Differential Expression of Genes Induced by Resveratrol in Human Breast Cancer Cell Lines

Ludovic Le Corre, Nasséra Chalabi, Laetitia Delort, Yves-Jean Bignon, and Dominique J. Bernard-Gallon

Abstract: The phytoalexin, trans-resveratrol (RES), is a polyphenolic compound found in plants and fruits that seems to have a wide spectrum of biological activities. It has been found to possess cancer chemopreventive effects by inhibiting diverse cellular events associated with tumor initiation, promotion, and progression. RES is also a phytoestrogen, which binds to and activates estrogen receptors (ERs) that regulate the transcription of estrogen-responsive target genes. We used two human breast tumor cell lines (MCF7 and MBA-MB-231) and one fibrocystic breast cell line (MCF10a) to examine whether RES altered mRNA expression of genes that are involved in biological pathways frequently altered during carcinogenesis. Two GEarray™ systems were used to screen the differentially expressed genes between RES-treated cells and control cells. The differentially expressed genes were analyzed further by quantitative reverse transcriptase polymerase chain reaction. Here, we demonstrate that RES regulates mRNA expression of several genes involved in cell cycle control, apoptosis, metastasis, cell–cell adhesion, and ER signaling pathway. This effect of RES on the gene expression appears in correlation with chemoprevention activities of RES described previously. RES is also found to be more active in ER+ than ER– cells.

Introduction

Trans-resveratrol (RES) (3,5,4′-trihydroxy-trans-stilbene) has been found in many plants, including grapes, peanuts, berries, Polygonum roots, and traditional oriental medicine plants (1). In recent years, research on RES has described several beneficial effects of this compound to human health; it has been reported to have both anticarcinogenic and cardioprotective activities (2–7). Many in vitro studies have addressed the activities of RES in breast cancer cells (8). RES exhibits anti-initiation, antipromotion, and antiprogession activities in breast cancer cells, and these properties seem to be related to regulation of xenobiotic carcinogen metabolism (9,10) and anti-inflammatory (11–13), antiproliferative, and proapoptotic (14–17) effects. RES has been shown to be a phytoestrogen able to bind to and activate the estrogen receptors (ERs) that regulate the transcription of estrogen-responsive target genes (18–20). Many studies provide evidence for the anticarcinogenic activity of RES, but the precise mechanism involved in the modulation of oncogenic precursors of breast carcinogenesis remains to be elucidated.

Breast cancer is a major cause of death in women. Several factors such as genetics, hormones, and environment are responsible for breast cancer development (21). In addition, the cellular processes that are involved principally in the proliferative advantage inherent to the malignant phenotype are aberrant signal transduction, DNA damage repair and cell cycle dysregulation, apoptosis, sustained angiogenesis, tissue invasion, metastasis, and immune tolerance (22–25). To increase our understanding of the cascade of molecular events in response to RES treatment of breast cancer cells, a comprehensive study on gene expression at the transcription level is required. In our study, we used two GEarray™ systems (SuperArray, Inc., Bethesda, MD), which allowed use to study the mRNA expression of 172 genes from two breast cancer cell lines (MCF-7 and MDA-MB-231) and one fibrocystic cell line (MCF10a) treated with 30 and 50 µM RES, respectively.

Materials and Methods

Cell Cultures

Fibrocystic breast cells MCF10a and breast cancer cells MCF-7 and MDA-MB-231 were purchased from the American Type Culture Collection (ATCC, Rockville, MD), Dulbecco’s Modified Eagle’s Medium/Ham’s F-12, Roswell Park Memorial Institute medium 1640, L-15, McCoy’s 5A media, fetal bovine serum, and horse serum were obtained from Gibco BRL (Life Technologies, Gaithersburg, MD). All cell lines were cultured as described by the ATCC. The status of estrogen receptor α and β was determined by immunocytochemistry by the Centre Jean Perrin’s

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anatomopathologist. MCF-7 was found to be ERα+/ERβ+, MDA-MB-231 was found to be ERα−/ERβ+, and MCF10a was found to be ERα−/ERβ− (26).

**Treatment of Cells**

Cell cycle phase distribution was studied by flow cytometry after treatments with 10, 30, and 50 µM RES (Sigma Chimie, St. Quentin Fallavier, France) for different times of exposure (24, 48, and 72 h) in MCF-7, MDA-MB-231 (27), and MCF10a (26). For the present study, MCF-7 and MDA-MB-231 cells were treated for 48 h with 30 µM RES and MCF10a cells were treated for 48 h with 50 µM RES. These doses corresponded to when the percentage of cells in S phase was considerably increased after treatment with RES, whereas the percentage of cells in G1 phase was decreased. It is well known that RES treatment causes an accumulation of cells in S phase (28). RES was dissolved in dimethylsulfoxide.

