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Expression of the aryl hydrocarbon receptor is not required for the proliferation, migration, invasion, or estrogen-dependent tumorigenesis of MCF-7 breast cancer cells

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Abstract

The AhR was initially identified as a ligand-activated transcription factor mediating effects of chlorinated dioxins and polycyclic aromatic hydrocarbons on cytochrome P450 1 (CYP1) expression. Recently, evidence supporting involvement of the AhR in cell-cycle regulation and tumorigenesis has been presented. To further define the roles of the AhR in cancer, we investigated the effects of AhR expression on cell proliferation, migration, invasion, and tumorigenesis of MCF-7 human breast cancer cells. In these studies, the properties of MCF-7 cells were compared with those of two MCF-7-derived sublines: AH^{R100}, which express minimal AhR, and AhR^{exp}, which overexpress AhR. Quantitative PCR, Western immunoblots, 17β-estradiol (E_2) metabolism assays, and ethoxyresorufin *O*-deethylase assays showed the lack of AhR expression and AhR-regulated CYP1 expression in AH^{R100} cells, and enhanced AhR and CYP1 expression in AhR^{exp} cells. In the presence of 1 nM E_2, rates of cell proliferation of the three cell lines showed an inverse correlation with the levels of AhR mRNA. In comparison with MCF-7 and AhR^{exp} cells, AH^{R100} cells produced more colonies in soft agar and showed enhanced migration and invasion in chamber assays with E_2 as the chemoattractant. Despite the lack of significant AhR expression, AH^{R100} cells retained the ability to form tumors in severe combined immunodeficient mice when supplemented with E_2, producing mean tumor volumes comparable to those observed with MCF-7 cells. These studies indicate that, while CYP1 expression and inducibility are highly dependent on AhR expression, the proliferation, invasion, migration, anchorage-independent growth, and estrogen-stimulated tumor formation of MCF-7 cells do not require the AhR.

Keywords

Aryl hydrocarbon receptor; breast cancer; MCF-7 xenograft

INTRODUCTION

The aryl hydrocarbon receptor (AhR) is a transcription factor that is activated by a variety of environmental, dietary, and endogenous ligands [1,2]. Xenobiotic AhR ligands include

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benzo(*a*)pyrene (BaP) and other polycyclic aromatic hydrocarbons (PAH), and halogenated aromatic hydrocarbons, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). AhR agonist and antagonist activities have been attributed to a number of dietary components, including various flavonoids [1], and a number of endogenous compounds, notably indole derivatives [3-8]. The ligand-bound AhR, in conjunction with its heterodimerization partner, the aryl hydrocarbon nuclear transporter, facilitates transcription of a number of genes including those of cytochromes P450 of the CYP1 family, which catalyze the metabolism of various xenobiotics and endogenous substrates [9]. While the concept of physiologically relevant AhR signaling involving an endogenous ligand has yet to be universally accepted, the rapid, AhR-mediated induction of CYP1A1 observed when cells were placed in suspension [10] established that the AhR can be activated in the absence of an exogenous ligand [10-13]. The recently reported activation of the AhR by the tryptophan-derived ligand, 6-formylindolo[3,2-*b*]carbazole, in response to ultraviolet irradiation supports the existence of a physiologic mechanism that ultimately leads to AhR-mediated activation of gene transcription [14].

In recent years, functions of the AhR in addition to those relating to the transcriptional regulation of genes encoding phase I and II enzymes have been identified [15-17]. Evidence supporting involvement of the AhR in immune regulation [18-21], control of the cell-cycle and cell proliferation [22-24], cross-talk of hormone and growth-factor signaling pathways, and physical interactions with other nuclear receptors impacting their function and their rates of degradation has been presented [24-26]. Many of the roles of the AhR that have been characterized are dependent on the context of specific tissue and cell type and the presence or absence of specific AhR ligands [27,28]. In MCF-7 human breast cancer cells, nuclear localization of the AhR subsequent to exposure to ligands increases AhR interaction with retinoblastoma protein [29] and induces cell-cycle arrest [30].

The involvement of the AhR in cancer has traditionally been viewed in terms of its role in the regulation of expression of the carcinogen-bioactivating CYP1 enzymes, leading to adductive and oxidative DNA damage and resulting in mutagenesis in the initiation phase of the disease [9,31]. In recent studies, additional roles of the AhR in the promotion and progression stages of breast cancer have been proposed; however, they are not consistent, particularly with regard to the role of the AhR in development of the invasive phenotype [27,32]. In light of the conflicting roles of the AhR that have been proposed in breast cancer, this study was undertaken to determine the involvement of the AhR in the post-initiation phases of estrogen receptor (ER)-positive breast cancer. To further define the roles of the AhR in human breast cancer, we used MCF-7 cells, which express ERα and are classified as luminal A breast cancer, and two MCF-7-derived sublines: AH^{R100} cells, which express minimal AhR, and AhR^{exp} cells, which overexpress AhR. The effects of differing AhR expression levels on CYP1 induction, metabolism of 17β-estradiol (E_2), cell proliferation, migration, invasion, anchorage-independent growth, and tumorigenesis were evaluated. Our results are consistent with the obligatory role of the AhR in CYP1 inducibility, but indicate that the AhR is not required for tumor formation and growth from MCF-7 cells.

