FOXA1 Is Essential for Aryl Hydrocarbon Receptor–Dependent Regulation of Cyclin G2

Shaimaa Ahmed, Sarra Al-Saigh, and Jason Matthews

Abstract

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor that mediates the effects of the environmental contaminant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Recently, AHR has emerged as a potential therapeutic target for breast cancer by virtue of its ability to modulate estrogen receptor-α (ERα) signaling and/or its ability to block cell proliferation. Our previous studies identified cyclin G2 (CCNG2), an inhibitor of cell-cycle progression, as an AHR target gene; however, the mechanism of this regulation is unknown. Chromatin immunoprecipitation assays in T-47D human breast cancer cells revealed a TCDD-dependent recruitment of AHR, nuclear co-activator 3 (NCoA3) and the transcription factor forkhead box A1 (FOXA1), a key regulator of breast cancer cell signaling, to CCNG2 resulting in increases in CCNG2 mRNA and protein levels. Mutation of the AHR response element (AHRE) and forkhead-binding sites abolished TCDD-induced CCNG2-regulated reporter gene activity. RNA interference–mediated knockdown of FOXA1 prevented the TCDD-dependent recruitment of AHR and NCoA3 to CCNG2 and reduced CCNG2 mRNA levels. Interestingly, knockdown of FOXA1 also caused a marked decrease in ERα, but not AHR protein levels. However, RNA interference–mediated knockdown of ERα, a negative regulator of CCNG2, had no effect on TCDD-dependent AHR or NCoA3 recruitment to or expression of CCNG2. These findings show that FOXA1, but not ERα, is essential for AHR-dependent regulation of CCNG2, assigning a role for FOXA1 in AHR action. Mol Cancer Res; 10(5): 636–48. © 2012 AACR.

Introduction

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor that mediates the toxic effects of both halogenated and polycyclic aromatic hydrocarbons, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and 3-methylcholanthrene (3MC). AHR plays important roles in development and differentiation, as well as in the immune and the reproductive systems (1, 2). Moreover, AHR has recently emerged as a therapeutic target for inflammatory and autoimmune diseases, as well as for the treatment of breast cancer (3, 4). We and others have shown that AHR activation inhibits estrogen receptor-α (ERα) signaling through direct interaction with ERα and through the modulation of ERα target genes (5–7). After ligand binding, AHR translocates into the nucleus where it binds to its dimerization partner AHR nuclear translocator (ARNT). This heterodimer then binds to specific DNA response elements termed AHR response elements (AHRE) in the regulatory regions of its target genes. DNA binding of the AHR/ARNT heterodimer results in the recruitment of coregulators (coactivators and corepressors) causing changes in gene expression. Well-studied AHR target genes include the phase I and II drug-metabolizing enzymes, such as cytochrome P450 1A1 (CYP1A1), CYP1B1, and glutathione S-transferases. Chromatin immunoprecipitation combined with microarrays (ChIP-chip) and gene expression microarray experiments revealed that AHR regulates numerous genes involved in diverse cellular pathways, including metabolism and cell-cycle regulation (8–10). In support of these findings, previous studies have shown that TCDD inhibits cell proliferation (11–13).

Cell proliferation is a highly regulated event mediated by the activation of cyclin-dependent kinases (CDK), which require the binding of cyclins for full activity. Mammalian cyclins are classified into 12 different types cyclins A to I based on sequence and functional similarities (14). Most cyclins have been shown to facilitate growth by either promoting G0–G1 to S-phase or the G2–M phase transition. The G-type cyclins (cyclin G1, G2, and I) on the other hand are associated with cell-cycle arrest (15). Cyclin G1 is involved in G2–M phase arrest, whereas cyclin I is thought to play a role in apoptosis (16). Cyclin G2 (CCNG2) has been shown to inhibit cell-cycle progression by preventing G1 to S-phase transition (14, 15, 17). This inhibition is achieved by interacting with protein phosphatase 2A (PP2A). The CCNG2-PP2A complex alters PP2A targeting and its substrate specificity leading to cell-cycle arrest (14). CCNG2 also stops cell-cycle progression by preventing the...
phosphorylation of CDK2 and retinoblastoma which are required for progression out of the quiescent state (G0). CCNG2 has been shown to be a critical gene in human epidermal growth factor receptor 2 (HER2)- and hormone-dependent breast cancers (18–21). Moreover, CCNG2 was among the few genes upregulated following anti-HER2 antibody trastuzumab treatment, indicating that CCNG2 might play a role in inhibiting HER2-dependent proliferation (22).

