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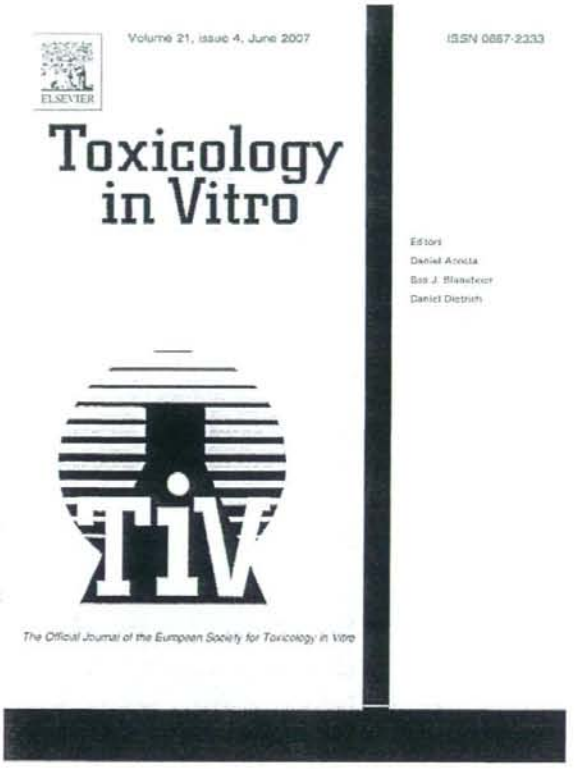

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Comparative profiling of the gene expression for estrogen responsiveness in cultured human cell lines

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Abstract

It is important to know the difference as well as the similarity in estrogen responsiveness among cell lines for understanding the effects of estrogenic chemicals. Here, using 120 estrogen responsive genes, we examined comparative expression profiles between the profile in breast cancer MCF-7 cells treated with 17β-estradiol and the profiles in other cell lines derived from breast (T-47D and HBC-4 cells), endometrium (Ishikawa cells) and kidney (RXF-631L cells) treated with estrogenic chemicals. First, comparative profiling between MCF-7 and T-47D cells showed similar (correlation coefficient or R value = 0.49–0.87) profiles for all chemicals examined: 17β-estradiol, estrone, estriol, diethylstilbestrol, bisphenol A, nonylphenol and genistein. The analysis using other cell lines indicated that significant correlations to the profile in MCF-7 cells treated with 17β-estradiol were observed for the profiles in Ishikawa cells treated with 17β-estradiol, diethylstilbestrol and bisphenol A, and HBC-4 cells treated with 17β-estradiol. The profiles for diethylstilbestrol and bisphenol A in HBC-4 cells and all three chemicals in RXF-631L cells did not show significant correlation with those in MCF-7 cells. Hierarchical cluster analysis revealed that there are cell-specific responses to estrogenic chemicals (T-47D and HBC-4 cells for example). Correlation analysis using six (proliferation, transcription, transport, enzymes, signaling and others) functionally-categorized gene groups indicated that the genes related to enzymes showed greater correlations for all chemicals tested in T-47D cells and some chemicals in Ishikawa and HBC-4 cells while those related to transcription contributed to variations.
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Keywords: Estrogen; Cultured cells; Focused microarray; Expression profile; Endocrine disruptor; Estrogenicity

1. Introduction

Estrogen is an essential hormone for the development of female organs that are responsible for metabolism and reproduction, and neuronal systems that affect the mind and behavior. Estrogen signaling is mediated mainly by estrogen receptors α and β (ERα and ERβ), which are expressed in a number of tissues, including breast, ovary, brain and bone, and regulate the transcription of their target genes

Abbreviations: E_2, 17β-estradiol; E_1, estrone; E_3, estriol; DES, diethylstilbestrol; BPA, bisphenol A; NP, nonylphenol; GEN, genistein; FBS, fetal bovine serum; DCC-FBS, FBS treated with dextran-coated charcoal; SSC, 0.15 M NaCl and 0.015 M sodium citrate; MAPK, mitogen-activated protein kinase; FMA, focused microarray analysis; SERM, selective estrogen receptor modulator.

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(Tang et al., 2004; Jin et al., 2005). Estrogen signaling is also mediated by other receptors, indicating a complex mechanism involving other signaling pathways. Therefore, the action of estrogen is mediated by a number of genes belonging to different gene networks or signaling pathways that vary among different tissues and cell lines. The ligands showing different effects (agonist or antagonist activity) in different tissues are called selective estrogen receptor modulators (SERMs), and have been considered to be effective therapeutic agents for hormone replacement therapy (Cuzick, 2000; Katzenellenbogen et al., 2000; Osborne et al., 2000; Jordan et al., 2001). However, as the number of genes related to the action of estrogen is limited, and as we now know that the pathways for different signaling molecules often share the same pathways (cross-talk), it is important to understand the involvement of the common, as well as the different, genes, and the degrees of their involvement in the action of estrogen. As different cell types represent different signaling pathways, which is at least partly due to the differences in expression levels of two ERs (ERα and ERβ) and their coregulators (Moggs and Orphanides, 2001), comparing the action of estrogen in different cell types will give us useful information as to the genes in the pathways.

The effect of estrogen can be detected at various steps in the signaling pathways (see a review by Tanji and Kiyama (2004), and the references therein) by means of various techniques. For example, competitive ligand-binding assays for the receptor/ligand binding step, reporter gene assays for the steps where the receptor/ligand complex interacts with co-regulators for transactivation or with estrogen-responsive elements at the promoter region of the target genes, enzyme linked immunosorbent assay (ELISA) or proteomic analysis for the steps where protein products of the target genes are produced and processed, and several types of bioassays such as cell growth assay, DNA microarray assay and animal life-cycle tests for the steps where the gene products are functioning as various enzymes, receptors, growth factors and transporters. Among them, expression profiling using DNA microarrays has been developed as one of the key technologies based on the information of genomes, gene annotations, gene networks and signaling cascades to monitor cellular activities. There are several trials for evaluation of physiological effects of estrogenic chemicals through gene expression profiling in human cultured cells (Wang et al., 2004b) or animals (Watanabe et al., 2004) using genome-wide microarrays. DNA microarray was also used for screening and identification of novel E_2-responsive genes (for example, see Fujimoto et al., 2004; Malyala et al., 2004). Meanwhile, we constructed a focused microarray (EstrArray) containing a total of 172 estrogen-responsive genes, which were selected from approximately 20,000 human genes through gene expression profiling of human breast cancer MCF-7 cells treated with 17β-estradiol for 72 h, using two sets of genome-wide comprehensive DNA microarrays (Inoue et al., 2002; Terasaka et al., 2004; reviewed by Inoue et al., 2006). The genes up- or down-regulated by the treatment with estrogen were selected after the treatment of the cells for 72 h instead of screening early responsive genes, based on the idea that late responsive genes are useful for profiling the effects of chemicals in human cells exposed to estrogen or estrogenic compounds. EstrArray was used for evaluation of estrogenic activity of natural and synthetic estrogens (Inoue et al., 2002; Terasaka et al., 2004), phenol derivatives (Terasaka et al., 2006), phytoestrogens (Ise et al., 2005), and other chemicals (Terasaka et al., 2004). By comparing the profiles between chemicals, we were able to characterize them according to their similarity in biological effects and functional specificity. For example, methoxychlor, a pesticide, showed a profile with a low, but distinct, correlation with that of estrogen (the correlation coefficient, or the R value, was 0.556), suggesting that it may be estrogenic within the cell (Terasaka et al., 2004). Besides pure chemicals, the profiles can be compared among crude extracts from soy beans, natural herbs and environmental samples, as well as polluted soils, giving the effects of chemicals in the mixtures and complexes.

In this report, we compared the expression profiles of the genes related to estrogen action among the human cell lines from several tissue origins to understand the biological effects at the level of gene functions and to examine the similarity as well as the difference between cell types. We found that some of the functionally categorized gene groups exhibited the similarity, while other groups showing the difference, in response to estrogen or estrogenic chemicals.

2. Materials and methods

2.1. Chemicals

Estrogen (17β-estradiol or E_2), estriol (E_3), estrone (E_1), genistein (GEN), diethylstilbestrol (DES), nonylphenol (NP), and bisphenol A (BPA) were purchased from Sigma–Aldrich (St. Louis, MO, USA). All chemicals were dissolved in ethanol.

2.2. Cell culture

Human cancer cell lines derived from breast (MCF-7, T-47D and HBC-4 cells) and kidney (RXF-631L cells) were maintained in RPMI 1640 medium supplemented with 10% (MCF-7 and T-47D cells) or 5% (other cell lines) fetal bovine serum (FBS). Human endometrial cancer Ishikawa (3H12) cells were cultured in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% FBS. For treatment of the cells with estrogen or other chemicals, each medium was replaced with phenol red-free RPMI 1640 medium or phenol red-free EMEM containing FBS treated with dextran-coated charcoal (DCC-FBS). All cells were incubated at 37 °C in humidified air containing 5% CO_2.

2.3. cDNA microarray assay

For the expression profiling of estrogen-responsive genes, human cancer cells were grown in the indicated phenol red-free medium with 5% or 10% DCC-FBS for 5 days and treated with 17β-estradiol or other estrogenic chemicals or ethanol (control) for 72 h. The final concentrations for the chemicals are as follows: 10 nM or 1 μM for E_2; 10 nM for E_1, E_3 and DES; 10 μM for BPA, NP and GEN. Then, mRNA was extracted and purified from the cells using the PolyATract System 1000 (Promega, Madison, WI, USA) according to the manufacturer's instructions.

A custom cDNA microarray (EstrArray) was manufactured by InfoGenes (Tsukuba, Japan) by mechanical spotting of cDNA (500 bp to 1.5 kb) of 172 estrogen-responsive genes, as described previously (Terasaka et al., 2004). Briefly, they were selected by DNA microarray assays from a total of approximately 20,000 genes using an Affymetrix oligonucleotide probe array and an IncyteGenomics human cDNA microarray, and consisted of 108 or 64 genes up- or down-regulated, respectively, by the treatment with estrogen. EstrArray contains a total of 203 genes consisting of 172 estrogen-responsive genes and 31 calibration/expression markers, whose cDNAs were spotted in duplicate. In this study, the 120 genes were selected from the 172 genes by selecting the genes with greater statistical stabilities based on DNA microarray assays repeated ten times for MCF-7 cells treated with 10 nM E_2 (Terasaka et al., 2006).

DNA microarray analysis was performed as follows: 2 μg of mRNA from the control and each test sample were used as templates for synthesis of cDNA probes labeled with Cy3-dUTP (test sample) or Cy5-dUTP (control) (both labeled dUTPs were purchased from Amersham Biosciences, Buckinghamshire, UK). Synthesis of cDNA was performed at 42 °C for 1.5 h using Superscript II (Invitrogen), and then, fluorescently labeled probes were denatured under alkaline conditions at 65 °C for 1 h. Free fluorescent nucleotides were removed using the CyScribe GFX Purification kit (Amersham Biosciences). Hybridization was performed at 65 °C in hybridization buffer (5 × SSC containing 0.5% SDS: 1 × SSC = 150 mM NaCl/15 mM Na citrate). The slides were washed with 2 × SSC/0.2% SDS for 5 min at room temperature, then with 0.2 × SSC/0.2% SDS for 5 min and with 0.05 × SSC at room temperature for 5 min. The slides were then scanned using a ChipReader (Virtek, Ont., Canada).

A total of three biologically independent assays were performed for the assays that needed close comparison among cell lines (MCF-7, T-47D and HBC-4 cells for the treatment with 1 μM E_2) or chemicals (E_2, DES and BPA for Ishikawa cells).

2.4. Data analysis

Image analysis was performed using IPLab (Scanalytics, Fairfax, VA, USA) according to the manufacturer's instructions, and the data was further analyzed with Microsoft Excel software. The measurements for Cy3 or Cy5 labels with the signal areas less than 50 were removed from the data processing, because the signal intensities with such small signal areas are often caused by poor hybridization and could lower the reliability of expression profiles. Removing such measurements actually improved the reliability of the data, as evaluated by correlation coefficients of $\log_2$-transformed Cy3/Cy5 ratios for all genes between two blocks on each microarray in this and the previous studies (Ise et al., 2005; Terasaka et al., 2006). For normalization of Cy3/Cy5 ratios, measurements for the internal control genes (28 genes in total) whose corresponding signal areas for both Cy3 and Cy5 were equal to or more than 100 were used. Averages of Cy3- and Cy5-signal intensities between duplicate spots were calculated for each probe. The ratio of mean signal intensities for Cy3 to Cy5 (Cy3/Cy5) was then calculated for each probe and normalized using the average of Cy3/Cy5 ratios for internal control genes, and the normalized ratios were $\log_2$-transformed. Average-linkage hierarchical clustering was applied using the Cluster program, and the results were displayed with the TreeView program (for both programs, see Eisen et al., 1998). Correlation coefficients (R-values) between gene-expression profiles and p-values for R-values were calculated using SPSS 12.0J (SPSS Japan, Tokyo, Japan).

A total of three biologically independent assays were performed for the assays that needed close comparison among cell lines (MCF-7, T-47D and HBC-4 cells for the treatment with 1 μM E_2) or chemicals (E_2, DES and BPA for Ishikawa cells) and the correlation coefficient with the least p-value was adopted.

