Analysis of estrogen-regulated genes in mouse uterus using cDNA microarray and laser capture microdissection

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Abstract

The steroid hormone, estrogen, plays an important role in various physiological events which are mediated via its nuclear estrogen receptors, ER α and ER β . However, the molecular mechanisms that are regulated by estrogen in the uterus remain largely unknown. To identify genes that are regulated by estrogen, the ovariectomized mouse uterus was exposed to 17 β -estradiol (E2) for 6 h and 12 h, and the data were analyzed by cDNA microarray. The present study confirms previous findings and identifies several genes with expressions not previously known to be influenced by estrogen. These genes include small proline-rich protein 2A, receptor-activity-modifying protein 3, inhibitor of DNA binding-1, eukaryotic translation initiation factor 2, cystatin B, decorin, secreted frizzled-related

protein 2, integral membrane protein 2B and chemokine ligand 12. The expression patterns of several selected genes identified by the microarray analysis were confirmed by RT-PCR. In addition, laser capture microdissection (LCM) was conducted to determine the expression of selected genes in specific uterine cell types. Analysis of early and late responsive genes using LCM and cDNA microarray not only suggests direct and indirect effects of E2 on uterine physiological events, but also demonstrates differential regulation of E2 in specific uterine cell types. These results provide a basic background on global gene alterations or genetic pathways in the uterus during the estrous cycle and the implantation period.

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Introduction

The steroid hormones, estrogen and progesterone, play a pivotal role in the development of the reproductive tract and the regulation of the implantation process. The cellular actions of these steroid hormones are mediated through their ability to bind nuclear receptors, which are basically ligand-activated transcription factors. Estrogen receptors (ERs) are known to exist in at least two types, ER α and ER β , in mammals (Green *et al.* 1986, Kuiper *et al.* 1996). The estrogen-ER complex directly binds to estrogen response elements on target genes and modulates gene transcription in a ligand-dependent manner, the so-called genomic actions of estrogen (Kousteni *et al.* 2001). However, some of the actions of estrogen are mediated by intracellular second messengers or various signal-transduction cascades through nongenomic actions (Angel *et al.* 2000, Ralf & Martin 2003). Together with the brain, the uterus is a major target of estrogen action, the heterogeneous cell types responding differentially to estrogen. To date, relatively few genes have been identified in the uterus that are regulated by estrogen, with the exact molecular pathways of estrogen

and the expression patterns of specific uterine cell types being largely unknown (McMaster *et al.* 1992, Das *et al.* 1994, Wang *et al.* 1994, Diane *et al.* 2000, Brannvall *et al.* 2002).

The aim of this study was to identify early and late responsive genes regulated by estrogen in the mouse uterus and to determine whether estrogen-responsive genes are differentially expressed according to the specific uterine cell types. For elucidation of estrogen-responsive genes in the uterus, we carried out cDNA microarray analysis. The technique of cDNA microarray has been used to profile the genome-wide analysis of gene expression in various uterine tissues such as implantation-interimplantation sites, progesterone-treated, and delayed-implanting uterus (Reese *et al.* 2001, Cheon *et al.* 2002). We also employed a combination of laser capture microdissection (LCM) and RT-PCR to quantify estrogen-responsive genes in specific cell types of the ovariectomized mouse uterus. Recently, LCM has been used to isolate specific cell types from tissue sections for the purpose of differential gene expression (Green *et al.* 2003). Using these techniques, we profiled both early and late responsive genes regulated by estrogen and demonstrated that several responsive genes

were differentially expressed in specific cell types of the estrogen-induced uterus.

Materials and Methods

Animals and estrogen treatment

ICR mice were housed within temperature- and light-controlled conditions under the supervision of a licenced veterinarian. Mice were maintained on a 12 h light:12 h darkness photoperiod and food and water were available *ad libitum*. Female mice (6–7 weeks of age) were ovariectomized (OVX) and rested for 14 days before receiving estrogen treatment. They were injected subcutaneously (0.1 ml/mouse) with sesame oil (control group) alone or with 17 β -estradiol (Sigma, St Louis, MO, USA; 300 ng/mouse; experimental group) dissolved in sesame oil. Mice were killed and uterine horns were collected 6 h or 12 h after injection. Uteri collected from both oil-treated and estrogen-treated mice were used for the microarray and LCM studies. Half the uterus was immediately frozen in liquid nitrogen for RNA isolation as described below and the remaining tissue was embedded in Tissue-Tek (Sakura Finetek, Torrance, CA, USA) for LCM. All animal experiments were performed in accordance with the Ulsan University Guide for the Care and Use of Laboratory Animals.

