Analysis of temporal changes in the expression of estrogen-regulated genes in the uterus

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Abstract

In order to understand early events caused by estrogen in vivo, temporal uterine gene expression profiles at early stages were examined using DNA microarray analysis. Ovariectomized mice were exposed to 17β-estradiol and the temporal mRNA expression changes of ten thousand various genes were analyzed. Clustering analysis revealed that there are at least two phases of gene activation during the period up to six hours. One involved immediate-early genes, which included certain transcription factors and growth factors as well as oncogenes. The other involved early-late genes, which included genes related to RNA and protein synthesis. In clusters of down-regulated genes, transcription factors, proteases, apoptosis and cell cycle genes were found. These hormone-inducible genes were not induced in estrogen receptor (ER) α knockout mice. Although expression of ERβ is known in the uterus, these findings indicate the importance of ERα in the changes in gene expression in the uterus.

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Introduction

The sex hormone, 17β-estradiol (E2), is a well-characterized steroid hormone that controls the development of the reproductive tract and mammary gland, regulates the estrous cycle, controls lactation, and exerts regulatory influences that are important for bone, liver and cardiovascular systems, as well as for behavior. These effects are mainly exerted by estrogen receptors (ERs) (Couse & Korach 1999). The critical regulatory functions of estrogen are mediated by ERs and their molecular mechanisms of transactivation have been well characterized. At least two types of estrogen receptors, ERα and ERβ, are known in mammals (Green et al. 1986, Kuiper et al. 1996). They bind to estrogen-response elements (EREs) within enhancer regions of target genes to regulate transcription in a ligand-dependent manner. The bound ERs then recruit cofactors related to p160 family members such as SRC-1 (Onate et al. 1995), GRIP1 and amplified in breast cancer 1 (AIB1) (Anzick et al. 1997), and histone acetylases such as CBP and p300 (Chakravarti et al. 1996) to reorganize chromatin structure and activate transcription.

Despite great progress in characterizing the molecular biology of ERs, the mechanisms underlying the physiological changes such as the uterotrophic effect induced by E2 remain largely unknown. This is partly because most of the genes known to be activated by estrogen have been determined by studying the effects of E2 on cultured cells and because our knowledge about the genes actually activated in vivo is rather limited. Especially, our knowledge of temporal changes of estrogen response genes in the rodent uterus is not sufficient to allow a full understanding of the long-term physiological effects of estrogen. For example, c-fos and c-jun are known to be activated...
within 30 min-2 h (Weisz & Bresciani 1988, Weisz et al. 1990) and c-myc is activated for 4–6 h after estrogen administration. On the other hand, expression of estrogen-responsive zinc finger protein is at its maximum around 10 h (Inoue et al. 1993) and lactoferrin and complement C3 (Sundstrom et al. 1989) are known to be activated after 24–78 h (Pentecost & Teng 1987). Although these genes are known as estrogen responsive genes, the mechanisms relating to the temporal control of the expression of these genes is largely unknown. Thus, determining the temporal changes of gene expression levels affected by estrogen and the identification of these genes are important for our understanding of the transcriptional cascade evoked by estrogen.

The uterus is a prominent E2 target organ, which also shows drastic changes during the estrous cycle. In order to clarify the mechanisms of the effect of estrogen on the reproductive tract, it is essential to identify the genes affected by estrogen, especially those whose expression is affected during the early stages of the uterine response. However, our knowledge of the overall number of uterine genes regulated by estrogen is rather limited except for information from microarray analysis (Watanabe et al. 2002), and only a limited number of genes, such as proto-oncogenes (Weisz & Bresciani 1988, Weisz et al. 1990) and growth factors (Liu et al. 1987, Murphy et al. 1987, Huet-Hudson et al. 1990), are known to be early induced genes, which are also activated by other stimuli such as mitogen and serum in vitro. As the ER is one of the transcription factors, an analysis of changes in mRNA expression patterns caused by E2 provides a powerful tool for understanding the molecular mechanisms underlying estrogen action. Thus, genome-wide gene expression profiling by E2 administration is important to fully understand the dramatic effects of estrogen on the uterus.