MATERIALS AND METHODS

Cell Lines and Culture

MCF-7 cells were those used in our previous studies [33]. AH^{R100} cells, which are deficient in AhR, were derived from MCF-7 cells after 6 to 9 months exposure to BaP [34]. Stock cultures of AH^{R100} cells were maintained in 0.8 μM BaP (Sigma, St. Louis, MO), which, unless otherwise indicated, was withdrawn for at least 2 weeks prior to the initiation of the experiments. AhR^{exp} cells were obtained by stable transfection of MCF-7 cells with the AhR-expression construct, pcDNA3.1(+)AhR [35]. AhR^{exp} cells were maintained in 0.3 mg/

mL Geneticin (Invitrogen, Carlsbad, CA), although the antibiotic was removed prior to initiation of the experiments. Stock cultures of MCF-7, AH^{R100}, and AhR^{exp} cells were maintained in DF_5 medium, which consisted of Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% (v/v) fetal bovine serum (Hyclone, Logan, UT), 100 μM nonessential amino acids, 2 mM L-glutamine, 10 μg/L insulin, 100 U/mL penicillin, and 100 μg/mL streptomycin. Unless otherwise indicated, all experiments were performed using DC_5 medium, which has minimal estrogen content [35]. The formulation of DC_5 differed from that of DF_5 in that it contained 5% (v/v) bovine calf serum (Cosmic calf serum; Hyclone Laboratories, Logan, UT) in place of the fetal bovine serum, and it was prepared with phenol-red-free DMEM. TMX2-28, an ERα-negative subline isolated from a MCF-7 culture that had been subjected to long-term tamoxifen exposure [36], was maintained in DC_5 medium. As indicated, cultures were exposed in DC_5 medium to the following compounds: IC182,780 (ICI; Tocris, Ellisville, MO), TCDD (Cambridge Isotope Laboratories, Andover, MA), and/or E_2 (Sigma) with dimethylsulfoxide (DMSO; Sigma) as the vehicle.

Ethoxyresorufin-O-deethylase (EROD) Assays

EROD assays were performed in 96-well plates as described [37]. Confluent cultures were exposed for 48 or 72 h to 10 nM TCDD, 1 nM E_2, and/or 100 nM ICI. After treatments, the medium was replaced with medium (100 μL/well) containing 4 μM ethoxyresorufin and 10 μM dicumarol (Sigma), and the plates were incubated at 37°C for 30 min. Fluorescence measurements were made using a Fusion Universal Microplate Analyzer (Packard Instrument Co., Meriden, CT) with 535-nm excitation and 590-nm emission filters. Data were normalized to total protein, which was determined using the BCA Protein assay reagent (Pierce, Rockford, IL).

Reverse-transcription and Quantitative Real-time PCR (qPCR)

Cultures at confluence in 6-well plates were exposed to 10 nM TCDD with or without 1 nM E_2 for 48 h. Isolation of total RNA from cell lysates and tumor homogenates, reverse-transcription of oligo-dT-primed RNA with Superscript III (Invitrogen), and qPCR using the LightCycler System with the FastStart DNA Master SYBR Green I kit (Roche Molecular Biochemicals, Indianapolis, IN) were performed as previously described [35]. Quantification of cDNA was by comparison of cycle numbers for unknowns to those of purified cDNA standards of known concentration. For qualitative analysis of vector-derived AhR cDNA, the AhR-reverse primer was substituted with the BGH-reverse universal primer (5′-TAGAAGGCACAGTCGAGG-3′).

Western Immunoblots

Western immunoblots of total cellular lysates for the analysis of ERα and AhR were performed as described [35]. Samples containing equal amounts of total protein were subjected to electrophoresis in 10% acrylamide NuPAGE Bis-Tris denaturing gels (Invitrogen). Proteins were transferred onto Immobilon-P membranes (Millipore, Bethesda, MD), and blots were probed with rabbit polyclonal anti-human AhR, rabbit polyclonal anti-human ERα, or rabbit polyclonal anti-GAPDH antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactive proteins were detected by using the West Pico Enhanced Chemiluminescence kit (Pierce).

Estrogen Metabolism Assays

Confluent cultures in 6-well plates were exposed to 10 nM TCDD for 48 h. Cultures were then exposed to 1 μM E_2 in 2 mL of medium for 6 h. Media were recovered, and 1 mL of each sample was hydrolyzed at 37 °C with sulfatase (SULF) from *Helix pomatia* (Sigma

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Type H-1; 24,190 units SULF and >300 units β-glucuronidase) for 6 h. Estrogen metabolites were recovered from the hydrolyzed and non-hydrolyzed samples by solid-phase extraction, converted to pyridyl-3-sulfonyl derivatives and analyzed by liquid chromatography-tandem mass spectrometry [38,39].

Cell Proliferation Assays

The Sulforhodamine B assay [40] was used for the determination of cell proliferation. To initiate the assay, cultures were seeded in 96-well plates at 6000 cells per well. At various times, plates were fixed with 10% trichloroacetic acid and stained for total protein with 0.4% Sulforhodamine B (Sigma, St. Louis, MO) in 1% acetic acid. After rinsing, the dye was dissolved by the addition of 10 mM Tris base, and the absorbance at 490 nm was determined.

Determination of Anchorage-independent Growth

Cells were trypsinized and suspended in DMEM with 0.1% bovine serum albumin and diluted to 3000 cells/mL in 0.3% soft agar (Difco, Becton Dickinson, Sparks, MD). Aliquots of the cell suspension (500 μL) were layered over a base layer of solidified agar (500 μL, 0.5%) in 24-well plates [41]. After solidification of the top layer, 1 mL of medium was added, and colonies were allowed to form for 2 weeks, after which colonies of 50 μm in 6 randomly selected fields were counted.

Invasion and Migration Assays

Cell migration and invasion assays were performed using Boyden chambers with inserts having 8.0 μm pores (BD Biosciences, Bedford, MA). Uncoated inserts were used for migration assays, whereas Matrigel-coated inserts were used for invasion assays [42,43]. Cells were suspended in DMEM containing 0.1% bovine serum albumin, and 10^5 cells per well were seeded in the upper chamber. The lower chamber contained 1 nM E_2 as the chemoattractant in DC_{10}, a DMEM-based medium with 10% bovine calf serum. After 40 h, inserts were scrubbed, fixed with methanol for 1 to 2 min, dried, and stained with 0.1% crystal violet for cell counting.

Xenograft Assays for Tumorigenicity

For the xenograft studies, groups of 15 mice received inoculations of cells with or without E_2 supplementation [44]. Each inoculation consisted of 1×10^6 cells in 50 μl of culture medium injected into the surgically exposed mammary fat pads of 6-to 8-week-old severe combined immunodeficient (SCID) mice from Taconic Farms (Germantown, NY). E_2 supplementation was accomplished by subcutaneous implantation of Silastic tubing capsules (2 mm in length) containing solid E_2, inserted on the day of tumor-cell implantation. Under this protocol, these implants produce serum E_2 levels of 100 pg/mL [45]. Mice were palpated daily, and emerging tumors were measured with Vernier calipers several times per week for 68 to 71 days. Tumor volume was calculated using the formula $V = (\pi/6)d^2D$, assuming the tumor shape to be ellipsoid with D as the long axis. The Albany Medical College Animal Care and Use Committee approved all work with animals.

