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5-Aza-2-deoxycytidine and trichostatin A increase COUP-TFII expression in antiestrogen-resistant breast cancer cell lines

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Abstract

COUP-TFII is reduced in endocrine-resistant breast cancer cells and is negatively associated with tumor grade. Transient re-expression of COUP-TFII restores antiestrogen sensitivity in resistant LCC2 and LCC9 cells and repression of COUP-TFII results in antiestrogen-resistance in MCF-7 endocrine-sensitive cells. We addressed the hypothesis that reduced COUP-TFII expression in endocrine-resistant breast cancer cells results from epigenetic modification. The *NR2F2* gene encoding COUP-TFII includes seven CpG islands, including one in the 5' promoter and one in exon 1. Treatment of LCC2 and LCC9 endocrine-resistant breast cancer cells with 5-aza-2'-deoxycytidine (AZA), a DNA methyltransferase (DNMT) inhibitor, +/- trichostatin A (TSA), a histone deacetylase (HDAC) inhibitor, increased COUP-TFII suggesting that the decrease in COUP-TFII is mediated by epigenetic changes. Methylation-specific PCR (MSP) revealed higher methylation of *NR2F2* in the first exon in LCC2 and LCC9 cells compared to MCF-7 cells and AZA reduced this methylation. Translational importance is suggested by Cancer Methylome System (CMS) analysis revealing that breast tumors have increased COUP-TFII (*NR2F2*) promoter and gene methylation versus normal breast.

Keywords

Breast cancer; Endocrine-resistance; COUP-TFII; Epigenetic modification; Estrogen receptor

1. Introduction

The orphan nuclear receptor (NR) chicken ovalbumin upstream promoter transcription factor II (COUP-TFII) regulates many biological processes such as metabolic and developmental programs including cell proliferation, survival, migration, and differentiation [1,2]. COUP-TFII modulates the expression of its target genes by forming homodimers that bind to a direct repeat motif 5'-AGGTCA-3' [3,4]. COUP-TFII can bind directly to

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Conflict of Interest

The authors declare no conflict of interest.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.canlet.2014.02.001>.

estrogen receptor α (ER α) and inhibit estradiol (E₂)-activated ER α transcriptional activity in transfected cells [5]. The expression of COUP-TFII is lower in endocrine-resistant breast cancer cell lines [6] and in ER α -negative compared with ER α -positive breast tumors [7]. Recently, COUP-TFII was identified as one of five NRs whose expression separates breast tumors from normal breast and COUP-TFII expression negatively associated with tumor histological grade [8]. Further, COUP-TFII was reported to be an independent predictor of metastasis-free survival in tamoxifen (TAM)-treated breast cancer patients [8]. These findings identify COUP-TFII as a good predictor for breast cancer patients who will respond favorably to TAM [9].

These and other data indicate that COUP-TFII may play a role in controlling the proliferation of ER α -positive breast cancer cells and in maintaining the sensitivity of breast cancer cells to TAM and other antiestrogens [2,6] [10]. Given that 40–50% of patients treated with antiestrogens develop acquired endocrine-resistant breast cancer [11], exploring the mechanisms for the decrease in COUP-TFII expression could lead to the development of ways to maintain endocrine sensitivity in breast cancer.

Epigenetic modifications play a significant role in alteration of gene expression in breast cancer [12,13]. Among the epigenetic modifications critical in controlling the transcriptional machinery, histone acetylation, methylation, phosphorylation, ubiquitinylation, sumoylation, and ribosylation [14] and DNA methylation play key roles [15,16]. Methylation of DNA, usually at CpG island sites in gene promoters, suppresses transcription by interfering with the binding of transcription factors [17,18]. Aberrant DNA methylation plays an important role in carcinogenesis with global hypomethylation linked to activation of proto-oncogenes and chromosomal instability while gene-specific hypermethylation is associated with inactivation of genes regulating apoptosis, DNA repair, tumor suppression, and the regulation of cell replication [19].

Many studies indicate an association between DNA methylation and histone deacetylation in silencing genes, supporting the importance of using DNA methyltransferase (DNMT) inhibitors and histone deacetylase inhibitors together to re-express silenced genes [20,21]. 5-aza-2'-deoxycytidine (AZA), a cytidine analog incorporated into DNA during the S phase that disrupts the interaction between DNA and DNMT and promotes its proteosomal degradation [22], and trichostatin A (TSA), a Class I and II histone deacetylase (HDAC) inhibitor (HDACi) [23,24], alone or in combination, are widely used to increase the expression of genes silenced by DNA methylation and chromatin condensation [25]. In general, cells are treated with AZA for ~3 d and then TSA is added in the last 12–24 h of treatment. For example, treatment of MDA-MB-231 and MDA-MB-435 breast cancer cells with AZA (0.8 μ M for 3 d) and TSA (100 ng/ml), alone or in combination, increased retinoic acid receptor β (RAR β) expression resulting from partial demethylation of the CpG-rich RAR β 2 region in these cell lines [23]. Likewise, treatment of ER α -negative MDA-MB-231 and MDA-MB-435 breast cancer cells with AZA (2.5 μ M for 96 h) and TSA (100 ng/ml for the last 12 h), alone or in combination, increased ER α and progesterone receptor (PR) expression [26] and caused the cells to be growth inhibited by 4-hydroxytamoxifen (4-OHT) [27]. Optimization of ER α re-expression was observed in cells treated with AZA (2.5 μ M for 80 h) with TSA (100 ng/ml) and tamoxifen (2.5 μ M) which simultaneously reduced

protein levels of the RNA binding protein HuR [28]. More recently, treatment of a TAM-resistant derivative of MCF-7 breast cancer cells with AZA (2 μ M for 60 h) + TSA (100 ng/ml for 12 h) increased ER β expression and subsequent treatment with 4-OHT reduced cell viability [29]. There is also evidence from treatment of HEK-293 cells with 5 μ M 5-azacytidine for 4 or 8 d that DNA methylation is involved in both activation, *e.g.*, increased expression of C2H2 zinc finger transcription factors, and repression, *e.g.*, mitochondrion-related genes, of gene transcription [30].

