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Convergent transcriptional profiles induced by endogenous estrogen and distinct xenoestrogens in breast cancer cells

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Estrogen receptors display high levels of promiscuity in accommodating a wide range of ligand structures, but the functional consequence of changing receptor conformations in complex with distinct agonists is highly controversial. To determine variations in the transactivation capacity induced by different estrogenic agonists, we assessed global transcriptional profiles elicited by natural or synthetic xenoestrogens in comparison with the endogenous hormone 17β-estradiol. Human MCF7 and T47D carcinoma cells, representing the most frequently used model systems for tumorigenic responses in the mammary gland, were synchronized by hormone starvation during 48 h. Subsequently, a 24 h exposure was carried out with equipotent concentrations of the selected xenoestrogens or 17β-estradiol. Analysis of messenger RNA was performed on high-density oligonucleotide microarrays that display the sequences of 33 000 human transcripts, yielding a total of 181 gene products that are regulated upon estrogenic stimulation. Surprisingly, genistein (a phytoestrogen), bisphenol-A and polychlorinated biphenyl congener 54 (two synthetic xenoestrogens) produced highly congruent genomic fingerprints by regulating the same range of human genes. Also, the monotonous genomic signature observed in response to xenoestrogens is identical to the transcriptional effects induced by physiological concentrations of 17β-estradiol. This striking functional convergence indicates that the transcription machinery is largely insensitive to the particular structure of estrogen receptor agonists. The occurrence of such converging transcriptional programs reinforces the hypothesis that multiple xenoestrogenic contaminants, of natural or anthropogenic origin, may act in conjunction with the endogenous hormone to induce additive effects in target tissues.

Introduction

Estrogen signaling is primarily mediated by two members of the nuclear steroid receptor superfamily, i.e. estrogen receptor alpha (ERα) and beta (ERβ). These receptors constitute ligand-stimulated transcription factors that associate with co-regulatory partners to remodel chromatin structure and recruit the general transcription machinery to downstream target genes (1–4). Additional estrogenic responses have been discerned with the discovery of novel ER pools that interact with membrane tyrosine kinases or other components of signal transduction pathways (5–7), which in turn lead to further gene expression changes through activation of transcription factors such as ELK1 or CREB (8–10). Thus, the vast majority of biological responses to estrogenic stimuli culminate in genome-wide transcriptional regulation, even though some of these effects are considered indirect or non-genomic.

Epidemiological studies have linked an increased risk of developing mammary or endometrial malignancies to prolonged estrogen exposure caused by early menarche, late menopause, late first-term pregnancy, oral contraceptives or an estrogen replacement therapy (4,11). There is also widespread concern that chemicals with estrogenic activity, for example bisphenol-A or organochlorine pollutants, may be associated with adverse health effects including cancer or other disorders of the female or male reproductive tract (9,12–16). On the other hand, phytoestrogens have been proposed to confer health benefits because the high dietary intake of plant-derived estrogens, such as genistein, appears to correlate with a lower incidence of breast and prostate cancer (17,18). Why prolonged exposure to synthetic estrogens should increase breast cancer risk, whereas natural phytoestrogens exert an opposite chemopreventive action, is not understood.

In the normal resting gland, ERs are expressed in only a small proportion of epithelial cells that are largely non-dividing (11). In contrast, enhanced expression of ERs is a critical event during breast cancer development and, accordingly, the growth of malignant tissue is estrogen-regulated in most cases (19,20). To analyze in detail the effects of ER agonists on gene expression, many laboratories have stimulated human breast cancer cells with 17β-estradiol and determined global transcriptional profiles using oligonucleotide-based microarrays. With few exceptions (21,22) these previous studies were performed at high 17β-estradiol concentrations of 1 nM (23) or even 10 nM (24–31), thereby exceeding the peak effect level, observed around a concentration of 0.1 nM (22), by one or more orders of magnitude. Previous reports also described transcriptional patterns induced by phytoestrogens, including genistein, at concentrations of 10 μM (29) or 100 μM (27,28), again exceeding by far the saturation level, which for genistein is reached at a concentration of ~1 μM (32). However, molecular insight into the effects of xenoestrogens in a lower subsaturating concentration would be important to assess health hazards or benefits at dose ranges that are more relevant for human exposure. Particular attention has been given to the question of whether the native hormone 17β-estradiol and exogenous estrogenic agents induce similar or different transactivation functions. This key issue has been analyzed in previous studies with highly conflicting results. For example, a gene expression

Abbreviations: BrdU, bromodeoxyuridine; DMSO, dimethyl sulfoxide; ER, estrogen receptor; ERα, estrogen receptor alpha; ERβ, estrogen receptor beta; FBS, fetal bovine serum; PCB54, polychlorinated biphenyl congener 54; TCDD, 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin.

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survey performed on the immature mouse uterus after 3 day exposures to 17β-estradiol, genistein or diethylstilbestrol (a synthetic estrogen derivative), yielded comparable transcriptional responses (33). Other reports concluded that there are significant differences between the transcriptional effects of 17β-estradiol and xenoestrogens in the uteri of immature mice (34) or in the reproductive tract of adult rats (35). Several studies came to the conclusion that there is only limited overlap between the expression patterns elicited by 17β-estradiol and xenoestrogens in human breast cancer cells (27–29,31). These contrasting results led us to undertake a large-scale comparative analysis of early gene expression changes in human MCF7 and T47D breast cancer cells treated with equipotent concentrations of 17β-estradiol, genistein, bisphenol-A and the polychlorinated biphenyl congener 54 (PCB54). This integrative study revealed that the expression fingerprint induced by many non-physiological estrogens coincides with the known response of these two carcinoma cell lines to the endogenous hormone.

Materials and methods

Chemicals

Genistein, bisphenol-A, 4-hydroxytamoxifen and 17β-estradiol were purchased from Fluka (Switzerland). PCB54, PCB126 and PCB153 were obtained from EGT (Switzerland); 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin (TCDD) was from the NCI Chemical Carcinogen Reference Standard Repository. The inhibitor ICI 182,780 was purchased from TOCRIS Bioscience (Avonmouth, UK).

Cell culture and treatments

Human T47D.Luc cells (BioDetection Systems, The Netherlands) were maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium supplemented with sodium bicarbonate, 1 mM L-glutamine and 7.5% fetal bovine serum (FBS; HyClone Laboratories, USA). The MCF7 cell line subtype BUS (provided by A. M. Soto and C. Sonnenschein, Tufts University, Boston, USA) was grown in DMEM supplemented with 10% FBS. The antibiotics used were 0.1 U/ml penicillin and 0.1 μg/ml streptomycin (Invitrogen). Both cell lines were cultured at 37°C in xenoestrogen-free plastic (Corning, Grand Island, USA) under humidified air containing 5% CO_2. Before each experiment, T47D.Luc and MCF7 cells were transferred to phenol red-free medium and cultured for 48 h in the presence of 5% charcoal/dextran-stripped FBS (DCC-FBS). Test compounds were dissolved in dimethyl sulfoxide (DMSO) and added to the culture medium as indicated. The final DMSO concentration was adjusted to 0.1% (v/v).

ER-CALUX assay

The ER-CALUX (estrogen receptor-mediated chemical-activated luciferase expression) assay was carried out following the standard operating procedure provided by BioDetection Systems. Briefly, T47D.Luc cells were seeded in microtiter plates at a density of 5000 cells per well in 0.1 ml phenol red-free medium containing 5% DCC-FBS. After 24 h, the medium was renewed and the cells were incubated for another 24 h followed by the addition of each test compound dissolved in DMSO. Solvent controls and a standard 17β-estradiol dose–response curve were included on each plate. After the indicated exposure times, cells were harvested, lysed and assayed for luciferase activity (32) on a Dynex microplate luminometer. All values were corrected for background luciferase expression detected in the presence of solvent alone.

DNA synthesis assay

T47D or MCF7 cells were seeded in microtiter plates, at a density of 5000 cells/well, in 0.1 ml phenol red-free medium containing 5% DCC-FBS. After 24 h, the medium was renewed and the cells were incubated for another 24 h followed by the addition of each test compound dissolved in DMSO. Solvent controls were included on each plate. DNA synthesis was measured after 24 h exposures using the Biotrak cell proliferation ELISA system (Amersham Biosciences, Piscataway, NJ). For that purpose, bromodeoxyuridine (BrdU) was added to the culture medium for 2 h and deoxyribonucleotide incorporation was quantified by the addition of peroxidase-labeled anti-BrdU antibodies. The resultant color development, proportional to DNA synthesis, was determined in a LS55 Luminescence Spectrometer (Perkin Elmer, Wellesly, MA) at 450 nm wavelength.