The UniGene names of the 120 genes (see the Entrez database: www.ncbi.nlm.nih.gov) and their categories (in parentheses) are as follows: ASNS, PSAT1, PCK2, WARS, ASS, PHGDH, MTHFD2, SERPINA3, SHMT2, CTSD, GOT1, GFPT1, CDIPT, PMPCA, SFTPB, ACO2, SCD, CPT1A, SORD, FBP1, FUT8, DHCR24, ENO2, ENO3 (enzymes); NPY1R, STC2, RPS6KA3, PGR, EDN2, GDF15, ULK1, TFF1, CDKN1A, SH3BP5, PIK3C3, PVR, ILK, PRKCSH, ERBB2, RHOC, PRKCD, LGALS3BP, ESR1, ARNT2, CTNND2, PTPN18, PCSK6, ARHGDIA, AGTR1, IGFBP5, IGFBP5, IGFBP5 (signaling); AREG, PDZK1, AREG, CCNA1, MGP, IGFBP4, LAMP3, KLF10, IFRD1, ISG20, FTH1, TP53I11, TACSTD2, TSPAN1, QSCN6, CAPNS1, PMP22 (proliferation); EGR3, ATF3, ATF3, FOS, CEBPB, RUNX1, NRIP1, TCEA1, TCEA1, TAF9, GTF2I, ENO1 (transcription); SLC7A11, LCN2, SLC7A5, SLC1A4, S100P, C1orf19, XPOT, GARS, CLIC4, SLC1A5, RCN1, TCN1, U5-116KD, EIF3S9, IMP4, CDH18, EFEMP1, SLC12A2 (transport); RBBP8, SH3BGR, SH3BGR, HSPA5, TPD52L1, PMAIP1, PEG10, TRA1, LOC401397, H3F3B, DAZAP2, KIAA0196, TM4SF1, AIM1, CBX1, HSPA1A, C19orf21, SYNGR2, SELENBP1 (others); TRIB3 and YARS (uncategorized). Category names shown above and gene functions for classification were based on the Gene

Ontology terms in the Entrez Gene database (www.ncbi.nlm.nih.gov/entrez/).

3. Results

3.1. The gene expression profiles for estrogenic chemicals in T-47D cells

We previously examined the expression profiles for phenol derivatives using DNA microarrays containing 120 estrogen responsive genes. The 120 genes were selected from the original 172 genes by selecting the genes with greater statistical stabilities based on DNA microarray assays repeated ten times for estrogen (Terasaka et al., 2004). The same set of genes was applied to compare between the profiles for the response to E_2 in T-47D and MCF-7 cells (Fig. 1a). A relatively high correlation ($R=0.69$) was observed between them for the treatment of these cells with 10 nM E_2, suggesting that they share common pathways or responses by the genes analyzed here and that the same set of genes can be used for analyzing the profiles for estrogenic chemicals in T-47D cells. We then compared the profiles for different chemicals (E_1, E_3, DES, BPA, NP and GEN) with that for E_2 in T-47D cells (Fig. 1b–g). In this study, we used the concentration of BPA, NP and GEN three magnitudes higher than that of E_2 as described previously (Terasaka et al., 2004), to increase the efficiency of ERα-binding to these chemicals to the levels similar to that to E_2; the affinities of those chemicals for ERα were lower than that of E_2 by at least three magnitudes (Blair et al., 2000; Matsumura et al., 2005), and the treatment of the cells with 10 nM of these chemicals is too low for comparative profiling of estrogen signaling pathways.

Very high correlations were observed for 10 nM of E_1 ($R=0.87$), E_3 ($R=0.83$) and DES ($R=0.87$), and for 10 μM BPA ($R=0.72$). Moderate correlations were observed for 10 μM of NP ($R=0.49$) and GEN ($R=0.52$). These results were similar with those observed for the same chemicals in MCF-7 cells, where the correlations were E_1 ($R=0.85$), E_3 ($R=0.93$), DES ($R=0.69$), BPA ($R=0.65$), NP ($R=0.86$) and GEN ($R=0.91$) for the 203 genes, including 172 E_2-responsive genes (Terasaka et al., 2004). The correlations of these chemicals for the 120 genes used here were E_1 ($R=0.90$), E_3 ($R=0.95$), DES ($R=0.78$), BPA ($R=0.69$), NP ($R=0.90$) and GEN ($R=0.94$) (data not shown), confirming that the relative responses of chemicals between these cell lines are similar.

The results obtained for the chemicals in T-47D cells were re-examined for six functional gene categories based on the annotations by Gene Ontology in the Entrez Gene database (Fig. 2). The genes related to signaling and transcription showed lower R values for several chemicals than the overall R values (signaling for E_2, E_3, BPA and

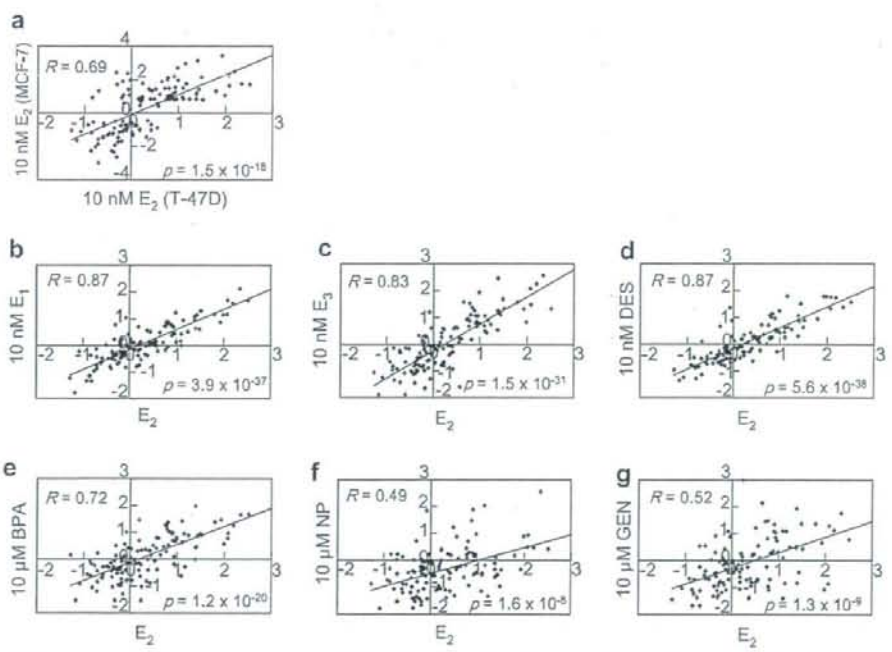

Fig. 1. The expression profiles for estrogen responsive genes after the treatment with various estrogenic chemicals in T-47D cells. The expression profile after the treatment with 10 nM E_2 was compared with that with 10 nM E_2 in MCF-7 cells (average of 10 assays, see Terasaka et al., 2006: Panel a), or with 10 nM E_1 (b), 10 nM E_3 (c), 10 nM DES (d), 10 μM BPA (e), 10 μM NP (f) or 10 μM GEN (g) in T-47D cells. Correlation coefficients (R-values) and p-values for testing the significance of the R-values are shown in each graph.

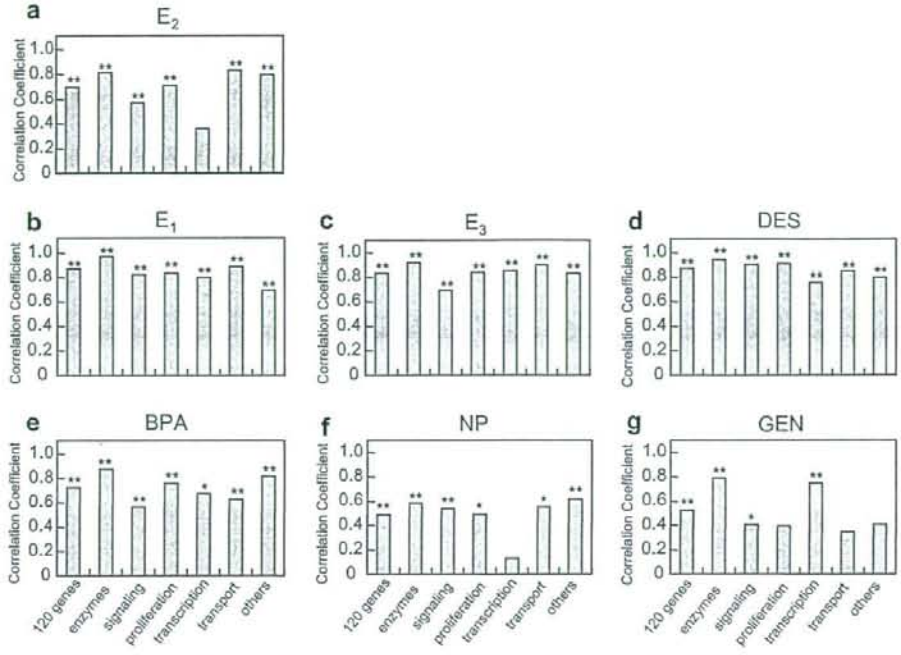

Fig. 2. Statistical evaluation of the effects of estrogenic chemicals on gene expression in T-47D cells based on the major categories of gene functions. The bars indicate coefficients of the correlation (R-values) between E_2 and each of the estrogenic chemicals analyzed in Fig. 1 for a total of 120 genes or for the genes categorized into six groups (enzymes, signaling, proliferation, transcription, transport and others) as described in Section 2. $^{*}p < 0.05$; $^{**}p < 0.01$, for testing the significance of the R-values.

GEN, and transcription for E_2, DES and NP). These genes showed high degrees of variation between the treatment of E_2 in T-47D and MCF-7 cells (Fig. 2a), suggesting that the mechanisms, pathways or activities for the genes related to signaling and transcription are highly different in the response to chemicals and in cell types compared with those for the genes related to enzymes, proliferation and transport.

3.2. The gene expression profiles for the estrogenic chemicals, E_2, DES or BPA in Ishikawa, HBC-4 and RXF-631L cells

We then examined the response to chemicals in other cell lines (Fig. 3). Ishikawa cells were derived from the endometrium, while HBC-4 and RXF-631L cells were derived from breast or kidney, respectively. As the responses of these cells to estrogenic chemicals were generally low, we used 1 μM of E_2. To compare the profiles, we also examined the treatment of MCF-7 (panel a) and T-47D (panel b) cells with 1 μM E_2, and the profiles were compared with that for the treatment of MCF-7 cells with 10 nM E_2. The profiles for the treatment of MCF-7 cells with 10 nM or 1 μM E_2 showed a very high correlation (panel a; $R = 0.87$) and those between 10 nM E_2 in MCF-7 cells and 1 μM E_2 in T-47D cells showed a moderate degree of correlation (panel b; $R = 0.51$). The same concentration was applied to Ishikawa (panel c), HBC-4 (panel d) and RXF-631L (panel e) cells. While Ishikawa and HBC-4 cells showed low ($R = 0.26$ for Ishikawa cells) to moderate ($R = 0.40$ for HBC-4 cells) degrees of correlation, RXF-631L cells did not show a positive correlation ($R = -0.04$). For the treatment of these cells with DES (panels f–h) or BPA (panels i–k), low or moderate degrees of correlation were observed in Ishikawa cells, although there was no correlation with the profiles for these chemicals in HBC-4 and RXF-631L cells. When the profiles for these chemicals in Ishikawa, HBC-4 and RXF-631L cells were analyzed by hierarchical clustering (Fig. 4), remarkably high similarity was observed for the cells with E_2 (see the positions of Ishikawa, T-47D and MCF-7 cells for the treatment with E_2); the profile for HBC-4 cells (E_2) is alternatively located between the profiles for Ishikawa and T-47D cells (E_2). The profiles for the treatment with DES or BPA were located next to each other for the cell lines examined here, except the cases for Ishikawa cells with DES and BPA, suggesting the similarity of the response to these chemicals in these cells.