RNA isolation for cDNA microarray

Uterine tissues collected from nine female mice in each group were pooled, snap frozen, and homogenized by mortar in liquid nitrogen. For cDNA microarray analysis, total RNA was extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) and purified using RNeasy total RNA isolation kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. DNA was digested using an RNase-free DNase set (Qiagen) during RNA purification. Total RNA was quantified by spectrophotometer and its integrity was assessed by running on a denaturing 0.8% agarose gel. Prior to use in cDNA microarray analysis, each uterine RNA sample obtained via the OVX/estrogen treatment/6-h/12-h protocols was validated by assaying for up-regulated or down-regulated genes which are known to be regulated by estrogen in the mouse uterus as markers of estrogen responsiveness.

cDNA microarray and data analysis

To analyze early and late estrogen-responsive genes in the uterus, total uterine RNA was extracted 6 h and 12 h after the injection of estrogen to ovariectomized mice (OVX/estrogen treatment/6-h and 12-h protocol). Profiling of estrogen-regulated gene expression was analyzed with a

TwinChip Mouse-7.4K (Digital Genomics, Seoul, Korea) consisting of 7616 mouse cDNA clones. Each total RNA sample (20 μ g) was reverse-transcribed with Cy3- (for oil-treated group) or Cy5 (for estrogen-treated group)-conjugated dUTP (Amersham Pharmacia Biotech, Piscataway, NJ, USA), using SuperScript II (Gibco BRL, Rockville, MD, USA) and oligo(dT)₁₈ primer (Ambion, Austin, TX, USA) in a reaction volume of 20 μ l according to the method suggested by the manufacturer. After the labeling reaction for 1 h at 42 °C, unincorporated fluorescent nucleotides were cleaned up using Microcon YM-30 column (Millipore, Bedford, MA, USA). The Cy3- and Cy5-labeled cDNA probes were mixed together and hybridized to a microarray slide. After overnight at 65 °C, the slide was washed twice with 2 $\times$ SSC containing 0.1% SDS for 5 min at 42 °C, once with 0.1 $\times$ SSC containing 0.1% SDS for 10 min at room temperature, and finally with 0.1 $\times$ SSC for 1 min at room temperature. The slide was dried by centrifugation at 650 r.p.m. for 5 min. Hybridization images on the slide were scanned by Scanarray lite (Packard Bioscience, Boston, MA, USA) and analyzed by GenePix Pro 3.0 software (Axon Instrument, Union City, CA, USA) to obtain gene expression ratios (oil-treated vs estrogen-treated). Logged gene expression ratios were normalized by LOWESS (locally weighted scatter plot smoother) regression (Yang *et al.* 2002).

The fluorescent intensity of each spot was calculated by local median background subtraction. We used the robust scatter-plot smoother LOWESS function to perform intensity-dependent normalization for gene expression. Scatter plot analysis was made by Microsoft Excel 2000 (Microsoft, Redmond, WA, USA). Significance analysis of microarray (SAM) was performed for the selection of the genes with significant expression changes (Tusher *et al.* 2001). The statistical significance of the differential expression was assessed by computing a q-value for each gene. To determine the q-value we used a permutation procedure, and for each permutation two-sample *t*-statistics were computed for each gene. Genes were considered differentially expressed when logarithmic gene expression ratios in three independent hybridizations were more than 1 or less than -1, i.e. a twofold difference in expression level, and when the q-value was <0.1.

Uterine sections and laser capture microdissection

Uterine horns were embedded in Tissue-Tek and frozen in liquid nitrogen. Cryosections (thickness, 6 μ m) were cut and mounted onto clean glass slides. After the sections were counterstained with Mayer's hematoxylin, each population of uterine cells (luminal epithelial, stromal and muscle cells) was isolated from these sections using P.A.L.M. Robot-Microbeam version 4.0 (P.A.L.M. Microlaser Technologies AG, Bernried, Germany). LCM was performed with a 15–30 μ m laser beam, laser power of

Figure 1 MA-plots (M, expression ratio; A, signal intensity) represent genes activated and repressed by estrogen in the ovariectomized mouse uterus. The MA-plot is used to represent the (R,G) data (R, red for Cy5; G, green for Cy3) where $M = \log_2 R/G$ and $A = \log_2(R \times G)$. (A) OVX/estrogen treatment/6-h protocol, (B) OVX/estrogen treatment/12-h protocol.