To analyze genome-wide gene expression changes, we employed the DNA microarray technique. DNA microarray technology has recently been developed and successfully applied to the genome-wide analysis of gene expression as influenced by various stimuli such as serum (Iyer et al. 1999), hormones (Feng et al. 2000) or chemicals (Marton et al. 1998). High-density oligonucleotide arrays (Lockhart et al. 1996) are especially suitable for genome-wide mapping of gene expression because a large number of genes can be analyzed at one time and it is readily scalable to the simultaneous monitoring of tens of thousands of genes.

In this study, the early time course changes in the expression of ten thousand genes were profiled and E2-regulated genes were clustered. We found that there are at least two phases in the gene activation pattern. As the change in gene expression was not evoked in the ERα-deficient mice, a major contribution of ERα but not ERβ in the transcription regulation in the uterus was indicated.

### Materials and methods

#### Animals

Animals were housed under a 12-h light, 12-h darkness cycle. ERα knockout (αERKO) mice and their wild-type counterparts (C57/BL6/J background; Lubahn et al. 1993) were ovariectomized at eight weeks of age. After two weeks, the ovariectomized mice were injected with 17βestradiol (E2; Sigma; 5 µg/kg body weight (b.w.)) intraperitoneally and total uterine RNA was prepared. Total uterine RNA from wild-type mice was prepared at 0 h, 1 h, 2 h, 6 h, 12 h, 24 h and 48 h after estrogen administration (5 µg/kg b.w.). Total uterine RNA from αERKO mice was prepared 6 h after E2 administration and processed as for the wild-type. All animal experiments were approved by the institutional animal committee.

#### Preparation of labeled cRNA and hybridization

Total uterine RNA was extracted using TRIZOL (Invitrogen, Tokyo, Japan) and purified using RNeasy (Qiagen, Tokyo, Japan). Total RNA was converted into double stranded cDNA using the SuperScript Choice System (Invitrogen) and labeled cRNA was synthesized according to the manufacturer’s protocol (Amersham Biosciences, Tokyo, Japan). Labeled cRNA was purified using RNeasy, denatured and subjected to limited fragmentation in fragmentation buffer (40 mM Tris–HCl (pH 8.1), 100 mM potassium acetate, 30 mM magnesium acetate) by heating at 94 °C for 35 min. Fragmented cRNA was mixed with hybridization buffer containing 100 mM 2-(N-Morpholino)ethane sulfonic acid, 1 M NaCl, 20 mM EDTA and 0.01 % Tween 20 and control oligonucleotides. High-density oligonucleotide
arrays (Mouse U74 A, Affymetrix, Amersham Biosciences) were hybridized for 16 h at 45 °C. Arrays were then washed, stained with streptavidin-phycocerythrin (Molecular Probes, Funakoshi, Tokyo, Japan) in an Affymetrix fluidix station and scanned with an argon-ion laser confocal scanner (Amersham Biosciences).

Data analysis
Scanned data were analyzed with GeneChip software (Affymetrix, Amersham Biosciences) and detailed methods for data analysis have already been described (Lockhart et al. 1996). Briefly, each gene is represented by the use of 20 perfectly matched (PM) and mismatched (MM) control oligonucleotides. The MM probes are used to detect background levels and cross-hybridization signals. The decision about the absence or presence of an mRNA is made by GeneChip software using values derived from these 20 sets of probes (Lockhart et al. 1996). To determine the quantitative RNA abundance, the average of the differences (called ‘average difference’) representing PM minus MM for each gene-specific probe is calculated. To normalize data, genes called ‘A’ (absence) during the 6 h were masked, negative average differences were adjusted to zero and rescaling factors were adjusted to produce an average intensity that equalled 2500.

To obtain convincing gene expression levels from the DNA microarray, we repeated the same experiment at least two times independently and the differences in intensities were calculated. In order to maintain reproducibility, we only selected the genes whose expression level changes were less than twofold between two independent experiments under the same conditions and average values were used as gene expression levels.

Clustering analysis
Clustering analysis was performed using GeneCluster software based on a self-organizing maps algorithm (Tamayo et al. 1999). Genes showing a max/min ratio greater than threefold in their gene expression levels were selected and their expression levels, represented by the average difference were normalized to a mean value of zero and a variance of one. The normalized data were then clustered using GeneCluster (http://www-genome.wi.mit.edu/cancer/software/software.html).