**RNA Isolation**

After 48 h of treatment, total RNA was extracted using RNA-Plus reagent (Q-Biogen, MP Biomedicals, Illkirch, France) according to the manufacturer’s protocol. RNA was recovered in 50 µl of nuclease-free water and either used immediately or stored at −80°C until further analysis. The quality of RNA was assessed using Bioanalyzer 2100 system (Agilent Technologies, Massy, France).

**Gene Expression Array**

For these experiments, we used the GEarray system (SuperArray). It includes reagents for probe generation and hybridization, and gene arrays containing 96 marker genes, four housekeeping genes (glyceraldehyde 3-phosphate dehydrogenase, β-actin, cyclophilin A, and ribosomal protein L13a), and PUC18 as negative control. Two different GEarray systems were used in our study: the Human Cancer Pathway Finder™ gene array and the Human Breast Cancer and Estrogen Receptor Signaling gene array.

The Human Cancer Pathway Finder gene array allows us to assess the status of six biological pathways frequently altered during carcinogenesis. The induction of marker genes suggested the activation of their associated pathways. The Human Breast Cancer and Estrogen Receptor Signaling gene array is designed to investigate the breast cancer–related regulation of genes and ER-dependent signal transduction.

The macroarrays were used according to the manufacturer’s instructions. In brief, cDNAs were prepared from total RNA by reverse transcription. Then, they were amplified and radiolabeled simultaneously with [α-32P]-deoxyctydine triphosphate (3,000 Ci/mmol) by using an AmpoLabeling-LPR kit (SuperArray). After hybridization and washing, the arrays were scanned with a Personal Imager FX Systems (Biorad, Marnes-la-Coquette, France). The expression of genes was measured using Scanalyze software (Lawrence Berkeley National Lab, Berkeley, CA) and normalized against the median value of the experiment. Analysis of results was performed as described previously (29,30). Adjusted signal intensity for each gene was calculated by subtracting the background from the spot intensity and bringing all values less than 1,000 up to low value of 1,000. RES treatment condition was compared to the nontreated condition using a normalized ratio. Each determination corresponds to the mean value ± SD from four independent assays to ensure reproducibility of the results. Only those genes for which expression was similarly changed in all four comparative analyses ($P < 0.02$, t-test) were considered to be RES-responsive genes.

**Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction**

To validate the GEarray results, differential expression observed was confirmed with quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) using specific Assay-On-Demands™ for selected genes: D1 cyclin (Hs00233498_m1), Caspase 8 (Hs00236278_m1), ERβ (Hs00230957_m1), and PGR (Hs00172183_m1). The sequence of forward primers, TaqMan® (Applied Biosystems, ZA Courtabeuf, Villebon/Yvette, France) probes and reverse primers were for KL 67 amplification: 5′-1790GCCC CAACCAAAAGAAAGTCT1810-3′, 5′-1846CATCAAGGAA CAGCCTCAACCATCAGGAA1873-3′, 5′-1922AGCTTTGTG CCTTCACTTCCA1902-3′, respectively. The synthesis of cDNA was performed using the High Capacity cDNA Archive kit (Applied Biosystems) following the manufacturer’s instructions. Twenty-five nanograms of cDNA was analyzed with quantitative PCR assays using an ABI Prism 7700 thermal cycler system (Applied Biosystems). PCR was performed in 96-well plates using Universal Master Mix (Applied Biosystems) comprising thermostable polymerase AmpliTaq Gold, uracil-DNA-N-glycosylase, nucleotides, and a passive internal reference dye, Rox. All cDNA samples and controls were assayed in triplicate. Simultaneous detection of both the target gene and the 18S rRNA internal reference were performed in the same tube. The result quantification is transformed to an exponential value, $2^{-\Delta\Delta Ct}$, where Ct is the cycle threshold. Real-time quantitative PCR consisted of 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min.

To ensure the reproducibility of determination of mRNA in each sample, two independent RNA extractions were done. With one RNA extraction, two independent reverse transcriptions were done to ensure the reverse transcription efficiency. With the second RNA extraction, only one reverse transcription was performed. For the three reverse transcriptions, one determination was done with the $\Delta Ct$ formula. Each determination was done in triplicate and expressed as mean ± SD (31).
TaqMan universal PCR Master Mix and 18S rRNA-labeled TaqMan probe and primers were purchased from Applied Biosystems. A mix including probes and primers termed Assay-On-Demands were used to study mRNA expression of cyclin D1, progesterone receptor, caspase 8, Ki67, and ERβ (Applied Biosystems). All probes were labeled with 6-carboxy-fluorescein, and the 18S rRNA probe was labeled with VIC®. TAMRA was used as the 3’ quencher in all cases.

Statistical Analysis

The GEarray and RT-PCR results were evaluated by the Student’s paired t-test. Two-tailed P values < 0.05 were considered statistically significant.

Results

We used the commercially available pathway-specific cDNA array (GEarray) system for analysis of basal gene expression altered by RES in breast tumor cell lines MCF-7 and MDA-MB-231 and fibrocystic breast cells MCF10a.

