Published in final edited form as:

Toxicol Appl Pharmacol. 2009 November 1; 240(3): 355–366. doi:10.1016/j.taap.2009.07.013.

Long-term estrogen exposure promotes carcinogen bioactivation, induces persistent changes in gene expression, and enhances the tumorigenicity of MCF-7 human breast cancer cells

Barbara C. Spink^a, James A. Bennett^b, Brian T. Pentecost^{a,c}, Nicole Lostritto^b, Neal A. Englert^{a,c}, Geoffrey K. Benn^a, Angela K. Goodenough^a, Robert J. Turesky^{a,c}, and David C. Spink^{a,c,*}

^aLaboratory of Molecular Toxicology, Wadsworth Center, New York State Department of Health, Albany, NY 12201-0509, USA

^bCenter for Immunology and Microbial Diseases, Albany Medical College, Albany, NY 12208-3479, USA

^cDepartment of Environmental Health Sciences, School of Public Health, University at Albany, State University of New York, Albany, NY, 12201-0509, USA

Abstract

The cumulative exposure to estrogens is an important determinant in the risk of breast cancer, yet the full range of mechanisms involving estrogens in the genesis and progression of breast cancer remains a subject of debate. Interactions of estrogens and environmental toxicants have received attention as putative factors contributing to carcinogenesis. Mechanistic studies have demonstrated interactions between estrogen receptor α (ER α) and the aryl hydrocarbon receptor (AhR), with consequences on the genes that they regulate. Many studies of ER α and AhR-mediated effects and crosstalk between them have focused on the initial molecular events. In this study, we investigated ER α - and AhR-mediated effects in long-term estrogen exposed (LTEE) MCF-7 human breast cancer cells, which were obtained by continuous culturing for at least 12 weeks in medium supplemented with 1 nM of 17 β -estradiol (E₂). With these LTEE cells and with parallel control cells cultured without E₂ supplementation, we performed an extensive study of cytochrome P450 (CYP) induction, carcinogen bioactivation, global gene expression, and tumorigenicity in immunocompromised mice. We found that LTEE cells, in comparison with control cells, had higher levels of AhR mRNA and protein, greater responsiveness for AhR-regulated CYP1A1 and CYP1B1 induction, a 6-fold higher initial level of benzo(a)pyrene-DNA adducts as determined by liquid chromatography tandem mass spectrometry, marked differences in the expression of numerous genes, and a higher rate of E₂-dependent tumor growth as xenografts. These studies indicate that LTEE causes adaptive responses in MCF-7 cells, which may reflect processes that contribute to the overall carcinogenic effect of E₂.

© 2009 Elsevier Inc. All rights reserved.

*Corresponding author. Fax: +1 518 473 2895 E-mail address: spink@wadsworth.org (D.C. Spink).

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Conflict of interest statement: The authors have nothing to disclose.

Keywords

breast cancer; MCF-7 cells; long-term estrogen exposure; benzo(a)pyrene; DNA adducts

Introduction

Estrogens have long been associated with breast cancer (Clemons and Goss, 2001; Eliassen et al., 2006; Tamimi et al., 2007; Yager and Davidson, 2006; Clamp et al., 2002). It is thought a woman's cumulative exposure to estrogens is an important determinant of her risk for the disease. A major proof of principle regarding the role of estrogen in breast cancer emerged from the Breast Cancer Prevention Trial with the selective estrogen receptor modulator, tamoxifen, showing a significant 45% decrease in incidence of breast cancer relative to the placebo-control group (Fisher et al., 2005). Despite some risks associated with the use of tamoxifen, these results were received with optimism, since for the first time a significant reduction in breast cancer incidence was enabled through pharmacologic intervention. Therapeutic or preventive approaches in use or in development for breast cancer that focus on modulation of estrogen function include the use of selective estrogen receptor modulators (Jordan and Brodie, 2007; Vogel et al., 2006; Goss et al., 2005), aromatase inhibitors (Jordan and Brodie, 2007; Goss et al., 2005; Freedman et al., 2006; Budzar et al., 2008), selective estrogen-receptor down-regulators (Whittmann et al., 2007), and ovarian ablation (The Adjuvant Breast Cancer Trials Collective Group, 2007).

The promotion and progression phases of carcinogenesis are thought to extend over many years, and the cumulative risk of the disease increases over the course of a woman's adult life. Long-term changes in the cellular, biochemical, and genetic properties of a tumor appear to be integral to the progression from carcinoma *in situ* to invasive carcinoma with metastatic potential. Estrogens cause the distinct developmental phenomena of genomic and gene imprinting, resulting in persistent alterations in gene expression that occur via non-mutagenic mechanisms (McLauchlan et al., 2001); recent studies suggest that estrogen-induced epigenetic changes similar to those that occur in early development are involved in breast cancer (Cheng et al., 2008).

The hypothesis that we consider here is that the spectrum of estrogen-induced responses in estrogen receptor (ER)-positive breast cancers, and the magnitudes of these effects, are not static, but vary over time during tumor development. We suggest that these changes alter the cellular responses to carcinogens. To investigate this hypothesis, we undertook studies with the ER-positive human breast carcinoma cell line, MCF-7, which is classified as a luminal A tumor, based on its gene expression pattern (Jonsson et al., 2007). We maintained MCF-7 cultures continuously in media that differed only by the presence or absence of physiologic concentrations of E₂. With these cultures, we performed an extensive study of cytochrome P450 (CYP) induction, carcinogen bioactivation, global gene expression, and tumorigenicity as xenografts in immunocompromised mice.

Materials and methods

Cell culture and treatments

The MCF-7 cells used in this study were those used previously (Spink et al., 1990; 1998; 2003a,b), and were subjected to a minimum of 12 weeks of long-term estrogen exposure (LTEE) in DC₅ medium supplemented with 0.01, 0.1, or 1 nM 17 β -estradiol (E₂), or in DC₅ with the solvent vehicle, 0.01% dimethyl sulfoxide (DMSO). DC₅ medium, which is very low in estrogens (Spink et al., 2003b), consisted of Dulbecco's modified Eagle's medium supplemented with 5% (v/v) bovine calf serum (Cosmic calf serum; Hyclone Laboratories,

Logan, UT), 10 mM nonessential amino acids, 2 mM L-glutamine, 10 µg/L insulin, 100 U/mL penicillin, and 100 µg/mL streptomycin. The DF₅ medium used in some experiments differed from DC₅ in that it contained 5% fetal bovine serum (Hyclone) rather than bovine calf serum, and in that phenol red was included. Cultures were passaged approximately every 9 days, using 1:10 subculturing. Unless otherwise indicated, experiments were initiated by seeding of cells in DC₅ medium and culturing to confluence, with one medium change. Cultures were then exposed, in DC₅ medium, to one or more of the following compounds: 100 nM ICI 182,780, or fulvestrant (Tocris, Ellisville, MO), 10 µM α-naphthoflavone (ANF; Sigma, St. Louis, MO), 200 nM 2,3',4,5'-tetramethoxystilbene (TMS; Cayman Chemicals, Ann Arbor, MI), 10 nM 2,3,7,8- tetrachlorodibenzo-*p*-dioxin (TCDD; Cambridge Isotope Laboratories, Andover, MA), and 0.01 or 0.1 µM benzo(*a*)pyrene (BAP; AccuStandard, New Haven, CT). LTEE cells, in our terminology, denote the cells cultured in DC₅ medium supplemented with 1 nM E₂ for 12 or more weeks, and control cells refer to those cells cultured in parallel, in DC₅ medium with vehicle. For short-term exposures, conditioned medium recovered from control cultures after ≥48 h of exposure was used as described previously (Spink et al., 2003a), to avoid the transient induction of CYP1 activity that occurs upon medium change (Öberg et al., 2005).

Ethoxyresorufin-O-deethylase (EROD) assay

LTEE cells and control cells were seeded in DC₅ medium and grown to confluence without a medium change before chemical exposures in DC₅ or conditioned medium. Cells were exposed for 72 h to 10 nM TCDD, or for 3 h to 0.01 or 0.1 µM BAP. EROD assays were performed using the 96-well plate procedure (Spink et al., 2003a). After treatments, the medium was replaced with medium (100 µL/well) containing 4 µM ethoxyresorufin and 10 µM dicumarol, and the plates were incubated at 37°C for 30 min. Fluorescence measurements were made using a Fusion Universal Microplate Analyzer 5 (Packard Instrument Co., Meriden, CT) with 535-nm excitation and 590-nm emission filters. Total protein was determined with the BCA Protein assay reagent (Pierce, Rockford, IL).

Reverse transcription and real-time PCR

LTEE and control cells were cultured to confluence in 6-well plates and treated with TCDD or BAP as described for the EROD assay. Isolation of total RNA with guanidine thiocyanate and phenol, reverse transcription of oligo-dT-primed RNA with Superscript II or III (Invitrogen, Carlsbad, CA), and real-time PCR using the LightCycler System with the FastStart DNA Master SYBR Green I kit (Roche Molecular Biochemicals, Indianapolis, IN) were performed as described (Spink et al., 2003b). cDNA templates corresponding to 10 to 20 ng total RNA were amplified with primers specific for the aryl hydrocarbon receptor (AhR), CYP1A1, CYP1B1, ERα, aryl hydrocarbon receptor nuclear translocator (ARNT), and 36B4 cDNAs, as described (Spink et al., 2003b). ABCG2 cDNA was amplified with the primers 5'-AACTTCTGCCCAGGACTCAA-3' and 5'-GATGGCAAGGGAACAGAAAA-3'. cDNA was quantified by comparison of cycle numbers for unknowns to those of cDNA standards of known concentration.