50 mV and a laser power duration of 4–6 ms. For each population of uterine cells, an average of 150 laser shots were transferred onto a 0.5 ml tube cap and stored at -70°C until utilized for total RNA extraction. Single laser shot means an approximate laser spot size of $30 \times 30 \mu\text{m}^2$ for luminal epithelial cells and $50 \times 50 \mu\text{m}^2$ for stromal and muscle cells.

Confirmation of microarray data with semiquantitative RT-PCR analysis

Total RNA from whole uterine tissues was extracted using TRIzol reagent and purified using an RNeasy total RNA isolation kit (Qiagen) following the manufacturer's instructions. One microgram RNA was reverse-transcribed at 42°C for 60 min in 20 μl reaction mixture consisting of oligo(dT)-adapter primer (Takara, Shiga, Japan) and AMV reverse transcriptase XL (Takara). The following PCR was performed in a total volume of 40 μl with 2 μl of the RT reaction mixture, 2 μl 25 mM MgCl_2 , 4 μl 10 $\times$ PCR buffer (100 mM Tris-HCl, 500 mM KCl, pH 8.3), 4 μl 2.5 mM dNTPs, 10 pmole forward and reverse primer and 1.25 U Taq polymerase (Takara).

Total RNA from LCM-captured luminal epithelial, stromal and muscle cells was extracted using RNeasy mini spin column (Qiagen) as described by the manufacturer. The caps were placed in microcentrifuge tubes with RLT buffer which contains denaturing guanidine isothiocyanate. The captured cell areas were collected by centrifugation at $8000 \times g$ for 3 min. After vortexing and centrifuging the tubes, the caps were removed and RNA was isolated according to the manufacturer's instructions. To

remove any potential genomic DNA contamination, an RNase-free DNase set (Qiagen) was used during RNA purification steps. Finally, 24 μl column-eluted RNA was all reverse-transcribed at 42°C for 60 min in 40 μl reaction mixture consisting of random hexamers (Takara) and AMV reverse transcriptase XL (Takara). The following PCR was performed in a total volume of 40 μl with 4 μl of the RT reaction mixture.

The sequences of the primers used in the RT-PCR investigation and the number of cycles (for whole uterus and LCM) tested to assess the best conditions for achieving linear amplification are listed in Table 3. RT-PCR from LCM samples was only performed on a few selected up-regulated genes (Fig. 4). The thermal cycling parameters consisted of denaturing (94°C , 30 s), annealing (60°C , 30 s), and extension (72°C , 30 s). The PCR products were separated by electrophoresis on 1.2% TBE agarose-ethidium bromide gels and visualized under UV light. The images were quantified by densitometric scanning followed by BioID image analysis software (Vilber-Lourmat, Mama La Vallee, Cedex, France), and gene expression was normalized against the density of the corresponding ribosomal protein L-7 (rpL7) PCR product as internal control.

Results

Global analysis of estrogen-regulated genes in the ovariectomized mouse uterus

To confirm reproducibility, experiments were repeated independently three times and relative changes were

Table 1 Genes up-regulated by estrogen in the ovariectomized mouse uterus. Uterine horns were collected 6 h (6H) or 12 h (12H) after estrogen treatment