Microarray hybridization, data acquisition and analysis

After a 24 h treatment with the test compounds, cells were collected by trypsinization and total RNA was extracted using the Rneasy kit (Qiagen, Hilden, Germany). Amount, purity and quality of the final RNA fractions were evaluated by UV spectrophotometry (260 and 280 nm wavelength) followed by examination of the probes by capillary electrophoresis on Agilent Bioanalyzers. Double-stranded complementary DNA was synthesized with the SuperScript kit from Invitrogen using a poly$(dT)_{24}$ primer from Microsynth (Switzerland), which has a T7 RNA polymerase promoter at the 5′ end. The synthesis of complementary RNA was performed with the Ambion MEGAScript T7 *in vitro* transcription kit in the presence of biotinylated CTP and UTP (Logo GmbH, Germany). The resulting biotin-labeled RNA was purified by the Rneasy kit, fragmented by hydrolysis and hybridized to human U-133 GeneChip DNA microarrays (Affymetrix) following the manufacturer's instructions. After hybridization (16 h), the microarrays were processed by an automated washing procedure on the Affymetrix Fluidics Station 400. Staining of the hybridized probes was performed with fluorescent streptavidin–phycoerythrin conjugates (1 mg/ml; Molecular Probes). The subsequent scanning of DNA microarrays was carried out on an Agilent GeneArray laser instrument. Data normalization and filtering were carried out by the dChip software version 1.3 (www.dchip.org). Finally, the results of triplicate experiments were imported into a Microsoft Excel file for SEM calculations, graphical representation and determination of correlation coefficients. The Gene Ontology database (www.geneontology.org) was consulted to verify the predominant molecular function of each transcript.

Real-time RT–PCR

PCR quantifications were carried out to validate the microarray hybridization results. Primers for the selected transcripts were obtained from Applied Biosystems. Briefly, 100 ng of complementary DNA were mixed with 100 nM of forward and reverse primers in a final volume of 25 μl. The reactions were performed in an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) for 45 cycles (95°C for 15 s, 60°C for 1 min) after an initial 10 min incubation at 95°C. The fold change in the expression of each gene was calculated using the $2^{-\Delta\Delta CT}$ method (36), with the abundant glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript as an internal standard.

Results

Dose-dependent transactivation from a minimal estrogen-responsive promoter

The dose range of xenoestrogens to be tested in the DNA microarray experiments has been assessed using a standard reporter gene assay. We took advantage of stably transfected T47D carcinoma cells that carry a chromosomally integrated luciferase gene. This synthetic reporter construct is under transcriptional control of a minimal promoter consisting of tandem repeats of palindromic estrogen response elements (5′-GGTCACTGTGACC-3′). To monitor estrogenic actions, cell lysates were examined for luciferase activity after a 24 h treatment with different concentrations of each test compound. In agreement with previous studies (32), the synthetic promoter in T47D cells mediated a detectable reporter gene induction in response to as low as 1 pM 17β-estradiol added to the cell culture medium. This estrogenic effect reached maximal levels at a hormone concentration of ~60 pM, yielding a nearly 100-fold induction relative to the solvent control (Figure 1A). Both genistein and bisphenol-A induced higher peak values of luciferase expression than the endogenous hormone. Instead, exposure to the estrogen-like PCB congener 54 resulted in lower levels of reporter gene induction compared with 17β-estradiol (Figure 1A). On the basis of these dose responses, concentrations in the near saturating range were selected for the subsequent transcriptome analyzes, i.e. 30 pM for 17β-estradiol, 1 μM for genistein as well as PCB54 and 10 μM for bisphenol-A. The induction of luciferase expression by all estrogenic chemicals was suppressed in the presence of the antagonists 4-hydroxytamoxifen or ICI 182,780 at a concentration of 0.1 μM (data not shown). The selectivity of this

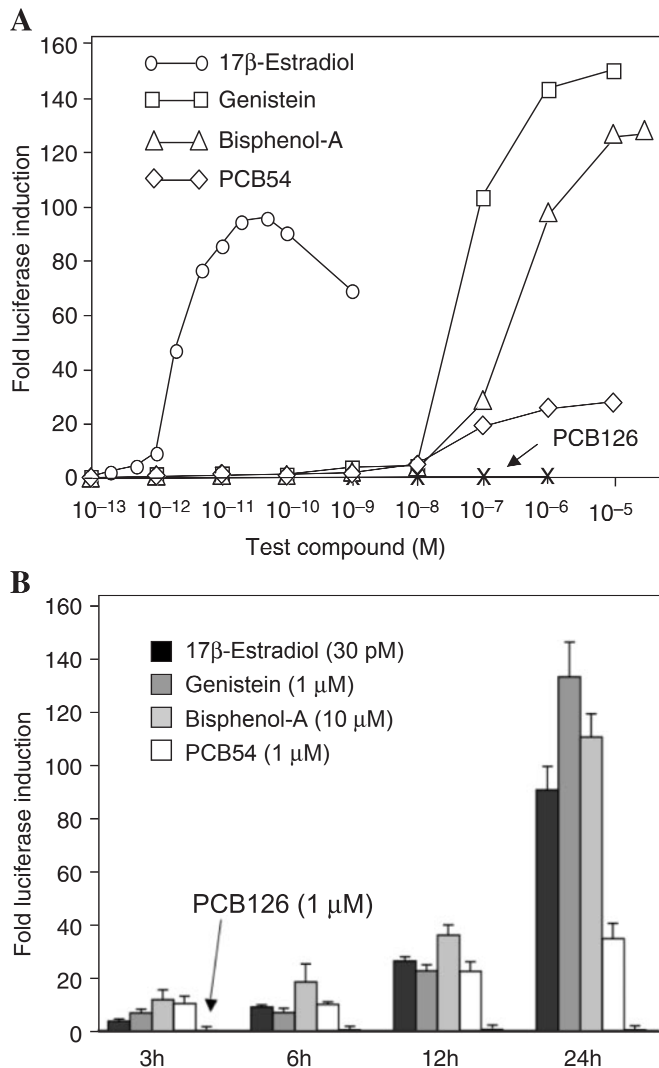

Fig. 1. Luciferase reporter gene expression. Stably transfected T47D cells were incubated with the indicated concentrations of each compound. ER activation was determined by measuring the luciferase induction from a minimal promoter containing repeats of estrogen response elements. (**A**) Dose dependence (mean values of 5–6 independent experiments done at different times). (**B**) Time course (mean values of three independent experiments ± SD). Results are shown as the fold induction relative to the solvent control.

reporter assay is further demonstrated by the lack of luciferase induction following treatment with the dioxin-like PCB congener 126 (Figure 1A). Similarly, 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin (TCDD; data not shown), as well as the non-dioxin-like PCB congener 153 (data not shown), were completely unable to induce this estrogen-specific reporter system.

Time course of synthetic promoter activation

The progression of reporter gene induction during the exposure period was determined in time course experiments. For that purpose, stably transfected T47D cells carrying the estrogen-dependent reporter construct were treated with 17β-estradiol (30 pM), genistein (1 μM), bisphenol-A (10 μM) or PCB54 (1 μM) and luciferase activity was measured in cell lysates after various time intervals (Figure 1B). In view of the steep increase of luciferase induction observed after 24 h estrogen exposures, this time point was used to compare in detail early

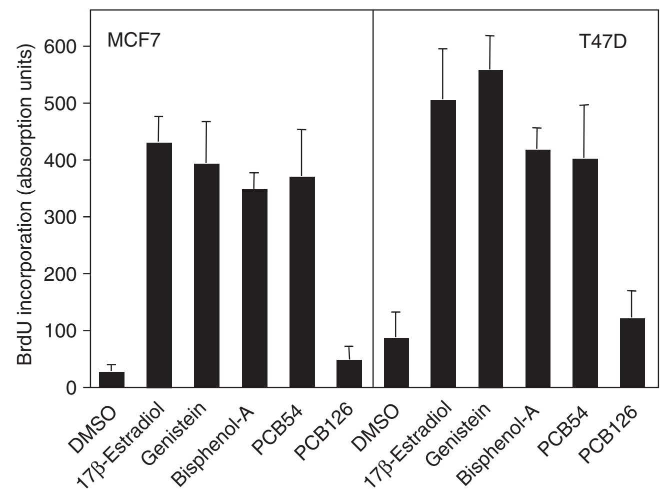

Fig. 2. Stimulation of DNA synthesis. MCF7 or T47D cells were incubated with near saturating concentrations of each test compound for 24 h. DNA synthesis rates were measured by monitoring the incorporation of BrdU (mean values of three independent determinations ± SD).

transcriptional changes in response to ER stimuli. The 24 h period also corresponds to the time of maximal induction of most estrogen-regulated genes according to previous experiments with 17β-estradiol (21), although transcripts that may be subjected to upregulation exclusively during a very early phase of the estrogenic response would be under-represented (27).