Western immunoblots

Parallel cultures to those for RNA analysis were analyzed for protein expression by Western immunoblot. Microsomes were prepared from cultures in 15-cm dishes for the analysis of CYP1A1 and CYP1B1 proteins, and total cellular lysates were prepared from cultures in 6-well plates for the analysis of ERα, AhR, and actin proteins. Total protein content in the microsome and lysate samples was quantified using the BCA Protein assay reagent (Pierce), and equal amounts of protein were subjected to electrophoresis in 10% acrylamide NuPAGE Bis-Tris denaturing gels (Invitrogen) as recommended by the manufacturer. Proteins were blotted onto Immobilon-P membranes (Millipore, Bethesda, MD), and blots were then

probed as described (Spink et al., 2003b) with goat polyclonal anti-rat CYP1A1/2 antibody (Daiichi, Tokyo, Japan), rabbit polyclonal anti-human CYP1B1 antibody (BD Gentest, Bedford, MA), goat polyclonal anti-human actin antibody, rabbit polyclonal anti-human AhR antibody, or rabbit polyclonal anti-human ER α antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactive proteins were detected by using the West Pico Enhanced Chemiluminescence kit (Pierce). For the analysis of ABCG2 protein, Western blots of microsomes were probed with mouse monoclonal anti-BCRP antibody (Chemicon, Temecula, CA) at 1:250 dilution.

CYP1A1 and CYP1B1 promoter activities

Plasmids for analysis of promoter activities were the human CYP1A1-promoter-luciferase construct, pHu-1A1-FL (Bessette et al., 2005; Spink et al., 2008), and the CYP1B1-promoter-luciferase construct, p1B1FLuc (Spink et al., 2003b). LTEE cells and control cells were seeded in 24-well plates with DC₅ medium so as to be 90% confluent the following day, on which they were transfected with plasmids using LipofectAMINE 2000 as recommended (Invitrogen). After 24 h, the cells were exposed to 10 nM TCDD, 1 nM E₂, or 100 nM ICI 182,780 for 6 h. The cells were lysed and assayed for luciferase activity using the Dual-Luciferase Reporter 1000 Assay System as described (Spink et al., 2003b), with the exception that the phRG-TK *Renilla* luciferase construct (Promega, Madison, WI), transfected at 50 ng/well, was used for normalization.

Incorporation of [³H]BAP into cellular DNA

The binding of BAP to cellular DNA was performed as described (Wen and Walle, 2007), with modifications. Confluent cultures in 6-well plates were treated with 0.1 μ M [³H]-BAP (78 Ci/mmol, 5 mCi/mL; Amersham Biosciences, Piscataway, NJ) with DMSO as the vehicle, in conditioned medium. After exposure to ³H-BAP, the cells were rinsed twice with PBS, lysed by addition of 0.4 mL of cell lysis solution, and genomic DNA was isolated by use of the Puregene DNA purification kit (Gentra Systems, Minneapolis, MN). The quantity and the purity of DNA were determined by UV absorbance, and the quantity of ³H-BAP bound to DNA was determined by liquid scintillation counting.

Liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI/MS/MS) analysis of BAP-DNA adduct formation

An LC-ESI/MS/MS method employing synthetic standards and quantitation by stable isotope dilution was used to determine BAP adducts at *N*² of dG, in DNA from LTEE and control cultures that had been exposed to 0.1 μ M BAP for 3 h. The ($\pm$)-anti-10-(deoxyquanosin-*N*²-yl)-7,8,9-trihydroxy-7,8,9,10-tetrahydro-benzo(*a*)pyrene (dG-*N*²-BAP) adduct was synthesized by reaction of ($\pm$)-*r*-7,*t*-8-dihydroxy-*t*-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPDE; 2.5 mg, 8.1 μ mol; NCI Chemical Carcinogen Reference Standard Repository, Midwest Research Institute, Kansas City, MO) with dG (25 mg, 88 μ mol) in 10 ml of 10 mM Bis-tris buffer (pH 7.5) as described (Beland et al., 2005). The ¹³C₁₀-labeled internal standard was synthesized in an analogous way, but using [¹³C₁₀]dG (>99% isotopic purity; Spectra Stable Isotopes, Columbia, MD). The standard was purified by preparative HPLC (Turesky et al., 1992), and its concentration was determined by UV spectroscopy (Pulkabek et al., 1979). Under these chromatographic conditions and the LC-ESI/MS/MS conditions described below, the four stereoisomers of dG-*N*²-BAP were unresolved. DNA (100 μ g) was digested with DNase I, nuclease P₁, alkaline phosphatase, and phosphodiesterase I as described (Goodenough et al., 2007). Cold ethanol was added to precipitate protein and salt. The supernatant was concentrated to dryness by vacuum centrifugation, and adducts were enriched by solid-phase extraction with HypersepTM C-18 Filter SpinTips (10 – 200 μ L; Thermo Fisher).

LC-ESI/MS/MS analyses were conducted on a Finnigan™ TSQ Quantum Ultra™ triple quadrupole mass spectrometer (Thermo Fisher, San Jose, CA), equipped with an Ion Max™ electrospray ionization source operating in positive ionization mode, and controlled with Xcalibur software (version 1.4; Thermo Electron). The instrument tuning parameters used were as follows: source spray voltage, 2.5 kV; nitrogen sheath gas, setting 1; capillary temperature, 250 °C; tube lens offset, 103 V; and in-source collision-induced dissociation offset, 5 V. For LC-ESI/MS/MS, the collision energy was 30 eV and the collision gas was argon at 1.5 mTorr. The MS/MS transitions recorded in the selected-reaction-monitoring mode were: m/z 570.2 • 257.2 and m/z 580.2 • 257.2, for dG- N^2 -BAP and [$^{13}\text{C}_{10}$]dG- N^2 -BAP, respectively, each with a dwell time of 0.05 s (Beland et al., 2005). Chromatography was performed with an Agilent (Palo Alto, CA) 1100 capillary LC equipped with an Aquasil C18 column (5 μm particle size, 0.5 $\times$ 250 mm; Thermo Fisher, Bellefonte, PA) using solvent A, consisting of 0.05% HCO_2H , 89.95% H_2O , and 10% CH_3CN , and solvent B, consisting of 0.05% HCO_2H , 5% H_2O , and 94.95% CH_3CN . The gradient program consisted of a hold at 100% A for 1 min, a linear gradient to 100% B over 30 min, a hold at 100% B for 5 min, regeneration to starting conditions for 2 min, and post-run re-equilibration for 8 min. The flow rate was 12 $\mu\text{L}/\text{min}$.

BAP-modified, calf thymus DNA with 1.15 adducts per 10^6 DNA nucleotides, a generous gift of Dr. Frederick Beland of the National Center for Toxicological Research, Jefferson AR (Beland et al., 2005), was diluted with salmon sperm DNA as carrier to 1.0 adduct per 10^7 nucleotides. A calibration curve was produced with a fixed amount of internal standard (5.8 pg) and 0, 1.7, 2.9, 5.7, 11.4, and 22.2 pg of dG- N^2 -BAP, corresponding to 0 to 4.3 adducts per 10^7 nucleotides. Its slope, obtained from the plot of the response ratio against the amount ratio (unlabeled adduct/internal standard), equaled 1.10, with $r^2 = 0.999$. Prior to analysis, cellular DNA (100 μg) was fortified with 17.6 pg of [$^{13}\text{C}_{10}$]-dG- N^2 -BAP, equivalent to 1 adduct per 10^7 nucleotides.

Microarray analysis

The microarray gene expression analyses consisted of a 2×2 array of samples in triplicate, with control cells, LTEE cells, control cells exposed to 10 nM TCDD for 72 h, and LTEE cells similarly exposed to TCDD. Total RNA was isolated, and its integrity was verified with an Agilent 2100 Bioanalyzer. Microarray analyses of RNA were performed at the Wadsworth Center Microarray Core using Affymetrix (Santa Clara, CA) HG U133A 2.0 arrays and the Affymetrix GeneChip system. Gene expression data were analyzed using GeneSpring GX 10 software (Agilent). Summarization of gene expression data was performed by implementing the robust multichip averaging algorithm, with subsequent baseline normalization of the log-summarized values for each probe set to that of the median log-summarized value for the same probe set in the control group. Expression data were then filtered to remove probe sets for which the signal intensities for all four of the treatment groups were in the lowest 20 percentile of all intensity values. The data were then subjected to analysis of variance (ANOVA), incorporating the Benjamini-Hochberg FDR multiple testing correction, with a significance level of $P < 0.05$. For Gene Ontology (GO) analyses, data sets were first normalized using the GeneTraffic Uno software package (version 3; Iobion); GenMAPP (version 2; <http://www.GenMAPP.org>) (Dalquist et al., 2002) was then used to sort the data. MAPPFinder (Doniger et al., 2003) was used to group genes that showed significant changes in expression ($P < 0.05$) according to the GO hierarchy based on biological process, cellular component, and molecular function.

MCF-7 xenograft assays

For the xenograft studies, four groups of 15 mice each received an inoculation as follows: group 1, LTEE cells; group 2, LTEE cells with E_2 supplementation; group 3, control cells;

and group 4, control cells with E₂ supplementation. Each inoculation consisted of 1×10^6 cells in 50 μ l of culture medium, injected into the surgically exposed mammary fat pads of 6- to 8-week-old severe combined immunodeficient (SCID) female mice from Taconic Farms (Germantown, NY), which were under light anesthesia with isoflurane. Mice in groups 2 and 4 received E₂ supplementation, which was accomplished by subcutaneous implantation of a silastic tubing capsule containing solid E₂ inserted on the day of tumor implantation; the capsule provided serum levels of 1 nM (Bennett et al., 2006). External palpation of mice was accomplished daily, and emerging tumors were measured with Vernier calipers several times per week for 61 to 72 days. Tumor volume was calculated using the formula $V = \pi/6(d)^2D$, assuming the tumor shape to be ellipsoidal with D as the long axis. The latency of tumor formation is the day at which the maximal incidence of tumor formation within the group is observed by palpation. The Albany Medical College Animal Care and Use Committee approved all work with animals.

Statistical evaluations

Statistical evaluations of biochemical determinations, including EROD activity, luciferase assays, RNA levels, and BAP adduct levels, 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.