Previously we reported that COUP-TFII is decreased in TAM/ endocrine-resistant breast cancer cell lines, *i.e.*, LCC2, LCC9, and LY2, derived from TAM-sensitive MCF-7 cells [6]. Others also observed reduced COUP-TFII expression in TAM-resistant (MCF7-T) and fulvestrant-resistant (MCF7-F) cell lines [31]. Further, the authors reported that MCF7-T cells have higher methylation density and the MCF7-F cells have lower methylation density in the promoter region of COUP-TFII when compared to MCF-7 cells [31]. These observations might explain the negative correlation detected between the expression of COUP-TFII and histological grade of breast cancer samples of patients treated with TAM [8]; however, no one has experimentally examined whether COUP-TFII can be re-expressed by blocking DNA methylation and/or histone deacetylation in TAM-resistant breast cancer cells. The goal of this study was to determine if treatment of TAM-resistant breast cancer cell lines LCC2 and LCC9 with 5-aza-2'-deoxycytidine (AZA) and trichostatin A (TSA), alone or in combination, increases the expression of COUP-TFII and restores endocrine sensitivity.

2. Materials and methods

2.1. Cell culture and treatments

MCF-7 cells were purchased from ATCC. LCC2 and LCC9 cells were kindly provided by Dr. Robert Clarke, Lombardi Cancer Center, Georgetown University [32,33]. Cells were maintained in IMEM supplemented with 5% fetal bovine serum (Atlanta Biologicals Lawrenceville, GA., USA) and 1% penicillin/streptomycin (Mediatech, Manassas, VA., USA). For RNA and whole cell lysate extractions, MCF-7 cells were plated in 6-well plates at 250,000 cells/well and LCC2 and LCC9 cells were plated at 200,000 cells/well and allowed to adhere overnight. Treatments included 5-aza-2'-deoxycytidine (AZA, 2.5 or 50 μ M), for 72 or 80 h; trichostatin A (TSA, 100 ng/ml) [28], and 4-hydroxytamoxifen (4-OHT, 100 nM, 1 or 2.5 μ M) (Sigma, S. Louis, MO., USA). Fresh AZA was added with a change of medium at the time of TSA addition, whether added or not, for Figs. 1–4, which is a limitation of this study since AZA is labile in aqueous media [34]. For Figs. 5–8, fresh medium and treatments were added. For all experiments, TSA was added only during the last 16 h of the 72 h treatment for many of the experiments and for the last 24 h of the 80 h treatment (Fig. 1).

2.2. RNA extraction and quantitative real-time-PCR (qPCR)

RNA was isolated from cells using RNeasy (Qiagen, Valencia, CA., USA) following manufacturer instructions. RNA quality and quantity were assessed using Nano-Drop (Thermo Scientific, Rockford, IL, USA). cDNA was synthesized from RNA using the High

Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA., USA). Quantitative real-time PCR (qPCR) for *ESR1*, *ESR2*, *BCL2*, *BAX*, and 18S was performed using Taqman primers and labeled probe sets specifically targeting these genes (Applied Biosystems). SYBR green was used to measure COUP-TFII (*NR2F2*) expression using COUP-TFII and *GAPDH* primers [35]. qPCR was performed in the ABI PRISM 7900 SDS 2.1 or ViiA7 (Applied Biosystems/Life Technologies). Fold change was calculated from the CT values with the formula 2^{-CT} and data are relative to DMSO (vehicle control)-treated cells (see Fig. 9).

2.3. Western Blotting

Nuclear extracts (NE) were harvested using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific) following manufacturer instructions. Bio-Rad DC protein assay kit was used to measure protein concentrations (Bio-Rad, Hercules, CA., USA). NE (30 µg protein) were resuspended in 1 × Laemmli loading buffer (Bio-Rad), separated by 10% SDS–PAGE, and transferred to a polyvinylidene difluoride membrane (PVDF, Bio-Rad). Proteins were probed using a monoclonal anti-COUP-TFII antibody (PP-H7147-00, R&D systems, Minneapolis, MN., USA) or polyclonal anti-ERα (HC-20, Santa Cruz Biotechnology, Santa Cruz, CA., USA) or anti-ERβ (H-150, Santa Cruz Biotechnology). Membranes were stripped and probed with antibody for β-actin (Sigma). Data were captured and analyzed by Carestream Image Station 4000 R Pro with Carestream Molecular Imaging Software, version 5.0, (Carestream Health, Inc., New Haven, CT, USA). The values from regions of interest (ROI) normalized to the loading control, *e.g.*, β-actin, and the normalized value of the control was set to 1 for comparison between separate experiments.

Whole cell extracts were prepared by lysing the cells directly in the wells of 12-well plates in 100 µl SDS sample buffer. The samples were boiled for 5 min. and 10 µl were loaded/lane in 15 well 4–20% gradient Tris–Glycine gels (Invitrogen). The proteins were transferred to PVDF membranes, blocked in 5% BSA-TBS-Tween, and probed with LC3A/B Rabbit mAb (cat # D3U4C from Cell Signaling Technology, Beverly, MA, USA). The membranes were stripped and re-probed for β-actin and then stripped again and re-probed for GAPDH (Santa Cruz Biotechnology). Data were captured and analyzed by Carestream Image Station 4000 R Pro with Carestream Molecular Imaging Software.