Statistical Evaluations

Statistical evaluations of biochemical determinations were performed in replicates of three or more by analysis of variance and the Bonferroni *t*-test for multiple comparisons. Statistical evaluations for comparisons of tumor volumes were performed with the Mann-Whitney rank sum test. Proliferation data were fit to the four parameter Chapman model: $y = y_0 + a(1-e^{-bx})^c$ using the SigmaPlot program (SPSS). Growth rates were calculated as the first derivative at the inflection point of the sigmoidal curve [46].

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RESULTS

Expression of AhR, ERα, and TCDD-inducible EROD activities in AH^{R100}, MCF-7, and AhR^{exp} cells

The AhR^{exp} clone was selected from among several clones as having the highest level of AhR expression. AhR mRNA and protein in MCF-7, AH^{R100}, and AhR^{exp} cells were examinedby PCR and Western immunoblot (Figure 1). When mRNA from AhR^{exp} cells was isolated and analyzed by PCR, but with substitution of the usual reverse primer that was homologous to the coding sequence with a primer that was homologous to the vector-derived 3′ untranslated RNA, the PCR product indicative of vector-derived expression was observed (Figure 1A; lane 2, 614-bp). None of this product was observed when RNA from MCF-7 cells was analyzed (Figure 1A; lane 5). PCR product representing mRNA encoded by the endogenous gene (Figure 1A; lane 4, 377-bp) was obtained from MCF-7 cDNA using the reverse primer homologous to the coding sequence. Total AhR cDNA representing endogenous plus heterologous AhR RNA from AhR^{exp} cells was analyzed using the primers that amplified the AhR coding sequence (Figure 1A; lane 3, 377-bp). The lack of PCR product formed in the negative-control amplification reaction of AhR^{exp} RNA in which reverse transcriptase was omitted (Figure 1A, lane 1) indicates that the heterologous cDNA was not derived from contamination of the RNA with vector DNA. Analysis by Western immunoblot did not show detectable AhR protein in AH^{R100} cells, whereas AhR^{exp} cells expressed higher levels of AhR protein than MCF-7 cells (Figure 1B). ERα was expressed in the MCF-7-derived sublines, AH^{R100} and AhR^{exp}. The MCF-7-derived cell line, TMX2-28 [36], was included as an ERα-negative control.

Because AH^{R100} cells were originally obtained from MCF-7 cultures after continuous exposure to BaP [34], it was necessary to confirm that AhR activity remained at a minimal level after the removal of BaP. The results presented in Figure 1C show that AhR-mediated, TCDD-induced EROD activity in AH^{R100} cells was less than 1% of that of the TCDD-induced activity of MCF-7 cells. After culture for 10 and 20 weeks in the absence of BaP, AH^{R100} cells maintained the very low response to TCDD in the EROD assay.

When the three cell lines were directly compared with regard to AhR-mediated CYP1 induction in the EROD assay (Figure 2), AhR^{exp} cells showed approximately twice the level of TCDD-induced CYP1 activity as MCF-7 cells, while AH^{R100} cells showed approximately 2% of the induced CYP1 activity in the EROD assay compared to that of MCF-7 cells, which is consistent with the results of Trapani et al. [34]. Induction of EROD activity, which is primarily a measure of CYP1A1 activity, was modestly inhibited by E_2. This inhibition was reversed by inclusion of the antiestrogen, ICI, indicating involvement of the ER in this effect.

Levels of ERα, CYP1A1, and CYP1B1 mRNAs in MCF-7-derived cell lines

Cultures of MCF-7, AH^{R100}, and AhR^{exp} cells were exposed to 1 nM E_2 and/or 10 nM TCDD for 48 h, and RNA was isolated and was analyzed by real-time qPCR for the following transcripts: AhR, ERα, CYP1A1, CYP1B1, and 36B4, which encodes acidic ribosomal phosphoprotein PO and is commonly used as a control mRNA that is not regulated by E_2 [47]. These mRNA levels are presented in Figure 3. AhR mRNA levels were 1.8 fold higher in AhR^{exp} cells than in MCF-7 cells, whereas AH^{R100} cells expressed approximately 2.5% of the AhR mRNA level of MCF-7 cells in the absence of E_2 treatment. In the absence of E_2 and TCDD, ERα mRNA levels in the three cell lines were consistent with ERα protein levels as determined by Western blot analysis (Figure 1B). In each of the three cell lines, E_2 exposure caused a significant down-regulation of ERα mRNA expression. Levels of the CYP1A1 and CYP1B1 mRNAs in TCDD-exposed cells correlated

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with the levels of expression of the AhR mRNA in the three cell lines. Levels of CYP1B1 mRNA in the absence of E_2 and TCDD also correlated with AhR mRNA levels. Interestingly, E_2 exposure resulted in a moderate down-regulation of TCDD-induced CYP1B1 mRNA levels in AhR^{exp} cells. Consistent with its effects on TCDD-induced EROD activity (Figure 2), E_2 modestly down-regulated TCDD-induced CYP1A1 mRNA levels in MCF-7 and AhR^{exp} cells.

Metabolism of E_2 in MCF-7, AH^{R100}, and AhR^{exp} cells

Having confirmed differential expression of AhR at the protein and mRNA levels in MCF-7, AH^{R100}, and AhR^{exp} cells, we then determined the effect of differential AhR expression on the TCDD-induced metabolism of E_2. The rates of formation of 2-, 4-, 6α, and 15α-hydroxyestradiol (OHE_2), and 2- and 4-methoxyestradiol ($MeOE_2$), with and without exposure to 10 nM TCDD and with and without hydrolysis of conjugates prior to analysis are shown in Figure 4. In MCF-7 and AhR^{exp} cells, basal E_2 metabolism at the C-2 and C-4 positions was detected, and this metabolism was greatly increased by TCDD exposure. Comparison of the levels of metabolites recovered with and without prior SULF treatment indicate that significant amounts of 2- and 4-OHE_2, and 2- and 4-$MeOE_2$ were present in conjugated form, whereas 6α- and 15α-OHE_2 were not conjugated to an appreciable extent. In contrast to E_2 metabolism in MCF-7 and AhR^{exp} cells, AH^{R100} cells did not show appreciable E_2 metabolism, with or without TCDD exposure.