Induction of DNA synthesis

Breast cancer cells that are deprived of estrogens or other growth factors accumulate early in the G1 stage of the division cycle. Treatment of estrogen-dependent cells with 17β-estradiol triggers cell cycle progression such that, 24 h after addition of the hormone, the majority of cells undergo DNA replication (21,37). To test whether all xenoestrogens, at the selected concentrations, elicit the same replicative response as the endogenous hormone, growth-arrested MCF7 or T47D cells were exposed to 17β-estradiol (30 pM), genistein (1 μM), bisphenol-A (10 μM) or PCB54 (1 μM) for 24 h. Entry into S phase was recorded by measuring DNA synthesis through the addition of BrdU to the culture medium. Specific antibodies directed against BrdU were employed to monitor incorporation of the deoxyribonucleoside analog following another 2 h of incubation. This quantitative assessment confirmed that all treatments induce comparable levels of DNA synthesis in both cell lines (Figure 2). Even PCB54 resulted in a similar rate of DNA polymerization as the other ER agonists despite the fact that this compound was much less efficient than 17β-estradiol in the induction of luciferase expression from the synthetic promoter (Figure 1). In contrast to PCB54, the dioxin-like PCB congener 126 (1 μM; Figure 2), TCDD (data not shown) and the non-dioxin-like PCB congener 153 (data not shown) were unable to stimulate DNA synthesis in human breast cancer cells.

Global expression profiles

MCF-7 or T47D cells were treated in triplicate experiments with equipotent concentrations of the estrogenic test compounds as outlined in the previous section. After 24 h of exposure, a fraction of RNA from each sample was analyzed using Affymetrix microarrays that display the sequences of 33 000 human transcripts. A total of ∼35% of the surveyed

gene products were called to be present at detectable levels by the Microarray Suite version 5.0 software. To identify transcripts that are susceptible to ER regulation, these results were normalized and subjected to statistical evaluation using the DNA-Chip Analyzer (dChip) open-source software (37). In a first step, all hybridization data were filtered using, as cut-offs, a fold change of >2.5 and a statistical significance of $P < 0.05$ (ANOVA) in at least one of the treatment groups. The number of genes whose transcription was regulated by estrogenic chemicals was considerably higher in MCF7 than in T47D cells. To facilitate direct comparisons between the two cell lines, a more stringent cut-off with a fold change of 3.0 was then applied to the positively regulated transcripts in MCF7 cells. Overall, this dChip analysis yielded a total of 134 transcripts in MCF7 cells, but only 76 transcripts in T47D cells, that are susceptible to estrogenic stimuli. The expression pattern in MCF7 and T47D cell lines is partially overlapping, resulting in a total of 181 ER-regulated human transcripts.

Concordance with real-time PCR values

Real-time RT–PCR was carried out on 14 sequences to validate the microarray hybridization results. The following transcripts were subjected to PCR analysis after exposure to bisphenol-A (10 μM): AREG (coding for amphiregulin), CCNG2 (cyclin G2), CDC2 (cell division cycle 2), CTSD (cathepsin D), CYFIP2 (cytoplasmic binding partner of fragile X protein), E2F1 (E2F transcription factor 1), IER3 (immediate early response 3), MYC (myelocytomatosis oncogene), OLFM1 (olfactomedin 1), RRM2 (ribonucleotide reductase M2 polypeptide), SDF1 (stromal cell-derived factor 1, also known as chemokine ligand 12), SNK (serum-inducible kinase), TFF1 (trefoil factor 1, also known as pS2) and KIAA0101 (predicted protein with unknown function). After normalization using the GAPDH (glyceraldehyde-3-phosphate dehydrogenase) transcript, expression values were transformed as the ratio of messenger levels between estrogen-treated and solvent-treated cells. A direct comparison of the microarray hybridization data with the respective RT–PCR values showed a high degree of correlation for transcripts that were significantly up- or downregulated by estrogenic stimuli (Figure 3). In some cases, the hybridization data tend to underestimate the fold changes induced by ER activation. For example, the most abundant transcript induced by xenoestrogens codes for TFF1, which is a primary marker of ER-positive breast tumors (38,39). According to the microarray hybridizations, TFF1 was 6.3-fold induced in MCF7 cells, but the subsequent analysis by RT–PCR yielded a 13.5-fold increase for the same transcript. In T47D cells, the transcription level of SDF1, another prominent marker of ER signaling (40), was 10.5-fold induced according to the microarray hybridizations but the subsequent PCR quantification revealed a 17.1-fold increment. No induction of SDF1 occurred in MCF7 cells in agreement with the report of Coser *et al*. (22), who showed that this transcript is increased in MCF7 cells only at highly saturating concentrations of the estrogenic stimulus. The upregulation of KIAA0101, which is pronounced in MCF7 cells although it can be detected in both cell lines (Figure 3), has not been reported before and hence represents a novel estrogenic response.

Estrogen-dependent transcripts in MCF7 and T47D cells

Most human transcripts that were significantly regulated by estrogenic treatment encode protein products with either a known or an inferred biological function. Accordingly, these transcripts were grouped in functional categories involving cell cycle, DNA metabolism and apoptosis (Tables I and II), growth stimulation, transcription and cell adhesion (Tables III and IV), as well as metabolism and transport systems (Tables V and VI). There were only few transcripts (included in Tables V and VI) that encode for predicted proteins with unknown function such as, for example, KIAA0101 or GREB1 (gene regulated by estrogen in breast cancer 1), which has been identified by virtue of its overexpression in estrogen-responsive breast carcinomas (41).

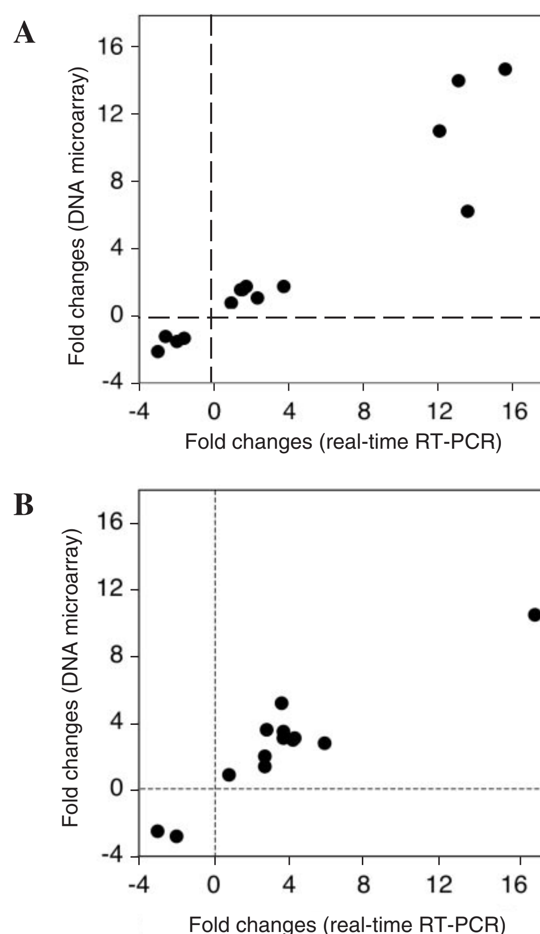

Fig. 3. Comparison between hybridization results and the corresponding RT–PCR values. Fold changes of messenger levels according to normalized and filtered hybridization data (dChip) were plotted against the corresponding real-time RT–PCR quantifications. This analysis was performed with RNA extracted from MCF7 (**A**) and T47D cells (**B**) exposed to 10 μM bisphenol-A.

Tables 1 and 2 display the estrogen-responsive transcripts whose protein products are involved in cell cycle, DNA metabolism and apoptosis. The majority of these genes have been identified before as being susceptible to transcriptional regulation by ERs (21–31), including HCAP-G (chromosome condensation protein G), the cell division cycle factors CDC2, CDC6, CDC20 and CCNA2 (cyclin A2), RRM2 (ribonucleotide reductase M2 polypeptide), PRIM1 (primase 1), TK1 (thymidine kinase 1), PCNA (proliferating cell nuclear antigen), DTYMK (deoxythymidylate kinase), FEN1 (flap structure-specific endonuclease 1), H2AFX (histone 2A family X) and UNG (uracil-DNA glycosylase). We also

