Development of cDNA microarray for expression profiling of estrogen-responsive genes

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Abstract

Estrogen plays an important role in many physiological events including carcinogenesis and the development of human breast cancer. However, the molecular mechanisms of estrogen signaling in cancers have not been clarified hitherto and accurate therapeutic prediction of breast cancer is earnestly desired. We first carried out estrogen-responsive expression profiling of approximately 9000 genes in estrogen receptor-positive human MCF-7 breast cancer cells. Based on the results, estrogen-responsive genes were selected for production of a custom-made cDNA microarray. Using a microarray consisting of the narrowed-down gene subset, we first analyzed the time course of the estrogen-responsive gene expression profiles in MCF-7 cells, resulting in subdivision of the genes up-regulated by estrogen into early-responsive and late-responsive genes. The expression patterns of several genes were confirmed by Northern blot analysis. We also analyzed the effects of the estrogen antagonists ICI 182,780 and 4-hydroxytamoxifen (OHT) on the estrogen-responsive gene expression profiles in MCF-7 cells. While the regulation of most of the genes by estrogen was completely abolished by ICI 182,780, some genes were partially regulated by estrogen even in the presence of OHT. Furthermore, the estrogen-responsive gene expression profiles of twelve cancer cell lines derived from the breast, ovary, stomach and other tissues were obtained and analyzed by hierarchical clustering including the profiles in MCF-7 cells. Several genes also showed up-regulation or down-regulation by estrogen in cell lines other than MCF-7 cells. The significance of the estrogen-responsive genes identified in these analyses concerning the nature of cancer is discussed.

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Introduction

Estrogen plays an important role in the biological events in many organs and is especially closely associated with both development and neoplasia of the mammary gland (Pike *et al.* 1993, Ciocca & Fanelli 1997, Anderson *et al.* 1998). In normal and malignant mammary cells, estrogen receptor alpha (ER α), one of the nuclear receptor transcription

factors, is the primary target of estrogen, and the enhanced expression of ER α is one of the most critical events in the development of breast cancer (Hayashi *et al.* 1997a, Shoker *et al.* 1999). Hence, the expression status of ER α is a primary determinant in the anti-hormone therapy of breast cancer using antagonists to ER α such as tamoxifen (McGuire 1978, Lapidus *et al.* 1988). We have been studying the molecular mechanisms of

estrogen-dependent breast carcinogenesis, specifically from the viewpoint of regulation of ER α gene expression (Hayashi *et al.* 1997a, Tanimoto *et al.* 1999, Yoshida *et al.* 2000) and functional modulation of ER α (Hayashi *et al.* 1997b, Saji *et al.* 2001) in breast cancer cells. For clinical application, however, the assay of ER α status is not at present completely predictive for responsiveness of the tumors to anti-estrogens; not all tumors of the patients diagnosed as ER-positive respond to anti-hormones.

There are many reports concerning the target genes transcriptionally activated by ER α such as *pS2* (Masiakowski *et al.* 1982) and cathepsin D (Westley & Rochefort 1980), but the entire mechanism of the pathway from ER α leading to the proliferation and progression of mammary tumors is far from being completely clarified. Furthermore, another estrogen-signaling pathway independent of nuclear receptors has been postulated (Pietras & Szego 1977, Nadal *et al.* 2000). For elucidation of the scheme of estrogen-signaling and improvement of clinical decisions, expression profiling analysis using cDNA microarray technology should be one of the most effective procedures.

Several laboratories have carried out cDNA microarray analysis of breast tumors from patients (Gruvberger *et al.* 2001, Sørli *et al.* 2001, West *et al.* 2001, van't Veer *et al.* 2002) and a novel gene whose expression status was highly correlated with the prognosis of patients was identified (Finlin *et al.* 2001). There has also been a report concerning gene expression profiling in human ZR75-1 breast cancer cells in the presence of estrogen or estrogen antagonists using oligonucleotide microarray (Soulez & Parker 2001), and several novel estrogen-responsive genes were identified. Nonetheless, there is little information on how many markers are sufficient and which markers are suitable for accurate prognosis and diagnosis of breast tumors, especially regarding sensitivity to anti-hormone therapy. Microarray systems used in previous reports consisted of several thousand clones and therefore are obviously powerful for basic cancer research, but to use such a microarray glass slide for the diagnosis of each patient is too expensive for clinical application.

In the present report we first analyzed the expression profiles of approximately 9000 genes in human MCF-7 breast cancer cells in response to

estrogen. Based on the results, we selected the estrogen-responsive genes in MCF-7 cells and developed custom-made microarray systems consisting of the selected genes. Using the microarray systems, we analyzed the time course of estrogen-responsive gene expression profiles and the effects of estrogen antagonists on the gene expression profiles in MCF-7 cells. Furthermore, we analyzed the estrogen-responsive gene expression profiles in other ER-positive cancer cell lines derived from the breast, ovary, stomach and other tissues

Materials and methods

Cell culture

Human cancer cell lines derived from breast (MCF-7, MCF-7 c9 (Toi *et al.* 1993), T-47D and HBC-4 cells), ovary (OVCAR-5 and SK-OV-3 cells), stomach (MKN-28 and MKN-45 cells), brain (SF-539 cells), renal (ACHN and RXF-631 L cells), and human melanoma LOX-IMVI cells were maintained in RPMI 1640 medium (NISSUI Pharmaceutical, Tokyo, Japan) supplemented with 10% (MCF-7, MCF-7 c9 and T-47D cells) or 5% (other cell lines) fetal bovine serum (FBS; Tissue Culture Biologicals, Turale, CA, USA). Human endometrium cancer Ishikawa-3H12 cells were cultured in Eagle's Minimum Essential Medium (EMEM; NISSUI Pharmaceutical) supplemented with 10% FBS. For treatment of the cells with estrogen or estrogen antagonists, each medium was replaced with phenol red-free RPMI 1640 medium (Sigma, St Louis, MO, USA) or phenol red-free EMEM (Sigma) containing FBS treated with dextran-coated charcoal (DCC-FBS). All the culture media contained 2 mM L-glutamine (Sigma) and 40 μ g/ml gentamicin (Schering-Plough, Osaka, Japan). All cells were incubated at 37 °C in humidified air containing 5% CO₂.