Proliferation, anchorage-independent growth, invasion, and migration of AH^{R100}, MCF-7, and AhR^{exp} cells

We next investigated the consequences of differential AhR expression on *in vitro* indices of tumorigenesis, with and without E_2 exposure. Cell proliferation of the two MCF-7-derived lines as compared with that of MCF-7 cells in the presence or absence of 1 nM E_2 is shown in Figure 5A. Growth rates derived from the data in Figure 5A are presented in Table 1. Proliferation without E_2 supplementation was highest for AH^{R100} cells and lowest for MCF-7 cells. MCF-7 cells, however, showed the greatest enhancement of proliferation in response to E_2 supplementation, and AhR^{exp} cells showed no enhancement of proliferation in response to E_2. Ah^{R100} cells showed the highest proliferation among the three cell lines with or without E_2 supplementation. In the presence of E_2, growth rates of the three cell lines (Table 1) were inversely correlated with AhR mRNA levels (Fig 3); $R^2 = 0.981$.

The three cells lines were then evaluated for colony formation in soft agar in the presence of DC_5 and DC_5 containing 1 nM E_2 (Figure 5B). Among the three cell lines, anchorage-independent growth was greatest for AH^{R100} cells with each medium tested. We then investigated migration and invasion of the three cell lines using Boyden chambers. The invasion assay, which requires that the cells degrade and transverse a layer of extracellular matrix, analogous to the penetration of the basement membrane by cancer cells in the initial events of metastasis [42], utilized Matrigel-coated inserts, whereas uncoated chamber inserts were used to assay for migration [43]. In both assays, the lower chamber contained medium supplemented with E_2 as the chemoattractant. Using these assays we found that AH^{R100} cells were >30-fold more invasive than MCF-7 or AhR^{exp} cells, and that migration of AH^{R100} cells was also highly elevated compared with that of AhR^{exp} and MCF-7 cells (Figure 5C).

Tumorigenesis of AH^{R100}, MCF-7, and AhR^{exp} cells in SCID mice

To evaluate *in vivo* tumorigenicity, AH^{R100}, MCF-7, and AhR^{exp} cells were inoculated into the mammary glands of SCID mice, with and without E_2 supplementation as a Silastic implant (Figure 6). In all of these experiments, tumors, although palpable, were not able to grow appreciably without E_2 supplementation. Included for comparison are ERα-negative

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TMX2-28 cells, which, despite the fact that they are highly proliferative and invasive *in vitro* [48], grew poorly as mammary-gland xenografts with or without E_2 supplementation (Figure 6, lower right panel). With E_2 supplementation, tumors from implanted AH^{R100} cells (Figure 6, upper left panel) grew to an extent comparable to MCF-7 cells, indicating that the AhR is not required for tumor formation and growth in SCID mice. AhR^{exp}-cell xenografts showed a trend toward slower tumor growth in comparison with those from MCF-7 cells; however, this difference was not statistically significant. Interestingly, with AhR^{exp} cells, tumors became palpable but did not grow to any significant extent, which was similar to the results found following inoculation of TMX2-28 cells.

To determine whether *AHR* gene expression in the xenograft tumors was comparable to that in the cells in culture, we examined the level of AhR mRNA in MCF-7 and AH^{R100} tumors dissected from animals after the final tumor-growth measurements were taken. AhR^{exp} tumors were of insufficient size for dissection. AhR cDNA and GAPDH cDNA for normalization were amplified with human-specific primers, which did not amplify the homologous targets from murine cDNA (results not shown). We found that the levels of AhR mRNA in tumors obtained from AH^{R100} xenografts remained low, at less than 7% the level in MCF-7 cells.

DISCUSSION

Studies have been conducted in a number of laboratories with the goal of deciphering the roles of the AhR in initiation, promotion, and progression phases of cancer, and from them a variety of roles of the receptor in cancer have been proposed [9,17,27-28,31]. Dependent on the species, tissue, and phase of carcinogenesis, evidence of both oncogenic [9,27,31,49-51] and tumor-suppressing functions [32,52,53] of the AhR have been reported. In this study, we show that, in the MCF-7 model of luminal A breast cancer, CYP1 expression and inducibility by TCDD in cultured cells are highly dependent on AhR expression, whereas cellular proliferation, invasion, migration, anchorage-independent growth, and estrogen-stimulated formation of xenograft tumors do not require the AhR. Our current studies with MCF-7 cells suggest that the AhR may have both pro- and anti-carcinogenic roles within ER-positive luminal breast cells during the various stages of carcinogenesis.

The importance of the AhR in the initiation phase of PAH-induced carcinogenesis is well established. The AhR-mediated induction of the CYP1 enzymes that catalyze the metabolic activation of PAH to DNA adductive forms is integral to the carcinogenic process [9]. AhR-null mice are refractory to PAH-induced carcinogenesis [49], and studies with CYP1 single- and double-knockout animals show the roles of these enzymes in differential organ carcinogenesis subsequent to PAH exposure [31]. These enzymes not only catalyze the metabolic activation of PAHs, but also heterocyclic aromatic amines [54] and endogenous estrogens [55]. The AhR may thus be involved in the initiation events from several exogenous and endogenous carcinogens. In our current study, the inducibility of CYP1A1 and CYP1B1 mRNAs, proteins, and CYP1 activities were consistent with the levels of AhR expression in AH^{R100}, MCF-7, and AhR^{exp} cells in culture. Increased rates of the 2- and 4-hydroxylation pathways of E_2 metabolism in response to TCDD exposure, which reflect the activities of CYP1A1 and CYP1B1, respectively [33], and the induction of EROD activity were not observed in the absence of AhR expression. The ultimate metabolites of E_2 in MCF-7 cells, the sulfate conjugates of 2- and 4-$MeOE_2$ [56], were not produced at appreciable levels in TCDD-treated AH^{R100} cells, nor were 6α-OHE_2 and 15α- OHE_2, which are products of CYP1A1-catalyzed metabolism [57]. These results indicate that the induction of E_2 metabolism, including the formation of the potentially carcinogenic 4-OHE_2 [55], is reliant on AhR expression in MCF-7 cells.