When the correlation was examined for the six categorized gene groups as in Fig. 2, the genes related to proliferation and transcription showed greater variations for many treatments (Fig. 5: 1 μM E_2 in T-47D cells, panel b; 1 μM E_2 in Ishikawa cells, panel c; 1 μM E_2 in HBC-4 cells, panel d;

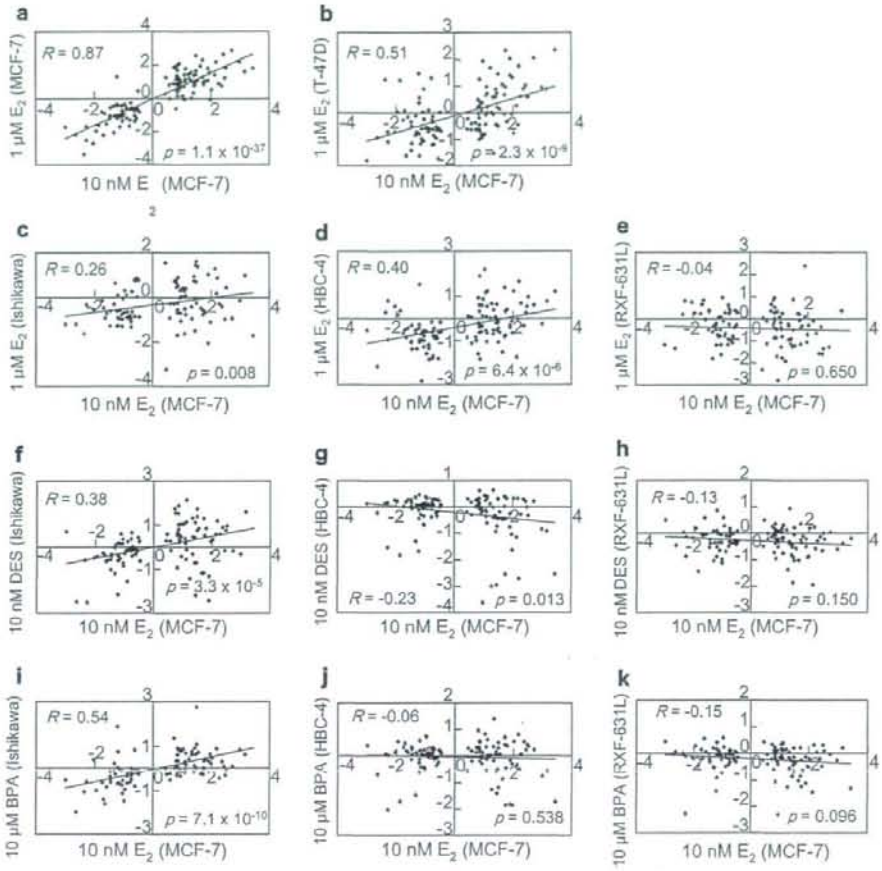

Fig. 3. The gene expression profiles after the treatment with estrogen and estrogenic chemicals in several cell lines. Gene expression profiles in MCF-7 (a), T-47D (b), Ishikawa (c), HBC-4 (d) and RXF-631L (e) cells treated with 1 μM E_2 were compared with that with 10 nM E_2 in MCF-7 cells (average of 10 assays) as in panel a of Fig. 1. DES-responsive gene-expression profiles in Ishikawa (f), HBC-4 (g) and RXF-631L (h), and BPA-responsive gene-expression patterns in Ishikawa (i), HBC-4 (j) and RXF-631L (k) were also compared with the same profile in MCF-7 cells (average of 10 assays) as in panel a of Fig. 1. Correlation coefficients (R-values) and p-values are shown in each graph.

10 nM DES in Ishikawa cells, panel e). The genes related to proliferation alone showed greater variations for the treatment with 1 μM E_2 in MCF-7 cells (panel a) and 10 μM BPA in Ishikawa cells (panel f), as compared to other gene groups and the total 120 genes.

4. Discussion

4.1. Comparative profiling of the gene expression in different cell lines

In this study, we examined the profiles of gene expression in response to estrogen in several established cell lines. Cell lines established from cancer specimens were widely used for charactering the response to chemicals at the level of genes due to technical merits. For example, the National Cancer Institute provides researchers with 60 cancer cell lines (NCI-60) derived from human tissues for expression profiling to understand their sensitivity to potential anticancer drugs (Staunton et al., 2001) in Developmental Therapeutics Program. In this program, it is explained that established cancer cell lines is good for eliminating the metabolic properties of donors. Besides the merits in handling and maintenance, a variety of cell types and molecular and cellular biological backgrounds, especially for the analysis of signaling pathways, are available. On the other hand, the prediction of outcomes by transcriptome analysis of tumors seems to be much more challenging than previously expected, as Ioannidis (2005) critically commented that the previous conclusions by many researchers about profiling and classification of tumors seem to be overoptimistic and need to be thoroughly re-examined.

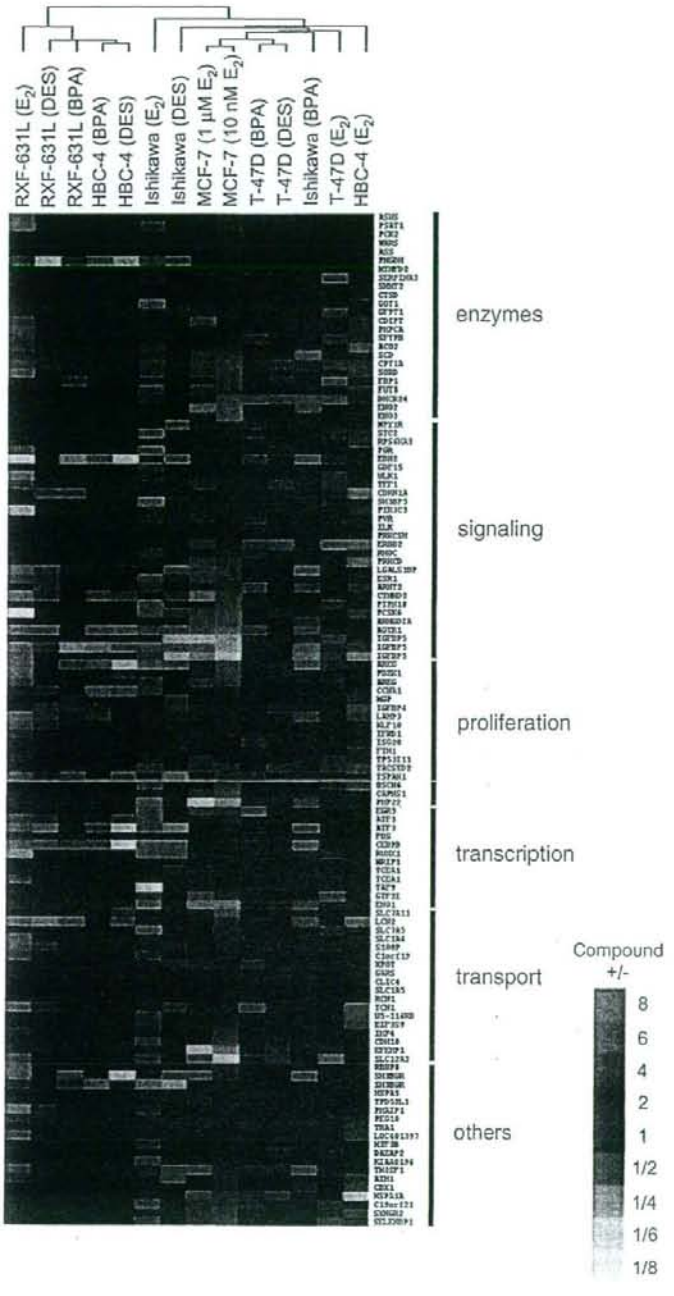

Fig. 4. Functional cluster analysis of EstrArray assays for E_2, DES or BPA in MCF-7, Ishikawa, HBC-4 and RXF-631L cells. The results of EstrArray assays shown in Fig. 3 and the results in T-47D cells treated with DES and BPA (Fig. 1) were analyzed to cluster the chemicals and gene functions. Gene expression profile in MCF-7 cells treated with 10 nM E_2 (average of 10 assays) was also included in the clustering. The gene expression profiles represent up-regulated genes (red) and down-regulated genes (green) after the treatment with E_2 or estrogenic chemicals. The color scale indicates the ratio of the signal for each chemical (Cy3 signal) to that for the vehicle (Cy5 signal). The UniGene name for each gene is shown on the right of the color matrix. Gene functions are categorized into six groups, as in Fig. 2.

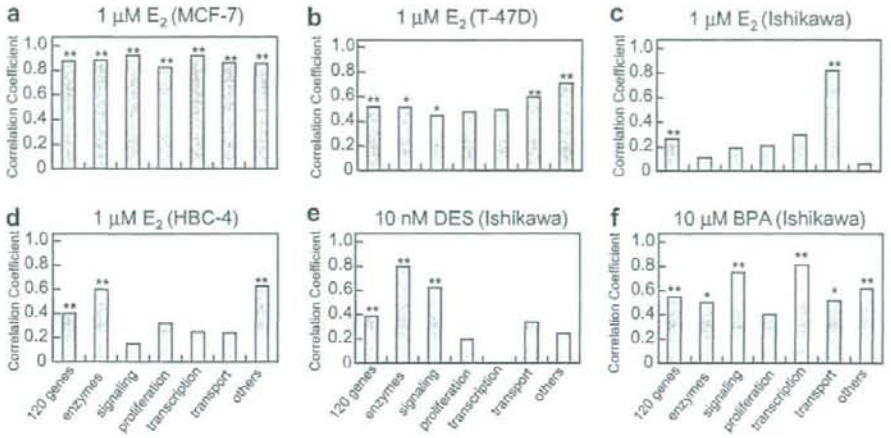

Fig. 5. Statistical evaluation of the effects of E_2, DES or BPA in MCF-7, T-47D and Ishikawa cells analyzed in Fig. 3 based on the major categories of gene functions. Correlation coefficients (R-values) were calculated between the profile in 10 nM E_2-treated MCF-7 (average of 10 assays) cells and the profiles in MCF-7 (a), T-47D (b), Ishikawa (c) or HBC-4 (d) cells treated with 1 μM E_2 or Ishikawa cells treated with 10 nM DES (e) or 10 μM BPA (f). The bars indicate the calculated correlation coefficients for a total of 120 genes, or for the genes categorized into six groups (enzymes, signaling, proliferation, transcription, transport and others) as described in Fig. 2. $^*p < 0.05$; $^{**}p < 0.01$.

Gene expression profiles have been examined for breast cancer cells in response to estrogen: MCF-7 cells (Inoue et al., 2002; Wang et al., 2004a) and ZR-75 cells (Soulez and Parker, 2001; Cicatiello et al., 2004). T-47D cells, which express ERα, were widely used for the study of estrogen responsiveness of breast cancer. Comparative analysis of gene expression profiling between MCF-7 and T-47D cells showed that subsets of human genes, those related to proliferation for example, were commonly used in the estrogen effects in these cell lines (Cunliffe et al., 2003; Lin et al., 2004). HBC-4 cells were derived from breast cancer, but were previously reported to be estrogen unresponsive (Furuya et al., 1989). Here, we detected a low but significant level of estrogen responsiveness of this cell line by DNA microarray assay (Fig. 3, panel d). However, no significant responsiveness to DES or BPA was observed (Fig. 3, panels g and j). As there is an expression of ERα in HBC-4 cells (data not shown), there seems to be a common estrogen-signaling pathway to the other breast cancer cell lines examined here, which may not respond to DES or BPA in HBC-4 cells. Comparative analysis of gene expression profiles using categorized gene sets suggested that E_2-responsiveness in HBC-4 cells similar to MCF-7 cells are quite limited to a small part of genes such as those for 'enzymes' (Fig. 5, panel d).

Ishikawa cells were derived from endometrial cancer and expected to show different responses to estrogenic chemicals. This cell line was used to identify and characterize the chemicals with the activity of SERMs (Bramlett and Burris, 2003; Mueller et al., 2003; Leong et al., 2004; Vivacqua et al., 2006), or to study the effects of estrogenic foods on health (Wober et al., 2002; Eason et al., 2005; Lehmann et al., 2005; Yoo et al., 2005). Gene expression profiling in response to chemicals in Ishikawa cells was also used for the study (Hanifi-Moghaddam et al., 2005). Our results for Ishikawa cells suggest that this cell line may respond to DES and BPA as well as E_2 through the pathways which are at least partly shared with MCF-7 cells, judged by correlation analysis using a total of 120 genes (Fig. 3, panels c, f and i). On the other hand, correlation analysis using categorized gene groups revealed prominent differences in responsiveness of the cells to these three chemicals (Fig. 5, panels c, e and f).

There were no significant correlations of the profiles for RXF-631L treated with all tested estrogenic chemicals with that for MCF-7 cells treated with E_2, suggesting that RXF-631L cells, which were derived from renal cancer, are not responsive to estrogens or have different estrogen signaling pathways from those for MCF-7 cells.

4.2. Gene functions and estrogen responsiveness

In the previous reports, we showed the 172 estrogen-responsive genes on EstrArrays were classified into several groups based on the information from literature search (Tanji and Kiyama, 2004; Ise et al., 2005). As estrogen activated or inactivated the signalings mediated by other growth factors, including EGF, TGF-β and IGF, a group of genes encoding receptors (IL1R, IL2Rβ and AT_2R), and those related to receptors, such as ligands (amphiregulin and trefoil factor), binding proteins (IGFBP4 and IGFBP5) and regulators (MGP and PACE4), showed a response. The genes responsible for signaling pathways, such as ras-superfamily genes (Rap1GAP, Rho and RhoGDI), MAPK-related genes (SH3BP5 and RSK), PI3K/Akt2-related genes (PRKCD, APPL and TRIB3) and AP-1

family genes (ATF3, FOS, JUN and FRA2) are included. The genes related to apoptosis (PRKCD and TP53I11) are negatively regulated, supporting the negative regulation of apoptosis.

In this study, we focused on 120 highly reproducible estrogen-responsive genes spotted on EstrArrays and categorized into six groups according to the annotations in the Gene Ontology database. Among the 120 genes analyzed here, many genes, if not all, were classified into several functional groups with more detailed annotations (Table 1). Among a total of 24 genes classified as enzymes, four belonged to the genes involved in lipid metabolism/biosynthesis and seven were related to amino acid metabolism/biosynthesis. Nine of the genes classified as signaling were related to signal transduction. The association of ERBB2 (Yarden and Sliwkowski, 2001) in estrogen signaling has been discussed before. Furthermore, groups of these genes are collaboratively used in the signaling initiated by small molecules, including hormones. ILK (Delcommenne et al., 1998), RPS6K (Manning, 2004) and PRKCSH (Hodgkinson et al., 2005) play major roles in the insulin signaling. The activation of mTOR by small molecules, such as amino acids, is mediated by class 3 (PIK3C3), but not through class 1 of PI3K (Nobukuni et al., 2005). PRKCD belongs to a family of serine- and threonine-specific protein kinases that can be activated by calcium and the second messenger diacylglycerol, and it plays a crucial role in the proliferation and anti-estrogen resistance of estrogen-dependent breast cancer cells (De Servi et al., 2005; Nabha et al., 2005).

