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Global Gene Expression in Mouse Vaginae Exposed to Diethylstilbestrol at Different Ages

ATSUKO SUZUKI,*,†,‡,§,|| HAJIME WATANABE,†,‡,¶ TAKESHI MIZUTANI,†,‡ TOMOMI SATO,§ YASUHIKO OHTA,*,‡,|| AND TAISEN IGUCHI†,‡,¶,[1]

The United Graduate School of Veterinary Science, Yamaguchi University, 1677-1, Yoshida, Yamaguchi, 753-8515, Japan; †Okazaki Institute for Integrative Bioscience, National Institute for Basic Biology, National Institutes of Natural Sciences, 5-1 Higashiyama, Myodaiji, Okazaki 444-8787, Japan; ‡Core Research for Evolutional Science and Technology, Japan Science and Technology, 4-1-8 Motomachi, Kawaguchi 332-0012, Japan; §Graduate School of Integrated Science, Yokohama City University, 22-2 Seto, Kanazawa-ku, Yokohama 236-0027, Japan; ||Laboratory of Experimental Animals, Department of Veterinary Medicine, Faculty of Agriculture, Tottori University, Koyama 680-8553, Japan; and ¶Department of Molecular Biomechanics, School of Life Science, Graduate University for Advanced Studies, 5-1 Higashiyama, Myodaiji, Okazaki 444-8787, Japan

Estrogens regulate proliferation and differentiation of cells in target organs such as the female reproductive tract. In mature mice, estrogens stimulate cell proliferation, whereas ovariectomy results in atrophy of the female reproductive tract. In contrast, perinatal exposure to estrogens, including diethylstilbestrol (DES), induces persistent, ovary-independent vaginal stratification and cervico-vaginal tumors later in life. These effects are due to altered cell fate following DES exposure during a critical developmental period. The detailed mechanisms underlying the reversible and irreversible cell proliferation in vaginae induced by DES at different ages has not been clarified. Therefore, we examined differences in gene expression pattern using DNA microarray analysis in mouse vaginae 6 hrs after a single injection of 2 μg DES per gram of body weight, and proliferation of vaginal epithelial and stromal cells 24 hrs after the injection at postnatal days (PNDs) 0, 5, 20, and 70. After DES stimulation, vaginal epithelial and stromal cells showed cell proliferation at PNDs 20 and 70, and at PNDs 0 and 5, respectively. DNA microarray analysis exhibited 54 DES-induced genes and 9 DES-repressed genes in vaginae at PND 0, whereas more than 200 DES-induced genes were found in vaginae at PNDs 5 and 20, and 350 genes at PND 70. Clustering analysis of DES-induced genes in the vaginae at different ages revealed that genes induced by DES at PND 5 were closer to the adult type than that of PND 0. Genes related to keratinocyte differentiation, such as Gadd45α, p21, 14–3–3 sigma, small proline-rich protein 2f (Sprr2f), and Krupple-like factor 4 (Klf4), were induced by DES. The number of DES-induced genes during the critical period, PND 0, was smaller than those found after the critical period. These results give insight toward understanding the molecular mechanisms underlying the critical period in mouse vaginae. Exp Biol Med 231:632–640, 2006

Key words: microarray; gene expression; diethylstilbestrol; vagina; mouse

Estrogens induce cell proliferation and differentiation, whereas estrogen depletion results in atrophy accompanied by apoptosis in adult female reproductive organs such as the uterus and vagina (1–3). Estrogen exposure during a critical period in early development induces persistent ovary-independent proliferation and keratinization in the vaginal epithelium during adulthood (4, 5). Diethylstilbestrol (DES), a synthetic estrogen used to prevent miscarriage during the 1940s to the early 1970s, induced vaginal clear cell carcinoma and uterine abnormalities in daughters of mothers exposed to DES during pregnancy (6). Similar abnormalities were reported in mice exposed to estrogens during a critical perinatal period (4, 5, 7). In female mice, various abnormalities such as polyovular follicles, oviductal tumors, uterine epithelial metaplasia, persistent vaginal stratification and keratinization, vaginal

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[1] To whom correspondence should be addressed at Okazaki Institute for Integrative Bioscience, National Institute for Basic Biology, National Institutes of Natural Sciences, 5-1 Higashiyama, Myodaiji, Okazaki 444-8787, Japan. E-mail: taisen@nibb.ac.jp

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adenosis, and cervico-vaginal carcinomas, were induced by perinatal exposure to estrogens including DES (4, 5, 7–13). Vaginal epithelial proliferation persists even after ovariectomy in adult mice exposed to a sufficient dose of DES during the early neonatal period (4, 5).

During the normal estrous cycle, vaginal epithelial cell proliferation and keratinization occur at the estrous stage (1), whereas keratin 1 (K1) and progesterone receptor expressions were induced at the proestrous stage (14, 15). DES exposure during a critical developmental period results in alteration of the response to estrogen in the vagina, leading to a set of subsequent abnormalities. Epithelial cells failed to undergo apoptosis even after ovariectomy, and persistent expression of various genes was observed in the persistently proliferated vagina (15–17). Reduced expression of estrogen receptor (ER) mRNA and persistent expression of c-fos and c-jun mRNAs were observed in the vaginae of mice exposed to DES at the neonatal stage, even after ovariectomy (15). Persistent phosphorylation of erbB receptors, including epidermal growth factor (EGF) receptor, and sustained expression of EGF-like growth factors were found in the vaginae of mice neonatally exposed to DES (16). Neonatal exposure to a fibroblast growth factor family member, keratinocyte growth factor (KGF), resulted in persistent vaginal epithelial stratification (18). The induction of EGF by estrogens may play important roles in the proliferation of epithelial cells in the uterus and vagina (18–21).

We used DNA microarray to analyze gene expression in neonatally DES-exposed mouse vaginae and observed persistent expression of interleukin-1 (IL-1), IL-1 receptor, insulin-like growth factor-I (IGF-I) mRNAs, and stress-activated protein kinase/c-jun N-terminal kinase (SAPK/JNK), as well as phosphorylation of downstream genes (17).

The critical periods for the induction of abnormalities by estrogenic chemicals during mouse development varies by organ (22). Analyses of the molecular mechanisms underlying the critical sensitive window in each organ is essential for understanding the etiology of the persistent changes induced in the reproductive tracts. Therefore, we examined global expression in vaginae of early genes elicited by DES treatment at different ages in order to understand the differences in estrogen-responsive genes during and after the critical period, and in adulthood.

Materials and Methods

Animals. C57BL/6J mice (CLEA, Tokyo, Japan) were used at postnatal days (PNDs) 0, 5, 20, and 70. Mice were maintained in a 12:12-hr light:dark cycle at 23–25°C. They were fed a commercial diet (CE-2; CLEA), and tap water was provided *ad libitum*. All experiments and animal husbandry protocols were approved by the animal care committee of the National Institutes of Natural Sciences.

Treatments. DES (Sigma Chemical Co., St. Louis, MO) was dissolved in sesame oil. Unless otherwise stated, all materials were obtained from Wako Pure Chemical Industries, Osaka, Japan. The day of birth was designated as Day 0. For microarray experiments, mice at PND 0 (7–12 mice from three litters), PND 5 (7–12 mice from three litters), PND 20 (8 mice), and PND 70 (8 mice) were given a single subcutaneous (sc) injection of 2 μg DES per gram of body weight (bw) or oil vehicle alone. Mice at PND 70 were ovariectomized at 56 days of age. Vaginae from DES-exposed and control mice were collected for DNA microarray analysis and quantitative real time–polymerase chain reaction (QRT-PCR). In order to identify early genes induced by DES, tissues were dissected 6 hrs after the injection as described previously (23–25).

In addition, five mice each were given a single sc injection of 2 μg DES/gram bw or oil vehicle alone for the bromodeoxyuridine (BrdU) experiment.

