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Research

Global gene profiling analysis of mouse uterus during the oestrous cycle

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Many genes related to the cyclic changes of the uterus during the oestrous cycle have been identified using a one-by-one approach. In the present study, cDNA microarray technology was applied to investigate the global profile of gene expression of mouse uterus at the oestrous and dioestrous stages. At a certain stage of the oestrous cycle, the uteri of mature CD-1 mice ($n = 10$) were removed, pooled and snap-frozen in liquid nitrogen. Total RNA was extracted to synthesize cDNA probes for microarray assay. By screening 8192 mouse genes and expressed sequence tags (ESTs), 51 upregulated and 51 downregulated genes were identified in oestrous uterus, of which 62 are well characterized and 40 are ESTs. The known genes were assigned to various gene categories according to their main function. The microarray was performed three times with three independent sets of uterine tissue pools. The results of northern blot analysis for small proline-rich protein 2 (Sprr2), 17β-hydroxysteroid dehydrogenase type 2 (17βHSD-2), high mobility group 2 (Hmg2), mitotic checkpoint component 2 (Mad2) and an EST AW555366 mRNA were consistent with that of microarray analysis. *In situ* hybridization was performed to localize the transcript of the EST AW555366. Most of the upregulated genes encode secreted immune-related proteins, proteinases and their inhibitors, indicating their potential involvement in sperm viability as well as capacitation. The downregulated genes mainly encode cell cycle-related factors, implying the active proliferation of uterus at dioestrus.

Introduction

In mammals, the uterus of a mature female undergoes a cyclic trophy–atrophy alternation. This is a steroid-driven event called the menstrual cycle in humans and the oestrous cycle in rodents, reflecting the cyclic change of the uterine endometrium in preparation for embryo implantation.

According to the typical characteristics of uterine structure and function, the oestrous cycle can be divided into four stages: pro-oestrus, oestrus, metoestrus and dioestrus. Dioestrus is characterized by the maintenance of basal oestrogen concentrations, atrophic endometrium and sexual inactivity. During the pro-oestrous stage, uterine oedema and proliferation of endometrial cells occur, accompanied by gradually increasing oestrogen concentrations. Ovulation and sexual receptivity are diagnostic characteristics of the oestrous stage when the uterus becomes trophic under peak level of oestrogen and other ovarian steroids. This is the crucial period for sperm capacitation and for the subsequent transition of the uterine milieu toward the receptive stage after mating. If no conceptus exists, the uterus regresses concomitant with cell apoptosis in all types of cell as serum oestrogen concentrations decrease and progesterone concentrations increase (Burroughs *et al*., 2000; Fata *et al*., 2001). Alteration of profiles of gene expression underlies these morphological and functional changes; however, this area of research is poorly represented in the literature.

Spermatozoa undergo capacitation in the female reproductive tract during the oestrous stage. Tulsiani *et al*. (1996) provided evidence that the carbohydrate moieties of glycoproteins on the sperm surface were modified during capacitation. It is thought that secretions from the uterus and oviduct can induce *in vitro* capacitation, but only a few inducers have been identified, including an endometrial sialic acid-binding protein (Banerjee and Chowdhury, 1995). It is likely that both positive and negative factors are present in the lumen of the oestrous uterus and oviduct that maintain the appropriate conditions for sperm mobility and viability, as well as prevent premature acrosome reaction. However, most of those factors have not been identified.

Recently, cDNA microarray analysis has proven to be a powerful molecular tool for the simultaneous monitoring of global profiles of gene expression, thereby providing overall information about molecular events. In the present study, cDNA microarray analysis was performed to investigate the global profile of gene expression of mouse uterus and to identify genes that were differentially expressed at oestrus and dioestrus. The

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research may contribute to understanding the molecular mechanisms involved in the cyclic changes of the uterus and in the regulation of sperm capacitation.

Materials and Methods

Tissue collection

Mature virgin female CD-1 mice were obtained from the Laboratory Animal Center, National Research Institute for Family Planning, Beijing. The mice were housed with 12 h light a day and food was available *ad libitum*. Vaginal smears were examined once a day to classify the phases of the oestrous cycle (Nelson *et al*., 1982). Animals with at least two consecutive 4-day cycles underwent cervical dislocation at known stages of the oestrous cycle. The uteri were collected and flash frozen in liquid nitrogen, and stored at −80°C.

RNA isolation

Uterine tissues at different stages of the oestrous cycle were homogenized with a polytron homogenizer (Kinematika, Littan Lucerne). Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The RNA samples were subjected to DNase I digestion, extracted with phenol:chloroform:isopropyl alcohol (25:24:1) (Sigma, St Louis, MO), and concentrated by ethanol precipitation.

cDNA microarray analysis

cDNA microarray M80S, consisting of 8192 cDNA fragments, which represent 5362 known murine genes (including 40 house keeping genes), and 2826 unique expressed sequence tags (ESTs), as well as four control genes (*Arabidopsis thaliana*), was generated by United Gene Holdings, Shanghai (a detailed list of these genes and ESTs is available in the manufacturer's manual). The cDNA fragments for generating the array were subcloned into plasmids, amplified by PCR and printed in duplicate on to the siliconized slides (CEL Associates, Houston, TX). Probe labelling, hybridization and signal scanning were conducted at the same company. In brief, 50 μg total RNA was used in an oligo(dT)-primed reverse transcription reaction to synthesize cy3 or cy5 fluorescent dye-labelled cDNA probes by using SuperScript II reverse transcriptase (Invitrogen) according to Schena *et al*. (1995, 1996). The cDNAs of dioestrous uteri were labelled with cy3 and the cDNAs of oestrous uteri were labelled with cy5. The labelled cDNA probes were purified with QIAquick PCR purification system (Qiagen, Hilden). The microarrays were prehybridized at 42°C for 6 h, and further hybridized at 42°C for 16 h with the mixed cy3- and cy5-labelled cDNA probes that had been denatured at 95°C for 5 min. After hybridization, the microarrays were washed and the fluorescent intensities of cy3 and cy5 were measured with a laser scanner GenePix4000B (Axon Instruments, Union City, CA) at 532 and 635 nm, respectively. After scanning, the data were analysed using GenePix Pro3.0 microarray analysis software (Axon Instruments). The spots with values of $\log_2 R/G$ (where R = cy5 intensity and G = cy3 intensity) between 0.1 and 10.0 were selected for further analysis. Data from individual arrays were normalized by setting a constant factor (k) as the mean value of $\log_2 R/G$. The ratio of R:kG was calculated for each gene in the selected set, and a criterion of twofold difference was applied to select the upregulated and downregulated genes, as described by Yang *et al*. (2002).