A significant number of genes belonged to the genes with growth factor/hormone/cytokine activity, growth factor/hormone/cytokine regulation, receptor/adaptor activity, or GTPase or related activity. TFF1 is one of the well-known target genes in transactivation by the estrogen receptor, but its role in estrogen signaling was not well-characterized (reviewed by Rio and Chambon, 1990). Some of them are related to the mechanisms of autocrine (EDN2; Grimshaw et al., 2002) or paracrine (PGR; Peyrat et al., 1988) effects of estrogen, including the mechanism mediated by insulin-like growth factor and epidermal growth factor (IGFBP4, IGFBP5, ERBB2 and AREG; Forsyth, 1996; Gee et al., 2005). The regulation of Ras GTP/GDP-exchange factors by RPS6K may be involved in the MAPK family-mediated

Table 1
List of genes categorized by functions

Lipid metabolism/biosynthesis
SERPINA3: a serine/cysteine proteinase inhibitor; SCD: stearoyl-CoA desaturase; SFTPB: pulmonary-associated protein B; CPT1A: carnitine palmitoyltransferase 1A

Amino acid metabolism/biosynthesis
ASNS: asparagine synthetase; PHGDH: phosphoglycerate dehydrogenase; PSAT1: phosphoserine aminotransferase; GOT1: glutamic-oxaloacetic transaminase; GFPT1: glutamine-fructose-6-phosphate transaminase; ASS: argininosuccinate synthetase; SHMT2: serine hydroxymethyltransferase

Signal transduction
PIK3C3: phosphoinositide-3-kinase, class 3; ULK1: unc-51-like kinase 1; ILK: integrin-linked kinase; RPS6KA3: ribosomal protein S6 kinase; CDKN1A: cyclin-dependent kinase inhibitor 1A; PRKCSH: protein kinase C substrate 80K-H; PRKCD: protein kinase C, delta; ERBB2: v-erb-b2; TRIB3: tribbles homolog 3

Growth factor/hormone/cytokine activity
TFF1: trefoil factor 1; EDN2: endothelin 2; STC2: stanniocalcin 2; GDF15: growth differentiation factor 15; AGTR1: angiotensin II receptor, type 1; PGR: progesterone receptor; PVR: poliovirus receptor; AREG: amphiregulin

Growth factor/hormone/cytokine regulation
ARNT2: aryl-hydrocarbon receptor nuclear translocator 2; IGFBP5: insulin-like growth factor binding protein 5; YARS: tyrosyl-tRNA synthtase; IGFBP4: insulin-like growth factor binding protein 4

Receptor/adaptor activity
NPY1R: neuropeptide Y receptor Y1; SH3BP5: SH3-domain binding protein 5; ERBB2: v-erb-b2; ESR1: estrogen receptor 1; LGALS3BP: lectin, galactoside-binding, soluble, 3 binding protein; SLC1A5: solute carrier family 1; SH3BGR: SH3 domain binding glutamic acid-rich protein

GTPase activity or related
RHOC: ras homolog gene family, member C; ARHGDIA: Rho GDP dissociation inhibitor alpha; U5-116KD: U5 snRNP-specific protein, 116 kD

Cell proliferation
PDZK1: PDZ domain containing 1; AREG: amphiregulin; LAMP3: lysosomal-associated membrane protein 3; ISG20: an interferon stimulated gene; IGFBP4: insulin-like growth factor binding protein 4; KLF10: Kruppel-like factor 10; FTH1: ferritin, heavy polypeptide 1; TACSTD2: tumor-associated calcium signal transducer 2; TP53I11: tumor protein p53 inducible protein 11; CAPNS1: a small subunit of calpain; TSPAN1: tetraspanin 1; PMP22: peripheral myelin protein 22; WARS: tryptophanyl-tRNA synthetase; QSCN6: quiescin Q6; FTH1: ferritin, heavy polypeptide 1

Transcription factor activity
EGR3: early growth response 3; FOS: v-fos homolog; ATF3: activating transcription factor 3; RUNX1: runt-related transcription factor 1; CEBPB: CCAAT/enhancer binding protein beta; ATF3: activating transcription factor 3; NRIP1: nuclear receptor interacting protein 1; TCEA1: transcription elongation factor A; TAF9: a TATA box binding protein-associated factor; ENO1: enolase 1; GTF2I: general transcription factor II; ARNT2: aryl-hydrocarbon receptor nuclear translocator 2; PGR: progesterone receptor; KLF10: Kruppel-like factor 10

Amino acid transporters
SLC7A11: solute carrier family 7; SLC1A4: solute carrier family 1; SLC7A5: solute carrier family 7; SLC1A5: solute carrier family 1; SLC12A2: solute carrier family 12

Ion transporters
TCN1: transcobalamin I; CLIC4: chloride intracellular channel 4; CPT1A: carnitine palmitoyltransferase 1A; FTH1: ferritin, heavy polypeptide 1

The genes in each category are listed by UniGene and common names.

estrogen signaling pathways, including FOS and JNK (Frodin and Gammeltoft, 1999). Transcription factors such as FOS and ATF3 are included in the 120 genes analyzed here.

A total of 15 genes belonged to cell proliferation and a total of 14 genes belonged to the genes with transcription factor activity. Among these, several genes are known to be associated with the action of estrogen or estrogen-related cellular functions. NRIP1 (or RIP140) is a nuclear receptor coregulator repressing the action of estrogen, and has a regulatory role in mediating anti-estrogenic effects of retinoic acid in estrogen-dependent breast cancer cells (White et al., 2005). Differential gene expression of KLF10 (or TIEG), a tumor suppressor gene, plays a significant role in the proliferation of breast cancer (Reinholz et al., 2004). EGR3 is a target for the estrogen receptor α and enhances the transcription of the Fas ligand promoter (Inoue et al., 2004). Additionally, RBBP8 forms a complex with BRCA1 and an adaptor protein, LMO4, and modulates the transcription of BRCA1 in the breast tissue (Sum et al., 2002).

Among the genes related to transporters, five belonged to amino acid transporters (SLC7A11, SLC1A4, SLC7A5, SLC1A5 and SLC12A2) and four belonged to ion transporters (TCN1, CLIC4, CPT1A and FTH1).

Categorizing the genes shown here may be useful for interpretation of the difference as well as the similarity in gene expression profiles of the cell lines described above. Categorizing the genes has an advantage of giving statistical reliability to the data analysis because fluctuation of data can be reduced by the data in the same group and the result by a single microarray assay was shown to match well with those by Northern hybridization (Inoue et al., 2002) and real-time RT-PCR (Terasaka et al., 2004; Ise et al., 2005). To increase statistical stability, we selected a total of 120 genes after correlation analysis of 10-times repeated assays (Terasaka et al., 2006). Using six gene groups having greater gene numbers and thus greater statistical powers than those in Table 1, we found gene groups representing similarity in estrogen responsiveness across T-47D cells treated with several compounds and several cell lines treated with E_2 (the group of 'enzymes' for example), and the gene groups reflecting variations between estrogenic chemicals or cell types (the group of 'transcription' for example). These results suggest the possibility that pathways related to lipid and amino acid metabolisms, half of which were categorized as enzymes, may have similar estrogen-responsiveness across different cell types and the diversity in cellular response to estrogen may be mainly due to the variations in estrogen responsiveness among the genes related to transcription.

The number of genes in each category will be increased in future as the progress of gene annotation and the improvement in the databases and search tools continues. The study of modulation in estrogen signaling mediated by different chemicals and by the different subsets of gene products in various cell lines and tissues, as examined here, will also contribute to gene annotation for a number of genes with still unknown or little-known functions.

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References

Blair, R.M., Fang, H., Branham, W.S., Hass, B.S., Dial, S.L., Moland, C.L., Tong, W., Shi, L., Perkins, R., Sheehan, D.M., 2000. The estrogen receptor relative binding affinities of 188 natural and xenochemicals: structural diversity of ligands. Toxicological Sciences 54, 138–153.

Bramlett, K.S., Burris, T.P., 2003. Target specificity of selective estrogen receptor modulators within human endometrial cancer cells. The Journal of Steroid Biochemistry and Molecular Biology 86, 27–34.

Cicatiello, L., Scafoglio, C., Altucci, L., Cancemi, M., Natoli, G., Facchiano, A., Iazzetti, G., Calogero, R., Biglia, N., De Bortoli, M., Sfiligoi, C., Sismondi, P., Bresciani, F., Weisz, A., 2004. A genomic view of estrogen actions in human breast cancer cells by expression profiling of the hormone-responsive transcriptome. Journal of Molecular Endocrinology 32, 719–775.

Cunliffe, H.E., Ringner, M., Bilke, S., Walker, R.L., Cheung, J.M., Chen, Y., Meltzer, P.S., 2003. The gene expression response of breast cancer to growth regulators: patterns and correlation with tumor expression profiles. Cancer Research 63, 7158–7166.

Cuzick, J., 2000. Future possibilities in the prevention of breast cancer: breast cancer prevention trials. Breast Cancer Research 2, 258–263.

Delcommenne, M., Tan, C., Gray, V., Rue, L., Woodgett, J., Dedhar, S., 1998. Phosphoinositide-3-OH kinase-dependent regulation of glycogen synthase kinase 3 and protein kinase B/AKT by the integrin-linked kinase. Proceedings of the National Academy of Sciences of the United States of America 95, 11211–11216.

De Servi, B., Hermani, A., Medunjanin, S., Mayer, D., 2005. Impact of PKCdelta on estrogen receptor localization and activity in breast cancer cells. Oncogene 24, 4946–4955.

Eason, R.R., Till, S.R., Velarde, M.C., Geng, Y., Chatman, L., Gu Jr., L., Badger, T.M., Simmen, F.A., Simmen, R.C., 2005. Uterine phenotype of young adult rats exposed to dietary soy or genistein during development. The Journal of Nutritional Biochemistry 16, 625–632.

Eisen, M.B., Spellman, P.T., Brown, P.O., Botstein, D., 1998. Cluster analysis and display of genome-wide expression patterns. Proceedings of the National Academy of Sciences of the United States of America 95, 14863–14868.

Forsyth, I.A., 1996. The insulin-like growth factor and epidermal growth factor families in mammary cell growth in ruminants: action and interaction with hormones. Journal of Dairy Science 79, 1085–1096.

Frodin, M., Gammeltoft, S., 1999. Role and regulation of 90 kDa ribosomal S6 kinase (RSK) in signal transduction. Molecular and Cellular Endocrinology 151, 65–77.

Fujimoto, N., Igarashi, K., Kanno, J., Honda, H., Inoue, T., 2004. Identification of estrogen-responsive genes in the GH3 cell line by cDNA microarray analysis. The Journal of Steroid Biochemistry and Molecular Biology 91, 121–129.

Furuya, Y., Kohno, N., Fujiwara, Y., Saitoh, Y., 1989. Mechanisms of estrogen action on the proliferation of MCF-7 human breast cancer cells in an improved culture medium. Cancer Research 49, 6670–6674.

Gee, J.M., Robertson, J.F., Gutteridge, E., Ellis, I.O., Pinder, S.E., Rubini, M., Nicholson, R.I., 2005. Epidermal growth factor receptor/HER2/insulin-like growth factor receptor signalling and oestrogen receptor activity in clinical breast cancer. Endocrine-Related Cancer 12 (Suppl. 1), S99–S111.

Grimshaw, M.J., Naylor, S., Balkwill, F.R., 2002. Endothelin-2 is a hypoxia-induced autocrine survival factor for breast tumor cells. Molecular Cancer Therapeutics 1, 1273–1281.

Hanifi-Moghaddam, P., Gielen, S.C., Kloosterboer, H.J., De Gooyer, M.E., Sijbers, A.M., van Gool, A.J., Smid, M., Moorhouse, M., van Wijk, F.H., Burger, C.W., Blok, C.W.L.J., 2005. Molecular portrait of the progestagenic and estrogenic actions of tibolone: behavior of cellular networks in response to tibolone. The Journal of Clinical Endocrinology and Metabolism 90, 973–983.

Hodgkinson, C.P., Mander, A., Sale, G.J., 2005. Identification of 80K-H as a protein involved in GLUT4 vesicle trafficking. The Biochemical Journal 388, 785–793.

Inoue, A., Yoshida, N., Omoto, Y., Oguchi, S., Yamori, T., Kiyama, R., Hayashi, S., 2002. Development of cDNA microarray for expression profiling of estrogen-responsive genes. Journal of Molecular Endocrinology 29, 175–192.

Inoue, A., Omoto, Y., Yamaguchi, Y., Kiyama, R., Hayashi, S.-I., 2004. Transcription factor EGR3 is involved in the estrogen-signaling pathway in breast cancer cells. Journal of Molecular Endocrinology 32, 649–661.

Inoue, A., Tanji, M., Kiyama, R., 2006. Focused microarray analysis: characterization of phenomes by gene expression profiling. Current Pharmacogenomics 4, 245–260.

Ioannidis, J.P.A., 2005. Microarrays and molecular research: noise discovery? Lancet 365, 454–455.