Gene name	Fold change		GenBank no.
	6H	12H	
Cell-cycle-related			
Nuclear factor I/C	− 1.15	5.48	U57635
Protamine 2	1.21	3.11	X14004
Cyclin-dependent kinase inhibitor 1A (P21)	1.28	2.51	BC002043
Ubiquitin-activating enzyme E1C	1.31	2.22	AY029181
FBJ osteosarcoma oncogene	− 1.05	2.22	BC029814
RAN, member RAS oncogene family	1.76	2.19	BC014829
Peroxisome proliferator activated receptor binding protein	1.04	2.07	AF000294
Serum-inducible kinase	− 1.12	2.01	M96163
Immune-related			
Protein tyrosine phosphatase, receptor type, C	1.59	2.89	NM011210
Small chemokine (C-C motif) ligand 11	1.79	2.55	BC0027521
Proteasome subunit, beta type 5 (Psmb5)	2.29	2.43	AF060091
Tumor necrosis factor (ligand) super family, member 13b	1.01	2.01	AF119383
Signal transduction-related			
Receptor (calcitonin) activity modifying protein 3 (Ramp3)	2.11	6.12	AF209907
Serine/arginine-rich protein specific kinase 2	1.26	5.96	BC020178
Chemokine orphan receptor 1	1.67	2.99	BC015254
Fibroblast growth factor 1	− 1.19	2.94	U67610
Guanine nucleotide binding protein, alpha 12	1.49	2.68	M63659
Inositol polyphosphate-5-phosphatase, 145 kDa	− 1.17	2.57	AF228679
Purinergic receptor P2Y, G-protein coupled 2	1.06	2.49	BC006613
Guanine nucleotide binding protein, alpha 11	1.93	2.23	M55411
Debrin-like	1.18	2.22	BC046430
Mitogen activated protein kinase 13	1.04	2.21	U81823
Endoglin	− 1.04	2.14	X77952
MAD homolog 2 (<i>Drosophila</i>)	1.06	2.09	BC021342
Phosphatidylinositol 3-kinase, C2 domain containing, gamma	1.08	2.07	AB008792
Cell division cycle 42 homolog (<i>S. cerevisiae</i>)	1.19	2.01	L78075
Transcription-related			
Homeo box A5	1.73	9.63	X16840
Activating transcription factor 5 (AFT5)	1.44	4.03	BC010195
Zinc finger protein 1	− 1.31	3.11	XM134378
Kruppel-like factor 4 (gut)	1.19	3.02	U20344
Paired-like homeodomain transcription factor 3	− 1.19	2.86	AF005772
Williams–Beuren syndrome chromosome region 14	1.02	2.54	AF245479
General transcription factor II H, polypeptide 2	1.29	2.31	BC016231
CpG binding protein	− 1.05	2.29	AY099096
Activating transcription factor 4 (ATF4)	− 1.04	2.28	M94087
Paired related homeobox 1	− 1.05	2.22	AK084387
Fibrillarin	1.16	2.21	Z22593
loquois related homeobox 3 (<i>Drosophila</i>)	1.14	2.17	Y15001
Tripartite motif protein 24	1.53	2.06	M64429
Activating transcription factor 1 (ATF1)	1.28	2.04	BC006871
Inhibitor of DNA binding 1 (Id-1)	1.23	2.01	M31885
Enzyme-related			
Branched chain ketoacid dehydrogenase E1	1.13	5.79	L16992
Branched chain aminotransferase 1, cytosolic	1.21	4.74	AK013888
Mevalonate (diphospho) decarboxylase	1.13	3.43	AJ309922
Mannosidase 1, beta	1.99	3.08	XM203179
Structure-related			
Small proline-rich protein 2A (Sprp2A)	1.95	75.86	BC010818
Small proline-rich protein 2H (Sprp2H)	1.27	12.07	AY158992
Procollagen, type I, alpha 1	− 1.64	4.11	U03419
Small proline-rich protein A (SprpA)	− 1.15	2.72	X91824
Procollagen, type III, alpha 1	− 1.58	2.39	X52046

Table 1 Continued

Gene name	Fold change		GenBank no.
	6H	12H	
Apoptosis-related			
Cytochrome c, somatic	1.87	2.79	BC034363
Sphingosine-1-phosphate phosphatase 1	– 1.15	2.14	AF247177
Programmed cell death 6 interacting protein	1.33	2.13	C026823
Others			
Pleckstrin homology, Sec7 and coiled/coil domain 3	1.25	7.04	NM011182
B-cell CLL/lymphoma 7B	1.52	4.77	AJ011145
Heat shock 70 kDa protein 4	– 1.19	3.38	D85904
Immediately early response 2	1.77	3.11	BC002067
SEC61, gamma subunit (<i>S. cerevisiae</i>)	2.85	2.52	U11027
Cystatin B	3.47	2.38	U59807
Eukaryotic translation initiation factor 2, subunit 2 (Eif2s2)	2.47	2.29	NM026030

calculated. The mean values of the intensities of each spot in the three experiments were calculated, and are plotted in Fig. 1. Of the 7616 genes examined in the OVX/estrogen treatment/6-h protocol, changes in mRNA expression were detected in 71 genes: 22 were activated and 49 were repressed (Tables 1 and 2). Only 0.9% of all genes were activated or repressed more than twofold, with estrogen causing no significant differences in the expression of the remaining 99.1% of the genes. However, in the OVX/estrogen treatment/12-h protocol, 638 genes showed a differential expression ratio of more than twofold or less than 50%: 351 were activated and 287 were repressed. About 8.4% of all genes were activated or repressed (Tables 1 and 2). Out of the 638 activated or repressed genes, 433 genes were listed as known genes in GenBank, and 205 genes were unknown. These genes were classified into eight functional categories based on biological functions: cell-cycle-related, immune-related, signal transduction-related, transcription-related, enzyme-related, structure-related, apoptosis-related, and others (including expressed sequence tags and unknown genes).