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Distinct from the roles of the AhR in CYP1 induction and the regulation of carcinogen metabolism, the AhR appears to have roles in post-initiation stages of carcinogenesis. Recently, transgenic and gene knockout animals have been used to specifically determine role of the AhR in the post-initiation events in carcinogenesis that do not rely on exogenous AhR ligands or CYP1-catalyzed carcinogen metabolism. Fritz et al. [52] used the transgenic adenocarcinoma of the mouse prostate (TRAMP) model, in which expression of the simian virus 40 large T and small t antigens are under the control of the androgen-dependent probasin gene promoter, to investigate the role of the AhR in tumor formation. They found that when TRAMP mice were crossed with *Ahr*$^{-/-}$ mice, the *Ahr*$^{+/-}$ and *Ahr*$^{-/-}$ TRAMP progeny showed greater prostate tumor incidence than in *Ahr*$^{+/+}$ TRAMP mice, indicating an inhibitory effect of AhR expression in this model of prostate carcinogenesis. Evidence of a tumor-suppressor function of the AhR was also reported by Fan et al. [53] in their study of *Ahr*$^{-/-}$ mice exposed to the direct-acting carcinogen, diethylnitrosamine. A significant increase in liver adenomas, which are thought to be the precursors of hepatocellular carcinomas, was observed in male *Ahr*$^{-/-}$ but not female mice in comparison with *Ahr*$^{+/+}$ mice after exposure to the carcinogen. Our results are consistent with these studies of prostate tumors *Ahr*$^{-/-}$ TRAMP mice [52] and liver adenomas [53] in *Ahr*$^{-/-}$ mice, as we found that AhR expression in MCF-7 cells is not necessary for xenograft tumor formation in SCID mice, and may in fact have a tumor-suppressor function in these cells.

A caveat that is associated with the use of AH^{R100} cells is that these cells were obtained after long-term exposure to BaP. Mutations and epigenetic modifications in AH^{R100} cells in addition to those directly related to the reduction of AhR expression and their ability to survive and proliferate in the presence of BaP may have occurred. However, in this regard we note that AH^{R100} cells retain ERα expression and remain dependent on estrogen for tumor growth as xenografts. They also retain epithelial-like morphology in culture. Recently, it was reported that vector-driven re-expression of the AhR in AH^{R100} cells restored the inducibility of the CYP1A1 and CYP1B1 mRNAs by TCDD [58], which supports the premise that these cells retain many characteristics of the parental cell line. Regardless of the genetic and/or epigenetic changes that may have occurred in the development of AH^{R100} cells and their loss of AhR expression, our conclusion that expression of the AhR is not required for the proliferation, migration, invasion, or estrogen-dependent tumorigenesis of MCF-7 breast cancer cells is entirely valid.

Rates of cell proliferation and alterations in cell-cycle control are recognized as key factors impacting carcinogenesis, and the AhR is known to affect them, albeit in cell-specific manner [17,24,59]. There are numerous studies indicating that, in the absence of an exogenous ligand, the AhR promotes progression through the cell cycle, whereas ligand activation of the AhR causes cell-cycle arrest in a number of cell types [17,22,29,30]. However, these effects are not universally observed; the role of the AhR in progression through the cell cycle and cell proliferation is dependent on cell type. Expression of the AhR in AhR-defective Hepa1c1 cells enhances the rate of cell proliferation in the absence of an exogenous AhR ligand [11]. Exposure to the AhR ligand, β-naphthoflavone, or overexpression of the AhR in A549 lung carcinoma cells caused enhanced cell proliferation [24]. The reduced rate of cell proliferation observed in AhR-null embryo fibroblasts appears be multi-factorial, but a significant role was attributed to the overproduction of TGF-β [23]. Conversely, our present study showed *enhanced* cell proliferation of AhR-deficient AH^{R100} cells, which is consistent with the increased G_0/G_1 phase progression that was observed when AhR expression was knocked down by small inhibitory RNA in MCF-7 cells [59].

An aspect of AhR activity in breast and other cancers that remains unresolved is the role of the AhR in development of the invasive phenotype that may be indicative of metastatic potential. Several studies suggest that the AhR, through regulation of Slug, initiates the

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down-regulation of E-cadherin and consequently the epithelial-to-mesenchymal transition and development of the invasive phenotype [27,50,51]. In contrast, Hall et al. [32] reported evidence for inhibitory roles of AhR expression and ligand activation of the AhR in cellular invasion and processes related to metastasis in a panel of breast cancer cell lines representing the major breast-cancer subtypes. Exogenous AhR agonists significantly inhibited cell invasiveness and motility and inhibited colony formation in soft agar regardless of ER, progesterone receptor, or human epidermal growth factor receptor 2 status. Knockdown of AhR expression using a small inhibitory RNA was also shown to be sufficient to increase, rather than decrease, the invasiveness of SKBR3 and MDA-MB-231 cells, and activation of the AhR by TCDD caused decreases in colonization in soft agar of SKBR3, MDA-MB-231, ZR-75-1 and MCF-7 cells [32].

The reasons for the differences in experimental observations regarding the role of the AhR in cellular invasiveness and anchorage-independent growth in breast cancer cells are not known, but they may be influenced by the specific developmental lineage of the tumor. The two main differentiation pathways in the mammary epithelium give rise to the luminal and basal/myoepithelial cell types [60]. We suggest that the AhR may have quite different roles in "basal-like" breast tumors versus "luminal-like" tumors. The epithelial-to-mesenchymal transition appears to occur within the specific genetic context of the basal phenotype [61]. If AhR-mediated regulation of Slug is a property of the basal-like tumor cells, then this could explain the differences in invasive properties of tumor cells that have been reported for the AhR. MCF-7 cells, which are luminal in phenotype, do not express significant levels of Slug [60], and our gene expression studies [44] did not show induction of the Slug transcript by TCDD exposure in MCF-7 cells.