Large-scale cDNA microarray analysis

Estrogen-responsive gene expression profiles were analyzed with a Human UniGEM v 2.0 microarray system (IncyteGenomics, Palo Alto, CA, USA) consisting of 9128 human cDNA clones covering 8502 unique gene/EST clusters. MCF-7 cells were cultured in the indicated phenol red-free medium with 10% DCC-FBS for 5 days and treated with 10 nM 17 β -estradiol for 72 h. From the cells,

mRNA was prepared using a PolyATtract mRNA Isolation System III (Promega, Madison, WI, USA), following total mRNA isolation using an RNeasy kit (Qiagen, Tokyo, Japan), according to the manufacturer's instructions. Preparation of Cy3- or Cy5-labeled cDNA, hybridization, quantification of Cy3 and Cy5 signal intensities, and data analysis were carried out with IncyteGenomics microarray system.

Production of custom-made cDNA microarrays

To produce a prototype of the custom-made cDNA microarray, a total of 148 genes were selected from the gene set included in Human UniGEM v 2.0, according to the results obtained using this large-scale microarray system. The selected gene subset consisted of 138 genes which showed up- or down-regulation by estrogen, and 10 genes which showed no response to estrogen (for internal control) in the large-scale microarray analysis. For production of a second version of the custom-made cDNA microarray (InfoArray; InfoGenes Co. Ltd, Tsukuba, Japan), new genes were added, resulting in a total of 204 genes including 27 genes for internal control (details are described in Results). For each gene, PCR-amplified cDNA fragments were obtained and spotted in duplicate on each glass slide.

Custom-made cDNA microarray hybridization

For estrogen-responsive gene expression profiling, human cancer cells were grown in the indicated phenol red-free medium with 5 or 10% DCC-FBS for 5 days and treated with 10 nM 17 β -estradiol for 72 h. For time-course analysis of estrogen-responsive gene expression profiles in MCF-7 cells, the duration of 17 β -estradiol treatment was 6, 12, 24, and 72 h. For the sample at 0 h, the cells were also similarly cultured in estrogen-deprived medium but without any treatment. For analysis of the effects of estrogen antagonists, MCF-7 cells were treated with 10 nM 17 β -estradiol and either 4-hydroxytamoxifen (OHT, 1 or 5 μ M) or ICI 182,780 (1 or 5 μ M) for 72 h, following estrogen-starved culture for 5 days. In every case, the cells used as references were treated with the same volume of ethanol. From the cells treated with those reagents, mRNA was isolated using PolyATtract system 1000 (Promega). The preparation of

Cy3- or Cy5-labeled cDNA and the hybridization procedure were performed according to a previous report (Arimura *et al.* 2000). Briefly, 2 to 4 μ g mRNA isolated from the cells treated with ethanol or the above-described ligands was reverse-transcribed in the presence of Cy5-dUTP or Cy3-dUTP (Amersham Pharmacia Biotech) respectively, using SuperScript II RNaseH-Reverse Transcriptase (Gibco BRL) with 6-mer and 9-mer random primers. In the case of mRNA from the cells without any treatment (0 h in time-course analysis), the same preparation of mRNA was divided and Cy3- or Cy5-labeled respectively. After the labeling reaction at 42 °C for 1 h, cDNA-RNA hybrids were denatured under alkaline conditions for 1 h. Unincorporated fluorescent nucleotides were removed by filtration through Microcon-30 columns (Millipore, Bedford, MA, USA). Cy3- and Cy5-labeled cDNA probes were mixed together, and after heat-denaturation the mixture was hybridized to a custom-made microarray slide in hybridization buffer (2 $\times$ SSC plus 0.2% SDS) under a cover slip. Hybridization was carried out overnight at 65 °C in a humidified hybridization chamber. The hybridized slides were washed three times with 2 $\times$ SSC/0.2% SDS for 5 min at room temperature (RT), three times with 0.2 $\times$ SSC/0.2% SDS for 5 min at RT, three times with 0.2 $\times$ SSC/0.2% SDS for 5 min at 60 °C, three times with 0.2 $\times$ SSC/0.2% SDS for 5 min at RT, then rinsed four times with 0.2 $\times$ SSC at RT. Finally, the remaining buffer on the slides was removed by centrifugation.

Scanning and data analysis

The fluorescent signals on the slides were scanned by ChipReader (Virtek, Ontario, Canada) and quantitative values for the signals were calculated using IPLab (Scanalytics, Fairfax, VA, USA) according to the manufacturer's instructions. Further data processing was carried out using Microsoft Excel software. For each spot, the ratio of Cy3 and Cy5 signal intensities (Cy3/Cy5) was calculated and log₂-transformed. Each log₂(Cy3/Cy5) value was normalized by subtracting the average of log₂(Cy3/Cy5) values for internal control genes, and the duplicated log₂(Cy3/Cy5) values for each gene were averaged. In the case of the analysis using InfoArray, the data for the spots with poor hybridization (signal areas of either Cy3

or Cy5 were below 100) were removed from the data processing described above, to improve the correlation coefficients between duplicated sets of $\log_2(\text{Cy3/Cy5})$ values. In the case of the analysis using the prototype microarray, there was actually no need to cut off the data for improvement of the correlation coefficients (details are described in Results). Average-linkage hierarchical clustering was applied using the CLUSTER program and the results were displayed using the TREEVIEW program (both programs were developed by Eisen *et al.* 1998).