2.4. Methylation-specific PCR (MSP)

Genomic DNA was isolated from cells using the Sigma kit G1N 10 (Sigma) following the manufacturer's instructions. DNA quality and quantity were assessed using NanoDrop (Thermo Scientific). DNA was bisulfite treated using the EZ DNA Methylation-Lightning™ Kit (Zymo Research, Irvine, CA., USA) following manufacturer's instructions. Following bisulfite conversion, DNA was quantified using Nano-Drop. PCR primers were designed for first 2000 bp of *NR2F2* (NM_021005.3) using Zymo Bisulfite Primer Seeker 12S (<<http://www.zymoresearch.com/tools/bisulfite-primer-seeker>>). Primers are detailed in Supplementary Table 1 and were purchased from Integrated DNA Technologies Inc. (IDT, Coralville, IA, USA).

The PCR reaction contained 1× PCR buffer (included in the Platinum Taq DNA polymerase from Invitrogen), 1.5 mM MgCl₂, primers (0.2 μM each per reaction), 200 μM dNTPs, 1 U Platinum Taq DNA polymerase (Invitrogen), and bisulfite DNA (30 ng) or unmodified DNA (30 ng). Reactions were hot started at 95 °C. Amplification was carried out in an Applied Biosystems 2720 thermocycler for 38 cycles (45 s. at 94 °C, 30 s at the annealing temperature selected for each primer pair (Supplementary Table 1), 45 s. at 72 °C), followed by 5 min at 72 °C. Twelve μl of the PCR reaction were electrophoresed in 2% agarose gels, stained with ethidium bromide, and visualized under UV light on a Carestream Imager and analyzed using Care-stream Molecular Imaging software (New Haven, CT, USA).

2.5. MTT assay

The viability of MCF-7, LCC2 and LCC9 cells was evaluated by Cell Titer 96 Aqueous One Solution cell proliferation assay (Promega, Madison, WI., USA) according to the manufacturer's protocol and as described previously [36]. In brief, cells were seeded in 96-well plates in IMEM +5% FBS. Twenty-four hours later, cells were treated for 72 h with DMSO (vehicle), 1 or 2.5 μM 4-OHT, 1 μM ICI 182,780 (fulvestrant, Tocris, Ellisville, MO., USA), and 50 μM AZA, with 100 ng/ml TSA added for the last 16 h. Fresh treatments were added with a medium change every 48 h. Each treatment was performed in quadruplicate within each experiment. All values were compared to vehicle (DMSO) control within each cell line and between the same treatment in MCF-7 cells using Student *t*-test in Graph Pad Prism (GraphPad Software, La Jolla, CA., USA).

2.6. Immunofluorescence staining

MCF-7, LCC2, and LCC9 cells were grown on coverslips overnight and treated with 50 μM AZA for 72 h with 100 ng/ml TSA added for the last 16 h. Cells were fixed with cold methanol for 5 min and washed 3 times with cold PBS. After blocking with 10% BSA for 1 h, cells were incubated with primary monoclonal anti-human COUP-TFII (PP-H7147-00, R&D Systems), diluted 1:200, for 2 h. The cells were stained with secondary anti-mouse antibody labeled with Alexa Fluor® 568 (Invitrogen), diluted 1:1000. Cells were incubated with Hoechst (2,59 -Bi-1H-benz- imidazole, Invitrogen) for 10 min. Images were captured using a Zeiss Axiovert 25 Inverted microscope with 63X objective lens (Fig. 4) or 10X and 63X/2.5 objective (Supplemental Figs. 7 and 8) and AxioVision Release 4.3 software. Mean pixel intensity was quantified using NIH Image J software.

3. Results

3.1. Treatment of endocrine-resistant LCC2 and LCC9 cells with inhibitors of DNMT and HDAC increases COUP-TFII expression

The expression of COUP-TFII is lower in TAM-resistant LCC2 and LCC9 breast cancer cells than in the parental TAM-sensitive MCF-7 breast cancer cells [6]. These two cell lines provide a model of increasing endocrine resistance since LCC9 cells are resistant to TAM and fulvestrant, whereas LCC2 are resistant to TAM and sensitive to fulvestrant [37]. To investigate if the reduced expression of COUP-TFII in TAM-resistant breast cancer cell lines was caused by gene methylation, cells were treated with an inhibitor of DNMT, 2.5 μM or 50 μM AZA for 72 h. Treatment of MCF-7 cells with AZA had a minimal, although

statistically significant, inhibitory effect on COUP-TFII expression (Fig. 1). Treatment of LCC2 and LCC9 cells with AZA increased COUP-TFII expression. The increase in COUP-TFII stimulated by AZA was significantly higher in LCC2 *versus* LCC9. Prolonged treatment with AZA for 96 h did not result in a further significant increase in COUP-TFII expression (Supplemental Fig. 1). Cells were treated with AZA for 72 h for subsequent experiments.

In previous studies in ER α -negative MDA-MB-231 breast cancer cells, re-expression of ER α (*ESR1*) required treatment of the cells with AZA plus the histone deacetylase (HDAC) inhibitor TSA [28]. Treatment of MCF-7 cells with TSA for 16 h had no significant effect on COUP-TFII expression (Fig. 1). Treatment of LCC2 or LCC9 cells with TSA alone for 16 h increased COUP-TFII expression (Fig. 1). Addition of TSA for the last 16 h of the 72 h incubation with 2.5 or 50 μ M AZA resulted in no further significant increase in COUP-TFII expression in LCC2 or LCC9 cells compared with TSA alone. Despite the increase in COUP-TFII mRNA expression with AZA + TSA treatment of LCC2 and LCC9, COUP-TFII levels were <2% of MCF-7 basal expression (Supplemental Fig. 2).

