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Constitutive Regulation of *CYP1B1* by the Aryl Hydrocarbon Receptor (AhR) in Pre-Malignant and Malignant Mammary Tissue

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Abstract The aryl hydrocarbon receptor (AhR) is a receptor/transcription factor which regulates cytochrome P450 (*CYP*) gene transcription and which is activated by environmental carcinogens, some of which are associated with increased breast cancer risk. Here, we show that the AhR is over-expressed and constitutively active in human and rodent mammary tumors, suggesting its ongoing contribution to tumorigenesis regardless of tumor etiology. AhR regulation of *CYP1A1* and *CYP1B1* was studied to determine if constitutively active AhR effects the same transcriptional outcomes as environmental chemical-activated AhR. Elevated *AhR* and *CYP1B1* but not *CYP1A1* before tumor formation in a rat model of mammary tumorigenesis suggested differential *CYP1B1* regulation by a constitutively active AhR. This hypothesis was tested with human mammary gland cell lines which hyper-express *AhR* and *CYP1B1* but which express little or no *CYP1A1*. *CYP1B1* expression was diminished by repression of AhR activity or by AhR knockdown, demonstrating AhR control of basal *CYP1B1* levels. ChIP assays demonstrated constitutive AhR binding to both *CYP1A1* and *CYP1B1* promoters, demonstrating that differential *CYP1A1* and *CYP1B1* regulation by constitutively active AhR does not result from different amounts of promoter-bound AhR. While increasing AhR binding to both *CYP1A1* and *CYP1B1*, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin induced *CYP1A1* mRNA in both a malignant and non-malignant line but increased only *CYP1B1* mRNA in the malignant line, again demonstrating that the level of promoter binding does not necessarily correlate with gene mRNA levels. These studies suggest that constitutively active AhR mediates different molecular outcomes than environmental chemical-activated AhR, and further implicate the AhR in mammary tumorigenesis. J. Cell. Biochem. 104: 402–417, 2008.

Key words: AhR; CYP1B1; mammary tumors; DMBA; TCDD

The aryl hydrocarbon receptor (AhR) is a cytosolic receptor/transcription factor activated by several classes of environmental chemicals including polycyclic aromatic hydrocarbons (PAHs), dioxins, and polychlorinated biphenyls [Poland et al., 1990; Dolwick et al., 1993; Chen and Perdew, 1994]. Several of these pollutants are carcinogenic and exposure to at least one class of environmental AhR ligands, the PAHs, is associated with breast cancer risk, particularly in certain genotypic subpopulations [Li et al., 1996, 1999; Terry et al., 2004; Santella et al., 2005; Shen et al., 2005, 2006; Brody et al., 2007]. Furthermore, preferential induction of breast cancers is seen in rats [Russo et al., 1989; Rogers and Conner, 1990; Trombino et al., 2000] and mice [Medina, 1974; Currier et al., 2005] following oral gavage with a prototypic AhR ligand, 2,4-dimethylbenz[α]anthracene (DMBA), suggesting that mammary tissue is particularly sensitive to PAH.

In the inactive state, the constitutively phosphorylated [Mahon and Gasiewicz, 1995], cytosolic AhR complexes with hsp90 molecules [Perdew and Bradfield, 1996], p23 [Kazlauskas

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et al., 1999], and an immunophilin-like accessory molecule, ARA 9/XAP-1 [Carver et al., 1998; Meyer et al., 1998]. In untransformed cells, exogenous ligands induce AhR translocation to the nucleus and dimerizaton with the AhR nuclear translocation protein (ARNT) through helix-loop-helix motifs present in both monomers [Ema et al., 1992; Hankinson, 1995; Swanson et al., 1995; Swanson, 2002]. The activated AhR complex binds target genes at specific DNA binding sites (aryl hydrocarbon response elements/xenobiotic response elements) (AhREs/XREs) [Hankinson, 1995] resulting in regulation of gene transcription. Chaperones, adapter molecules, and transcriptional cofactors associate with the AhR complex and play a critical role in dictating the level and specificity of its transcriptional activity [Swanson and Yang, 1998; Kumar and Perdew, 1999; Nguyen et al., 1999; Kazlauskas et al., 2000; Wei et al., 2004; Hankinson, 2005; Chen et al., 2006].

Perhaps the most commonly studied outcome of AhR activation with xenobiotics is the induction of genes such as those encoding the cytochrome P4501A1, 1A2, and 1B1 (CYP1A1, CYP1A2, CYP1B1) monooxygenases [Carrier et al., 1992; Hayes et al., 1996; Larsen et al., 1997; Spink et al., 1998, 2002, 2003a; Burdick et al., 2003; Burchiel et al., 2007]. Ligand-induced, AhR-dependent up-regulation of genes encoding these enzymes results in metabolism of some environmental AhR ligands and the production of carcinogenic intermediates [Buters et al., 1999; Dertinger et al., 2001; Burdick et al., 2003; Burchiel et al., 2007]. Notably, prototypic AhR target genes in the "AhR gene battery," such as *CYP1A1* and *CYP1B1*, are not always coordinately expressed [Spink et al., 1994; Dohr et al., 1995], indicating that they may perform non-overlapping functions. Of note, CYP1B1 hyper-expression is a characteristic of many human cancers [Maecker et al., 2003]. For example, our laboratory [Trombino et al., 2000; Maecker et al., 2003; Currier et al., 2005], as well as others [Eltom et al., 1998; Spink et al., 1998; Larsen et al., 2004; Chang et al., 2007], have demonstrated that AhR and CYP1B1 hyper-expression correlate in several cell types, including breast carcinomas. *CYP1B1* gene induction in mammary and ovarian tumors is particularly important since CYP1B1 is a highly efficient 17β-estradiolhydroxylase which metabolizes endogenous estrogen into mutagenic 4-hydroxy-estradiol [Hayes et al., 1996; Liehr and Ricci, 1996; Belous et al., 2007]. Indeed, the extent to which CYP1B1 is expressed and activated correlates with human breast [Watanabe et al., 2000] and ovarian [Sellers et al., 2005] cancer risk.

While the AhR is most frequently studied in the context of environmental carcinogen exposure, a growing number of studies have evaluated AhR up-regulation and constitutive activation, particularly in transformed cells, in the absence of exogenous ligands [Corton, 1996; Ma and Whitlock, 1996; Chang and Puga, 1998; Wang et al., 1999; Abdelrahim et al., 2003; Hayashibara et al., 2003; Levine-Fridman et al., 2004; Yang et al., 2005]. Relatively little is known about the function and gene targeting of constitutively active AhR, although some studies implicate the AhR in critical cell functions such as regulation of cell growth [Ma and Whitlock, 1996; Abdelrahim et al., 2003; Schlezinger et al., 2006], apoptosis [Robles et al., 2000; Caruso et al., 2006], and morphogenesis [Larsen et al., 2004]. Specifically, it is not known if constitutively active AhR effects the same transcriptional outcomes as environmental chemical-activated AhR. To address this issue, constitutively active and environmental chemical-activated AhR control of two prototypic AhR target genes, *CYP1A1* and *CYP1B1*, was studied in a rat model of mammary tumorigenesis and in immortalized human mammary epithelial cell lines that represent distinct phases in mammary tumorigenesis. These experiments demonstrate for the first time high level *AhR* and *CYP1B1* expression prior to tumor formation and confirm the correlate that *AhR* and *CYP1B1* expression is up-regulated in pre-malignant cell lines. Furthermore, results indicate that a constitutively active AhR contributes to basal *CYP1B1* but not *CYP1A1* mRNA levels. In contrast, AhR hyper-activation with an environmental chemical (2,3,7,8-tetrachlrodibenzo-*p*-dioxin) activates both genes. The results have important implications for the differential regulation of target genes in mammary tumors and for the presumption that activation of the AhR with environmental chemicals recapitulates physiologic AhR activity.