Confirmation of estrogen-regulated genes

The expression patterns of several genes up- or down-regulated by estrogen were confirmed to verify the results of the microarray analysis using semiquantitative RT-PCR. Several genes were selected in different categories of genes: small proline-rich protein 2A (Sprp2A), cystatin B, receptor-activity-modifying protein 3 (Ramp3), proteasome subunit beta type 5 (Psb5), eukaryotic translation initiation factor 2 (Eif2s2), and inhibitor of DNA binding-1 (Id-1) (up-regulated); and decorin, integral membrane protein 2B (Imp2B), secreted frizzled-related sequence protein 2 (SFR-2), Bach2, chemokine ligand 12 (CXCL12), and Id-2 (down-regulated). The primer sets used in the RT-PCR investigation are listed in Table 3,

and the results of semiquantitative RT-PCR analysis are shown in Fig. 2. Densitometric analysis was performed and intensities were normalized to rpL7 for samples on the same gels. Ratios between oil- and estrogen-treated groups were calculated for each PCR product, and mean values of relative intensities were then calculated from three different experiments. These data validate the up- or down-regulation of the above selected genes, and are exactly consistent with the microarray expression profiles.

Analysis of selected gene expression patterns in specific uterine cell types using LCM

To further analyze our findings we investigated gene expression of the selected up-regulated genes in specific uterine cell types using the LCM technique. LCM allows the isolation of specific uterine cell types without contamination from other cell types. Green *et al.* (2003) showed that mRNA from cells could be successfully isolated by LCM from frozen sections with high efficiency, producing RNA with excellent quality and quantity. We isolated populations of specific uterine cell types from frozen sections stained with Mayer's hematoxylin: luminal epithelial, stromal and muscle cells. Figure 3A shows a representative frozen section (stained with hematoxylin) of an ovariectomized mouse uterus, and subsequent microdissections of specific uterine cells – luminal epithelial cells, muscle cells and stromal cells – are shown in Fig. 3B–D respectively. While Sprp2A, Eif2s2, Id-1, and Psb5 mRNAs were strongly activated only in luminal epithelial cells, Ramp3 and cystatin B were activated in both luminal epithelial and stromal cells (Fig. 4). Our qualitative RT-PCR data demonstrate clearly the differential expression of selected genes in specific uterine cell types.

Table 2 Genes down-regulated by estrogen in the ovariectomized mouse uterus. Uterine horns were collected 6 h (6H) or 12 h (12H) after estrogen treatment