3.2. AZA + TSA inhibits ER α (*ESR1*) expression in breast cancer cells

We observed no significant difference in ER α protein expression between MCF-7, LCC2, and LCC9 cells [6]. Basal levels of ER α (*ESR1*), ER β (*ESR2*), and *GAPDH* are similar in these 3 cell lines (CT values shown in Supplemental Fig. 3). The expression of *ESR1* was unaffected by 2.5 μ M AZA treatment in MCF-7 or LCC2 cells (Fig. 2A). 50 μ M AZA reduced *ESR1* in LCC2 cells, but not MCF-7 cells. Both 2.5 and 50 μ M AZA reduced *ESR1* transcript expression in LCC9 cells. TSA reduced *ESR1* transcript levels in all 3 cell lines. The combination of AZA + TSA further reduced *ESR1* transcript levels in LCC2 and LCC9 cells.

3.3. AZA + TSA increases ER β (*ESR2*) transcript expression levels in breast cancer cells

ER β expression is reduced in high tumor grades [38]. AZA was shown to increase ER β mRNA expression in ER β -negative SkBr3 and MDA-MB-435 breast cancer cells [39] as well as in breast cancer cells with low endogenous ER β expression, *e.g.*, BT20, HBL100, and HMT3522 [40], and MDA-MB-231 [41]. TSA alone increased ER β mRNA and protein and stimulated nuclear translocation of ER β in MDA-MB-231 cells [41]. AZA alone (2.5 μ M) did not increase *ESR2* transcript levels in MCF-7, LCC2, or LCC9, but 50 μ M AZA significantly increased *ESR2* in MCF-7 and LCC2 cells (Fig. 2B). TSA increased *ESR2* in MCF-7 cells, but not LCC2 or LCC9. The combination of AZA + TSA increased *ESR2* transcript levels in all 3 cell lines.

3.4. AZA + TSA increased COUP-TFII protein in LCC9 cells

In agreement with changes in mRNA transcript expression, AZA + TSA increased COUP-TFII protein expression in LCC9 cells (Fig. 3A). Also in agreement with reduced *ESR1* mRNA transcript levels, AZA + TSA decreased ER α protein in MCF-7, LCC2, and LCC9 cells (Fig. 3B). In agreement with the increase in *ESR2* mRNA transcript levels, a ~20 and 25% increase in ER β was detected in LCC2 and LCC9 cells (Fig. 3C and Supplemental Fig.). In contrast, a ~20% decrease was detected in ER β protein with AZA + TSA treatment

of MCF-7 (Fig. 3C). Since fresh AZA was added only after 56 h and AZA is unstable in aqueous medium [34], the greater increase in COUP-TFII protein with 50 μ M AZA may result from a lack of active AZA in the medium. This is a limitation of this study.

3.5. Immunofluorescent imaging of COUP-TFII in MCF-7, LCC2, and LCC9 cells

We also examined the effect of 50 μ M AZA + 100 ng/ml TSA on the expression and cellular distribution of COUP-TFII in MCF-7, LCC2, and LCC9 cells (Fig. 4). For these studies, the cells were grown on coverslips and, due to the toxicity of AZA + TSA, most of the dead cells went into the medium. Hence, these results were obtained on ~30% of the initial population (Fig. 6). Endogenous COUP-TFII was nuclear in MCF-7 cells and COUP-TFII signal was increased ~16% with AZA + TSA treatment. For both LCC2 and LCC9 cells, there was lower COUP-TFII nuclear signal in the control-treated cells. COUP-TFII staining intensity was lower in LCC2 than LCC9, but increased COUP-TFII signal (~40%) was seen in the AZA + TSA-treated LCC2 cells and LCC9 cells (~26%), reflecting the increase in mRNA for COUP-TFII (Fig. 1) and COUP-TFII in LCC9 seen in the western blot (Fig. 3). AZA + TSA treatment appeared to result in decreased clumping of all three cell lines (Supplemental Fig. 5), with more cell projections and disrupted nuclei with apparent apoptotic features, *i.e.*, Hoechst-positive extra-nuclear staining and condensed and fragmented nuclei, in LCC2 and most notably in LCC9 cells (Supplemental Fig. 6). The decrease in cell clumping may reflect changes in cell surface protein expression commensurate with apoptosis [42].

3.6. Global DNA methylation is not different in MCF-7, LCC2, and LCC9 cells

Supplemental Fig. 7 indicates no significant global difference in DNA methylation between MCF-7, LCC2, and LCC9 cell lines. We detected a significant decrease in DNA methylation in cells treated with AZA, but not AZA + TSA (Supplemental Fig. 7).

3.7. CpG island methylation in the NR2F2 gene

To examine potential CpG islands in the *NR2F2* gene for COUP-TFII, NCBI Reference Sequence NC_000015.9 for *NR2F2* from base 96867006 to 96885641 was submitted to the CpGPLOT program from the European Bioinformatics Institute website <http://www.ebi.ac.uk/emboss/cpgplot>. This program defines a CpG island as 200 bp with P50% C + G content and 0.6 CpG observed/CpG expected. The 18.64 kB region for *NR2F2* contained seven CpG islands (Supplemental Fig. 8A). NM_021005 for *NR2F2* was also submitted to the CpGPLOT program. The 5110 bp from *NR2F2* contained two CpG islands: 51-606 and 1027-2132 (Supplemental Fig. 8B). NM_021005.3 lists the translated, coding sequence (CDS) for *NR2F2* = 1225..2469, indicating that the first CpG island is in the promoter whereas the second CpG island is within exon 1. NM_021005.3 was also submitted to MethPrimer [43], a program for designing bisulfite-conversion-based Methylation PCR Primers for MSP, which also identified the same CpG islands (Supplemental Fig. 8C). In accordance with the regulation of gene transcription by DNA methylation [19], these data suggest that transcription of *NR2F2* may be regulated by DNA methylation.