MATERIALS AND METHODS

Animal Treatment

Four-week-old female Sprague–Dawley rats were purchased from Charles River Laboratories

(Wilmington, MA). Animals were housed individually in environmentally controlled animal quarters and maintained according to NIH Guidelines and with the approval of the Boston University Institutional Animal Care and Use Committee. Rats were fed a nutritionally complete diet (AIN-75A) ad libitum and given deionized drinking water. Rats were randomized to control and DMBA-treated groups. At 56 days of age the experimental group was given 15 mg/kg DMBA in 0.2 ml sesame oil by oral gavage and the control group was given only 0.2 ml oil vehicle. Rats were observed and mammary glands palpated weekly. Nine weeks after treatment, rats were sacrificed and mammary tissue excised. Portions of freshly isolated tissue were fixed in 10% formalin, embedded in paraffin, and sectioned at 4 μm for histological (hematoxylin and eosin staining) analyses. The remainder of the tissue was snap frozen for mRNA analyses (below).

Cell Lines

The 184S, 184L, 184A1, and 184B5 cell lines were the generous gift of Dr. M. Stampfer (Stanford University). MCF-10F and BP1 cells were generously provided by Dr. J. Russo (Fox Chase). All other lines were obtained from the ATCC (Manassas, VA). The epithelial lines 184S and 184L were derived from a primary reduction mammoplasty [Stampfer, 1985; Stampfer and Bartley, 1985]. Both lines have a finite life span and senesce at approximately the 5th and 10th passage respectively. Lines 184A1 and 184B5 are immortalized, nonmalignant breast epithelial cell lines derived from benzo[*a*]pyrene (B[*a*]P)-treated 184S cells [Stampfer, 1985; Stampfer and Bartley, 1985]. MCF-10F is a spontaneously immortalized premalignant human breast epithelial cell line established from mammary tissue from a patient with fibrocystic breast disease [Soule et al., 1990]. BP1 cells were generated by treatment of MCF-10F cells with B[*a*]P [Calaf and Russo, 1993]. MDA-MB-231 is a human breast ductal carcinoma cell line established from pleural effusion [Cailleau et al., 1978]. CAMA-1 is a human breast adenocarcinoma cell line established from pleural effusion [Yu et al., 1981]. Hs578Bst is a breast myoepithelial cell line derived from the same patient as the malignant carcinosarcoma Hs578T line [Hackett et al., 1977]. MCF-10F and BP1 cells were grown in DMEM/F12 (Sigma-Aldrich, St. Louis, MO) and supplemented with 5% horse serum, 5 mg insulin, 10 μg/μl human rEGF, 100 μl/ml streptomycin, 100 U/ml penicillin, 2 mM L-glutamine, 250 μg hydrocortisone and 0.1 ml plasmosin. All other lines were grown in Dulbecco's modified Eagles medium (DMEM; Sigma-Aldrich) supplemented with 10% FCS, 5 μg/ml insulin, 100 U/ml penicillin, 50 μg/ml streptomycin, and 2 mM L-glutamine. Cells were maintained at subconfluency at 37°C in humidified air containing 10% CO_2 by splitting cultures 1:4 every 3–4 days.

Semi-Quantitative RT-PCR

Mammary tissue was frozen and pulverized into a fine powder. Total cellular RNA was isolated using RNAzol as described by the manufacturer (Leedo Medical Laboratories, Houston, TX). RNA was quantified with a spectrophotometer at ODs of 260 and 280 nm. Each RT-PCR reaction was performed with 5 μg RNA as described by the RT-PCR kit manufacturer (SuperScript Preamplification System; Gibco/BRL, Gaithersburg, MD). Equal sample loading was confirmed by comparison with the *β-actin* gene transcript.

The following cross-species reactive primers and conditions were used for rat mammary gland and human cell line studies: *AhR*: 5′-CTGGCAATGAATTTCCAAGGGAGG and 5′-CTTTCTCCAGTCTTAATCATGCG in 1.5 mM $MgCl_2$ for 30 cycles to yield a 334 base pair product; *CYP1A1*: 5′-TCTGGAGACCTTCCGGCATT and 5′-CCGTTCGCTTTCACATGCCG in 1.5 mM $MgCl_2$ for 36 cycles to yield a 260 base pair product; *CYP1B1*: 5′-TCAACCGCAACTTCAGCAACTTC and 5′AGGTGTTGGCAGTGGTGGCAT in 1.5 mM $MgCl_2$ for 36 cycles to yield a 404 base pair product; *β-actin*: 5′GTCGTCGACAACGGCTCCGGCATGTG and 5′-CATTTGTAGAAGGTGTGGTGCCAGATC, 1 mM $MgCl_2$ for 30 cycles to yield a 256 base pair product. Amplified cDNAs were electrophoresed through 3% agarose gels (3:1 NuSieve:Le agarose, FMC, Rockland, ME) and visualized by ethidium bromide staining. The number of PCR cycles was adjusted such that resulting band densities fell on the linear portion of the logarithmic amplification curve. *AhR*, *CYP1A1*, and *CYP1B1* cDNA band densities were determined by image analysis with Kodak Digital Science 1D imaging software and presented as the average ratio of experimental band density/β-actin band density ± standard error. A minimum of three

experiments was performed with each sample yielding comparable results.

For studies with human cell lines, adherent cells were lifted from tissue culture flasks by treatment with 0.25% trypsin. Viability was determined by trypan blue exclusion. Cell pellets were lysed with RNAzol (Leedo Medical Laboratories) and pipetted to collect cell lysates. After 5 min on ice, chloroform (20% of lysate volume) was added. Samples were vigorously shaken and incubated on ice for 5 min, then centrifuged for 15 min at 14,000*g* at 4°C. RNA was precipitated from the aqueous phase with isopropanol (50% of volume). Samples were centrifuged at 14,000*g* for 10 min at 4°C and the pellet washed in 75% ethanol centrifuged and washed again n 100% cold ethanol. The pellet was resuspended in DEPC-water and semi-quantitative RT-PCR was performed using cross-species reactive *AhR*, *CYP1A1*, *CYP1B1*-specific primers and human *β-actin* primers and conditions as described for rat studies.

Quantitative Real-Time PCR

RNA was extracted using either the Stratagene Mini Prep Kit (La Jolla, CA) or the Qiagen RNeasy Mini Kit (Valencia, CA) according to the manufacturers' directions. RNA was quantified by UV absorbance or with the RiboGreen reagent (Molecular Probes, Eugene, OR). First-strand cDNA was synthesized using 2 μg of each total RNA, random hexamers, and SuperscriptII reverse transcriptase according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Real-time PCR amplification mixtures (25 μl) contained 1 μl template cDNA, 2× SYBR Green I Master Mix buffer (12.5 μl; Applied Biosystems, Inc., Foster City, CA), and 1.25 μl Assays-on-Demand Gene Expression Reagent (Applied Biosystems, Inc.) for either *CYP1A1* or *CYP1B1*, or for *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) as an endogenous control. The proprietary *CYP1A1* and *GAPDH* primer sequences were obtained from Applied Biosystems, Inc. The *CYP1B1* primer sequences were: 5′-CTGCTCCTCCTCTTCACCAG-3′ and 5′-ATACAAGGCAGACGGTCCCT-3′. qPCR was performed with an ABI Prism 7000 Sequence Detection System. The initial step was for 10 min at 95°C followed by 40 cycles (95°C for 15 s, 60°C for 60 s). Background signal was eliminated and Ct values were determined using the SDS version 1.1 analysis software (Applied Biosystems, Inc.) according to the formula $2^{[Ct(GAPDH)-Ct(CYP)]}$. For data presented in Figures 3 and 7, RT reactions were performed with the PCR primers and amplification was performed in the same tube.