Northern blot analysis

For Northern blot analysis, MCF-7 cells were grown in estrogen-starved medium as in the case of microarray analysis and treated with ethanol or 10 nM 17 β -estradiol for 6, 12, 24 or 72 h. For the sample at 0 h, the cells were also similarly cultured in the estrogen-deprived medium but without any treatment. Total RNA was prepared from each culture of the cells according to the method of Chomczynski and Sacchi (1987). For each sample, 20 μ g total RNA were used for Northern analysis. DNA fragments (420–570 bp) for hybridization probes were prepared by RT-PCR using total RNA from MCF-7 cells as a template and using an RNA PCR kit (AMV) ver. 2.1 (Takara Shuzo, Otsu, Japan). The sequences of the primers used in PCR amplification were as follows: 5'-GAG GCA ATG GCC ACC ATG G-3' and 5'-GTA GTC AAA GTC AGA GCA GTC-3' for *pS2* (Jakowlew *et al.* 1984); 5'-GTG GGG GCA AGA TGA AGG TC-3' and 5'-TTA CCC CAA GGG CAC ACC C-3' for insulin-like growth factor (IGF)-binding protein-4 (IGFBP4) (Kiefer *et al.* 1991b); 5'-TTC GAG AGC AAG TGG AAC CC-3' and 5'-AGC TCC TCC TGA ATG TGG TC-3' for KIAA1051 (Kikuno *et al.* 1999); 5'-AGC TGT GGA AGC CCT AAC TC-3' and 5'-TCG TAG CCG GTT AAC GCC AG-3' for retinoblastoma-binding protein 8 (Fusco *et al.* 1998); 5'-AAT GGC GGT TCT CAT GCT GG-3' and 5'-ATC TGG TTG ACT TTG AGC AGG-3' for *c-myc* promoter-binding protein 1 (Ray & Miller 1991); 5'-ACG AAA AGA GCT ACC GCG AG-3' and 5'-TTG CTG CTG TCG AAG GTG TG-3' for insulin-like growth factor-binding protein-5 (IGFBP5) (Kiefer *et al.* 1991a); 5'-TGG AGG CAC GGA CCA CTG C-3' and 5'-AGA CAG TCC CCT GCG

GTG G-3' for solute carrier family 7 member 5 (Gaugitsch *et al.* 1992); 5'-GCA CAG AGC CTC GCC TTT G and 5'-CAT CAC GAT GCC AGT GGT A-3' for β -actin (Nakajima-Iijima *et al.* 1985). Twenty-five nanograms of cDNA fragments were labeled with [α -³²P]dCTP using Megaprime DNA labeling systems (Amersham Pharmacia Biotech), and hybridization was carried out using ExpressHyb Hybridization solution (Clontech) according to the manufacturer's instructions.

Results

Large-scale DNA microarray analysis for screening the estrogen-responsive genes

First, we analyzed the estrogen-responsive gene expression profiles in human MCF-7 breast cancer cells using a Human UniGEM v 2.0 microarray system (IncyteGenomics) consisting of 9128 human cDNA clones covering 8502 unique gene/EST clusters. One of the purposes of our present research was to find the molecular markers which reflect the physiological status of ER-positive breast tumors supplied with a large amount of estrogen from themselves or from surrounding stromal cells for a long period. If such markers actually exist, these expression patterns should be quite valuable information for clinical application such as the diagnosis of estrogen- and anti-estrogen-responsiveness of mammary tumors. As a model cell line, we chose the MCF-7 cell line because it is ER α -rich, highly responsive to estrogen and therefore has been extensively investigated as one of the standard models of ER-positive breast cancer. After MCF-7 cells were cultured in estrogen-starved medium for 5 days, the cells were treated with 10 nM 17 β -estradiol, which is considered to be a saturating estrogen-rich condition for the MCF-7 cells but physiologically relevant, and is also a possible condition present in the mammary glands. We set the duration time of estrogen treatment at 72 h, with the aim of finding the genes exhibiting up- or down-regulation after such a long time of exposure to estrogen, which we think is one of the most important characteristics of a candidate for the markers we seek.

The ratios of differential expression of the genes between the MCF-7 cells with and without estrogen treatment are shown in Fig. 1A. Among a total of 9128 clones, the data for 1846 clones were cut off

Figure 1 The results from gene expression profiling of MCF-7 cells treated with (E_2+) or without (E_2-) 10 nM 17 β -estradiol for 72 h using the large-scale microarray by IncyteGenomics. (A) Expression profiles of 7282 genes in MCF-7 cells. After filtrating the data for a total of 9128 genes loaded on the microarray according to the cut-off threshold defined by IncyteGenomics, the $\log_2$ -transformed ratios of the expression levels of the remaining 7282 genes between the cells with or without estrogen (E_2+/E_2-) were plotted in order of the E_2+/E_2- ratios. The broken lines corresponding to E_2+/E_2- ratios equal to 2.0 or 0.5 are also indicated. (B) Expression profiles of 286 potentially estrogen-responsive genes in MCF-7 cells. From the 7282 genes plotted in panel A, 181 genes which showed E_2+/E_2- ratios equal to or >2.0 and 105 genes which showed E_2+/E_2- ratios equal to or <0.5 were selected and plotted in order of the E_2+/E_2- ratios. The broken lines corresponding to E_2+/E_2- ratios equal to 2.0 or 0.5 are also indicated.

Table 1 Genes induced or repressed by estrogen treatment for 72 hours in MCF-7 breast cancer cells

Gene name and category	Ratio of differential expression (E_2+/E_2- or $-(E_2-/E_2+)$) ^a				
	Incyte 1	Incyte 2	Custom	InfoArray	Accession no.
Cancer-related genes					
Trefoil factor 1	11.7	2.7	3.2	2.4	X52003
Tumor protein D-52 like 1	8.1	5.0	2.7	3.7	U44427
Retinoblastoma-binding protein 8 (CtIP)	3.9	2.2	5.1	2.3	NM_002894
Tumor rejection antigen (gp96) 1	3.3	2.6	1.8	2.8	X15187
c-myc promoter-binding protein 1	-2.6	-4.5	-6.0	-3.5	A1338641
Growth-related genes					
Solute carrier family 7, member 5	5.3	8.3	8.0	5.5	M80244
Insulin-like growth factor binding protein-4	3.5	3.1	3.4	4.0	M62403
Cyclin A1	3.4	2.1	2.8	2.5	U66838
TGFB inducible early growth response	3.4	2.5	2.4	2.3	AF050110
Solute carrier family 12, member 2	-4.3	-4.8	-4.8	-2.1	NM_001046
EGF-containing fibulin-like extracellular matrix protein	-6.7	-2.4	-4.1	-1.8	U03877
Insulin-like growth factor binding protein-5	-7.0	-2.8	-3.6	-2.1	AW157548
Transcription-related genes					
Activating transcription factor 3	4.9	4.5	2.4	4.3	N39944
Nuclear receptor interacting protein 1	3.0	2.6	3.6	2.4	X84373
Estrogen receptor 1 (ER α)	-3.0	-1.5	-1.9	-1.9	X03635
Others					
PDZK1	3.4	6.0	6.4	1.8	AF012281
Stanniocalcin 2	5.5	3.1	2.3	2.5	AB012664

^a'Ratio of differential expression' means the ratio of expression levels of each gene in MCF-7 cells treated with (E_2+) and without (E_2-) 17 β -estradiol. For E_2 -upregulated or E_2 -downregulated genes, the values are shown as the ratios E_2+/E_2- or $-(E_2-/E_2+)$ respectively. These data were obtained using the microarray of IncyteGenomics (Incyte 1 and Incyte 2, from two-times hybridization), prototype customized microarray (custom) or InfoArray. TGFB, transforming growth factor β ; EGF, epidermal growth factor.

due to low signal intensities as defined by the manufacturer (IncyteGenomics). Among the remaining 7282 clones, 181 genes showed differential expression ratios equal to or more than 2.0, and 105 genes showed differential expression ratios equal to or less than 0.5 (shown in Fig. 1B); the remaining 96% of the genes revealed no significant differences in their expression levels.