Effect of RES on Gene Expression Profile

Two different gene arrays, each containing 96 marker genes monitoring the activation of six signal transduction pathways and genes specifically related with breast cancer were used to hybridize with labeled probes obtained from total RNA of breast cell lines treated or not treated with RES. Thus, we investigated mRNA levels of genes implicated in apoptosis, cell cycle, cell–cell adhesion, metastasis, angiogenesis, and ER pathway. The hybridization signal detected for each gene was normalized against the median value. Each determination corresponds to the mean value ± SD from four independent assays to ensure reproducibility of the results. Only those genes for which expression was similarly changed in all four comparative analyses (P < 0.02, t-test) were considered to be RES-responsive genes (Tables 1 and 2). To validate the GEarray results, differential expression observed was confirmed with quantitative real-time RT-PCR using specific Assay-On-Demands for selected genes. RES treatment produced a markedly different yet consistent response between the three breast cell lines, which suggested a breast cell line–specific gene expression signature.

RES-Regulated Genes and Cell Growth

In MCF-7 and MCF10a cells, RES induced increased expression of proapoptotic genes APAF-1, caspase 8, and more weakly, Fas (CD95), implicated in caspase-mediated apoptosis. Conversely, the proapoptotic gene Bax remained unchanged, whereas the antiapoptotic gene Bcl-x was increased. Thus, in both cell lines, RES seems to activate the gene of the caspase-dependent apoptosis pathway. Interestingly, in MDA-MB-231, no apoptotic genes presented variations in their mRNA expression after RES treatment.

Results from the GEarray analysis also showed that RES acts in breast cells by an alteration in expression of the genes involved in cell cycle control. We observed an altered expression of genes that regulate G1/S transition phases. However, RES-induced genes had different functions depending on the breast cell lines. In MCF-7, we obtained an increase of Cyclin D1 and Cdk4 mRNA expression. In MCF10a, we observed an overexpression of ATM and CDC25A mRNA. In MDA-MB-231, Cyclin E1 and CDC25A mRNA levels appeared to increase after RES treatment.

RES-Regulated Transcription Factors and Signal Transducers

RES appears to regulate expression of genes encoding to transcription factors and signal transducers involved in the cell growth control pathway. However, we observed a divergence among RES-altered genes according to the cell line. In MCF-7 and MCF10a, RES increased the p38 MAPK mRNA level, and for each cell line inhibited or enhanced Erb-2 and β-catenin gene expression, respectively. In contrast, expression of these genes remained stable in MDA-MB-231, whereas mRNA expression of c-myc, NF-κB, and GAP (RASA1) was downregulated.

RES-Regulated Genes and Cell Invasion

As shown in Table 2, we observed an alteration in the expression of genes related to the cell adhesion, metastasis, and cell invasion in the three breast cell lines. In particular, RES regulated expression of genes encoding to integrins such as α2, α3, αv, and β1, which are known to be involved in invasiveness of breast cancer. We observed a decrease of α2, αv, and β1 mRNA levels in MDA-MB-231 and an increase of α2, α3, and αv in MCF10a and MCF-7. In MCF10a, RES increased mRNA expression of a metastasis suppressor gene Nm23-E4 and decreased the urokinase-type plasminogen activator receptor (uPAR/PLAUR) involved in the degradation of the extracellular matrix. In contrast, in MDA-MB-231 cells, RES modified expression of several genes involved in an antimetastatic effect. Thus, it reduced the expression of PLAU, PLAUR, PAI 1, MMP1, and MMP2 genes and activated TIMP1 (MMP1 inhibitor) and BAPG1 genes.

RES-Regulated Genes and Angiogenesis

The effects of RES on mRNA gene expression implicated in angiogenesis mechanisms suggest a stimulation of angiogenesis by an increased expression of transforming growth factor β receptor 1 (TGFBR1), epidermal growth factor receptor (EGFR), and interleukin 8 (IL-8) in MCF10a and reduced mRNA levels of thrombospondins 1 and 2 (THBS1 and 2) in MDA-MB-231 cells. Conversely, in MCF-7 cells, RES seemed to have an inverse effect by suppressing gene
expression of TGFβ1 and TGFBR1 and stimulating the expression of THBS1.

**RES-Regulated Genes and the ER Pathway**

The phytoestrogenic properties of RES were investigated by studying genes involved in the ER pathway. In the three breast cell lines, mRNA levels of ERβ were increased after RES treatment. In MCF-7 cells, RES induced an increase of progesterone-receptor (PR) mRNA expression, suggesting the activation of the ER pathway. Conversely, the PR gene appeared not to be expressed in MCF10a cells (PR−) and no change in mRNA level was observed in MDA-MB-231 cells (PR−). In addition, the expression of an estrogen-related gene encoding to heat-shock protein HSP28 was inhibited in MCF-7 and MDA-MB-231 cells but not in MCF10a cells. The HSP28 protein interacts directly with ERβ and its expression is inversely correlated with estrogen-mediated transcription activity. In MCF10a, no genes presented variations in their mRNA expression after RES treatment.