Transient Transfections and Luciferase Assays

The *Fundulus heteroclitus AhRR* expression vector (*AhRR*) [Karchner et al., 2002] was provided by Dr. M. Hahn (Woods Hole Oceanographic Institution). We previously demonstrated that transfection of this expression vector efficiently inhibits human *AhR* activity [Yang et al., 2005; Murray et al., 2006]. The *AhR* siRNA sequence, synthesized by Qiagen, was 5′-AAGUCGGUCUCUAUGCCGCTT-3′ as previously described [N'Diaye et al., 2006]. Control *lamin* siRNA was obtained from Qiagen. For transfections, BP1 cells (3×10^4/well) were plated in 6-well culture plates and cultured to 80% confluence. Lipofectamine 2000 transfection reagent (Invitrogen) was used according to the manufacturer's instructions. The renilla luciferase vector *phRL-TK* (0.1 μg) was co-transfected with 0.2 μg *pGL3-basic*, a control plasmid containing a minimal promoter sequence, or 0.2 μg *pGudLuc6.1* firefly luciferase reporter construct, kindly provided by Dr. M. Denison (U.C. Davis). *pGudLuc* activity is driven by four AhR response elements (AhREs/XREs) [Han et al., 2004]. Where indicated, *pcDNA-AhRR* (1.7 μg), control *pcDNA3.1* (1.7 μg; Invitrogen), *AhR* siRNA (20 μg) or control *lamin A/C* (20 μg) siRNA was added to the transfection mixture. For each experiment, the amount of total DNA transfected was equilibrated with parental expression vectors. Cells were incubated overnight, washed twice with phosphate-buffered saline (pH 7.2), and resuspended in 180 μl RPMI prior to luciferase analysis. Luciferase activity was determined with the Dual Glo Luciferase system (Promega, Madison, WI) which allowed sequential reading of the firefly and renilla signals. Cells were lysed according to the manufacturer's directions (Promega), transferred to 96-well white wall plates in triplicate, and analyzed using a Reporter Luminometer (Promega). The renilla signal was read after quenching the firefly output, thus allowing normalization between sample wells. The normalized firefly luciferase signal is expressed relative to the renilla signal. Reporter activity in *pGL3-basic*-transfected

cells did not differ significantly from background levels.

GFP-AhRR Lentivirus Transduced MCF-10F

The *AhRR* vector was cloned into a GFP-expressing HPV570 lentivirus plasmid and transfected, along with plasmids essential to virus assembly, into the 293T/17 packaging line. Infectious supernatants containing the *AhRR-GFP*-lentivirus, or a control *GFP*-lentivirus, were used to transduce MCF-10F cells. Briefly, 10^4 cells were added to each well of a 6-well plate and incubated overnight to reach 40–50% confluency. On the day of transduction, viral solution was diluted 1:10 in DMEM. Culture medium was changed 1–2 h prior to transduction. Prepared virus solution (1 ml) was added to each well along with 1 μl of 6 mg/ml hexadimethyrine bromide (polybrene; Sigma-Aldrich). Cells then were incubated at 37°C at 10% CO_2 for 5 h and virus solution was discarded. Cells were washed extensively and cultured as above. Cells expressing high levels of GFP were obtained by sorting in a DAKO MoFlo cell sorter operated by the Boston University Medical Campus Flow Cytometry Core Facility and cells subsequently maintained as long-term stable lines. Western blotting using AhRR-specific antibody (provided by Dr. Hahn) confirmed successful *AhRR* transfection and expression (not shown). Cells were regularly monitored by flow cytometry for GFP fluorescence and periodically resorted to assure high level GFP and AhRR expression.

Chromatin Immunoprecipitation (ChIP)

ChIP and DNA quantification were performed as we previously described [Hestermann and Brown, 2003; Yang et al., 2005]. ChIP DNA (5 μl) was amplified by PCR for the *CYP1A1* promoter with primers 5′-CGGCCCCGGCTCTCT-3′ and 5′-GTGTCGGAAGGTCTCCCAGGAT-3′ for 40 cycles at 95° for 15 s and 58° for 1 min. For the *CYP1B1* promoter, the region 790–890 bp upstream of the start site was amplified using the primers 5′-ATATGACTGGAGCCGACTTTCC-3′ and 5′-GGCGAACTTTATCGGGTTGA-3′ under the same amplification protocol. In our hands, DNA is sheared within approximately 1 kb of the amplified fragment. Therefore, ChIP detects protein-bound DNA within approximately 1 kb of the region spanned by the primers. For quantification, equal amounts of DNA from each sample were amplified by real-time PCR using the same primers with SYBR Green master mix (Applied Biosystems, Inc.) in a DNA Engine Opticon 2 (MJ Research, Waltham, MA). Recovery of *CYP1A1* or *CYP1B1* regulatory regions in immunoprecipitated samples was normalized to the recovery of non-specific DNA from input controls ($2^{-\Delta\Delta CT}$).

TCDD Treatment

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) was obtained from UltraScientific (Kingston, RI) at >99% purity and was maintained as a 1,000× stock solution in anhydrous tissue culture grade dimethylsulfoxide (DMSO). TCDD (10^{-8} M) in DMSO or DMSO alone (final volume 0.1%) was added to cells grown to 90% confluence in 150 mm dishes. Cells were harvested 45 min later for ChIP studies and 4 h later for mRNA quantification.

Data Analyses

Statistical analyses were performed with Statview (SAS Institute, Cary, NC) or Excel. Data from a minimum of three experiments are presented as means ± standard errors (SE). One-factor ANOVAs and a Fisher PLSD post hoc comparisons test or the Student's *T*-test were used to determine significant differences.

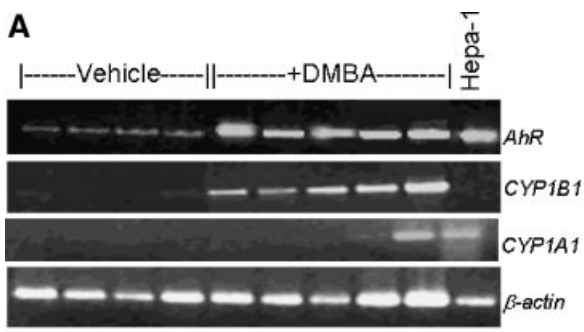
RESULTS

Up-Regulation of *AhR* and *CYP1B1* But Not *CYP1A1* mRNA Occurs Prior to DMBA-Induced Tumor Formation

Our previous studies demonstrated that DMBA-induced rat and mouse mammary tumors express extremely high AhR levels [Trombino et al., 2000; Currier et al., 2005]. Nuclear AhR localization in these tumors suggested constitutive AhR activation. High level *CYP1B1* mRNA expression in PAH-induced rodent tumors and in primary "spontaneous" human breast tumors [Murray et al., 1997; Maecker et al., 2003; Schlezinger et al., 2006] suggested that CYP1B1, as an activator of the 17β-estradiol procarcinogen, may contribute to early stages of mammary epithelial cell transformation. If this were the case, then it would be predicted that elevated *CYP1B1* levels would precede overt tumor formation. To test this prediction, female Sprague–Dawley rats were treated by a single oral gavage with 15 mg/kg DMBA (five rats) or oil vehicle (four rats).