In the total of 286 genes which proved to be potentially estrogen-responsive genes by this analysis, there were some genes which had previously been reported to be induced by estrogen, such as *pS2* (trefoil factor 1, Brown *et al.* 1984), PDZK1 (Ghosh *et al.* 2000), IGFBP4 (Qin *et al.* 1999) and nuclear receptor interacting protein 1 (Thenot *et al.* 1999), indicating the reliability of this analysis. These genes are listed in Table 1 with the differential expression ratios in response to 17 β -estradiol (shown as 'Incyte 1'). Other potentially estrogen-responsive genes which we considered to be related to estrogen-dependent growth

and/or tumor progression were selected from the 286 genes referring to the background information of those genes, and are also listed in Table 1.

The estrogen-responsive gene expression profiling of MCF-7 cells by the microarray of IncyteGenomics was carried out once more, using mRNA isolated from the cells of another culture. The correlation coefficient between the expression ratios of the 286 genes that resulted from two-times microarray analysis was 0.819, and concerning the genes listed in Table 1, not identical but similar estrogen-responsive expression patterns were obtained by both analyses (shown as 'Incyte 1' and 'Incyte 2').

Time course of estrogen response

From the 286 potentially estrogen-responsive genes mentioned above, a total of 138 genes were selected for production of a prototype of the custom-made microarray, removing the genes which showed

relatively low ratios of estrogen-responsive expression (near or equal to 2.0 or -2.0) and were inferred to have little relation to the nature of breast cancer according to the background information on the genes.

Using this customized microarray prototype, we analyzed the time course of estrogen response of the genes in MCF-7 cells. The estrogen-starved MCF-7 cells were treated with ethanol (E_2 -) or 10 nM 17 β -estradiol (E_2 +) for 6, 12, 24 or 72 h and mRNA isolated from each culture of the cells was used for the microarray analysis. The mRNA was also isolated from the estrogen-starved cells but without any treatment (shown as 0 h). On the custom microarray, cDNA fragments for each gene were spotted in two blocks, yielding a pair of data sets of expression ratios from one hybridization. The correlation coefficients between pairs of gene expression ratios from one hybridization were 0.603, 0.835, 0.949, 0.833 and 0.978 at 0, 6, 12, 24 and 72 h of estrogen treatment respectively, without filtering the data. The relatively lower correlation coefficient at 0 h was considered to be due to random scattering of the Cy3/Cy5 ratios that resulted from technical variation in fluorescent labeling and/or hybridization. Filtration of the data of ratios according to the criterion used in the case of InfoArray analysis (see Materials and methods) resulted in no or little increase in these correlation coefficients (not shown). Therefore, further data analysis was performed without filtration.

The expression ratios of several genes at the final time point (72 h) are shown in Table 1 (as 'Custom'). At least for the genes listed in Table 1, similar results to those from large-scale microarray analysis were obtained. The estrogen-responsive expression patterns of a total of 148 genes were analyzed by hierarchical clustering and represented as a pseudo-color visualization matrix (Fig. 2). As shown in Fig. 2, hierarchical clustering clearly highlighted the gene clusters of estrogen-induced and estrogen-repressed genes, and made it possible to subdivide the cluster of estrogen-induced genes into two subgroups; one group contained the genes which showed expression ratios above 2.0 after 12 h of estrogen treatment (early-responsive estrogen-induced genes, group A in Fig. 2) and the other group contained the genes which showed significant expression after 24 h or, in most cases, 72 h of estrogen treatment (late-responsive estrogen-induced genes, group B in Fig. 2). On the

other hand, the cluster of estrogen-repressed genes (Group C in Fig. 2) did not show any apparent subgroups.

Figure 3 shows the time course of estrogen-responsive expression of the several genes selected from groups A, B and C in Fig. 2. As expected, *pS2* (trefoil factor), *IGFBP4* and cathepsin D, which were reported to be the target genes of *ER* α (Brown *et al.* 1984, Qin *et al.* 1999 and Augereau *et al.* 1994 respectively), were found in the group of early-responsive estrogen-induced genes (Fig. 2A). Solute carrier family 7 member 5 (*SCF7*, also named *E16*) was reported to be induced by estrogen (Soulez & Parker 2001) and was also found in the same group as *pS2* etc. in our analysis, but the expression pattern was different from those of other genes in that the expression level increased persistently up to 72 h. Insulin-like growth factor-binding protein-5 (*IGFBP5*), which was reported to be down-regulated by estrogen (Huynh *et al.* 1996), was found in the group of estrogen-repressed genes (Fig. 2C). These observations indicate the reliability of this customized microarray for analyzing the estrogen-responsive genes, and the usefulness of the time-course study by this microarray for characterization of the estrogen-regulated genes.