In estrogen-dependent breast cancer, cell proliferation is facilitated by the binding of estrogen to ERα resulting in either increased expression of genes associated with proliferation or suppression of genes that block cell-cycle progression. ERα represses CCNG2 expression in response to estrogen by recruiting a complex containing nuclear corepressor (NCoR) and histone deacetylases to the CCNG2 promoter region resulting in the displacement of RNA polymerase II (21). Our previous ChIP-chip studies identified an AHR-bound AHRE-containing sequence in the upstream regulatory region of CCNG2. In contrast to estrogen, the AHR agonists, TCDD and 3MC, induced AHR recruitment to CCNG2 causing an increase in CCNG2 mRNA levels (8, 9). However, the molecular mechanisms and characterization of the AHR-dependent regulation of CCNG2 remain elusive.

Another protein shown to be important in the regulation of genes in breast cancer is the forkhead box A1 (FOXA1) transcription factor, a member of the winged-helix transcription factors (23). The FOXA family is composed of 3 closely related members, FOXA1, A2, and A3, which have critical roles in development and differentiation (23). High expression of FOXA1 is observed in ERα-positive breast tumors and its expression is a positive prognostic factor which correlates with sensitivity to endocrine therapy and inhibition of tumor growth (19, 24). FOXA1 acts as a "pioneer factor" by altering chromatin structure and facilitating ERα binding to its target genes (25). FOXA1 also modulates androgen receptor function, indicating that it has a more general role in regulating gene expression (26, 27). The role of FOXA1 in AHR transactivation or cross-talk between AHR and ERα has not been determined.

AHR activation inhibits breast cancer cell proliferation and induces cell-cycle arrest through a number of different pathways (2, 10, 28). We report here that FOXA1, but not ERα, is essential for AHR-dependent regulation of CCNG2, assigning a role for FOXA1 in AHR action and characterizing a new mechanism by which AHR inhibits breast cancer cell proliferation.

Materials and Methods
Chemicals

Antibodies used for ChIP and Western blotting experiments include ERα (HC-20), AHR (H-211), and NCoA3 (M-397) from Santa Cruz Biotechnology and FOXA1 (ab23738) from Abcam. TCDD was purchased from Accustandard and dimethyl sulfoxide (DMSO) was purchased from Sigma. Cell culture media, FBS, and trypsin were all purchased from Wisent Bioproducts. All other chemicals and biochemicals were of the highest quality available from commercial vendors.

ChIP assays

T-47D human breast carcinoma cells were cultured in 1:1 mixture of Dulbecco’s Modified Eagle’s Medium (DMEM) and Ham’s F12 nutrient mixture medium supplemented with 10% (v/v) FBS and 1% penicillin/streptomycin. Cells were maintained at 37°C and 5% CO2. T-47D cells were subcultured every 2 to 3 days or when they reached 80% to 90% confluency. For the ChIP studies, 3 million T-47D cells were seeded in 10-cm dishes in 1:1 mixture of DMEM: F12 phenol red–free media supplemented with 5% (v/v) dextran-coated charcoal (DCC)-striped serum and 1% penicillin/streptomycin. After 72 hours, cells were treated with DMSO (final concentration, 0.1%), 10 nmol/L TCDD, 10 nmol/L E2, or E2 + TCDD (10 nmol/L) for 0.75 hours. ChIP and sequential ChIP assays were conducted as previously described (8). Enrichment levels (relative to 100% total input) were determined by quantitative real-time PCR (qPCR) using the following primers: CCNG2 AHRE2 (enhancer): 5'-TGGTTTACCAAGGCCAA
gAA-3' and 5'-CCAGAGGTTGTAGTGCTGTGTGT-3';
CCNG2 AHRE1: 5'-AAGTCCTCCGTGCTGAAA and
5'-CGCCCGGCTTCTCCTAA-3' CCNG2 TATA: 5'-GGAGGCGCGAGAGA-3' and 5'-TCCCTCACG
GACTTTAAAAAGAC-3'.

RNA isolation and real-time PCR

T-47D cells were seeded in 6-well plates and grown in a 1:1 mixture of DMEM:F12. Cells were treated with either 10 nmol/L TCDD or pretreatment for 1 hour with 1 µmol/L CH223191 for 0.75, 1.5, 3, 6, or 24 hours. RNA was isolated using illustra RNAspin minicolumns (GE Healthcare) and reverse-transcribed as previously described (8). All target gene transcripts were normalized to ribosomal 18S RNA and fold inductions were calculated using time-matched DMSO-treated samples and the comparative C_T (ΔΔC_T) method used for data analysis. qPCR primers used in the study have been described elsewhere (8) with exception of the following primers: FOXA1 mRNA 5'-GAAGATGGAAGGCATGAAA-3' and 5'-GCCTGAG
TTCTGTTTAAAAAGAC-3'.

