Global gene expression profiles induced by phytoestrogens in human breast cancer cells

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Abstract

The nutritional intake of phytoestrogens seems to reduce the risk of breast cancer or other neoplastic diseases. However, these epidemiological findings remain controversial because low doses of phytoestrogens, achievable through soy-rich diets, stimulate the proliferation of estrogen-sensitive tumor cells. The question of whether such phytochemicals prevent cancer or rather pose additional health hazards prompted us to examine global gene expression programs induced by a typical soy product. After extraction from soymilk, phytoestrogens were deconjugated and processed through reverse- and normal-phase cartridges. The resulting mixture was used to treat human target cells that represent a common model system for mammary tumorigenesis. Analysis of mRNA on high-density microarrays revealed that soy phytoestrogens induce a genomic fingerprint that is indistinguishable from the transcriptional effects of the endogenous hormone 17β-estradiol. Highly congruent responses were also observed by comparing the physiologic estradiol with daidzein, coumestrol, enterolactone, or resveratrol, each representing distinct phytoestrogen structures. More diverging transcriptional profiles were generated when an inducible promoter was used to reconstitute the expression of estrogen receptor β (ERβ). Therefore, phytoestrogens appear to mitigate estrogenic signaling in the presence of both ER subtypes but, in late-stage cancer cells lacking ERβ, these phytochemicals contribute to a tumor-promoting transcriptional signature.

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Introduction

Epidemiological studies have linked an increased risk of developing mammary or endometrial malignancies to prolonged estrogen exposure due to early menarche, oral contraceptives, nulliparity, late first-term pregnancy, delayed menopause, or an estrogen replacement therapy (McPherson et al. 2000, Clemons & Gross 2001, Nelson et al. 2002). In contrast, the dietary intake of soy isoflavones such as genistein and daidzein correlates with a lower incidence of breast and prostate cancers (Ingram et al. 1997, Branca & Lorenzetti 2005). However, why endogenous estrogen hormones or synthetic xenoestrogens increase cancer risk, whereas natural phytoestrogens appear to exert an opposite preventive action, is not understood.

In the normal resting mammary gland, estrogen receptors (ERs) are expressed in only a small proportion of epithelial cells that are largely non-dividing (Ali & Coombes 2002). In contrast, enhanced expression of ERs is a critical event in the pathogenesis of a majority (~70%) of breast cancers and, accordingly, the growth of malignant mammary tumors is estrogen dependent in most cases (Hayashi et al. 1997, Gruvberger et al. 2001, Rice & Whitehead 2006). Like other ER agonists, phytoestrogens stimulate the proliferation of estrogen-sensitive tumor cells in various experimental systems (Hsie et al. 1998, Allred et al. 2001), and this growth-promoting activity has raised concerns that soy products, or similar phytochemicals, may represent an additional health hazard for vulnerable risk groups (Messina et al. 2006). More skepticism regarding the true benefits of
phytoestrogens came from the observation that hyperplasia of the epithelium and other markers of cell proliferation are detectable in breast biopsies of pre- and postmenopausal women after a period of dietary soy supplementation (Petrakis et al. 1996, McMichael-Phillips et al. 1998, Hargreaves et al. 1999). Thus, establishing the consequences of soy intake in populations at high risk for breast or prostate cancer is an important public health issue (Messina et al. 2006).

The present study was instigated by the notion that essentially all cellular responses to estrogenic stimuli culminate in transcriptional regulation, even though some of the known effects are considered indirect or non-genomic (Duan et al. 2001, McDonnell & Norris 2002, Levin 2003). Thus, oligonucleotide-based microarrays, which are able to detect global expression profiles at the transcriptional level, provide a convenient molecular approach to analyze biological endpoints of phytoestrogen exposure. Particular attention has been given to the question of whether the native hormone 17β-estradiol and phytoestrogens induce similar or different transactivation functions. For example, a gene expression survey performed on immature mice treated with genistein or 17β-estradiol yielded identical transcriptional responses in the uterus (Moggs et al. 2004). Other reports concluded that there are significant differences between the transcriptional profiles elicited by genistein or other estrogenic compounds in the reproductive tract of female rats (Naciff et al. 2002, Hong et al. 2006). Additional analyses were carried out under more tightly controlled cell culture conditions. One of these studies concluded that there is a partial overlap between the expression patterns elicited by 17β-estradiol and phytoestrogens, including genistein, daidzein, and coumestrol, in human MCF7 breast cancer cells (Ise et al. 2005). Another contrasting study with MCF7 cells yielded no substantial relationship between the transcriptional effects of genistein and the endogenous hormone 17β-estradiol (Wang et al. 2004).