Quantitative real time-PCR (Q-RT-PCR)
Total RNA was extracted from the uterus using TRIZOL and purified with RNeasy. cDNA was synthesized from purified total RNA by Superscript II RT(−) (Invitrogen) with random primers at 42 °C for 60 min. PCR reactions were performed in the PE Prism 5700 sequence detector (PE Biosystems, Tokyo, Japan) using SYBR-Green (Molecular Probes, Eugene, OR, USA) in the presence of appropriate primers according to the manufacturer’s instructions. The assay uses fluorescence emitted by SYBR-Green to quantify double-stranded DNA produced during the PCR reaction. The use of a sequence detector allows the continuous measurement of fluorescent spectra of all 96 wells of the thermal-cycler during PCR amplification. The model 5700 software constructs amplification plots from extension phase fluorescent emission data collected during PCR amplification. C\textsubscript{T} (threshold) values were calculated by determining the point at which fluorescence exceeds a threshold limit (usually 10 times the s.d. of the baseline).

For the standard curves of the genes, serial dilutions of a known amount of a cDNA sample were used. The C\textsubscript{T} values of each gene were plotted on these standard curves to obtain the number of copies present in the initial cDNA sample. Each PCR amplification was performed in triplicate, using the following conditions: 2 min at 50 °C and 10 min at 95 °C, followed by a total of 40 two-temperature cycles (15 s at 95 °C and 1 min at 60 °C). Gene expression levels were normalized to that of 28S ribosomal RNA. Gel electrophoresis and melting curve analyses were performed to confirm correct amplicon size and absence of nonspecific bands.

Primers were chosen with the assistance of the Primer Express (PE) computer program, and were NM_010234 (c-fos): ACGCACAGACCA CACAAACAG, GCTAGCAGCCACAGCTCG TG; U83902 (Mad2): GCCGTCGTCCGGTGA GG, TCGGGCAGCTGCTGT; U19118 (LRG-21, ATF3): GGGTCCTA TGCAAAGCAG GAT, TCAAGGTAGACTTCTGCTGAA; U77630 (adrenomedullin): ATAAGGCTACATTA CTACTTGAACT, TTGCAGGTCTCCTGCTGA; M28845 (EGR-1): GAGTACCCGTTCCT GCCTAAAA, GGTAGGACTCAGGGCTGAAA AACA; NM_010849 (Myc): ACCTTCCCTTTCT
Figure 1 Temporal changes of gene expression pattern. Scatter plot of gene expression levels between each time point (Y axis) and 0 h (X axis). Each dot corresponds with each gene, whose X value and Y value indicate the gene expression level represented by average difference at 0 h and at the indicated time point respectively. Correlation coefficients and formula used for the derivation of the correlation curve are also indicated.

GACAGA ACTGA, GGTTAGGCTTTGGCAT GCAT; AF009246 (DEXRAS1): GGAAGTAGA GCAGCGGGAGA, CGAAGTAGGCACAACG CTGA; L07264 (HB-EGF): TGGAGAATCCC CTATACACA, CAGCACCACAGCCAAGACT; M96163 (serum inducible kinase): ACCAGAC CAGCCTAAGGTGTAGAG, GACAGGTATCT CAGGGAAAACTGC; M59821 (pip92): TTGAATCTCAGGGTCGAACTCT, ATGGTAGT GAAACGGCCTTGA; NM_146217 (alanyl-tRNA synthetase): TTGACAGCAACCCCAACCA, CT CCACCTCCTCCTCCAGGAT; XM_133968 (cysteinyl-tRNA synthetase): GGGCCACTTGAC GATAGCA, AAGTTTTTCAGTGACTTTGGAC ATCT; XM_123283 (histidyl tRNA synthetase): ATTTTGACATTGCCGGACAGT, GGCACTC TGATCAGGAATCA; AF123263 (phenylalanyl tRNA synthetase beta subunit): GGAGGACG CAGCTATTGCTTAT, GTACGTTTTCGGCA GAGTCATCT; BC008612 (seryl-aminoacyl-tRNA synthetase 1): CCCAGAGAAGCTGTCT AGTTTC, TGAGACTTTCAGGGCAGCTA GC; U95826 (cyclin G2): ACTGCCGAGGGTCTTCTCCAA, GCACAGAGCTAATA CACTGGTTCT; U42467 (leptin receptor): CTTTGAAAGCCCTGACGAA, CGTACCTCCTCACA CACTACAGT; M61215 (ferrochelatase) TG GTGAAGCTGCTGGATGAG, GGGATGGAC GTACCGGAATC; Y13090 (caspase 12): ATT GGCCCATGAATCAGTCTAAT, GGACAGGGCTTCAGTGTATCTTGAG; AJ007970 (guanylate nucleotide binding protein 2): CAGCCACCTGTCAGTAACGCAGT, GCCAACCA GCTTCAGTGTATCTTGAGA; AJ007970 (guanylate nucleotide binding protein 2): CAGCCACCTGTCAGTAACGCAGT, GCCAACCA
Results