Western blotting

Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membrane. The membrane was blocked in 2% (w/v) ECL-Advanced blocking agent for 1 hour at room temperature with constant rocking and then incubated with anti-ERα (HC-20), anti-AHR (H-211), or anti-FOXA1 (ab23738) overnight at 4°C with constant rocking. The membrane was then washed 3 times and incubated with an anti-rabbit secondary antibody for 1 hour. For detection of β-actin, a primary mouse anti-β-actin antibody (Sigma) was incubated for 2 hours at room temperature followed by a 1-hour washing with PBS/0.1% Tween-20 before incubation with anti-mouse secondary antibody for 1 hour. After
washing, the bands were visualized using ECL-Advanced (GE Healthcare).

**Plasmid construction and mutagenesis**

The plasmid pGL4-CCNG2 containing -1.402 kb to +179 of the upstream regulatory region of CCNG2 as well as plasmids pGL4-CCNG2 ΔAHRE1 and ΔAHRE2 were generously provided by Dr. Chun Peng (York University, Toronto, ON, Canada). The numbering of the cloned CCNG2 regulatory region was relative to CCNG2 mRNA (NM_004354). Site-directed mutagenesis targeting the AHRE site in CCNG2 binding sites upstream from AHRE2 was done using pGL4-CCNG2 as well as plasmids pGL4-CCNG2 ΔAHRE1 and ΔAHRE2 were generously provided by Dr. Chun Peng (York University, Toronto, ON, Canada). The numbering of the cloned CCNG2 regulatory region was relative to CCNG2 mRNA (NM_004354). Site-directed mutagenesis targeting the AHRE site in CCNG2 was completed as previously described (29). For mutation of AHRE2 the PCR primers, 5'-CTCATCAGCCCGCTAAGTTTT-3' and 5'-AAAACCTTAGCCGCTGTAGAG-3' were used to mutate pGL4-CCNG2, the mutated residues underlined. Mutation of the 2 forkhead-binding sites upstream from AHRE2 was done using pGL4-CCNG2 as a template and the following PCR primers for FHk3: 5'-TAGGAGGGAG AGAGTGCTCCAAA TAAATTGTCGGACAGG-3' and 5'-CTGGAACTTTTTTTTG GCCGCTTAAGTTTT-3' and 5'-CACGCTGTAGAG-3'. All mutations were verified by DNA sequencing.

**Transient transfection and reporter gene assay**

T-47D cells were plated in 12-well dishes in a 1:1 DMEM:F12 mixture containing 5% DCC-stripped serum. Twenty-four hours after plating, cells were transfected with 200 ng of luciferase reporter vectors using Lipofectamine LTX (Invitrogen). All reactions included 100 ng pCH110-β-gal (GE Healthcare) to normalize for transfection efficiency. The cells were dosed 24 hours posttransfection with either 0.1% DMSO (vehicle control) or 10 nmol/L TCDD. The following day, cells were lysed and luciferase activity was determined using the ONE-Glo system according to manufacturer’s recommendations (Promega). The firefly luciferase activity was normalized to β-gal levels and the normalized data were presented relative to vehicle control.

**Co-immunoprecipitation**

For co-immunoprecipitation (co-IP) studies, T-47D cells were seeded in 10-cm dishes in 1:1 mixture of DMEM:F12 supplemented with 5% DCC-stripped serum. After 72 hours, cells were treated with either 10 nmol/L TCDD or DMSO (final concentration, 0.1%) for 1 hour and cross-linked using 1% formaldehyde and quenched using 125 mmol/L glycine. Cell lysates were precleared using Protein A, and protein complexes were immunoprecipitated using 2 μg of rabbit IgG (Sigma), AHR (H-211), FOXA1 (Abcam 23738), or NCoA3 (M-397) for 2 hours. Beads were washed 4 times with wash buffer (10 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, 10% glycerol, 1% NP-40, and 2 mmol/L EDTA). Eighty microliters of sample buffer was added to the beads and samples were heated to 70°C for 10 minutes. Samples were loaded on an 8% SDS-PAGE gel, transferred to nitrocellulose membrane, and visualized using the Clean Blot anti-rabbit HRP (Thermo Scientific) and ECL-Advanced.