These conflicting results prompted us to examine genome-wide expression profiles in human MCF7 and T47D breast cancer cells exposed to either single phytoestrogens or the natural phytoestrogen mixture extracted from a representative soy product. Soymilk has been selected for this study because it represents a widespread nutritional supplement and it is increasingly used as a basic component of food products for newborns. As a reference control, an analogous extract from cow milk has been included in view of its much lower phytoestrogen content. The comparison of expression data comprising 47 400 human transcripts revealed that the particular fingerprint induced by soymilk phytoestrogens coincides with the known transcriptional response of breast cancer cells to the endogenous 17β-estradiol hormone. In addition, we found that soy phytoestrogens induce nearly identical expression fingerprints as other structurally distinct phytoestrogens from different sources. A phytoestrogen signature that deviates from the characteristic transcriptional fingerprint of 17β-estradiol was observed when an inducible genetic construct was used to express, in addition to ERα, also the ERβ subtype.

Materials and methods

Chemicals

Daidzein and resveratrol were purchased from Sigma–Aldrich; 17β-estradiol, coumestrol, and enterolactone were from Fluka (Buchs, Switzerland). The inhibitor ICI 182 780 was purchased from TOCRIS Bioscience (Avonmouth, UK). All solvents and reagents were of analytical grade quality.

Extraction and analysis of milk samples

The sample preparation procedure developed for extracting phytoestrogens from milk, including the removal of endogenous estrogen hormones, is fully described elsewhere (Antignac et al. manuscript in preparation). Briefly, the samples (cow milk or soymilk obtained from a local retailer) were extracted in 10 ml aliquots with acetate buffer (2 M, pH 5.2) and acetone. After centrifugation, the supernatants were collected and the acetone phase was evaporated under a nitrogen stream. Subsequently, an enzymatic hydrolysis was carried out by overnight incubation (52 °C) with a purified Helix pomatia preparation (Sigma). This deconjugation step was followed by purification through two successive cartridges combining a reverse (C18) and a normal (silica) stationary phase (SDS, Peypin, France). After evaporation of the methanol eluates, the remaining residues were reconstituted in 30 μl dimethyl sulfoxide (DMSO) for cell culture experiments. Separate cow milk and soymilk samples were subjected to the same procedure, except that a deuterated compound (daidzein-d3) was included as internal standard. These samples were analyzed by liquid chromatography–tandem mass spectrometry for the quantitative determination of phytoestrogens (Antignac et al. 2003). The 17β-estradiol measurements were performed according to a previously described method (Courant et al. 2007).
Cell culture and treatments

Human T47D.Luc cells (BioDetection Systems, Amsterdam, 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; Invitrogen). The T47Dβ derivatives (Ström et al. 2004) were grown in the presence of 1 μg/ml tetracycline. This antibiotic was removed for a period of 48 h to induce the expression of ERβ. The MCF7 cell line subtype BUS (Soto et al. 1995) was maintained in DMEM supplemented with 10% FBS. The other antibiotics used were 0.1 U/ml penicillin and 0.1 μg/ml streptomycin (Invitrogen). All cell lines were cultured at 37 °C in xenoestrogen-free plastic (Corning Inc., Grand Island, New York, USA) under humidified air containing 5% CO2. Before each experiment, the cells were transferred to phenol red-free medium and cultured for 48 h in the presence of 5% dextran/charcoal-stripped FBS (DCC-FBS). DMSO stocks of each test compound were added to the culture medium. Unless otherwise indicated, the final solvent concentration was adjusted to 0.1% (v/v).

Cytotoxicity assays

A commercial kit was used to measure intracellular ATP levels. Briefly, MCF7 cells were grown in multi-well plates as outlined before and exposed to the indicated fractions of soymilk or cow milk extracts. After 24 h, the CellTiter-Glo reagent (Promega) was added and the luminescent signal was recorded in a microplate reader following the manufacturer’s instructions. Additionally, the CellTiter 96 and CytoTox 96 assays (Promega) were used to monitor the overall metabolic activity and the release of lactate dehydrogenase.