Northern blot analysis of representative estrogen-induced or -repressed genes

To confirm the estrogen-responsive gene expression patterns obtained by custom microarray analysis, we selected several genes from those shown in Fig. 3 and carried out Northern blot analysis concerning those selected genes. As shown in Fig. 4, *IGFBP4*, *pS2*, *KIAA1051* and *SCF7* in the early-responsive estrogen-induced gene group showed similar expression patterns to those obtained by microarray analysis. All four genes were rapidly and significantly induced by the addition of 17 β -estradiol (20-fold, 2-fold, 2-fold and 3-fold E_2 +/ E_2 - expression ratios at 6 h in *IGFBP4*, *pS2*, *KIAA1051* and *SCF7* respectively). In the case of *SCF7*, the mRNA level was increased continuously up to 72 h, resulting in 6-fold (6.1 kb transcript) and 9-fold (4.1 kb transcript) E_2 +/ E_2 - ratios at 72 h, which is consistent with the induction pattern depicted in Fig. 3. In contrast, the mRNA of retinoblastoma-binding protein 8 (*RbBP8*), which was found in the late-responsive estrogen-induced gene group, was detected only

Figure 2 Gene expression patterns in MCF-7 cells at different times after estrogen stimulation. MCF-7 cells incubated in estrogen-deprived medium were treated with 10 nM 17 β -estradiol (E_2+) or ethanol (E_2-) for 6, 12, 24 and 72 h; mRNA was isolated from each cell culture and from the cells without any treatment (shown as 0 h), and was then used for prototype customized cDNA microarray analysis as described in Materials and methods. The expression patterns of 148 genes were analyzed by hierarchical clustering and were represented as a pseudo-color visualization matrix using CLUSTER and TREEVIEW (Eisen *et al.* 1998). Gene clusters denoted by the bars and letters A, B and C are the groups containing early-responsive estrogen-induced genes, late-responsive estrogen-induced genes and estrogen-repressed genes respectively. The gene groups A and C are zoomed and the expression patterns with gene names are shown on the right. The color scale used to represent the expression ratios is shown on the lower right.

at 72 h after the estrogen treatment. At 72 h, RbBP8 mRNA appeared both under E_2+ and E_2- conditions, but the E_2+/E_2- ratio of the expression levels was 3.8, which is consistent with the data obtained by microarray analysis (Fig. 3B). IGFBP5 and *c-myc* promoter-binding protein 1 (MYC-BP1) in the estrogen-repressed gene group showed repression by estrogen treatment, and at 72 h, 7-fold (IGFBP5) and 3-fold (MYC-BP1) reductions between E_2- and E_2+ conditions were observed, similar to the data shown in Fig. 3C. The similar results obtained by microarray and Northern analysis emphasize the usefulness and reliability of the customized microarray system

for analyzing the expression profiles of the estrogen-regulated genes.

Estrogen-responsive gene expression profiles in the presence of estrogen antagonists

Next, we developed a second version of the custom-made cDNA microarray, with a larger number of genes loaded on it. On this microarray (named InfoArray), new genes were added resulting in a total of 204 genes, including 27 genes for internal control. The newly added gene group included the genes found as estrogen-responsive or not estrogen-responsive (as internal control genes)

Figure 3 Expression profiles of several genes in MCF-7 cells at different times after estrogen stimulation analyzed by prototype customized microarrays. From the gene groups A, B and C in Fig. 2, several genes were selected and the time courses of the ratios of expression levels between the cells treated with 17 β -estradiol (E_2^+) and with ethanol (E_2^-) were plotted. Panels A, B and C show the expression patterns of early-responsive estrogen-induced genes, late-responsive estrogen-induced genes and estrogen-repressed genes respectively. EGF, epidermal growth factor.

by large-scale microarray analysis but not loaded on the prototype custom microarray. The group also included the genes for which there was no experimental evidence to consider them estrogen-responsive but whose expression levels would be critical for the physiology of breast cancer, such as aromatase and estrogen sulfatase. The cDNA fragments for each gene were spotted in duplicate as in the case of the prototype customized microarray. Using this microarray (InfoArray), we analyzed the effects of anti-estrogens, that is, OHT and ICI 182,780, on the estrogen-responsive gene expression profiles of MCF-7 cells. One of the purposes of this analysis was to subdivide the estrogen-responsive genes loaded on InfoArray into those genes regulated through the pathway involving ER α and those genes regulated independently of ER α . MCF-7 cells cultured in estrogen-deprived medium were treated with ethanol (E_2 -), 10 nM 17 β -estradiol (E_2 +), 10 nM E_2 and 1 μ M or 5 μ M OHT, or 10 nM E_2 and 1 μ M or 5 μ M ICI 182,780 for 72 h, then mRNA was isolated from each cell culture. The mRNA from the E_2 - cells was used for Cy5-labeling and the mRNA from the cells treated with above-mentioned ligands was used for Cy3-labeling, and the fluorescent-labeled cDNA probes were hybridized to InfoArray.

Filtration of the data for the E_2 +/ E_2 -, (E_2 +OHT)/ E_2 - and (E_2 +ICI)/ E_2 - ratios was performed using our own criteria (described in Materials and methods), cutting off the data for the spots with poor hybridization (signal areas for either Cy3 or Cy5 below 100). By filtrating the data, all correlation coefficients between duplicated sets of the ratios obtained from a total of five microarray experiments increased significantly, resulting in the values ranging from 0.876 to 0.948.

The expression ratios of several genes in MCF-7 cells stimulated by 17 β -estradiol only are shown in Table 1 (as 'InfoArray'). At least for the genes listed in Table 1, we obtained similar results to those

from large-scale microarray analysis and the prototype custom microarray analysis.

From the total of 204 genes on InfoArray, 168 genes for which there were remaining data in at least three culture conditions were selected and those expression patterns were analyzed by hierarchical clustering (Fig. 5). In the presence of 1 μ M or 5 μ M ICI 182,780, almost all the estrogen-responsive genes showed no regulation by estrogen. Some genes (cluster d in Fig. 5) showed lower expression levels in the presence of 17 β -estradiol and ICI 182,780 than under E_2 - conditions, which was possibly due to the low level of induction of those genes by traces of estrogen under E_2 - conditions and suppression of the induction by ICI 182,780. Group d contains well-known estrogen-responsive genes such as trefoil factor (*pS2*) and IGFBP4, suggesting that these genes may have a lower threshold of estrogen concentration for induction than other estrogen-regulated genes. The estrogen-repressed genes in group f showed higher expression levels in the presence of ICI 182,780 than under E_2 - conditions, which is possibly due to the low level of repression of those genes by traces of estrogen under E_2 - conditions and suppression of this E_2 - repression by ICI 182,780. Group f contains IGFBP5, which has been reported to be down-regulated by estrogen and up-regulated by ICI 182,780 (Huynh *et al.* 1996). Because ICI 182,780 is recognized as a pure anti-estrogen and is considered to repress all estrogen-responsive promoter contexts (Hall *et al.* 2001), these observations suggest that almost all the estrogen-responsive genes are regulated by estrogen through ER-dependent mechanisms or pathways.