Table I. Estrogen-regulated transcripts in MCF7 cells

Gene	Control	17β-Estradiol	Genistein	Bisphenol-A	PCB54
Centromer protein J	18 ± 3	53 ± 8	55 ± 9	38 ± 12	49 ± 12
Kinesin family member 14	27 ± 0	84 ± 27	132 ± 16	80 ± 22	111 ± 6
Kinesin family member 23	27 ± 1	110 ± 42	176 ± 8	125 ± 47	127 ± 4
Chromosome condensation protein G	27 ± 1	71 ± 21	92 ± 6	69 ± 18	75 ± 4
RA-regulated nuclear matrix-associated protein	27 ± 1	95 ± 7	81 ± 9	89 ± 21	85 ± 5
Checkpoint 1 homolog	28 ± 3	65 ± 13	91 ± 12	77 ± 17	85 ± 6
Cell division cycle 6	28 ± 2	92 ± 6	121 ± 26	91 ± 11	124 ± 10
Minichromosome maintenance deficient 10	28 ± 3	106 ± 21	144 ± 33	113 ± 17	139 ± 15
M-phase phosphoprotein 9	30 ± 4	54 ± 8	92 ± 7	61 ± 10	85 ± 2
Abnormal spindle-like microcephaly associated	30 ± 4	128 ± 23	186 ± 8	129 ± 32	158 ± 12
Kinesin-like 7	31 ± 6	83 ± 8	121 ± 6	78 ± 11	100 ± 5
RAD51 interacting protein 1	32 ± 4	95 ± 26	118 ± 6	99 ± 32	110 ± 8
Cyclin-dependent kinase inhibitor 2C	35 ± 5	102 ± 11	120 ± 16	87 ± 9	101 ± 5
Cell division cycle 2	35 ± 5	351 ± 88	362 ± 23	389 ± 99	304 ± 10
Structural maintenance of chromosome 2-like 1	35 ± 8	104 ± 34	159 ± 24	114 ± 33	141 ± 10
Kinesin family member 4A	37 ± 9	164 ± 22	213 ± 8	144 ± 26	173 ± 6
Ribonucleotide reductase M2 polypeptide	37 ± 9	502 ± 44	552 ± 65	521 ± 75	474 ± 47
Kinesin family member 20A	39 ± 14	272 ± 111	331 ± 34	280 ± 107	259 ± 13
APOBEC3B[a]	40 ± 9	133 ± 14	153 ± 9	98 ± 20	137 ± 6
RAD51 homolog	40 ± 8	254 ± 33	373 ± 30	188 ± 59	248 ± 11
Cyclin A2	41 ± 4	157 ± 35	304 ± 35	151 ± 37	223 ± 25
Mitotic arrest deficient-like 1	42 ± 10	239 ± 89	221 ± 38	253 ± 90	199 ± 6
Centromere protein A	43 ± 7	144 ± 38	163 ± 6	145 ± 49	132 ± 8
Kinesin family member 11	44 ± 7	114 ± 22	150 ± 10	114 ± 24	136 ± 4
Cyclin-dependent kinase inhibitor 3	47 ± 8	267 ± 90	309 ± 21	275 ± 111	227 ± 28
Transforming acidic coiled-coil containing protein 3	48 ± 8	228 ± 21	268 ± 42	213 ± 33	207 ± 23
CDT1 DNA replication factor	53 ± 11	153 ± 11	214 ± 75	184 ± 13	168 ± 58
Survivin	59 ± 8	164 ± 13	181 ± 11	166 ± 22	140 ± 6
Centromer protein F	59 ± 8	178 ± 23	234 ± 11	169 ± 24	190 ± 10
PFS2 DNA replication complex protein	62 ± 14	419 ± 29	330 ± 19	335 ± 63	289 ± 10
High-mobility group box 2	63 ± 21	658 ± 226	807 ± 104	658 ± 252	602 ± 53
Protein regulator of cytokinesis 1	64 ± 15	554 ± 50	844 ± 124	505 ± 68	642 ± 103
Cyclin B2	67 ± 13	402 ± 119	431 ± 32	354 ± 143	334 ± 15
CNAP1[a]	67 ± 7	146 ± 22	227 ± 38	151 ± 28	171 ± 28
Geminin	71 ± 15	337 ± 38	270 ± 15	300 ± 65	240 ± 14
Primase 1	73 ± 9	226 ± 30	228 ± 10	215 ± 56	191 ± 10
Cell division cycle 20	75 ± 14	323 ± 90	376 ± 42	323 ± 110	273 ± 19
BUB1B[a]	78 ± 17	191 ± 30	268 ± 33	205 ± 31	154 ± 20
Structural maintenance of chromosomes 4-like 1	79 ± 15	216 ± 68	270 ± 32	223 ± 69	225 ± 7
Thymidine kinase 1	80 ± 16	494 ± 53	475 ± 58	444 ± 72	367 ± 51
Cell division cycle 45-like	84 ± 14	221 ± 19	256 ± 26	204 ± 18	209 ± 27
ZW10 interactor	90 ± 21	490 ± 67	561 ± 59	481 ± 62	481 ± 44
Minichromosome maintenance deficient 6	92 ± 17	390 ± 52	339 ± 54	374 ± 70	324 ± 12
Proliferating cell nuclear antigen	104 ± 20	487 ± 88	482 ± 52	440 ± 72	433 ± 22
Deoxythymidylate kinase	105 ± 13	355 ± 30	337 ± 18	296 ± 38	242 ± 22
Flap structure-specific endonuclease	106 ± 22	442 ± 14	369 ± 39	407 ± 72	336 ± 19
Minichromosome maintenance deficient 2	108 ± 16	471 ± 22	481 ± 92	396 ± 59	407 ± 67
Thymidylate synthase	118 ± 32	803 ± 82	1061 ± 215	785 ± 86	786 ± 134
CDC28 protein kinase regulatory subunit 1B	120 ± 23	583 ± 44	630 ± 38	489 ± 80	470 ± 30
Ribonuclease H1 large subunit	137 ± 15	664 ± 54	608 ± 34	586 ± 43	463 ± 30
Minichromosome maintenance deficient 7	209 ± 19	730 ± 59	658 ± 152	714 ± 87	533 ± 131
RAD51 homolog C	264 ± 57	760 ± 94	970 ± 196	797 ± 50	963 ± 125
Defender of cell death 1	269 ± 17	649 ± 174	821 ± 50	564 ± 141	661 ± 93
Histone 2A family X	272 ± 49	1174 ± 106	1196 ± 191	1289 ± 118	828 ± 143
Growth arrest and DNA damage-inducible alpha	285 ± 37	101 ± 36	84 ± 16	119 ± 29	89 ± 19
Histone 2A family Z	433 ± 53	1455 ± 245	1923 ± 206	1690 ± 299	1563 ± 164
Zinc finger protein 36 type-like 1	494 ± 95	143 ± 58	125 ± 21	233 ± 95	95 ± 20
Histone 1 H2ac	533 ± 44	369 ± 20	234 ± 97	194 ± 23	284 ± 85
Histone H2BE	760 ± 31	454 ± 78	310 ± 102	278 ± 55	400 ± 89
B-cell translocation gene 1	1019 ± 107	514 ± 46	345 ± 69	475 ± 79	279 ± 25
Cyclin-dependent kinase inhibitor 1	1209 ± 74	575 ± 204	341 ± 90	558 ± 180	338 ± 45

Target genes involved in cell cycle, DNA metabolism and apoptosis. Hybridization data were normalized and filtered using dChip (mean values of three independent experiments ± SEM). The relative expression values are shown in light intensity units. Transcripts are listed in ascending order of their constitutive expression in control cells treated with the DMSO solvent.

[a]*Abbreviations*: APOBEC3B, apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3B; BUB1B, Buddding inhibited by benzimidazoles 1 homolog beta; CNAP1, chromosome condensation-related SMC-associated protein 1.

found that several members of the minichromosome maintenance deficient family are upregulated by estrogen treatment, including MCM2, MCM6, MCM7 and MCM10, all of which have roles in promoting DNA replication. As noted in a previous study (26), the induction of transcripts that drive cell proliferation was accompanied in MCF7 cells by the upregulation of survivin (BIRC5), an inhibitor of apoptosis. This antiapoptotic response was accompanied in MCF7 cells