ER-CALUX assay

The ER-mediated chemical-activated luciferase expression (ER-CALUX) assay was carried out following the instructions 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 the indicated test compounds dissolved in DMSO. Solvent controls and a standard 17β-estradiol dose response were included on each plate. After 24 h exposure times, cells were harvested, lysed, and assayed for luciferase activity on a Dynex microplate luminometer (Legler et al. 1999).

Microarray hybridization, data acquisition, and analysis

After a 24 h treatment with test compounds, cells were collected by trypsinization and total RNA was extracted using the RNeasy kit (Qiagen). Amount and quality of the RNA fractions were evaluated by u.v. spectrophotometry (260 and 280 nm wavelength) followed by examination of the probes by capillary electrophoresis on Agilent Bioanlyzers. The GeneChip expression and IVT (in vitro transcription) labeling kits (Affymetrix) were used for the synthesis of cDNA and complementary RNA respectively. The biotin-labeled RNA was fragmented and hybridized on human genome U133 plus 2.0 microarrays (Affymetrix, Santa Clara, CA, USA) following the manufacturer’s instructions. After hybridization (16 h), the microarrays were processed by automated washing on the Affymetrix Fluidics Station 400. Staining of the hybridized probes was performed with fluorescent streptavidin–phycoerythrin conjugates (1 mg/ml; Invitrogen). The scanning of DNA microarrays was carried out on an Affymetrix laser instrument. Microarray quality assessment, condensing of the probe sets, data normalization, and filtering were conducted using the Expressionist software (Genedata AG, Basel, Switzerland). The t-tests were performed between controls and treated cells to assess the statistical significance of differentially expressed genes. False discovery rates were determined according to the Benjamini–Hochberg method (Benjamini & Hochberg 1995). Finally, the means of three to five replicates were imported into a Microsoft Excel file for graphical representation and determination of correlation coefficients. The ‘Gene Ontology’ database (www.geneontology.org) was consulted for the molecular function of each transcript and, for simplicity, only gene products with a known or inferred function are displayed in the figures.

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 (Foster City, CA, USA). Briefly, 100 ng cDNA were mixed with 100 nM 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) 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 $2^{-\Delta\Delta C_{t}}$ method (Livak & Schmittgen 2001), with the abundant glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript as an endogenous control.

**Results**

**Sample preparation and analysis**

After liquid extraction and enzymatic deconjugation, the soymilk and cow milk samples were subjected to a two-step fractionation procedure using reverse-phase (C18) and normal-phase (SiOH) cartridges. The quantitative analysis by liquid chromatography coupled to tandem mass spectrometry confirmed that soymilk contains large quantities of the isoflavones genistein and daidzein, whereas cow milk is characterized by the presence of low levels of enterolactone together with trace amounts of other phytoestrogens (Table 1). An additional determination by gas chromatography–mass spectrometry was used to verify that endogenous estrogen hormones, including 17β-estradiol, were removed from the cow milk sample during the final solid-phase extraction step (data not shown). For the subsequent cell culture experiments, each isolate from a 10 ml sample was reconstituted in 30 μl DMSO, which proved to be compatible with the solubility properties of the various phytoestrogens.

**Dose-dependent transactivation from a minimal estrogen-responsive promoter**

Initially, human MCF7 and T47D breast cancer cells were exposed to increasing concentrations of the soymilk and cow milk extracts. Cell viability was tested 24 h later by measuring the intracellular ATP pool, which is used as an indicator of metabolic activity. The resulting dose responses demonstrated that no cytotoxic reactions were triggered when the cell culture medium contained up to 0.5% (v/v) milk extracts dissolved in DMSO. This lack of cytotoxicity was confirmed when the metabolic activity was assessed by measuring the capacity to reduce a tetrazolium reagent or when the cell integrity was measured by monitoring the release of lactate dehydrogenase (data not shown).