Ise, R., Han, D., Takahashi, Y., Terasaka, S., Inoue, A., Tanji, M., Kiyama, R., 2005. Expression profiling of the estrogen responsive genes in response to phytoestrogens using a customized DNA microarray. FEBS Letters 579, 1732–1740.

Jin, V.X., Sun, H., Pohar, T.T., Liyanarachchi, S., Palaniswarny, S.K., Huang, T.H.M., Davuluri, R.V., 2005. ERTargetDB: an integral information resource of transcription regulation of estrogen receptor target genes. Journal of Molecular Endocrinology 35, 225–230.

Jordan, V.C., Gapstur, S., Morrow, M., 2001. Selective estrogen receptor modulation and reduction in risk of breast cancer, osteoporosis, and coronary heart disease. Journal of the National Cancer Institute 93, 1449–1457.

Katzenellenbogen, B.S., Choi, I., Delage-Mourroux, R., Ediger, T.R., Martini, P.G., Montano, M., Sun, J., Weis, K., Katzenellenbogen, J.A., 2000. Molecular mechanisms of estrogen action: selective ligands and receptor pharmacology. The Journal of Steroid Biochemistry and Molecular Biology 74, 279–285.

Lehmann, L., Esch, H.L., Wagner, J., Rohnstock, L., Metzler, M., 2005. Estrogenic and genotoxic potential of equol and two hydroxylated metabolites of Daidzein in cultured human Ishikawa cells. Toxicology Letters 158, 72–86.

Leong, H., Riby, J.E., Firestone, G.L., Bjeldanes, L.F., 2004. Potent ligand-independent estrogen receptor activation by 3,3'-diindolylmethane is mediated by cross talk between the protein kinase A and mitogen-activated protein kinase signaling pathways. Molecular Endocrinology (Baltimore, Md.) 18, 291–302.

Lin, C.Y., Strom, A., Vega, V.B., Kong, S.L., Yeo, A.L., Thomsen, J.S., Chan, W.C., Doray, B., Bangarusamy, D.K., Ramasamy, A., Vergara, L.A., Tang, S., Chong, A., Bajic, V.B., Miller, L.D., Gustafsson, J.A., Liu, E.T., 2004. Discovery of estrogen receptor alpha target genes and response elements in breast tumor cells. Genome Biology 5, R66.

Malyala, A., Pattee, P., Nagalla, S.R., Kelly, M.J., Ronnekleiv, O.K., 2004. Suppression subtractive hybridization and microarray identification of estrogen-regulated hypothalamic genes. Neurochemical Research 29, 1189–1200.

Manning, B.D., 2004. Balancing Akt with S6K: implications for both metabolic diseases and tumorigenesis. The Journal of Cell Biology 167, 399–403.

Matsumura, A., Ghosh, A., Pope, G.S., Darbre, P.D., 2005. Comparative study of oestrogenic properties of eight phytoestrogens in MCF7 human breast cancer cells. The Journal of Steroid Biochemistry and Molecular Biology 94, 431–443.

Moggs, J.G., Orphanides, G., 2001. Estrogen receptors: orchestrators of pleiotropic cellular responses. EMBO Reports 2, 775–781.

Mueller, S.O., Hall, J.M., Swope, D.L., Pedersen, L.C., Korach, K.S., 2003. Molecular determinants of the stereoselectivity of agonist activity of estrogen receptors (ER) alpha and beta. The Journal of Biological Chemistry 278, 12255–12262.

Nabha, S.M., Glaros, S., Hong, M., Lykkesfeldt, A.E., Schiff, R., Osborne, K., Reddy, K.B., 2005. Upregulation of PKC-delta contributes to antiestrogen resistance in mammary tumor cells. Oncogene 24, 3166–3176.

Nobukuni, T., Joaquin, M., Roccio, M., Dann, S.G., Kim, S.Y., Gulati, P., Byfield, M.P., Backer, J.M., Natt, F., Bos, J.L., Zwartkruis, F.J., Thomas, G., 2005. Amino acids mediate mTOR/raptor signaling through activation of class 3 phosphatidylinositol 3OH-kinase. Proceedings of the National Academy of Sciences of the United States of America 102, 14238–14243.

Osborne, C.K., Zhao, H., Fuqua, S.A., 2000. Selective estrogen receptor modulators: structure, function, and clinical use. Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology 18, 3172–3186.

Peyrat, J.P., Bonneterre, J., Beuscart, R., Djiane, J., Demaille, A., 1988. Insulin-like growth factor 1 receptors in human breast cancer and their relation to estradiol and progesterone receptors. Cancer Research 48, 6429–6433.

Reinholz, M.M., An, M.W., Johnsen, S.A., Subramaniam, M., Suman, V.J., Ingle, J.N., Roche, P.C., Spelsberg, T.C., 2004. Differential gene expression of TGF beta inducible early gene (TIEG), Smad7, Smad2 and Bard1 in normal and malignant breast tissue. Breast Cancer Research and Treatment 86, 75–88.

Rio, M.C., Chambon, P., 1990. The pS2 gene, mRNA, and protein: a potential marker for human breast cancer. Cancer Cells (Cold Spring Harbor, N.Y.: 1989) 2, 269–274.

Soulez, M., Parker, M.G., 2001. Identification of novel oestrogen receptor target genes in human ZR75-1 breast cancer cells by expression profiling. Journal of Molecular Endocrinology 27, 259–274.

Staunton, J.E., Slonim, D.K., Coller, H.A., Tamayo, P., Angelo, M.J., Park, J., Scherf, U., Lee, J.K., Reinhold, W.O., Weinstein, J.N., Mesirov, J.P., Lander, E.S., Golub, T.R., 2001. Chemosensitivity prediction by transcriptional profiling. Proceedings of the National Academy of Sciences of the United States of America 98, 10787–10792.

Sum, E.Y., Peng, B., Yu, X., Chen, J., Byrne, J., Lindeman, G.J., Visvader, J.E., 2002. The LIM domain protein LMO4 interacts with the cofactor CtIP and the tumor suppressor BRCA1 and inhibits BRCA1 activity. The Journal of Biological Chemistry 277, 7849–7856.

Tang, S.H., Han, H., Bajic, V.B., 2004. ERGDB: estrogen responsive genes database. Nucleic Acids Research 32, D533–D536.

Tanji, M., Kiyama, R., 2004. Expression profiling of estrogen responsive genes using genomic and proteomic techniques for the evaluation of endocrine disruptors. Current Pharmacogenomics 2, 255–266.

Terasaka, S., Aita, Y., Inoue, A., Hayashi, S., Nishigaki, M., Aoyagi, K., Sasaki, H., Wada-Kiyama, Y., Sakuma, Y., Akaba, S., Tanaka, J., Sone, H., Yonemoto, J., Tanji, M., Kiyama, R., 2004. Using a customized DNA microarray for expression profiling of the estrogen-responsive genes to evaluate estrogen activity among natural estrogens and industrial chemicals. Environmental Health Perspectives 112, 773–781.

Terasaka, S., Inoue, A., Tanji, M., Kiyama, R., 2006. Expression profiling of estrogen-responsive genes in breast cancer cells treated with alkylphenols, chlorinated phenols, parabens, or bis- and benzoylphenols for evaluation of estrogenic activity. Toxicology Letters 163, 130–141.

Vivacqua, A., Bonofiglio, D., Recchia, A.G., Musti, A.M., Picard, D., Ando, S., Maggiolini, M., 2006. The G protein-coupled receptor GPR30 mediates the proliferative effects induced by 17-estradiol and hydroxytamoxifen in endometrial cancer cells. Molecular Endocrinology (Baltimore, Md.) 20, 631–646.

Wang, D.Y., Fulthorpe, R., Liss, S.N., Edwards, E.A., 2004a. Identification of estrogen-responsive genes by complementary deoxyribonucleic acid microarray and characterization of a novel early estrogen-induced gene: EEIG1. Molecular Endocrinology (Baltimore, Md.) 18, 402–411.

Wang, D.Y., McKague, B., Liss, S.N., Edwards, E.A., 2004b. Gene expression profiles for detecting and distinguishing potential endocrine-disrupting compounds in environmental samples. Environmental Science & Technology 38, 6396–6406.

Watanabe, H., Suzuki, A., Goto, M., Lubahn, D.B., Handa, H., Iguchi, T., 2004. Tissue-specific estrogenic and non-estrogenic effects of a xenoestrogen, nonylphenol. Journal of Molecular Endocrinology 33, 243–252.

White, K.A., Yore, M.M., Deng, D., Spinella, M.J., 2005. Limiting effects of RIP140 in estrogen signaling: potential mediation of anti-estrogenic effects of retinoic acid. The Journal of Biological Chemistry 280, 7829–7835.

Wober, J., Weisswange, I., Vollmer, G., 2002. Stimulation of alkaline phosphatase activity in Ishikawa cells induced by various phytoestrogens and synthetic estrogens. The Journal of Steroid Biochemistry and Molecular Biology 83, 227–233.

Yarden, Y., Sliwkowski, M.X., 2001. Untangling the ErbB signalling network. Nature Reviews. Molecular Cell Biology 2, 127–137.

Yoo, H.H., Kim, T., Ahn, S., Kim, Y.J., Kim, H.Y., Piao, X.L., Park, J.H., 2005. Evaluation of the estrogenic activity of Leguminosae plants. Biological & Pharmaceutical Bulletin 28, 538–540.

Aromatase Localization in Human Breast Cancer Tissues: Possible Interactions between Intratumoral Stromal and Parenchymal Cells

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Abstract

Aromatase is a key enzyme in intratumoral estrogen production required for the production of estrogens through the conversion of serum androgens in postmenopausal breast cancer patients. There have been, however, controversies regarding the intratumoral localization of aromatase in human breast carcinoma tissues. Therefore, we have first examined the intratumoral localization of aromatase mRNA/protein in 19 breast carcinomas using laser capture microdissection/quantitative reverse transcription-PCR (RT-PCR) and immunohistochemistry. Aromatase mRNA and protein were detected in both intratumoral stromal and parenchymal cells in breast carcinoma tissues. Subsequent microarray expression profiling and clustering analyses, in addition to quantitative RT-PCR studies, showed a significant positive correlation between aromatase and estrogen-related receptor α mRNA expression in isolated carcinoma cells. We further examined an interaction between stromal cells isolated from human breast carcinoma tissues and breast carcinoma cell lines using a coculture system to study the biological characteristic of aromatase expression in carcinoma cells. Aromatase mRNA and enzyme activity and 17β-hydroxysteroid dehydrogenase type 1 mRNA in breast carcinoma cell lines, including MCF-7 and SK-BR-3 cells, were up-regulated in the presence of patient-derived 32N or 74T intratumoral stromal cells. The results from steroid conversion assays were also consistent with the findings above. The results of our study also showed that aromatase inhibitors were more effective in inhibiting aromatization induced by coculture in MCF-7 than that in stromal 32N. The examination of the localization of aromatase and its regulation, including the interactions existing between different cell types in human breast carcinoma tissues, may provide important information as to achieving better clinical response to aromatase inhibitors in breast cancer patients. [Cancer Res 2007;67(8):3945–54]

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Introduction

Estrogens play important roles in the growth and invasion of estrogen-dependent human breast carcinomas. While postmenopausal women have low levels of circulating plasma estrogens, the local synthesis or intratumoral production of estrogens that takes place in breast carcinoma tissue itself can lead to higher estrogen levels in the tumor (1, 2). Intratumoral production of estrogens occurs as a result of aromatization of C19 steroids such as androstenedione and testosterone into estrogens, and this is catalyzed by the cytochrome P450 aromatase enzyme (3–5). Previously, the localization of aromatase has been mostly examined using immunohistochemistry with the reported results demonstrating the presence of aromatase protein predominantly in tumoral stromal cells and adipocytes of breast carcinoma tissues (6, 7). However, there have been controversies regarding the cellular localization of intratumoral aromatase with other studies demonstrating aromatase immunoreactivity in carcinoma or parenchymal as well as stromal cells of human breast carcinoma tissues (8, 9).

Aromatase expression is well known to be regulated by various transcriptional factors, including nuclear receptors and their putative ligands in several types of human cells and tissues (10, 11). Both interleukins, such as interleukin (IL)-1, IL-6 and IL-11 released from carcinoma, and/or inflammatory cells have been shown to potently induce aromatase expression in adipose fibroblast cells (12). However, the correlation between nuclear receptors and aromatase in parenchymal or carcinoma cells of breast carcinoma tissues has remained largely unknown. In addition, there have been no studies reported examining whether the factors released from human intratumoral stromal cells affect aromatase expression of breast carcinoma or parenchymal cells.

Intratumoral aromatase has been considered a viable clinical target for the treatment of estrogen receptor–positive postmenopausal breast cancer patients (13). However, routine evaluation methods for the detection of intratumoral aromatase expression in clinical specimens have not been established. Therefore, in this study, we have first examined the localization of aromatase mRNA in 19 breast carcinoma tissues using laser capture microdissection (LCM), together with quantitative reverse transcription-PCR (RT-PCR), and then examined their correlation with clinicopathologic parameters of the patients. The expression and localization of the aromatase protein were also confirmed by immunohistochemistry using the aromatase monoclonal antibody termed 677 (14, 15). Microarray expression profiling and clustering analyses were also done on both isolated carcinoma and stromal cells obtained from the 19 breast carcinoma cases to identify possible

aromatase-regulating nuclear receptors in human breast carcinoma cells. We then examined the possible effects of isolated stromal cells from breast carcinoma tissues on both aromatase enzymatic activity and mRNA transcripts in breast carcinoma cell lines. Cocultured intratumoral stromal 32N or 74T cells, established from breast carcinoma tissues by primary culture, were used to evaluate the potential effects of carcinoma/stromal cell interactions on aromatase expression and enzyme activity in the carcinoma cells. Effects of coculture of MCF-7 cells with stromal 32N cells on MCF-7 cell proliferation and the inhibitory effects of the aromatase inhibitors on cell proliferation were subsequently investigated to further characterize the biological features of aromatase function in carcinoma or parenchymal cells.