DNA Microarray Analysis. Total RNA from vaginae was extracted using TRIZOL (Invitrogen, Tokyo, Japan) and purified using an RNeasy mini kit (Qiagen, Tokyo, Japan). Quality and quantity of total RNA were confirmed by the Agilent 2100 bioanalyzer (Agilent, Tokyo, Japan). cRNA probes were prepared from the purified RNA using an Affymetrix cRNA probe kit (Affymetrix, Inc., Santa Clara, CA) according to the manufacturer's protocol. All preparations met the recommended criteria of Affymetrix for use on their expression array. The amplified cRNA was hybridized to high-density oligonucleotide arrays (Mouse U74A; Affymetrix, Inc.) containing approximately 12,500 genes, and the scanned data were analyzed with GeneChip software (Affymetrix, Inc.) and processed as described previously (23). To confirm the estrogen-related changes in gene expression revealed by DNA microarray analysis, we independently repeated the same experiment at least twice. The expression data were analyzed with GeneSpring software (Agilent, Palo Alto, CA) as described previously (26).

For the clustering analysis, genes activated more than 2-fold by DES were selected, and similarities between experiments and expression levels were measured by standard correlation using the GeneSpring program as described (23–26). Gene expression change was estimated by the value of control PND 0 as one. Putative target genes were validated by QRT-PCR.

QRT-PCR. Total RNA was purified as described above. cDNA was synthesized from purified total RNA with Superscript II RT (–) (Invitrogen) with random primers at 42°C for 60 mins. PCR reactions were performed in the PE Prism 5700 sequence detector (PE Biosystems, Tokyo, Japan) using SYBR-Green PCR core reagents (PE Biosystems) in the presence of appropriate primers according to the manufacturer's instructions. PCR amplification was performed in triplicate under the following conditions: 2 mins at 50°C, 10 mins at 95°C, followed by a total of 40 cycles of 15 secs at 95°C and 1 min at 60°C.

Gene expression levels were normalized to the expression levels of ribosomal protein L8 mRNA

Table 1. Sequences for Primers Used for QRT-PCR

Genbank accession No.	Name	Forward primer	Reverse primer
U20344	Klf4	ACACAGGCGAGAAACCTTACCA	AATTTCCACCCACAGCCGT
AF058798	14-3-3 sigma	ACAAGGACAGCACCCTCATCA	ACAGCGTCAGGTTGTCTCTCAG
AJ005559	Sprr2a	TCCTGTAGTGTGCTATGAGCAATG	TTGCACAGGAGGGCATGTT
AJ005564	Sprr2f	TGAGGCTTCAGCAACAATGTCTT	TTGGTGGTGGACACACAGGA
AB003502	Gspt1[a]	CAAGTATGCATTGCGCGTTTA	CCCATCTGAGGGAAGTCCTTAA
AI121305	EST	TTATGTCCTCAGTCCGCAGCT	TAGTGTTGCAGGTCTGTGGTCC
AW048937	p21	TGAGACGCTTACAATCTGAGTGG	AACATGTATTGTGGCTCCCTCC
U00937	Gadd45α	GAAGAAGGAAGCTGCGAGAAAA	CCTGGCCATCCTAAATTAGCAGT
U67771	Ribosomal protein L8	ACAGAGCCGTTGTTGGTGTTG	CAGCAGTTCCTCTTTGCCTTGT

[a] Gspt1, G1 to S phase transcript 1.

(U67771), and gel electrophoresis and melting curve analyses were performed to confirm correct amplicon size and the absence of nonspecific bands. The primers were chosen to amplify short PCR products of less than 100 base pairs and their sequences are listed in Table 1.

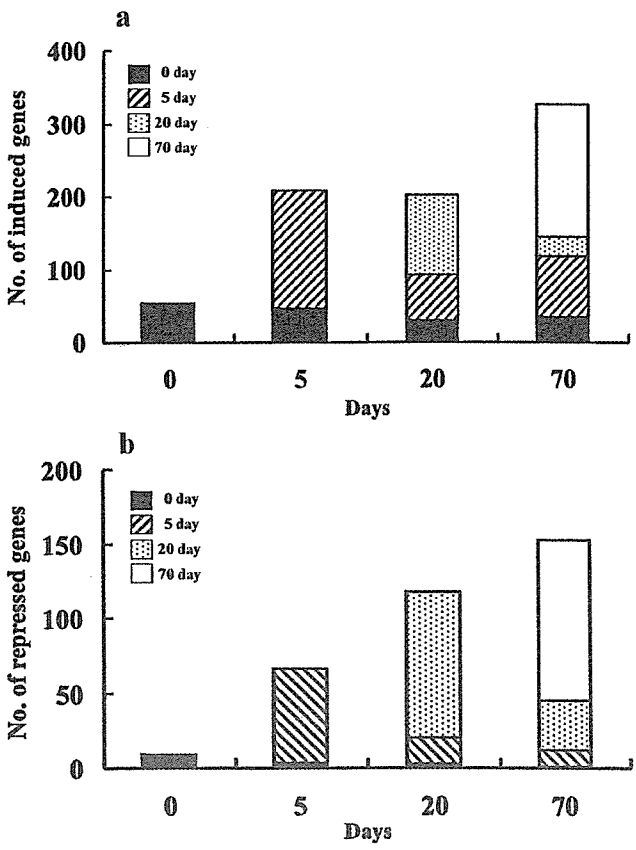

Figure 1. (a) The number of induced genes in vaginae 6 hrs after a single injection of 2 μg DES/g bw at PNDs 0, 5, 20, and 70. Mice at PND 70 were ovariectomized 2 weeks before. The number of DES-induced genes was small at PND 0, but it increased drastically at PND 5. (b) The number of repressed genes in vaginae 6 hrs after a single injection of 2 μg DES/g bw at PNDs 0, 5, 20, and 70. The numbers of DES-repressed genes increased linearly with age. Each pattern in the bar indicates genes commonly induced (a) or repressed (b) genes in respective ages. Days indicate ages given a single injection of DES.

Gene expression levels in DES-treated groups were normalized using control PND 0 as one. Parametric variables were analyzed by one-way analysis of variance (ANOVA) with post-hoc Student's *t* test or Welch's *t* test.

Immunostaining of BrdU. Five mice each given a single sc injection of 2 μg DES were killed 24 hrs after the injection. Two hours before dissection, 0.05 mg/g BrdU (Sigma, Tokyo, Japan) was injected intraperitoneally. Tissues fixed with neutral-buffered 10% formalin were embedded in paraffin. Sections cut at 8 μm were incubated with 0.3% H_2O_2 in methanol for 30 mins at room temperature (RT) to block endogenous peroxidase activity. They were washed with 0.5% Tween-20 in phosphate-buffered saline (PBS), incubated with 2 *N* HCl in water for 20 mins at RT. They were washed with 0.5% Tween-20 in PBS, incubated with 1% bovine serum (BSA) for 20 mins at RT, and with anti-BrdU antibody (Roche, Mannheim, Germany) at a dilution of 1:15 in 1% BSA at 4°C overnight. Washing with 0.5% Tween-20 in PBS, sections were incubated with mixture of 3,3′-diaminobenzidine tetrahydrochloride (DAB) and H_2O_2. Counterstain was conducted with methyl green. The number of BrdU-positive cells in 300 epithelial cells in the middle part of vagina and that in 500 stromal cells were recorded. Proliferation rate (%) was estimated as a percentage of BrdU-positive cells in epithelial cells and stromal cells, separately.

Results

Gene Expression in the Vaginae of Mice Treated with DES at Different Ages. The number of detected genes in the mouse vagina from oil-injected controls was not very different among animals examined at different ages: newborn (PND 0) = 4988 genes, PND 5 = 4937 genes, PND 20 = 4881 genes, and PND 70 = 4903 genes. We selected genes showing at least a 2-fold change in expression in response to DES treatment for further analysis (listed at http://www.nibb.ac.jp/bioenv1/suzuki/). The number of genes induced or repressed by DES was modest at PND 0, but showed a sharp increase with age (Fig. 1). DES exposure induced 54, 208, 202, and 326 genes and repressed 9, 66, 117, and 152 genes in vaginae at PNDs

Table 2. Induced or Repressed Genes in Vaginae 6 Hrs After a Single Injection of DES at Only PND 0

Genbank accession No.	Fold change	Name
AV170770	2.0	EST
AI837116	2.4	Solute carrier family 41, member 1
AI851565	2.5	RIKEN cDNA 1500034J01 gene
U58887	3.3	SH3-domain GRB2-like 3
D50646	0.4	Stromal cell–derived factor 2
AI425990	0.4	RIKEN cDNA C530046L02 gene
AI646638	0.5	Frequently rearranged in advanced T-cell lymphomas 2
M12347	0.5	Actin, alpha 1, skeletal muscle
AI841689	0.5	Chemokine-like factor superfamily 3

0, 5, 20, and 70, respectively (Fig. 1). The number of genes (208) induced by DES at PND 5 was similar to that of PND 20 (202). Ninety-two of 208 (44%) genes induced by DES at PND 5 were also induced at PND 20 by DES. Fifty-eight (28%) PND 5–specific genes were induced by DES (Fig. 1). Four genes (including two enhanced sequence tags [ESTs]) were specific to PND 0 (Table 2 and Fig. 1).