In the presence of 5 μ M OHT, the regulation of many genes (group c, d, and f in Fig. 5) by estrogen was completely abolished by OHT, similar to the case of ICI 182,780 treatment. But several genes showed residual regulation by estrogen even in the presence of a high concentration of OHT. For

Figure 4 Northern blot analysis of several genes selected from the genes shown in Fig. 3. Estrogen-starved MCF-7 cells were treated with 17 β -estradiol or ethanol for 6, 12, 24, and 72 h and total RNA was isolated from each culture of the cells and from the cells without any treatment (shown as 0 h). From each sample, 20 μ g total RNA were taken for Northern blot analysis, and cDNA fragments of the genes listed on the left were used as probes. The sizes of the transcripts are shown on the right. Abbreviations are as follows: IGFBP4, insulin-like growth factor-binding protein-4; SCF7, solute carrier family 7 member 5; RbBP8, retinoblastoma-binding protein 8; MYC-BP1, c-myc promoter-binding protein 1; IGFBP5, insulin-like growth factor-binding protein-5. Northern blot analysis of β -actin was also carried out as an internal control.

Figure 5 Gene expression patterns in MCF-7 cells in the presence of 10 nM 17 β -estradiol (E_2), or 10 nM E_2 and either 4-hydroxytamoxifen (OHT) or ICI 182,780 (ICI). MCF-7 cells cultured in estrogen-deprived medium were treated with ethanol (E_2 -), 10 nM 17 β -estradiol (E_2 +) alone or 10 nM E_2 together with either OHT (1 μ M or 5 μ M) or ICI 182,780 (1 μ M or 5 μ M) for 72 h, then mRNA was isolated from each cell culture. The mRNA from the E_2 -cells was used for Cy5-labeling and mRNA from the cells treated with the ligands was used for Cy3-labeling, and gene-expression profiles were analyzed using InfoArray. After filtering the data of expression ratios according to the criterion described in Materials and methods, 168 genes for which there were remaining data in at least three culture conditions were selected from a total of 204 genes loaded on InfoArray, and analyzed by hierarchical clustering. The results are represented as a pseudo-color visualization matrix as in Fig. 2. The characters of gene clusters denoted by the bars and letters a to f are as follows: a, group containing the genes whose differential expression ratios exceeded 2.0 only in the presence of both estrogen and OHT; b, group containing the genes which were induced by estrogen both in the presence and absence of OHT; c, group including the genes which were estrogen-induced and where the presence of ICI 182,780 or OHT completely abolished the regulation by estrogen; d, group containing the genes which showed similar expression patterns as group c, but are different in that the presence of ICI 182,780 resulted in apparent down-regulation; e, group containing the genes which were repressed by estrogen both in the presence and absence of OHT; f, group including the genes which were estrogen-repressed and where the presence of OHT or ICI 182,780 completely abolished the regulation. All the gene groups are zoomed and the expression patterns with gene names are shown on the right. The color scale used to represent the expression ratios is shown on the lower right. The gray color means that there are no reliable data after filtering due to poor hybridization.

example, nuclear receptor interacting protein 1 in group b, which showed 2.4-fold induction by estrogen, showed 2.2-fold induction in the presence

of 5 μ M OHT. *c-myc* promoter-binding protein 1 in group e, which showed 3.5-fold reduction by estrogen, was also reduced by 1.9-fold in the

presence of estrogen and 5 μ M OHT. In group a, one gene (FOS-like antigen 2) and one clone (cDNA FLJ12748 fis, clone NT2RP2001137) showed expression ratios above 2.0 only in the presence of OHT. The significance of the expression patterns of these gene/clones in relation to breast cancer is unknown, but there is a possibility that induction of these genes reflects the activation of other mechanisms of the effects of tamoxifen on breast cancer apart from inhibition of ER α , such as apoptosis (Mandlekar & Kong 2001).

In the presence of 1 μ M OHT, most of the estrogen-induced genes (group b, c and d in Fig. 5) showed above 2.0-fold induction by estrogen, suggesting that this concentration of OHT is not sufficient for complete inhibition of ER α function, which was quite different from the effects of the same concentration of ICI 182,780.

These observations on the effects of OHT suggest that there is a small number of genes whose regulation by estrogen is not completely abolished by OHT, but that most of the genes lost induction or repression by estrogen, which is consistent with the fact that tamoxifen inhibits the proliferation of at least a part of ER-positive breast tumors.

Estrogen-responsive gene expression profiles in other cancer cell lines

Finally, we analyzed the estrogen-responsive gene expression profiles of twelve cancer cell lines other than MCF-7 cells using InfoArray. MCF-7 c9 cells were cloned and established as a tamoxifen-resistant sub-line of MCF-7 cells by Toi *et al.* (1993). Other cell lines were derived from breast, ovary, endometrium, stomach, brain, renal or melanoma cells (details are in Materials and methods). ER α mRNA was detected by RT-PCR in all the cell lines, although most of the cell lines other than MCF-7 cells showed low level expression of ER α (data not shown). Each cell line was cultured in estrogen-deprived medium and treated with ethanol (E_2 -) or 10 nM 17 β -estradiol (E_2 +) for 72 h. Gene expression patterns of these cell lines were obtained using InfoArray. After cutting off the data for the spots with poor hybridization according to the criterion as described in a previous section and in Materials and methods, correlation coefficients between duplicated sets of expression ratios obtained by

hybridization increased significantly (data not shown). From a total of 204 genes loaded on InfoArray, 179 genes for which there were remaining data in at least seven cell lines were selected and analyzed by hierarchical clustering, including the gene expression profiles of MCF-7 cells treated with estrogen (the same data as shown in Fig. 5).