In order to get an overview of the temporal expression of genes affected by estrogen in the uterus, total uterine RNA was prepared at 1 h, 2 h, 6 h, 12 h, 24 h and 48 h after estrogen administration. To evaluate temporal changes in gene expression, we compared gene expression levels at each time point with 0 h. As shown in Fig. 1, the changes in gene expression reached a maximum between 6 and 12 h after estrogen administration. After 12 h, the number of estrogen-affected genes decreased and no obvious changes remained at 48 h. This result indicates that a single dose of estrogen, 5 µg/kg b.w., which is sufficient to provoke a uterotrophic effect by repetitive administration, can affect uterine gene expression up to 12–24 h after administration. Thus, we used this dose of estrogen to study early gene expression during the period up to 6 h after estrogen administration.

Clustering of changes in gene expression

In order to examine the temporally regulated genes, we selected the genes whose expression levels were changed more than threefold during 6 h and their temporal gene expression patterns were clustered. When the clustering analysis based on the self-organizing map algorithm (Tamayo et al. 1999) was applied to the filtered genes induced during the period up to 6 h, temporal changes in estrogen-affected gene expression could be clustered into six groups. This result indicates that temporal changes of estrogen response genes show at least six gene expression patterns up to 6 h. Figure 2 shows the centroids in the clustered pattern when six (3 × 2) nodes were used for the clustering analysis. The number of genes in each cluster is also indicated in Fig. 2. By clustering analysis, three clusters of induced genes (clusters A-C) and three clusters of repressed genes (clusters D-F) were revealed.
In the case of induced genes, at least three groups of gene expression patterns were revealed (A-C). The genes in the first group (immediate-early genes, cluster A) were activated within a few hours of estrogen administration and their induction was transient. The second group of genes were induced in a linear fashion (cluster B). Genes in the third group (early-late induced genes, cluster C) were activated during the decline in the expression of the early genes.

Among the clusters of repressed genes (D-F), cluster D was characteristic because only three genes were selected in this cluster. On the other hand, the number of gradually repressed genes or genes repressed at the early-late stage (clusters E and F respectively) were comparable to the number of induced genes (clusters B and C respectively). In contrast to the limited number of genes known to be repressed by E2, our findings show that a relatively large number of genes are repressed by E2. The list of clustered genes is indicated in Fig. 4.

**Functions of the genes in each cluster**

Based on the clustering result, we examined the functions of the genes and the percentage representation of each gene function in each cluster. When gene functions were categorized into 13 groups (names of the categories are indicated in Fig. 3), it was found that the dominant gene functions were different in each cluster (Fig. 3). In cluster A, there was a notable occurrence of a large number of immediate early genes together with a limited number of known genes, such as heparin-binding epidermal growth factor (Wang et al. 1994). Their expression patterns and names are indicated in Fig. 5 (upper panel). In cluster C (early-late genes), genes related to RNA processing, such as capping, poly-adenylation and splicing were activated and genes responsible for protein synthesis such as initiation factors, elongation factors, releasing factors and tRNA-synthetases were found. Most of the genes related to RNA and protein synthesis that were analyzed by the array showed similar temporal changes, which are indicated in Fig. 5 (lower panel). This result indicates that a part of functionally related genes are regulated in a similar fashion at the transcriptional level.