Construction and labelling of the cDNA probe for northern blot analysis

A sample (1 μg) of total RNA was reverse transcribed in a 20 μl reaction mixture with oligo(d)T primers (Promega, Madison, WI) by SuperScript II reverse transcriptase as specified by the manufacturer. An aliquot (1 μl) from the reverse transcription was amplified by PCR with specific primers (SBS Genetech, Beijing) that were designed according to the cDNA sequences derived from the GenBank database. The primer sequences are shown (Table 1). The PCR products were verified by sequencing and subcloned into pGEM-T easy vector (Promega) to generate the recombinant plasmids. Specific cDNA probes for northern blot analysis were labelled with [α-^{32}P]dCTP (Yahui, Beijing) by Prime-a-gene system (Promega) and purified by nick column (Amersham Ciosciences AB, Uppsala).

Northern blot analysis

Total uterine RNAs (30 μg) of four stages of the oestrous cycle were subjected to electrophoresis on 1% (w/v) formaldehyde–agarose gels and transferred by vacuum to Hybond$^+$ nylon membranes (Pharmacia). Wet membranes were crosslinked in a violet crosslinker for 8 s. The membranes were prehybridized for 4 h at 65°C in prehybridization buffer (0.2 mol sodium phosphate l^{-1}, pH 7.4, 0.1 mmol EDTA l^{-1}, 7% (w/v) SDS, 1% (w/v) BSA and 15% (v/v) formamide) and hybridized overnight at 65°C in fresh hybridization buffer containing 10% (w/v) dextran sulphate (Sigma) and a specific ^{32}P-labelled cDNA probe. After hybridization, the membranes were washed and exposed to Fuji film (Fuji Photo Film Co., Tokyo). The signals were quantified by densitometric scanning and normalized by comparison with the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene.

Preparation of tissue for in situ *hybridization*

The uterine tissues were washed twice with PBS buffer and fixed immediately in 4% (w/v) paraformaldehyde at

Table 1. Primers used in RT–PCR

Gene symbol	Primer sequence	Product size (bp)	GenBank accession no.
Sprr2A	F: 5′-AAGTAAAAGAGGCAATCCAGG R: 5′-CATCATAGGCACATGGAGG	580	MMAJ5559
Hmg2	F: 5′-GGAAGGAAGTCTCTCTGC R: 5′-GCCTGAGCAAATATACTCC	744	Z46757
17β-HSD2	F: 5′-AAGGCCAGTGGAGAATGAGC R: 5′-CAAGGTCGGGATGATGAGC	947	MM17BDTII
Mad2	F: 5′-CGATCTAGTGATGGCACAGC R: 5′-GTAAATCAGGAGCCCAAACG	526	NM_019499
EST AW555366	F: 5′-TTCTCCAAAGACTATCAAGTG R: 5′-CAAACAGTAAAGGGGAAA	432	AW555366
GAPDH	F: 5′-ACGACCCCTTCATTGACC R: 5′-TCAGATGCCTGCTTCACC	700	M32599

Sprr2A: small proline-rich protein 2A; Hmg2: high mobility group 2; 17β-HSD2: 17β-hydroxysteroid dehydrogenase type 2; Mad2: mitotic checkpoint component 2; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.
F: forward; R: reverse.

Table 2. Genes upregulated in mouse uterus at the oestrous stage

GenBank no.	Fold up	Gene description	Functional category
AJ005559	9.8	Small proline-rich protein 2A (Sprr2A)	Structural protein
X81627	7.99	24p3	Unspecified function
M75718	7.61	Serine protease inhibitor 1-4 (α-1 protease inhibitor 4, α-1 PI-4)	Protein turnover
M75717	7.04	α-1 protease inhibitor 5 (α-1 PI-5, API)	Protein turnover
M75721	5.89	α-1 protease inhibitor 1 (α-1 PI-1)	Protein turnover
NM_010884	5	N-myc downstream regulated 1 (Ndr1)	Oncogenes and tumour suppressors
K02782	4.64	Complement component C3	Immune-related
M12660	4.62	Complement component factor h	Immune-related
AF039663	4.62	AC133 antigen homologue	Haematopoietic
J03298	4.6	Lactotransferrin	Immune-related
AJ005567	4.52	Small proline-rich protein 2I (Sprr2I)	Structural protein
AF110764	4.42	RS21-C6 (Tdrg-TL1)	Unspecified function
M57890	4	Histocompatibility 2, complement component factor B	Immune-related
L10244	3.9	Spermidine/spermine N1-acetyl transferase	Enzyme
X06086	3.84	Cathepsin L	Protein turnover
M55253	3.6	Myosin Vb	Cytoskeleton and mobility
AB001489	3.45	Phosphatidylinositol glycan, class R	Immune-related protein
M75720	3.21	α-1 protease inhibitor 3 (α-1 PI-3)	Protein turnover
X93035	3.12	BRP39 protein	Oncogenes and tumour suppressors
NM_010362	3.01	Glutathione-*S*-transferase like (Gsttl-pending)	Stress response protein
M29009	2.84	Complement factor H-related protein	Immune-related
AB027138	2.79	Tektin-t	Cytoskeleton and mobility
D78264	2.77	Pancortin-3	Nerve-related protein
NM_008670	2.58	Neuronal apoptosis inhibitory protein 1 (Naip1)	Apoptosis-associated protein
AB031386	2.54	Clast1	Unspecified function
D26123	2.45	Carbonyl reductase 2	Enzyme
M21050	2.45	Lysozyme M gene	Protein turnover
U06119	2.41	Cathepsin H	Protein turnover
D86232	2.21	Lymphocyte antigen 6 complex, locus C	Immune-related
AJ002386	2.15	Cathepsin S	Protein turnover
U24674	2.15	Max interacting protein 1 alternatively spliced long form (Mxi1)	Oncogenes and tumour suppressors
Y09517	2.12	17β-hydroxysteroid dehydrogenase type 2 (17β-HSD2)	Enzyme
U67187	2.1	G protein signalling regulator RGS2 (RGS2)	Cell signalling

4°C for 16 h. The fixed tissues were gradually dehydrated in ethanol and embedded in paraffin wax. Sections of 6 μm thickness were collected on Super Frost+/Plus glass slides (Menzel, Braunschweig).