Next, the soymilk and cow milk extracts were tested for their overall estrogenic activity using a standard reporter gene assay. For that purpose, we exploited a stably transfected carcinoma cell line (T47D.Luc) that carries a chromosomally integrated reporter gene sequence (Legler et al. 1999). In this genetically modified cell line, the firefly (Photinus) luciferase gene is under transcriptional control of a minimal promoter displaying tandem repeats of estrogen response elements (5'-GGTCACTGTGACC-3'). This artificial construct drives the expression of firefly luciferase in response to ER activation. Thus, to monitor estrogenic activity, cell lysates were examined for luciferase activity after a 24 h treatment with progressively increasing concentrations of 17β-estradiol or the different extracts added to the cell culture medium. In all these treatments, the final concentration of the DMSO solvent was 0.1% (v/v).

In a series of control reactions, the synthetic promoter mediated a dose-dependent luciferase induction in response to the 17β-estradiol standard. This expected estrogenic effect reached peak levels at a hormone concentration of 60 pM (Fig. 1). A similar level of reporter gene induction was observed in the cells incubated with soymilk extract. In agreement with its limited phytoestrogen content, however, the corresponding cow milk isolate resulted in only minor reporter gene induction when compared with the soy product (Fig. 1). These responses to the treatment with 17β-estradiol or soymilk extract were completely suppressed by the addition of the ER antagonist ICI 182 780 at a concentration of 0.1 μM (data not shown).

**Global expression profiles elicited by natural phytoestrogen mixtures**

Previously, we found that the MCF7 breast cancer cell line is markedly more responsive than T47D cells to estrogenic stimuli, thus yielding a wider range of estrogen-regulated genes as well as larger amplitudes of expression changes (Buterin et al. 2006). As a consequence, the MCF7 cells were used herein to perform genome-wide analyses of endogenous

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<td>Soymilk</td>
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<td>Daidzein</td>
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<td>Genistein</td>
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nd, not detected at the limit of detection, which varied from 0.05 to 0.7 ng/ml depending on the compound.
transcripts after a treatment with phytoestrogen mixtures reconstituted in culture medium. The target cells were incubated in triplicates with soymilk or cow milk extracts to reach a final solvent concentration of 0.1% (v/v). After 24 h exposures, a fraction of RNA from each sample was analyzed using Affymetrix microarrays that display the sequences of 47,400 human transcripts. To identify genes that are susceptible to ER regulation, these microarray data were normalized and subjected to statistical analysis. Also, in view of the large number of regulated genes, the expression data were subjected to a filter for transcripts that exhibited at least a fivefold change relative to the solvent control, thereby eliminating the vast majority of gene products that are more moderately affected or not altered at all following the phytoestrogen treatment. The significance threshold was \( P < 0.01 \), yielding false discovery rates in the range of 0.02–0.1 (Benjamini & Hochberg 1995). According to these criteria, a total of 358 different transcripts were up- or down-regulated by more than fivefold in response to incubation with the soymilk extract. In contrast, the gene expression profile induced by the cow milk sample deviated only marginally from the background transcriptional pattern observed in the solvent control group. In this case, only six transcripts were affected by more than fivefold changes, thus reflecting the much lower phytoestrogen concentration in cow milk.

Figure 2A shows the transcripts displaying the highest amplitude of regulation in response to the treatment with soymilk phytoestrogens. The majority of these transcripts encode for proteins involved in DNA metabolism (ribonucleotide reductase M2 (RRM2), thymidylate synthetase, thymidine kinase 1; see legend to Fig. 2 for abbreviations), DNA replication or recombination (minichromosome maintenance-deficient 10, CDT1, topoisomerase II-\( \alpha \) (TOP2A), primase 1, RAD51), cell division cycle (cyclin A2, cell division cycle 2 (CDC2), CDC6, TTK), chromosome segregation and centromere function (aurora kinase B, kinesin-like 7 (KNSL7), Spc24, Spc25, cell division cycle associated 1, kinetochore associated 2, kinesin family member 2C, extra-spindle poles like 1, abnormal spindle-like microcephaly), or inhibition of apoptosis (baculoviral IAP repeat-containing 5). Together with the over-expressed proto-oncogenes myeloblastosis oncogene-like 1 (MYBL1) and MYBL2 as well as two different proliferation markers (antigen identified by monoclonal antibody Ki-67 and Opa-interacting protein 5), this transcriptional profile reflects the typical mitotic signature observed in estrogen-stimulated breast cancer cells (Lobenhofer et al. 2002, Coser et al. 2003, Frasor et al. 2003, Vendrell et al. 2004, Buterin et al. 2006, Lavigne et al. 2007).