Table II. Estrogen-regulated transcripts in T47D cells

Gene	Control	17β-Estradiol	Genistein	Bisphenol-A	PCB54
CTP synthase	62 ± 8	157 ± 23	173 ± 26	205 ± 22	162 ± 17
Minichromosome maintenance deficient 10	73 ± 7	201 ± 14	326 ± 82	414 ± 31	180 ± 18
Cell division cycle 6	89 ± 12	188 ± 22	303 ± 61	412 ± 15	268 ± 62
Centromer protein A	93 ± 20	254 ± 13	310 ± 22	230 ± 30	271 ± 14
Chromosome condensation protein G	98 ± 26	305 ± 24	336 ± 38	254 ± 13	265 ± 91
RAD51 interacting protein	115 ± 27	201 ± 28	333 ± 53	284 ± 22	280 ± 25
Cyclin A2	159 ± 29	395 ± 24	521 ± 45	460 ± 33	401 ± 137
Checkpoint 1 homolog	190 ± 29	389 ± 39	553 ± 74	499 ± 16	380 ± 43
Sac3 homology domain 1	200 ± 39	622 ± 46	350 ± 28	540 ± 62	559 ± 152
Ribonucleotide reductase M2 polypeptide	214 ± 35	661 ± 60	980 ± 127	766 ± 83	826 ± 193
Geminin	240 ± 19	577 ± 64	656 ± 153	574 ± 107	671 ± 114
Tubulin beta 6	270 ± 61	594 ± 80	781 ± 67	676 ± 57	658 ± 188
Cyclin-dependent kinase inhibitor 3	271 ± 53	719 ± 74	1160 ± 68	702 ± 52	879 ± 176
RNA binding motif protein 24	277 ± 78	672 ± 72	566 ± 183	1244 ± 268	817 ± 94
Cyclin-dependent kinase 2	283 ± 39	464 ± 48	756 ± 81	579 ± 100	570 ± 50
RAD51	300 ± 52	551 ± 37	763 ± 90	673 ± 55	817 ± 177
Minichromosome maintenance deficient 7	307 ± 25	421 ± 65	909 ± 150	861 ± 25	634 ± 131
Uracil-DNA glycosylase	307 ± 53	829 ± 73	688 ± 113	692 ± 155	697 ± 64
High-mobility group box 2	335 ± 110	843 ± 85	1034 ± 84	835 ± 43	837 ± 258
Cell division cycle A5	354 ± 46	899 ± 133	892 ± 163	1004 ± 57	1160 ± 167
Minichromosome maintenance deficient 2	361 ± 42	820 ± 123	1057 ± 240	1273 ± 59	1120 ± 198
Thymidine kinase 1	387 ± 72	663 ± 43	884 ± 189	1040 ± 68	874 ± 77
Minichromosome maintenance deficient 6	427 ± 35	975 ± 104	1184 ± 258	1418 ± 164	1124 ± 77
Topoisomerase 2A	433 ± 115	928 ± 89	1235 ± 97	1067 ± 114	933 ± 41
MAC30 regulator of cell growth	481 ± 66	1244 ± 70	1282 ± 135	1314 ± 141	1315 ± 87
Thymidylate synthase	641 ± 176	1170 ± 101	2038 ± 204	1685 ± 57	1791 ± 305
Flap structure-specific endonuclease 1	682 ± 48	1404 ± 132	1896 ± 184	1570 ± 68	1756 ± 172
Polymerase delta 4	1121 ± 186	469 ± 80	614 ± 72	713 ± 78	415 ± 152

Target genes involved in cell cycle, DNA metabolism and apoptosis. Hybridization data were normalized and filtered using dChip (mean values of three independent experiments ± SEM). The relative expression values are shown in light intensity units. Transcripts are listed in ascending order of their constitutive expression in control cells treated with the DMSO solvent.

by the induction of DAD1 (defender against apoptotic death 1). In addition to these known responses, an appreciable number of novel targets were identified, including RAD51, RAD51C (RAD51 homolog C) and RAD51AP1 (RAD51 interacting protein 1), which are subunits of the homologous recombination machinery. Another cluster of estrogen-induced factors comprises the centromeric proteins CENPA, CENPF, CENPJ and TACC3 (transforming acidic coiled–coil 3) (42). On the other hand, GADD45A (growth arrest and DNA damage inducible 45A), BTG1 (B cell translocating gene 1) and cyclin-dependent kinase inhibitors CDKN1, CDKN2C and CDKN3, known to inhibit cell cycle progression, were downregulated. In addition, we identified transcripts involved in chromosome segregation that have not been associated with estrogenic signaling in previous studies, as for example PRC1 (protein regulator of cytokinesis 1), RAB6KIFL (kinesin family member 20A), KNSL5 (kinesin family member 23), KIF4A (kinesin family member 4A), KIF14 (kinesin family member 14), KIF11 (kinesin family member 11) and KNSL7 (kinesin like 7).

Estrogen-responsive transcripts involved in growth stimulation, transcription and cell adhesion are listed in Tables III and IV. In agreement with former DNA microarray studies (21–28,31), the production of TFF1, TFF3 (trefoil factor 3), IGFBP4 (insulin-like growth factor binding protein 4), SDF1, STC2 (stanniocalcin 2), AREG, OLFM1 and OLFML3 (olfactomedin-like 3) was induced upon estrogen treatment. THBS1 (thrombospondin 1) is also upregulated in an estrogen-dependent manner in T47D cells. Concomitantly, the expression of inhibitors of cell growth such as TGFB2 (transforming growth factor beta 2) or EFNB2 (ephrin B2) is suppressed (Table III). GFRA1 (glial cell line-derived neurotrophic factor family receptor alpha 1) represents a growth factor receptor that is susceptible to positive estrogenic regulation in both cell lines, whereas PTGER3 (prostaglandin E receptor 3) was upregulated only in T47D cells (Table IV). The induction of signal transduction pathways is illustrated by an increased level of transcripts coding for ITPK1 (inositol 1,3,4-triphosphate 5/6 kinase). As noted previously (30), the estrogen-dependent reprogramming of breast cancer cells was further characterized by the downregulation of tight junction and adhesion molecules including CLDN4 (claudin 4), L1CAM (L1 cell adhesion molecule) and JUP (junction plakoglobin), implying that ER activation may predispose to anchorage loss. Cell adhesion could be further reduced by the suppression of TIMP3 (tissue inhibitor of metalloproteinase 3), leading to increased metalloproteinase activity. We observed that many transcription factors and proto-oncogenes were suppressed following estrogenic stimulation (Tables III and IV). In fact, ATF3 (activating transcription factor), ETS2 (erythoblastosis E26 oncogene homolog 2), KRAS (Kirsten rat sarcoma oncogene), MAXD4 (MAX dimerization protein 4), NCOA3 (nuclear receptor coactivator 3) and other related transcripts were downregulated in MCF7 cells. The SOX4 (SRY-box 4) transcript was suppressed in both MCF7 and T47D cells. The tumor suppressor DOC-1R (deleted in oral cancer-related 1) was suppressed only in T47D cells. The downregulation of several proto-oncogenes is compensated by the induction of MYBL2 (also known as B-Myb) in both cell types. Other prominent transcripts that were induced in the course of this estrogen-dependent program include the MYC (myelocytomatosis), MYB (myeloblastosis)