**Effects of RES on Altered Expression of Breast Cancer Markers**

As shown in Table 2, using the Human Breast Cancer and Estrogen Receptor Signaling gene array, we investigated ex-

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**Table 1. Differentially Expressed Genes in Breast Cells Exposed to trans-Resveratrol From the Human Cancer Pathway Finder Gene Array**

<table>
<thead>
<tr>
<th>Gene Class</th>
<th>Accession No.</th>
<th>Genes</th>
<th>MCF-7</th>
<th>MCF10a</th>
<th>MDA-MB-231</th>
</tr>
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<tbody>
<tr>
<td>Apoptosis</td>
<td></td>
<td></td>
<td>1.79 ± 0.39*</td>
<td>2.2 ± 0.2*</td>
<td>1.46 ± 0.78</td>
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<td>L22474</td>
<td></td>
<td>Apaf-1</td>
<td>0.68 ± 0.13*</td>
<td>0.7 ± 0.17*</td>
<td>1.14 ± 0.47</td>
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<td>Z23115</td>
<td></td>
<td>Bax</td>
<td>2.17 ± 1.18*</td>
<td>2.26 ± 0.19*</td>
<td>1.15 ± 0.36</td>
</tr>
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<td>NM 001228</td>
<td></td>
<td>Caspase 8</td>
<td>1.55 ± 0.68*</td>
<td>1.68 ± 0.27*</td>
<td>1.31 ± 0.45</td>
</tr>
<tr>
<td>X63717</td>
<td></td>
<td>Fox (Cdx5)</td>
<td>2.69 ± 1.58*</td>
<td>1.37 ± 0.41</td>
<td>0.87 ± 0.24</td>
</tr>
<tr>
<td>Cell cycle control</td>
<td></td>
<td>Cyclin D1*</td>
<td>4.27 ± 3.10*</td>
<td>0.73 ± 0.28</td>
<td>0.96 ± 0.19</td>
</tr>
<tr>
<td>M64349</td>
<td></td>
<td>Cyclin E1</td>
<td>0.93 ± 0.13</td>
<td>1.07 ± 0.22</td>
<td>1.68 ± 0.64*</td>
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<tr>
<td>NM 000051</td>
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<td>ATM</td>
<td>0</td>
<td>1.62 ± 0.18*</td>
<td>1.05 ± 0.54</td>
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<td>NM8001789</td>
<td></td>
<td>CDC25a</td>
<td>0.88 ± 0.40</td>
<td>1.55 ± 0.29*</td>
<td>1.78 ± 0.70*</td>
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<tr>
<td>M14505</td>
<td></td>
<td>Cd4</td>
<td>1.76 ± 0.25*</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Transcription factors and signal transducers</td>
<td>NM 001315</td>
<td>P38 MAPK</td>
<td>1.83 ± 0.63*</td>
<td>1.34 ± 0.18*</td>
<td>0.75 ± 0.27</td>
</tr>
<tr>
<td>M11730</td>
<td></td>
<td>Erb-2</td>
<td>0.47 ± 0.05*</td>
<td>1.68 ± 0.48*</td>
<td>1.04 ± 0.36</td>
</tr>
<tr>
<td>NM 001904</td>
<td></td>
<td>β-catenin</td>
<td>0.66 ± 0.22*</td>
<td>1.67 ± 0.56*</td>
<td>1.59 ± 1.08</td>
</tr>
<tr>
<td>X00364</td>
<td></td>
<td>c-myc</td>
<td>0</td>
<td>0</td>
<td>0.68 ± 0.09*</td>
</tr>
<tr>
<td>M58603</td>
<td></td>
<td>c-κB</td>
<td>0</td>
<td>0.70 ± 0.12*</td>
<td>0.64 ± 0.22*</td>
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<tr>
<td>Cell adhesion</td>
<td></td>
<td>CDC44</td>
<td>0.89 ± 0.55</td>
<td>1.33 ± 0.48</td>
<td>0.64 ± 0.22*</td>
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<tr>
<td>M59040</td>
<td></td>
<td>0.21 ± 0.90*</td>
<td>0.12 ± 0.22</td>
<td>0.65 ± 0.25*</td>
<td></td>
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<tr>
<td>X17033</td>
<td></td>
<td>Integrin a2</td>
<td>2.28 ± 0.26*</td>
<td>0</td>
<td>0.67 ± 0.22*</td>
</tr>
<tr>
<td>M59911</td>
<td></td>
<td>Integrin a3</td>
<td>2.54 ± 1.26*</td>
<td>1.59 ± 0.42*</td>
<td>1.27 ± 0.17</td>
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<tr>
<td>L12002</td>
<td></td>
<td>Integrin a4</td>
<td>3.68 ± 2.95*</td>
<td>0</td>
<td>1.20 ± 0.10</td>
</tr>
<tr>
<td>NM 002210</td>
<td></td>
<td>Integrin α1</td>
<td>1.43 ± 0.18*</td>
<td>0</td>
<td>0.58 ± 0.11*</td>
</tr>
<tr>
<td>NM 002211</td>
<td></td>
<td>Integrin b1</td>
<td>0.96 ± 0.07</td>
<td>1.05 ± 0.21</td>
<td>1.01 ± 0.55</td>
</tr>
<tr>
<td>J02703</td>
<td></td>
<td>Integrin β5</td>
<td>0.54 ± 0.25*</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Y16801</td>
<td></td>
<td>MUC-18</td>
<td>0.78 ± 0.17</td>
<td>1.66 ± 0.55*</td>
<td>1.01 ± 0.55</td>
</tr>
<tr>
<td>Metastasis</td>
<td></td>
<td>MMP-1</td>
<td>0</td>
<td>0.61 ± 0.25*</td>
<td>0.43 ± 0.14*</td>
</tr>
<tr>
<td>J03210</td>
<td></td>
<td>MMP-2</td>
<td>0</td>
<td>0</td>
<td>0.57 ± 0.18*</td>
</tr>
<tr>
<td>NM 005009</td>
<td></td>
<td>Nm23-E4</td>
<td>0.79 ± 0.25</td>
<td>1.47 ± 0.21*</td>
<td>1.05 ± 0.04</td>
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<tr>
<td>D00244</td>
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<td>PLA2</td>
<td>0</td>
<td>0</td>
<td>0.44 ± 0.04*</td>
</tr>
<tr>
<td>NM 002659</td>
<td></td>
<td>PLAU</td>
<td>0.55 ± 0.07*</td>
<td>0</td>
<td>0.65 ± 0.22*</td>
</tr>
<tr>
<td>M16006</td>
<td></td>
<td>PAI-1</td>
<td>0</td>
<td>0</td>
<td>1.45 ± 0.36*</td>
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<tr>
<td>NM 003254</td>
<td></td>
<td>TIMP-1</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>J02685</td>
<td></td>
<td>PAI-2</td>
<td>0</td>
<td>1.09 ± 0.07</td>
<td>0</td>
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<tr>
<td>Angiogenesis</td>
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<td>EGFR</td>
<td>0</td>
<td>1.71 ± 0.45*</td>
<td>0.94 ± 0.20</td>
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<tr>
<td>X00588</td>
<td></td>
<td>IL-8</td>
<td>0</td>
<td>2.47 ± 0.61*</td>
<td>1.87 ± 0.97*</td>
</tr>
<tr>
<td>M17017</td>
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<td>TGFβ1</td>
<td>0.55 ± 0.05*</td>
<td>0.85 ± 0.22</td>
<td>0</td>
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<tr>
<td>L11695</td>
<td></td>
<td>TGFBR1</td>
<td>0.63 ± 0.28*</td>
<td>1.78 ± 0.52*</td>
<td>0.95 ± 0.32</td>
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<tr>
<td>NM 003246</td>
<td></td>
<td>THBS1</td>
<td>1.86 ± 1.15</td>
<td>1.37 ± 0.24*</td>
<td>0.54 ± 0.20*</td>
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<tr>
<td>L12350</td>
<td></td>
<td>THBS2</td>
<td>0</td>
<td>0</td>
<td>0.49 ± 0.16*</td>
</tr>
</tbody>
</table>