3.8. NR2F2 gene methylation in MCF-7, LCC2, and LCC9 breast cancer cells

To examine if the COUP-TFII (*NR2F2*) gene was methylated and if this methylation was linked with COUP-TFII expression differences between MCF-7, LCC2, and LCC9 breast cancer cells, DNA was isolated from cells treated with vehicle control, AZA, or AZA + TSA. Following bisulfite treatment, which converts all Cs to Us except for those Cs that are methylated in CpG islands, methylation-specific PCR (MSP) was performed using primers to distinguish between methylated and unmethylated alleles after bisulfite treatment [44]. Non-bisulfite converted samples were not recognized by any of the *NR2F2* primer pairs tested (a negative control, data not shown). MSP indicates lower methylation of the promoter region of *NR2F2* in LCC2 compared to MCF-7 or LCC9 cells (Fig. 5A and B). This methylation is reduced by 50 μ M AZA. MSP indicates that 2.5 μ M AZA reduced methylation in the promoter of MCF-7 and LCC9 cells. An increase in methylation was seen with 50 μ M AZA and 2.5 μ M AZA + TSA in LCC9 cells. MSP indicates higher methylation in the first exon of *NR2F2* in LCC2 and LCC9 compared to MCF-7 cells (Fig. 5A and B). AZA decreased methylation in this region. These data suggest higher methylation within the first exon of *NR2F2* in LCC2 and LCC9 cells relative to MCF-7 cells, in agreement with lower COUP-TFII expression (Fig. 3 and Supplemental Fig. 4) and our previous reports [6,7].

3.9. Endocrine-resistant LCC2 and LCC9 cells are more sensitive to inhibition by AZA and TSA than endocrine-sensitive MCF-7 cells

MTT assays were performed to determine if AZA and TSA increased the sensitivity of the endocrine-resistant LCC2 and LCC9 cells to growth inhibition by 4-OHT or ICI 182,780 (fulvestrant). Results were compared to identical treatment of MCF-7 cells. Based on the previous results (Figs. 1 and 2), a concentration of 50 μ M AZA was selected for these experiments. Cells were treated with 4-OHT, ICI 182,780, and AZA + TSA alone or in combination (Fig. 6) for a total of 5 days. As expected, LCC2 and LCC9 showed less growth inhibition than MCF-7 cells in response to 4-OHT or ICI 182,780. Treatment of LCC2 and LCC9 with AZA + TSA reduced viability by ~70% and neither 4-OHT nor ICI 182,780 further inhibited cell viability. MCF-7 cells were less sensitive to inhibition by AZA + TSA, and AZA + TSA did not enhance the inhibition of MCF-7 cell viability by 4-OHT or ICI 182,780. The greater sensitivity of LCC2 and LCC9 to AZA + TSA agrees with the changes in cell appearance detected in these cells (Fig. 4).

3.10. BAX/BCL2 ratio with AZA + TSA treatment

To examine the possible contribution of apoptosis to the observed decrease in LCC2 and LCC9 cell viability with TSA or AZA + TSA treatment, we measured the expression of *BAX* (proapoptotic) and *BCL2* (anti-apoptotic) in MCF-7, LCC2, and LCC9 cells treated with vehicle, AZA, TSA, or AZA + TSA (Fig. 6). Basal *BCL2* expression was higher in the endocrine-resistant LCC2 and LCC9 cell lines compared to parental, endocrine-sensitive MCF-7 cells (Fig. 6A). *BAX* expression was also higher in LCC2 and LCC9 cells, but less elevated relative to MCF-7 cells. An increased *BAX/BCL2* is an indicator of apoptosis [45]. TSA increased the *BAX/BCL2* ratio in MCF-7 and LCC2, but not LCC9 cells. AZA + TSA did not increase the *BAX/BCL2* ratio in LCC2 cells and had a minor, but statistically

significant effect in LCC9 cells, suggesting that the decrease in cell viability with TSA or AZA + TSA in LCC2 and LCC9 cells (Fig. 6) may not be mediated by apoptosis.

3.11. TSA increases LC3, an autophagy marker, in breast cancer cells

LC3 (microtubule-associated protein 1 light chain 3) serves as the most widely monitored primary autophagy-related protein [46]. LC3 is processed to LC3-I and modified to PE-conjugated LC3II which is associated with both completed autophagosomes and phagophores [46]. Increases in LC3 and specifically an increase in LC3-II relative to LC3-I reflects autophagy, although there are numerous caveats, including the fact that LC3-II may be rapidly degraded [46]. To examine the possible contribution of autophagy to the observed decrease in MCF-7, LCC2, and LCC9 cell viability with TSA or AZA + TSA treatment, LC3 protein levels were measured by western blot (Fig. 7). 5 μ M 4-OHT served as a positive control for autophagy induction [47] and the expected increase in the LC3-II band with a reduction in the LC3-I band was detected in all three ER α + breast cancer cell lines, despite the resistance of LCC2 and LCC9 to treatment with 'physiological' levels of 4-OHT, *e.g.*, ~100 nM that would be found in the breast tissue of a patient on oral tamoxifen therapy [48]. We noted that the loss of LCC2 and LCC9 cells in the wells treated with 5 μ M 4-OHT was greater than for MCF-7 cells (observational data not shown). This observation is reflected in the reduction in both β -actin and GAPDH in those wells (Fig. 7A). We note that cytoskeletal β -actin is degraded by the auto-phagosome [47] and studies in the literature rarely quantitate LC3 relative to a loading control. Instead, some reports show data of LC3-I and LC3-II [49]; as plotted in Fig. 7B. Plots of LC3-I, LC3-II, and total LC3 relative to GAPDH are shown in Supplemental Fig. 9. Treatment of MCF-7 cells with AZA decreased LC3-I, possibly indicating increased autophagy. AZA reduced LC3-I and LC3-II, and thus total LC3, in the endocrine-resistant LCC9 cells. TSA increased total LC3 in MCF-7 and LCC2 cells, possibly reflecting increased autophagy.