Gene name	Fold change		GenBank no.
	6H	12H	
Cell-cycle-related			
Nuclear factor I/A	−1.04	−3.79	Y07690
Epidermal growth factor receptor (EGFR)	−1.33	−2.44	BC023729
Checkpoint kinase 1 homolog (<i>S. pombe</i>)	−1.44	−2.28	AF16583
Topoisomerase (DNA) I	−2.11	−1.23	D10061
Immune-related			
Interleukin 15	ND	−7.74	U14332
Chemokine (C-X-C motif) ligand 12 (CXC-12)	−3.19	−6.49	BC046827
Serine (or cysteine) proteinase inhibitor, Clade G, member 1	−1.54	−3.63	BC002026
Histocompatibility 2, class II antigen E beta	−1.23	−3.09	M36940
Fc receptor, IgG, alpha chain transporter	1.19	−2.84	D37874
Immunoglobulin (CD79A) binding protein 1	−1.11	−2.75	XM196586
Complement component factor h	−2.19	−2.65	NM009888
Proteasome (prosome, macropain) subunit, beta type 9	−1.19	−2.16	BC032210
Chemokine (C-X-C motif) ligand 14 (CXC-14)	−1.54	−2.13	AF192557
Chemokine (C-C motif) receptor 7	1.21	−2.04	L31580
Signal transduction-related			
Lymphoid blast crisis-like 1	−1.46	−9.73	U28495
Secreted frizzled-related sequence protein 2 (SFR-2)	−1.41	−6.03	AF337040
Taste receptor, type 1, member 1	−1.29	−4.63	U36757
Coagulation factor II (thrombin) receptor	−1.04	−3.37	BC047086
Protein tyrosine phosphatase, receptor type, B	−1.30	−2.91	M84607
Platelet derived growth factor receptor, alpha polypeptide	1.23	−2.81	NM080428
F-box and WD-40 domain protein 7, archipelago homolog	−1.89	−2.63	D14340
Tight junction protein 1	−1.34	−2.57	AB028143
Endothelial differentiation, sphingolipid G-protein	−1.27	−2.41	U06924
Signal transducer and activator of transcription 1	−1.28	−2.29	AF194871
Interferon-inducible GTPase	−1.48	−2.25	U07617
Growth factor receptor bound protein 2	−1.29	−2.24	BC037696
Platelet-derived factor, C polypeptide	−1.25	−2.04	AF062484
Sorting nexin 12	−1.37	−2.03	BC014722
Transcription-related			
Neurogenic differentiation 1 (NeuroD)	−2.28	ND	U28068
Transcription factor Dp1	−2.05	−3.15	X72310
Snail homolog 3 (<i>Drosophila</i>)	−1.62	−3.27	AF133714
Trichorhinophalangeal syndrome I (human)	−1.11	−2.89	AF346836
Y box protein 2	1.01	−2.63	AF073954
Histone deacetylase 5	−1.05	−2.41	AF207748
Ring finger protein 12	1.02	2.33	AF069992
Eleven-nineteen lysine-rich leukemia gene	−1.14	−2.31	U80227
Lymphoid enhancer binding factor 1	−1.06	−2.29	X58636
Inhibitor of DNA binding 2 (Id-2)	−1.26	−2.26	M69293
Homeo box D9	−1.21	−2.13	BC019150
BTB and CNC homology 2 (Bach2)	−1.19	−2.08	D86604
WD40 protein Cio 1	−1.01	−2.07	BC004089
Musculin (Msc)	−1.38	−2.04	AF087035
Enzyme-related			
UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase	−2.49	−11.97	BC046322
Putative phosphatase	1.24	−8.49	U96724
Thioether S-methyltransferase	−1.19	−3.52	M88694
Ubiquitin specific protease 21	−1.23	−2.99	BC021903
Cysteine dioxygenase 1, cytosolic	−1.72	−2.59	BC013638
Structure-related			
Decorin	−4.75	−7.03	X53929
Fibromodulin	−1.37	−4.31	X94998
Tetranectin (plasminogen binding protein)	−1.51	−3.13	U08595
Syndecan 2	1.03	−2.13	U00674

Table 2 Continued

Gene name	Fold change		GenBank no.
	6H	12H	
Apoptosis-related			
Programmed cell death 5 (Pcd5)	-3.25	ND	AF161074
Tumor necrosis factor (ligand) superfamily, member 12	-1.55	-2.31	AF030100
Others			
Selenoprotein P, plasma, 1	-1.91	-7.99	X99807
Integrin alpha 9	-1.23	-4.18	AJ344342
RNA binding motif protein 5	-1.01	-3.09	AY309168
Integral membrane protein 2B (Imp2B)	-2.71	-2.91	U76253
Plexin B1	-1.02	-2.35	AB072381

ND, not detected.

Discussion

Estrogen plays an important role in various physiological events, but the molecular mechanisms that are regulated by estrogen in the uterus remain largely unknown. Therefore, the identification of novel estrogen-regulated gene pathways is essential to understanding how estrogen regulates various aspects of uterine physiological events, such as the estrous cycle and the implantation process. In the

present study, we identified genes that are regulated by estrogen in the ovariectomized mouse uterus exposed to estrogen for 6 h or 12 h using a cDNA microarray. Prior to use in cDNA microarray analysis, each uterine RNA sample obtained via the OVX/estrogen treatment/6-h/12-h protocols was validated by assaying for up-regulated (asparagine synthetase and lactoferrin) or down-regulated (glutathione S-transferase and SFR2) genes which are known to be regulated by estrogen in the mouse uterus