Preparation of digoxigenin (DIG)-labelled probes for in situ *hybridization*

The DIG-labelled RNA probes were used for *in situ* hybridization. In brief, using total RNA extracted from mouse uteri as template, specific cDNA fragments of certain genes were amplified by RT–PCR with primer sets designed according to the sequences from GenBank (Table 1). pGEM-T easy vector was used to construct the recombinant plasmids. After confirmation of insert by sequencing, the recombinant plasmids were linearized with *Sal*I or *Nco I* (Promega) and purified with QIAquick Spin Columns (Qiagen), and used as templates for *in vitro* transcription reactions to synthesize DIG-labelled sense or antisense RNAs according to the manufacturer's instruction for the DIG-RNA labelling system (Roche Diagnostics, Mannheim). DIG-labelled RNA probes were stored at −80°C at 0.1 μg μl^{-1}.

In situ *hybridization*

In situ hybridization was performed as described by Braissant and Wahli (1998). In brief, paraffin wax sections were routinely deparaffinized and rehydrated. After treatment with 0.2 mol HCl l^{-1} for 15 min, the slides were denatured at 70°C in 2 × SSC for 15 min and digested with 4 μg proteinase K ml^{-1} (Invitrogen) for another 15 min. Post-fixation was performed in 4% (w/v) paraformaldehyde at room temperature (25°C) for 10 min, followed by incubation twice in PBS containing 0.1% (v/v) active DEPC (Sigma-Aldrich, Steinheim) for 15 min. Acetylation was performed in triethanolamine buffer containing 0.5% (v/v) acetic anhydride for 10 min and subsequent equilibration was performed in 5 × SSC for 15 min. The slides were prehybridized for 4 h at 58°C in prehybridization buffer (50% formamide, 20 mmol Tris–HCl l^{-1}, 50 mmol EDTA l^{-1}, 0.5 mg *Escherichia coli* tRNA ml^{-1}, 100 mmol dithiothreitol l^{-1}) and further hybridized for 18 h at 58°C in fresh hybridization buffer containing 1 ng antisense DIG-RNA probes μl^{-1}. After washing in consecutive baths of 2 × SSC for 1 h and 0.1 × SSC for 1 h at 65°C, the slides were blocked with Tris–HCl buffer containing 0.5% (w/v) blocking reagent (Boehringer Mannheim, Mannheim) and incubated at room temperature for 2 h with alkaline phosphatase-coupled anti-digoxigenin antibody (dilution 1:500). Colour development was performed in buffer II (100 mmol Tris–HCl l^{-1}, 100 mmol NaCl l^{-1}, 50 mmol $MgCl_2$ l^{-1}, pH 9.5) containing 4.5 μl 4-Nitro blue tetrazolium chloride and 3.5 μl x-phosphate/5-bromo-4-chloro-3-indolyl-phosphate (Boehringer Mannheim). After washing with tap water, the slides were mounted in Clearmount (Zymed, San Francisco, CA) without counterstaining. The negative control was performed with the sense RNA probe in hybridization buffer in place of the antisense RNA probe.

Statistical analysis

The microarray hybridization was repeated three times with three independent RNA pools, each derived from the uteri of ten mice at oestrus and ten mice at dioestrus. Values for the mean and SE of the ratio *R*:k*G* from the three microarrays were calculated for each gene or EST on the Genechip. Comparison of the values for various genes or ESTs with that of the housekeeping gene (GAPDH) was performed by Student's *t* test and $P < 0.05$ was considered as significant.

The northern blot analysis for each selected gene was performed in triplicate with three independent RNA pools, each generated from uteri at the pro-oestrous, oestrous, metoestrous and dioestrous stages, with ten mice per stage. Values for the mean and SE of the triplicate difference scores from the three experiments were calculated. Comparison between the normalized relative level at a specific stage with that at dioestrus was performed by Student's *t* test and $P < 0.05$ was considered as significant.

Results

Altered gene expression profile in oestrous and dioestrous uteri

A representative result for the comparative analysis of gene expression between oestrous uteri and dioestrous uteri is shown (Fig. 1). Of the 8192 genes on the chip, most showed equal expression in oestrous and dioestrous uteri, visible as red points. A small number of genes showed at least a twofold difference in expression between the two stages. The green points above the band of red points indicate upregulated genes; those below the band are downregulated genes in oestrous uterus. After intensity normalization and data analysis from three independent experiments, the genes with significantly differential expression were identified (Tables 2–5).

In total, there were 51 upregulated and 51 downregulated genes or ESTs in oestrous uterus, of which 62 are well characterized and 40 are ESTs according to the gene description shown in the company's user manual. Of the known 33 upregulated genes, the expression of 20 was more than threefold higher and the expression of 13 was two- to threefold higher in oestrous uterus than that of the corresponding genes in dioestrous uterus (Table 2). The most significantly induced gene was *sprr2A*, with tenfold higher expression in oestrous uterus, followed by *24p3*, serine protease inhibitor α-1 PI-1, α-1 PI-4 and α-1 PI-5, all with fivefold higher expression. Of the known 29 downregulated genes, the expression of two