The total number of DES-regulated genes was 781. Some genes showing drastic expression change by DES were selected for further study (Table 3). Many of these genes showed upregulation by a single injection of DES from PND 0 to PND 70. Twenty-five genes, including MAD2, G1 to phase transition 1, c-fos, early growth response 1 (Egr-1), and Kruppel-like factor 4 (Klf4) were induced by DES exposure at all ages examined. MAD2 is an estrogen-responsive gene and assembles the mitotic spindle at the G2/M checkpoint (27). c-fos and Egr-1 were reported to be estrogen-responsive genes in the mouse uterus or mammary gland (or both) (15, 23, 28). Klf4, an inhibitor of the G1/S phase, plays a role in keratinocyte differentiation (29, 30), and is identified as an estrogen-responsive gene in the present study (Table 3).

The number of DES-repressed genes showed an age-dependent increase (Fig. 1). Twenty of 66 (30%) DES-repressed genes at PND 5 were also found at PND 20. Thirty-nine of 117 (33%) DES-repressed genes at PND 20 were also found at PND 70, although the number of age-specific DES-repressed genes was 5 (56%), 39 (59%), 64 (55%), and 107 (70%) at PNDs 0, 5, 20, and 70, respectively (Table 2 and Fig. 1). One of the common DES-repressed genes at all ages was flavin-containing monooxygenase 1 (Fmo 1; Table 3), which regulates metabolism of chemicals (31).

Clustering Patterns and Category of DES-Regulated Genes in the Vaginae of Mice at Different Ages. The 729 genes showing more than a 2-fold change following a single injection of DES at PNDs 0, 5, 20, and 70 were used for clustering analysis. These genes can be grouped as PND 0, PND 5, and PND 70 in controls, and as PND 0 and PND 5–70 in DES-exposed vaginae (Fig. 2) because the clustering patterns of genes in DES-exposed vaginae at PND 5 was more similar to those of PND 20 and PND 70 than that of PND 0 (Fig. 2).

These genes could be categorized into several groups. Genes involved in cell proliferation (15%) and protein modification (17%) were found in the DES-induced genes at PND 0 (Table 4). DES-repressed genes at PND 0 included those involved in cell tissue structure (11%), defense response (11%), transcription (11%), and transport (22%) compared with other groups. DES-regulated genes categorized in organogenesis were repressed by DES at PNDs 5, 20, and 70.

Confirmation of Gene Expression by QRT-PCR. Expression of several genes showing upregulation by DES in mouse vaginae at different ages was confirmed using QRT-PCR. The fold change in gene expression was estimated based on the value of each gene in control PND 0 as one (Fig. 3). Expressions of 14–3–3 sigma , Klf4, Sprr2f, EST (AI121305), and Gadd45α, which promotes the G1 phase and acts at the G2/M checkpoint (32, 33), were increased with age in control mice. Expression of p21 and G1 to phase transition 1 mRNAs were increased at PND 5 in control mice, whereas expression of Sprr2a was decreased at PND 5 in control mice. Expression of these genes, except for Klf4 and EST (AI121305), were induced by DES at PNDs 5, 20, and 70, but not at PND 0. Expression of Klf4 and EST (AI121305) was upregulated by DES at PND 0.

Immunostaining of BrdU. In oil-treated controls, ratios of BrdU-positive cells were not different among postnatal ages either in epithelial cells or in stromal cells. BrdU-positive cells in the vaginal epithelium were increased at PNDs 5, 20, and 70 in DES-treated vaginae as compared with the oil controls. In contrast, a higher number of BrdU-positive cells were found in the stroma in PND 0 and PND 5 mice (Fig. 4).

Discussion

Estrogen, androgen, and KGF exposure for 5 days from the day of birth induces persistent vaginal epithelial stratification in mice (4, 7, 11, 16–18). The persistent vaginal epithelial stratification with superficial keratinization induced by perinatal estrogen exposure was reported to be accompanied by persistent expression of several growth

Table 3. Induced or Repressed Genes in Vaginae 6 Hrs After a Single Injection of DES at PNDs 0, 5, 20, and 70 Using DNA Microarray and QRT-PCR[a]

Genbank accession No.	Gene[b]	Age (days) 0	5	20	70
Induced genes		Ratio by microarray (by QRT-PCR)			
Cell proliferation					
AB003502	G1 to S phase transition 1 (Gspt1)	2.2 (1.4)	2.8 (1.6)	2.1 (2.4)	3.3 (1.2)
V00727	c-fos	4.0	14.5	6.4	9.4
U83902	MAD2	2.1	2.7	3.7	13.8
X59846	Gas6	4.0	3.9	2.1	3.7
AF058798	14-3-3 sigma	–(1.1)	3.4 (3.6)	3.4 (5.3)	2.7 (5.1)
AW048937	p21	–(1.5)	2.4 (1.6)	3.2 (3.9)	3.7 (2.5)
U00937	GADD45α	–(1.8)	6.0 (3.9)	4.5 (4.1)	4.5 (3.6)
Protein modification					
AB013848	Peptidyl arginine deiminase type I	4.1	6.8	3.3	4.5
L02526	Map2k1	2.1	2.1	2.2	2.0
X04591	Creatine kinase, brain	2.1	2.5	2.1	4.3
X59274	Protein kinase C, beta	2.6	2.7	2.1	2.2
Transcription					
M28845	Early growth response 1 (Egr-1)	2.8	4.7	2.9	3.5
U20344	Kruppel-like factor 4 (Klf4)	3.8 (3.5)	4.2 (5.0)	2.1 (4.2)	2.7 (2.0)
Signal cascade					
M63801	Gap junction membrane channel protein alpha 1	2.4	3.5	2.1	2.1
AI596360	RIKEN cDNA 4930422J18 gene	3.1	7.2	2.6	2.8
Unknown					
X67644	Immediate early response 3	2.7	5.3	3.7	2.7
AI121305	RIKEN cDNA 1600029D21 gene	3.8 (2.4)	15.1(14.9)	3.7 (4.3)	3.6 (2.4)
Cell structure					
K02108	K 2, basic, gene 6a	—	6.3	3.0	5.2
AB012042	K 2, basic, gene 6g	—	2.9	7.7	4.1
M36120	K 1, acidic, gene 19	—	—	—	2.6
AJ005559	Sprr2a	–(0.4)	2.2 (4.5)	–(1.3)	4.9 (4.0)
AJ005560	Sprr2b	—	—	—	4.4
AJ005564	Sprr2f	–(1.7)	15.0(14.2)	4.0 (8.0)	7.5 (3.9)
Repressed genes					
Transport					
D16215	Flavin-containing monooxygenase 1 (Fmo1)	0.5	0.3	0.3	0.5

[a] Gene expression change was calculated based on the value of PND 0 as one. QRT-PCR data appear in parentheses; — indicates no change.
[b] Gas6, growth arrest–specific 6; Map2k1, mitogen-activated protein kinase kinase 1; p21, cyclin-inhibitor 21; GADD45α, growth and DNA damage 45α; MAD2, mitotic arrest–deficient; Sprr, small proline-rich protein; K, keratin complex.

factors (16–19, 34, 35). However, the precise mechanism of estrogen effects on the vaginal epithelial proliferation at different ages has not been clearly demonstrated, although several studies demonstrated that neonatal DES exposure induced persistent expression of EGF and EGF-like growth factors (35, 36) and phosphorylation of ERα, EGF receptor, and erbB in mouse vaginae (16, 17).