Table 3 Primer sequences for RT-PCR

Gene	Forward (F) and reverse (R) primer sequences		bp	Cycles	
				Whole	LCM
Sprp2A	F	GTGTGGCCTGGGCCTTGTCG	240	22	36
	R	GAGTCGGTGAGCTGGTGAG			
Cystatin B	F	CGCGCCATCTGCACAAT	225	22	36
	R	GGCTTGTTCATGGGGGAG			
Ramp3	F	GGTGGTGTGGCGCAGCAAGC	427	24	40
	R	GCAGGGGTCAGGGTCAGGAC			
Psmb5	F	GGCTGGGGTGCAGCGGAT	350	24	40
	R	GGTAGATGGCTCGGCGGG			
Eif2s2	F	CGTGATGGGGTAAGGAGG	489	24	40
	R	CTAGAGCACAGGTTGGAG			
Id-1	F	GATCATGAAGGTCGCCAGTG	476	28	40
	R	TCCATCTGGTCCTCAGTGC			
Decorin	F	CCCCTACCGATGCCAGTGTC	423	24	
	R	GCTCCGTTTTCAATCCCAGA			
Imp2B	F	GTGGCGGTGGATTGCAAGGA	432	22	
	R	GGGCGGCATACGATGGAAG			
SFR-2	F	TGGCTTATACGTGCACT	295	24	
	R	TATTGAGGGCATCATGCAA			
Bach2	F	CCCATGTCACAAACCCTATC	471	28	
	R	TGCTCACCTGACACCGTTCG			
CXC12	F	CGTGGGAGATGCAAGGGCAG	247	22	
	R	GAGGAGAATGGGGATGAAGC			
Id-2	F	GTGACCAAGATGGAAATCCT	236	24	
	R	TTTATTTAGCCACAGATAC			
rPL7	F	TCAATGGAGTAAGCCCAAAG	246	22	36
	R	CAAGAGACCGAGCAATCAAG

Figure 2 Semiquantitative RT-PCR analysis confirming estrogen-regulated genes. (A) Expression patterns of estrogen-activated genes. Upper panel, representative result of the up-regulated genes. Lower panel, representative result of the down-regulated genes. (B) Expression patterns of estrogen-repressed genes. Values of each band were normalized to rpl7 for the same sample. Data are means $\pm$ S.E.M. from three replicate experiments. C, oil-treated group; 6H, post-estrogen 6 h; 12H, post-estrogen 12 h.

(Das *et al.* 1998, 2000, Watanabe *et al.* 2002). Watanabe *et al.* (2002) listed the early estrogen-responsive genes in the ovariectomized mouse uterus. In their microarray data using the OVX/estrogen treatment/6-h protocol, asparagine synthetase is up-regulated (4.4-fold) and glutathione S-transferase is down-regulated (10-fold). Glutathione S-transferase was the most down-regulated gene by estrogen in their study. Das *et al.* (1998, 2000) also showed the differential regulation of lactoferrin and SFR-2 in response to estrogen in the ovariectomized mouse uterus: while uterine levels of lactoferrin mRNA were strongly enhanced at 12 h after estrogen administration, SFR-2 levels were reduced over the same period. We therefore used these genes as markers of estrogen responsiveness (data not shown).

Our microarray results showed that only 0.9% (71 genes) of all genes spotted on the microarray slide were activated or repressed more than twofold in the OVX/estrogen treatment/6-h protocol, which suggests that direct or fast responses by estrogen are not widespread at the mRNA level in the genes contained in our microarray.

Therefore, if a much larger cohort of cDNAs is used in this experiment, we may obtain a different expression outcome. On the other hand, 638 genes showed a differential expression ratio more than twofold or less than 50% in the OVX/estrogen treatment/12-h protocol. About 8.4% of all genes were activated or repressed. It is possible that, in addition to direct or genomic pathways, many indirect or nongenomic pathways affected the patterns of mRNA expression in the latter protocol. Watanabe *et al.* (2002) reported that generally 5% of all genes were activated or repressed more than threefold by estrogen in an OVX/estrogen treatment/6-h protocol: 616 genes were determined as estrogen-affected genes, of which 299 were activated and 317 were repressed. It is likely that this difference between their results and ours is due to different strains (they used the C57/BL6/J strain) used in the microarray analysis and the different genes spotted in the microarray techniques.

Even though our results identifying estrogen-regulated genes are quite different from those of Watanabe *et al.* (2002), two genes did show the same expression pattern:

Figure 3 Laser capture microdissection (LCM) procedure. (A) Uterine sections stained with hematoxylin before LCM. LE, luminal epithelial cells; S, stromal cells; M, muscle cells. Capture sites of each of the uterine cell types are indicated by an arrow. (B–D) Capture of (B) luminal epithelial cells, (C) muscle cells, and (D) stromal cells.

chemokine orphan receptor 1 and Ramp3; they were slightly increased in the OVX/estrogen treatment/6-h protocol and highly increased in the OVX/estrogen treatment/12-h protocol. Calcitonin, calcitonin-gene-related peptide, adrenomedullin, and amylin are related hormones and neuropeptides. Ramp-1, Ramp-2 and Ramp-3, through interaction with calcitonin receptor, are required for the recognition of calcitonin-gene-related peptide, adrenomedullin, and amylin by these receptors (Gangula *et al.* 2000, Fischer *et al.* 2002). These relationships are enhanced by estrogen, suggesting a role for these genes in uterine estrogen responses.