Table 3. Genes downregulated in mouse uterus at the oestrous stage

GenBank no.	Fold up	Gene description	Functional category
M13441	5.03	α-tubulin isotype M-α-6	Cytoskeleton and mobility
M13443	3.9	α-tubulin isotype M-α-7	Cytoskeleton and mobility
Z46757	2.9	High mobility group 2 protein (HMGb2)	DNA-binding proteins
AB018575	2.82	Cell division cycle 7-like 1	Cell cycle regulators
D10464	2.78	Kallikrein	Protein turnover
X12944	2.72	High mobility group 17 (HMG-17)	DNA-binding proteins
U05809	2.71	Transketolase	Enzyme
M13445	2.62	Tubulin α1	Cytoskeleton and mobility
D55720	2.6	Karyopherin (importin) α2	Nuclear transporter
M13446	2.55	Tubulin α2	Cytoskeleton and mobility
M13444	2.49	α-tubulin isotype Mα-4	Cytoskeleton and mobility
AF053232	2.48	SIK similar protein	Kinase
X58251	2.43	Procollagen, type I, α2	Cytoskeleton and mobility
X70887	2.42	P59 immunophilin	Chaperonin protein
U86338	2.41	Zinc finger protein Png-1 (Png-1)	Transcription factors
M96163	2.39	Serum inducible kinase (SNK)	Protein kinase
U27830	2.35	Murine homologue of the stress-inducible phosphoprotein (mSTI1)	Stress response protein
X57800	2.25	Proliferating cell nuclear antigen (PCNA)	DNA synthesis
X52046	2.24	Procollagen, type III, α1	Cytoskeleton and mobility
X66091	2.22	Alternative splicing factor (ASF)	RNA splicing-related protein
M29395	2.21	Orotidine-5′-monophosphate decarboxylase	DNA synthesis
X15666	2.21	Ribonucleotide reductase M2	DNA synthesis
Z14044	2.17	Valosin-containing protein, VCP	Cytoskeleton and mobility
J02870	2.17	Laminin receptor	Cell receptor
U83902	2.15	Mitotic checkpoint component 2 (Mad2)	Cell cycle regulator
X52101	2.15	Polypyrimidine tract-binding protein	RNA splicing-related protein
U43918	2.13	Proliferation-associated protein 1, PTB	DNA synthesis
D17666	2.05	Mitochondrial stress-70 protein (PBP74/CSA)	Stress response protein
X04663	2.02	β-tubulin isotype Mβ5	Cytoskeleton and mobility

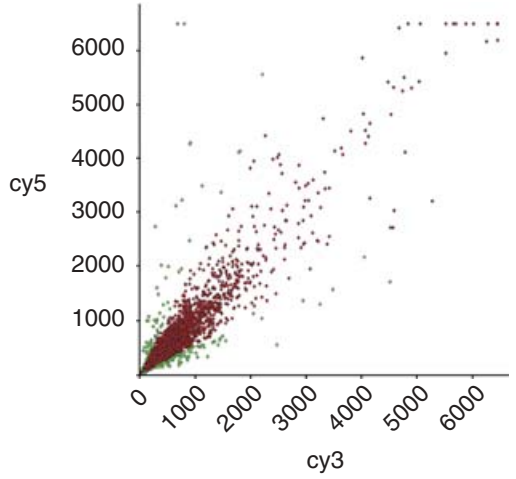

Fig. 1. Scatter plot of the composite normalized data. Fluorescence-labelled mouse cDNAs were hybridized to biostar M80s GeneChip. Three microarray hybridizations were performed independently. The cDNAs of dioestrous uteri were labelled with cy3 fluorescent dye and the cDNAs of oestrous uteri were labelled with cy5. The green points above the band of red points indicate upregulated genes; those below the band are downregulated genes in oestrous uterus compared with dioestrous uterus.

was more than threefold lower and the expression of 27 was two- to threefold lower in oestrous uterus than that of their counterparts in dioestrous uterus (Table 3). The most significantly downregulated gene was the α-tublin isotype M-α-6 gene, which had a fivefold lower expression in oestrous uterus. Of the differentially expressed ESTs, six of 18 upregulated ESTs and four of 22 downregulated ESTs displayed a more than threefold difference in expression relative to the corresponding ESTs in dioestrous uterus (Tables 3 and 4).

Differentially expressed genes were categorized according to their biological function; those with multiple functions were put in a separate category. Of the upregulated genes listed, eight were related to protein turnover, one to cell signalling, six to immune reactions, one to stress response, one to apoptosis, three to oncogenes and tumour suppressors, three to metabolic enzymes, three to cytoskeleton and structural proteins, one to nerve-related proteins, and two had unspecified functions (Table 2). Of the proteins encoded by the downregulated genes listed, nine were cytoskeleton and structural proteins, two were DNA-binding proteins, two were regulators of cell cycle, one was a nuclear

Table 4. Expressed sequence tags (ESTs) upregulated in mouse uterus at the oestrous stage

GenBank no.	Fold up	Homological gene by BLAST analysis	*E* value	Presumed functional category
AW536164	8.49	XM_148407: *Mus musculus*, peptidylprolyl isomerase (cyclophilin)-like 2 (Ppil2)	0.0	Protein folding
AI593561	3.57	BC025165: *Mus musculus*, glycoprotein m6b	0.0	Neuro-specific protein
AI116859	3.56	XM_134374: *Mus musculus*, similar to tyrosine minotransferase (TAT)	0.0	Enzyme
AA896021	3.44	BC010789: *Mus musculus*, placenta-specific 8	0.0	Unknown function
AW546453	3.24	AK013287: *Mus musculus* 10, 11 day embryo whole body cDNA, RIKEN full-length enriched library	0.0	Unknown function
AW912899	3.03	AK037080: *Mus musculus*, adult female vagina cDNA	0.0	Unknown function
AW489972	2.86	XM_204171: *Mus musculus*, expressed sequence AI790744	0.0	Unknown function
C77646	2.50	BC005695: *Mus musculus*, expressed sequence AL024210	0.0	Unknown function
		AF172642: *Mus musculus*, ocular albinism type 1 protein (Oa1)	e^{-174}	Neuro-specific protein
AW556696	2.48	AL606982: mouse DNA sequence from clone RP23-354C15 on chromosome 4	0.0	Unknown function
AU067636	2.44	AL772275: mouse DNA sequence from clone RP23-308P6 on chromosome 4	0.0	Unknown function
AA986889	2.41	BC003470: *Mus musculus*, phospholipase A1 member A	0.0	Enzyme
AU067716	2.40	XM_130448: *Mus musculus*, solute carrier family 24, member 3 (Slc24a3)	0.0	Cell trafficking
AW048109	2.37	No significant homology with the known DNA fragments in GenBank	–	Unknown function
BB012137	2.25	BC018512: *Mus musculus*, similar to IRA1 protein	0.0	Cell signalling
AI508687	2.14	NM_028756: *Mus musculus*, solute carrier family 35, member A5	0.0	Unknown function
AI327419	2.08	AK002574: *Mus musculus*, adult male kidney cDNA. Hypothetical serpin-containing protein	0.0	Unknown function
AI526768	2.07	NM_027464: *Mus musculus*, RIKEN cDNA 5730469M10 gene	0.0	Unknown function
AU015314	2.06	AL627184: mouse DNA sequence from clone RP23-125F21 on chromosome 4	0.0	Unknown function

transporter, three were stress response proteins and chaperonin, one was a transcription factor, two were protein kinases, one was a cell receptor, one was a metabolic enzyme, four were associated with DNA synthesis, two were associated with RNA splicing and one was associated with protein turnover (Table 3).