In the present study, global gene expression in vaginae was analyzed at different ages using DNA microarray analysis. We demonstrated age differences in vaginal responses to estrogen in the induction of gene expression from PND 0 to PND 70. ER staining was found in vaginal stromal cells in CD-1 mice at PND 0 (37), and in both vaginal epithelial and stromal cells, but in only stromal cells in uterine epithelial cells in C57BL/Tw mice at PND 0 (38). Epithelial expression of ER in the reproductive tracts occurs later in C57BL/6 mice than in CD-1 mice (39). Developmental effects of estrogens, including DES on neonatal mouse vaginae, are mediated through stromal ER (40). In the present study, we used C57BL/6 mice. Thus, neonatal mouse vaginae seem to be responsive to estrogen at PND 0. However, in the present study, the number of DES-induced genes in vaginae at PND 0 was smaller compared with those in PNDs 5, 20, and 70 mice. Moreover, vaginal epithelia in mice at PNDs 5, 20, and 70 were proliferated by DES, but not at PND 0 in the present study (Fig. 4). The PND 0 mouse vagina is still under development even without estrogenic stimulation (4, 41). Thus, in terms of gene expression, the vagina at PND 0 is less sensitive to estrogenic stimulation than it is at later stages. It has been shown that the response to DES is different between the Müllerian and urogenital sinus vaginal epithelium; proliferation in the vaginal fornix (Müllerian vagina) is inhibited by neonatal DES and the cell morphology is altered (7). Further

Table 4. Functional Categories of DES-Responsive Genes in Mouse Vaginae Selected in the Present Study[a]

	Age (in days)							
	0		5		20		70	
Category	Induced	Repressed	Induced	Repressed	Induced	Repressed	Induced	Repressed
Biosynthesis	6	0	11	3	13	5	11	6
Cell proliferation	15	0	10	10	10	8	9	6
Cell tissue structure	2	11	9	10	9	5	9	3
Defense response	7	11	5	6	3	4	4	6
Metabolism	7	0	7	3	5	8	6	5
Mitochondrion	0	0	0	0	0	0	1	1
Organogenesis	0	0	0	4	0	3	1	3
Protein modification	17	0	12	7	12	7	9	5
Ribosome	2	0	1	1	0	1	1	0
Signal transduction	7	0	6	6	6	6	6	7
Transcription	7	11	11	10	10	14	12	16
Transport	7	22	7	9	13	13	11	7
Unclassified	23	45	21	31	19	26	20	35
Total (%)	100	100	100	100	100	100	100	100

[a] Total number of clustered genes in each category was 100%. Induced, % of categorized genes in DES-induced gene at each age; Repressed, % of categorized genes in DES-repressed gene at each age.

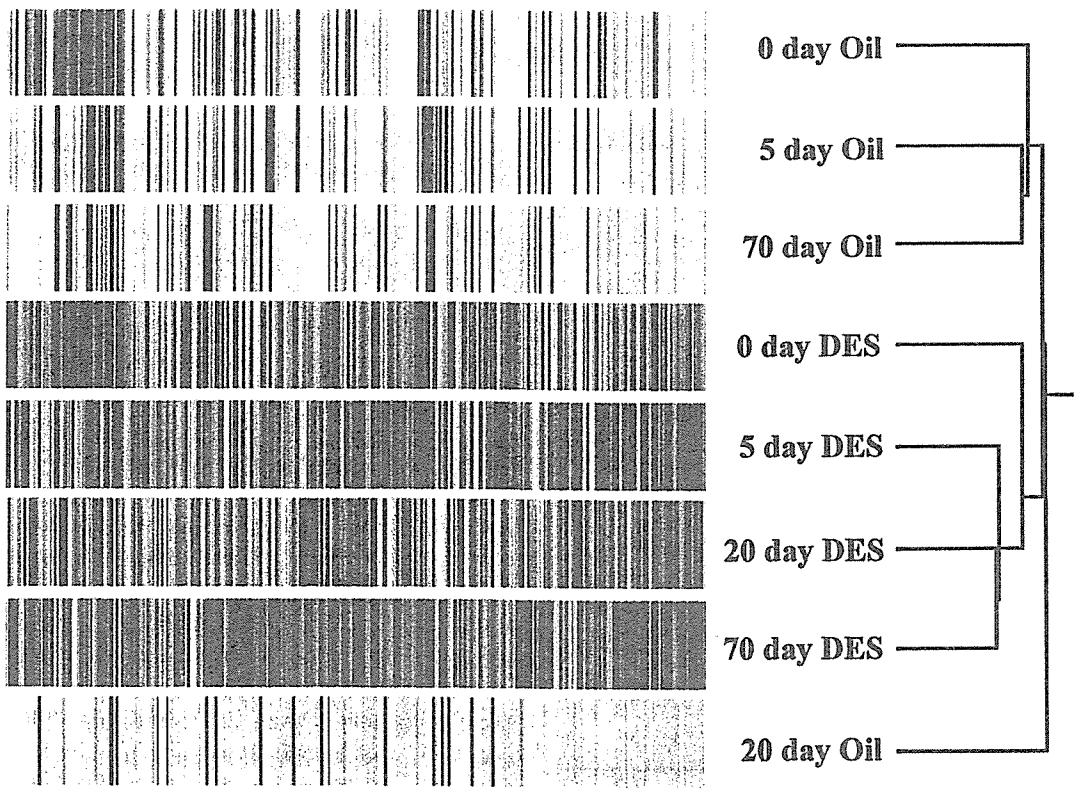

Figure 2. Clustering analysis of DES-responsive genes in mouse vaginae selected in the present study. Mice at PNDs 0, 5, 20, and 70 were stimulated by a single injection of DES. Each color bar indicates the expression level of one gene: red, induction; green, repression; yellow, average expression in eight groups; gray, not detected. Genes showing more than a 2-fold change in expression 6 hrs after a single injection of 2 μg DES/g bw at all ages were used for clustering analysis. Seven-hundred seventy-nine genes were selected. Control mice exhibited separated trees between the neonatal period (PNDs 0 and 5) and adulthood (PNDs 20 and 70). Branching of clustered genes in DES-exposed vaginae at PND 5 was closer to that of PND 0.

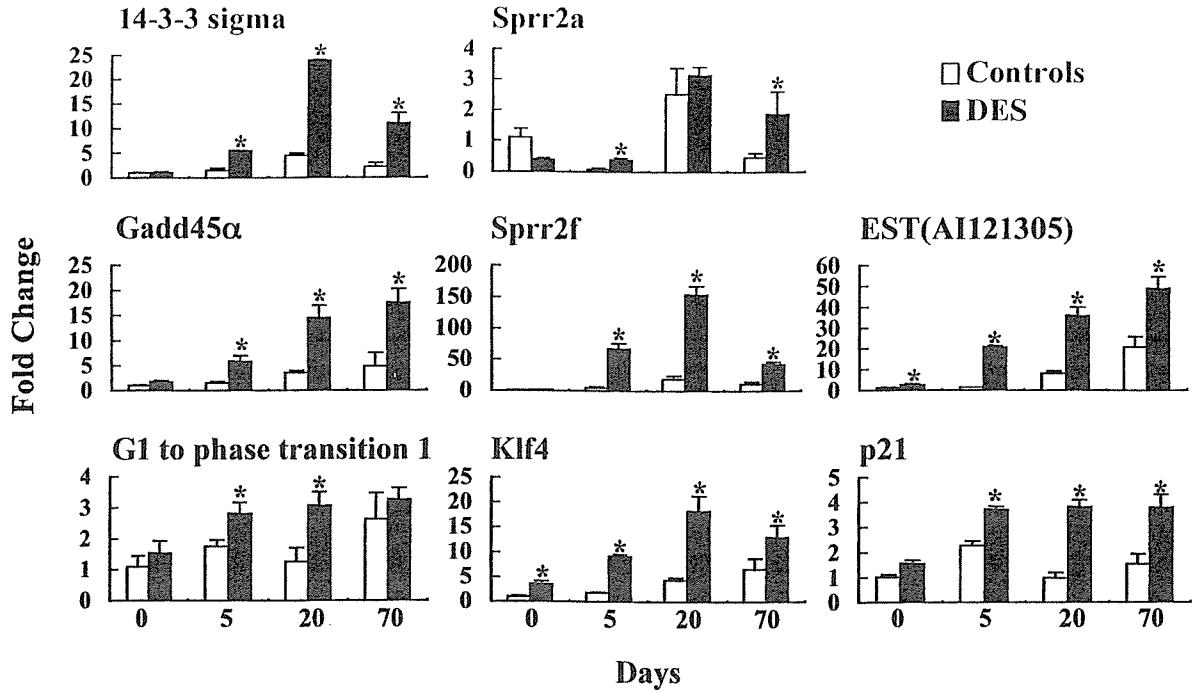

Figure 3. Changes in gene expression of cell cycle and keratinocyte differentiation regulators were confirmed by QRT-PCR. Fold change of gene expression by QRT-PCR was estimated based on the value of each gene at PND 0 as one. Results are the mean and SEM of three experiments. Each experiment was performed in triplicate. $P < 0.05$ vs. age-matched controls.