3.12. NR2F2 methylation and expression in breast tumors and normal breast from breast cancer patients

To investigate whether COUP-TFII DNA methylation occurs in human breast tumors and to compare the level of DNA methylation in breast tumors *versus* normal breast, we used the Cancer Methy-lome System (CMS) [50] (Fig. 8). Data in the Cancer Methylome System were generated by MBDCap-seq technology, which couples immunoprecipitation of methylated DNA using the methyl-CpG binding domain of the MBD2 protein followed by high-throughput sequencing [51,52]. We observed that breast tumors have increased methylation in the 5' promoter of the COUP-TFII (*NR2F2*) gene as well as within the coding and intron sequences (Fig. 8). A statistically significant increase in DNA methylation upstream and within the COUP-TFII gene was observed in breast tumors *versus* normal tissue (Table 1). While the tumor grades for the cancer methylome data are unknown, these data indicate that methylation of the *NR2F2* gene region is increased upon oncogenic transformation. Further study is necessary to determine if this mechanism explains the negative correlation detected between COUP-TFII protein expression and tumor grade [8].

4. Discussion

Acquired resistance to endocrine therapies is a major problem leading to disease recurrence in breast cancer survivors [11,37]. We and others reported that the expression of COUP-TFII is lower in endocrine-resistant breast cancer cell lines [6,31] and in ER α -negative compared with ER α -positive breast tumors [7]. COUP-TFII expression is negatively associated with tumor histological grade [7,8]. Further, COUP-TFII was recently reported to be an independent predictor of metastasis-free survival in TAM-treated breast cancer patients, independent of ER α expression [8]. These and other data indicate that COUP-TFII may play a role in maintaining the sensitivity of breast cancer cells to TAM and other antiestrogens.

In this study we investigated if COUP-TFII expression is repressed in endocrine-resistant breast cancer cell lines by epigenetic reprogramming, *i.e.*, DNA methylation and histone deacetylation, that would cause chromatin condensation and repression of gene transcription. Recent studies revealed wide-scale chromatin landscape reprogramming, as revealed by micrococcal nuclease-ChIP-sequencing, in endocrine-sensitive versus resistant MCF-7 cells [53]. Whole genome DNA methylation profiling of 8 human breast cancer cell lines, including MCF-7, revealed global hypomethylation compared to a normal human mammary epithelial cells (HMECs), but the breast cancer cells had a fourfold increase in hypermethylation at CpG-rich gene regions, including promoters, exons, and introns, compared to HMECs [54]. Inhibition of HDAC activity by treating breast cancer cells with TSA along with AZA to inhibit DNMT has been shown to increase ER α and ER β expression in ER-negative breast cancer cells [27,41,55,56] and here we demonstrated that these epigenetic treatments increased COUP-TFII expression in endocrine-resistant breast cancer cells. Supporting our suggestion, we note that supplementary data in a paper showing lower COUP-TFII expression in tamoxifen (T)- and fulvestrant (F)- resistant MCF-7 cells showed that MCF7-T cells have higher methylation density and the MCF7-F cells have lower methylation density in the promoter region of COUP-TFII when compared to MCF-7 cells [31].

Conversely, TSA inhibited *ESR1* transcription in MCF-7 as well as endocrine-resistant LCC2 and LCC9 cells. These data agree with a previous report that TSA inhibited ER α expression in MCF-7 cells [41,57]. An earlier report demonstrated that ER α mRNA stability is decreased by AZA + TSA treatment of MCF-7 cells through reduction of the RNA binding protein, HuR [56].

While our data suggest that the reduction in COUP-TFII expression, like that of ER β , in endocrine-resistant LCC2 and LCC9 cell lines may be caused by epigenetic changes, whether this is a direct effect on DNA or histone methylation on the COUP-TFII gene (*NR2F2*) is unknown. However, the correlation between CpG islands of *NR2F2* and DNA methylation was examined by MSP and showed methylation within the first exon of *NR2F2* was higher in LCC2 and LCC9 compared to MCF-7 cells, corresponding to the lower level of COUP-TFII expression in these cell lines. AZA treatment reduced *NR2F2* methylation, corresponding to the increase in COUP-TFII expression detected in our experiments. This is consistent with a previous study showing that AZA reduced MSP *ESR1* methyl-specific product appearance in A549 lung adenocarcinoma cells, a result correlated with increased

ER α transcript expression [58]. Further detailed study of the methylation of the *NR2F2* gene will be necessary to fully dissect which CpG residues contribute to decreased gene expression.

There are alternative explanations, *e.g.*, the effect of AZA + TSA may alter the epigenome of a transcription factor(s), repressor (s), or other protein, *e.g.*, a coactivator, that regulates COUP-TFII transcription. We note that AZA not only inhibits DNA methylation but removed H3K9me3 and H3K27me3 on ~2000 genes with initially high H3K9me3 and H3K27me3, corresponding to initially silenced genes [30]. The greater increase in COUP-TFII mRNA transcript expression with TSA +/– AZA in LCC2 cells compared with LCC9 cells might indicate less *NR2F2* promoter methylation/ deacetylation/chromatin condensation in LCC2 *versus* LCC9 cells. This suggestion may reflect the fact that LCC2 cells are less resistant to TAM and other antiestrogens than LCC9 [37,33]. For example, LCC2 are resistant to TAM but not to fulvestrant, whereas LCC9 are resistant to both antiestrogens. This is reflected in the MTT assay data presented here. Further experiments will be required to examine the methylation of the COUP-TFII promoter in these two cell lines compared to MCF-7 cells.