**Flow cytometry**

Cell-cycle analysis by bromodeoxyuridine (BrdUrd) and propidium iodide (PI) double staining was completed on T-47D cells transiently transfected with universal negative control or 50 nmol/L of siRNA against CCNG2 (L-003271-00-0005). Twenty-four hours after transfection, cells were treated with DMSO (final concentration, 0.1%) or 10 nmol/L TCDD and left for another 48 hours. Cells were then collected and fixed in 70% ethanol for 20 minutes at –20°C. Cells were then rinsed in wash buffer (PBS + 0.5% bovine serum albumin) and resuspended in 2N HCl for 20 minutes, washed, incubated with 0.1 mol/L sodium borate, pH 8.5, for 2 minutes, washed again, then incubated with fluorescein isothiocyanate (FITC)-conjugated anti-BrdUrd (BD Biosciences) in PBS + 0.5% bovine serum albumin + 0.5% Tween-20 and left in the dark for 30 minutes. Cells were then washed with wash buffer and stained with 50 μg/mL PI for another 30 minutes. FACS analyses and data acquisition were done by the Faculty of Medicine Flow Cytometry Facility using a FACSCalibur flow cytometer (BD Biosciences) and FlowJo software (TreeStar).

**Statistical analysis**

All results were expressed as means ± SEM. Statistical analysis was calculated using GraphPad Prism 5 statistical software. One-way ANOVA followed by Tukey’s multiple comparison tests and the Student two-tailed t tests were used when appropriate. Statistical significance was assessed at P < 0.05.

**Results**

**TCDD- and AHR-dependent regulation of CCNG2**

To investigate the mechanism of the AHR-dependent regulation of CCNG2, we first conducted time course mRNA expression analysis of TCDD-treated T-47D cells cultured for 72 hours in medium containing 5% DCC-treated FBS to
synchronize the cells in G0-G1 (30) and to reduce the concentration of potential AHR activators in the FBS. As shown in Fig. 1A, CCNG2 mRNA levels were increased following 1.5 hours of treatment and remained elevated until 24 hours. TCDD-dependent increases in CCNG2 mRNA levels were reduced after cotreatment with the selective AHR antagonist CH223191 (31) at all time points examined. Western blotting confirmed TCDD-dependent increases in CCNG2 protein levels after 6 hours of treatment (Fig. 1B).

To assess the impact of CCNG2 upregulation and its role in the TCDD-dependent cell-cycle inhibition, we conducted cell-cycle analysis. T-47D cells transiently transfected with RNA interference (RNAi) targeting CCNG2 (~70% knockdown was achieved at the mRNA level, data not shown) were double-stained with BrdUrd and PI after 48
hours of treatment with DMSO or 10 nmol/L TCDD and analyzed using FACS. We observed that cells transfected with universal negative control and treated with TCDD resulted in an increase in the number of cells in G1 when compared with DMSO (Fig. 1C and E). However, in cells transfected with RNAi-targeting CCNG2, there was an increased amount in the S-phase (Fig. 1D) but TCDD treatment did not alter the distribution of cells (Fig. 1F).

The ability of TCDD to increase the number of cells in G1 and reduce the number of cells in S-phase was lost following knockdown of CCNG2 (Fig. 1G and H), suggesting that CCNG2 is an important contributor to the TCDD-dependent cell-cycle inhibition in T-47D cells.

**TCDD induces the recruitment of AHR and FOXA1 to CCNG2**

To identify AHREs and other transcription factor–binding sites that might be important in the TCDD-dependent regulation of CCNG2, we conducted transcription factor–binding site analysis on the −1.4 kb CCNG2 regulatory region using MatInspector (Genomatix). This analysis identified 2 AHRE sequences that were positioned in close proximity to multiple forkhead (FKH) sites. We designated the AHRE sites, AHRE1 and AHRE2, and the FKH sites, FKH1–4 (Fig. 2D). Because FOXA1 is an important transcription factor in ERα-positive breast cancer cells, we then determined the role of FOXA1 as well as each of the individual AHREs in the AHR-dependent regulation of CCNG2. To examine AHR and FOXA1 recruitment to the CCNG2 promoter in T-47D cells, we conducted ChIP assays. Cells were treated with DMSO or 10 nmol/L TCDD for 1 hour, cross-linked and protein–DNA complexes were immunoprecipitated with antibodies directed against AHR, FOXA1, H3K4Me2, or H3K9Ac. H3K9Ac was examined because this modification correlates with actively transcribed genes (32). H3K4Me2 was tested to identify functional FOXA1-binding sites, as H3K4Me2 has been reported to correlate with FOXA1 binding to its FKH site (33). As shown in Fig. 2A, TCDD treatment significantly and preferentially induced the recruitment of AHR and FOXA1 to AHRE2, whereas only a modest, albeit significant, increase in AHR recruitment to AHRE1 was observed (Fig. 2A). ChIP studies revealed constitutive binding of FOXA1 to the enhancer region, which was increased after ligand treatment. Treatment with TCDD resulted in increased levels of H3K4Me2 or H3K9Ac at AHRE2 but not at AHRE1 (Fig. 2B). These findings suggest that AHRE2, but not AHRE1, is the dominant AHRE driving the TCDD-dependent regulation of CCNG2. In agreement with TCDD-dependent increases in CCNG2 mRNA levels, ChIP assays confirmed increases in the recruitment of RNA polymerase II to the proximal promoter region of CCNG2 after TCDD treatment (Fig. 2C). Although the expression of FOXA2 was comparable with FOXA1 levels, FOXA2 was not recruited to CCNG2, whereas FOXA3 was not detected in T-47D cells (data not shown).