*a:* Abbreviations are as follows: RES, trans-resveratrol; RT-PCR, reverse transcriptase polymerase chain reaction.

*b:* Each determination corresponds to the mean value ± SD from four independent assays. Boldface signifies statistically significant. Statistical analysis was performed using the Student’s t-test (*P < 0.02).

*c:* Quantitative RT-PCR confirmed.
expression of marker genes of breast cancer, its prognosis, and response to chemotherapy. Among these genes, Erb-2, ERβ, and KLK5 are important markers to define breast cancer prognosis. In MCF-7 cells, RES induced an increase of ERβ mRNA level and a decrease of Erb-2 and KLK5. In contrast, in MCF10a and MDA-MB-231 cells, we observed an augmentation of Erb-2, ERβ, and KLK5.

Validation of Differential Expression With Quantitative Real-Time RT-PCR

To validate the GEarray results, differential expression observed was confirmed with quantitative real-time RT-PCR using specific Assay-On-Demands for selected genes. A number of selected genes (CCND1, caspase 8, Ki 67, ERβ, and PGR) were chosen for this analysis. Thus, relative gene expression was determined using the comparative Ct method, which consists of the normalization of the number of target gene copies to an endogenous reference gene (18S rRNA). Then, mRNA expression in RES treated cells was normalized to their expression levels in untreated cells (normalized to 1). Each measure was performed on two extractions and three reverse transcription analyses. In all cases, the RT-PCR data confirmed those obtained by GEarray analyses (Fig. 1). All mRNA determinations with treated cells were expressed as mean ± SD, with a Student’s t-test (P < 0.05).