The sequences of differentially expressed ESTs were subjected to BLAST analysis and data on the available gene most closely related in sequence are shown (Tables 4 and 5) and were used for clarification base on gene ontology. Of the upregulated ESTs, one was related to protein folding, one to cell trafficking, one to cell signalling, two to metabolic enzymes, two to neural-specific proteins and one to immuno-related protein (Table 4). Of the downregulated ESTs, two were associated with growth factors and their binding proteins, one with cellular trafficking, four with enzymes, one with cytoskeleton, one with transcription factors, one with cell cycle and one with DNA-binding protein (Table 5). The others are of unknown function.

Patterns of gene expression revealed by northern blot analysis and in situ *hybridization*

Two upregulated genes including Sprr2A and 17β-HSD2 and three downregulated genes that consisted of Hmg2, Mad 2 and an EST sequence (GenBank No. AW555366) in oestrous uterus were selected for northern blot assay in the normal cyclic uteri to confirm the changes in gene expression detected by microarray assay. Three independent experiments showed consistent results. A representative set of data is shown (Fig. 2). After densitometric scanning and GAPDH normalization, the expression of sprr2A and 17β-HSD2 showed 16- and sixfold upregulation in oestrous uteri, respectively, compared with dioestrous uteri. In contrast, the expression of Hmg2 and Mad2 in oestrous uteri was downregulated by about 16- and 14-fold, respectively, compared with that in dioestrous uteri. The expression of EST AW555366 was much lower at the oestrous stage compared with the other stages, and was 40-fold lower than at the dioestrous stage. *In situ* hybridization of EST AW555366 further revealed its mRNA localization in uteri. The signal was highest at the dioestrous and metoestrous stages, and moderate at the pro-oestrous stage, whereas it was much lower at the oestrous stage. In pro-oestrous uterus, the mRNA of AW555366 was mainly expressed by the stromal cells beneath the luminal epithelium. At the metoestrous stage, positive signals were shown by the stromal cells around the glandular epithelium. The basal stroma adjacent to the myometrium showed transcription of the gene at the dioestrous stage (Fig. 3).

Table 5. Expressed sequence tags (ESTs) downregulated in mouse uterus at oestrous

GenBank no.	Fold up	Homological gene by BLAST analysis	*E* value	Presumed functional category
AW555366	3.93	S43941: *Rattus norvegicus,* insulin-like growth factor I (exon 6)	$1e^{-67}$	Growth factors and binding proteins
C87819	3.76	AK077038: *Mus musculus*, adult male testis cDNA, hypothetical protein	0.0	Unknown function
W34067	3.52	BC019836: *Mus musculus*, insulin-like growth factor binding protein 4	0.0	Growth factors and binding proteins
AW548568	3.19	AK079430: *Mus musculus*, ATPase, aminophospholipid transporter (APLT), class I, type 8A, member 1 (Atp8a1)	0.0	Cell trafficking
AW554526	2.93	NM_133755: *Mus musculus*, tubulin, gamma complex-associated protein 2 (Tubgcp2)	0.0	Cytoskeleton and mobility
AW475908	2.83	HSU35612: *Homo sapiens*, SOX22 protein mRNA	$2e^{-60}$	Transcription factors
AA033369	2.82	NM_029568: *Mus musculus*, RIKEN cDNA 1110007F23 gene	0.0	Unknown function
AW543487	2.71	AC110302: *Mus musculus*, clone RP24-194G21	$4e^{-09}$	Unknown function
C79736	2.59	XM_135010: *Mus musculus*, NADPH cytochrome B5 oxidoreductase (Ncb5or)	0.0	Unknown function
W13431	2.53	S83436: *Rattus norvegicus*, glutathione *S*-transferase subunit 13	$2e^{-92}$	Enzyme
AU017342	2.37	BC003252: *Mus musculus*, similar to transforming, acidic coiled-coil containing protein 3 (TACC3)	$3e^{-40}$	Cell cycle
AW544168	2.36	XM_050988: *Homo sapiens*, KIAA1795 protein (KIAA1795)	e^{-122}	Unknown function
AU080846	2.32	AK076084: *Mus musculus*, similar to HNOEL-isoprotein	0.0	Unknown function
AA068786	2.28	BC016534: *Mus musculus*, nudix (nucleotide diphosphate linked moiety X)-type motif 3	$1e^{-98}$	Enzyme
AA763560	2.28	BC029165: *Mus musculus*, similar to ubiquitin-specific protease 24	0.0	Enzyme
AW611405	2.26	BC002133: *Mus musculus*, mitofusin 1	0.0	Unknown function
AA290483	2.24	AK018618: *Mus musculus*, adult male caecum cDNA	0.0	Unknown function
AI649097	2.23	AK081406: *Mus musculus*, day 16 embryo head cDNA	0.0	Unknown function
AI892207	2.18	NM_007453: *Mus musculus*, peroxiredoxin 6 (Prdx6)	0.0	Enzyme
W36443	2.16	XM_131776: *Mus musculus*, RIKEN cDNA 2610012O22 gene	0.0	Unknown function
AU040133	2.06	MMU404329: *Mus musculus*, partial mRNA for hypothetical protein (ORF1)	0.0	Unknown function
AW261702	2.06	XM_130271: *Mus musculus*, structure specific recognition protein 1 (Ssrp1), HMG-box protein, mRNA	0.0	DNA-binding proteins

Discussion

In the present study, a group of genes differentially expressed in the uterus at oestrus versus dioestrus was identified by cDNA microarray, and the credibility of microarray data was validated by northern blot analysis and *in situ* hybridization. The genes were subsequently categorized according to their biological functions. In this discussion, we will focus on the regulation of these genes by cyclic hormones, and the potential gene functions associated with immunoreaction and sperm capacitation, as well as the trophy–atrophy alteration of the uterus.