Results

LTEE MCF-7 cells show enhanced aryl hydrocarbon (Ah) responsiveness

LTEE of MCF-7 cells using physiologic levels of E₂ resulted in significantly higher basal and TCDD-induced EROD activities, as compared with the activities for MCF-7 cultures without E₂ supplementation (Fig. 1A). This result indicates a persistent effect of LTEE on CYP1-catalyzed EROD activity, given that the added E₂ was present during 12 weeks of culture prior to the experiment, whereas all of the manipulations, including subculture, proliferation to confluence, TCDD exposure, and the EROD assay were performed with DC₅ medium without added E₂. LTEE with 1.0 nM E₂ caused approximately 3-fold higher basal and TCDD-induced EROD activities. LTEE with 0.01 and 0.1 nM E₂ showed lesser, although statistically significant, elevations in EROD activity. The enzyme activities of cultures continuously maintained in DF₅ medium, containing fetal serum rather than calf serum, are shown for comparison. Consistent with the findings of a previous study (Spink et al., 2003b), MCF-7 cells continuously maintained in DC₅ medium showed significantly lower basal and TCDD-induced EROD activities than did cultures maintained in DF₅ medium.

To investigate whether the observed effects of LTEE on basal and TCDD-induced EROD activity reflect changes at the level of mRNA expression, we analyzed levels of several mRNAs relevant to the Ah response for CYP1 induction. Control and LTEE cultures undergoing continuous exposure to 1 nM E₂ were treated for 72 h with either 10 nM TCDD or vehicle, 0.1% DMSO. Real-time PCR of reverse-transcribed RNA isolated from the LTEE cultures showed significantly higher AhR mRNA levels (> 4-fold) compared with the levels for the controls (Fig. 1B). In both control and LTEE cultures, the levels of AhR mRNA were not affected by treatment with TCDD (Fig. 1B). In contrast to the effects on expression of AhR mRNA, expression of mRNA encoding the AhR dimerization partner, ARNT, was not significantly affected by LTEE. The level of ER α mRNA was approximately 45% lower in LTEE cells than in control cells, and was not significantly affected by TCDD. LTEE resulted in higher expression levels of the TCDD-induced CYP1A1 and CYP1B1 mRNAs, by 3.3- and 3.7-fold, respectively. Levels of the 36B4

mRNA, which encodes a ribosomal protein and is commonly used as an E₂-independent control transcript, showed little difference in expression among the treatment groups.

Having established that LTEE results in greater levels of EROD activity in response to TCDD exposure and elevates the levels of AhR, CYP1A1, and CYP1B1 mRNA, we then investigated whether similar changes were evident at the level of protein expression. Analyses by Western blot confirmed that AhR expression was up-regulated by LTEE; continuous exposure to 0.01, 0.10 or 1.0 nM E₂ for 12 weeks induced higher levels of AhR protein expression, relative to the control (Fig. 1C). Under each of the long-term culture conditions, exposure to 10 nM TCDD for 72 h caused down-regulation of AhR protein expression, presumably due to ligand-induced, proteasomal degradation of the AhR (Davarinos and Pollenz, 1999). Induction of CYP1A1 and CYP1B1 proteins by TCDD was enhanced by LTEE with 0.01, 0.10, and 1.0 nM E₂.

Effects of LTEE and the antiestrogen, ICI 182,780, on TCDD-induced CYP1A1 and CYP1B1 promoter activity

We next investigated whether the AhR-mediated activation of *CYP1A1* and *CYP1B1* gene transcription in LTEE cells requires the continued presence of estrogen and is subject to inhibition by the antiestrogen, ICI 182,780. For these experiments, control and LTEE cultures were subcultured in DC₅ medium without added E₂, and were transfected with CYP1-promoter-luciferase constructs. Cultures then were treated with E₂, ICI 182,780, or TCDD for 6 h. Short-term exposure of control or LTEE cell cultures to E₂ resulted in a modest up-regulation of TCDD-induced CYP1B1-promoter activity and a modest down-regulation of CYP1A1-promoter activity, whereas LTEE enhanced the TCDD-induced CYP1A1- and CYP1B1-promoter activities by 3.9- and 2.7-fold, respectively (Fig. 2A). The modest increases in CYP1B1 promoter activity as a result of short-term exposure to E₂ were reversed by co-exposure to ICI 182,780. To further evaluate the persistence of LTEE on AhR-regulated promoter activities, we examined the effect of ICI 182,780 in the absence of added E₂ on TCDD- inducible CYP1 promoter activities in control cells, LTEE cells, and LTEE cells that had been depleted of E₂ for 19 days, during which time the cells underwent approximately 5 doublings. CYP1A1- promoter activity was not affected by ICI 182,780, and CYP1B1 promoter activity was only modestly inhibited by the antiestrogen (Fig. 2B). In LTEE cells depleted of E₂ for 19 days, there was some diminution of CYP1 promoter activities, but the activity levels remained significantly higher than in control cells. These results show that the effects of LTEE on TCDD-induced CYP1 promoter activities (i) greatly exceed those of short-term E₂ exposure, (ii) are weakly sensitive to ICI 182,780, and (iii) persist after E₂ depletion.

LTEE results in increased initial levels of ³H-BAP-DNA adducts

CYP1A1 and CYP1B1 are prominent enzymes in the metabolic activation of BAP and other polycyclic aromatic hydrocarbon (PAH) carcinogens (Shimada et al., 1996); we therefore investigated the effect of LTEE on BAP-DNA adduct formation. For these experiments, LTEE cells and control cells were subcultured using DC₅ medium without added E₂. At confluence, cultures were exposed to 0.1 μM ³H-BAP in conditioned medium. At the times indicated, DNA was isolated, and the levels of DNA-bound ³H-BAP were determined (Fig. 3A). A higher initial level of ³H-BAP bound to DNA was observed in the LTEE cultures than in the control cultures. At 2 h and 3 h of exposure to ³H-BAP, levels of DNA-bound ³H-BAP in LTEE cells were respectively 5.4- and 4.2-fold higher than levels at the same time points in control cells. A higher maximal level of DNA-bound ³H-BAP was observed in LTEE cultures than in control cultures, at 8 h. However, the adduct level decreased more rapidly in LTEE cultures, in the period from 8 to 42 h after ³H-BAP exposure. In other studies, similar reductions in BAP adducts with time (Kuljukkka-Rabb et

al., 2001) were associated with p53-dependent activation of DNA repair (Lloyd et al., 2000), and, in MCF-7 cells, expression of p53 protein is correlated with BAP DNA adducts (Ramet et al., 1995).

We then investigated the role of CYP1 enzymes in BAP-DNA adduct formation during a 3-h exposure of cells to 0.1 μ M 3 H-BAP. Dependence of adduct formation on CYP1 activity was evidenced in cultures that were co-exposed to the pan-CYP1 enzyme inhibitor, ANF; inclusion of this agent resulted in 84 and 95% inhibition of DNA-adduct formation, in control and LTEE cultures, respectively (Fig. 3B). Exposure to the CYP1B1-specific inhibitor, TMS (Chun et al., 2001), resulted in reduction in levels of DNA adduct formation in both control and LTEE cultures, by 53 and 43%, respectively. The effect of a low concentration of BAP on the induction of CYP1 enzymes was evident in the levels of mRNA expression (Fig. 4A) in cultures treated for 3 h with BAP. Exposure to 0.1 μ M BAP caused 8.7- and 4.9-fold elevations of the levels of CYP1A1 and CYP1B1 mRNAs in LTEE cells, over the levels in cultures without BAP. The BAP-induced levels of CYP1A1 and CYP1B1 mRNAs in LTEE cultures were 4.1- and 5.0-fold higher than the BAP-induced levels in control cultures. ANF or TMS were also found to significantly induce the CYP1A1 and CYP1B1 mRNAs in LTEE cultures. In co-exposure experiments, ANF significantly inhibited induction of both the CYP1A1 and CYP1B1 mRNAs by BAP in LTEE cultures, consistent with the previously reported AhR antagonist activity of ANF. TMS caused a slight enhancement of BAP-induced CYP1A1 mRNA expression, and a modest diminution of BAP-induced CYP1B1 mRNA expression.

Concomitant with the rapid rise in DNA-adduct formation after BAP exposure, there was significant induction of CYP1 activity when measured 3 h after BAP administration, by EROD assay (Fig. 4B). Similar to the pattern observed for the CYP1A1 and CYP1B1 mRNAs, the level of BAP-induced EROD activity in LTEE cultures greatly exceeded the level in the control cultures, by 12.3- or 7.4-fold, when exposure was to 0.01 or 0.1 μ M BAP, respectively. The basal EROD activity and the activity induced by 0.01 or 0.1 μ M BAP were completely inhibited by inclusion of ANF, whereas TMS inhibited the EROD activity induced by 0.1 μ M BAP in LTEE cells by 15%.

LTEE enhances the initial formation of dG-N²-BAP adducts

Having established that LTEE causes an increase in the initial level of BAP-DNA adducts, and that this enhancement is subject to inhibition by CYP1A1 and CYP1B1 inhibitors, we next investigated whether the observed DNA adduction occurred via the well-characterized pathway of metabolic activation of BAP leading to BPDE formation (Fig. 5). Analysis by LC-ESI/MS/MS that was specific for dG-N²-BAP adduct detection (Fig. 5A, 5B) showed a highly significant effect of LTEE on adduct formation at 3 h of BAP exposure (Fig. 5C). The enhancement of dG-N²-BAP adduct formation in LTEE cultures, relative to the rate of formation in control cultures, was 6.7-fold, an effect similar in magnitude to the 5.4-fold increase of 3 H-BAP adduct formation in LTEE cells, relative to control cells (Fig. 3B). However, the absolute levels of adducts quantified by the two methods differed greatly. Levels of adducts determined by incorporation of 3 H-BAP into DNA were approximately 10-fold higher than those determined by LC-ESI/MS/MS analysis of dG-N²-BAP.