**TCDD-dependent interactions between FOXA1 and AHR**

To determine whether FOXA1 and AHR were present in the same protein complex, we carried out sequential ChIP and co-IP experiments. Sequential ChIP analyses revealed that AHR and FOXA1 were recruited simultaneously to CCNG2 (Fig. 3A). Furthermore, AHR and FOXA1 were shown to be part of the same protein complex in co-IP assays completed in the presence or absence of TCDD (Fig. 3B).

**AHR mediates the TCDD-dependent regulation of CCNG2 using FOXA1**

To determine how AHR regulates CCNG2 transcription and to identify the key response element(s) involved in this regulation, we conducted promoter deletion and site-directed mutagenesis analyses. Treatment with 10 nmol/L TCDD resulted in an approximate 1.5-fold increase in activity of the full-length promoter, pGL4-CCNG2 (Fig. 4A). Deletion of AHRE2 abolished the TCDD-dependent regulation of CCNG2 (Fig. 4A). Site-directed mutagenesis of AHRE2 inhibited the TCDD-mediated luciferase activity (Fig. 4B). These findings suggest that AHRE1 is not required for AHR-dependent regulation of CCNG2. We then mutated the FKH3 and FKH4 sites to evaluate the role of FOXA1 in modulating the AHR-dependent regulation of CCNG2. Mutation of either FKH site significantly decreased, but did not abolish, the TCDD-dependent increase in luciferase activity (Fig. 4B). However, mutation of both FKH sites prevented the TCDD-dependent increase in luciferase activity. Taken together, these data show that AHRE2 and FOXA1 play key roles in the AHR-dependent regulation of CCNG2. The AHR binding observed at AHRE1 in the ChIP analysis (Fig. 2A) may just represent larger immunoprecipitated DNA fragments containing AHRE2, as the resolution of our ChIP assay is 500 to 800 bp.

**FOXA1 but not ERα is required for the AHR-dependent regulation of CCNG2**

FOXA1 is an important modulator of ERα and androgen receptor transactivation in breast and prostate cancer cells, respectively (25, 26, 34–36). Because FKH sites were found to be important in the TCDD-mediated regulation of the CCNG2 luciferase reporter plasmid, we hypothesized that FOXA1 may have a similar role with AHR–chromatin interactions at CCNG2. To test this hypothesis, we used RNAi-mediated knockdown of FOXA1 and measured mRNA expression, as well as the recruitment patterns of AHR, FOXA1, and ERα. ERα is known to negatively regulate CCNG2 (21). Following transient transfection of 2 distinct siRNA oligos into T-47D cells, we determined that 48 hours posttransfection, FOXA1 protein levels were greatly reduced (Fig. 5A) and mRNA expression was reduced to 20% compared with control cells (data not shown). Interestingly, the loss of FOXA1 caused a marked decrease in ERα protein levels, which has been previously reported (34), but did not cause any changes in AHR protein levels. Similar findings were observed in MCF-7 ERα-positive breast carcinoma cells (data not shown). RNAi-mediated
knockdown of FOXA1 inhibited the TCDD-dependent gene expression supporting our promoter deletion and mutagenesis results described above (Fig. 5A). As indicated by our ChIP studies, treatment with 10 nmol/L of TCDD resulted in increased recruitment of FOXA1, AHR, and ERα (Fig. 5B–D). Interestingly, we observed constitutive binding of both FOXA1 and ERα when compared with IgG controls. RNA-mediated knockdown of FOXA1 abolished the TCDD-dependent recruitment of both AHR and ERα (Fig. 5C and D). The reduced recruitment of ERα to CCNG2 was most likely due to reduced protein expression levels rather than the loss of FOXA1. FOXA1, however, was necessary for the AHR-mediated regulation of CCNG2, although we cannot exclude the possibility that the reduced ERα protein levels influence AHR transactivation, as our laboratory and others have shown that ERα modulates AHR activity (7, 8, 29).

We then conducted RNAi-mediated ERα knockdown studies to determine the role of ERα in AHR-mediated regulation of CCNG2 expression. The loss of ERα had no effect on either FOXA1 or AHR protein levels (Fig. 5E). In agreement with our previous findings, the knockdown of ERα significantly increased the constitutive levels of CCNG2 mRNA levels but did not affect the TCDD-mediated increase in CCNG2 mRNA levels (ref. 8; Fig. 5E). ChIP analysis showed that ERα was
recruited to CCNG2 in the absence of ligand, suggesting that under these conditions, ERα modulates CCNG2 gene expression (Fig. 5E and F). Knockdown of ERα did not affect the TCDD-dependent recruitment of AHR or FOXA1 to CCNG2 (Fig. 5G and H). Together, these data provide evidence that FOXA1 is driving the AHR-mediated regulation of CCNG2 irrespective of ERα levels.