Ovarian steroid hormones are thought to be the factors underlying the rhythmic changes of uterus during the oestrous cycle. In mice, serum oestrogen concentrations reach a peak at the pro-oestrous stage, remain high during the oestrous stage, decrease to relatively low concentrations at metoestrus and remain at a slightly increased basal concentration during dioestrus. In contrast, serum progesterone concentration was low at the oestrous stage, and increased at the metoestrous and dioestrous stages (Fata *et al*., 2001). 17β-HSD is the key enzyme responsible for the metabolism of oestrogen. Types II and IV 17β-HSD primarily catalyse the oxidative reaction of oestradiol to oestrone, and thus attenuate the effect of oestradiol (Penning, 1997). It has been reported that progesterone stimulates the expression of 17β-HSD II via progesterone receptor, so the induced expression of progesterone receptor by oestrogen in pro-oestrous and oestrous uteri might account for the upregulation of 17β-HSD II expression (Tibbetts *et al*., 1998; Yang *et al*., 2001). The decrease in aromatase activity after the preovulatory gonadotrophin surge is an important factor in the decrease in oestradiol concentration after ovulation (Banks *et al*., 1991; Fitzpatrick and Richards, 1991). The upregulation of 17β-HSD II mRNA in oestrous uterus as indicated in the present

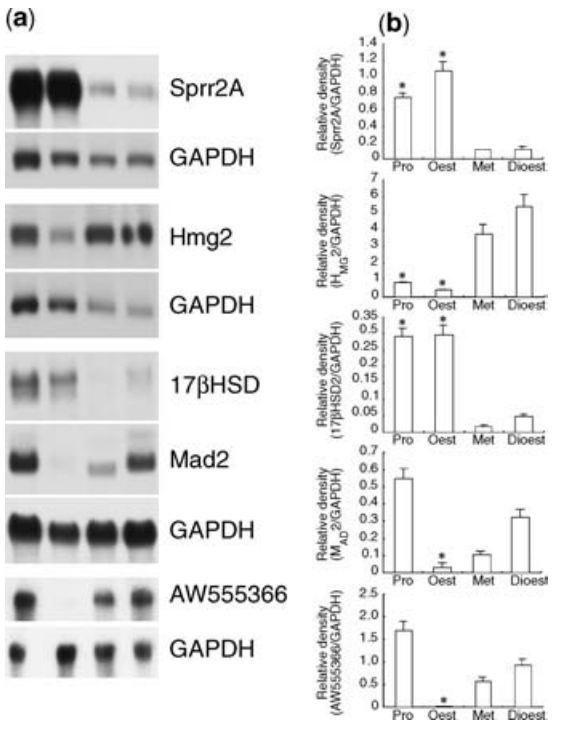

Fig. 2. Northern blot analysis demonstrating the changes in small proline-rich protein 2A (Sprr2A), 17β-hydroxysteroid dehydrogenase type 2 (17βHSD-2), high mobility group 2 (Hmg 2), mitotic checkpoint component 2 (Mad2) and EST AW555366 expression in mouse uterus during the oestrous cycle. (a) A representative result of the autoradiogram. (b) Densitometric analysis of autoradiograms. The relative amounts of the unique mRNA were standardized with that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Data were expressed as the ratio change, mean ± SD from the three separate experiments. *Significant difference compared with dioestrus, $P < 0.05$). Sprr2A expression was highly induced at the pro-oestrous (Pro) and oestrous (Oest) stages, and was suppressed at the metoestrous (Met) and dioestrous (Dioest) stages. The expression of Hmg2 was much lower at pro-oestrus and oestrus compared with its high expression at metoestrus and dioestrus. Expression of 17βHSD-2 was upregulated at pro-oestrus and oestrus, and downregulated at metoestrus and dioestrus, whereas Mad2 expression was downregulated at oestrus. Expression of EST AW555366 was greatly reduced at the oestrous stage.

study might contribute, together with the decreased aromatase expression, to the rapid decrease in oestrogen concentration observed after ovulation. However, there was no obvious change in 17β-HSD IV expression (data not shown), indicating the divergent transcriptional regulation of these two types of enzyme.

Many oestrogen-inducible genes have been identified in uterus including the progesterone receptor, vascular endothelial growth factor (VEGF), uteroglobin and retinoic acid receptor α genes (Inoue *et al*., 1993; Rishi *et al*., 1995; Scholz *et al*., 1998; Mueller *et al*., 2000). However, progesterone receptor, VEGF, uteroglobin and retinoic acid receptor α were not included in the microarray used in the present study. Some of the differentially expressed genes at oestrus versus dioestrus determined in the present study have been described previously, and some of the upregulated genes have been found to possess the oestrogen response element (ERE) in their promoters and, thus, to respond to oestrogen induction. Lactotransferrin secreted by uterine epithelial cells is one such example (Yang *et al*., 1996; Tibbetts *et al*., 1998); progesterone could antagonize the prolonged induction of this gene by oestradiol in uterus (McMaster *et al*., 1992). Another such gene is 24p3 (lipocalin/uterocalin); the protein encoded by 24p3 is mainly secreted by uterine epithelial cells as well as mammary gland and is thought to be an acute phase protein related to involution of the reproductive tissues (Ryon *et al*., 2002). In the uterus, oestrogen and retinoic acid can induce expression of 24p3 in the endometrial luminal and glandular epithelial cells (Garay-Rojas *et al*., 1996). Consistent with our data, Huang *et al*. (1999) found that the mRNA expression of this protein in the uterus was increased at pro-oestrus and oestrus but decreased sharply at metoestrus and dioestrus. Specific EREs have also been found in some complement components and factors including C3 and H genes (Munoz-Canoves *et al*., 1990). The pattern of expression of complement C3 and factor B during the oestrous cycle in mice in the present study is consistent with the results of Li *et al*. (2002). The activity and content of carbonyl reductase (CR) in rat ovaries were reported to be stimulated by LH, and oestrogen enhanced the stimulation of LH (Inazu *et al*., 1992). It remains uncertain whether LH and oestrogen can regulate CR in the uterus, although LH/hCG receptors have been found (You *et al*., 2000; Zhang *et al*., 2001). The temporal regulation of other genes in the uterus during the oestrous cycle and whether they have steroid hormone response elements are not yet clear. Watanabe *et al*. (2002) used microarray to analyse the gene expression cascades induced or repressed in response to oestrogen treatment in the uteri of ovariectomized mice. These workers found that there were several functionally closely related subgroups in the oestrogen-activated genes: sterol biosynthesis, tRNA ligase, RNA maturation, receptors (that is, adrenomedullin/calcitonin gene-related peptide (CGRP) receptors and leptin receptors) and growth factors (insulin-like growth factor I (IGF-I) and kallikrein). Activation of these genes suggested a mechanism for the marked uterotrophic effects of oestrogen, and the findings revealed the diverse effects of oestrogen via oestrogen receptor (ER) activation on the content of cellular transcripts *in vivo*. Some genes induced by oestrogen, including kallikrein and serum inducible kinase (SIK), were repressed in oestrous uterus as indicated also in the present study. These data may indicate that the expression of the uterine genes during the normal oestrous cycle was under the control of several factors, which consist of not only oestrogen, but also progesterone and various local growth factors.