LTEE markedly alters global gene expression in MCF-7 cells

To investigate the impact of LTEE on global gene expression, we performed microarray analyses with mRNA from LTEE cells and control cells, with and without exposure to 10 nM TCDD for the final 72 h. The RNA preparations used for the microarray studies were those used in the real time RT-PCR studies presented in Fig. 1B; the LTEE cultures contained E₂ continuously until the time of RNA isolation. Analysis of the microarray data

identified a subset of 824 probe sets for which significant differences in expression among the 4 groups (control, control plus TCDD, LTEE, and LTEE plus TCDD) were indicated (ANOVA, $P < 0.05$), and for which there was variation of ≥ 2 -fold in at least one of the pairwise comparisons among the four groups. A heat map of mRNA expression evaluated with these 824 probe sets is shown in Fig. 6A, displaying the effects of LTEE and TCDD exposure. Although exposure of control cells to TCDD caused changes in the expression of a number of transcripts, the effects of LTEE on gene expression without or with TCDD exposure were much more striking. In contrast to mRNAs elevated by LTEE, some other mRNAs showed decreases in level in response to LTEE; notable was the mRNA that encodes ABCG2, also known as the breast cancer resistance protein. While LTEE depressed the level of ABCG2 expression, TCDD highly induced ABCG2 expression in both control and LTEE MCF-7 cells, as determined by microarray (Fig. 6B), real-time PCR (Fig. 6C), and Western immunoblot (Fig. 6D).

Gene expression data limited to the subset of 824 probe sets described above and in Fig 6A are summarized as (i) responses that were elevated or depressed ≥ 2 -fold in LTEE cells, as compared with responses in control cells (Supplementary Table 1); (ii) responses that were elevated or depressed ≥ 2 -fold by exposure to TCDD (10 nM, 72 h) as compared with responses in control cultures without TCDD-exposure (Supplementary Table 2); and (iii) responses that were elevated or depressed ≥ 2 -fold by TCDD exposure in LTEE cells as compared with responses in LTEE cells (Supplementary Table 3). In LTEE cultures, data for 345 probe sets indicated expression increases by ≥ 2 -fold, and 156 depressions by ≥ 2 -fold, relative to the controls (Supplemental Table I). There were numerous indications of AhR/ER α interactive effects on gene expression: of the 345 probe sets that indicated ≥ 2 -fold elevated expression in LTEE cultures, 13 showed further enhancement of expression by TCDD exposure at least 2-fold over the expression observed with LTEE only, and 89 of these showed an anti-estrogenic effect of TCDD, defined as $\geq 50\%$ diminution of the enhancement of expression of genes from LTEE cells, when co-exposed to TCDD. Among the genes that were more highly expressed in LTEE cultures compared with control cultures (≥ 2 -fold), a number of human genes or their rodent orthologs have been previously reported to be regulated by estrogen: *S100A7* (Moog-Lutz et al., 1995), *AREG* (Martinez-Lacaci et al., 1995), *IFI27* (Rasmussen et al., 1993), *PDZK1* (Ghosh et al., 2000), *SCNN1A* (Soulez and Parker, 2001; Bourdeau et al., 2004), *SERPINA5* (Soulez and Parker, 2001; Bourdeau et al., 2004), *C3* (Middleton and Linder, 1993; Li et al., 2002), *CP* (Middleton and Linder, 1993; Stuckey et al., 2006), *WISP2* (Inadera et al., 2000), *MGP* (Sheikh et al., 1993), *PLAT* (Ryan et al., 1984), *SERPINE1* (Smith et al., 2004), *BF* (Li et al., 2002), *SERPINA3* (Yamamura et al., 2004), *ABCC3* (Vendrell et al., 2004), *NPY1R* (Amlal et al., 2006) *SLPI* (Shimoya et al., 2006), *ISG20* (Pentecost, 1998), *PGR* (Nardulli et al., 1988; Coser et al., 2003), *GJA1* (Petrocelli and Lye, 1993) *PRSS23* (Cunliffe et al., 2003; Coser et al., 2003) *FRK* (Coser et al., 2003), *CYP1B1* (Spink et al., 2003b; Labhart et al., 2005), *EGR3* (Inoue et al., 2004), *TGFA* (Kenney et al., 1993), *CTSD* (Cavaillès et al., 1988), and *GREB1* (Ghosh et al., 2000). In LTEE cultures, there were also genes that are down-regulated in expression, relative to their transcript abundances in the control cultures. Human genes or their rodent orthologs that have been previously reported to be down-regulated in expression by E₂, and that were down-regulated in our studies (≥ 2 -fold), include: *GRB14* (Kairouz et al., 2005); *TNFRSF11B* (Rachner et al., 2008), *CAVI* (Zschocke et al., 2002), *CAV2* (Zschocke et al., 2002), *CRY2* (Nakamura et al., 2004), *CLU* (So et al., 2005), *IL1R1* (Santen et al., 2005; Coser et al., 2003), *IGFBP5* (Inoue et al., 2002), *EFEMP1* (Inoue et al., 2002), *PSCA* (Coser et al., 2003) *KCNJ8* (Coser et al., 2003), and *CTNND2* (Coser et al., 2003).

Differences in gene expression patterns among treatment groups were analyzed with the MAPPFinder tool (Dalquist et al., 2002). Only two GO terms were found in the first pairwise comparison, which was between control cells and control cells exposed to TCDD.

In contrast, 60 GO terms were found in the second comparison, which evaluated RNA expression in LTEE cells and in control cells, and 75 GO terms were found in the third comparison, which evaluated RNA expression in LTEE cells and in TCDD-exposed LTEE cells. For the second and third comparisons, several similar GO terms were observed, reflecting the effects of LTEE and, in some instances, the anti-estrogenic effects of TCDD (Spink et al., 1990; Safe et al., 2000). In general, expression of genes related to the cell cycle, including regulation of mitosis and the mitotic checkpoint, was up-regulated by LTEE, and was down-regulated by the addition of TCDD to LTEE cells. Expression of genes related to the response to reactive oxygen species and DNA polymerization was also elevated by LTEE. Expression of genes related to cellular development and morphogenesis was down-regulated by LTEE; however, expression of genes related to cellular morphogenesis was also down-regulated in LTEE cells exposed to TCDD. Expression of genes that regulate cell death, apoptosis, and immune response was up-regulated in LTEE cells, whereas the addition of TCDD to LTEE cells resulted in the down-regulation of the expression of genes involved in the negative regulation of apoptosis and the up-regulation of gene expression related to prostaglandin metabolism. GO terms that were unique to TCDD effects in LTEE cells included many cellular metabolic processes. The complete list of GO terms and analysis can be found in Supplementary Table 4.

LTEE enhances the rate of estrogen-dependent tumor growth in SCID mice

Xenograft studies using SCID mice showed differences in development of tumors, between mice inoculated with LTEE MCF-7 cells and mice inoculated with the control MCF-7 cells (Fig. 7). When the animals received E₂ supplementation, both the LTEE-cell and control-cell groups showed 100% tumor incidence; *i.e.*, all of the animals in the E₂ supplementation groups developed palpable tumors in the mammary glands. Among the groups that received E₂ supplementation, there was a shorter latency to attainment of 100% tumor incidence in mice inoculated with LTEE cells than in mice inoculated with control cells. However, among the groups without E₂ supplementation, there was a greater incidence of palpable tumors in the control-cell mice, 15/15 (100%), than in the LTEE-cell mice, 9/15 (60%). There were also differences in the E₂-induced rates of tumor growth, dependent on whether the animals were inoculated with LTEE cells or control cells. In mice inoculated with LTEE cells and that received E₂ supplementation, the tumors had grown to a mean volume of 440 mm³ by day 62, which was significantly greater than the tumor volumes in mice inoculated with control cells and that received E₂ supplementation ($P < 0.05$); these tumors grew to only a mean volume of 62 mm³ at day 63. When the experiment was extended to day 71, the mean volume was still only 137 mm³. While the majority of the animals in the LTEE-cell and the control-cell groups that did not receive E₂ supplementation developed palpable tumors, these tumors failed to grow beyond 2 mm³; these results establish that tumor growth of both LTEE cells and control cells is dependent on the continued presence of E₂.

Discussion

The incidence of breast cancer and the progression of the disease have been linked to lifetime estrogen exposure. Even in post-menopausal women, the majority of diagnosed breast cancers are estrogen-dependent. Most current studies of estrogenic effects in breast cancer cells focus on the very early events after E₂ exposure. However, it is well established that estrogen exposure can have long-term effects. For example, estrogens can cause genetic imprinting in neonates (McLachlan et al., 2001), and these effects can carry over to the next generation. We suggest that pre-cancerous, nascent, and dormant tumor cells are subject to similar induction of persistent changes in gene expression elicited by long-term exposure to estrogens, and posit that these changes may affect both the initiation and promotion phases of carcinogenesis.

MCF-7 cells provide a model system for the study of estrogen-responsive breast cancer and the molecular biology of estrogen action. MCF-7 cells are commonly employed for *in vitro* studies because no ER-positive breast epithelial cell lines, normal or immortalized, of the luminal differentiation lineage are currently available. While it is known that estrogen imprinting occurs *in vivo*, it is not known whether the effects of LTEE observed in our study represent a directly comparable process. The propensity of the long-term presence or absence of estrogen to alter signaling pathways and gene expression in MCF-7 cells has been known for some time (Katzenellenbogen et al., 1987). Long-term estrogen deprivation of MCF-7 cells, a treatment in which estrogen is essentially eliminated from the cultures by use of charcoal-stripped serum in medium formulations, leads to estrogen hypersensitivity, changes in gene expression, and up-regulation of growth factor pathways (Katzenellenbogen et al., 1987; Santen et al., 2005). Our studies differed from this approach in that we cultured MCF-7 cells in DC₅ medium prepared with bovine calf serum, which is naturally low in estrogens. We previously observed that long-term culture of MCF-7 cells in DC₅ medium resulted in a marked reduction of Ah responsiveness for induction of CYP1A1 and CYP1B1 compared with cells maintained long-term in DF₅ medium (Spink et al., 2003b). To clearly differentiate between E₂ and serum effects, the current study was performed with DC₅-based media that differed only by the inclusion or omission of physiologic concentrations of E₂. The effects of LTEE were greater in magnitude than those previously observed for short-term E₂ exposure on CYP1B1 expression (Spink et al., 2003b) and estrogen induction of ³H-BAP-DNA adduct formation (Kang and Lee, 2005) in MCF-7 cells.