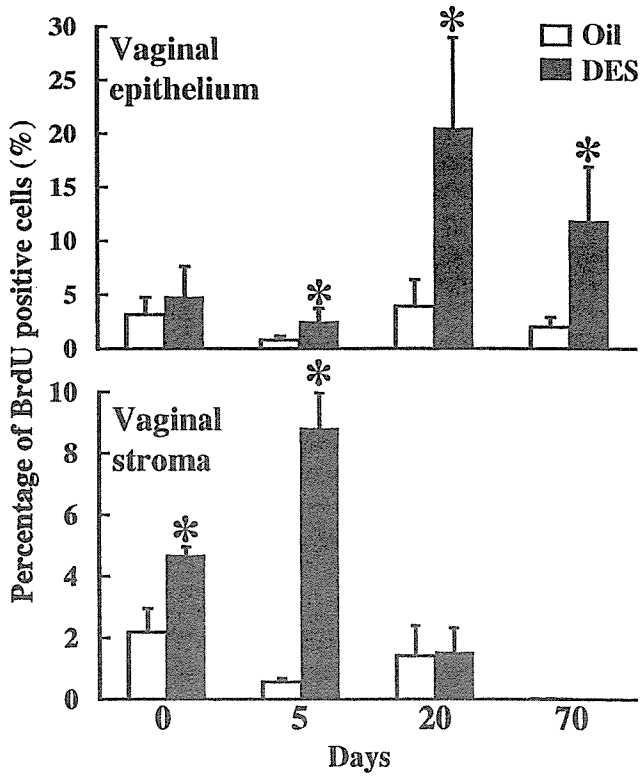

Figure 4. Percentage of BrdU-positive cells in vaginal epithelial cells and stromal cells at different ages. Mean and SEM. Note reverse of vaginal cell proliferation in epithelium and stroma after PND 5. *$P <$ 0.05 vs. age-matched controls.

studies examining differences in gene expression in Müllerian and urogenital sinus vagina in response to DES are essential.

Clustering analysis of estrogen-responsive genes in DES-exposed mouse vaginae showed that they could be broadly categorized into two types; a neonate type (PND 0) and an adult type (PNDs 5, 20, and 70). Vaginal stromal cells showed proliferative response to DES only at PNDs 0 and 5, but not at PNDs 20 and 70. In contrast, vaginal epithelial cells showed proliferative response to DES at PND 20 and PND 70 in the present study. The critical window for induction of estrogen-independent persistent changes in vaginae is within 3–5 days after birth (22). The underlying mechanism of the differences in responsiveness between vaginal epithelial cells and stromal cells at different ages needs to be analyzed in the near future to understand the molecular basis of the critical window. In mouse vaginae, proliferative response to DES in epithelial cells and stromal cells were reversed after PND 5, which may indicate the critical window of the mouse vagina.

Estrogens induce expression of genes related to cell cycle regulators, chromatin remodeling, IGF-I signaling, apoptosis, and keratin expression in mouse uteri (23–25, 42). Increased expression of mRNAs of cell cycle regulators were reported after 17β-estradiol treatment in the uteri of adult ovariectomized mice (42). In the present study, these cell cycle regulators except for cyclin G1 and E1, were induced in adult vaginae; thus vaginae responded to estrogen similar to uteri at the gene expression level.

From PND 5 onward, DES induced the following cell regulatory genes: p21, which delays S phase progression (43); Gadd45α, which acts at the G2/M checkpoint; and 14–3–3 sigma, which inhibits activation of cyclin B (32, 43, 44). Thus, induction of these cell cycle regulators at the mitotic phase checkpoint 6 hrs after DES stimulation may play a role in DNA synthesis required for vaginal cell proliferation.

Induction of keratinocyte differentiation regulators, such as Sprr1a, Sprr2a, and keratin complexes, was reported in estrogen-exposed uteri (45). Sprr family genes are expressed in all squamous cells, such as epithelial cells of skin, vagina, and digestive tract (46, 47). Sprr2a, Sprr2b, and Sprr2f are expressed in uteri and vaginae (48). Sprr2f is expressed most intensely in uteri and vaginae (48). Sprr2f and Keratin complex 2 (K2) mRNAs were elevated in vaginae 6 hrs after DES exposure from PND 5 to PND 70. Genes related to epithelial cell differentiation responded to DES earlier than genes related to proliferation at PND 5. The increase in expression of Sprr2f and K2 genes at PND 20 and PND 70 is probably related to proliferation of vaginal epithelial cells. Sprr2a and Sprr2b may be correlated with keratinization in vaginal epithelial cells.

Klf4 is a transcription factor of Sprr2 (47, 49) and plays a role in keratinocyte differentiation (30). DES induced Klf4 expression in vaginae even at PND 0 in the present study. The inductions of Klf4 and Sprr2a expressions in DES-exposed uteri at PND 5 were reported previously (45). In vaginae, Klf4 is an early estrogen-responsive gene and a candidate for persistent vaginal stratification by perinatal DES exposure.

14–3–3 Sigma also regulates the cell cycle by inhibiting G2/M progression-dependent p53 (44) and is induced in squamous cell carcinoma of the urinary bladder (50). Expression of 14–3–3 sigma was found in DES-stimulated mouse vaginae at PND 5 in the present study and also in neonatally DES-exposed vaginae (17), suggesting that this gene will be a candidate for further study in the persistent vaginal stratification and keratinization induced by perinatal estrogen exposure.

In conclusion, vaginal epithelial cells and stromal cells showed proliferation after a single injection of DES at PNDs 20 and 70, and at PNDs 0 and 5, respectively. The number of genes induced 6 hrs after DES exposure in mouse vaginae at PND 0 was lower than those induced at PNDs 5, 20, and 70. The DES-induced gene expression pattern in vaginae at PND 5 was closer to the adult type. Several cell cycle regulators such as Gadd45α, G1 to S phase transition 1, and p21; and keratinocyte differentiation factors, 14–3–3 sigma and Sprr2f, were induced by DES in vaginae from PND 5 to adult. Thus, microarray analysis revealed that the gene expression pattern in vaginae during the critical period was different from that after the critical period. Further studies are essential to examine the time course of gene expression to discover late genes induced in mouse vaginae by DES exposure at different ages.

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Chromatin immunoprecipitation-mediated target identification proved aquaporin 5 is regulated directly by estrogen in the uterus

Mika Kobayashi, Eri Takahashi, Shin-ichi Miyagawa, Hajime Watanabe* and Taisen Iguchi

Okazaki Institute for Integrative Bioscience, National Institutes of Natural Sciences, 5-1 Higashiyama, Myodaiji, Okazaki, 444-8787, Japan

Estrogens play a central role in the reproduction of vertebrates and affect a variety of biological processes. The major target molecules of estrogens are nuclear estrogen receptors (ERs), which have been studied extensively at the molecular level. In contrast, our knowledge of the genes that are regulated directly by ERs remains limited, especially at the level of the whole organism rather than cultured cells. In order to identify genes that are regulated directly by ERs *in vivo*, we used estrogen treated mouse uterus and performed chromatin immunoprecipitation. Sequence analysis of a precipitated DNA fragment enabled alignment with the mouse genomic sequence and revealed that the promoter region of the gene encoding aquaporin 5 (*AQP5*) was precipitated with antibody against ERα. Quantitative PCR and DNA microarray analyses confirmed that *AQP5* is activated soon after administration of estrogen. In addition, the promoter region of *AQP5* contained a functional estrogen response element that was activated directly by estrogen. Although several *AQP* genes are expressed in the uterus, only direct activation of *AQP5* could be detected following treatment with estrogen. This chromatin immunopreciptation-mediated target identification may be applicable to the study of other transcription factor networks.

