A focused DNA microarray analysis of estrogen-responsive genes in COS-7 cells over-expressing the estrogen receptor α

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ABSTRACT The COS-7 cell line, derived from the kidney cells of African green monkeys, is often used in studies involving the production of recombinant proteins. Using a focused DNA microarray (EstrArray™), we examined the expression profile of estrogen-responsive genes in COS-7 cells which over-express the estrogen receptor α. This was undertaken *via* transfection of the expression vector in the absence or presence of 17β-estradiol (E_2). The genes which were up- or down-regulated over 1.5-fold ($+E_2/-E_2$ ratio or $-E_2/+E_2$ ratio, respectively) in the 17β-estradiol-stimulated COS-7 cells were shown. The data was compared with that of the MCF-7 breast cancer cells. They were mostly the members necessary for gene expression and metabolism of amino acids and polynucleotides.

(Accepted on May 1, 2009)

Key words : **DNA microarray, estrogen-responsive genes, COS-7 cell, estrogen receptor, MCF-7 cell**

INTRODUCTION

The estrogen receptors (ERs) belong to a superfamily of nuclear receptors including those of steroid, thyroid hormones, vitamin D and retinoic acid. Estrogen receptors α and β generally mediate the biological effects of estrogen in their target cells by binding to estrogen response elements (EREs) in the promoter region of the target genes. Ligand-dependent transcriptional activation is mediated by the interaction of nuclear receptors with coactivators such as SRC-1/p160, p300/CBP, ARA70, Tip60 and RIP140. This kind of coactivator recruits histone acetyltransferase (HAT) or bears HAT activity within itself. The recruitment of HAT allows the local decondensation of chromatin, followed by the binding of other types of coactivators. Thus, these coactivators facilitate the transcriptional process. In the absence of ligands, certain kinds of nuclear receptors bind to corepressors, which in turn recruit histone deacetylase (HDAC). The chromatin-modifying complexes, chromatin-remodeling complexes, and the HAT or HDAC regulate chromatin structures and gene transcription. The estrogen receptors can modulate the transcription of genes by various means including the classical interaction of activated receptors with estrogen response elements on the DNA and the indirect effects of estrogen receptors on transcription

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interactions through protein-protein interaction with Sp1, AP-1 and NF-κB. The non-genomic effects of estrogen *via* membrane receptors may be involved in the control of apoptosis, cell proliferation, and differentiation[1-4].

Two subtypes of COS cell lines, COS-1 and COS-7 cells, were obtained by transformation of an origin-defective mutant of Simian virus 40 (SV 40) which codes for the wild-type T antigen to the CV-1 cells derived from the African green monkey kidney cells. The COS cell lines have been used to produce recombinant proteins[5]. In estrogen receptor-negative COS-7 cells, transiently transfected with the reporter plasmid bearing ERE in a certain promoter, 17β-estradiol (E_2) could induce promoter activities in the cells after cotransfection with expression vectors of the estrogen receptors. The experimental system using COS-7 cells cotransfected both with reporter plasmids and expression vectors of the estrogen receptors has long been used in our laboratory[3,4] and others[6,7]. Except for the reporter plasmids above, however, it is unclear what genes could be up- or down-regulated by 17β-estradiol in the COS-7 cells over-expressing the estrogen receptor α.

A DNA microarray has been developed to examine the gene expression in high-throughput and time/cost-saving fashion[8,9]. Expression profiling is one of the most advanced transcriptomic techniques available to monitor and evaluate the effect of chemicals.

In the present study, utilizing a focused DNA microarray of newly selected estrogen-responsive genes, we examined the expression profiling of estrogen-responsive genes in COS-7 cells which were over-expressing the estrogen receptor α. We found several genes were up- or down-regulated by 17β-estradiol in the COS-7 cells which were cotransfected both with the reporter plasmid and the expression vector of the estrogen receptor α.

MATERIALS AND METHODS

Construction of plasmids

The construction of the expression vector of human-origin (h)ERα, pcDNA-hERα, the reporter plasmid for monitoring the transcriptional activation ability of the estrogen receptor, pEREtkCAT, and the expression vector of β-galactosidase (β-gal), pcDNAβ-gal, was described previously[3,4].