In numerous studies it has been suggested that the AhR could be a therapeutic target in cancer. However, the results of the present study, notably those obtained with AH^{R100} cells, indicate that breast cancer cells can in some cases obtain a growth advantage when AhR expression is diminished. We found that cell proliferation, migration, invasion, and anchorage-independent growth do not correlate with AhR expression in MCF-7 cells and its sublines, AH^{R100} and AhR^{exp}. Over-expression of AhR in AhR^{exp} cells did not confer increased anchorage-independent growth, invasiveness, or tumorigenicity. As has been reported in numerous previous studies, tumor formation from MCF-7 xenografts was highly dependent on the presence of E_2 in the host animal. Mice without E_2 supplementation did not have measurable tumor growth as xenografts from MCF-7, AH^{R100}, or AhR^{exp} cells. Surprisingly, even the highly invasive and proliferative MCF-7-derived cell line, TMX2-28 [36,48], which is unresponsive to E_2, was unable to sustain appreciable tumor growth in SCID mice.

In summary, our results show that, in the MCF-7 model of luminal A breast cancer, CYP1 expression and inducibility by TCDD are highly dependent on AhR expression, whereas cellular invasion, migration, anchorage-independent growth, and estrogen-stimulated formation of tumors do not require the AhR. Our experiments with AH^{R100} cells show that highly proliferative, invasive, and tumorigenic cells can arise from AhR-deficient tumor cells. The studies reported here indicate that the AhR and the *AHR* gene may represent chemoprevention targets that are focused on reducing the cancer-initiating events due to bioactivation of exogenous and endogenous carcinogens, but they are not likely to represent universal targets in breast cancer therapeutics aimed at inhibiting the growth and invasiveness of the developing tumor.

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Abbreviations

AhR	aryl hydrocarbon receptor
BaP	benzo(*a*)pyrene
PAH	polycyclic aromatic hydrocarbons
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
CYP	cytochrome P450
ER	estrogen receptor
E_2	17β-estradiol
DMEM	Dulbecco's modified Eagle's medium
ICI	IC1-182,780 or Fulvestrant
DMSO	dimethylsulfoxide
EROD	ethoxyresorufin-*O*-deethylase
qPCR	quantitative real-time PCR
SULF	sulfatase
SCID	severe combined immunodeficient
$MeOE_2$	methoxyestradiol
OHE_2	hydroxyestradiol
TRAMP	transgenic adenocarcinoma of the mouse prostate

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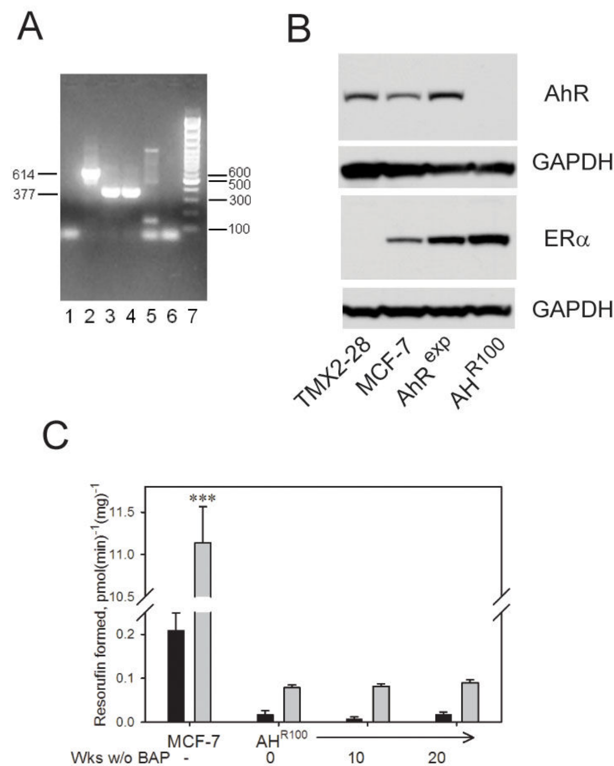

Figure 1.

Expression of AhR and ERα in MCF-7 and MCF-7-derived cell lines. (A) Heterologous AhR mRNA expression in AhR^{exp} cells. Total RNA from AhR^{exp} (lanes 1-3) and MCF-7 cells (lanes 4-5) was isolated, and PCR was performed with primers specific for the coding sequence of AhR mRNA representing total AhR mRNA (377-bp product) or specific for the vector-derived mRNA (614-bp product). Lane 1, reverse transcriptase-negative control using vector-specific primers; lanes 2 and 5, PCR with vector-specific primers; lanes 3, 4, and 6, PCR with primers specific for the AhR coding sequence; lane 6, minus-RNA control using primers specific for the AhR coding sequence; lane 7, 100-bp ladder. (B) AhR and ERα protein levels in AH^{R100}, MCF-7, and AhR^{exp} cells. Western blots of whole cell lysates from MCF-7, AhR^{exp}, and AH^{R100} cells were probed with anti-AhR and -ERα antibodies and detected with enhanced chemiluminescence. Lysates from TMX2-28 cells are included as an ERα-negative control. GAPDH was probed as a loading control for each blot. (C) The loss of TCDD-inducible EROD activity in AH^{R100} cells is persistent. BaP was withdrawn from AH^{R100} cultures for 10 or 20 weeks, and compared with cells without BaP withdrawal (designated as 0 weeks without BaP), after which cells were exposed for 72 h to 10 nM TCDD (gray bars) or the vehicle, 0.1% (v/v) DMSO (black bars). CYP1 activity was then measured by EROD assay. EROD activity of MCF-7 cells is shown for comparison; note the difference in scale. Data are presented as mean + SE; n = 5. Significant differences for TCDD-treated cultures in comparison with the TCDD-treated control group (AH^{R100} cells without BaP withdrawal) are indicated (***P<0.001).