As shown in Fig. 6, several characteristic genes or gene clusters were observed although most of the genes exhibited regulation by estrogen only in MCF-7 cells. For example, all the genes in group a showed above 2.5-fold induction by estrogen in MCF-7 cells but not in other cells (and not in MCF-7 c9 cells). The gene denoted as 'mal, T-cell differentiation protein' in group b showed 2.8-fold induction by estrogen only in stomach MKN-28 cells. The genes in group c were induced by 2.0- or above 2.0-fold after estrogen stimulation in both MCF-7 and T-47D breast cancer cells. The genes in group d showed higher induction ratios in MCF-7 c9 than in MCF-7 cells; for example, KIAA0018 was induced 1.6-fold in MCF-7 cells but 9.8-fold in MCF-7 c9 cells. Ribosomal protein L35 in group e was repressed by estrogen in several cell lines; 2.0-, 2.4-, 2.8- and 2.0-fold reduction in MCF-7 c9, SK-OV-3, MKN-28 and MKN-45 respectively.

In contrast with MCF-7 cells, there was too small a number of estrogen-regulated genes in other cell lines to be highlighted as gene groups by hierarchical clustering, and therefore these gene expression patterns were apparently far from reflecting the effects of estrogen on each organ from which each cell line was derived. But in the pseudo-color visualized matrix shown in Fig. 6, there are a few genes showing expression ratios of 2.0-fold or above 2.0-fold in each cell line, suggesting that these expression profiles imply the cell-type specific response to estrogen treatment.

Discussion

We report here on our on-going cDNA microarray analysis to specify estrogen-responsive genes, some of which would be critical for the development of breast cancer and also for the prediction of individual responses to anti-hormone therapy. Our first goal is the integrated and precise selection of patients for anti-hormone therapy, which is

Figure 6 Gene expression patterns in MCF-7 and twelve other cancer cell lines in the presence of 10 nM 17 β -estradiol (E_2). The cancer cell lines cultured in estrogen-deprived medium were treated with ethanol (E_2^-) or 10 nM 17 β -estradiol (E_2^+) for 72 h and mRNA was isolated from each culture of the cells. The mRNA from the E_2^- and E_2^+ cells was used for Cy5- and Cy3-labeling respectively, and gene-expression profiles were analyzed using InfoArray. After filtrating the data of expression ratios according to the criterion described in Materials and methods, 179 genes for which there were remaining data in at least seven cell lines were selected from a total of 204 genes loaded on InfoArray, and analyzed by hierarchical clustering. The results are represented as a pseudo-color visualization matrix as in Fig. 2. The characters of gene clusters denoted by the bars and letters a to e are as follows: a, group containing the genes estrogen-induced only in MCF-7 cells (not induced by estrogen in MCF-7 c9 cells); b, group containing one gene which was induced by estrogen only in MKN-28 stomach cancer cells; c, group including the genes induced by estrogen both in MCF-7 and T-47D breast cancer cells; d, group containing the genes highly estrogen-induced in MCF-7 c9 cells; e, group containing one gene repressed by estrogen in several cancer cell lines. All the gene groups are zoomed and the expression patterns with gene names are shown on the right. The color scale used to represent the expression ratios is shown on the lower right. The gray color means that there are no reliable data after filtrating due to poor hybridization.

urgently needed for the clinical treatment of breast cancer.

For all cDNA microarray analyses except time-course analysis, MCF-7 cells (and also other cancer cells) were treated with 10 nM 17 β -estradiol for 72 h. As described in Results, we decided on an estrogen exposure duration of 72 h because we considered that stable or persistent estrogen-responsive expression of the genes is one of the most important criteria for the selection of

candidates for prognostic and/or diagnostic markers for breast tumors supplied with estrogen for a long time in patients. Conversion from ER-positive to ER-negative status in breast cancer *in vivo* along with loss of sensitivity to anti-hormone therapy is one of the most important clinical problems, and anti-hormone therapy will be most effective in those tumors in the status saturated with estrogen and heavily estrogen-dependent for growth. According to this speculation, we decided

to perform gene expression profiling of ER-rich breast cancer MCF-7 cells stimulated with a saturating but physiologically relevant concentration (10 nM) of estrogen for 72 h.

First, screening of the estrogen-responsive genes in MCF-7 cells using a large-scale cDNA microarray revealed that a limited number of genes were sufficient for profiling estrogen responsiveness. The fact that 96% of the genes loaded on the microarray of IncyteGenomics exhibited no or little regulation by estrogen even in ER-rich MCF-7 cells prompted us to develop a custom-made cDNA microarray system containing only a narrowed-down estrogen-responsive gene subset.

The 138 estrogen-responsive genes/ESTs selected contained the genes previously reported to be early-responsive estrogen-induced genes such as *pS2* (trefoil factor) and IGFBP4 (Qin *et al.* 1999). From this fact we inferred that in the total of 138 genes there would be some unidentified early-responsive genes closely related to the physiology of breast cancer. Therefore, we analyzed the time course of estrogen-responsive gene expression profiles in MCF-7 cells using the custom-made microarray and the obtained results were confirmed by Northern blot analysis.

As expected, we identified KIAA1051, which is one of the functionally unidentified genes, as a novel early-responsive gene. But clarification of the function of the gene and the significance of estrogen-responsiveness in relation to breast cancer depend on further investigation. Solute carrier family 7 member 5 (SCF7), another identified early-responsive gene, was also reported to be induced by estrogen in ZR75-1 breast cancer cells (Soulez & Parker 2001), suggesting that the regulation mechanism of SCF7 by estrogen might be universal in breast cancer. Retinoblastoma-binding protein 8 (RbBP8, also named CtIP), which we identified as a late-responsive estrogen-induced gene, was also reported to be induced by estrogen in ZR75-1 cells (Soulez & Parker 2001), although this gene is apparently an early-responsive gene in ZR75-1 and different from the case in MCF-7. RbBP8 may be regulated by estrogen through at least partly different mechanisms or pathways in MCF-7 and ZR75-1 cells, but in any case we think the estrogen-responsiveness of RbBP8 is an interesting observation regarding breast cancer. RbBP8, identified as a binding protein to retinoblastoma, was reported to bind to