Cell culture

COS-7 cells were purchased from The Institute of Physical and Chemical Research (RIKEN, Wako, Japan). They were maintained in Dulbecco's modified Eagle's minimal essential medium (DMEM, Sigma Co., St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Medical & Biological Laboratories Co., Nagoya, Japan). The cells were maintained in 5% CO_2 at 37 °C.

Transfection of plasmids

The medium for COS-7 cells in a screw-capped flask (25 cm^2) was replaced by phenol red-free MEM (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with a 10 % dextran-coated charcoal-treated (DCC) FBS 48 h before transfection[3]. The COS-7 cells were cotransfected with plasmids by using Lipofectamine™ 2000 (Invitrogen Co., Groningen, The Netherlands) as indicated (Fig. 1), and incubated for 48 h in the absence or presence of 10^{-7} M 17β-estradiol after transfection. The cells were then submitted to a chloramphenicol acetyltransferase assay and DNA microarray assay.

Chloramphenicol acetyltransferase assay

The chloramphenicol acetyltransferase (CAT) assay and the β-gal assay were performed as mentioned previously[3,4]. Briefly, a FAST CAT Yellow (deoxy) CAT Assay Kit (Molecular Probes, Eugene, OR, USA) was utilized in the CAT assay. The cell extract was prepared according to the manufacturer's manual. A portion of the

cell extract was incubated with a fluorescent deoxychloramphenicol substrate and acetyl CoA at 37 °C for 2 h. Quantification of the acetylated derivative and the substrate was undertaken by measuring the absorbance with a fluorescence spectrophotometer, model F-3010 (Hitachi Co., Tokyo, Japan). For the β-gal assay, the other portion of the cell extract was incubated with o-nitrophenyl β-galactopyranoside at 37 °C for 30 min. The relative CAT activities were calculated according to the manufacturer's manual and normalized by β-gal activities. The background CAT activity of the cells cotransfected with the parental plasmid, pcDNA3 (Invitrogen Co.) instead of pcDNA-hERα was subtracted from each value of CAT activity.

cDNA microarray assay

A focused cDNA microarray (EstrArray™) was manufactured by InfoGenes Co., Ltd. (Kashiwa, Japan) through mechanical spotting of cDNA fragments (500 bp to 1.5 kb) for 203 genes including a set of 172 estrogen-responsive genes as described previously[10]. Briefly, the 172 genes were selected based on the results from expression profiling of approximately 20,000 genes of MCF-7 breast cancer cells using Human U95A oligonucleotide probe arrays (Affymetrix, Santa Clara, CA, USA) and a Human UniGEM ver. 2.0 microarray system (IncyteGenomics, Palo Alto, CA, USA). They consisted of 108 up- and 64 down-regulated genes in the 17β-estradiol-stimulated MCF-7 cells. EstrArray™ contains a total of 203 genes including of 172 estrogen-responsive genes, three expression marker genes and 28 control genes used for normalization of Cy3/Cy5 ratios of signal intensities.

The COS cells were cotransfected with pcDNA-hERα (0.01 μg) and pEREtkCAT (1 μg) in a flask (25 cm^2) and incubated as mentioned above (designated as COS-7 + ERα) cells. For the EstrArray™ assay, total RNA was isolated from the cells according to the manufacturer's instruction using ISOGEN (Wako Pure Chemical, Tokyo, Japan). Antisense RNA (aRNA) was prepared according to the manufacturer's protocol from 5 mg of total RNA using a RiboAmp RNA Amplification Kit (Takara Bio, Otsu, Japan). cDNA labeled with either Cy3 or Cy5 was synthesized from 4 μg of aRNA which was derived from 17β-estrtadiol-treated cells (corresponding to Cy3) or 17β-estrtadiol-untreated cells (Cy5) using an Atlas PowerScript Fluorescent Labeling Kit (Takara Bio, Otsu, Japan). The labeled cDNA probes were purified using a CyScribe GFX Purification Kit (GE Healthcare, Tokyo, Japan) and then utilized for co-hybridization onto the EstrArray™ overnigh at 65 °C in a hybridization buffer (5 x SSC containing 0.5% SDS; 1x SSC consists of 150 mM sodium chloride and 15 mM sodium citrate). The slides were washed for 5 min at room temperature with 2x SSC/0.2% SDS, followed by washing for 5min with 0.2x SSC/0.2% SDS and with 0.2x SSC. The slides were scanned using a ChipReader (Virtek, Ont., Canada) and the image was analyzed with IPLab software (Scanalytics, Fairfax, VA, USA). The EstrArray™ assay was performed twice using independent cell cultures.