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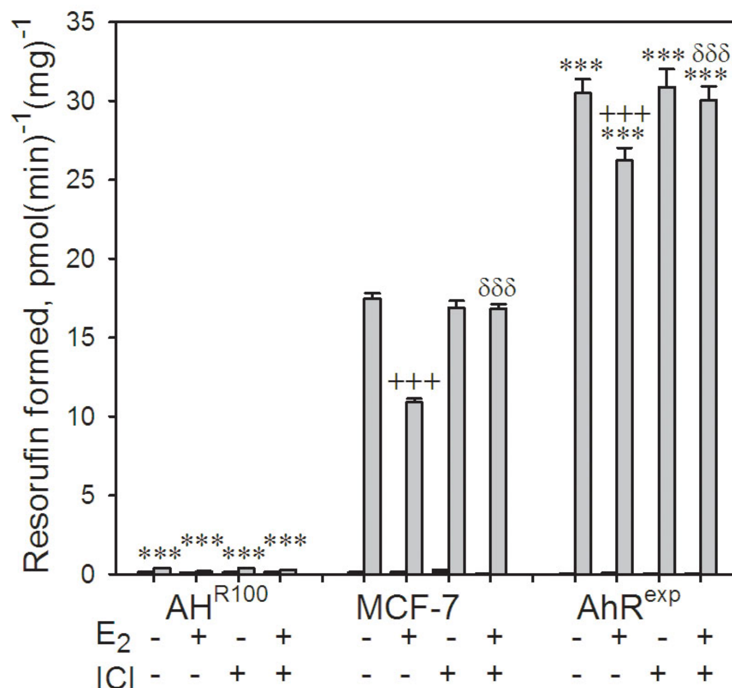

Figure 2.
Ah-responsiveness in AH^{R100}, MCF-7, and AhR^{exp} cells as measured by the EROD assay. Confluent cultures of AH^{R100}, MCF-7, and AhR^{exp} cells were exposed for 48 h to 10 nM TCDD (gray bars), or the vehicle, 0.16% (v/v) DMSO (black bars), with or without 1 nM E_2 and 100 nM ICI, as indicated. CYP1 activity was then measured by the EROD assay. Data are represented as the mean + SE; n = 8. Significant differences between TCDD-exposed AH^{R100} or AhR^{exp} cultures and MCF-7 cultures in which all other treatments were identical ($^{***}P<0.001$); between groups differing only in E_2 exposure ($^{+++}P<0.001$); and between groups differing only in ICI exposure ($^{+++}P<0.001$) are indicated.

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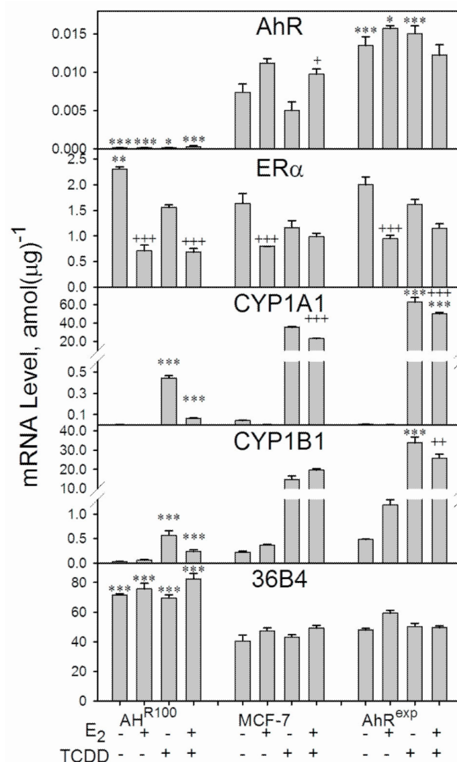

Figure 3.

Effect of AhR expression on ERα mRNA levels and the inducibility of CYP1 mRNAs. Confluent cultures of AH^{R100}, MCF-7, and AhR^{exp} cells were exposed to 10 nM TCDD, 1 nM E_2, or the vehicle, 0.16% (v/v) DMSO, for 48 h, and the RNA isolated. qPCR was performed with primers specific for the transcripts as indicated. Data are represented as the average +/- SEM; n=3, and are normalized to total RNA. Significant differences between TCDD-exposed AH^{R100} or AhR^{exp} cultures in comparison with MCF-7 cultures in which all other treatments were identical ($^{*}P<0.05$; $^{***}P<0.001$); and between E_2 exposure and the respective group differing only in the absence of E_2 exposure ($^{++}P<0.01$; $^{+++}P<0.001$) are indicated.

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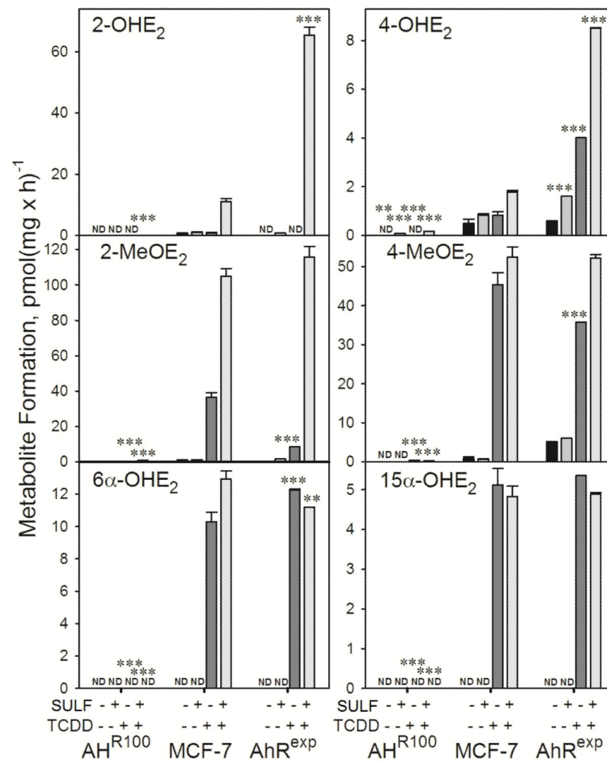

Figure 4.