LTTEE would be expected to affect numerous pathways in addition to that of the Ah response. Zou and Matsumura (2003) also chronically exposed MCF-7 cells to E₂ and found *in vitro* increases in anchorage-independent growth and invasiveness. Our results showing that LTTEE cells had greater estrogen-stimulated tumor growth in SCID mice than did control cells are consistent with the transformation-related biochemical changes described by (Zou and Matsumura 2003), and they likely reflect changes in multiple signaling pathways. Indeed, cells subjected to LTTEE in our study showed profound changes in global gene expression, profound not only in the magnitudes of the changes but also in the number of genes that showed differential expression. In comparison with the lesser changes seen in previous microarray studies aimed at determining the genes that are directly activated by short-term (3 to 24 h) estrogen exposure (Frasor et al., 2003; Coser et al., 2003; Ochsner et al., 2009), our data essentially reflect long-term, steady-state differences in gene expression. Recently, (Sadler et al. 2009) also observed very large changes in gene expression in MCF-7 cells that were chronically exposed to E₂. In their study and in ours, some of the observed changes may have been secondary to the initial events elicited by estrogen exposure, but they nonetheless are potentially important in the development and advancement of breast cancer.

PAHs are known mammary carcinogens in rodents, and are suspected of having the same effect in humans (Brody et al., 2007). The enhancement of the AhR-mediated induction of CYP1A1 and CYP1B1 by LTTEE, leading to higher capacity for metabolic activation of PAH to ultimate carcinogens, may have important implications for cancer. Our results show higher initial levels of BAP-adduct formation in LTTEE cells than in control cells; however, determination of the absolute levels was method-dependent. Much higher absolute levels of DNA adducts were observed when ³H-BAP incorporation was measured, as compared to the levels of dG-N²-BAP adducts determined by LC-ESI/MS/MS. Similar quantitatively inconsistent results have been previously obtained by Beland *et al.* (2005), who found that measurements of ³H-BAP incorporation gave much higher adduct levels than did LC-ESI/MS/MS analysis. Those authors suggest that dG-N²-BAP adducts contribute only a small fraction of the total BAP-DNA adducts, and our results are consistent with this contention. While it is not clear what accounts for the discrepancy in adduct levels as determined by the

two techniques, our results raise the possibility that pathways of metabolic activation other than the BPDE pathway are responsible for the formation of DNA adducts from BAP. Two additional pathways of PAH metabolism that could be responsible for formation of BAP-DNA adducts are (i) the aldo-keto reductase-catalyzed pathway that converts PAH dihydrodiols into DNA-adductive *ortho*-quinones (Quinn et al., 2008; McCoull et al., 1999; Balu et al., 2004), and (ii) the one-electron oxidation of PAHs to adductive intermediates (Chen et al., 1996). In both the BPDE and the *ortho*-quinone pathways of BAP metabolic activation, the initial oxidation is catalyzed by CYP1-family enzymes. The potent inhibition of the incorporation of ³H-BAP incorporation into DNA by ANF is evidence of the involvement of CYP1 enzymes in the metabolic pathway(s) that led to adduct formation. Furthermore, the marked enhancement, by LTEE, of the initial levels of both ³H-BAP incorporation into DNA and the formation of dG-N²-BAP adducts is consistent with the involvement of CYP1 enzymes, since, by both measures, BAP-DNA adduct formation was elevated to roughly the same magnitude as were the increases of CYP1A1 and CYP1B1 mRNAs, proteins, and enzyme activities in LTEE cells

A notable finding in this study is that changes induced by LTEE persisted after the removal of E₂. AhR-regulated CYP1A1- and CYP1B1-promoter activities, CYP1A1 and CYP1B1 mRNAs levels, EROD activities, and BAP-DNA adduct levels were all elevated in LTEE cells that were subcultured in DC₅ medium without E₂ supplementation, and inclusion of the antiestrogen, ICI 182,780, did not block effects of LTEE on CYP1A1 and CYP1B1 transcriptional activation. These results suggest that persistent changes in gene expression accompany LTEE of MCF-7 cells. Through enhancement of the Ah response, E₂ could play a role in the propensity of tumors to continually develop the spectrum of mutations that is observed in breast cancer (Sjöblom et al., 2006; Wood et al., 2007) via the metabolic activation of carcinogens. If this is so, E₂ may participate in the initiation of breast cancer, as well as in the promotion and progression phases of the disease. However, it remains to be determined whether normal ER-positive luminal cells and nascent cancer cells within the human breast respond similarly to LTEE as do MCF-7 tumor cells. The studies reported here indicate that LTEE causes adaptive responses in MCF-7 cells, possibly reflecting processes that contribute to the overall carcinogenic effect of E₂.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Dr. Frederick Beland of the National Center for Toxicological Research, Jefferson AR, for the generous gift of BAP-modified, calf-thymus DNA. The authors gratefully acknowledge use of the Wadsworth Center's Tissue Culture Facility and the Biochemistry, Molecular Genetics, and Microarray Core Facilities.

Funding

This research was supported by National Institutes of Health grants CA801243 (to DCS) and ES014438 (to RJT).

References

- Amlal H, Faroqui S, Balasubramaniam A, Sheriff S. Estrogen up-regulates neuropeptide Y Y1 receptor expression in a human breast cancer cell line. *Cancer Res.* 2006; 66:3706–3714. [PubMed: 16585197]
- Balu N, Padgett WT, Lambert GR, Swank AE, Richard AM, Nesnow S. Identification and characterization of novel stable deoxyguanosine and deoxyadenosine adducts of benzo[*a*]pyrene-7,8-quinone from reactions at physiological pH. *Chem. Res. Toxicol.* 2004; 17:827–838. [PubMed: 15206904]

- Beland FA, Churchwell MI, Von Tungeln LS, Chen S, Fu PP, Culp SJ, Schoket B, Gyorffy E, Minarovits J, Poirier MC, Bowman ED, Weston A, Doerge DR. High-performance liquid chromatography electrospray ionization tandem mass spectrometry for the detection and quantitation of benzo[*a*]pyrene-DNA adducts. *Chem. Res. Toxicol.* 2005; 18:1306–1315. [PubMed: 16097804]
- Bennett JA, DeFreest L, Anaka I, Saadati H, Balulad S, Jacobson HI, Andersen TT. AFPep: an anti-breast cancer peptide that is orally active. *Breast Cancer Res. Treat.* 2006; 98:133–141. [PubMed: 16538538]
- Bessette EE, Fasco MJ, Pentecost BT, Kaminsky LS. Mechanisms of arsenite-mediated decreases in benzo[*k*]fluoranthene-induced human cytochrome P4501A1 levels in HepG2 cells. *Drug Metab. Dispos.* 2005; 33:312–320. [PubMed: 15576448]
- Bourdeau V, Deschenes J, Metivier R, Nagai Y, Nguyen D, Bretschneider N, Gannon F, White JH, Mader S. Genome-wide identification of high-affinity estrogen response elements in human and mouse. *Mol Endocrinol.* 2004; 18:1411–1427. [PubMed: 15001666]
- Brody JG, Moysich KB, Humblet O, Attfield KR, Beehler GP, Rudel RA. Environmental pollutants and breast cancer: epidemiologic studies. *Cancer.* 2007; 109(12 Suppl):2667–2711. [PubMed: 17503436]
- Buzdar AU, Coombes RC, Goss PE, Winer EP. Summary of aromatase inhibitor clinical trials in postmenopausal women with early breast cancer. *Cancer.* 2008; 112:700–709. [PubMed: 18072256]
- Cavaillès V, Augereau P, Garcia M, Rochefort H. Estrogens and growth factors induce the mRNA of the 52K-pro-cathepsin-D secreted by breast cancer cells. *Nucleic Acids Res.* 1988; 16:1903–1919. [PubMed: 3282224]
- Chen L, Devanesan PD, Higginbotham S, Ariese F, Jankowiak R, Small GJ, Rogan EG, Cavalieri EL. Expanded analysis of benzo[*a*]pyrene-DNA adducts formed in vitro and mouse skin: their significance in tumor initiation. *Chem. Res. Toxicol.* 1996; 9:897–903. [PubMed: 8828927]
- Cheng AS, Culhane AC, Chan MW, Venkataramu CR, Ehrich M, Nasir A, Rodriguez BA, Liu J, Yan PS, Quackenbush J, Nephew KP, Yeatman TJ, Huang TH. Epithelial progeny of estrogen-exposed breast progenitor cells display a cancer-like methylome. *Cancer Res.* 2008; 68:1786–1796. [PubMed: 18339859]
- Chun YJ, Kim S, Kim D, Lee SK, Guengerich FP. A new selective and potent inhibitor of human cytochrome P450 1B1 and its application to antimutagenesis. *Cancer Res.* 2001; 61:8164–8170. [PubMed: 11719446]
- Clamp A, Danson S, Clemons M. Hormonal risk factors for breast cancer: identification, chemoprevention, and other intervention strategies. *Lancet Oncol.* 2002; 3:611–619. [PubMed: 12372723]
- Clemons M, Goss P. Estrogen and the risk of breast cancer. *N. Engl. J. Med.* 2001; 344:276–285. [PubMed: 11172156]
- Coser KR, Chesnes J, Hur J, Ray S, Isselbacher KJ, Shioda T. Global analysis of ligand sensitivity of estrogen inducible and suppressible genes in MCF7/BUS breast cancer cells by DNA microarray. *Proc. Natl. Acad. Sci. U. S. A.* 2003; 100:13994–13999. [PubMed: 14610279]
- Cunliffe HE, Ringner M, Bilke S, Walker RL, Cheung JM, Chen Y, Meltzer PS. The gene expression response of breast cancer to growth regulators: patterns and correlation with tumor expression profiles. *Cancer Res.* 2003; 63:7158–7166. [PubMed: 14612509]
- Dahlquist KD, Salomonis N, Vranizan K, Lawlor SC, Conklin BR. GenMAPP, a new tool for viewing and analyzing microarray data on biological pathways. *Nat. Genet.* 2002; 31:19–20. [PubMed: 11984561]
- Davarinos NA, Pollenz RS. Aryl hydrocarbon receptor imported into the nucleus following ligand binding is rapidly degraded via the cytoplasmic proteasome following nuclear export. *J. Biol. Chem.* 1999; 274:28708–28715. [PubMed: 10497241]
- Doniger SW, Salomonis N, Dahlquist KD, Vranizan K, Lawlor SC, Conklin BR. MAPPFinder: using Gene Ontology and GenMAPP to create a global gene-expression profile from microarray data. *Genome Biol.* 2003; 4:R7. [PubMed: 12540299]