In the present study, some significant changes in mRNA level were noticed. Activating transcription factors (ATF)-1, ATF-4 and ATF-5 were activated by estrogen in the OVX/estrogen treatment/12-h protocol. It is known that ATF activates gene expression by binding as a homo- or heterodimer to the cAMP response element in the regulatory region of target genes. Although the ATF family is not well characterized in uterine physiological events, such as the estrous cycle and the implantation

process, its functions in other tissues suggest an important role in cell proliferation and various signaling mechanisms (Sabbah *et al.* 1999, Bleckmann *et al.* 2002). In addition, several genes included in the helix-loop-helix family, such as neuroD, musculin, Id-1, and Id-2, were also identified as estrogen-responsive genes in our microarray analyses. Although the associated changes in mRNA levels were not strongly activated or repressed by estrogen, the estrogen-responsiveness of these genes may indicate their involvement in uterine physiological events based on other recent studies. In the mouse, the main sign of blastocyst attachment sites during the implantation period is angiogenesis. Recent work has shown that the expression of Id-1 and Id-3 is required for correct angiogenesis in the neuroectoderm during embryo development; in particular the partial reduction of Id-1 dosage results in an angiogenic defect in adult mice which blocks the vascularization of tumor xenografts (Lyden *et al.* 1999). More interestingly, extracellular matrix surrounding endothelial cells was thickened in Id-knockout mice, and thus Id expression may regulate the expression of integrin and matrix metal-

Figure 4 Expression patterns of up-regulated genes by estrogen in specific uterine cell types obtained with LCM. C, oil-treated group; E 6H, post-estrogen 6 h; E 12H, post-estrogen 12 h; LE, luminal epithelial cells; S, stromal cells; M, muscle cells.

loproteinase MMP2 which are required for tumor angiogenesis. Furthermore, the loss of Id function leads to a decrease in vascular endothelial growth factor expression in endothelial cells (Brooks *et al.* 1994). These results strongly suggest that endometrial angiogenesis during the estrous cycle or implantation period is directly or indirectly regulated by Id expression under the control of ovarian steroid hormones, prime modulators of cyclic endometrial changes and inducers of local factors produced at the time of implantation.

Interestingly, Sprp2A showed the highest level of up-regulation by estradiol in the OVX/estrogen treatment/12-h protocol. This gene is reportedly expressed mainly in stratified squamous epithelial cells and controls the differentiation of several cell types (Fischer *et al.* 1996, Song *et al.* 1999). To date, three families of Sprps have been described and differentially expressed in various types of epithelia (Kartasova *et al.* 1996, Fujimoto *et al.* 1997). Song *et al.* (1999) isolated 11 mouse Sprp2 genes and examined the expression patterns of Sprp2 (A–K) genes in mouse epithelial tissues. They found that Sprp2A, Sprp2D and Sprp2F were strongly expressed in the uterus, whereas Sprp2C, Sprp2H, Sprp2I, Sprp2J, and Sprp2K were not detected. In our study, Sprp2A mRNA was strongly activated in luminal epithelial cells compared with stromal and muscle cells using LCM techniques. This regulation suggests that the expression of Sprp2A in luminal epithelial cells has a significant effect on the relationship between epithelial cell function and developing embryos. Although

clarification of the function of Sprp2A and the significance of estrogen responsiveness in relation to uterine physiological events have not been identified, recent studies have suggested that the Sprp2A gene family plays a pivotal role in the estrous cycle and the implantation process.

To the best of our knowledge this is the first study to profile early and late responsive genes in mouse using cDNA microarray and LCM techniques. We used the LCM technique to isolate specific uterine cell types from frozen sections stained with Mayer's hematoxylin. Using this technique, we determined the differential expression of several up-regulated genes in specific uterine cell types. In conclusion, our results indicate differential spatiotemporal expression of genes regulated by estrogen, and implicate various genes not previously known to be expressed in ovariectomized mouse uterus. Further investigations in regard to progesterone regulation, spatiotemporal expression, protein expression, and the function of these genes may be necessary to understand the exact mechanisms of estrogen action on the estrous cycle and the implantation process.

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