Figure 3. FOXA1 and AHR are part of the same protein complex. A, sequential ChIP analyses were conducted with the indicated antibodies. Immunoprecipitated DNA was measured by qPCR using the CCNG2 enhancer primers. Quantification of binding was determined as a fold induction above IgG DMSO. Each error bar represents the SEM of 3 independent replicates. *, statistically significant differences compared with IgG DMSO control samples (P < 0.05, one-way ANOVA). B, co-IP studies were completed in T-47D cells. Cells were treated for 1 hour with either DMSO or TCDD and then cross-linked using formaldehyde. Cell lysate was immunoprecipitated using antibodies against AHR and FOXA1. IgG was used as the negative control. Western blotting was then completed using the reciprocal antibody.

Figure 4. AHR mediates the TCDD-dependent regulation of CCNG2. A, deletion fragments of pGL4-CCNG2 were tested to deduce the functional significance of AHRE1 and AHRE2. Two hundred nanograms of each vector was transfected in T-47D cells and luminescence was measured following a 24-hour 10 nmol/L TCDD treatment. B, site-directed mutagenesis of AHRE2 as well as to the FKH recognition sites was generated in pGL4-CCNG2. T47-D cells were transfected with 200 ng of the single and double response element mutants and treated with either DMSO or 10 nmol/L TCDD for 24 hours. Results represent the mean of 3 independent replicates. *, luciferase (luc) activity that was statistically different compared with DMSO pGL4-CCNG2 control; #, luciferase activity statistically different compared with TCDD pGL4-CCNG2 (P < 0.05, one-way ANOVA).
Figure 5. FOXA1, but not ERα, is essential for the TCDD-dependent recruitment of AHR to CCNG2. A, gene expression profiles were completed on T-47D cells transfected for 48 hours with siRNA and then treated for 6 hours with TCDD. RNA was isolated and reverse-transcribed. mRNA expression was then determined using qPCR. Data were normalized against time-matched DMSO and to ribosomal 18S levels. Data represent the mean of 3 independent replicates and * is compared with TCDD negative (neg.) control (P < 0.05, one-way ANOVA). Inset, analysis of FOXA1 knockdown in T-47D cells after 48 hours. Cell extracts were probed with rabbit antibody against AHR, ERα, and FOXA1. β-Actin was used as loading control. Recruitment of FOXA1 (B), AHR (C), and ERα (D) following siRNA-mediated knockdown of FOXA1 using the ChIP assay. Briefly, T-47D cells were transfected for 48 hours with siRNA and then treated for 45 minutes with TCDD and immunoprecipitated using the antibodies indicated. The immunoprecipitated DNA was measured by qPCR with primers targeting the enhancer region (relative to 100% total input). Each graph represents the mean of 3 independent replicates with * indicating statistically significant differences compared with DMSO negative control whereas the # indicates statistically significant differences compared with TCDD negative control (P < 0.05, one-way ANOVA). E, CCNG2 mRNA expression levels were completed on T-47D cells transfected for 48 hours with siERα and then treated for 6 hours with TCDD. Data represent the mean of 3 independent replicates and the * are compared with DMSO negative control (P < 0.05, one-way ANOVA). Inset, analysis of ERα knockdown in T-47D cells after 48 hours. Cell extracts were probed with rabbit antibody against AHR, ERα, and FOXA1. β-Actin was used as loading control. Recruitment of ERα (F), FOXA1 (G), and AHR (H) was determined following RNAi-mediated knockdown of ERα using ChIP assays. Each graph represents the mean of 3 independent replicates with the * indicating statistically significant differences compared with DMSO negative control (P < 0.05, one-way ANOVA).
TCDD-dependent recruitment of NCoA3 to CCNG2

Previous studies showed that CCNG2 was negatively regulated by ERα through the recruitment of an NCoR complex leading to the hypoacetylation of histones and the release of RNA polymerase II (21). On the basis of these results, we hypothesized that the TCDD-dependent positive regulation of CCNG2 must overcome this inhibition through the recruitment of nuclear coactivators to promote gene expression. Because NCoA3/AIB1 is overexpressed in breast cancer, we determined the ability of TCDD, E2, and E2 + TCDD to induce recruitment of NCoA3 to CCNG2 in T-47D in the presence or absence of RNAi-mediated knockdown of FOXA1 or ERα. TCDD treatment resulted in increased NCoA3 recruitment to CCNG2, which was significantly reduced only after knockdown of FOXA1 but not ERα (Fig. 6A and B). The co-IP studies provided further evidence that NCoA3 is part of the activated multiprotein AHR-containing complex, as it was found to interact with both AHR and FOXA1 (ref. 37; Fig. 6C).