A side-by-side comparison indicated that the observed expression changes resulting from exposure to the soy phytoestrogens are similar to the transcriptional effects of the endogenous hormone 17\( \beta \)-estradiol. In fact, all transcripts that were increased by incubation with the soymilk extract were also up-regulated following the treatment with 17\( \beta \)-estradiol (Fig. 2A). To analyze this presumed relationship in more quantitative terms, the mRNA profile induced by the soymilk extract was plotted against the corresponding values obtained with 17\( \beta \)-estradiol. All transcripts were included in this analysis that showed at least a fivefold up- or down-regulation in the phytoestrogen treatment group. The threshold for statistical significance was \( P < 0.01 \). Figure 2B shows that the data points in this comparison grouped in two distinct clusters reflecting those genes that were over-expressed and those that were under-expressed relative to the solvent control. A linear regression analysis of all 358 pairs of data yielded an overall correlation coefficient of \( R = 0.87 \), thereby exceeding the values (\( R = 0.6 \)) found in another similar study that
compared soy extracts with 17β-estradiol (Ise et al. 2005). The close correspondence of expression values, demonstrated in Fig. 2B, lends support to the notion that soy phytoestrogens and 17β-estradiol induce nearly identical transcriptional responses in MCF7 cells. Thus, in contrast to previous reports (Wang et al. 2004, Ise et al. 2005), we found that the transcriptional machinery of this breast cancer cell line responds in a very monotonous manner to distinct estrogenic stimuli.

**Figure 2** Comparison of genome-wide transcriptional changes in MCF7 cells exposed to milk extracts or 17β-estradiol. (A) Transcripts with the highest amplitude of induction following a treatment with soymilk extract. The level of the same transcripts has also been determined for the treatment with 17β-estradiol (30 pM) or an analogous cow milk extract. The extracts were dissolved in DMSO and added to the cell culture medium to a final solvent concentration of 0.1%. In each case, the fold changes were calculated against the corresponding values obtained in control experiments with solvent alone (mean values of three independent experiments). (B) Linear regression analysis of pairs of data obtained from the experiments with soymilk extract and 17β-estradiol (30 pM). A fivefold change (up- or down-regulation) in the soymilk-treated samples relative to the controls was used as the cut-off to filter the data. A total of 358 transcripts passed the statistical threshold of $P < 0.01$. RRM2, ribonucleotide reductase M2; AURKB, aurora kinase B; MCM10, minichromosome maintenance-deficient 10; TYMS, thymidylate synthetase; CCNA2, cyclin A2; KNSL7, kinesin-like 7; Spc25, kinetochore protein Spc25; UHRF1, ubiquitin-like, containing PHD and RING finger domains 1; TK1, thymidine kinase 1; FOXM1, forkhead box 1; GAJ, GAJ protein; MYBL1, myeloblastosis oncogene-like 1; MYBL2, myeloblastosis oncogene-like 2; TOPK, T-LAK cell-originated protein kinase; FKSG14, leucine zipper protein FKSG14; KNTC2, kinetochore associated 2; CDC2, cell division cycle 2; CDT1, chromatin licensing and DNA replication factor 1; TOP2A, topoisomerase II-α; CDC6, cell division cycle 6; KIF1C, KSHV latent nuclear antigen interacting protein 1; TMPRSS3, transmembrane protease serine 3; MKI67, antigen identified by monoclonal antibody Ki-67; DLG7, discs large homolog 7; Spc24, kinetochore protein Spc24; OIP5, Opa-interacting protein 5; NUSAP1, nucleolar and spindle associated protein 1; CDCA1, cell division cycle associated 1; BIRC5, baculoviral IAP repeat-containing 5; ANLN, anilin; BRRN1, barren homolog; PRO2000, PRO2000 protein; KIF2C, kinesin family member 2C; PRIM1, primase 1; ESPL1, extra-spindle poles like 1; ASPM, abnormal spindle-like microcephaly; TTK, TTK protein kinase; RAD51, radiation sensitive 51; TCF19, transcription factor 19; SHCBP1, ortholog of mouse Shc SH2-domain binding protein 1.
Validation using real-time PCR