The expressions patterns of some of the genes were further confirmed by Q-RT-PCR as shown in Fig. 6. Although temporal changes in gene expression patterns as determined by the two systems were not completely identical, most of the expression patterns determined by DNA microarray analysis agreed well with those determined by Q-RT-PCR. As shown in Fig. 6, temporal changes of gene expression were confirmed not only for induced genes but also for repressed genes.

**Effect of estrogen in a αERKO mouse**

In order to determine if these genes are affected by E2 in an ERα-dependent manner, we examined gene expression changes induced by E2 in αERKO mice. The fold changes of wild-type mice were calculated and compared with those of ERKO mice and indicated by scatter plot (Fig. 7). Most genes affected by E2 in wild-type mice (X-axis) were not affected in αERKO mice (Y-axis), indicating that E2 does not affect E2-mediated gene expression in mice lacking the ERα receptor. This result indicates that uterine gene expression changes induced by estrogen are mainly mediated by ERα at the transcriptional level. The fact that neither induced genes nor repressed genes were affected by E2 in αERKO mice suggests that ERα also regulates most of the estrogen-repressed genes. Our results are consistent with previous observations in αERKO mice showing that αER disruption causes severe uterine defects (Lubahn et al. 1993).

![Figure 3](image1.png)  
**Figure 3** Functional classification of clustered genes. Gene functions of the clustered genes were categorized into thirteen functions (right panel) and the percentage representation of each function is indicated. Each cluster corresponding to Fig. 2 is indicated on the left. Cluster D was omitted because it contained only three genes. EST genes were also categorized based on gene similarity. ECM, extra cellular matrix.
and that ERβ cannot compensate for ERα function. Although ERβ, another weakly expressed ER in the uterus, is another candidate for E2-dependent changes in gene expression, it was revealed that the contribution of ERβ in transcriptional regulation is very small, if it occurs at all.

**Discussion**

In this study, we examined temporal gene expression changes at early stages using the DNA microarray technique. Clustering analysis of estrogen-affected genes indicated that some functionally related genes show similar temporal expression levels, which suggests a coordinated response to estrogen signaling. This finding is consistent with the hypothesis that estrogen has pleiotropic effects on gene regulation through ERα and ERβ.

### Figure 4

List of clustered genes. The gene names and GenBank accession numbers in each cluster are indicated. Only the genes with annotations are indicated with their gene names. Changes in expression levels are also indicated by color based on normalized values (green indicates low and red indicates high values in normalized values) with time points (in hours) indicated above.

**Up Regulated Genes**

<table>
<thead>
<tr>
<th>Accession</th>
<th>Gene Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>U20735</td>
<td>Transcription factor jumbl (mimB)</td>
</tr>
<tr>
<td>U83902</td>
<td>Mtoric checkpoint component Mad2</td>
</tr>
<tr>
<td>AF902180</td>
<td>hair keratin basic 5 (Hd5)</td>
</tr>
<tr>
<td>M28845</td>
<td>Early growth response 1</td>
</tr>
<tr>
<td>U35374</td>
<td>purine nucleoside phosphorylase (Np-b)</td>
</tr>
<tr>
<td>M17979</td>
<td>Epidermal growth factor binding protein type A</td>
</tr>
<tr>
<td>U11918</td>
<td>transcription factor LRG-23</td>
</tr>
<tr>
<td>M32490</td>
<td>Insulin-like growth factor binding protein 10</td>
</tr>
<tr>
<td>M59821</td>
<td>growth factor-inducible protein (pip92)</td>
</tr>
<tr>
<td>U31993</td>
<td>Interleukin 17 receptor</td>
</tr>
<tr>
<td>M89979</td>
<td>Wingless-related MMTV integration site 4</td>
</tr>
<tr>
<td>X79903</td>
<td>Integrin alpha 5 (fibronectin receptor alpha)</td>
</tr>
<tr>
<td>U17264</td>
<td>Hepatocyte nuclear factor 4</td>
</tr>
<tr>
<td>M59163</td>
<td>serum-inducible kinase (SNK)</td>
</tr>
<tr>
<td>AF809246</td>
<td>ras-related protein (Dexras1)</td>
</tr>
<tr>
<td>Y77630</td>
<td>Adrenomedullin</td>
</tr>
</tbody>
</table>