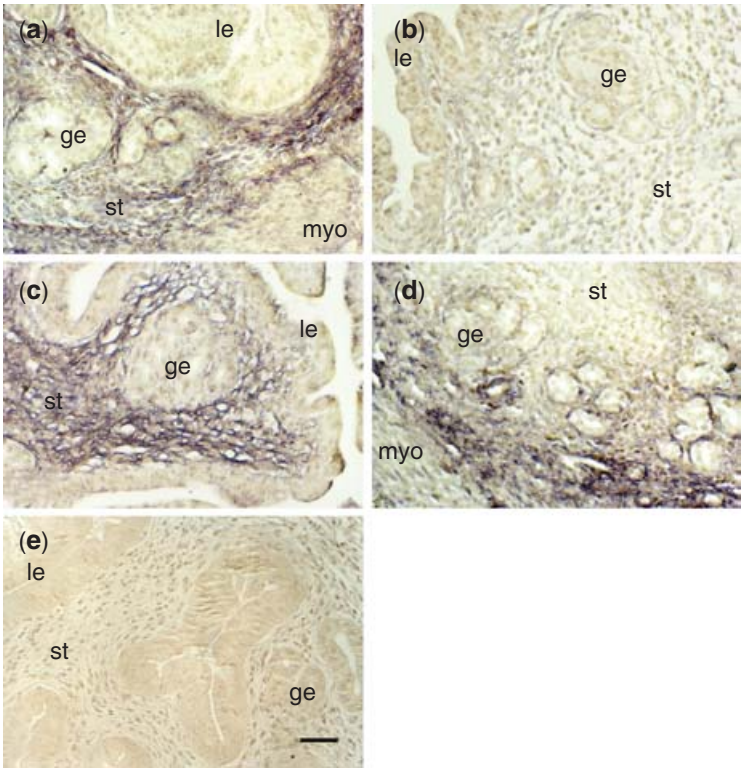

Fig. 3. *In situ* hybridization of EST AW555366 in mouse uterus during the oestrous cycle. (a) The signal was mainly localized in the stroma (st) beneath the luminal epithelum (le) at the pro-oestrous stage. (b) The stroma staining was weak at the oestrous stage. (c) At the metoestrous stage, a strong signal was presented by the stromal cells around the glandular epithelium (ge) that showed atrophic morphology. (d) At the dioestrous stage, the signal was mainly localized in basal stroma adjacent to myometrium (myo). (e) Hybridization with the sense RNA probe was used as negative control. Scale bar represents 40 μm.

In the present study, immune-related genes that were upregulated in the oestrous uterus included lactotransferrin, lysozyme, the complement component C3, factor B, factor H and 24p3. Lactotransferrin is a stimulator of proliferation of uterine stromal cells and is also an important factor in host mucosal immunity, exerting antibacterial activities by chelating iron and immune regulatory effects by binding to cells (Singh *et al*., 2002). Lysozyme is another important factor for natural mucosal immunity. It is possible that lysozyme and lactotransferrin may act in a cooperative way to inhibit and kill pathogens effectively (Leitch and Willcox, 1999). Lysozyme has also been proven to be an immune regulator; when cleaved by cathepsin D it releases peptides to stimulate T cells (van Noort and Jacobs, 1994). Complement component C3 is an important mediator of acute inflammation and its active form could induce the production of interleukin 8 (IL-8) in epithelial cells (Monsinjon *et al*., 2001). IL-8 was able to induce the proliferation of stromal cells and the apoptosis of cytotoxic T lymphocytes in the endometrium (Selam *et al*., 2002). 24p3 induced apoptosis in a wide variety of leucocytes by regulating bcl-2 family members through the 24p3 receptor (Devireddy *et al*., 2001). There is evidence that 24p3 may be related to spermatogenesis in the testis (Tanaka *et al*., 2002) and to the maturation of spermatozoa in the epididymis of male mice (Chu *et al*., 2000). It is not clear whether 24p3 can directly influence spermatozoa in the uterus after copulation; however, it may protect spermatozoa from immuno-attack by inducing the apoptosis of infiltrating leucocytes. An increased number of immune cells including macrophages are recruited to uterine stroma at the oestrous stage (Kaushic *et al*., 1998) and, therefore, *in situ* hybridization will be essential to localize the transcripts the cellular origin of which was formerly unidentified, in order to clarify whether the increased expression of these genes is just the result of the recruitment of the immune cells at the oestrous stage, or of changes in gene expression. The upregulation of these immuno-related factors in oestrous uterus might indicate their involvement in proper immuno-protection against pathogens introduced by copulation, or in the balance between immune

tolerance and immune rejection with respect to possible seminal antigens.