Real-time reverse transcriptase-PCR (RT-PCR) assays were carried out on representative sequences to confirm the tight correlation between the expression profiles induced by soy phytoestrogens and 17β-estradiol. The following transcripts were tested: RRM2 polypeptide, CDC2, TTK (a protein kinase), and UDP-N-acetylglucosaminyltransferase 5. The exposure with soy phytoestrogens was performed with three different proportions of extract in the cell culture medium between 0.001 and 0.1% (v/v). In view of the phytoestrogen contents listed in Table 1, these residue levels translate to genistein concentrations ranging from 0.1 to 10 μM. Incubations with the 17β-estradiol reference were performed using the standard concentration of 30 pM. After normalization with the constitutive GAPDH transcript, expression values are indicated as the ratio between treated cells and solvent controls. A linear regression analysis of the resulting RT-PCR values yielded correlation coefficients of $R=0.92–0.98$, thus confirming that phytoestrogens and the physiologic estradiol hormone induce very similar transcriptional responses (Fig. 3).

Expression patterns induced by single phytoestrogens

We already demonstrated that the most abundant soy phytoestrogen, i.e., genistein, induces global gene expression profiles in both MCF7 and T47D cells that are indistinguishable from the transcriptional changes resulting from 17β-estradiol treatments (Buterin et al. 2006). A similar convergence was now obtained when we assessed the response of MCF7 cells to another major soy phytoestrogen, i.e., daidzein tested at a concentration of 1 μM (Fig. 4A). Subsequently, this study was extended to different categories of phytoestrogens including coumestrol (found in white clover), resveratrol (found in grape skins and red wine), and enterolactone (an endogenous metabolite generated from plant lignans). The criteria for inclusion of the transcripts into the correlation analyses of Fig. 4 were again a fold change >5 and a corresponding $P$ value <0.01. Compared with the daidzein treatment, the number of significantly regulated transcripts was slightly higher in the coumestrol experiment (Fig. 4B) and markedly reduced in the resveratrol and enterolactone treatments (Fig. 4C and D). However, a direct comparison with the effects of 17β-estradiol on the same human genes yielded correlation coefficients of $R=0.85–0.92$, further supporting the idea that, at least in a low-dose range, all kinds of phytoestrogens generate expression profiles in human breast cancer cells that are superimposable with the transcriptional pattern elicited by a 17β-estradiol stimulus.

Modulation of expression fingerprints by ERβ

RT-PCR quantifications with oligonucleotide primers specific for each ER subtype showed that the predominant mRNA in MCF7 and T47D cells is the one coding for ERα, whereas ERβ transcripts remained undetectable (data not shown). These findings are consistent with previous studies reporting the presence of only trace amounts of ERβ transcripts in T47D and essentially no such transcripts in MCF7 cells (Legler et al. 1999, Lobenhofer et al. 2002). By immunoblotting methods, it has also been shown that there is no detectable ERβ protein in T47D cells (Ström et al. 2004). The lack of an appropriate breast cancer cell line containing significant amounts of ERβ protein can be circumvented by taking advantage of a genetic system (T47Dβ) in which a tetracycline-regulated construct drives the expression of a full-length human ERβ sequence (Ström et al. 2004). Here, these stably transfected T47Dβ derivatives have been used to examine the contribution of ERβ to the transcriptional reprogramming triggered by phytoestrogens.

T47Dβ cells were exposed to coumestrol (at a concentration of 1 μM) because this particular phytochemical displays the highest affinity for ERβ among...
all phytoestrogens tested to date (Kuiper et al. 1997, 1998, Bovee et al. 2004). The transcriptional changes were tested in the presence of tetracycline, i.e., under conditions that suppress the expression of ERβ, as well as after tetracycline withdrawal, resulting in promoter activation and ERβ expression. In the absence of tetracycline, the level of mRNA coding for ERβ is four to five times higher than the corresponding ERα transcripts, thus leading to substantial quantities of both receptor subtypes (Ström et al. 2004). To eliminate possible confounding effects due to the antibiotic used for induction, the fingerprints obtained in the presence of tetracycline were analyzed against appropriate solvent controls containing the same level of antibiotic. Conversely, the fingerprints obtained after tetracycline withdrawal, promoting ERβ expression, were determined against corresponding controls without tetracycline in the medium.