Introduction

Estrogens play crucial roles in reproduction and other biological processes via two pathways. Firstly, they exert their effects genomically through the estrogen receptors (ERs), which function as ligand-dependent transcription factors that bind DNA at conserved estrogen response elements (EREs). Two ERs (ERα and ERβ) have been identified in mammals and since ERα knockout mice are sterile, the former is considered to play an essential role in reproduction (Lubahn *et al.* 1993). Secondly, estrogens function in a non-genomic manner by rapid activation of membrane-initiated kinase cascades such as the MAPK (Migliaccio *et al.* 1996) and phosphatidylinositol-3-OH kinase (Simoncini *et al.* 2000) signaling pathways. As the net effect of estrogen represents the integration of both genomic and non-genomic activities (Kahlert *et al.* 2000; Wong *et al.* 2002), it is important to distinguish between these pathways when attempting to understand its functions.

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**Correspondence*: E-mail: watanabe@nibb.ac.jp

Membrane binding assays (Inoue *et al.* 1993), computational analyses (Bourdeau *et al.* 2004; Kamalakaran *et al.* 2005) and chromatin immunoprecipitation (ChIP) (Carroll *et al.* 2005; Laganiere *et al.* 2005) have been used to identify ER target genes in a non-biased manner. In particular, the recently developed ChIP technique has enabled investigation of genes at a genome-wide level. However, these approaches have been applied primarily to cultured cell lines such as MCF-7. Consequently, the estrogen target genes of whole organisms remain poorly understood in comparison to cultured cell lines.

The uterus is known to be a major target organ for estrogen and its condition both physiologically and morphologically depends upon estrogen levels. ERα is expressed at high levels in the uterus and in ERα knockout mice the effects of estrogen are canceled. From studies using ERα and ERβ null mice (Lubahn *et al.* 1993; Krege *et al.* 1998), it is known that the former plays an essential role in reproduction.

Although the influence of estrogen on the uterus is both well recognized and studied, our understanding of estrogen-activated transcriptional networks remains limited. Several groups have examined the uterine gene

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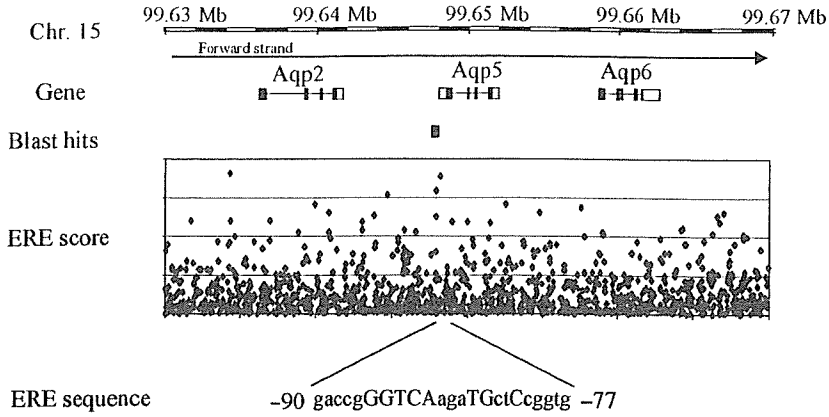

Figure 1 Genome map around *AQP5*. The solid box below the mouse genome map indicates the region identified by BLAST as matching the DNA fragment precipitated by anti-ERα antibody. The locations of *AQP2*, *AQP5* and *AQP6* are mapped on chromosome 15, with exons and flanking regions indicated by solid black and open boxes, respectively. ERE scores were calculated using the PATSER program (Hertz & Stormo 1999), and the putative ERE sequence in the 5′ flanking region of *AQP5* is indicated.

expression profile (Watanabe *et al.* 2002; Hewitt *et al.* 2003; Moggs *et al.* 2004), but none have determined whether these genes were activated directly by ER. Thus, it is not clear to what extent these genes are activated as a direct result of genomic action by estrogen in the target organ.

One of the prominent effects of estrogen is water imbibition in the uterine endometrium. This estrogen-stimulated water transport is considered to be important in the periimplantation period to change the uterine environment. For the regulation of water transportation, water channels termed aquaporins (AQP) play a critical role. AQPs are a family of transmembrane channel proteins that have six transmembrane domains. Thirteen AQPs (AQP0–AQP12) have been identified in mammals and the expression of AQPs in the uterus has also been studied at the protein and RNA level (Offenberg *et al.* 2000; Jablonski *et al.* 2003; Richard *et al.* 2003), although there has not been a study to address whether the AQP genes could be directly activated by estrogen.

In order to identify the genes that are regulated directly by estrogen in the whole organism, we used ChIP to select activated genes in the mouse uterus. A DNA fragment that was recognized by the ER was precipitated and identified as the 5′ region flanking *AQP5*. We analyzed its function and response to estrogen as well as the responses of other *AQP* genes.

Results

AQP5 is a target of ERα

We used ChIP to investigate the uterine genes targeted by ERα. The mouse uterus was fixed 1 h after estrogen treatment, after which genomic DNA was fragmented and precipitated using anti-ERα antibody. This DNA was amplified by PCR and sequenced and compared to the mouse genome. One of the sequences of a precipitated DNA fragment mapped to Chr:15 99647562-99647991, located only 6 bases upstream of *AQP5* (Chr:15 99,647,997-99 652 041; Fig. 1). In contrast, no specific DNA fragments that could be assigned to mouse genome sequence were recovered from ChIP of the untreated uterus (data not shown).

Although a canonical ERE was not identified in the precipitated sequence, a highly conserved motif was found upstream of *AQP5* (between −85 and −73; Fig. 1). To confirm binding of ERα to the putative ERE, we performed a conventional ChIP with fragments from the 5′ flanking and coding regions of *AQP5* (Fig. 2A and Table 1). Only fragments containing the putative ERE and its adjacent sequences could be precipitated following administration of estrogen (Fig. 2B). The other fragments could not be precipitated with anti-ERα antibody, even after administration of E2. Similar results could be

Table 1 Primer sequences used for ChIP analysis of *AQP5*

Primer set	Position	Direction	Primer sequence
1	−1885	F	AATCTGCTTGTCTCTGCCTC
	−1691	R	CCTTCTCTTTCCCAGCTAAC
2	−946	F	GACAAGAGGAAGCTGGGAAC
	−818	R	CCGGCCTATCTCACTTTCTA
3	−506	F	GAAAGACCAACAGGGACAAG
	−318	R	TTCTCAGTGGTAGCCCTTGG
4	−338	F	CCCAAGGGCTACCACTGAGA
	−101	R	ACGGACGGGTCAGAGTGATG
5	−161	F	GGGCGGATAAGGAGCTAAGA
	76	R	GTGCGTGCTGGGCTCTCCTG
6	1026	F	CCTGGCTTCTCTTCACATTC
	1228	R	GTTGCTCCAGACCTCCATCC
7	1996	F	CTCCCCAGCCTTATCCATTG
	2269	R	GTCTCTGTGCTCGCCCTCCC

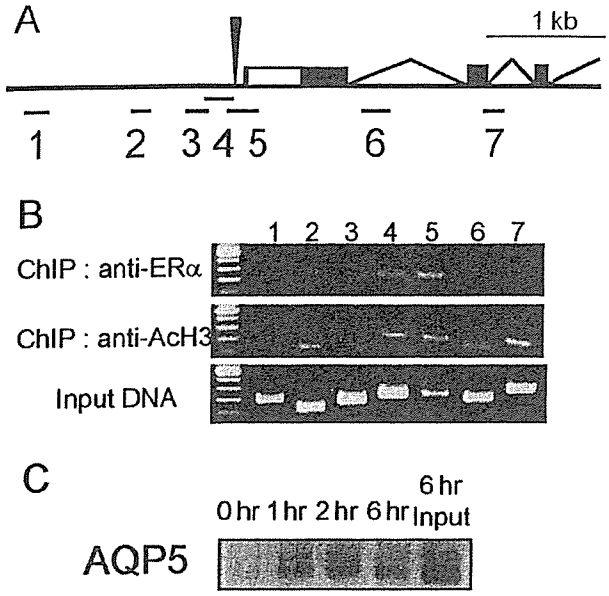

Figure 2 ChIP of the 5′ flanking region of *AQP5*. (A) Schematic of the 5′ region of *AQP5*. The transcriptional initiation site and exons are indicated by an arrowhead and solid black boxes, respectively. Numbered bars indicate the genomic regions examined by PCR following ChIP. (B) ChIP analysis of the 5′ region of *AQP5*. ChIP was performed using either anti-ERα or anti-acetylated histone H3 antibodies, on samples of uterine tissue that were prepared 1 h after the administration of estrogen. Precipitated DNA fragments were amplified by PCR and the lane numbers correspond to the location of the PCR products indicated in (A). The presence of a PCR product indicates precipitation of a DNA fragment by the specified antibody. (C) Temporal analysis of binding between ERα and the *AQP5* promoter region. ChIP was performed on uterine tissue samples harvested 0, 1, 2 and 6 h following administration of estrogen. DNA fragments were precipitated with anti-ERα antibody, then amplified by PCR using primers for the ERE region (#4 in Fig. 2A). The presence of a PCR product indicates binding between ERα and the *AQP5* promoter region.