**Down Regulated Genes**

<table>
<thead>
<tr>
<th>Accession</th>
<th>Gene Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>U19641</td>
<td>Small inducible cytokine A2</td>
</tr>
<tr>
<td>X65128</td>
<td>Growth arrest specific 1</td>
</tr>
</tbody>
</table>

**CLASS B**

- AF800203: Chorionic gonadotropin receptor 1
- AB011030: FSHR
- AF064740: type VI collagen alpha 3 subunit
- U53142: Nitric oxide synthase 3, endothelial cell
- L1797: HIF-1 alpha |

**CLASS C**

- U11274: RNA-binding protein AUF1
- M12547: Actin, alpha 1, skeletal muscle
- U10185: B lymphocyte induced maturation protein
- AF041892: T-box 15
- AB099933: collaggen a(V)

**CLASS D**

- U08875: anti-DNA antibody light chain variable region
- X9206: ubiquitin-conjugating enzyme UevM2
- U38940: asparagus syntaphase
- X58842: Wingless-related MMTV integration site 3A
- L22121: Potassium voltage-gated channel, shaker-related, member 5
- M65254: inhibitor protein of AMP-dependent protein kinase

**CLASS E**

- U07861: Zinc finger protein 101
- U43938: NAD(P)-dependent 15-20hydroxysteroid dehydrogenase
- Y84693: Testis nuclear RNA-processing protein
- U76372: CBP or p300 activator protein
- M13226: GsyntX4
- U28405: Chemokine (C-C) receptor 1, like 1
- U63841: Neurogenic differentiation 3
- AF107780: potassium channel Kv4.2
- AF027133: Mucin 5, mucosal
- X40673: Adipin
- U39545: Bone morphogenetic protein 8b
- M21285: Stathmin-coyntese A destratase 1
- U51865: Liver arginase
- AF108054: monocarboxylate transporter 2 (mMCT2)
- AB10829: growth differentiation factor-9 (GDF-9)

**CLASS F**

- U34557: Fucosyltransferase 4
- U34267: Lepin receptor
- M24417: P glycoprotein 3
- T76440: Necdin
- M23632: Soluble binding protein 1
- X8070: Brevicin
- M88694: transmembrane serine protease
- Y1779: Dna1 protein
- U12791: 3-hydroxy-3-methylglutaryl coenzy A dehydrogenase 2
- U023957: EGF 180 mRNA for endothelial inducible gene product
- U95224: cyclin G2
- Y92688: RecA-like protein (mRERE2)
- U43541: Laminin, beta 2
- X69810: Esp6-fl2
- Y1628: Basigin
- X16664: Adenosine receptor, beta 2
- Y75478: Acidic nuclear phosphoprotein 32
- A962688: S6K1
- M70642: Fibroblast inducible secreted protein

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changes with each other. For example, genes related to signal transduction were mainly found in cluster A, whereas only a few signal transduction genes were categorized in cluster F. Genes related to biosynthesis were mainly found in clusters B and C.

For the clustering analysis, we used six (3 × 2) node numbers. This node number was optimal for Figure 5 Temporal changes in the expression levels of genes categorized in cluster A (upper panel) and genes related to protein and RNA synthesis (lower panel). X-axis indicates time (h) and Y-axis indicates gene expression levels (average differences) evaluated from DNA microarrays. Genes that were induced more than threefold were selected and plotted.
the clustering of the type of gene expression obtained in this study because it gave independent clustering patterns. As an increment of the node number did not produce any new patterns, it can be concluded that these clusters are representative of the temporal changes in gene expression after estrogen administration.

The fact that the expression pattern of the c-fos (Weisz & Bresciani 1988) and c-jun (Weisz et al. 1990) gene, a typical immediate-early gene, showed a very similar pattern to that of other genes clustered in A (Fig. 6 and data not shown) confirms that these genes also belong to the immediate-early group of genes, although they were maximally induced 1–2 h after estrogen administration. The transcription factors and signal transduction genes-clustered in A may play an important role in the gene activation or repression of late genes.