Nine weeks later rats were sacrificed and mammary glands excised. Whole tissue was analyzed histologically for tumor formation and extracts were evaluated by semi-quantitative RT-PCR for *AhR* and *CYP1B1* mRNA levels.

No palpable mammary tumors were detected in either the control or DMBA-treated groups (not shown). Of the five mammary tissue samples from DMBA-treated rats, none showed histologic evidence of tumors. This result was not surprising given our finding that rats treated with 15 mg/kg DMBA typically do not develop mammary tumors until approximately 15–16 weeks after DMBA gavage [Trombino et al., 2000]. Despite the absence of detectable numbers of tumor cells, *AhR* mRNA was significantly elevated approximately threefold (Fig. 1A, first row; Fig. 1B, $P < 0.05$). This result suggests that AhR up-regulation represents a field effect that occurs prior to overt tumor formation. Coincident with AhR up-regulation, a significant ~12-fold increase in *CYP1B1* was noted (Fig. 1A, second row; Fig. 1B, $P < 0.01$).



B

	β-Actin Normalized mRNA	
Gene	Vehicle	DMBA
AhR	0.36 ± 0.01	0.97 ± 0.11*
CYP1B1	0.05 ± 0.03	0.69 ± 0.10*

Fig. 1. Up-regulation of *AhR* and *CYP1B1* but not *CYP1A1* mRNA occurs prior to DMBA-induced tumor formation. Female Sprague–Dawley rats were treated by oral gavage with vehicle (sesame oil) (four rats) or 15 mg/kg DMBA in vehicle (five rats). Nine weeks later rats were sacrificed, mammary glands excised, and evaluated histologically for tumor formation. No tumors were palpable at this time and histologic analysis did not reveal the presence of microtumors. Mammary gland RNA extracts also were evaluated by semi-quantitative RT-PCR for *AhR*, *CYP1B1*, *CYP1A1*, and as a control, *β-actin* gene expression. **A**: Representative data from one of three experiments of *AhR*, *CYP1B1*, *CYP1A1*, and *β-actin* expression. **B**: *AhR*, *CYP1B1*, and *CYP1A1* band densities normalized to *β-actin* band densities. Data pooled from three experiments are expressed as means ± standard errors. An asterisk (*) indicates a significant difference in gene expression in mammary tissue from vehicle-treated as compared with DMBA-treated rats, $P < 0.05$.

Since DMBA administered by oral gavage is metabolized and eliminated within 5 days [Lee et al., 1986], increases in *CYP1B1* at the 9-week time point are unlikely to result from continuous DMBA-dependent AhR activation. These results are consistent with the hypothesis that AhR up-regulation results in increased baseline *CYP1B1* levels and they suggest that co-ordinate up-regulation of *AhR* and *CYP1B1* precedes tumor formation and may represent an early marker in the transformation process.

Treatment of rats with DMBA by oral gavage results in a rapid (within 6 h) up-regulation of both *CYP1A1* and *CYP1B1* in breast tissue [Trombino et al., 2000]. In contrast, DMBA-induced breast tumors constitutively express only *CYP1B1* [Trombino et al., 2000], suggesting differential regulation of these two AhR gene targets when the AhR is acutely activated by exogenous ligands, as opposed to when it is constitutively active. To determine if the relative levels of *CYP1A1* and *CYP1B1* expression prior to tumor formation, but well after DMBA elimination, resemble relative *CYP1A1* and *CYP1B1* levels in mammary tumors (i.e., high *CYP1B1*, little or no *CYP1A1*) or untransformed mammary tissue shortly after PAH exposure (i.e., high levels of both *CYP1A1* and *CYP1B1*), mammary tissue from rats treated 9 weeks previously with vehicle or DMBA was evaluated for *CYP1A1* expression. RNA extract from the murine hepatoma, Hepa-1, was used as a positive control for *CYP1A1* detection. Significant *CYP1A1* levels were detected in only one of the five mammary gland samples expressing high *AhR* and *CYP1B1* levels (Fig. 1A, third row). These results suggest a transition from DMBA-dependent co-induction of *CYP1A1* and *CYP1B1* in short term, 6-h experiments to preferential up-regulation of only *CYP1B1* at 9 weeks, as is found in breast tumors.

Preferential Up-Regulation of *CYP1B1* But Not *CYP1A1* mRNA in Immortalized or Malignant Human Breast Cell Lines

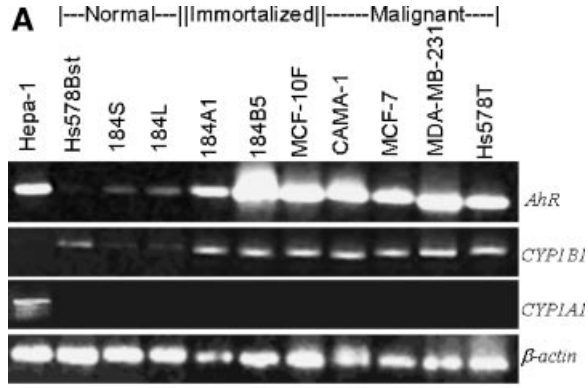
Results in the rat system indicate a transition from up-regulation of both *CYP1A1* and *CYP1B1* to up-regulation of just *CYP1B1* during malignant PAH-induced carcinogenesis. To determine if a similar pattern of *AhR*, *CYP1B1*, and *CYP1A1* expression is manifest in human cells immortalized/transformed with PAH or in cells "spontaneously" immortalized/transformed

in the absence of PAH, expression of these genes in a panel of untransformed ("normal"), immortalized but non-malignant, and malignant human breast-derived cell lines was evaluated.

The cell lines selected were representative of human mammary cells at various points in transformation. With the exception of Hs578Bst, a breast myoepithelial cell line, and Hs578T, a carcinosarcoma (mixed epithelial and soft tissue carcinoma) derived from the same patient as the Hs578Bst cells [Hackett et al., 1977], all lines were epithelial in origin. The 184A1 and 185B5 lines were immortalized by treatment of 184S cells with B[a]P [Walen and Stampfer, 1989], a PAH carcinogen and an AhR agonist. All other lines were established without in vitro carcinogen exposure.

Low but detectable *AhR* mRNA levels were observed in the finite life-span Hs578Bst, 184S, and 184L cell lines (Fig. 2). *AhR* expression was significantly increased approximately 10-fold in immortalized but non-malignant 184A1, 184B5, and MCF-10F cells and approximately 8-fold in the fully malignant CAMA-1, MCF-7, MDA-MB-231, and Hs578T cell lines (Fig. 2A, top row; Fig. 2B, $P < 0.05$). No significant differences were observed between estrogen receptor positive (CAMA-1, MCF-7) and negative (MDA-MB-231, Hs578T) cell lines. Furthermore, no differences were observed between the immortalized but non-malignant and the fully malignant cell lines suggesting, as in the rat model, that the commitment to high level *AhR* expression occurs prior to full malignancy. Furthermore, *AhR* up-regulation marks both PAH-induced and "spontaneous" tumors, suggesting that the AhR plays an important role in tumorigenesis regardless of tumor etiology. These data are consistent with previous studies with 184A1, MDA-MB-231, and other human mammary epithelial cell lines demonstrating high *AhR* mRNA levels in immortalized and malignant mammary epithelial cell lines [Spink et al., 1998].