Materials and Methods

Patients and tissue preparation. A total of 42 specimens of invasive ductal carcinoma of the breast were obtained from Japanese female patients from 2002 to 2005 at the Department of Surgery, Tohoku University Hospital and Tohoku Kosai Hospital (Sendai, Japan). The number of subjects examined in each experiment were as follows: 19 cases [54.2 years (range, 37–86; SD, 12.9)] for LCM/quantitative RT-PCR (qPCR) to investigate aromatase localization, 23 cases [55.0 years (range, 36–74; SD, 10.7)] for LCM/microarray studies, and 11 cases [53.1 years (range, 36–77; SD, 10.1)] for LCM/qPCR to validate results of microarray analysis. Nonpathologic breast and adipose tissues adjacent to the carcinoma were also available for examination in 12 out of 23 cases used in LCM/microarray analysis. Relevant clinical data were retrieved from the review of the patient's files. The histologic grade of each specimen was independently evaluated by three of the authors (T. Suzuki, T. Moriya, and H. Sasano), based on the modified methods of Bloom and Richardson (16), according to Elston and Ellis (17). The ethics committees at Tohoku University School of Medicine and Tohoku Kosai Hospital approved the research protocols (2004-144, 2005-068, and 2006-042, respectively), with informed consent being obtained from these patients before surgery in each institution.

Immunohistochemistry. For immunohistochemistry of aromatase, sequential frozen tissues, also used in the LCM analyses, were taken to examine the correlation between mRNA and protein in individual cellular compartments of the breast cancer tissues.

The aromatase monoclonal antibody 677 was raised against native recombinantly expressed human aromatase protein, with details of its characterization and utilization for immunohistochemistry being previously reported by the authors (14, 15). Tissue sections were immunostained by a biotin-streptavidin method with Histofine kit (Nichirei Co. Ltd., Tokyo, Japan). Breast carcinomas tissues were rapidly embedded in Tissue-Tek optimal cutting temperature compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan) and frozen materials sectioned at a thickness of 3 μm. The antigen-antibody complex was then visualized with 3,3′-diaminobenzidine solution and counterstained with hematoxylin. Evaluation of aromatase immunohistochemistry was done as previously reported using 10% formalin-fixed and paraffin-embedded tissue specimens (14). The approximate percentage of cells staining (proportion score) were classified into the following four groups: 0, <1%; 1, −25%; 2, −50%; and 3, >50% immunopositive cells. Relative intensity of aromatase immunopositive cells was classified as follows: 0, no immunoreactivity; 1, weak; 2, moderate; and 3, intense immunoreactivity. Aromatase immunoreactivity was evaluated as a total score composed of the proportion score + relative immunointensity score.

Other antibodies used in this study for characterizing clinicopathologic parameters of the cases are as follows: monoclonal antibodies: ERα (ER1D5; Immunotech S.A., Marseilles, France), progesterone receptor (MAB429; Chemicon International Inc., Temecula, CA), and Ki-67 (MIB1; DakoCytomation Co. Ltd., Kyoto, Japan); and rabbit polyclonal antibody: HER-2/*neu* (Ao485; DakoCytomation). The rabbit polyclonal antibody for 17β-HSD1, 17β-HSD5, and monoclonal antibody for steroid sulfatase (STS) were kindly provided by Dr. Poutanen (University of Oulu, Finland), Dr. Lu (Laval University Hospital Center, Québec, Canada) and Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan), respectively. These antibodies were used for immunohistochemistry in 10% formalin-fixed and paraffin-embedded tissue specimens of the cases. To score estrogen receptor (ER), progesteron receptor (PR), and Ki-67, more than 1,000 carcinoma cells from each case were counted independently by the same three authors as described above (Y.Miki, T. Suzuki, and H. Sasano), and the percentage of immunoreactivity as a labeling index (LI) was subsequently determined. The cases with <10% ERα LI or PR LI were designated ER- or PR-negative breast carcinomas according to the report by Allred et al. (18). For scoring of HER-2/*neu*, 17β-HSD1, 17β-HSD5, and STS (19–22), two groups were tentatively identified (0, no immunoreactivity and 1, positive carcinoma cells).

Total RNA extraction from breast tissues and cDNA synthesis. Total RNA was carefully extracted from 12 breast carcinoma specimens from both carcinomas and adipose compartments of the breast cancers in addition to nonpathologic breast tissues adjacent to the carcinoma using the TRIzol method (Invitrogen Corporation, San Diego, CA). A reverse transcription kit (Superscript II Preamplification system; Invitrogen) was used in the synthesis of cDNA.

Real-time RT-PCR. Real-time PCR was carried out using the LightCycler System (Roche Diagnostics GmbH, Mannheim, Germany). The primer sequences of aromatase, *ERRα*, and *RPL13A* were shown previously (23). Primer sets of *GCNF, HNF-4α, VDR, TRβ, TR4, HSD17B1, HSD17B2, HSD17B3, HSD17B4,* and *HSD17B5* were designed using OLIGO Primer Analysis Software (Takara Bio Inc., Shiga, Japan). cDNAs of known concentrations for target genes and the housekeeping gene, ribosomal protein L13a *(RPL13A)* were used to generate standard curves for real-time quantitative PCR to determine the quantity of target cDNA transcripts. The mRNA level in each case was represented as a ratio of *RPL13A* (%; refs. 20, 21, 23).

Laser Capture Microdissection. Nineteen breast carcinoma cases were frozen-sectioned at a thickness of 8 μm. Approximately 5,000 cells were laser-transferred from the carcinoma cells and the intratumoral stromal cells under light microscopic examination. For LCM/microarray expression profiling, after initial recovery and resuspension of the RNA pellet, a DNase digestion was done for 2 h at 37°C using 10 units of DNase (GenHunter, Nashville, TN) in the presence of 10 units of RNase inhibitor (Invitrogen), followed by extraction and precipitation. The pellet was resuspended in 27 μL of RNase-free H_2O and used for high-density cDNA array analysis.

Microarray analysis in isolated carcinoma cells. Twenty-three breast carcinomas were available for examination of gene expression patterns using microarray analysis following isolation by laser capture microscopy. Total RNA was extracted from ~5,000 carcinoma cells prepared by LCM procedures as described above. Sample preparation and processing were done essentially as described in the Affymetrix GeneChip Expression Analysis Manual (Affymetrix, Inc., Santa Clara, CA), with the exception that the labeled cRNA samples were hybridized to the complete human U133 GeneChip set (Affymetrix), including 22,215 and 22,577 genes. Relative levels of gene expression were calculated by global normalization. All gene expression data were clustered, and results were visualized using GeneSpring 7.2 (Agilent Technologies, Inc., Santa Clara, CA).

In this study, we focused on nuclear receptors that may modulate aromatase expression in carcinoma cells. Out of 44,792 genes, 88 genes were selected from the gene expression profiling for further analysis by reference to the web database of Nuclear Receptor.[11] Each case of breast carcinoma was ordered according to the level of aromatase gene expression determined by microarray and clustering analysis between each gene. Data from these categories and aromatase were subjected to hierarchical clustering analysis and visualization using the Cluster and TreeView programs (Stanford University, Palo Alto, CA; ref. 24) to generate tree structures based on the degree of similarity, as well as matrices comparing the levels of expression of individual genes in each sample examined. In addition, we further examined the correlations between the levels of aromatase mRNA expression and seven genes that were most closely

[11] NucleaRDB (April 2005, release 5.0); http://www.receptors.org/NR/index.html.

associated with aromatase in carcinoma cells isolated by LCM from 11 cases of human breast carcinoma.

Breast cancer cell lines and culture conditions. Human breast carcinoma cell lines MCF-7, T-47D, ZR-75-1, and SK-BR-3 and the human choriocarcinoma cell line BeWo were provided from the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). Human breast carcinoma cell lines MDA-MB-231 and MDA-MB-468 and mouse preadipocyte 3T3-L1 cells were purchased from the American Type Culture Collection (Manassas, VA). The MCF-7, T-47D, ZR-75-1, and 3T3-L1 cell lines were maintained in RPMI 1640 (Sigma-Aldrich Co., St. Louis, MO) or Leivobitz's L-15 medium (Invitrogen) for the MDA-MB-231 and MDA-MB-468 cells and supplemented with 10% fetal bovine serum (FBS; SAFC Biosciences, Lenexa, KS). BeWo placental cells were maintained in Ham's F12 medium (Invitrogen) supplemented with 15% FBS. Primary stromal cells employed in this study were designated 74T and 32N and were isolated using collagenase treatment from human breast carcinoma tissues (25) and maintained in RPMI 1640 with 10% FBS.

Coculture system. For physical separation of stromal and carcinoma cell lines, transwell cultures were established in six-well plates or 100-mm dishes using Transwell Permeable Supports (0.4 μm pore; Corning, Incorporated, New York, NY). MCF-7 and SK-BR-3 cells were cultured in transwell chambers in the absence or presence of 32N, 74T, and 3T3-L1 cells and were cultivated on the bottom of the plates or dishes. After 24 h of cultivation using this coculture system, carcinoma and stromal cells were separated, and each component was examined in the aromatization assay, estrogen production assays, or by real-time RT-PCR. The cells after coculture with other cells were designated with subscript CO (i.e., MCF-7_{CO}, $32N_{CO}$). After these assays, viable cells were counted by the trypan blue exclusion (TBE) assay, and the total RNA was extracted using the TRIzol method described above.

Aromatization assay. Several previous studies have shown that the aromatase enzyme activity and/or mRNA levels were low or not detectable in the breast carcinoma cells such as MCF-7 (26–29). Therefore, in this study, we employed 6α-methylandrost-4-ene-3,17-dione (MeAD) assay as a quantitative evaluation of aromatization activity (30). The conversion of MeAD, an androgen analogue, into estrogen analogue (6α-methylestradiol) by aromatase was shown to be highly specific, and an evaluation of accurate aromatase activity could become possible with the measurement of its estrogen analogue produced (30). From the results of comparative studies using rat ovary tissue, the quantitative limit of detection of the MeAD assay was approximately 2-fold higher than that of the ^{3}H-water release assay (data not shown).

The aromatization assay using MeAD was done as described previously (30). MeAD and 6α-methylestradiol (MeE2) were generously provided by Dr. Numazawa (Tohoku Pharmaceutical College, Sendai, Japan). Estradiol-3-pentafluorobenzyl-17β-methylpyridinium ether (E2-PFBY) was synthesized at Teizo Medical Co., Ltd. (Kanagawa, Japan). We used LC-MS–grade water, methanol, and acetonitrile for liquid chromatography–tandem mass spectrometry (LC-MS/MS). The LC-MS/MS devices and measurement conditions were as follows: an API 4000 electrospray ionization mass spectrometer (MDS SCIEX; Applied Biosystems (Foster City, CA)) was used. For high-performance liquid chromatography (HPLC), an Agilent 1100 chromatograph (Agilent Technologies) was employed. HTC PAL autosampler (CTC Analytics AG Industriestrasse, Zwingen, Switzerland) and an X Terra MS C18 column (3.5 μm, 2.1 × 150 mm) were employed. Measurement was done in the positive ion mode. For the quantification of MeE2-PFBY, we used a product ion (m/z 353.2) produced from a precursor ion (m/z 558.3). On the other hand, product ion of ^{13}C-E2-PFBY was m/z 109.9 produced from precursor ion at m/z 548.2. For the quantification of MeE2, after estrone, $^{13}C_4$ (Hayashi Pure Chemical Industries, Inc., Osaka, Japan) was added to the medium, and the mixture was mixed. The extract was dissolved in methanol and reduced with 1% $NaBH_4$. The sample was dried and treated with pentafluorobenzyl bromide under 0.8% KOH ethanol. Then, the reactive solution was diluted with purified water, and a derivative was extracted with ether. The sample was dried under reduced pressure and mixed with 2% 2-fluoro-1-methylpyridinium-*p*-toluenesulfonate/dichloromethane and 10% triethylamine. E2-PFBY derivative was eluted with 10% formic acid solution/acetonitrile (2, 8). The elution vehicle was removed under reduced pressure, dissolved in 0.1 mL of the mobile phase, and used as a sample for LC-MS/MS.