BRCA1, regulating this activity, and to have an association with the ataxia telangiectasia mutated (ATM) (Li *et al.* 1999, 2000). The estrogen responsiveness of this gene may implicate its involvement in the cell cycle regulation of the estrogen-dependent cells. Down-regulation of IGFBP5 by estrogen in MCF-7 cells has been reported (Huynh *et al.* 1996) and similar results were obtained by our cDNA microarray and Northern analysis. IGFBPs including IGFBP4 and IGFBP5 are considered to bind to IGF-I and IGF-II in the extracellular space, regulating access of IGFs to IGF receptors (reviewed by Sachdev & Yee 2001), which is one of the most critical steps for proliferation of breast tumor cells. But the physiological meaning of the opposite regulation of IGFBP4 and IGFBP5 by estrogen is unknown. Since IGFBP5 was also reported to be localized to the nucleus (Schedlich *et al.* 2000), repression of IGFBP5 by estrogen might have some influence on the functions of molecules in the nucleus such as nuclear receptors and cofactors. We newly identified *c-myc* promoter-binding protein 1 (MYC-BP1) as another estrogen-repressed gene. Since MYC-BP1 is a transcriptional repressor of the proto-oncogene *c-myc* (Ray & Miller 1991), down-regulation of this gene by estrogen may bring about up-regulation of *c-myc*, and, in turn, the gene product c-Myc may induce many downstream genes involved in proliferation and progression of tumor. On the other hand, there is another possibility that MYC-BP1 represses transcription of some unknown genes other than *c-myc* (Ghosh *et al.* 1999). Estrogen-responsive repression of MYC-BP1 may influence the expression levels of many genes and not only the downstream genes of *c-myc*.

The time courses of estrogen-responsive expression of these above-mentioned genes were analyzed by both customized cDNA microarray and Northern blot, and we obtained quite similar results for all of the genes. These observations emphasize the usefulness and reliability of the customized microarray system for analyzing the expression profiles of the estrogen-regulated genes.

We think comprehensive analysis of the effects of estrogen antagonists on breast cancer is no less important than analysis of the effects by estrogen. At present, many estrogen antagonists have been developed for anti-hormone therapy, and many reports have accumulated on the potency of

antagonists such as tamoxifen in clinical application. Nevertheless, it has not been completely elucidated how these anti-estrogens affect the regulation of ER-target genes. Therefore, we performed microarray analysis of gene expression profiles in MCF-7 cells treated with OHT or ICI 182,780. For this experiment, we developed an upgraded custom-made cDNA microarray (named InfoArray) with a larger number of estrogen-responsive genes than the prototype microarray and supplemented it with several genes involved in metabolism of estrogen such as aromatase and estrogen sulfatase. There is no evidence that these genes are regulated by estrogen or ER, but these genes are considered to be closely associated with the physiology of estrogen-dependent breast tumors. Thus, InfoArray is a more specific version of the customized microarray for clinical application including the viewpoint of intracrinology of estrogen. To examine the anti-estrogen effects, we decided to treat the estrogen-starved MCF-7 cells with 1 or 5 μM OHT or ICI 182,780 together with 10 nM 17β -estradiol. These defined conditions are different from those adopted for the treatment of ZR75-1 cells in a previous report (Soulez & Parker 2001) in that we added these antagonists together with a saturating concentration of estrogen. Our experimental design attached much importance to the purpose of gaining useful information for clinical application, for example, to screen the markers which mirror the status of breast tumors provided with estrogen from stroma but treated with anti-hormone therapy.

ICI 182,780 completely suppressed the estrogen-responsive regulation of almost all of the genes loaded on InfoArray including the genes identified through time-course analysis mentioned above, which suggests these genes are induced or repressed by estrogen through ER-dependent mechanisms or pathways. There may be no or few genes regulated by estrogen through ER-independent pathways. OHT also completely blocked the regulation of many estrogen-responsive genes, consistent with the fact that tamoxifen is considered to be an effective inhibitor of breast tumor proliferation. But, in contrast with ICI 182,780, the extent of suppression of estrogen-responsiveness by OHT was apparently much smaller than by ICI 182,780 and several genes such as MYC-BP1 and nuclear receptor interacting protein 1 appeared to show residual regulation by estrogen. Tamoxifen is

recognized as a SERM (selective estrogen receptor modulator) and has been demonstrated to work as an agonist in some promoter contexts and some organs. Our results concerning the effects of OHT suggest that some genes in MCF-7 possess an unidentified estrogen-responsive regulation mechanism on which tamoxifen acts as a partial agonist.

We think InfoArray is one of the most useful tools to examine the potency and specific effects of other estrogen antagonists, such as raloxifene and newly-designed anti-estrogen drugs, and estrogen-like endocrine disrupters on breast cancer. Using InfoArray and several cell lines, we may be able to develop an assay system which can evaluate the dose effects of diverse estrogen-like compounds including anti-hormones, and find the markers to discriminate the effects of certain compounds from others with high specificity.

As is generally known, the effects and functions of estrogen are not limited to the physiology of hormone-dependent cancer and there have been several reports concerning the effects of estrogen on organs and cells other than breast cancer. For example, in glomerular mesangial cells of the kidney, estrogen suppressed collagen synthesis (Dubey & Jackson 2001), and in ER-positive human melanoma cells the production of interleukin-8 was inhibited by estrogen (Kanda & Watanabe 2001).

We were intrigued as to how potent the InfoArray is for evaluating estrogen-responsiveness of cells derived from organs other than breast. In other words, we thought it important to investigate how many genes among the gene set loaded on InfoArray would also exhibit regulation by estrogen in other cells. Therefore, using InfoArray, we analyzed the gene expression profiles in twelve cells other than MCF-7 cells treated with estrogen. Unfortunately, all of the twelve cells exhibited much lower responsiveness to estrogen treatment than MCF-7 cells, so we could not find any gene expression patterns that were characteristic or available to distinguish the organ- or tissue-specific responsiveness to estrogen. But some genes were suggested to be induced or repressed by estrogen in one cell line or several cell lines. Further analysis will be needed to clarify the functions of these genes regarding the estrogen-responsiveness of each cell line or organ.

We are now trying gene expression profiling of breast cancer tissues from patients using InfoArray.

This analysis will also provide useful information for our goal, but in the case of analysis using tissue samples there are a lot of limitations regarding design of experimental conditions such as duration and dosage of estrogen and/or anti-estrogen treatment, treatment with newly-developed drugs which are not approved yet for clinical application, and exposure to hormone-like endocrine disrupters. Gene expression profiling analysis of both tissue samples and cell lines cultured in diverse conditions using the same cDNA microarray system with narrowed-down estrogen-responsive genes will complement the advantages and disadvantages of each type of analysis, and it will contribute to clarification of the physiology of breast cancer and to the establishment of the best clinical diagnosis.

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