In oestrous uterus, upregulated genes that might regulate sperm activity were spermidine/spermine N1-acetyl transferase, carbonyl reductase, serine protease inhibitors α-1 PI-1, -3, -4 and -5, and some protease genes. Spermidine/spermine N1-acetyl transferase was implicated in spermine metabolism by catalysing the acetylation of spermidine and spermine (Della Ragione *et al*., 1983). It has been reported that spermine can inhibit the acrosome reaction, thereby preventing premature capacitation (Rubinstein and Breitbart, 1991), but its role in uterus has not been reported before. In the present study, the upregulation of spermidine/spermine N1-acetyl transferase expression at oestrus may adjust the ratio of acetylized spermine in the uterine cavity, thereby exerting a regulatory effect on sperm state. Cytosolic carbonyl reductase belongs to the family of short chain dehydrogenases/reductases and catalyses the reductive reactions of many carbonyl compounds including endogenous prostaglandins and steroids (Forrest and Gonzalez, 2000). Its activity at the surface of spermatozoa was required for sperm–zona pellucida binding in hamsters, and this binding was specifically blocked by carbonyl reductase inhibitors (Montfort *et al*., 2002). Whether the increased expression of carbonyl reductase 2 in the uterus at oestrus affects sperm activity needs to be further studied. It is possible that only the carbonyl reductase 2 expressed on the apical plasma membrane of luminal epithelial cells has the potential to regulate sperm activity. Serine proteases and their inhibitors are implicated in tissue remodelling, cellular invasiveness, matrix degradation and tumour growth (Lockwood and Schatz, 1996; Van den Steen *et al*., 2001). Several serine protease inhibitors including α-1 PI-1, -3, -4 and -5 were found to be induced in oestrous uterus, whereas expression of serine protease inhibitors -1, -2, -3, -4, -6, -14 and -17 did not change markedly (data not shown). This finding indicates that various members of a certain family can be differentially regulated to play roles in different contexts. Elisen *et al*. (1998) reported that protein C inhibitor (PCI), a heparin-binding plasma serine protease inhibitor on the acrosomal cap of human spermatozoa, could inhibit sperm protease acrosin and block sperm–egg binding, which indicated that PCI might protect spermatozoa against premature acrosome reaction by modulating acrosin activity to coincide with binding to the oocyte. Noticeably, some proteases, such as cathepsins H, L and S, were also induced in the uterus during the same period. Uterine cathepsins can participate in many extracellular events including degradation of extracellular matrix (ECM) during the oestrous or menstrual cycle, implantation and placentation (Afonso *et al*., 1997; Jokimaa *et al*., 2001). Complement component factor B was also found to possess serine protease activity in addition to its immunological functions (Christie *et al*., 1980). The coordination of upregulated proteases and protease inhibitors indicates their possible involvement in balancing the uterine milieu for appropriate sperm activity and the periodic tissue remodelling of the uterine endometrium. However, information on the cellular localization or source of these enzymes is essential for clarifying their physiological significance in the uterus. This needs further investigation.

In the results of the present study, sprr2 was the most highly expressed gene at the oestrous stage, whereas sprr1 and sprr3, which belong to the same family, showed no differential expression between the two stages (data not shown). Members of the sprr family are known to serve as precursor proteins for stratified squamous epithelia to construct the cornified cell envelope (CE), a unique protective shield of squamous epithelia that acts against environmental insults such as trauma, wear-and-tear and loss of body water (Steinert, 2000). Therefore, they were considered differentiation markers of squamous epithelia. *In situ* hybridization indicated that sprr2 was also expressed in the epithelium of the uterus (Song *et al*., 1999). As uterine epithelium does not have CE structure, the high expression of sprr2 at the pro-oestrous and oestrous stages may indicate that it has novel functions in the uterus.

Information relevant to the relationship between ovarian steroid hormones and the downregulated genes found at oestrus in the present study is limited. High mobility group b2 (hmgb2) is one member of a high mobility group protein (HMG) family. It was thought that hmgb1 and hmgb2 could act as steroid receptor co-activators to interact with the receptors of oestrogen, progestin, androgen and glucocorticoids, and thus promote the binding of steroid–receptor complexes to their corresponding response elements (Boonyaratanakornkit *et al*., 1998). Oestrogen enhances acetylation of nuclear hmgb2 and -17 proteins in the uteri of newborn guinea-pigs, thereby altering their binding properties to chromatin (Pasqualini *et al*., 1989). The relatively upregulated expression of hmgb2 and hmg17 in dioestrous uterus may amplify the effects of oestrogen at this stage, causing the uterus to enter pre-proliferation status. The other downregulated genes in the oestrous uterus were related to cell proliferation, including cell cycle effectors and regulators (including Mad2), DNA synthesis- and RNA splicing-related factors, transcription factors and DNA binding proteins, chaperonins, kinases and transketolase. The pattern of expression of these genes was consistent with the status of uterine endometrial cells, which begin to actively proliferate at mid-dioestrus under the slightly increased oestrogen concentration, and which are less proliferative but more functional at oestrus.

One of the downregulated ESTs, which was named AW555366 and is homologous to exon 6 of rat IGF-I, was also downregulated at the oestrous stage but greatly induced at the metoestrous stage. *In situ* hybridization further demonstrated its localization in stromal cells.

As ESTs represent partial sequences of known and unknown transcripts by randomly selecting and one-pass sequencing cDNA clones from libraries, analysing ESTs helps in the discovery of novel genes (Hsu and Hsueh, 2000). However, cloning of full-length sequences of the cDNAs represented by the ESTs and clarification of their biological functions requires considerable effort. Further investigation is required to determine whether EST AW555366 is regulated by the progesterone that is highly concentrated in serum at the metoestrous stage and whether it is involved in the apoptosis that occurs at this stage.

In conclusion, the global profile of gene expression in mouse uterus at oestrus versus dioestrus was investigated, and the genes potentially susceptible to cyclic sex hormones were identified. These results may enable better understanding of the mechanisms underlying the cyclic alteration of uterine trophy–atrophy that occurs in preparation for sperm capacitation and endometrial receptivity after mating and resisting pathogen induced by copulation. Further efforts are needed to define the derivation and roles of those differentially expressed genes that were formerly unrecognized in reproduction.

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