As in the rodent system, AhR up-regulation correlated with significant increases in *CYP1B1* (Fig. 2A, second row, Fig. 2B, $P < 0.05$). Little or no *CYP1A1* was detected in either the immortalized or fully malignant lines (Fig. 2A, third row). To more accurately quantify the baseline levels of *CYP1A1* and *CYP1B1* in immortalized or malignant cell lines, quantitative PCR was performed on extracts from immortalized MCF-10F cells and from malignant BP1 cells. The



B

Gene	β-Actin Normalized mRNA		
	Normal	Immortalized	Malignant
AhR	0.10 ± 0.03	1.06 ± 0.34*	0.8 ± 0.06*
CYP1B1	0.45 ± 0.12	2.11 ± 0.34*	1.75 ± 0.02*

Fig. 2. *CYP1B1* but not *CYP1A1* mRNA is increased in human mammary cell lines. RNA was extracted from subconfluent hepatoma cells (Hepa-1), limited life-span (''normal''), immortalized, and malignant mammary gland cell lines and evaluated by semi-quantitative RT-PCR for *AhR, CYP1B1, CYP1A1,* and *β-actin* gene expression as described in Materials and Methods Section. **A**: Representative data from one of three experiments of *AhR, CYP1B1, CYP1A1,* and *β-actin* expression. **B**: *AhR, CYP1B1, CYP1A1* band densities normalized to *β-actin* band densities. Data pooled from three experiments are expressed as means ± standard errors. An asterisk (*) indicates a significant difference between gene expression in mammary tissue from vehicle-treated as compared with DMBA-treated rats, $P < 0.05$.

invasive, malignant BP1 line was generated by transformation of non-invasive, non-malignant MCF-10F cells with B[a]P [Calaf and Russo, 1993]. Consistent with the semi-quantitative data, little or no *CYP1A1* was detected in these lines (Fig. 3). In contrast, readily detectable baseline *CYP1B1* levels were observed in both lines, a result consistent with differential regulation of these two gene targets by constitutively active AhR.

Constitutively Active AhR Maintains High Baseline Levels of *CYP1B1* in Mammary Epithelial Cell Lines

Since elevated *CYP1B1* and *AhR* levels correlated in the rat and human model systems, two approaches were taken to determine if constitutively active AhR drives *CYP1B1* transcription. First, *CYP1B1* levels were quantified in MCF-10F or BP1 cells either stably transduced or transiently transfected with a plasmid encoding an AhR repressor (AhRR) which we have shown significantly decreases AhR activity in human mammary tumor cells [Yang et al., 2005; Murray et al., 2006]. In the second

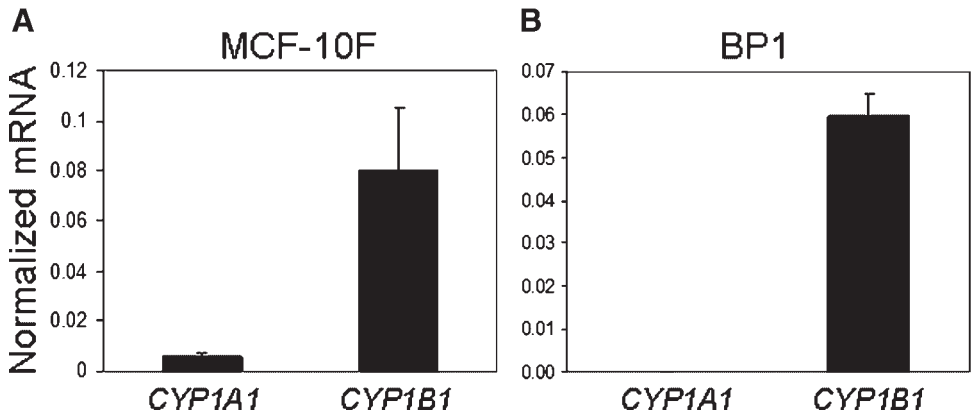


Fig. 3. Preferential expression of *CYP1B1* in immortalized (MCF-10F) and DMBA-transformed, malignant (BP1) human breast epithelial cell lines. RNA was extracted from vehicle-treated MCF-10F (**A**) and BP1 (**B**) cells and evaluated by qPCR for *CYP1A1* and *CYP1B1* expression. *GAPDH* was used as an internal control. Data pooled from three experiments are presented as the means of *GAPDH*-normalized *CYP1A1* or *CYP1B1* mRNA levels + standard errors.

approach, *CYP1B1* levels were quantified in BP1 cells transiently transfected with *AhR*-specific siRNA. In both cases, the objective was to determine if baseline *CYP1B1* levels decrease with a decrease in constitutive AhR activity or expression.

To monitor the efficacy of transfected *AhRR*, MCF-10F cells, stably transduced with control *GFP*- or *AhRR-GFP*-expressing lentivirus vectors, or BP1 cells transiently transfected with control plasmid (*pCS2*) or *AhRR* were co-transfected with *pGL3-basic*, a minimal luciferase reporter construct, or *pGudLuc*, a luciferase reporter driven by multiple AhREs, and renilla luciferase vector *phRL-TK*. Cells were harvested 24 h later and lysates assayed for firefly and renilla luciferase activity.

pGL3-basic reporter activity did not differ significantly from background levels and consistently represented less than 0.25 units of renilla luciferase-normalized activity (not shown). In contrast, significant background *pGudLuc* luciferase activity, an indicator of constitutive AhR activity, was observed in control *GFP*-transduced MCF-10F or *pCS2*-transfected BP1 cells (Fig. 4A,C, left bars). These data are consistent with previous experiments demonstrating constitutive AhR (*pGudLuc* luciferase) activity in Hs578T cells [Yang et al., 2005; Murray et al., 2006]. Stable transduction of MCF-10F cells with *AhRR* or transient transfection of BP1 cells with *AhRR* significantly reduced *pGudLuc* activity in both lines (Fig. 4A,C, right bars), again demonstrating a background level of AhR transcriptional activity. A proportional decrease in *CYP1B1* mRNA levels was observed concomitant with *AhRR*-mediated AhR down-regulation in both cell lines (Fig. 4B,D).

Similarly, in a second series of experiments, a considerable background of *pGudLuc* activity was seen in BP1 cells transiently transfected with control *lamin*-specific siRNA (Fig. 5A, left bar). Transfection with *AhR*-specific siRNA significantly inhibited *pGudLuc* activity (Fig. 5A, right bar) and proportionally decreased *CYP1B1* expression (Fig. 5B, right bar). The data obtained with the *AhRR* plasmid and *AhR*-specific siRNA demonstrate that maximal levels of baseline *CYP1B1* expression are dependent on constitutively active AhR in both immortalized, non-malignant MCF-10F and in fully malignant BP1 cells.