Table III. Estrogen-regulated transcripts in MCF7 cells

Gene	Control	17β-Estradiol	Genistein	Bisphenol-A	PCB54
Serine-threonine kinase 18	27 ± 1	72 ± 20	84 ± 5	65 ± 17	74 ± 5
Highly expressed in cancer	30 ± 9	155 ± 33	169 ± 10	144 ± 48	132 ± 8
Pituitary tumor-transforming 3	33 ± 6	125 ± 38	124 ± 12	118 ± 33	100 ± 8
GDNF family receptor alpha 1	39 ± 4	229 ± 63	256 ± 15	218 ± 80	278 ± 14
Myeloid leukemia factor 1	40 ± 9	315 ± 54	290 ± 23	300 ± 97	253 ± 14
Serine-threonine kinase 12	45 ± 18	340 ± 50	382 ± 56	298 ± 79	257 ± 42
Transmembrane protein 38B	51 ± 10	88 ± 29	164 ± 11	101 ± 35	125 ± 9
Maternal embryonic leucine zipper kinase	61 ± 17	319 ± 43	341 ± 33	307 ± 55	281 ± 12
Thyroid hormone receptor interactor 13	72 ± 11	212 ± 14	228 ± 20	199 ± 32	201 ± 7
Rac GTPase activating protein 1	72 ± 23	454 ± 119	439 ± 51	454 ± 126	353 ± 14
Epithelial cell transforming sequence 2 oncogene	73 ± 9	186 ± 45	244 ± 21	183 ± 50	208 ± 7
Thymopoietin	78 ± 17	290 ± 54	269 ± 25	279 ± 89	234 ± 13
Stathmin 1/oncoprotein 18	87 ± 28	432 ± 62	357 ± 17	414 ± 77	313 ± 13
Serine-threonine kinase 6	116 ± 22	573 ± 91	596 ± 41	488 ± 144	452 ± 24
Trefoil factor 3	138 ± 21	935 ± 207	845 ± 61	722 ± 224	705 ± 36
Anterior gradient 2 homolog	142 ± 29	1093 ± 220	1938 ± 417	1020 ± 304	1823 ± 180
Erythroblastosis virus E26 oncogene	150 ± 20	58 ± 7	73 ± 6	87 ± 14	73 ± 4
Phosphatidic acid phosphatase type 2B	151 ± 5	81 ± 9	60 ± 11	93 ± 10	59 ± 12
Transforming growth factor beta 2	155 ± 26	55 ± 9	51 ± 12	51 ± 6	58 ± 14
Myeloblastosis viral oncogene homolog-like 2	157 ± 19	465 ± 61	682 ± 185	515 ± 75	498 ± 142
Tissue inhibitor of metalloproteinase 3	170 ± 9	86 ± 14	97 ± 7	59 ± 12	121 ± 11
MAX dimerization protein 4	197 ± 16	126 ± 15	77 ± 7	113 ± 14	73 ± 9
Insulin-like growth factor binding protein 4	198 ± 21	379 ± 27	804 ± 257	713 ± 125	881 ± 137
NEDD9[a]	214 ± 33	114 ± 21	81 ± 12	92 ± 16	98 ± 24
Interleukine enhancer binding factor 2	214 ± 11	481 ± 91	647 ± 47	429 ± 162	517 ± 66
Kirsten rat sarcoma viral oncogene	221 ± 28	155 ± 21	78 ± 9	144 ± 18	69 ± 9
Epithelial membrane protein 2	229 ± 23	479 ± 63	696 ± 696	320 ± 77	617 ± 55
Ephrin B2	232 ± 41	88 ± 13	51 ± 51	96 ± 20	51 ± 7
CCAAT-enhancer binding protein delta	299 ± 38	148 ± 36	102 ± 102	136 ± 25	104 ± 17
Transmembrane prostate androgen-induced	301 ± 38	128 ± 26	97 ± 13	136 ± 27	98 ± 11
Sex determining region Y-box 4	377 ± 58	191 ± 52	84 ± 19	215 ± 57	80 ± 22
Tropomyosin 3	381 ± 53	893 ± 143	1364 ± 209	828 ± 261	1096 ± 119
Retinoic acid-induced protein 3	396 ± 36	197 ± 45	165 ± 57	156 ± 28	168 ± 50
Claudin 4	447 ± 62	279 ± 22	174 ± 12	276 ± 49	184 ± 17
Activating transcription factor 3	488 ± 72	167 ± 64	89 ± 19	204 ± 76	93 ± 22
L1 cell adhesion molecule	630 ± 62	258 ± 97	216 ± 22	262 ± 94	211 ± 16
Brain abundant membrane-attached signal protein 1	634 ± 25	336 ± 103	149 ± 43	369 ± 90	145 ± 45
Tripartite motif-containing 16	654 ± 49	234 ± 97	173 ± 24	308 ± 94	191 ± 22
Basic helix-loop-helix domain containing class B2	744 ± 77	302 ± 64	280 ± 46	331 ± 107	298 ± 27
Nuclear receptor coactivator	850 ± 113	333 ± 59	291 ± 87	443 ± 66	273 ± 94
Junction plakoglobulin	866 ± 80	362 ± 88	295 ± 52	377 ± 89	304 ± 86
Trefoil factor 1	871 ± 79	5626 ± 250	6109 ± 526	5452 ± 597	5798 ± 248
Inhibitor of DNA binding 3	1307 ± 154	700 ± 150	446 ± 110	613 ± 181	434 ± 24

Target genes involved in growth stimulation, transcription and cell adhesion. Hybridization data were normalized and filtered using dChip (mean values of three independent experiments ± SEM). The relative expression values are shown in light intensity units. Transcripts are listed in ascending order of their constitutive expression in control cells treated with the DMSO solvent.
[a]*Abbreviation*: NEDD9, neural precursor cell expressed developmentally down-regulated 9.

and ECT2 (epithelial cell transforming 2) oncogenes, as well as TMPO (thymopoietin), HEC (highly expressed in cancer), LAP18 (stathmin 1) and MLF1 (myeloid leukemia factor), which are known markers of malignant cell proliferation (43).

Tables V and VI display the estrogen-responsive transcripts involved in metabolism and transport sytems. Interestingly, CYP1A1 (cytochrome P4501A1) is downregulated in MCF7 cells (Figure 9A) but strongly induced in T47D cells. CYP1B1 is another P450 enzyme induced in T47D cells. Also, several genes associated with carrier function were susceptible to estrogen-dependent regulation. Induction was observed for the solute carrier family members SLC6A14 and SLC39A8 while the transcript coding for SLC7A11 was repressed. Finally, different factors involved in protein ubiquitylation, including FBX05 (F-box only protein 5), UBCH10 and HSPC150 (coding for ubiquitin conjugating enzymes) were positively regulated after estrogen stimulation. An estrogen-dependent induction of the immunophilin FKBP4 (FK506 binding protein 4), a factor involved in protein folding, has already been described before (30). Another estrogen-inducible factor with a function in protein folding is the chaperone ATAD2 (ATPase family, AAA domain containing 2).

Convergence of estrogenic transcriptional profiles

The only criterion for the inclusion of transcripts in Tables I–VI was their significant up or downregulation in at least one treatment group, without any bias for overlaps with the response to the other tested estrogenic agents. Surprisingly, this extensive comparison performed with two distinct cell lines revealed a high degree of similarity between the expression profiles elicited by natural and synthetic estrogenic compounds. Transcripts that were regulated by a 17β-estradiol stimulus turned out to be modulated in the same direction, and to a similar extent, by genistein, bisphenol-A and PCB54. Conversely, transcriptional induction or repression mediated by these xenoestrogens was accompanied by a

Table IV. Estrogen-regulated transcripts in T47D cells

Gene	Control	17β-Estradiol	Genistein	Bisphenol-A	PCB54
Glial-axonal junction protein	47 ± 4	170 ± 37	154 ± 34	142 ± 13	214 ± 63
Microtubule-associated serine-threonine kinase-like	50 ± 7	158 ± 18	154 ± 40	165 ± 20	185 ± 54
GDNF family receptor alpha 1	50 ± 8	119 ± 15	151 ± 9	168 ± 27	178 ± 13
Chemokine ligand 12	61 ± 15	363 ± 40	582 ± 168	704 ± 192	550 ± 179
Purkinje cell protein 4	78 ± 27	172 ± 34	187 ± 30	176 ± 49	222 ± 34
Collagen type 12 alpha 1	87 ± 15	180 ± 24	172 ± 29	170 ± 19	261 ± 60
DEP domain containing 1B	89 ± 18	219 ± 19	216 ± 31	272 ± 57	258 ± 66
KNTC2a	90 ± 33	271 ± 16	308 ± 28	245 ± 40	271 ± 31
Myelocytomatosis viral oncogene	100 ± 22	336 ± 22	283 ± 26	278 ± 36	250 ± 49
Trefoil factor 1	103 ± 18	231 ± 25	407 ± 26	365 ± 18	202 ± 24
Thrombospondin 1	120 ± 18	283 ± 24	211 ± 55	170 ± 51	241 ± 33
T-LAK cell-originated protein kinase	138 ± 51	329 ± 26	602 ± 38	386 ± 49	353 ± 36
Amphiregulin	143 ± 18	564 ± 48	219 ± 57	793 ± 176	251 ± 96
Stanniocalcin 2	157 ± 34	195 ± 16	568 ± 52	219 ± 24	37 ± 98
PPP2R2C[a]	174 ± 37	346 ± 31	292 ± 47	505 ± 76	435 ± 65
Serine-threonine kinase 12	177 ± 31	454 ± 48	580 ± 30	506 ± 32	493 ± 141
Sex determining region Y box 2	238 ± 90	128 ± 31	64 ± 9	72 ± 25	66 ± 3
Prostaglandin E receptor 3	260 ± 30	562 ± 81	573 ± 160	761 ± 170	648 ± 62
Myeloid leukemia factor 1 interacting protein	265 ± 53	620 ± 92	700 ± 249	693 ± 137	761 ± 149
Myeloblastosis viral oncogene	281 ± 97	573 ± 41	509 ± 43	833 ± 66	681 ± 134
Olfactomedin 1	381 ± 76	1187 ± 104	1267 ± 96	1193 ± 162	92 ± 177
Myeloblastosis viral oncogene homolog-like 2	421 ± 71	641 ± 106	1165 ± 62	1192 ± 132	1013 ± 115
Inositol 1,3,4-triphosphate 5/6 kinase	423 ± 99	566 ± 38	912 ± 238	1040 ± 190	1087 ± 78
Six transmembrane epithelial antigen of the prostate	469 ± 24	315 ± 61	291 ± 97	199 ± 15	201 ± 40
G protein-binding protein 4	491 ± 137	1473 ± 106	1083 ± 144	1039 ± 131	1167 ± 380
Olfactomedin-like 3	519 ± 145	1200 ± 248	1866 ± 394	1832 ± 608	1791 ± 94
Tumor suppressor deleted in oral cancer-related 1	896 ± 144	464 ± 47	537 ± 68	539 ± 67	362 ± 117
Insulin-like growth factor binding protein 4	922 ± 228	2354 ± 291	3698 ± 141	3787 ± 170	3729 ± 127
Phosphatidic acid phosphatase type 2C	1029 ± 140	331 ± 67	617 ± 138	527 ± 61	415 ± 118
Sex determining region Y box 4	2489 ± 330	1587 ± 175	1287 ± 183	1207 ± 154	1021 ± 82