Estrogen production assays. MCF-7 and MCF-7_{CO} cells were incubated at 37°C in FBS-free RPMI 1640 containing 10 nmol/L of androstenedione or testosterone as substrates for 24 h. Concentrations of estrone and estradiol were evaluated by LC-MS/MS analysis (31). All the cells treated with substrates were counted by TBE assay. After addition of 100 pg of androstenedione-$^2H_7$ (C/D/N Isotope Inc., Quebec, Canada), estrone-$^{13}C_4$, and estradiol-$^{13}C_4$ (Hayashi Pure Chemical Industries) as internal standards, steroids were extracted with diethyl ether from the media. The separated organic layer was evaporated and then dissolved in picolinoic anhydride in

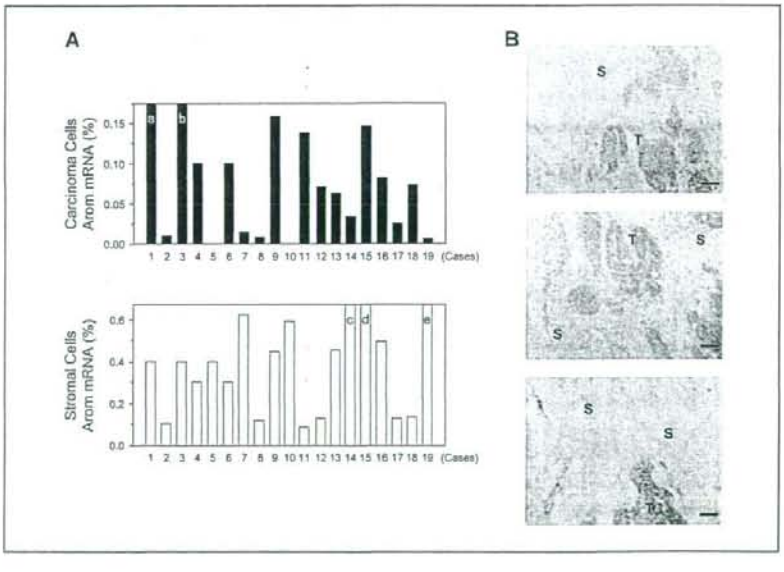

Figure 1. *A*, aromatase mRNA level in each case (1 to 19) of breast carcinoma (*top*) and stromal (*bottom*) cells. There were no detectable levels of aromatase in the breast carcinoma cells from two cases (5 and 10). For the following samples, the values of aromatase mRNA were a = 0.20%, b = 0.30%, c = 0.91%, d = 6.23%, and e = 1.02%. Aromatase immunoreactivity scores for each case (1 to 19) of breast carcinoma (*black*) and stromal (*white*) cells. There were no aromatase immunoreactivities observed in breast carcinoma cells from four cases (7, 12, 16, and 18). *B*, immunohistochemical localization of aromatase in human breast carcinoma tissues. Aromatase immunoreactivity was detected predominantly in carcinoma cells (*top*), only in stromal cells (*middle*), or in both carcinoma and stromal cells (*bottom*). *S*, stromal cells; *T*, carcinoma cells. *Bar*, 50 μm.

tetrahydrofran solution with triethylamine. After application to a Bond Elut C_{18} column, steroid derivatives were eluated with 80% acetonitrile solution. In this study, we used liquid chromatography (Agilent 1100; Agilent Technologies) coupled with an API 4000 triple-stage quadrupole mass spectrometer (Applied Biosystems) operated with electron spray isonization in the positive-ion mode, and the chromatographic separation was done on Cadenza CD-C_{18} columns (3 × 150 mm, 3.5 μm; Imtakt Corporation, Kyoto, Japan). Ion spray voltage was 4.5 kV, and turbo gas temperature was 450°C in ionization conditions. The estrogen levels in each case are presented as picograms per milliliter per 10^6 cells.

Cell proliferation assay. After coculture with stromal cells for 24 h, MCF-7 cells treated with 10^{-9} to 10^{-7} mol/L testosterone or androstenedione for 24 h were trypsinized and harvested in phenol red- and FBS-free medium in 96-well plates (3×10^4 cells/mL). Androstenedione and testosterone (10^{-9} to 10^{-7} mol/L) were added for 24 h. Cell proliferation was evaluated using the WST-8 method (Cell Counting Kit-8; Dojindo Inc., Kumamoto, Japan; ref. 32). We also examined the effects of aromatase inhibitors on cell proliferation by methods described above. Both steroidal (exemestane 10^{-8} mol/L; Pfizer Inc., New York, NY) and nonsteroidal (letrozole 10^{-8} mol/L; Novartis Pharma AG, Basel, Switzerland.) aromatase inhibitors were used in the androgen-treated MCF-7_{CO} and MCF-7 cells for 24 h.

Statistical analysis. Statistical analysis was done using the StatView 5.0 J software (SAS Institute Inc., Cary, NC). Values for patient's age, tumor size, Ki-67 LI, and mRNA levels for aromatase are represented as the mean ± SD. Simple regression analysis was employed to assess the correlations between aromatase and ERα mRNA expression levels. An association between the degree of mRNA expression of aromatase and these parameters for each individual case was evaluated using one-way ANOVA and the Bonferroni test. Statistical differences between aromatase mRNA expression and ER status, PR status, menopausal status, stage, lymph node status, histologic grade and HER-2/*neu*, 17β-HSD1, and 17β-HSD5 immunoreactivities were all evaluated in a cross-table using the χ^2 test.

Results

Distributions of aromatase mRNA transcripts in breast tissues. The level of aromatase mRNA expression (mean ± SD; $n = 12$) was significantly higher in both carcinoma (1.327 ± 1.394%) and adipose tissues (2.103 ± 1.790%) than in non-neoplastic breast tissue (0.106 ± 0.095%) adjacent to carcinoma. The aromatase level in placenta tissues as a positive control was 80.770 ± 31.867% (mean ± SD; $n = 3$; data not shown).

Localization of intratumoral aromatase mRNA transcripts in breast carcinoma tissues. From 19 breast carcinoma cases, aromatase mRNA transcripts were detected in intratumoral stromal cells from all 19 cases and in the carcinoma cells from 17 of these cases (Fig. 1*A*). The mean value of aromatase mRNA transcript level was significantly higher ($P = 0.009$) in intratumoral stromal cells (0.348 ± 0.238) than in the carcinoma cells (0.112 ± 0.090). No significant correlation was detected in the aromatase mRNA level between stromal and carcinoma cells ($r = 0.132$, $P = 0.592$). Aromatase immunoreactivity was detected in the cytoplasmic compartment of both intratumoral stromal and carcinoma cells (Fig. 1*B* and *C*). Aromatase immunoreactivity was absent in the carcinoma cells of four cases.

Correlation between aromatase mRNA levels in intratumoral stromal and carcinoma cells and clinicopathologic status in breast carcinoma patients. An association between the intratumoral aromatase mRNA levels in stromal and/or parenchymal/carcinoma cells and clinicopathologic parameters in 19 breast carcinoma cases are summarized in Table 1. Two cases of aromatase-negative carcinoma cells were assigned as 0.000%. Aromatase mRNA levels in stromal cells were positively correlated with histologic grade ($P = 0.032$). Aromatase mRNA levels in

Table 1. Correlation between aromatase expression levels and clinicopathologic parameters in 19 breast carcinomas

Parameter	*n*	Carcinoma cells		Stromal cells	
		Mean ± SD	*P*	Mean ± SD	*P*
Histologic grade					
I + II	14	0.098 ± 0.085		0.342 ± 0.228	
III	5	0.014 ± 0.014	0.072	2.002 ± 2.847	0.032*
ER status					
+	11	0.116 ± 0.087		0.374 ± 0.239	
−	8	0.029 ± 0.031	0.015*	1.146 ± 2.080	0.234
PR status					
+	12	0.107 ± 0.089		0.337 ± 0.228	
−	7	0.033 ± 0.032	0.051	1.320 ± 2.190	0.134
17β-HSD1 status					
+	10	0.134 ± 0.090		0.413 ± 0.230	
−	9	0.028 ± 0.029	0.007*	1.101 ± 2.100	0.373
17β-HSD5 status					
+	11	0.096 ± 0.104		0.361 ± 0.164	
−	8	0.062 ± 0.052	0.437	1.266 ± 2.225	0.24
STS status					
+	12	0.074 ± 0.068		0.435 ± 0.292	
−	7	0.102 ± 0.135	0.592	1.723 ± 3.012	0.138
EST status					
+	8	0.068 ± 0.066		0.462 ± 0.337	
−	11	0.087 ± 0.094	0.683	0.891 ± 1.790	0.609

*Significantly different; in percent.

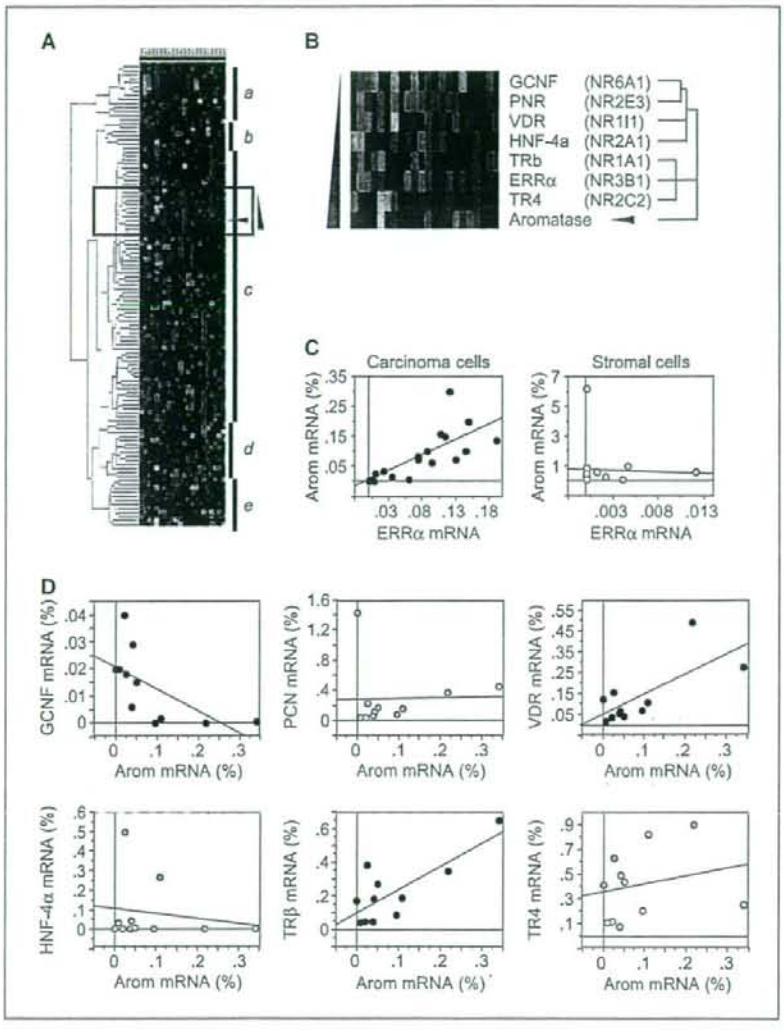

Figure 2. *A* and *B*, clustering analysis of microarray expression profiles of nuclear receptor genes. The cluster associated with the aromatase gene was composed of 59 genes (*A*, *group c*). More closely associated with the aromatase gene (*A*, *square*) were 12 genes (*B*). *C*, a statistically significant positive correlation was detected between aromatase and ERRα gene in 19 cases of (*left*) carcinoma cells ($r = 0.748$, $P = 0.002$), but not in (*right*) stromal cells ($r = 0.044$, $P = 0.860$). *D*, statistically significant correlations were detected between aromatase and ERRα ($r = 0.74$, $P = 0.01$; data not shown), VDR ($r = 0.73$, $P = 0.02$), TRβ ($r = 0.62$, $P = 0.04$), and GCNF ($r = -0.64$, $P = 0.03$) genes in 11 cases of carcinoma cells. No statistically significant correlations were detected between aromatase and PNR ($r = 0.02$, $P = 0.95$), HNF-4α ($r = -0.17$, $P = 0.61$), and TR4 ($r = 0.24$, $P = 0.48$) gene in 11 cases of carcinoma cells.

parenchymal/carcinoma cells were positively correlated with ER status ($P = 0.015$) and 17β-HSD1 status ($P = 0.007$), but not with histologic grade ($P = 0.072$). No significant association was detected between aromatase mRNA level in stromal or carcinoma cells and age, tumor size, lymph node status, PR status, HER-2/*neu* status, Ki-67 LI, 17β-HSD5 status, and STS status in this study.

Microarray analysis evaluated by hierarchical clustering. The results of focused clustering analysis subclassified 88 genes into five well-defined expression profiles or groups (Fig. 2*A*, *groups a–e*). The aromatase gene was included in group c (59 genes). The following seven genes were most closely associated with aromatase in group c (Fig. 2*B*): GCNF (NR6A1), PNR (NR2E3), VDR (NR1I1), HNF-4α (NR2A1), TRβ (NR1A1), ERRα (NR3B1), and TR4 (NR2C2).

Correlation between aromatase and nuclear receptors in isolated breast carcinoma cells. A statistically significant positive correlation was detected between aromatase and ERRα ($r = 0.74$, $P = 0.01$), VDR ($r = 0.73$, $P = 0.02$), and TRβ ($r = 0.62$, $P = 0.04$) in parenchymal/carcinoma cells of 11 human breast carcinoma cases (Fig. 2*D*). A statistically significant negative correlation was also detected between aromatase and GCNF ($r = -0.64$, $P = 0.03$) in these isolated parenchymal/carcinoma cells (Fig. 2*D*).

There were no significant increases or decreases of mRNA levels for the nuclear receptors GCNF, PNR, VDR, HNF-4α, TRβ, ERRα, and TR4 in MCF-7_{CO} compared with MCF-7 cells (data not present).