### Discussion

Gene expression analysis by DNA microarray is part of our ongoing research on phytoestrogen-regulated genes in breast cancer and its response to therapy.
Figure 1. Comparison between GEarrays and quantitative real-time reverse transcriptase quantitative polymerase chain reaction (RT-QPCR) results in A: MCF10a, B: MCF-7, and C: MDA-MB 231 after 48 h of trans-resveratrol (RES) treatment. Relative gene expression of CCND1, caspase 8, Ki 67, ERβ, and PGR was determined using the comparative Ct (cycle threshold) method, which consists of the normalization of the number of target gene copies to an endogenous reference gene (18S rRNA). Then, mRNA expression in treated cells was normalized to untreated controls (corresponding to arbitrary value of 1). Each measure was performed on two extractions and three RTs, and is expressed as mean ± SD. Statistic analysis was performed using the Student’s t-test (*P < 0.05).
human breast cancer. Several reports evaluated the effects of RES on gene expression in major cancers such as breast (8,16,26), prostate (32,33), colon (34,35), and leukemias (36). Such approaches are crucial in understanding the effect of RES on different tissues and eventually in predicting their physiologic effects. Although many RES-responsive genes have been characterized, the complete set of RES-regulated genes is unknown in breast cancer models. DNA microarray technology permits the transcriptional analysis of a large number of genes simultaneously. Here, we used two different GEarray gene expression array systems to determine the effect of RES on 172 genes. The Human Cancer Pathway Finder gene array allows us to assess the status of six biological pathways that frequently are altered during carcinogenesis: apoptosis, cell cycle, cell–cell adhesion, metastasis, angiogenesis, and signal transduction molecules. The induction of marker genes suggested the activation of their associated pathways.

The Human Breast Cancer and Estrogen Receptor Signaling gene array is designed for investigate the breast cancer–related gene regulation and ER-dependent signal transduction. In our study, we used two breast cancer cell lines MCF-7 and MDA-MB-231, and fibrocystic breast cells MCF10a. The choice of these cells was based on their ER status. Thus, MDA-MB-231 cells exhibited ERβ only, MCF-7 cells presented ERα and ERβ, and MCF10a cells did not express ER (26). The RES treatment used in our study (48 h; 30 and 50 µM, respectively, to each cell line) correlates with the induction of S-phase cell cycle arrest in these three cell lines as described previously (26,27).

RES has been reported to inhibit the three major stages of carcinogenesis: initiation, promotion, and progression (8,37). In our study, we demonstrated alterations of mRNA expression of genes associated with cell cycle arrest and apoptosis. Effects of RES on cell growth seem to involve a combination of induction of gene involved in apoptosis and cell cycle control. However, induction of cell death seems to be cell type–specific, given that apoptopic gene expression was induced in MCF-7 and MCF10a cells but not in MDA-MB-231 cells. This result correlated with previous study showing that although RES decreased cell viability and proliferation in both breast cancer cell lines MCF-7 and MDA-MB-231, apoptosis was only induced in MCF-7 (16). This divergence could be due to nonfunctional p53 in MDA-MB-231 (38), suggesting a p53-dependent cell death in MCF-7 and MCF10a cells. This hypothesis is supported by our previous study, which reported an RES-induced increase of TP53 mRNA expression in these cells (26). This result was also observed at the protein level in breast tumor cell lines (39). In addition, RES seems to activate caspase-dependent apoptosis pathway by increasing expression of APAF-1, caspase 8, and Fas (CD95) in MCF10a and MCF-7 cells. This result supports previous reports, which described an activation of caspase 3 in MKL-F breast cancer cell line, which is a derivative of MCF-7 (15), and an induced activity of caspase 9 in MCF-7 cells (40). In contrast, expression of proapoptotic Bax gene was unchanged, suggesting that mito-

chondrial-mediated apoptosis pathway would not be induced by RES treatment in both cell lines. However, these observations are in contradiction with previous studies that reported that RES (12.5 to 74 µM) increased the expression of apoptotic proteins Bax and Bak in MCF-7 cells (15,40). This discrepancy could be explained by an effect of RES at post-transcriptional or translational levels.

First, RES has been described to cause an accumulation of promyelocytic cell line HL-60 in S phase (28). In our study, treatment of MCF-7 and MDA-MB-231 cells with 30 µM, and treatment of MCF10a with 50 µM RES corresponded to doses inducing an accumulation of these cells in S phase (26,27). The cell cycle normally is under the control of a number of proteins including the cyclin-dependent kinases (cdks) and their activators, the cyclins. We observed an increased expression of genes that regulate G1/S transition phases. Cyclin D1 functions as a regulatory subunit in the complexes formed with CDK4 or CDK6, the activities of which are required for cell cycle G1/S transition. In addition, DNA damages induce the phosphorylation of the Chk2 kinase by ATM. Chk2, once activated, targets the CDC25A phosphatase for ubiquitin-dependent degradation. Consequently, the CDK2/cyclin E and CDK2/cyclin A complexes remain inactive, thus preventing completion of DNA synthesis (41,42). Pozo-Guisado et al. reported that RES (10–150 µM) differentially affect the protein expression and kinase activity of G1/S (cyclin D1/CDK4 and cyclin E) and G2/M (cyclin B1/cdc2) regulators in MCF-7 and MDA-MB-231 cells. In MDA-MB-231 cells, RES induced a concentration-dependent inhibition of the expression and kinase activity of G1/S and G2/M regulators. In contrast, MCF-7 cells exhibited a transient increase in the expression and kinase activity of these proteins, with a peak at 50 µM RES (16). Conversely, a recent report described a strong decrease levels of D-type cyclins (cyclins D1 and D2) and Cdk4 in MCF-7 treated with 50 µM RES (40).