Histone deacetylase inhibitors (HDACi) are an emerging class of anticancer agents that restore chromatin structure to a more open state leading to re-expression of silenced genes and resulting in cell cycle arrest and apoptosis by various mechanisms [59]. Here we observed that LCC2 and LCC9 endocrine-resistant cells are more sensitive to growth inhibition by AZA + TSA compared to MCF-7 cells. The precise reason for this difference is unknown, but clearly of interest for further testing and exploration of other HDACi as possible therapies for endocrine-resistant breast cancer.

We observed that basal *BCL2* expression was higher in the endocrine-resistant LCC2 and LCC9 cell lines compared to parental, endocrine-sensitive MCF-7 cells. Robert Clarke's group had previously reported higher *BCL2* in LCC9 relative to tamoxifen-sensitive LCC1 breast cancer cells and that elevated *BCL2* is a mediator of resistance to several chemotherapeutic drugs as well as antiestrogens, *i.e.*, tamoxifen and fulvestrant [60]. TSA increased the *BAX/BCL2* ratio in MCF-7 and LCC2 cells but not in LCC9 cells. AZA + TSA did not increase the *BAX/BCL2* ratio in LCC2 or LCC9 cells. The lack of increase in the *BAX/BCL2* ratio with the decrease in cell viability also led us to investigate autophagy, a suggestion consistent with a report that treatment of SKBr3 HER2+/ER α - endocrine-resistant breast cancer cells with TSA increased autophagy [61] and with a report that MCF-7 cells resistant to paclitaxel switch from apoptotic to autophagic cell death [62]. Resistance of breast cancer cells to tamoxifen is associated with increased autophagy as a mechanism of cell survival [47]. Further, our data agree with a report that tamoxifen-resistant breast cancer cells are sensitive to the induction of autophagy by other drugs, *e.g.*, U0126 which blocks p70S6K [63,64].

We observed that treatment of MCF-7 cells with AZA increased LC3-II and decreased LC3-I, possibly indicating increased autophagy. AZA reduced LC3-I and LC3-II, and thus total LC3, in the endocrine-resistant LCC2 and LCC9 cells. TSA increased total LC3 in MCF-7 and LCC2 cells, possibly reflecting increased autophagy. All three breast cancer cell lines

showed the expected increase in the LC3-II band with a reduction in the LC3-I band with 4-OHT treatment. These findings are inconsistent with a previous report that 1 μ M 4-OHT treatment of MCF-7-LCC1 (endocrine-sensitive) or LCC9 cells for 72 h had no effect on LC3-I [65]. The precise reason is unclear. In a previous report, TSA increased LC3 and specifically LC3-II in HCT116 colon cancer cells [66], but no studies appear to have examined TSA's effect on autophagy in breast cancer cells. There is one report showing that incubation of MCF-7 cells with 1 μ M TSA for 24 h induced dephosphorylation of Akt, resulting in activation of GSK3 β and cytotoxicity [67]. We note that the concentration of TSA used in our studies was much lower, i.e., 0.3 μ M for 16 h. The combination of AZA with TSA had no effect relative to TSA alone on LC3 levels in MCF-7 or LCC2 cells, but decreased LC3-II, and hence total LC3, in LCC9 cells. Future studies examining other autophagy markers and double-membrane auto-phagosomes within treated cells will be required to confirm whether AZA and TSA increase autophagy in MCF-7, LCC2, and LCC9 cells.

In conclusion, the results presented here suggest that the expression of COUP-TFII in endocrine-resistant LCC2 and LCC9 breast cancer cells is lower relative to endocrine-sensitive MCF-7 cells because of epigenetic changes in LCC2 and LCC9 cell lines. Using the Cancer Methyloome System (CMS) [50], we report that COUP-TFII DNA methylation occurs in human breast tumors versus normal breast tissue. Together, our data may explain the negative correlation detected between COUP-TFII and breast tumor grade [8]. Further, our data suggest that HDACi may be useful to inhibit the proliferation of endocrine-resistant breast cancer cells and tumors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Stimulation of COUP-TFII expression by inhibitors of DNA methyltransferase and HDAC in breast cancer cells. MCF-7 (endocrine-sensitive) and endocrine-resistant LCC2 and LCC9 breast cancer cells were treated with 2.5 or 50 μ M 5-aza-2'-deoxycytidine (AZA) for 72 h +/- 100 ng/ml trichostatin A (TSA), an inhibitor of histone deacetylase activity, for the last 16 h. Fresh AZA was added at the time when TSA was added, whether TSA was added or not. COUP-TFII gene expression was normalized by *GAPDH* and the fold relative to DMSO (vehicle control) was set to one in each cell line. Values are the average of 4–6 separate experiments. * $p < 0.05$ vs. DMSO. CT values for *NR2F2* mRNA are ~19.9, 32.0, and 31.2 for MCF-7, LCC2, and LCC9, respectively.

Fig. 2.

Inverse pattern of ER α and ER β expression with inhibition of DNA methyltransferase and HDAC activity in breast cancer cells. MCF-7 (endocrine-sensitive) and endocrine-resistant LCC2 and LCC9 breast cancer cells were treated with 2.5 or 50 μ M 5-aza-2'-deoxycytidine (AZA) for 72 h +/- 100 ng/ml trichostatin A (TSA) for the last 16 h. Fresh AZA was added at the time when TSA was added, whether TSA was added or not. (A) ER α (*ESR1*) and (B) ER β (*ESR2*) gene expression was normalized by *GAPDH* and the fold relative to DMSO (vehicle control) was set to one in each cell line. Values are the average of 4–6 separate experiments. * p < 0.05 vs. DMSO. CT values for *ESR1* mRNA are ~19.7, 19.8, and 20.3 for MCF-7, LCC2, and LCC9, respectively. CT values for *ESR2* mRNA are ~31.6, 31.9, and 32 for MCF-7, LCC2, and LCC9, respectively.