Basal and TCDD-induced E_2-metabolite formation in AH^{R100}, MCF-7, and AhR^{exp} cells. Confluent cultures of AH^{R100}, MCF-7, and AhR^{exp} cells were exposed for 48 h to 10 nM TCDD or the solvent vehicle, 0.1% (v/v) DMSO, as indicated. Metabolite formation was determined with (+SULF) and without (-SULF) prior hydrolysis of conjugates as described in Materials and Methods. Data are represented as the mean + SE and are normalized to total cellular protein; n = 3. Significant differences between TCDD-exposed AH^{R100} or AhR^{exp} cultures and MCF-7 cultures, where all other treatments were identical (***$P<0.001$) are indicated; ND denotes that the metabolites were not detected.

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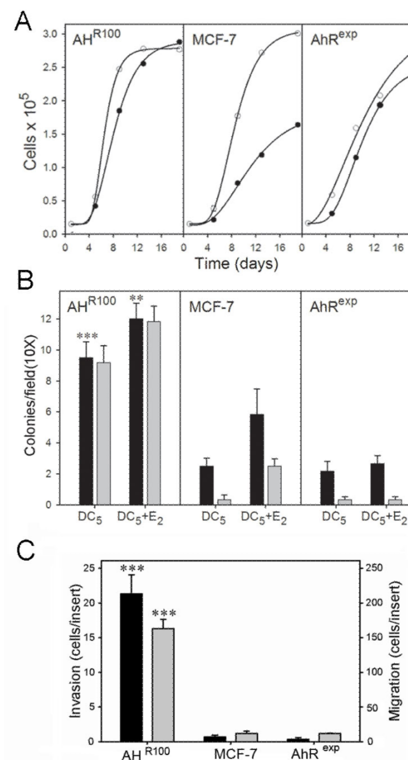

Figure 5.

Proliferation, migration, and invasion of AH^{R100}, MCF-7, and AhR^{exp} cells. (A) Proliferation: AH^{R100}, MCF-7, and AhR^{exp} cells were seeded in 96-well plates at 6000 cells per well, and the media were replenished every 3 or 4 days with DC_5 (closed circles) or DC_5 containing 1 nM E_2 (open circles). Proliferation was measured at the indicated times using the Sulforhodamine B assay. Data are presented as mean + SE; n = 8; however, for all points the error bar falls within the symbol. Curves were obtained by fitting data to the four parameter Chapman model. (B) Anchorage-independent growth: AH^{R100}, MCF-7, and AhR^{exp} cells were suspended in 500 μL 0.3% soft agar in 24-well plates at 1500 cells per well. The cells were cultured for 2 weeks in DC_5 or DC_5 containing 1 nM E_2 as indicted. Anchorage-independent growth was assessed under 10X magnification by counting colonies larger than 50 μm (black bars) or larger than 100 μm (gray bars) in diameter. Significant differences between AH^{R100} or AhR^{exp} cultures in comparison with MCF-7 cultures when all other treatments were identical (***P*<0.01; ****P*<0.001) are indicated. Data are represented as mean colonies per field + SE; n = 6. Significant differences for colonies 50 μm are shown. (C) Migration and invasion: AH^{R100}, MCF-7, and AhR^{exp} cells were suspended in DMEM containing 0.1% bovine serum albumin, and 10^5 cells per well were seeded in Matrigel-coated chambers for measurement of invasion (black bars) or in control inserts for measurement of migration (gray bars). The lower chamber contained 1 nM E_2 in DC_{10} as a chemoattractant. After 40 h, inserts were fixed and stained with crystal violet. Data are represented as mean cells per insert + SE; n = 3. Significant differences between AH^{R100} or AhR^{exp} cultures in comparison with MCF-7 cultures (***P*<0.01; ****P*<0.001) are indicated.

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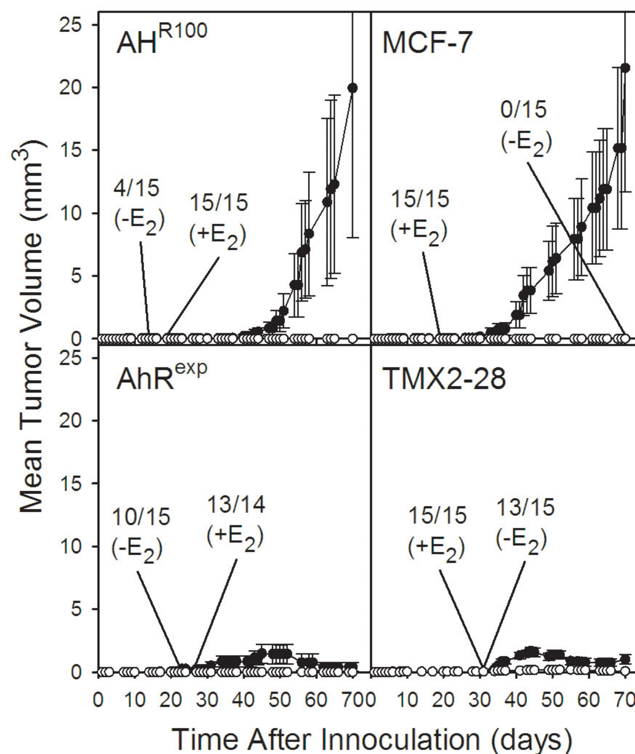

Figure 6.
Tumorigenicity of the MCF-7-derived cell lines with differential expression of AhR and ERα. AH^{R100}, MCF-7, AhR^{exp} cells, or TMX2 cells, as indicated, were implanted into the mammary glands of SCID mice, with (closed circles) or without (open circles) E_2 supplementation as a Silastic implant. Tumor growth was monitored by palpation at the times indicated. The latency of tumor formation is indicated in the plots by denotation of the day at which the maximal incidence of tumor formation was observed, with the number of mice with palpable tumors/total number of mice. Data are presented as mean tumor volume + SE of the mice with measurable tumors.

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Table 1

Proliferation rates of AH^{R100}, MCF-7, and AhR^{exp} cells

	Cell line		
	AH^{R100}	**MCF-7**	**AhR^{exp}**
Medium			
DC_5	0.382 (0.353-0.429)[a]	0.137 (0.129-0.150)	0.2446 (0.2443-0.2449)
DC_5 + 1 nM E_2	0.659 (0.623-0.744)	0.398 (0.363-0.433)	0.215 (0.167-0.275)

[a]Rates are expressed as 10^5 cells/day, with 95% confidence intervals given in parenthesis.