obtained with monoclonal anti-ERα antibodies. These results suggest that the putative ERE plays a key role in the estrogen response. In contrast, all DNA fragments could be precipitated with anti-acetylated histone H3 antibody (anti-AcH3). It is known that the modification states of the N-terminal tails of hitones H3 and H4 play important role in chromatin formation (Richards & Elgin 2002) and the fact that Lys9 of H3 was acetylated indicated that the chromatin state of the 5′ flanking region of *AQP5* was active.

We examined the temporal changes in binding between ERα and the putative ERE following administration of estrogen. Binding could be detected 1 h after estrogen administration and continued for at least 6 h (Fig. 2C). In contrast, no PCR product was detected from samples precipitated with IgG (data not shown).

Identification of an ERE in the 5′ flanking region of AQP5

In order to determine whether the putative ERE plays a functional role in estrogen-dependent transcriptional activation, we used three luciferase reporter constructs (p611AQP5-luc, p103AQP5-luc and p13AQP5-luc) containing deletions of the 5′ flanking region of *AQP5* (−611, −103 and −13 to +74, respectively).

Only the positive control (containing 3 × canonical ERE) and the two constructs containing the putative ERE (p611AQP5-luc and p103AQP5-luc) were activated by estrogen, whereas the construct that did not contain the putative ERE (p13AQP5-luc) and a construct in which the element was mutated (pm103AQP5-luc; GGTCA→aaTCA), were unaffected (Fig. 3). These results suggest that there is a functional ERE element in the 5′ region of *AQP5* that is regulated directly by ERα.

DNA microarray analysis

In order to obtain a uterine gene expression profile following treatment with estrogen, and to identify gene targets that were regulated directly by ERα rather than via secondary effects of estrogen, we performed a DNA microarray analysis of mouse uterus isolated 1 h following treatment. At 1 h following administration of estrogen, we identified a more than twofold up- or down-regulation in 216 and 204 genes, respectively. As *AQP5* is located between *AQP2* and *AQP6*, we examined whether other *AQP* genes were affected by estrogen. At 1 h post-treatment, expression of *AQP5* and *AQP8* was activated, whereas expression of the *AQP* genes in the proximity of the former (*AQP2* and *AQP6*) was unaffected (Fig. 4).

Quantitative PCR analysis of AQP genes

As the DNA microarray indicated early activation of *AQP5* and *AQP8* by estrogen, we examined temporal expression changes of all AQP genes using quantitative PCR, because it has been reported several AQP genes are expressed in uterus and spatiotemporally regulated during the periimplantation (Richard *et al.* 2003). We confirmed that *AQP5* and *AQP8* were activated within 1 h of estrogen administration and that activation continued for at least 2 h. Although *AQP5* activation declined after 6 h relative to untreated samples, the levels of transcript remained elevated at 24 h (Fig. 5). The other *AQP* genes, including *AQP2* and *AQP6*, were not activated by estrogen.

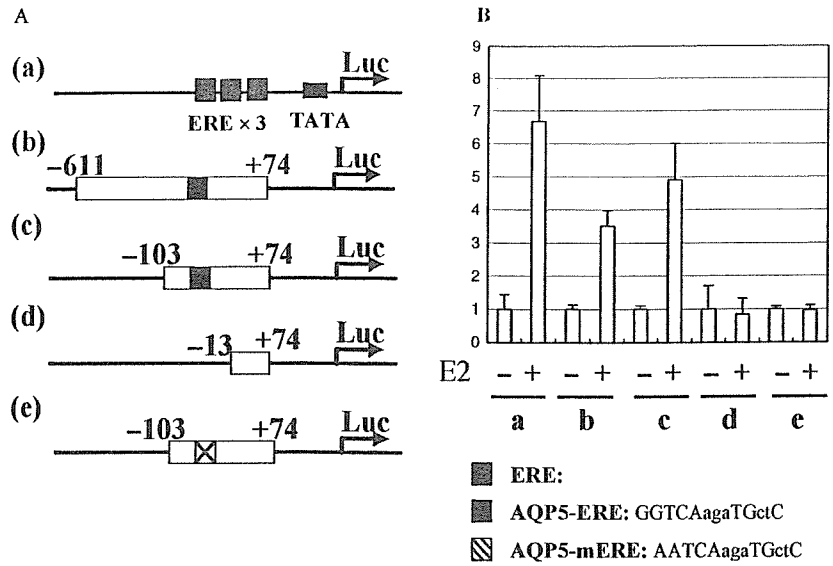

Figure 3 Presence of a functional ERE in the 5′ flanking region of *AQP5*. (A) Luciferase reporter constructs. The positive control (a) contained three canonical ERE elements. Three constructs contained deletions of the 5′ flanking region of *AQP5* (b) p611AQT5-luc, (c) p103AQT5-luc, and (d) p13AQP5-luc and one contained a mutation of the putative ERE sequence (e) pm103AQP5-luc. Black boxes indicate the presence of a putative ERE motif in the *AQP5* promoter region (-86GGTCAagaTGctC-74). Crossed boxes indicate a mutation in the putative ERE motif (AATCAagaTGctC). Gray boxes indicate the canonical ERE motif used as a positive control. (B) The response of 5′ flanking regions of *AQP5* to estrogen stimulation. Luciferase reporter constructs were transfected into HEK293 cells with an ERα expression vector and a control plasmid, then examined in the presence or absence of estrogen. Luciferase intensities detected in the presence of estrogen were divided by those in the absence of estrogen and the relative differences calculated.

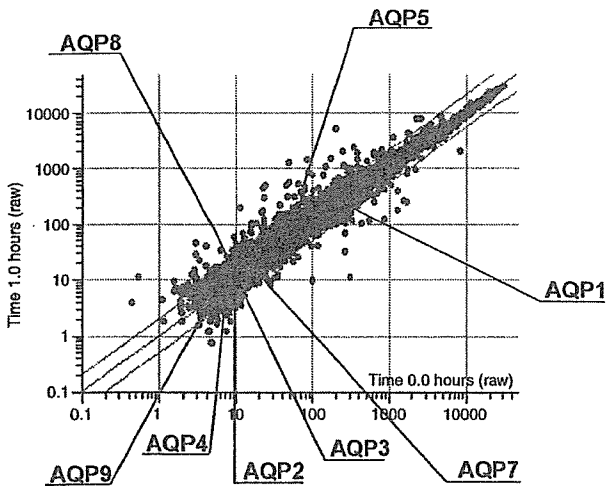

Figure 4 DNA microarray analysis of the expression profile of *AQP* genes. Gene expression levels (intensity of fluorescence) of *AQP* genes determined 1 h after the administration of estrogen (*x*-axis) compared with those at 0 h (*y*-axis). *AQP* genes on the DNA microarray are indicated.