Correlation between aromatase and ERRα expressions in breast carcinoma cells. A statistically significant positive correlation was detected between aromatase and the ERRα gene (Fig. 2*C*) in 19 cases of parenchymal/carcinoma cells ($r = 0.748$, $P = 0.002$), but not in stromal cells ($r = 0.044$, $P = 0.860$).

In breast carcinoma cell lines, ERRα mRNA was detected in all cell lines examined in this study (Fig. 3*A*). Relatively low levels of

ERRα mRNA were detected in both stromal 32N and 74T cells. ERRα mRNA was also detected in placental BeWo cells.

Aromatase mRNA expression levels in human intratumoral stromal and carcinoma cells. Aromatase mRNA was detected in both stromal 32N and 74T cells isolated from human breast cancer tissues examined in this study, but the levels were lower than that of placental BeWo cells (Fig. 3*B*). Relatively low levels of aromatase mRNA was detected in mouse preadipocyte 3T3-L1 cells. Both 32N and 74T cells also exhibit aromatase enzyme activity, and there were no significant differences found between the aromatization levels in the 32N and 74T cells ($P = 0.140$).

Aromatase mRNA was detected in all breast carcinoma cell lines examined in this study (Fig. 3*B*). High levels of aromatase mRNA were detected in SK-BR-3 and MDA-MB-468 breast carcinoma cell lines, but the levels were lower than that of the 74T and 32N stromal cells and placental BeWo cells. We therefore used the higher aromatase mRNA expressing SK-BR-3 cells and the low aromatase mRNA expressing MCF-7 cells for further examinations. The aromatase mRNA level of native MCF-7 cells was significantly lower than that of native SK-BR-3 cells ($P = 0.041$). However, the levels in native SK-BR-3 cells were significantly lower than those of 32N ($P = 0.032$) or 74T cells ($P = 0.044$).

Effects of coculture on aromatase mRNA and activity levels in human intratumoral stromal and carcinoma cells. The results of the effects of coculture of breast cancer cell lines on the 32N and 74T cell aromatase activity are summarized in Fig. 3*C* to *E*. Aromatase mRNA level and activity in both 32N and 74T were significantly increased by cocultivation with MCF-7 but not with SK-BR-3 cells.

The results of the effects of coculture of stromal cells on MCF-7 and SK-BR-3 aromatase level are summarized in Fig. 3*D* and *E*. Both aromatase enzyme level (Fig. 3*D*) and mRNA level (Fig. 3*E*) in MCF-7_{CO} (after coculture with both 32N and 74T) were significantly higher than the levels found in monocultures of MCF-7 cells. There were no significant differences between native SK-BR-3 cells alone and SK-BR-3_{CO} (Fig. 3*D* and *E*). There were also no significant differences found in the aromatization levels between MCF-7_{CO} and SK-BR-3_{CO}. Aromatase activity/mRNA expression in both MCF-7_{CO} and 3T3-$L1_{CO}$, however, was not increased after coculture with MCF-7 and 3T3-L1 (Fig. 3*C–E*).

Effects of aromatase inhibitors on MCF-7 or stromal cells. Both 10^{-8} mol/L exemestane and 10^{-8} mol/L letrozole inhibited the increase in aromatization activity of the MCF-7_{CO} (Fig. 4*A*). Both 10^{-8} mol/L exemestane and 10^{-8} mol/L letrozole also inhibited the increase in aromatase activity of $32N_{CO}$ compared with the aromatase activity level of the 32N cells alone (Fig. 4*A*). The aromatization activity levels of MCF-7_{CO} following treatment with the aromatase inhibitors in MCF-7_{CO} were significantly lower than the aromatase activity level found in native MCF-7 cells (Fig. 4*A*).

The results of the cell proliferation assays are summarized in Fig. 4*B* to *D*. In monocultures of MCF-7 cells, there were no changes in the number of cells after 24 h incubation with 10^{-7} to 10^{-9} mol/L androstenedione, whereas 10^{-7} mol/L testosterone significantly increased the number of cells after 24 h. In MCF-7_{CO}, there were significant increments in the number of cells after 24 h treatment with 10^{-7} mol/L androstenedione and 10^{-8} to 10^{-7} mol/L testosterone. The cell numbers of MCF-7_{CO} treated with 10^{-8} mol/L androstenedione and 10^{-8} mol/L testosterone were significantly higher than those found in monocultures of MCF-7 cells. All of these increases of MCF-7_{CO} cell proliferation were inhibited following treatment with 10^{-8} mol/L exemestane (Fig. 4*C*) or 10^{-8} mol/L letrozole (Fig. 4*D*).

Estrogen production and expression levels of 17β-hydroxysteroid dehydrogenases in MCF-7. The results of estrogen production assays are summarized in Table 2*A* and *B*. Following the treatment with androstenedione (10^{-9} mol/L; 286.4 mg/mL) as the aromatase substrate, the rate of conversion into both estrone and estradiol in MCF-7_{CO} was higher than that in MCF-7 cells alone. The rate of conversion into estradiol but not into estrone in

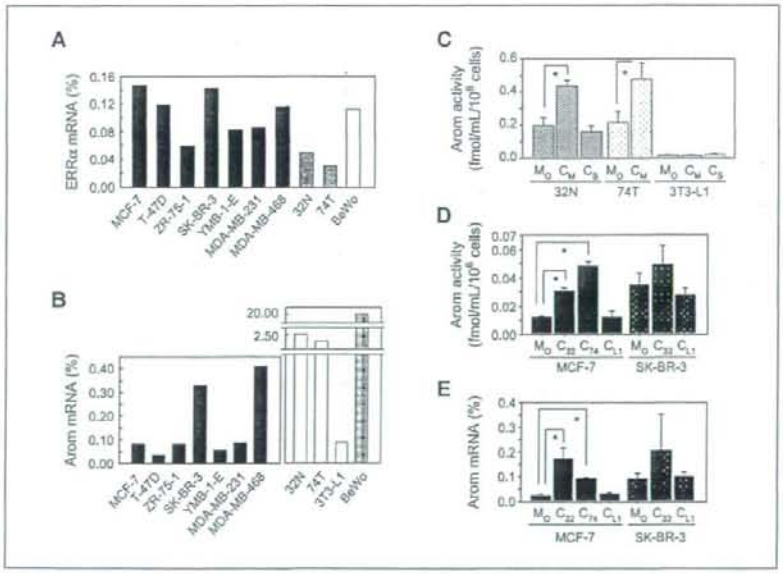

Figure 3. *A*, ERRα mRNA was detected in all breast carcinoma cell lines examined in this study, stromal 32N and 74T, and BeWo (in percent of RPL13A). *B*, aromatase levels in breast carcinoma cell lines and breast cancer-derived stromal 32N and 74T cells, mouse preadipocyte 3T3-L1 cells, and placental BeWo cells. *C–E*, the effects of coculture on aromatase mRNA level and enzyme activity in breast carcinoma and stromal cells. *C*, aromatase mRNA level in 32N, 74T, and 3T3-L1 cells; *D*, aromatase enzyme activity in MCF-7 and SK-BR-3 cells; *E*, aromatase mRNA level in MCF-7 or SK-BR-3 cells; M_O, monoculture; *C*, after coculture with 32N (C_{32}), 74T (C_{74}), 3T3-L1 (C_{L1}), MCF-7 (C_M), or SK-BR-3 (C_S) cells.

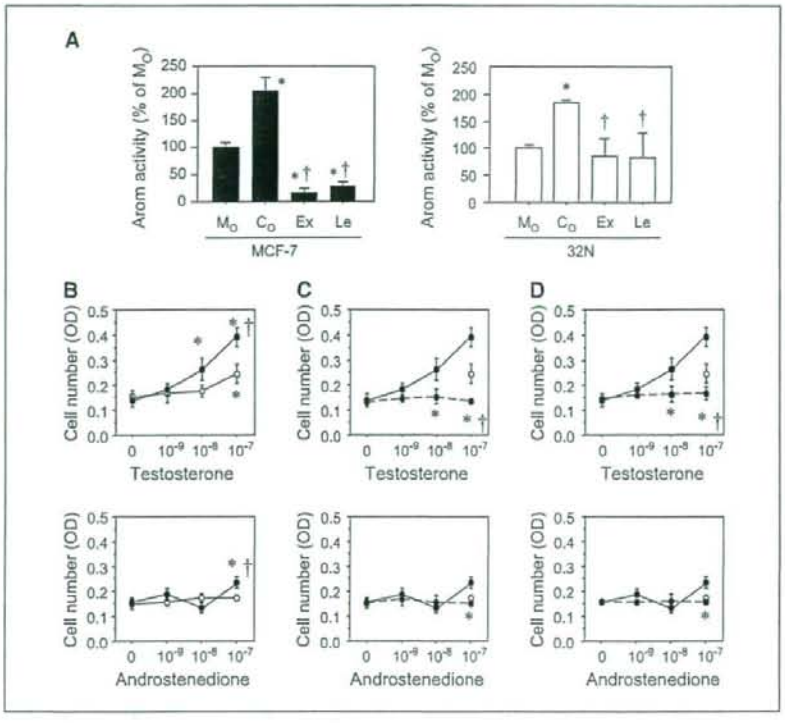

Figure 4. *A*, the effects of aromatase inhibitors on aromatase enzyme activities in MCF-7 (*left*) and 32N cells (*right*). *Ex*, treatment with 10^{-8} mol/L exemestane; *Le*, treatment with 10^{-8} mol/L letrozole; *, $P < 0.05$ versus M_O; †, $P < 0.05$ versus C_{32} C_{32} or C_M; in percent of RPL13A (aromatase mRNA level) or femtomoles per milliliter per 10^6 cells (aromatase enzyme activity). *B*, cell proliferation of $MCF\text{-}7_{CO}$ and MCF-7 cells treated with testosterone (*top*) and androstenedione (*bottom*). ●, $MCF\text{-}7_{CO}$; ○, MCF-7 cells; *, $P < 0.05$ versus vehicle control (0 nmol/L); †, $P < 0.05$ versus MCF-7 treated with 10 or 100 nmol/L androgens. *C* and *D*, cell proliferation following treatment with the aromatase inhibitors, (*C*) exemestane and (*D*) letrozole. ●, *solid line*, $MCF\text{-}7_{CO}$; ●, *dashed line*, $MCF\text{-}7_{CO}$ treated with aromatase inhibitor; ○, MCF-7; *, $P < 0.05$ versus $MCF\text{-}7_{CO}$; †, $P < 0.05$ versus MCF-7.

$MCF7_{CO}$ was also higher than that observed with MCF-7 cells alone, following the treatment with testosterone (10^{-9} mol/L; 288.4 mg/mL) as the aromatase substrate.

The results of 17β-hydroxysteroid dehydrogenases mRNA levels in cocultures and monocultures of MCF-7 cells are summarized in Table 2*C*. The 17β-HSD1 mRNA level in $MCF\text{-}7_{CO}$ was significantly higher than that found in MCF-7 cells. The 17β-HSD2 mRNA level in $MCF7_{CO}$ was significantly lower than that found in MCF-7 cells. There were no significant increases or decreases of other types of 17β-HSD types such as types 3, 4, and 5 in $MCF\text{-}7_{CO}$.

Discussion

Lu et al. (33) previously reported localization of aromatase protein and mRNA using immunohistochemistry and mRNA *in situ* hybridization, respectively, in the same breast cancer specimens. They showed that the aromatase protein and mRNA expression was predominantly detected in parenchymal cells (33). Further studies have shown the localization of aromatase protein using immunohistochemistry in human breast tissues (6–9), but the reported results have been markedly different between the different laboratories. These discrepancies in the cellular localization of intratumoral aromatase expression in human breast carcinomas may be due to the different aromatase antibodies and probe sequences employed in these different studies. This is the first study to show aromatase mRNA expression in the different cellular compartments of human breast carcinoma tissues following isolation using laser capture microscopy and subsequent qPCR analysis. The results of the combined LCM/qPCR study in our study showed that intratumoral aromatase in human breast cancer is expressed in both stromal and carcinoma or parenchymal components of the tissue. This finding confirms results of previous immunohistochemical study using the monoclonal antibody 677 done in 10% formalin-fixed and paraffin-embedded materials. We have also done immunohistochemistry using this monoclonal antibody 677 in frozen tissue sections adjacent to those in which LCM/qPCR analysis was conducted and have evaluated the immunoreactivity using the scoring system developed on 10% formalin-fixed and paraffin-embedded tissue specimens (14). Parenchymal/carcinoma cells are the major cell types of breast cancer tissues, and estrogens produced *in situ* by carcinoma cells could effectively activate the ER in the nuclei of carcinoma cells via an autocrine mechanism. Stromal cells also express aromatase, and thus, this source of intratumoral estrogen biosynthesis and subsequent estrogen-dependent cell proliferation is considered significant. An important aspect is the potential interplay that may exist between the carcinoma and stromal cell compartments. Therefore, we did further characterization of the potential regulation of aromatase in parenchymal or carcinoma cells in human breast cancer tissues.

Aromatase mRNA in adipose stromal cells was shown to be increased by coculture with MCF-7 cells (27). The results of previously reported studies all showed that various aromatase-stimulating factors (ASF), such as IL-1, IL-6, IL-11, IL-6 soluble receptor, tumor necrosis factor-α and prostaglandin E_2, etc. (10, 34, 35), are released from parenchymal or carcinoma cells, which resulted in the up-regulation of aromatase expression in stromal cells (including adipostromal cells, preadipocytes, or fibroblasts) within