Data analysis

For data processing, we used the values of Cy3- and Cy5-signals when their intensities were equal to or greater than 30, and their corresponding signal areas were equal to or greater than 50. The calculation of expression ratios was carried out using Microsoft Excel software. The average signal intensities for Cy3 or Cy5 between duplicated spots were calculated and the ratio of the mean signal intensity for Cy3 to that of Cy5 was then calculated for each gene. For normalization, the ratios of Cy3 to Cy5 signal intensities for 28 control genes were averaged after removing the genes whose signal areas for either Cy3 or Cy5 were less than 100. Finally, the ratios of Cy3/Cy5 for all genes were normalized by dividing the ratios with the mean

Cy3/Cy5 ratio for 28 control genes.

RESULTS AND DISCUSSION

Transcriptional activation ability of the estrogen receptor α over-expressed in the COS-7 cell

First we evaluated whether the estrogen receptor α over-expressed in COS-7 cells, could mediate transcriptional activities in a cell. The COS-7 cells were transiently cotransfected with the reporter plasmid bearing EREs in the tk promoter and the expression vector of the estrogen receptor α, pcDNA-hERα, as shown in the bottom of Fig. 1 and incubated in the absence or presence of 17β-estradiol. In the presence of 17β-estradiol, the estrogen receptor α enhanced the transcriptional activity (relative CAT activity) by 167-fold over

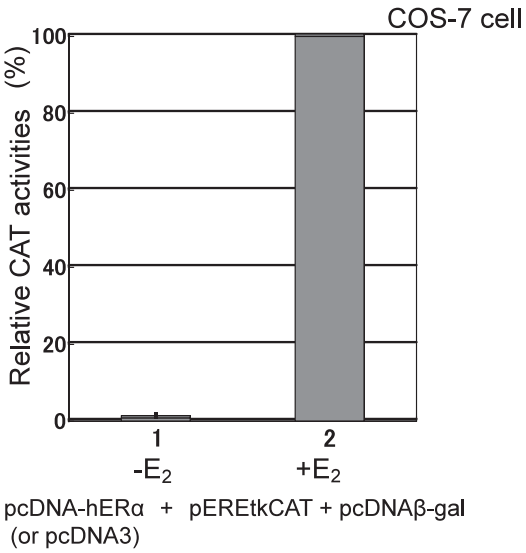

Fig. 1. Evaluation of transcriptional activation ability of estrogen receptor α over-expressed in COS-7 cells by the reporter plasmid bearing ERE in the tk promoter The COS-7 cells were transiently transfected with plasmids as indicated at the bottom. pcDNA3 (a parental plasmid of pcDNA-hERα) was added to obtain the same input DNA amount. pcDNAβ-gal was cotransfected to normalize transfection efficiency. After transfection, the cells were incubated for 48 h in the absence (No.1) or presence (No.2) of 10^{-7} M 17β-estradiol (E_2). In each graph, the background CAT activity values in cells transfected with pcDNA3 instead of pcDNA-hERα were subtracted from each CAT activity values. The CAT activity in the presence of 17β-estradiol (No.2) is represented as 100 %. Values are the means ± SD of three independent experiments.

activity in the absence of 17β-estradiol. Therefore, the estrogen receptor α was transcriptionally active in the COS-7 cells