AHR prevents the ERα-dependent negative regulation of CCNG2

In support of a previous report, estrogen-bound ERα inhibited CCNG2 mRNA expression levels (ref. 21; Fig. 7A and B). However, this repression was overcome by cotreatment with TCDD and required FOXA1 but not ERα (Fig. 7A and B). Co-treatment of TCDD + E2 prevented the E2-dependent removal of NCoA3 from the CCNG2 resulting in increased recruitment of both AHR and FOXA1 (Fig. 7C–E). The TCDD-induced recruitment of NCoA3 was dependent on FOXA1 (Fig. 7C–E). RNAi-mediated knockdown of ERα had no effect on the ability of AHR to block the repression caused by E2 treatment (Fig. 7G–I). Overall, our findings show that FOXA1 facilitated the binding of TCDD-induced AHR and NCoA3 to CCNG2, leading to increased CCNG2 gene expression and preventing the previous described repressive actions of ERα on CCNG2 expression (ref. 21; Fig. 8).

Discussion

AHR has emerged as an important therapeutic target for breast cancer, as its activation has been reported to inhibit the growth of ER-positive, ER-negative, and HER2-positive breast cancer cells (7, 8, 28, 38, 39). In the present study, we show that ligand-activated AHR together with FOXA1 increases the expression of CCNG2 in ERα-positive T-47D breast cancer cells. We also report that activation of AHR prevented the ability of ERα to repress CCNG2 expression, providing another example where activation of the AHR pathway opposes the actions of ERα. Our findings provide a new mechanism by which AHR can inhibit human breast cancer cell proliferation. The increase in expression of CCNG2 by AHR further supports the notion that targeting AHR might be an effective therapy for breast cancer treatment (4, 38). Although the clinical importance of AHR-dependent activation of CCNG2 remains to be investigated, trastuzumab treatment has also been reported to increase CCNG2 levels (22). RNAi-mediated knockdown of CCNG2 resulted in a slight reduction in trastuzumab-dependent growth inhibition, suggesting that CCNG2 is required, but not necessary, for the inhibitory action of trastuzumab (18, 40). However, the mechanism of CCNG2 upregulation by trastuzumab most likely does not require FOXA1, as it is not expressed in ER-negative breast cancer (41). Nonetheless, these findings suggest that modulating CCNG2 expression might be an important mechanism to inhibit cancer cell growth.

TCDD-dependent activation of AHR blocks cell-cycle progression through the G1 phase in several cell lines and under many different conditions, including mouse...
hepatoma Hepa1c1c7 (42), rat hepatoma 5L cells (43), and in estrogen-induced MCF-7 cell proliferation (44). Potential mechanisms include the AHR-dependent induction of p27Kip1 and p21WAF1, inhibition of CDK function, inhibition of retinoblastoma phosphorylation, and repression of E2F-regulated genes through interactions with retinoblastoma and displacement of the coactivator p300 (10, 12, 45). The TCDD-dependent increase in CCNG2 expression reported here provides another mechanism by which ligand-activated AHR regulates cell-cycle progression in the G1 phase, as increases in CCNG2 levels result in G1 to S-phase arrest (17). In support of these findings, we

Figure 7. AHR can overcome the ERα-dependent negative (neg.) regulation of CCNG2. CCNG2 mRNA expression levels were determined from T-47D cells transfected for 48 hours with siFOXA1 (A) or siERα (B) and then treated for 6 hours with 10 nmol/L E2 or 10 nmol/L E2 + 10 nmol/L TCDD. RNA was isolated and reverse-transcribed, and mRNA expression levels were determined using qPCR. Data were normalized against time-matched DMSO and to ribosomal 18S levels. Data represent the mean of 3 independent replicates with the * representing statistically significant differences compared with DMSO and the # represents statistically significant differences compared with treatment-matched samples (P < 0.05, one-way ANOVA). Cells were treated with 10 nmol/L E2 or 10 nmol/L E2 + 10 nmol/L TCDD for 45 minutes and the recruitment of AHR (C), FOXA1 (D), NCoA3 (E), and ERα (F) was determined 48 hours post-siFOXA1 transfection using ChIP assays and qPCR. Similar experiments were also carried out 48 hours after siERα transfection using antibodies against AHR (C), FOXA1 (H), NCoA3 (I), and ERα (J).
report that the TCDD-dependent increase in the number of T-47D cells in G1 phase arrest is lost after knockdown of CCNG2.