AhR Constitutively Binds to *CYP1A1* and *CYP1B1* Promoters in Immortalized MCF-10F and Malignant BP1 Human Breast Epithelial Cell Lines

Data presented in Figures 1–3 demonstrate preferential expression of *CYP1B1* as compared with *CYP1A1* mRNA. Since constitutively active AhR contributes to baseline *CYP1B1* levels in at least two human mammary epithelial cell lines (MCF-10F and BP1; Figs. 4 and 5), the results collectively raise the question of how constitutively active AhR differentially regulates these two similar gene targets in immortalized/malignant mammary cell lines. To determine if preferential *CYP1B1* expression reflects preferential binding of constitutively active AhR to the *CYP1B1* gene promoter,

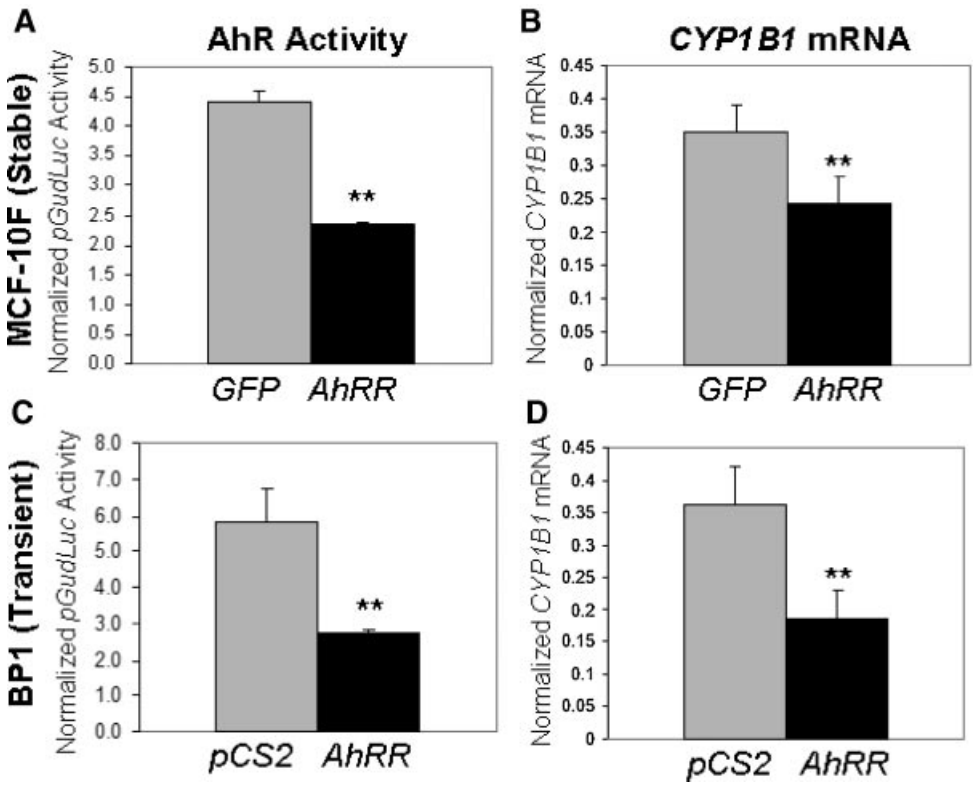


Fig. 4. Suppression of constitutively active AhR with AhRR down-regulates high baseline *CYP1B1* levels. MCF-10F cells were stably transduced with a lentivirus construct encoding GFP alone or a construct encoding both GFP and *Fundulus heteroclitus*-derived AhRR. BP1 cells were transiently transfected with control vector (*pCS2*) or vector encoding AhRR. Subconfluent MCF-10F (**A**) or BP1 (**C**) cells were transfected with 0.1 μg of a control vector (*pGL3-basic*) or with 0.1 μg of an AhRE-driven firefly luciferase gene (*pGudLuc*) and 0.5 μg of the renilla-luciferase vector *phRL-TK*. Cells were harvested 24 h later and lysates assayed for firefly and renilla luciferase activity. Subconfluent layers of MCF-10F (**B**) or BP1 (**D**) cells were harvested, RNA extracted, and *CYP1B1* mRNA levels determined by qPCR. Data pooled from four to fourteen experiments are expressed as *GAPDH*-normalized means + standard errors. A double asterisk (**) indicates a significant decrease in renilla-normalized *pGudLuc* activity (A,C) or in GAPDH-normalized *CYP1B1* mRNA levels (B,D), $P < 0.02$.

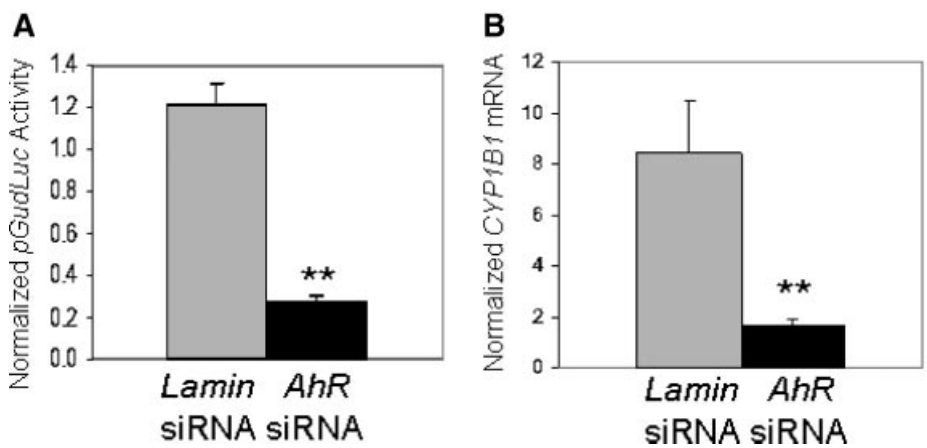


Fig. 5. Suppression of constitutively active AhR with AhR-specific siRNA in BP1 cells down-regulates baseline *CYP1B1* levels. BP1 cells were transfected with 20 μg *lamin*- or *AhR*-specific siRNA with (**A**) or without (**B**) 0.2 μg *pGudLuc* and 0.1 μg *phRL-TK*. Cells were harvested 24 h later and lysates assayed for firefly and renilla luciferase activity (A) or for *CYP1B1* and *GAPDH* expression (B). Data are presented as *GAPDH*-normalized means + standard errors from six (A) or seven (B) experiments. A double asterisk (**) indicates a significant decrease in renilla-normalized *pGudLuc* activity or *GAPDH*-normalized *CYP1B1* expression, $P < 0.001$.

ChIP assays were performed with extracts from MCF-10F and BP1 cells and using AhR-specific antibody and *CYP1A1* and *CYP1B1*-specific primers.

Significant constitutive binding of AhR to both *CYP1A1* and *CYP1B1* was seen in MCF-10F (Fig. 6A) and BP1 (Fig. 6B) cells. Although one cannot directly compare the *CYP1A1* and *CYP1B1* signals in anti-AhR antibody immunoprecipitates from a given cell line because of different primer efficiencies, it is possible to conclude that there is, relative to *CYP1A1*-bound AhR, a higher level of *CYP1B1*-bound AhR in malignant BP1 cells than in non-malignant MCF-10F cells and that there is an overall higher signal for both gene targets in BP1 cells as compared with MCF-10F cells. (Note the difference in scales in Fig. 6A,B.) These data indicate that it is unlikely that the level of promoter binding alone by constitutively active AhR accounts for preferential *CYP1B1* expression in both lines.

Hyper-Activation of the AhR With an Environmental Agonist Increases AhR Binding to Both *CYP1A1* and *CYP1B1* Promoters But Does Not Necessarily Increase Gene Expression

Since AhR activation with environmental ligands such as TCDD generally results in induction of both *CYP1A1* and *CYP1B1*, the preferential increase in *CYP1B1* by constitutively active AhR in human cell lines could reflect either differences in gene targeting by environmental ligand-activated and constitutively activated AhR or an inherent characteristic of AhR regulation in immortalized/transformed cells. To distinguish between these two possibilities, the ability of TCDD, a potent environmental AhR agonist, to up-regulate AhR-*CYP1* promoter binding and *CYP1* mRNA expression in immortalized/transformed cells was evaluated. To this end, MCF-10F and BP1 cells were treated with vehicle or 10^{-8} M TCDD and assayed 45 min later for AhR-*CYP1A1* and -*CYP1B1* promoter occupancy by ChIP and 4 h later for *CYP1A1* and *CYP1B1* mRNA levels by qPCR.