Estrogens are also known to stimulate cell cycle progression, particularly in early G1, and the induction of cellular proliferation correlates with the induction of cyclin D1 expression (43,44). Mitogenic effects of estrogens seem to be the consequence of an activation of the cell cycle progression through G1 phase, which therefore promotes the S phase entry. Interestingly, RES and estrogens act on the same genes implicated in G1/S transition. In MCF-7 cell line, 17β-estradiol (E2) increased c-myc, cyclin D1 expression, and activated cyclin D1/Cdk4 and cyclin E/Cdk2 complexes (45). In the present study, we described a stimulation of cyclin D1, Cdk4, and cyclin E1 mRNA expression. These data suggest that RES as well as E2 induces an accumulation of cell in S phase by increasing the expression of cyclin D1 and Cdk4 in MCF-7. In contrast, the effects of RES on gene expression of cell cycle in MCF10a and MDA-MB-231 remain to be elucidated.

The effects of RES on cell growth could be mediated by some transcription factors and signal transducers. p38 MAPKs act as an integration point for multiple biochemical signals, and are involved in a wide variety of cellular pro-
cesses such as proliferation, differentiation, transcription regulation, and development. The substrates of this kinase include transcription regulator ATF2, MEF2C, and MAX, cell cycle regulator CDC25B, and tumor suppressor p53, which suggest the roles of this kinase in stress-related transcription and cell cycle regulation (46). Its overexpression in MCF10a and MCF-7 could be related to effects of RES on cell cycle and apoptosis genes. Likewise, the decreased expression of c-myc, NF-κB, and RASS1, which are involved in cell cycle progression (47–49) could be in accordance with effects of RES on the cell cycle and the lack of effect on apoptosis gene expression in MDA-MB-231.

To date, no study has reported an effect of RES on genes implicated in invasion and metastasis processes. In particular, integrins have been implicated in several aspects of tumor progression, including tumor cell survival, induction and activation of matrix-degrading enzymes, migration, and anchorage at the sites of metastasis (50). Landström et al. reported an important role for the α2β1 and α3β1 integrins during the initial attachment “anchoring” process in MDA-MB-231 cells. The authors concluded that these receptors appear to be the key receptors used by cancer cells for the initial attachment to cortical bone, and this could facilitate the localization of α2β1– and α3β1–expressing cancer cells to the skeleton (51). Moreover, high levels of α3β1 integrin in mammary epithelial cell lines are associated with high levels of migration, invasive potential, and MMP-9 production (52). Likewise, It has been suggested that αvβ3 integrin is involved in the development of bone metastasis and that this integrin accelerates the formation of osteolytic lesions, given that tumor cells expressing αvβ3 adhere to bone extracellular matrix and display an increased ability to invade bone (53). Thus, RES-induced inhibition of mRNA expression of these integrins in MDA-MB-231 and their increase in MCF10a and MCF-7 suggest that RES decreases the invasiveness of MDA-MB-231. In addition, effects of RES on genes regulating metastasis and cell invasion appeared more efficient in MDA-MB-231 cells than in the other cell lines. Interestingly, these results are in agreement with the invasive capacity of MDA-MB-231 cells in contrast to noninvasive cells MCF-7 and MCF10a (54). Thus, efficacy of RES could be more important in invasive cells than in noninvasive cells.

In a previous report, RES (6–100 µM) was shown to inhibit angiogenesis in vitro by inhibiting the growth of bovine aorta endothelial cells (55). The inhibition of angiogenesis in vivo can attenuate tumor growth and metastasis (56). In this study, we showed a regulation of genes involved in tumor angiogenesis, such as THBS1, THBS2, EGF, and TGFBR1. Altered expression of these marker genes suggests that RES could stimulate angiogenesis in MDA-MB-231 and MCF10a, and inhibit angiogenesis in MCF-7 cells. This divergence can be related to the ER status of each cell line and to the expression of Erb-2. Signal transduction mediated by Erb-2 and EGFRs increases the secretion of vascular endothelial growth factor (VEGF) protein by solid tumors (57) and treatment targeted to block VEGF activity can stop tumor progression (58). VEGF plays a critical role in breast cancer progression (56,59). Thus, decreased of Erb-2 expression in MCF-7 and its increase in MCF10a could be related to the anti- and proangiogenic effects of RES, respectively. In addition, when the activation status of the ERα was affected, a number of studies showed that estradiol deprivation or the use of an antiestrogen in ERα-positive breast cancer cell lines resulted in increased expression of EGFR and the induction of cell growth (60,61).