FOXA1 has been implicated in both ERα and androgen receptor signaling (26, 35). Previously reported ChIP-chip data from our laboratory revealed that in both human cells and mouse tissue, FKH sites are significantly enriched in AHR-bound regions supporting a possible role for forkhead proteins in AHR signaling (8, 9, 46). In support of these data, we show that FOXA1 is critical for the AHR-dependent induction of CCNG2 levels. We observe that FOXA1 is present at CCNG2 in the absence of AHR activation. Following AHR ligand treatment, the levels of FOXA1, AHR, NCoA3, and H3K4Me2 are increased at CCNG2, suggesting that FOXA1 primes the CCNG2 for AHR recruitment and subsequent transcriptional activation. AHR and FOXA1 interact in co-IP and re-chip experiments, showing that they are part of the same multiprotein complex, which agrees with other reports showing that forkhead protein family members interact with other transcription factors (26). FOXA1 has been previously reported to interact with androgen receptor (47) and was shown to enhance glucocorticoid receptor transactivation (48). Similarly, FOXA3 interacts with glucocorticoid receptor where by the binding of FOXA3 provides a favorable DNA conformation for glucocorticoid receptor binding to its target genes (49). FOXA1 may use both the direct interaction with AHR and altered DNA conformation to enhance AHR binding to CCNG2. We hypothesize that FOXA1 stabilizes the AHR-activated complex at CCNG2 and therefore is required for maximal gene activation by AHR.

The regulation of CCNG2 by other members of the forkhead protein family has been previously reported, with one group showing that FoxO transcription factors increased CCNG2 expression in NIH3T3 mouse embryonic fibroblasts (14). Recently, Nodal, a member of the TGF-β family, was found to increase CCNG2 mRNA expression by increasing the expression of FOXO3a, which then forms a complex with Smad proteins at the CCNG2 promoter region (50). They found that the more proximal FKH sites (FKH1 and FKH2 in Fig. 2) were required for FOXO3a-mediated induction of CCNG2, rather than the distal FKH sites (FKH3 and FKH4 in Fig. 2) that are required for AHR-dependent induction of CCNG2 reported here. Interestingly, the antiproliferative effect of Nodal on ovarian cells was found to be partly mediated by CCNG2 (51). These findings show the important role of the forkhead protein family in the regulation of CCNG2 but reveal that the regulation of CCNG2 by FOXA1 or FOXO3a occurs via distinct FKH sites.

Recent RNAi-mediated knockdown of FOXA1 followed by ChIP-sequencing studies revealed that FOXA1 is required for almost all ERα binding to its genomic target regions irrespective of the proximity of FKH sites to ERα-binding sites (35). Although the RNAi-mediated knockdown of FOXA1 was reported to not affect ERα protein levels (35), we and others report that knockdown of FOXA1 has been shown to decrease ERα mRNA expression and protein levels (34). The reason for the discrepancies between the studies is unclear, but we observe that RNAi-mediated knockdown of FOXA1 reduces ERα protein levels in 2 different cell lines (T-47D, Fig. 5; MCF-7, data not shown) using 2 unique siRNA oligos targeting FOXA1. We also observe that RNAi-mediated knockdown of FOXA1 reduced the TCDD-dependent induction of CYP1A1 and CYP1B1. But unlike what was observed for CCNG2, RNAi-mediated knockdown of ERα did reduce the TCDD responsiveness of both CYP1A1 and CYP1B1 in T-47D.
cells (S. Ahmed and J. Matthews unpublished findings; ref. 8). This suggests that for certain genes the reduced AHR transactivation following RNAi-mediated knockdown of FOXA1, may be due to reduced ERα levels and not reduced FOXA1 expression. Therefore, it will be important to distinguish the effects of FOXA1 knockdown on AHR transactivation compared with those mediated by ERα. Further studies investigating the recruitment patterns of AHR and FOXA1 using ChIP-sequencing and RNAi-mediated knockdown approaches will be helpful in determining whether FOXA1 is a general modulator of AHR signaling or a gene-specific regulator. In summary, we report that the AHR-dependent regulation of CCNG2 requires FOXA1 to stabilize the binding of activated AHR and aiding in the recruitment of other co-regulatory proteins, such as NCoA3. Our findings also show that activated AHR is able to overcome the repressive actions of ERα at CCNG2, providing further evidence supporting the antiestrogenic effects of AHR. Although additional studies are needed to fully characterize the depth and role of FOXA1 in AHR signaling, the data presented here provide new insight into the antiproliferative action of AHR in breast cancer cells and further support AHR as a therapeutic target for breast cancer treatment.

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

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References


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Shaimaa Ahmed, Sarra Al-Saigh and Jason Matthews


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