- Eliassen AH, Missmer SA, Tworoger SS, Spiegelman D, Barbieri RL, Dowsett M, Hankinson SE. Endogenous steroid hormone concentrations and risk of breast cancer among premenopausal women. *J. Natl. Cancer Inst.* 2006; 98:1406–1415. [PubMed: 17018787]
- Fisher B, Costantino JP, Wickerham DL, Cecchini RS, Cronin WM, Robidoux A, Bevers TB, Kavanah MT, Atkins JN, Margolese RG, Runowicz CD, James JM, Ford LG, Wolmark N. Tamoxifen for the prevention of breast cancer: current status of the National Surgical Adjuvant Breast and Bowel Project P-1 study. *J. Natl. Cancer Inst.* 2005; 97:1652–1662. [PubMed: 16288118]
- Frasor J, Danes JM, Komm B, Chang KC, Lyttle CR, Katzenellenbogen BS. Profiling of estrogen up- and down-regulated gene expression in human breast cancer cells: insights into gene networks and pathways underlying estrogenic control of proliferation and cell phenotype. *Endocrinology.* 2003; 144:4562–4574. [PubMed: 12959972]
- Freedman OC, Verma S, Clemons MJ. Pre-menopausal breast cancer and aromatase inhibitors: Treating a new generation of women. *Breast Cancer Res. Treat.* 2006; 99:241–247. [PubMed: 16752075]
- Ghosh MG, Thompson DA, Weigel RJ. PDZK1 and GREB1 are estrogen-regulated genes expressed in hormone-responsive breast cancer. *Cancer Res.* 2000; 60:6367–6375. [PubMed: 11103799]
- Goodenough AK, Schut HA, Turesky RJ. Novel LC-ESI/MS/MS⁽ⁿ⁾ method for the characterization and quantification of 2'-deoxyguanosine adducts of the dietary carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine by 2-D linear quadrupole ion trap mass spectrometry. *Chem. Res. Toxicol.* 2007; 20:263–276. [PubMed: 17305409]
- Goss PE, Ingle JN, Martino S, Robert NJ, Muss HB, Piccart MJ, Castiglione M, Tu D, Shepherd LE, Pritchard KI, Livingston RB, Davidson NE, Norton L, Perez EA, Abrams JS, Cameron DA, Palmer MJ, Pater JL. Randomized trial of letrozole following tamoxifen as extended adjuvant therapy in receptor-positive breast cancer: updated findings from NCIC CTG MA.17. *J. Natl. Cancer Inst.* 2005; 97:1262–1271. [PubMed: 16145047]
- Inadera H, Dong HY, Matsushima K. WISP-2 is a secreted protein and can be a marker of estrogen exposure in MCF-7 cells. *Biochem. Biophys. Res. Commun.* 2002; 294:602–608. [PubMed: 12056810]
- Inoue A, Omoto Y, Yamaguchi Y, Kiyama R, Hayashi SI. Transcription factor EGR3 is involved in the estrogen-signaling pathway in breast cancer cells. *J. Mol. Endocrinol.* 2004; 32:649–661. [PubMed: 15171706]
- Jonsson G, Staaf J, Olsson E, Heidenblad M, Vallon-Christersson J, Osoegawa K, de Jong P, Oredsson S, Ringner M, Hoglund M, Borg A. High-resolution genomic profiles of breast cancer cell lines assessed by tiling BAC array comparative genomic hybridization. *Genes Chromosomes Cancer.* 2007; 46:543–558. [PubMed: 17334996]
- Jordan VC. SERMs: meeting the promise of multifunctional medicines. *J. Natl. Cancer Inst.* 2007; 99:350–356. [PubMed: 17341726]
- Jordan VC, Brodie AM. Development and evolution of therapies targeted to the estrogen receptor for the treatment and prevention of breast cancer. *Steroids.* 2007; 72:7–25. [PubMed: 17169390]
- Kairouz R, Parmar J, Lyons RJ, Swarbrick A, Musgrove EA, Daly RJ. Hormonal regulation of the Grb14 signal modulator and its role in cell cycle progression of MCF-7 human breast cancer cells. *J. Cell. Physiol.* 2005; 203:85–93. [PubMed: 15372466]
- Kang SC, Lee BM. Effect of estrogen receptor (ER) on benzo[*a*]pyrene-DNA adduct formation in human breast cancer cells. *J. Toxicol. Environ. Health A.* 2005; 68:1833–1840. [PubMed: 16207632]
- Katzenellenbogen BS, Kendra KL, Norman MJ, Berthois Y. Proliferation, hormonal responsiveness, and estrogen receptor content of MCF-7 human breast cancer cells grown in the short-term and long-term absence of estrogens. *Cancer Res.* 1987; 47:4355–4360. [PubMed: 3607768]
- Kenney NJ, Saeki T, Gottardis M, Kim N, Garcia-Morales P, Martin MB, Normanno N, Ciardiello F, Day A, Cutler ML, et al. Expression of transforming growth factor alpha antisense mRNA inhibits the estrogen-induced production of TGF alpha and estrogen-induced proliferation of estrogen-responsive human breast cancer cells. *J. Cell. Physiol.* 1993; 156:497–514. [PubMed: 8360257]

- Kuljucka-Rabb T, Peltonen K, Isotalo S, Mikkonen S, Rantanen L, Savela K. Time- and dose-dependent DNA binding of PAHs derived from diesel particle extracts, benzo[a]pyrene and 5-methylchrysene in a human mammary carcinoma cell line (MCF-7). *Mutagenesis*. 2001; 16:353–358. [PubMed: 11420405]
- Labhart P, Karmakar S, Salicru EM, Egan BS, Alexiadis V, O'Malley BW, Smith CL. Identification of target genes in breast cancer cells directly regulated by the SRC-3/AIB1 coactivator. *Proc. Natl. Acad. Sci. U. S. A.* 2005; 102:1339–1344. [PubMed: 15677324]
- Li SH, Huang HL, Chen YH. Ovarian steroid-regulated synthesis and secretion of complement C3 and factor B in mouse endometrium during the natural estrous cycle and pregnancy period. *Biol. Reprod.* 2002; 66:322–332. [PubMed: 11804945]
- Lloyd DR, Hanawalt PC. p53-dependent global genomic repair of benzo[a]pyrene-7,8-diol-9,10-epoxide adducts in human cells. *Cancer Res.* 2000; 60:517–521. [PubMed: 10676627]
- Martinez-Lacaci I, Saceda M, Plowman GD, Johnson GR, Normanno N, Salomon DS, Dickson RB. Estrogen and phorbol esters regulate amphiregulin expression by two separate mechanisms in human breast cancer cell lines. *Endocrinology*. 1995; 136:3983–3992. [PubMed: 7649107]
- McCoull KD, Rindgen D, Blair IA, Penning TM. Synthesis and characterization of polycyclic aromatic hydrocarbon *o*-quinone depurinating N7-guanine adducts. *Chem. Res. Toxicol.* 1999; 12:237–246. [PubMed: 10077486]
- McLachlan JA, Burow M, Chiang TC, Li SF. Gene imprinting in developmental toxicology: a possible interface between physiology and pathology. *Toxicol. Lett.* 2001; 120:161–164. [PubMed: 11323173]
- Middleton RB, Linder MC. Synthesis and turnover of ceruloplasmin in rats treated with 17 β -estradiol. *Arch. Biochem. Biophys.* 1993; 302:362–368. [PubMed: 8489241]
- Moog-Lutz C, Bouillet P, Regnier CH, Tomasetto C, Mattei MG, Chenard MP, Anglard P, Rio MC, Basset P. Comparative expression of the psoriasin (S100A7) and S100C genes in breast carcinoma and co-localization to human chromosome 1q21-q22. *Int. J. Cancer*. 1995; 63:297–303. [PubMed: 7591220]
- Nakamura TJ, Shinohara K, Funabashi T, Kimura F. Effect of estrogen on the expression of Cry1 and Cry2 mRNAs in the suprachiasmatic nucleus of female rats. *Neurosci. Res.* 2001; 41:251–255. [PubMed: 11672838]
- Nardulli AM, Greene GL, O'Malley BW, Katzenellenbogen BS. Regulation of progesterone receptor messenger ribonucleic acid and protein levels in MCF-7 cells by estradiol: analysis of estrogen's effect on progesterone receptor synthesis and degradation. *Endocrinology*. 1988; 122:935–944. [PubMed: 3342760]
- Öberg M, Bergander L, Håkansson H, Rannug U, Rannug A. Identification of the tryptophan photoproduct 6-formylindolo[3,2-*b*]carbazole, in cell culture medium, as a factor that controls the background aryl hydrocarbon receptor activity. *Toxicol. Sci.* 2005; 85:935–943. [PubMed: 15788723]
- Ochsner SA, Steffen DL, Hilsenbeck SG, Chen ES, Watkins C, McKenna NJ. GEMS (Gene Expression MetaSignatures), a Web resource for querying meta-analysis of expression microarray datasets: 17 β -estradiol in MCF-7 cells. *Cancer Res.* 2009; 69:23–26. [PubMed: 19117983]
- Pentecost BT. Expression and estrogen regulation of the HEM45 mRNA in human tumor lines and in the rat uterus. *J. Steroid Biochem. Mol. Biol.* 1998; 64:25–33. [PubMed: 9569007]
- Petrocelli T, Lye SJ. Regulation of transcripts encoding the myometrial gap junction protein, connexin-43, by estrogen and progesterone. *Endocrinology*. 1993; 133:284–290. [PubMed: 8391423]
- Pulkabek P, Leffler S, Grunberger D, Weinstein IB. Modification of deoxyribonucleic acid by a diol epoxide of benzo[a]pyrene. Relation to deoxyribonucleic acid structure and conformation and effects on transcriptional activity. *Biochemistry*. 1979; 18:5128–5134. [PubMed: 387082]
- Quinn AM, Penning TM. Comparisons of (+/-)-benzo[a]pyrene-trans-7,8-dihydrodiol activation by human cytochrome P450 and aldo-keto reductase enzymes: effect of redox state and expression levels. *Chem. Res. Toxicol.* 2008; 21:1086–1094. [PubMed: 18402469]