In the immediate-early gene cluster, several genes that are known to be induced by growth factors or serum were identified. For example, early growth response gene (egr-1, M28845) (Janssen-Timmen et al. 1989), serum inducible kinase gene (M96163) (Simmons et al. 1992), growth factor-inducible protein gene (pip92, M59821) (Charles et al. 1990) and serum/glucocorticoid regulated kinase gene (AW046181) (Webster et al. 1993) are known to be activated by serum induction. Among these immediate-early genes, the serum response element is known to be important for the induction of c-fos (Duan et al. 2001), egr-1 (McMahon & Monroe 1995, de Jager et al. 2001) and pip92 (Chung et al. 1998) but no ERE has been reported in the promoter sequences of these genes. Although a more detailed analysis will be necessary, common mechanisms between serum induction and estrogen

**Figure 6** Confirmation of the temporal changes of gene expression levels by quantitative real-time PCR. The fold changes were evaluated by quantitative real-time PCR based on the gene expression levels at 0 h. Representative fold change at each time point is indicated. Names of the examined genes are labeled in each panel.
may be important for the activation of the immediate-early genes. In this context, nonclassical transcription activation via Sp1 or Ap1 may also play an important role in the gene activation.

Clustering analysis also revealed that large numbers of genes were temporally repressed by E2. Some of the repressed genes indicate characteristic features of the estrogen effect. For example, we found that cyclin G2 was repressed. As this gene is known to arrest cell cycle by interacting with protein phosphatase 2A (Bennin et al. 2002), down-regulation of cyclin G2 may indicate the progression of the cell cycle. We also identified caspase-12 as a temporally repressed gene. This gene product evokes apoptosis by stress in the endoplasmic reticulum (Nakagawa et al. 2000). As suggested by the induced genes, many genes related to translation and transportation were activated by estrogen, which may cause stress in the endoplasmic reticulum. Thus repression of the gene related to endoplasmic reticulum stress-induced apoptosis may be responsible for rapid protein synthesis and secretion. Down-regulation of leptin receptor gene is consistent with other gene expression changes which direct sterol synthesis (Watanabe et al. 2002), as it is known that leptin represses the gene expression related to fatty acid and cholesterol synthesis (Liang & Tall 2001).

Our analysis could not cluster all the genes activated by estrogen probably because the time window was rather narrow, the threshold of our analysis was too high and/or some genes were poorly represented on the array. For example, complement C3 (Sundstrom et al. 1989), epidermal growth factor (EGF) (DiAugustine et al. 1988), EGF receptors (Gardner et al. 1989), muc-1 (Surveyor et al. 1995), lactoferrin (Teng et al. 1989) and the progesterone receptor (Manni et al. 1981) could not be selected because the expression levels of these genes did not change during the 6-h period after E2 administration. These genes are known to be activated at 12 h or one or two days after E2 administration. Ornithine decarboxylase was not selected by the clustering analysis because of the rather strict criteria for gene selection. It was activated nearly threefold at 6 h (data not shown). Estrogen-responsive finger protein is also known to be activated by estrogen within 10 h (Inoue et al. 2000).

Figure 7 Comparison of the effect of estrogen on gene expression in wild-type (WT) and ERKO (KO) mice. The genes induced or repressed more than threefold at 6 h were selected from the 182 filtered genes and their fold changes in ERKO mice were calculated based on the ratios of average differences between E2-treated mice and control. The fold changes (log2) in the wild-type are indicated by the X-axis and those in ERKO are indicated by the Y-axis.
expression of the genes. In this study, early events preceding the uterotropic effect could be identified at the transcription level, resulting in the identification of novel gene clusters whose members are most likely responsible for the triggering of uterotropic effects. As several transcription factors and genes related to growth signaling were clustered as immediate-early genes, these genes may play an important role in subsequent changes in gene expression. Functional analysis of these clustered genes and analysis of their regulation will provide new insights into the estrogen effect in vivo.

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