Target genes involved in growth stimulation, transcription and cell adhesion. Hybridization data were normalized and filtered using dChip (mean values of three independent experiments ± SEM). The relative expression values are shown in light intensity units. Transcripts are listed in ascending order of their constitutive expression in control cells treated with the DMSO solvent.
[a]*Abbreviations*: KNTC2, Highly expressed in cancer rich in leucine heptad repeats; PPP2R2C, protein phosphatase 2 regulatory subunit B gamma isoform.

corresponding change in the level of the same transcripts following exposure to the native hormone. This striking similarity between the responses to diverse estrogenic agents is illustrated for example by the RRM2 (ribonucleotide reductase M2 polypeptide) messenger, which encodes the rate-limiting enzyme for deoxyribonucleotide production during DNA synthesis (22). The RRM2 transcript was induced in MCF7 cells 13.5-fold after treatment with 17β-estradiol and between 12.8- and 14.9-fold after treatment with the different xenoestrogens (Table I). The RRM2 transcript was also increased (between 3.1- and 4.6-fold) in T47D cells following all kinds of estrogenic stimuli (Table II). Another prominent example is the cyclin A2 transcript, whose induction level ranges from 2.5- to 7.4-fold in both MCF7 and T47D cells following treatment with 17β-estradiol as well as the different xenoestrogens (Tables I and II). To delineate the degree of similarity in quantitative terms, the messenger RNA profiles induced by the tested xenoestrogens in MCF7 cells were plotted against the corresponding values obtained for 17β-estradiol. In these comparisons, all 134 data points representing estrogen-dependent transcripts cumulated along the diagonal axis of the graphs (Figure 4) and the resulting correlation coefficients were in the range of $R = 0.98$–0.99. This quantitative analysis thus confirms that the transcriptional responses induced by the distinct estrogenic agents are nearly identical. When the levels of these estrogen-dependent transcripts were plotted against the amount of the same transcripts following treatment with TCDD (0.1 μM), the resulting correlation coefficient was reduced to $R = 0.11$, reflecting the distinct transactivation pattern elicited by this aromatic hydrocarbon receptor agonist.

Discussion

Estrogenic regulation plays an important role not only in the development of normal mammary glands but also in the initiation and progression of breast cancer, which has become the most common malignancy among American and European women (11). Epidemiological studies suggest a positive correlation between blood levels of chemicals with estrogenic activity, such as organochlorine pollutants, and breast cancer incidence among women, implying that the growing risk of contracting mammary cancer may be linked to the wide distribution of synthetic xenoestrogens (12,13,44–47). On the other hand, beneficial health effects have been attributed to the dietary intake of natural phytoestrogens in food of plant origin (17,18).

The two ER subtypes (ERα and ERβ) are unique among the steroid receptor family in their ability to interact with a wide variety of ligands that exhibit remarkably diverse structural features (48). Several lines of evidence apparently support the view that the biological action of different ER agonists may diverge significantly. First, the endogenous hormone and various xenoestrogens display differences in the binding affinity for ERα and ERβ (48). Second, ERα and ERβ

Table V. Estrogen-regulated transcripts in MCF7 cells

Gene	Control	17β-Estradiol	Genistein	Bisphenol-A	PCB54
Solute carrier family 39 member 8	31 ± 3	99 ± 11	85 ± 6	96 ± 22	90 ± 17
Procollagen lysine 2-oxoglutarate 5-dioxygenase	34 ± 8	110 ± 27	86 ± 9	95 ± 38	105 ± 18
Dehydrogenase-reductase (SDR family) member 2	35 ± 4	103 ± 33	148 ± 16	62 ± 17	121 ± 4
F-Box only protein 5	35 ± 7	103 ± 15	119 ± 5	102 ± 13	114 ± 11
Solute carrier family 6 member 14	36 ± 9	397 ± 126	299 ± 25	354 ± 110	332 ± 39
Nuclear spindle-associated protein	42 ± 16	410 ± 115	473 ± 50	415 ± 132	370 ± 19
CRP2 binding protein	48 ± 5	142 ± 22	159 ± 17	96 ± 29	156 ± 54
gamma-Glutamyl hydrolase	49 ± 5	132 ± 19	159 ± 4	142 ± 32	137 ± 6
ATPase family AAA domain containing 2	50 ± 7	155 ± 59	240 ± 38	206 ± 49	216 ± 28
HSPC150 ubiquitin conjugating enzyme isolog	53 ± 10	511 ± 167	761 ± 74	543 ± 34	564 ± 20
Gene regulated by estrogen in breast cancer 1	57 ± 12	340 ± 62	317 ± 87	420 ± 75	366 ± 17
FLJ10719	58 ± 7	291 ± 47	357 ± 49	293 ± 19	276 ± 41
HADHSC[a]	71 ± 20	211 ± 19	241 ± 17	178 ± 31	233 ± 26
Adaptor-related protein complex 1 sigma 2 subunit	75 ± 10	214 ± 45	233 ± 34	118 ± 25	196 ± 56
KIAA0186	75 ± 15	323 ± 19	354 ± 39	330 ± 36	319 ± 14
BM039	87 ± 12	306 ± 78	338 ± 33	303 ± 95	280 ± 16
Solute carrier family 7 member 11	92 ± 8	41 ± 14	27 ± 2	35 ± 6	31 ± 5
KIAA0101	97 ± 44	1212 ± 216	1390 ± 94	1438 ± 140	1100 ± 70
Ubiquitin conjugating enzyme E2C	131 ± 32	844 ± 123	1144 ± 145	872 ± 176	810 ± 106
ADP-ribosylation factor-like 3	140 ± 13	431 ± 44	330 ± 14	373 ± 96	317 ± 27
UDP glycosyltransferase 1 family polypeptide A3	212 ± 23	71 ± 12	61 ± 9	104 ± 25	57 ± 6
YIPPEE protein	256 ± 34	157 ± 20	156 ± 52	101 ± 12	180 ± 51
Myosin 6	306 ± 25	193 ± 33	108 ± 23	142 ± 21	114 ± 29
PRO1489	358 ± 50	161 ± 42	186 ± 55	136 ± 53	201 ± 39
Karyopherin alpha 2	435 ± 104	1131 ± 148	1460 ± 248	1190 ± 175	1195 ± 1195
Cytochrome P450 1B1	463 ± 35	164 ± 63	141 ± 36	289 ± 96	116 ± 26
Beta subunit of the Na,K-ATPase pump	466 ± 31	195 ± 59	51 ± 6	248 ± 28	53 ± 6
Cytochrome P450 1A1	647 ± 148	219 ± 130	88 ± 12	346 ± 132	96 ± 10
Sequestosome 1	1882 ± 112	775 ± 198	579 ± 85	921 ± 236	643 ± 89

Target genes involved in metabolism and transport systems, as well as transcripts with unknown function. Hybridization data were normalized and filtered using dChip (mean values of three independent experiments ± SEM). The relative expression values are shown in light intensity units. Transcripts are listed in ascending order of their constitutive expression in control cells treated with the DMSO solvent.
[a]HADHSC, L-3-hydroxyacyl-coenzyme A dehydrogenase short chain.

Table VI. Estrogen-regulated transcripts in T47D cells

Gene	Control	17β-Estradiol	Genistein	Bisphenol-A	PCB54
F-box only protein 5	82 ± 13	169 ± 11	203 ± 26	242 ± 24	182 ± 36
ATP-binding cassette subfamily C member 13	87 ± 15	180 ± 24	172 ± 29	170 ± 19	261 ± 60
Cytochrome P450 1B1	117 ± 53	472 ± 28	1089 ± 178	698 ± 20	877 ± 146
Chondroitin synthase 1	137 ± 41	275 ± 19	313 ± 20	375 ± 41	293 ± 99
Gene regulated by estrogen in breast cancer 1	152 ± 21	240 ± 41	326 ± 77	437 ± 39	326 ± 53
ATPase family AAA domain containing 2	164 ± 31	496 ± 128	341 ± 45	421 ± 47	436 ± 112
Chloride intracellular channel 6	214 ± 61	556 ± 93	593 ± 192	1000 ± 282	664 ± 106
UHRF1[a]	247 ± 24	652 ± 62	575 ± 120	731 ± 34	752 ± 117
Acyl-coenzyme A cholesterol acyltransferase	330 ± 35	832 ± 63	666 ± 95	701 ± 68	708 ± 147
KIAA0256	338 ± 35	130 ± 24	149 ± 27	218 ± 29	147 ± 42
FLJ14299	342 ± 35	539 ± 69	560 ± 124	958 ± 137	607 ± 76
Carbonic anhydrase 12	367 ± 42	616 ± 82	1107 ± 171	1110 ± 62	1017 ± 226
Emopail binding protein	426 ± 75	1438 ± 83	753 ± 66	938 ± 62	878 ± 80
KIAA1049	542 ± 58	251 ± 89	250 ± 47	348 ± 19	159 ± 49
Sideroflexin 2	592 ± 87	1988 ± 127	2167 ± 553	2526 ± 543	2908 ± 109
FK506 binding protein 4	960 ± 206	3674 ± 291	3698 ± 141	3787 ± 170	5133 ± 429
KIAA0101	1060 ± 216	1771 ± 266	2806 ± 202	2238 ± 225	2099 ± 400
F-box only protein 32	1921 ± 331	1260 ± 128	1214 ± 368	719 ± 102	906 ± 52