Thus, in this study, the antiangiogenic activity of RES in MCF-7 cells could be explained by the activation of the ERα pathway, as indicated by the upregulation of PR expression. The effect of RES on the ER pathway in our cell lines could be explained by its phytoestrogenic properties and ERα/β status of cell lines. First, RES is structurally similar to the synthetic estrogen, diethylstilbestrol, which exhibits estrogenic activity. Gehm et al. reported that RES activated transcription of estrogen-responsive reporter genes transfected into human breast cancer cells (18). RES binds ERα and ERβ with comparable affinity. However, RES-liganded ERβ has higher transcriptional activity than 17α-estradiol–liganded ERβ. This indicates that the cells that uniquely express ERβ or that express higher levels of ERβ than ERα may be more sensitive to the estrogen-agonist activity of RES (19). Moreover, RES showed estrogen-agonist activity in MCF-7 cells by activating the expression of two estrogen-responsive genes, PR and pS2 (62). Previously, we also found an increase in mRNA of an estrogen-responsive gene pS2 in MCF-7 (ERα +/β+) and MDA-MB-231 (ERα–/β+) but not in MCF10a (ERα–/β–) (26). Here, we observed an increase of pS2 mRNA expression in MCF-7 (PR+), a stability in MDA-MB-231 (PR–), and a lack of expression in MCF10a (PR–). These data correlate with PR expression in cell lines: MCF-7 cells are PR+, whereas MDA-MB-231 and MCF10a are PR– (63). In addition, the expression of an estrogen-related gene encoding to heat-shock protein HSP28 was inhibited in MCF-7 and MDA-MB-231 cells but not in MCF10a cells. The HSP28 protein interacts directly with ERβ and its expression is inversely correlated with estrogen-mediated transcription activity (64). In conclusion, these results suggest an activation of ER pathway in MCF-7 (ERα +/β+) and MDA-MB-231 (ERα–/β+) but not in MCF10a (ERα–/β–).

RES was also without effect on ERβ gene expression in MDA-MB-231. This result is compatible with other studies using mammary cells treated with RES (65). The stability of ERβ gene expression may be due to an inhibition by ligand-bound estrogen receptors (66). Previously, we reported that MCF10a has been found ERβ+ by Le Corre et al. (26). Here, we observed expression of ERβ mRNAs. Possible explanations are either that mRNAs are not translated or estrogen receptors are denatured. Their increased expression in response to RES may be due to a stimulation of their expression, which is not repressed because ERs are not functional. For MCF-7, RES increased ERβ expression. The cytoplasmic location of ERβ (26) could explain this result, given that the lack of ERβ in the nucleus would not inhibit ERβ gene expression.

An additional benefit of Human Breast Cancer and Estrogen Receptor Signaling gene array is the identification of...
genes that might be associated with the prognosis of breast cancers. Many potential prognostic/predictive markers have been identified, such as Erb-2 (67,68), ERβ (69–71), and KLK5 (72). Erb-2 was described to be overexpressed in >30% of breast tumors (73). HER-2/neu overexpression is linked with increased tumor aggression, rates of recurrence, and mortality in node-positive patients (74,75). Hopp et al. reported that high levels of ERβ predicted an improved disease-free and overall survival in patients treated with adjuvant tamoxifen therapy. Thus, the authors concluded that ERβ may be an independent predictor of response to tamoxifen in breast cancer (69). Likewise, Yousef et al. studied the quantitative expression of KLK5 in breast tumors and found that higher KLK5 expression is associated with decreased disease-free and overall survival (72). In our study, RES appeared to improve prognosis in MCF-7 cells but not in MCF10a and MDA-MB-231 cells. However, Sotiriou et al. reported that overexpression of NTFYB, RPL27, and HMG1 were found in good responders to chemotherapy and such overexpression might confer an increased sensitivity to the cytotoxic effect of chemotherapy (76). In the present study, RES induced an increase of mRNA levels of these three genes in MCF10a and MDA-MB-231 cells. However, larger studies seem to be necessary to confirm these data and notably to establish the clinical value of these biomarkers. Thus, it seems that ER+ cells (MCF-7) are good responders to RES treatment, whereas ER– cells (MCF10a and MDA-MB-231) are poor responders.

In conclusion, RES acts on expression of genes involved in biological pathways frequently altered during carcinogenesis. RES regulated genes of cell cycle control, apoptosis, metastasis, cell–cell adhesion, angiogenesis, and the ER signaling pathway. These results appear in correlation with RES activities described at the cellular level, such as apoptosis, cell cycle arrest, and angiogenesis. This study also showed an implication of RES in expression of genes involved in metastasis and cell invasion processes. Additional functional experiments should be performed to ensure this observation. We also observed that effects of RES occurred in ER+ cells as well as in ER– cells, but RES appeared to be more efficient in the ER+ cells.

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