AhR binding to both gene promoters in both cell lines increased significantly after TCDD treatment (Fig. 7A,C), demonstrating that the AhR in both the non-malignant and malignant cells is responsive to an environmental chemical. For both cell lines, TCDD significantly increased *CYP1A1* mRNA levels (Fig. 7B,D), although the induced *CYP1A1* levels in BP1 cells were extremely low (Fig. 7D inset). These results indicate that there is nothing inherently defective in the AhR in these cell lines that precludes it from transactivating the *CYP1A1* gene. Interestingly, while TCDD induced *CYP1B1* levels in BP1 cells (Fig. 7D) in parallel with increased AhR-*CYP1B1* promoter binding (Fig. 7C), it did not increase *CYP1B1* mRNA levels in MCF-10F cells (Fig. 7B), despite the profound increase in AhR-*CYP1B1* promoter binding in MCF-10F cells (Fig. 7A). If anything, TCDD treatment tended to decrease *CYP1B1* levels in MCF-10F cells, although statistical significance was not reached. These results suggest that AhR activation with an environmental chemical may not always mimic gene regulation by constitutively active AhR, at least

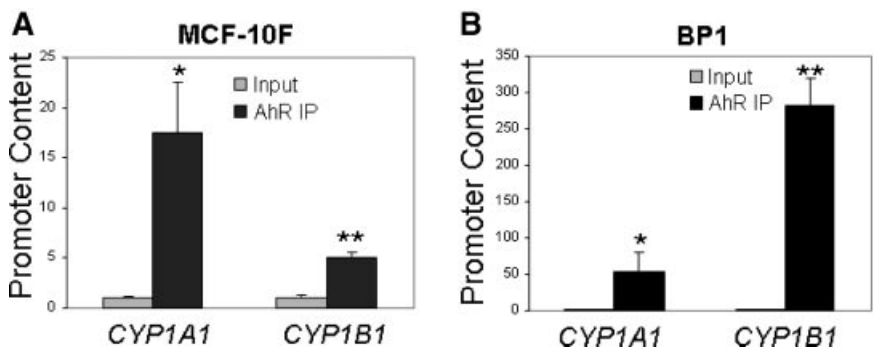


Fig. 6. AhR constitutively binds to *CYP1A1* and *CYP1B1* promoters in immortalized and B[*a*]P-transformed human breast epithelial cell lines. Subconfluent MCF-10F and BP1 cells were harvested and ChIP assays performed as described in Materials and Methods Section using AhR-specific antibody for immunoprecipitation and *CYP1A1*- and *CYP1B1*-specific primers to amplify the respective gene promoters. Data pooled from three experiments are presented as means + standard errors. An asterisk (*) indicates a significant increase in promoter content of AhR antibody precipitated samples as compared with input fractions, $P < 0.05$. A double asterisk (**) indicates a significant increase, $P < 0.01$.

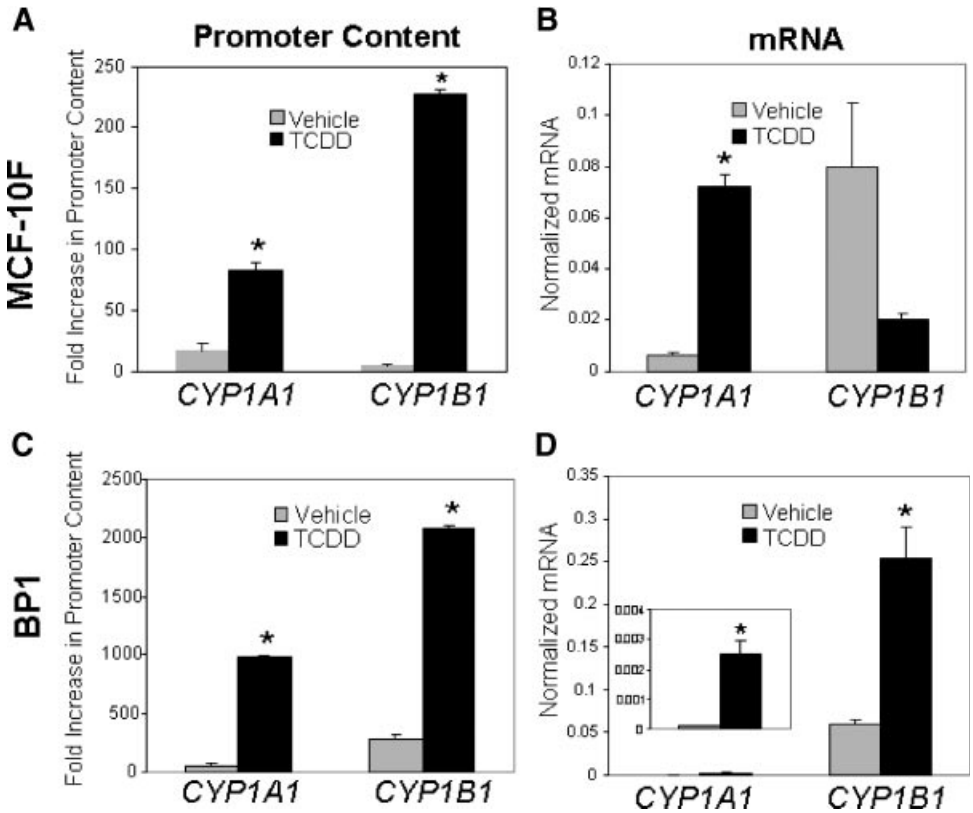


Fig. 7. Hyper-activation of the AhR with TCDD increases AhR binding to both *CYP1A1* and *CYP1B1* promoters but does not necessarily increase gene expression. Subconfluent monolayers of MCF-10F and BP1 cells were treated with vehicle (DMSO) or 10^{-8} M TCDD. Aliquots of cells were harvested 45 min later and evaluated by ChIP for AhR binding to *CYP1A1* and *CYP1B1* promoters (**A**,**C**) or 4 h later and evaluated by qPCR for *CYP1A1* and *CYP1B1* mRNA levels (**B**,**D**). (The *CYP1A1* and *CYP1B1* mRNA levels in DMSO-treated cells also were presented in Fig. 3 to facilitate comparison of baseline gene expression levels.) Data pooled from three experiments are presented as means of promoter content + standard errors or *GAPDH*-normalized means + standard errors. An asterisk indicates a significant change in either promoter content or *GAPDH*-normalized *CYP1* mRNA levels, $P < 0.05$.

in immortalized cells, and that the preferential augmentation of *CYP1B1* by constitutively active AhR likely reflects qualitative differences between environmental chemical-activated and constitutively active AhR.