- Rachner TD, Schoppet M, Niebergall U, Hofbauer LC. 17 β -Estradiol inhibits osteoprotegerin production by the estrogen receptor- α -positive human breast cancer cell line MCF-7. *Biochem. Biophys. Res. Commun.* 2008; 368:736–741. [PubMed: 18252198]
- Ramet M, Castren K, Jarvinen K, Pekkala K, Turpeenniemi-Hujanen T, Soini Y, Paakko P, Vahakangas K. p53 protein expression is correlated with benzo[*a*]pyrene-DNA adducts in carcinoma cell lines. *Carcinogenesis*. 1995; 16:2117–2124. [PubMed: 7554063]
- Rasmussen UB, Wolf C, Mattei MG, Chenard MP, Bellocq JP, Chambon P, Rio MC, Basset P. Identification of a new interferon-alpha-inducible gene (p27) on human chromosome 14q32 and its expression in breast carcinoma. *Cancer Res.* 1993; 53:4096–4101. [PubMed: 8358738]
- Ryan TJ, Seeger JJ, Kumar SA, Dickerman HW. Estradiol preferentially enhances extracellular tissue plasminogen activators of MCF-7 breast cancer cells. *J. Biol. Chem.* 1984; 259:14324–14327. [PubMed: 6542103]
- Sadler AJ, Pugazhendhi D, Darbre PD. Use of global gene expression patterns in mechanistic studies of oestrogen action in MCF7 human breast cancer cells. *J. Steroid Biochem. Mol. Biol.* 2009; 114:21–32. [PubMed: 19167489]
- Safe S, Wormke M, Samudio I. Mechanisms of inhibitory aryl hydrocarbon receptor-estrogen receptor crosstalk in human breast cancer cells. *J. Mammary Gland Biol. Neoplasia*. 2000; 5:295–306. [PubMed: 14973392]
- Santen RJ, Lobenhofer EK, Afshari CA, Bao Y, Song RX. Adaptation of estrogen-regulated genes in long-term estradiol deprived MCF-7 breast cancer cells. *Breast Cancer Res. Treat.* 2005; 94:213–223. [PubMed: 16258703]
- Sheikh MS, Shao ZM, Chen JC, Fontana JA. Differential regulation of matrix Gla protein (MGP) gene expression by retinoic acid and estrogen in human breast carcinoma cells. *Mol. Cell. Endocrinol.* 1993; 92:153–160. [PubMed: 8319825]
- Shimada T, Hayes CL, Yamazaki H, Amin S, Hecht SS, Guengerich FP, Sutter TR. Activation of chemically diverse procarcinogens by human cytochrome P-450 1B1. *Cancer Res.* 1996; 56:2979–2984. [PubMed: 8674051]
- Shimoya K, Zhang Q, Temma K, Kimura T, Tsujie T, Tsutsui T, Wasada K, Kanzaki T, Koyama M, Murata Y. Secretory leukocyte protease inhibitor levels in cervicovaginal secretion of elderly women. *Maturitas*. 2006; 54:141–148. [PubMed: 16289563]
- Sjöblom T, Jones S, Wood LD, Parsons DW, Lin J, Barber TD, Mandelker D, Leary RJ, Ptak J, Silliman N, Szabo S, Buckhaults P, Farrell C, Meeh P, Markowitz SD, Willis J, Dawson D, Willson JK, Gazdar AF, Hartigan J, Wu L, Liu C, Parmigiani G, Park BH, Bachman KE, Papadopoulos N, Vogelstein B, Kinzler KW, Velculescu VE. The consensus coding sequences of human breast and colorectal cancers. *Science*. 2006; 314:268–274. [PubMed: 16959974]
- Smith LH, Coats SR, Qin H, Petrie MS, Covington JW, Su M, Eren M, Vaughan DE. Differential and opposing regulation of PAI-1 promoter activity by estrogen receptor alpha and estrogen receptor beta in endothelial cells. *Circ. Res.* 2004; 95:269–275. [PubMed: 15217907]
- So A, Sinnemann S, Huntsman D, Fazli L, Gleave M. Knockdown of the cytoprotective chaperone, clusterin, chemosensitizes human breast cancer cells both in vitro and in vivo. *Mol. Cancer Ther.* 2005; 4:1837–1849. [PubMed: 16373699]
- Soulez M, Parker MG. Identification of novel oestrogen receptor target genes in human ZR75-1 breast cancer cells by expression profiling. *J. Mol. Endocrinol.* 2001; 27:259–274. [PubMed: 11719280]
- Spink BC, Hussain MM, Katz BH, Eisele L, Spink DC. Transient induction of cytochromes P450 1A1 and 1B1 in MCF-7 human breast cancer cells by indirubin. *Biochem. Pharmacol.* 2003a; 66:2313–2321. [PubMed: 14637189]
- Spink DC, Katz BH, Hussain MM, Pentecost BT, Cao Z, Spink BC. Estrogen regulates Ah responsiveness in MCF-7 breast cancer cells. *Carcinogenesis*. 2003b; 24:1941–1950. [PubMed: 12970067]
- Spink DC, Lincoln DW 2nd, Dickerman HW, Gierthy JF. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin causes an extensive alteration of 17 β -estradiol metabolism in MCF-7 breast tumor cells. *Proc. Natl. Acad. Sci. U. S. A.* 1990; 87:6917–6921. [PubMed: 2395886]

- Spink DC, Spink BC, Cao JQ, DePasquale JA, Pentecost BT, Fasco MJ, Li Y, Sutter TR. Differential expression of CYP1A1 and CYP1B1 in human breast epithelial cells and breast tumor cells. *Carcinogenesis*. 1998; 19:291–298. [PubMed: 9498279]
- Spink DC, Wu SJ, Spink BC, Hussain MM, Vakharia DD, Pentecost BT, Kaminsky LS. Induction of CYP1A1 and CYP1B1 by benzo(k)fluoranthene and benzo(a)pyrene in T-47D human breast cancer cells: roles of PAH interactions and PAH metabolites. *Toxicol. Appl. Pharmacol.* 2008; 226:213–224. [PubMed: 17919675]
- Stuckey R, Aldridge T, Lim FL, Moore DJ, Tinwell H, Doherty N, Davies R, Smith AG, Kimber I, Ashby J, Orphanides G, Moggs JG. Induction of iron homeostasis genes during estrogen-induced uterine growth and differentiation. *Mol. Cell. Endocrinol.* 2006; 253:22–29. [PubMed: 16684588]
- Sundstrom SA, Komm BS, Ponce-de-Leon H, Yi Z, Teuscher C, Lyttle CR. Estrogen regulation of tissue-specific expression of complement C3. *J. Biol. Chem.* 1989; 264:16941–16947. [PubMed: 2674144]
- Tamimi RM, Byrne C, Colditz GA, Hankinson SE. Endogenous hormone levels, mammographic density, and subsequent risk of breast cancer in postmenopausal women. *J. Natl. Cancer Inst.* 2007; 99:1178–1187. [PubMed: 17652278]
- The Adjuvant Breast Cancer Trials Collaborative Group. Ovarian ablation or suppression in premenopausal early breast cancer: results from the international adjuvant breast cancer ovarian ablation or suppression randomized trial. *J. Natl. Cancer Inst.* 2007; 99:516–525. [PubMed: 17405996]
- Turesky RJ, Rossi SC, Welti DH, Lay JO Jr, Kadlubar FF. Characterization of DNA adducts formed in vitro by reaction of N-hydroxy-2-amino-3-methylimidazo[4,5-f]quinoline and N-hydroxy-2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline at the C-8 and N2 atoms of guanine. *Chem. Res. Toxicol.* 1992; 5:479–490. [PubMed: 1391614]
- Vendrell JA, Magnino F, Danis E, Duchesne MJ, Pinloche S, Pons M, Birnbaum D, Nguyen C, Theillet C, Cohen PA. Estrogen regulation in human breast cancer cells of new downstream gene targets involved in estrogen metabolism, cell proliferation and cell transformation. *J. Mol. Endocrinol.* 2004; 32:397–414. [PubMed: 15072547]
- Vogel VG, Costantino JP, Wickerham DL, Cronin WM, Cecchini RS, Atkins JN, Bevers TB, Fehrenbacher L, Pajon ER Jr, Wade JL 3rd, Robidoux A, Margolese RG, James J, Lippman SM, Runowicz CD, Ganz PA, Reis SE, McCaskill-Stevens W, Ford LG, Jordan VC, Wolmark N. Effects of tamoxifen vs raloxifene on the risk of developing invasive breast cancer and other disease outcomes: the NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 trial. *Jama*. 2006; 295:2727–2741. [PubMed: 16754727]
- Wen X, Walle T. Cytochrome P450 1B1, a novel chemopreventive target for benzo[a]pyrene-initiated human esophageal cancer. *Cancer Lett.* 2007; 246:109–114. [PubMed: 16530937]
- Wittmann BM, Sherk A, McDonnell DP. Definition of functionally important mechanistic differences among selective estrogen receptor down-regulators. *Cancer Res.* 2007; 67:9549–9560. [PubMed: 17909066]
- Yager JD, Davidson NE. Estrogen carcinogenesis in breast cancer. *N. Engl. J. Med.* 2006; 354:270–282. [PubMed: 16421368]
- Yamamura J, Miyoshi Y, Tamaki Y, Taguchi T, Iwao K, Monden M, Kato K, Noguchi S. mRNA expression level of estrogen-inducible gene, alpha 1-antichymotrypsin, is a predictor of early tumor recurrence in patients with invasive breast cancers. *Cancer Sci.* 2004; 95:887–892. [PubMed: 15546506]
- Zou E, Matsumura F. Long-term exposure to beta-hexachlorocyclohexane (beta-HCH) promotes transformation and invasiveness of MCF-7 human breast cancer cells. *Biochem. Pharmacol.* 2003; 66:831–840. [PubMed: 12948864]
- Zschocke J, Manthey D, Bayatti N, van der Burg B, Goodenough S, Behl C. Estrogen receptor alpha-mediated silencing of caveolin gene expression in neuronal cells. *J. Biol. Chem.* 2002; 277:38772–38780. [PubMed: 12138116]

Fig. 1.