Focused DNA microarray (EstrArray™) analysis

By using a focused DNA microarray (EstrArray™), we examined the genes up- or down-regulated in the presence or absence of 17β-estradiol in the COS-7 cells which over-express the estrogen receptor α by transient transfection of the expression vector of the estrogen receptor α, pcDNA-hERα, as shown in the bottom of Fig. 1. The genes which were up- or down-regulated over 1.5-fold ($+E_2/-E_2$ ratio or $-E_2/+E_2$ ratio, respectively) in the 17β-estradiol-stimulated COS-7 cells were shown in Table 1. The data of COS-7 cells was compared with that of the MCF-7 cell (Table 1). They were mostly the members necessary for gene expression and metabolism of amino acids and polynucleotides. Transcription elongation factor A is necessary for RNA chain elongation[11, 12]. Solute carrier family 7 members 11 and 5 are for amino acid transport[13]. Methylenetetrahydrofolate dehydrogenase ($NADP^+$ dependent) 2, methylenetetrahydrofolate cyclohydlase is for folic acid derivative biosynthesis[14]. The function of retinoblastoma binding protein 8 is a potential modulator of transcriptional regulation of BRACA1, DNA repair and cell cycle checkpoint control[15]. Phorbol-12-myristate-13-acetate-induced protein 1 promotes activation of caspases and apoptosis[16]. Thus the growth of COS-7 cells could be inhibited. The estrogen receptor α influenced not only gene expression of the transient transfected reporter gene bearing ERE in the tk promoter (Fig. 1), but also the intrinsic gene expression (Table 1).

Comparison between the genes responding to 17β-estradiol in COS-7 cells and MCF-7 cells

The mean magnitudes of up- or down-regulation of genes in response to 17β-estradiol in the COS-7 cells which over-express the estrogen receptor α

Table 1. The genes which were up- or down-regulated over 1.5-fold in response to 17β-estradiol in the COS-7 cells which over-express the estrogen receptor α By using a focused DNA microarray (EstrArray™), we examined the genes up- or down-regulated in the COS-7 cells which over-express the estrogen receptor α by transient transfection of plasmids as shown in the bottom of Fig. 1. The genes which were up- or down-regulated over 1.5-fold in means of independent duplicate experimental data [+17β-estradiol (E_2)/ − E_2 ratio or − E_2/+E_2 ratio, respectively] were shown and compared with the results of MCF-7 cell (the data by InfoGenes Co., Ltd.). The results (fold induction) are shown as r; the ratio of Cy3 signal intensity from 17β-estradiol-treated cells to Cy5 from 17β-estradiol-untreated cells. r = Cy3/Cy5 (in the case of 1 ≦ Cy3/Cy5) or r = -Cy5/Cy3 (in the case of Cy3/Cy5<1)

(fold induction)

Acc. No.	Gene Synbol	Human UniGene Name	COS-7 1st	COS-7 2nd	COS-7 Means	MCF-7
NM 021127	PMAIP1	Phorbol-12-myristate-13-acetate-induced protein1	1.9	1.7	1.8	2.2
NM 006756	TCEA1	Transcription elongation factor A (SII), 1	2.1	1.6	1.8	1.8
NM 014331	SLC7A11	Solute carrier family 7 (cationic amino acid transporter, y+ system) member11	2.0	1.4	1.7	8.8
NM 006636	MTHFD2	Methylenetetrahydrofolate dehydrogenase ($NADP^+$ dependent)2, methenyltetrahydrofolate cyclohydrolase	1.5	1.4	1.5	3.3
NM 002894	RBBP8	Retinoblastoma binding protein 8	-1.2	-1.7	-1.5	5.2
NM 003486	SLC7A5	Solute carrier family7 (cationic amino acid transporter, y+ system) member5	-1.3	-1.9	-1.5	4.0

Acc.No. : Accession No. of GeneBank.