Figure 5 illustrates the distinctly different transcriptional profiles induced by coumestrol in the absence or in the presence of ERβ. To simplify the representation of data, the graph of Fig. 5 shows only those transcripts that were up-regulated by a fold change >10 in at least one of the treatment groups (P<0.01 for all transcripts). The response obtained in T47Dβ cells containing ERα alone involves, for example, an overexpression of interleukin 20 (IL 20), chemokine ligand 12 (CXCL2), insulin-like growth factor-binding protein-4 (IGFBP4), MYB or trefoil factor 1 (TFF 1 also known as pS2). The range of regulated target genes did not change when, in the absence of tetracycline, both ERα and ERβ were expressed in the same cell line. However, the induction of many transcripts was attenuated in the presence of ERβ (Fig. 5), indicating that this additional receptor subtype is able to partially suppress some transactivation functions mediated by ERα. Conversely, in the presence of ERβ, other transcripts were regulated with larger amplitudes of induction than in the cells expressing only ERα. This second category of genes,
which become more responsive in the presence of ERβ, include, for example, tripin, TOP2A (see legend to Fig. 5 for abbreviations), the cyclin-dependent kinase inhibitors CDKN3 and CDKN2C as well as multiple members of the kinesin family (KNSL7, KIF20A, KIF14).

Toward a distinctive phytoestrogen signature

Many phytoestrogens display a selective binding to ERβ whereas 17β-estradiol has approximately the same affinity for both major ER subtypes (Kuiper et al. 1997, 1998). Thus, we explored the hypothesis that the preferential interaction of these phytochemicals with ERβ may mediate a more distinctive transactivation function. These experiments were again carried out with coumestrol because of its superior affinity for ERβ compared with other phytoestrogens (Bovee et al. 2004). The expression changes were subjected to a filter for transcripts that exhibit at least a threefold induction relative to untreated controls. A statistical threshold of \( P < 0.05 \) was applied to all differentially expressed transcripts.

In T47Dβ cells containing only ERα, in the presence of tetracycline, we observed the usual tight correlation between the expression profiles generated by 17β-estradiol and coumestrol, with 79 transcripts matching the filtering criteria (Fig. 6A). Upon linear regression analysis, the correlation coefficient between the 17β-estradiol and coumestrol data reached a value of \( R = 0.90 \). For the same transcripts, however, this correlation coefficient was reduced to \( R = 0.70 \) when the cells, in the absence of tetracycline, were able to express the ERβ subtype (Fig. 6B). Also, the slope of the linear regression decreased substantially from 0.7
decreases both the correlation coefficient and the slope of the absence of tetracycline). These graphs illustrate that ER
A) Comparison of gene expression profile in cells containing as the reference, solvent controls with or without tetracycline. The fold changes of each transcript (mean values of three independent experiments) have been calculated using, tetracycline. The fold changes of each transcript (mean values

Figure 6 Diverging expression profiles in the presence of ERβ. T47Dβ cells were challenged with 17β-estradiol (30 pM) or coumestrol (1 μM), either in the presence or in the absence of tetracycline. The fold changes of each transcript (mean values

Discussion
Breast cancer has become the most common malignancy among American and European women (Rice & Whitehead 2006) but, in eastern countries such as Japan, the incidence of breast cancer is only about one-third that of western populations. This difference has often been attributed to a much higher dietary intake of soy phytoestrogens (Konstantakopoulos et al. 2006, Martinez et al. 2006, McCarty 2006, Messina et al. 2006). However, despite the large amount of research conducted in the last years, no clear consensus has emerged regarding the preventive action of phytoestrogens against cancer. There is still no conclusive evidence that the ingestion of phytoestrogens is directly related to a reduced incidence of breast cancer, or whether phytoestrogens rather represent a biomarker of generally healthy diets (Martinez et al. 2006, Rice & Whitehead 2006).

At low physiologic serum concentrations that are normally achieved by nutritional intake, phytoestrogens are likely to act through modulation of estrogen signaling. In fact, these lower serum concentrations appear insufficient to inhibit tyrosine kinases or other enzymes that may provide alternative targets of phytoestrogen effects (McCarty 2006). Most estrogenic responses are mediated by two members of the nuclear steroid receptor superfamily, i.e., ERα and ERβ. Both receptors constitute ligand-stimulated transcription factors that associate with co-regulatory partners to remodel chromatin and recruit the general transcription machinery to downstream genes (Katzenellenbogen & Katzenellenbogen 2000, Hall et al. 2001, Mogs & Orphanides 2001, Safe 2001). Although both receptors bind to the same consensus estrogen-responsive element within gene promoters, ERα and ERβ have been shown to exert partially antagonistic effects (Omoto et al. 2003, Ström et al. 2004).