Target genes involved in metabolism and transport systems, as well as transcripts with unknown function. Hybridization data were normalized and filtered using dChip (mean values of three independent experiments ± SEM). The relative expression values are shown in light intensity units. Transcripts are listed in ascending order of their constitutive expression in control cells treated with the DMSO solvent.
[a]Abbreviation: UHRF1, ubiquitin-like containing PHD and RING finger domains 1.

expression levels vary among different cells, indicating that the biological activity of estrogens is modulated by tissue-specific ER patterns (49). Third, ERα and ERβ have been shown to exert, at least in part, antagonistic biological effects (50,51). Finally, a conformational change in the ER protein is required for activation or repression of responsive genes but it has been observed that 17β-estradiol and genistein induce distinct changes in the receptor fold (52), prompting the hypothesis that different ER agonists may exert distinct transactivation functions. In apparent agreement with this expectation, numerous studies reported that the transcriptional patterns induced by genistein or bisphenol-A in human breast cancer cells

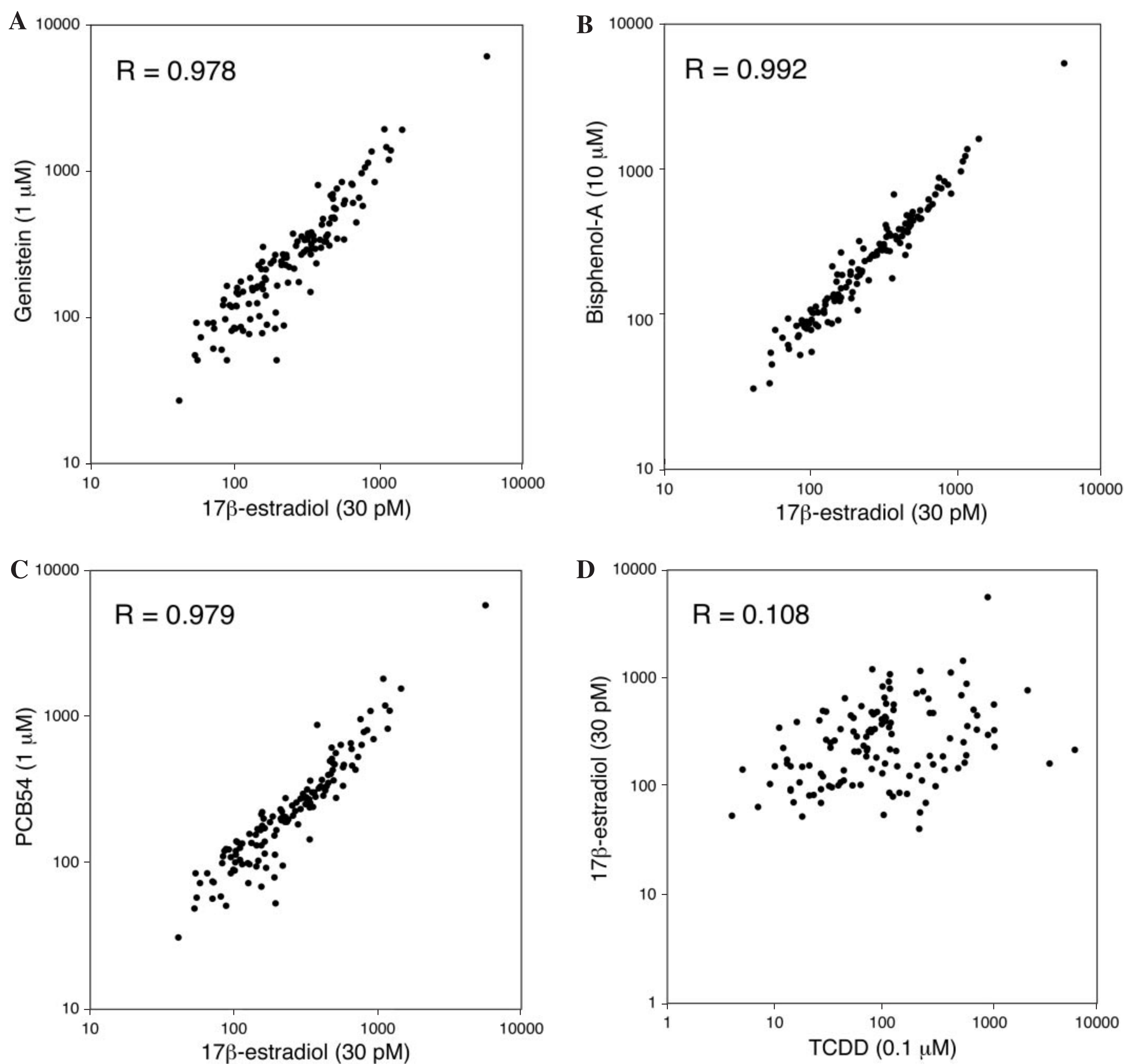

Fig. 4. Relationship between gene expression profiles resulting from exposure to different estrogenic agents. Transcriptional fingerprints after treatment with xenoestrogens were compared with the expression pattern induced by 17β-estradiol in scatter blot graphs. The axes indicate $\log_{10}$ expression levels of transcripts in units of light intensity. The *R*-values were calculated for each relationship on the basis of the linear regression between each pair of data. (**A**) Comparison between genistein and 17β-estradiol. (**B**) Comparison between bisphenol-A and 17β-estradiol. (**C**) Comparison between PCB54 and 17β-estradiol. (**D**) Comparison between 17β-estradiol and TCDD (0.1 μM). Higher concentrations of TCDD (≥1 μM) exerted cytotoxic effects.

are only in part similar to the characteristic expression fingerprint of 17β-estradiol (27–29,31).

Previous comparative analyses have been performed with highly saturating levels of estrogenic agents, reaching concentrations of up to 10 nM for 17β-estradiol and 100 1 μM for genistein. Therefore, the goal of our study was to employ subsaturating and equipotent levels of each ER agonist to determine transactivation patterns in MCF7 and T47D cells. The resulting expression signatures have been compared with the emerging transcriptional profile elicited by the endogenous hormone 17β-estradiol in the same cancer cell lines. Indeed, the gene expression changes that we observed in response to 17β-estradiol include a large number of transcripts that were previously known to be susceptible to estrogenic regulation, thus substantiating the validity of our transcriptomic analysis. In contrast to previous reports (27–29,31), we unexpectedly found that the transcriptional machineries of MCF7 and T47D breast cancer cells respond in a very monotonous manner to estrogenic stimuli. Presumably, the differential transcription profiles documented in previous studies arise from dose-dependent variations in the magnitude of gene expression, rather than from distinct mechanisms of gene regulation. For example, it has been demonstrated that some estrogen-responsive transcripts are induced only when the hormone

level is raised to concentrations that exceed the saturation range (22). On the other hand, the induction of similar expression patterns in response to distinct ER ligands, including 17β-estradiol and genistein, has already been reported for the mouse uterus (33). Thus, there is growing evidence that, at least in some susceptible target tissues, phytoestrogens and synthetic estrogenic chemicals elicit the same monotonous transcriptional program as the endogenous hormone.

The existence of congruent expression profiles help to explain the discrepancy between the high concentrations of estrogenic chemicals that are needed in most cases to elicit an effect and the low level of these compounds in the diet or environment. In fact, it has been demonstrated in a simple experimental set-up, consisting of a reporter gene assay in yeast transfected with human ERα, that the multiple components of xenoestrogen mixtures can act together to yield measurable responses when combined at concentrations which individually produce undetectable effects (53). Thus, the induction of identical transcriptional signatures, both with respect to the precise endpoints (gene targets) and the quality of response (gene induction or repression), supports the view that distinct estrogens can act in a cumulative manner even in complex systems covering a multitude of genomic targets at higher levels of biological organization. On the basis of our results, it appears that non-saturating concentrations of 17β-estradiol, genistein, bisphenol-A, PCB54 and other xenoestrogens may cooperate to transactivate or repress the same spectrum of genes, thereby inducing an additive transcriptional response that is characteristic for ER agonists. Therefore, we propose that the multitude of estrogenic chemicals to which the population is exposed involuntarily, in conjunction to changes in endogenous hormone levels, may constitute the cumulative cause for an increased risk of breast cancer or other malignancies of estrogen-dependent tissues.

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