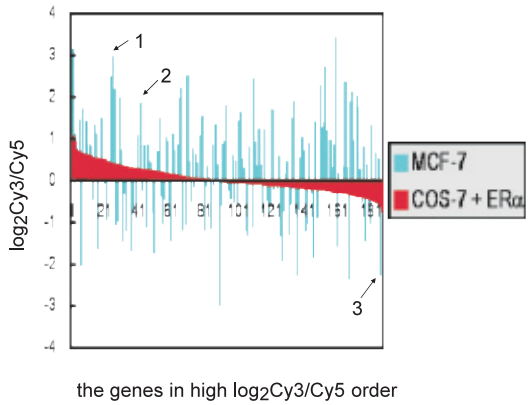

Fig. 2. Comparison between the genes responding to 17β-estradiol in COS-7 cells and the corresponding genes of MCF-7 cells The genes which were up- or down-regulated in the COS-7 cells (red vertical bars in the graph) over-expressing the estrogen receptor α by transfection of the expression vector pcDNA-hERα were arranged according to order from high expression values (left) to low expression values (right). They were compared with the corresponding genes in the presence or absence of 17β-estradiol in a human breast cancer cell line, MCF-7 cells (blue vertical bars in the graph) expressing an intrinsic estrogen receptor α. The y-axis represents $\log_2$Cy3/Cy5. Cy3: the value from the 17β-estradiol-treated cells and Cy5: the value from the 17β-estradiol-untreated cells. The previously identified estrogen-responsive genes, No.1: PDZK1, No.2: EGR3, No.3: IGFBP5, were indicated by the arrows and the numbers[10].

were arranged in order from high expression (left) to low expression (right). They were compared to the gene expression profile of a human breast cancer cell line, MCF-7 cells expressing an intrinsic estrogen receptor α (InfoGenes Co., Ltd.) (Fig.2). The genes which were up- or down-regulated in the COS-7 cells which over-express the estrogen receptor α (red vertical bars in the graph) were very different from the genes which were up- or down-regulated in MCF-7 cells which express an intrinsic estrogen receptor α (blue vertical bars in the graph). The previously identified estrogen-responsive genes, No.1: PDZK1, No.2: EGR3, and No.3: IGFBP5[10] (indicated by the arrows and the numbers in the graph), showed prominent differences in estrogen-responsiveness between the two cell lines. In the COS-7 cells, in the presence of 17β-estradiol, the estrogen receptor α influenced mainly the gene expression of the reporter gene (CAT) bearing EREs in the tk promoter.

The regulation mechanisms for the genes on EstrArray™ were not fully investigated. The expression of genes under the control of eukaryotic promoters may depend on the chromatin structure surrounding DNA and the accessibility of the transcription factors to the promoter. The

methylation of the promoter has been demonstrated to correlate with gene silencing[17]. The COS-7 cell is not a target cell for estrogen. The cell does not have intrinsic estrogen receptors and was assumed not to have the machineries to respond to the estrogen receptor signal. Many of the estrogen-responsive genes in MCF-7 cells might require cofactors or other proteins to control chromatin remodeling in responding to estrogen, but some of these factors may not have been available in COS-7 cells. The effects of estrogen on the reporter gene (CAT) bearing ERE in the tk promoter were extremely amplified (Fig. 1). The genomic EREs in COS-7 cells may not have been exposed to the estrogen receptor to bind, and many genes did not participate in biological effects. The estrogen receptors can modulate transcription of reporter genes in various modes including the estrogen receptor-ERE interaction and the protein-protein interaction as mentioned above. The effects of estrogen observed in this study might be caused by estrogen receptor-protein interactions, but not by interactions between estrogen receptors and genomic EREs. The estrogen receptor expressed in the COS-7 cells may exercise positive or negative effects on the metabolism of amino acids and polynucleotides, cell cycles or apoptosis (Table 1). The effects of estrogen on MCF-7 cells were more numerous, more complicated, and more potent (Fig. 2). In summary, estrogen causes the cell growth in MCF-7 cells expressing intrinsic estrogen receptor α[18], but not in the COS-7 cells with the estrogen receptor α over-expressed (data not shown).

A focused DNA microarray (EstrArray™) containing estrogen-responsive genes from human cells was previously developed by some of the authors. They analyzed the effects of estrogens, phytoestrogens, and estrogen antagonists and reported that all genes examined showed similar responses in both DNA microarray and RT-PCR analysis. This confirms the results of the DNA microarray assay[19]. Others also demonstrated that the accuracy of data for a focused DNA microarray based on RT-PCR analysis, thus confirming the reliability of data where gene expressions were up- or down-regulated 1.3-fold as compared to the control data[20, 21]. The gene-expression analysis using a focused microarray is accurate enough to examine whether the selected genes are up- or down-regulated over 1.5-fold, and the cost and time needed for the analysis are effectively reduced.

ACKNOWLEDGMENTS

This study was supported in part by Research Project Grants (No. 20-105N) from Kawasaki Medical School.

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