DISCUSSION

Historically, the AhR has been studied in the context of its up-regulation of genes encoding cytochrome P450 enzymes in response to environmental chemicals and some potential endogenous substrates [Heath-Pagliuso et al., 1998; Phelan et al., 1998; Burdick et al., 2003; Spink et al., 2003a; Henry et al., 2006; Burchiel et al., 2007]. Some of the resulting metabolites modulate important mammary epithelial cell functions including cell growth and death [Burdick et al., 2003, 2006]. However, more recent work has revealed that the AhR may be constitutively active, either in developing tissue or in tumors [Ma and Whitlock, 1996; Singh et al., 1996; Chang and Puga, 1998; Shehin et al., 2000; Hayashibara et al., 2003; Ito et al., 2004; Murray et al., 2005]. Tumors generally are characterized by an increase in total *AhR* mRNA levels [Spink et al., 1998], a tendency for AhR to localize to the nucleus [Trombino et al., 2000; Yang et al., 2005; Murray et al., 2006], and a concomitant increase in at least one AhR target, *CYP1B1* [Spink et al., 1998; Shehin et al., 2000]. This high level AhR expression appears to occur in "spontaneous" human mammary tumor cells lines [Spink et al., 1998; Yang et al., 2005], rodent tumors induced with *N*-methyl-*N*-nitrosourea [Chan et al., 2005], and primary lung adenocarcinomas [Chang et al., 2007], suggesting that AhR up-regulation may characterize many tumors regardless of etiology and possibly regardless of tumor type.

Evaluation of histologically normal mammary glands 9 weeks after DMBA gavage revealed a modest but significant increase in *AhR* mRNA expression (Fig. 1). A corresponding increase in *CYP1B1* further exemplified the association between elevated *AhR* and *CYP1B1* expression. Since extensive histological analyses of these glands failed to detect microtumors, *AhR* and *CYP1B1* up-regulation appeared to represent a more global "field effect" induced by PAH exposure in which the mammary gland microenvironment may be more conducive to the outgrowth and/or progression of mammary tumors [Petersen et al., 2003]. This postulated field effect is consistent with our immunohistochemical studies of DMBA-induced rat tumors in which both neoplastic epithelial cells and surrounding fibroblast-like cells in the tumor microenvironment were shown to express elevated levels of AhR with nuclear localization [Trombino et al., 2000].

The elevated levels of *AhR* and *CYP1B1* mRNA expression or AhR activity seen in the present study (Fig. 2) and elsewhere [Spink et al., 1998] in immortalized but nontumorigenic human mammary tumor cell lines is consistent with *AhR* and *CYP1B1* up-regulation prior to full malignancy. At the very least, the up-regulation of *AhR* and *CYP1B1* prior to overt malignancy suggests two biomarkers of early mammary tumorigenesis. The observation that CYP1B1 is up-regulated in most if not all human cancers [Maecker et al., 2003], its ability to regulate cell metabolism [Hayes et al., 1996; Liehr and Ricci, 1996; Belous et al., 2007; Chambers et al., 2007], including its capacity to generate mutagenic metabolites from both endogenous and exogenous substrates [Liehr et al., 1995] and to regulate retinol metabolism [Chambers et al., 2007], and its association with increased cancer risk [Watanabe et al., 2000; Sellers et al., 2005], underscore the importance of defining the mechanisms through which CYP1B1 expression is modulated during tumorigenesis. Although several factors likely regulate *CYP1B1* transcription, including endogenous estrogen levels [Christou et al., 1995], BRCA1 activity [Chang et al., 2007], and *CYP1B1* promoter methylation status [Tokizane et al., 2005], the presence of seven AhR binding sites within 1,500 base pairs of the human *CYP1B1* start site and the aforementioned high levels of both constitutively active AhR and CYP1B1 in tumors, suggests that the AhR participates in the maintenance of high baseline *CYP1B1* levels in tumors. This hypothesis is strongly supported by: (1) the demonstration that inhibition of AhR expression or activity with *AhR*-specific siRNA or AhRR, respectively, proportionally reduces baseline *CYP1B1* expression, and (2) the constitutive association of the AhR with the *CYP1B1* promoter in both the immortalized MCF-10F line and its PAH-transformed counterpart, BP1. That AhR regulates *CYP1B1* in other cancers is supported by the demonstration that AhR down-regulation in a lung adenocarcinoma line similarly reduces *CYP1B1* expression [Chang et al., 2007]. Interestingly, AhR down-regulation also was associated with the loss of anchorage-independent growth [Chang et al., 2007], suggesting that AhR target genes play an important role in tumor progression. Our data similarly suggest that AhR inhibition suppresses mammary tumor growth in Matrigel (data not shown).

While constitutive association of the AhR with *CYP1B1* likely promotes *CYP1B1* transcription, AhR association with the *CYP1A1* promoter is not sufficient to drive *CYP1A1* transcription in the human tumor cell lines, despite the presence of five AhREs within 1,500 base pairs of the human *CYP1A1* start site. That is, little or no *CYP1A1* mRNA was detected in any of the untreated normal, immortalized, or malignant cell lines evaluated herein. Similarly, little or no *CYP1A1* was detected in primary DMBA-induced rat [Trombino et al., 2000] or mouse [Currier et al., 2005] mammary tumors. While the molecular mechanism(s) through which the AhR may preferentially transactivate *CYP1B1* is still under investigation, it seems likely that this qualitative control of AhR transcriptional activity is mediated in part by differential recruitment of a growing list of co-regulators that interact with the AhR complex. These factors include the non-p160 family co-activator RIP 140 [Kumar et al., 1999; Nguyen et al., 1999], p160 family members with histone acetyltransferase activity [Beischlag et al., 2002; Hestermann and Brown, 2003], ATPase-dependent histone modifiers [Wang and Hankinson, 2002], mediator adapter complex proteins [Wang et al., 2004], co-repressors such as HDAC-1 or SMRT [Nguyen et al., 1999; Wang et al., 2004], and general transcription factors such as TFIIB [Swanson and Yang, 1998]. This is not to say that the level

of AhR-*CYP1A1* promoter complexing never contributes to control of *CYP1A1* transcription. Indeed, we previously demonstrated that, using the same ChIP conditions employed herein, constitutively active AhR does not associate with the *CYP1A1* promoter in the Hs578T mammary carcinosarcoma cell line to any appreciable extent [Yang et al., 2005] and hence, no *CYP1A1* transcript is detectable in that line (Fig. 2).

Finally, results presented here raise the question of whether activation of the AhR with environmental ligands accurately models the "physiologic" in vivo function of this receptor/transcription factor. The contrast between the significant induction of both *CYP1A1* and *CYP1B1* in mammary tissue 6 h after DMBA exposure [Trombino et al., 2000] and the preferential up-regulation of *CYP1B1* but generally not *CYP1A1* 9 weeks later (Fig. 1) or in tumors [Trombino et al., 2000], suggests that environmental chemical-liganded AhR does not necessarily behave in the same fashion as constitutively active AhR. This conclusion is further supported by the absence of *CYP1A1* transcript in untreated MCF-10F or BP1 cells but an increase in AhR-*CYP1A1* promoter binding and *CYP1A1* transcription in both lines after TCDD exposure (Fig. 7). These results demonstrate that there is nothing inherently defective in the ability of the AhR complex present in immortalized or fully transformed cells that prevents it from binding to and transactivating the *CYP1A1* gene. Importantly, even the target gene preference of TCDD-activated AhR may differ from mammary gland cell line to cell line according to estrogen receptor status and estrogen availability [Christou et al., 1995; Spink et al., 1998, 2003b]. These important results imply that AhR activation with environmental chemicals may have diverse outcomes that may or may not represent the endogenous activity of the AhR in normal or malignantly transformed cells. Collectively, the data suggest that the transcriptional specificity of constitutively active and environmental chemical-activated AhR is regulated by cofactors associated with the AhR complex and that it is these cofactors which dictate the physiologic function of the AhR during mammary tumorigenesis.

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