LTEE enhances the induction of CYP1A1 and CYP1B1 by TCDD. In panel A, MCF-7 cells were cultured long-term in DF₅, DC₅, or DC₅ medium supplemented with 0.01, 0.1, or 1.0 nM E₂ as indicated. Cultures were then seeded in DC₅, and at confluence they were exposed to 10 nM TCDD or the solvent vehicle, 0.1% DMSO, in DC₅ for 72 h; then, EROD activities were determined. Data shown are the mean values $\pm$ SE; $n = 4$ wells; significant differences from the control (DC₅) group (** $P < 0.01$; *** $P < 0.001$) are indicated. In panel B, LTEE cultures and control cultures were exposed to 10 nM TCDD or the solvent vehicle in their respective long-term culture media for 72 h. RNA was isolated, reverse-transcribed, and analyzed by real-time PCR for the indicated mRNAs. Data are presented as amol of target mRNA per μ g of total RNA and are the means $\pm$ SE; $n = 3$. Significant differences between LTEE and the control group, or between LTEE with TCDD exposure and the control group with TCDD exposure (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$); and between TCDD exposure in control cells and control cells without TCDD exposure, or between TCDD exposure in LTEE cells and LTEE cells without TCDD exposure (++ $P < 0.01$; +++ $P < 0.001$) are indicated. In panel C, effects of LTEE and TCDD on CYP1A1, CYP1B1, and AhR protein expression in MCF-7 cells. Cells were cultured long-term in DF₅, DC₅, or DC₅ medium supplemented with 0.01, 0.1, or 1.0 nM E₂ as indicated. Cultures were then seeded in DC₅, and at confluence they were exposed to 10 nM TCDD or the solvent vehicle, 0.1% DMSO, in DC₅ medium or 72 h. Microsomes, for analysis of CYP1A1 and CYP1B1, and whole-cell lysates, for the analysis of AhR and actin proteins, were analyzed by Western immunoblot with chemiluminescence detection.

Fig. 2.

Effects of TCDD, E₂, and ICI 182,780 on CYP1A1 and CYP1B1 promoter activities, in control cells and LTEE cells. In panel A, control cells and LTEE cells, as indicated, were seeded in DC₅ so as to attain confluence the following day and were then transfected with pHu-1A1FL (upper panel), or p1B1Fluc (lower panel). On the day following transfection, cultures were exposed in a total 0.2% DMSO vehicle to combinations, as indicated, of 1 nM E₂ (E₂), and 100 nM ICI 182,780 (ICI), with (gray bars) or without (black bars) 10 nM TCDD in conditioned medium for 6 h. Firefly luciferase activities were then determined and normalized to activities of *Renilla* luciferase. In panel B, control cells (control), LTEE cells (LTEE), and LTEE cells cultured for an additional 19 days, subculturing twice with 7 medium changes without E₂ (LTEE-E₂), were seeded, transfected with pHu-1A1-FL (upper panel), or p1B1Fluc (lower panel), and exposed to ICI 182,780 with (gray bars) or without (black bars) 10 nM TCDD as in panel A. Data are the mean values $\pm$ SE for three transfections. Significant differences between LTEE cells and control cells, where all other treatments were identical (*** P <0.001); between TCDD exposure and the respective group differing only in the absence of TCDD exposure (++ P <0.01; +++ P <0.001); between ICI 182,780 exposure and the respective group differing only in the absence of ICI 182,780 exposure (δ P <0.05; $\delta\delta\delta$ P <0.001); between short-term E₂ exposure in panel A and the respective group differing only in the absence of short-term E₂ exposure (θ P <0.05; $\theta\theta$ P <0.01; $\theta\theta\theta$ P <0.001); between the effects of E₂ withdrawal (LTEE-E₂) in panel B and the respective LTEE group, where all other treatments were identical ($\#$ P <0.01; $\#\#\mathcal{P}$ <0.001); and between the effects of E₂ withdrawal (LTEE-E₂) in panel B and the respective control group, where all other treatments were identical ($\Delta\Delta\Delta$ P <0.001) are indicated.

Fig. 3.

Effects of LTEE on ³H-BAP-DNA adduct formation. In panel A, control cells (filled circles) and LTEE cells (open circles) were seeded in DC₅ medium, and, at confluence, cultures were exposed to 0.1 μM ³H-BAP or the solvent vehicle (0.1% DMSO) in conditioned medium, for the indicated lengths of time. DNA was then isolated, and adducts were determined by liquid scintillation counting. In panel B, control cells (black bars) and LTEE cells (gray bars) were seeded in DC₅, and at confluence cultures were exposed to 0.1 μM ³H-BAP in conditioned medium for 3 h, with or without the addition of 10 μM ANF or 200 nM TMS as indicated.

Fig. 4.

Effects of LTEE on the induction of the CYP1A1 and CYP1B1 mRNAs and EROD activity by short-term exposure to BAP. In panel A, control (black bars), and LTEE cultures (gray bars) were exposed to 0.1 μM BAP, 10 μM ANF or 200 nM TMS as indicated for 3 h in conditioned medium. RNA was then isolated, reverse-transcribed, and analyzed by real-time PCR for CYP1A1, CYP1B1, and 36B4 mRNA levels. Data are represented as the means ± SE; n = 3. In panel B, cultures were exposed to 0.01 or 0.1 μM BAP, and ANF or TMS in conditioned medium as in panel A, and EROD activities in control cells (black bars), and LTEE cells (gray bars) were then determined. Data are represented as the means ± SE; n = 8. In panels A and B, significant differences between LTEE cells and control cells, when all

other treatments were identical (* $P<0.05$; ** $P<0.01$; *** $P<0.001$); between BAP exposure and the respective group differing only in the absence of BAP exposure (⁺⁺ $P<0.01$; ⁺⁺⁺ $P<0.001$); between ANF exposure and the respective group differing only in the absence of ANF exposure (^{δδ} $P<0.01$; ^{δδδ} $P<0.001$); and between TMS exposure and the respective group differing only in the absence of TMS exposure ([#] $P<0.05$; ^{##} $P<0.01$; ^{###} $P<0.001$) are indicated.

Fig. 5.

LTEE increases the levels of dG- N^2 -BAP adducts during exposure of MCF-7 cells to BAP, as determined by LC-ESI/MS/MS. Control cells and LTEE cells were seeded in DC₅, and at confluence cultures were exposed for 3 h to 0.1 μ M BAP in conditioned medium; DNA was then isolated and hydrolyzed, and adducts were analyzed by LC-ESI/MS/MS. In panel A, the MS/MS product-ion spectrum of the dG- N^2 -BAP standard with collisional activation of the $[M+H]^+$ ion at m/z 570.2 is shown. In panel B, representative selected-reaction monitoring chromatograms from the analysis of dG- N^2 -BAP and [$^{13}\text{C}_{10}$] dG- N^2 -BAP, monitoring the MS/MS transitions m/z 570.2 $\rightarrow$ 257.2 and m/z 580.2 $\rightarrow$ 257.2, respectively

are shown. In panel C, dG- N^2 -BAP adduct levels in LTEE cells and control cells are presented as the means $\pm$ SE; $n = 3$; (***) $P < 0.001$).

Fig. 6.

LTEE and TCDD induce changes in global gene expression in MCF-7 cells. In panel A, mRNA expression evaluated using a subset of 824 probe sets, in which significant differences in expression among the groups was indicated (ANOVA, $P < 0.05$), and with a variation of at least 2-fold in at least one of the pairwise comparisons of the control, control plus TCDD (10 nM, 72 h), LTEE, and LTEE plus TCDD groups, is presented as a heat map, with log₂-normalized values arranged by hierarchical clustering based on similar expression profiles in response to LTEE and TCDD exposure. The color key for the log₂-normalized values is in the lower right of the figure. ABCG2 mRNA levels as determined by microarray analysis (panel B), and by RT-PCR (panel C), and ABCG2 protein levels as analyzed by

Western immunoblot (panel D) are shown. Data in panels B and C are presented as the means $\pm$ SE; $n = 3$. Significant differences in panel C between the control group and TCDD exposure, or between the LTEE group and LTEE with TCDD exposure (***) $P < 0.001$ are indicated.

Fig. 7.

LTEE enhances the rate of E₂-stimulated tumor growth in SCID mice. LTEE cells or control cells were implanted into the mammary glands of SCID mice, either with (filled circles) or without E₂ (open circles) supplementation as a silastic implant. Tumor growth was monitored at the times indicated, yielding the data shown for the control cells (upper panel) and for LTEE cells (lower panel). 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, as indicated for control and LTEE cells, without (-E₂) and with (+E₂) supplementation.