Several findings converge on the idea that the proliferative stimulus mediated by activation of ERα can be opposed by the expression of ERβ. First, the knockout mice lacking ERβ are more susceptible than wild-type controls to develop markers of epithelial hyperplasia in the mammary gland ( Förster et al. 2002). Second, the mRNA coding for ERα is up-regulated during cancer progression, whereas the ERβ transcript is reduced in part via promoter methylation (Iwao et al. 2000, Rutherford et al. 2000, Rody et al. 2005, Park et al. 2006). Third, the continued expression of ERβ in breast tumors is associated with low aggressiveness and improved survival rates compared with ERβ-negative counterparts (Hopp et al. 2004). Fourth, the activation of ERα promotes the growth of breast cancer cells both in culture and in animal models (Soto et al. 1995, Hsie et al. 1998, Allred et al. 2001, Ju et al. 2006) but, when ERβ is restored using an appropriate expression vector, it exerts a negative effect on cell proliferation or even induces apoptosis (Omoto et al. 2003, Skliris et al. 2003, Ström et al. 2004). This antiproliferative action of ERβ correlates with the down-regulation of several factors involved in DNA replication and the cell cycle machinery (Lin et al. 2007).

Contrary to 17β-estradiol, which does not discriminate between ERα and ERβ, phytoestrogens bind to ERβ with up to five times higher affinities compared with ERα ( Kuiper et al. 1997, 1998). This finding appears relevant in view of the possible action of ERβ as a tumor suppressor because phytoestrogens may be able to trigger beneficial responses through their preferential interaction with the ERβ subtype. However, such a protective effect is abrogated in cells that specialize on the expression of ERα with minimal residual amounts of ERβ. This possible mechanism was supported when, in the present report, we analyzed the transcriptional fingerprints induced by soymilk extracts in human breast cancer cells. This type of soy product has been tested in the context of
this study because of its high content in isoflavones with estrogenic activity, such that the findings may be extrapolated to a wide range of other soy-based foods and supplements. Unlike previous reports (Wang et al. 2004, Ise et al. 2005), we observed that this natural mixture of soy phytoestrogens, as well as other types of phytoestrogens, induces a stereotyped expression fingerprint in breast cancer cells containing high levels of ERα but essentially no ERβ. Thus, in the absence of ERβ, all tested phytoestrogens result in essentially the same expression changes as those induced by the endogenous 17β-estradiol, and this recurrent genomic profile reflects the proliferative response mediated through ERα activation. This particular signature includes the up-regulation of multiple factors involved in cell cycle, DNA replication, chromosome segregation, and inhibition of apoptosis. However, when the expression of ERβ is reconstituted using an inducible genetic system, the same breast cancer cells react in a different manner to phytoestrogen stimulation. First, the induction of many growth-promoting transcripts involved in the cell division cycle is attenuated compared with the genuine 17β-estradiol response and second, there is a stronger induction of factors that arrest cell proliferation, such as, for example, inhibitors of cyclin-dependent kinases, thus further contributing to the inhibition of cell proliferation.

We conclude that this attenuation of ERα-induced expression fingerprints may account for the presumed chemopreventive activity of phytoestrogens since, as indicated before, many of these compounds display particularly strong affinities for ERβ. In view of our findings, we propose a biphasic activity of phytoestrogens during cancer development in estrogen-sensitive tissues. Thus, the presumed beneficial effects of phytoestrogens depend on the timing of exposure. Initially, phytoestrogens are able to slow down cell growth by activating ERβ, thereby generating an anti-proliferative expression signature. Due to the genetic instability of malignant tumor cells, however, the expression of ERβ may be abrogated by gene deletion or promoter methylation. In such late-stage cancer cells, phytoestrogens, in conjunction with other estrogenic chemicals, induce a transcriptional profile that promotes the proliferation of those clones that exhibit high amounts of ERα but little ERβ. In light of these considerations, the potentially beneficial effect of phytoestrogens should be reevaluated, particularly in relation to risk groups that are susceptible to the development of ERα-positive tumors arising from steroid hormone-dependent tissues.

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