Fig. 3.

Treatment of endocrine-resistant LCC9 breast cancer cells with AZA + TSA increased COUP-TFII and ER β while inhibiting ER α protein expression. (A) MCF-7 cells were untreated or treated with vehicle (DMSO) for 72 h. LCC9 cells were treated with 2.5 or 50 μ M AZA with 100 ng/ml TSA added during the last 16 h of a total of 72 h incubation, as in Figs. 1 and 2. NE (30 μ g protein) were separated on 10% SDS PAGE gels and proteins western blotted. (B) MCF-7, LCC2, and LCC9 cells were treated with vehicle (DMSO) or 2.5 M AZA + 100 ng TSA for the last 16 h of a total 72 h incubation. For all panels, each blot was stripped and re-probed for β -actin. Values are the ratio of the indicated protein/ β -actin in that blot.

Fig. 4.

Immunostaining of COUP-TFII. Cells were treated with DMSO or 50 μM AZA for 72 h with 100 ng/ml TSA added for the last 16 h, as in Figs. 1 and 2. Cells were stained for COUP-TFII (red). Hoechst dye indicates nuclei (blue) and the merged image is shown at the right.

Fig. 5.

NR2F2 methylation analysis by MSP in breast cancer cell lines. MSP was performed using bisulfite-treated DNA with primers that recognize methylated (M) *NR2F2* in the promoter (F480 M + R179 M) or coding (F1163 M + R1503 M) regions (Supplemental Fig. 8, Supplemental Table 1). Cells were treated with vehicle control (DMSO), 2.5 or 50 μ M AZA or 2.5 μ M AZA + 100 ng/ml TSA for the last 16 h of a total 72 h treatment with fresh AZA added every 24 h with a change in medium. (A) Thirty ng of DNA was separated on 2% agarose gels and EtBr stained. (B) Quantitation of the PCR product pixel density is shown. Arrows indicate a decrease in MSP product with AZA or AZA + TSA compared to control samples in the same cell line.

Fig. 6.

Endocrine-resistant LCC2 and LCC9 cells are more sensitive to growth inhibition by AZA and TSA than MCF-7 endocrine-sensitive cells. Cells were treated for 5 days with the concentrations of 4-OHT or ICI 182,780 (fulvestrant) +/- 50 μ AZA and 100 ng/ml TSA (added for the last 48 h), as indicated. Cell viability was determined by MTT assays. Values are the mean ± SEM of quadruplicate determinations within one representative experiment. * $P < 0.05$ in that cell line. * $P < 0.05$ versus the same treatment in MCF-7 cells.

Fig. 7.

AZA + TSA do not increase the *BAX/BCL2* ratio in endocrine-resistant LCC2 and LCC9 cells. MCF-7 (endocrine-sensitive) and endocrine-resistant LCC2 and LCC9 breast cancer cells were treated with 2.5 or 50 μM AZA for 72 h +/- 100 ng/ml TSA for the last 16 h, as in Figs. 1 and 2. (A) *BAX* and *BCL2* mRNA transcript expression was normalized by *GAPDH* and the fold relative to DMSO (vehicle control) was set to one in MCF-7 cells. Values are the average +/- SEM of triplicate determinations within one experiment. (B) The ratio of *BAX/BCL2* expression is plotted. **p* < 0.05 vs. DMSO in that cell line.

Fig. 8.

Immunoblot analysis of LC3 autophagy marker. MCF-7, LCC2 and LCC9 cells were treated with DMSO (vehicle control), 5 μ M 4-OHT, or 2.5 or 50 μ M AZA for 72 h $\pm$ 100 ng/ml TSA for the last 16 h, as in Figs. 1 and 2. (A) Whole cell lysates were collected and separated on 4–20% gradient Tris–Glycine gels. Proteins were transferred to PVDF membranes and probed with LC3A/B antibody. The membranes were stripped and reprobed for β -actin and then stripped again and reprobed for GAPDH. Cytoskeletal β -actin is degraded by the autophagosome. (B) Values of the integrated optical density (IOD) from Carestream analysis of the bands for LC3-I and LC3-II were plotted.

Fig. 9.

NR2F2 methylome analysis. Methylation of CpG islands near the promoter of *NR2F2* was compared between 10 normal breast and 77 breast tumor samples using the Cancer Methylome System (<http://cbbiweb.uthscsa.edu/KMethylomes/>), which contains data from 10 normal breast and 77 breast tumor samples generated by MBDCap-seq. 10 normal breast and 10 breast tumor samples are pictured, with darker color indicating increased methylation.

Table 1

COUP-TFII/*NR2F2* methylome analysis. The methylation of the *NR2F2* gene was compared between 10 normal breast and 77 breast tumor samples using the Cancer Methylome System (<http://cbbiweb.uthscsa.edu/KMethylomes/>). A statistically significant increase in methylation was found in breast tumors compared to normal breast. Methylation difference (Diff): average reads number difference in differentially methylated region (DMR) between breast tumor and normal breast samples; Methylation ratio: methylation difference divided by average reads number of normal breast samples.

Chromosome	Start	End	<i>p</i> value	Methylation diff	Methylation ratio
chr15	94665500	94666500	0	17.9	483.71
chr15	94666500	94667500	9.00E-04	53.02	282.01
chr15	94671000	94672000	0.0026	4.79	684.79
chr15	94674000	94675000	0.007	4.27	853.25
chr15	94676000	94677000	0.006	10.64	887.01
chr15	94677000	94678000	7.00E-04	7.6	542.86
chr15	94678000	94679000	0.0041	28.75	239.61