Cells through Multiple Pathways of Estrogen Signaling