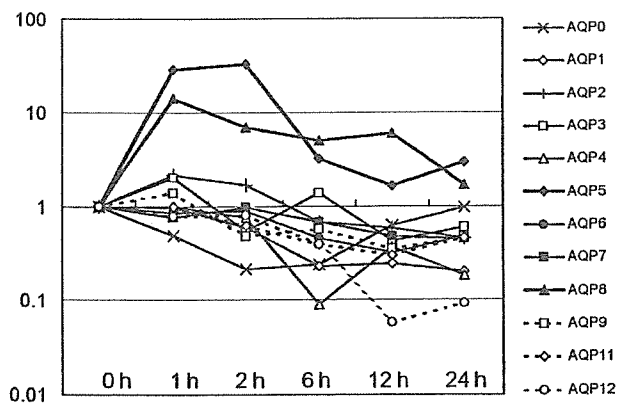

Figure 5 Temporal changes in expression of *AQP* genes following estrogen treatment. Total RNAs were prepared from mouse uteri at 0, 1, 2, 6, 12 and 24 h following estrogen administration, and expression levels of *AQP* genes were estimated using quantitative PCR. Changes relative to expression at 0 h are indicated using a log scale.

Table 2 ERE-like motifs identified in the promoter regions of *AQP* genes and primer sequences for ChIP analysis of *AQP* genes

Gene	Position	Putative ERE sequence	Score	Position	Direction	ChIP primer sequence
AQP1	−348/−330	TCAGGCAcCCTTGAgCTTT	72	−467	F	TCAGAGTGGGATGGGACAGG
	−810/−828	GAAtGACAGCTgGACCTGA	72	−296	R	GGCCAGAGAAATCCAGGTGT
AQP2	−327/−355	GCtGtGTcCAGTGACCTTC	72	−430	F	CAGAAGAAAGACCATCCAGT
				−268	R	CCAGCCCCCACAATGACCAC
AQP3	−581/−563	TCcAGACgGTGTGACCaGG	76	−617	F	AAGGAGAAAGCCCAGGTATC
				−355	R	TGCGATGACTGGATAGAACA
AQP4	-			−174	F	CTGAAATGCCCTGTGTCTAT
				37	R	AGCTCTGTCACTCATGCCTT
AQP5	−123/−105	TCtcaTCACTCTGACCCGT	78	−163	F	GGGCGGATAAGGAGCTAAGA
	−70/−88	CCgGaGCATCTTGACCCGG	78	74	R	GTGCGTGCTGGGCTCTCCTG
AQP6	−843/−825	AAgAGGCACGGTGACaCTT	70	−945	F	CCAGGTGCAGCCAGGGTTAG
				−748	R	CTTCGGGACCTTGTTCTCAG
AQP7	−714/−696	TCAGcTCcCTCTGACCTCA	83	−751	F	CTGAACCCAGACAAACCATT
				−602	R	TAGGAAAAGTATGCCCAAGG
AQP8	−490/−472	TGcAGGCAGTGTGAgCaGA	71	−569	F	TGCCGATGAAACAGTGAAAG
	−571/−553	AAcAGTGAAAGTGACtCGA	71	−419	R	GACCTACGGGCTTACCTACC
AQP9	+22/+4	ATtAGGAACTGTGACtTAA	72	−57	F	GCAAACAAATAGCAATGAGC
				74	R	ATCTCTGGAGGCGACTAAAG
AQP11	−717/−735	ATtGGGCACCTTtACCTGC	74	−199	F	CGGAGTGTGCAAAGATCAAG
				17	R	AGGGGACAACGGTCTGTAGA
AQP12	−92/−74	ACttGGCtCTGTGACCaAT	76	−105	F	TCCTCTGTGGGTGTTCCTTG
				79	R	TCAGTCTGGGTTCTACAAGG
consensus		NNARGNNANNNTGACCYNN				

The highest-scoring putative ERE-like motifs that were identified in the 5′ flanking regions (within 1 kb) of *AQP* genes (Heinemeyer *et al.* 1999).

Although uterine expression of *AQP* genes has been examined by other groups at the protein and RNA levels, not all of these observations were in agreement. Weak ERE motifs were found in a proximal promoter region (< 1 kb) upstream of *AQP8* and similar motifs were identified upstream of other *AQP* genes (Table 2). Thus, we examined whether the motifs upstream of the various *AQP* genes were functional EREs.

Using a reporter system, we examined whether the regions flanking the *AQP* genes could respond to estrogen stimulation. We prepared transcriptional fusions comprising 1 kb of sequence upstream of each *AQP* gene and the gene encoding luciferase. With the exception of *AQP5*, no *AQP* genes were activated by estrogen (Fig. 6), including *AQP8*, which had been activated by the administration of estrogen in earlier experiments (Figs 4 and 5).

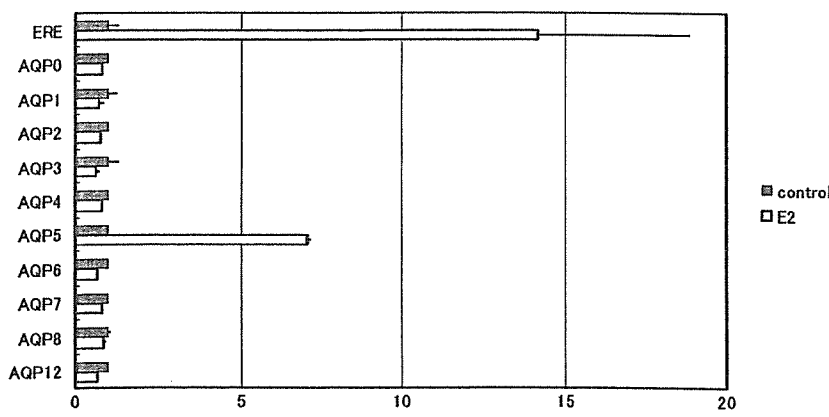

Figure 6 Luciferase reporter assay of *AQP* genes. Estrogen activation of *AQP* genes was examined using luciferase reporter constructs containing the 5′ flanking region of each gene. The luciferase activities of constructs in the presence (open) or absence (gray) of estrogen are indicated relative to a control reporter. ERE indicates the positive control containing three repeats of a canonical ERE motif.

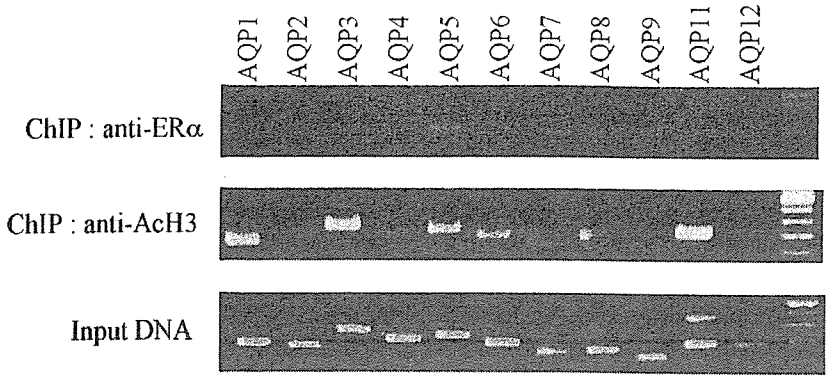

Figure 7 ChIP of the 5′ flanking regions of *AQP* genes. ChIP was performed using either anti-ERα or anti-acetylated histone H3 antibodies, on samples of uterine tissue that were prepared 1 h after administration of estrogen. Precipitated DNA fragments were amplified by PCR using primers (Table 1) designed to amplify the putative EREs (Table 3) of each *AQP* gene.

Next, in order to examine if weak EREs might be recognized by ERα in the uterus, we performed a chromatin immunoprecipitation using anti-ERα. PCR primers were designed to amplify putative ERE sequences in the 5′ flanking region of each *AQP* gene (Table 2). As the only DNA fragment that could be amplified by PCR contained the ERE of *AQP5* (Fig. 7), these results suggest that ERα cannot bind to the flanking region of other *AQP* genes, although some other AQPs have half ERE (AQP2,3,7, and 12).

Discussion

Methodology

In this study, we identified *AQP5* as an ERα target gene through a combination of ChIP and DNA microarray analysis. Previously, we identified hundreds of genes in the mouse uterus that were activated by exposure to estrogen (Watanabe *et al.* 2002), but it was not clear whether those genes were activated directly by estrogen via ERα. We have identified a direct target of ERα using chromatin immunoprecipitation and DNA microarray analysis, and this methodology can be applied to other transcription factors, including other nuclear receptors.

Identification of the target genes of transcription factors is critical to our understanding of transcriptional networks. For example, as canonical EREs were rarely found in promoter regions (O'Lone *et al.* 2004), it was difficult to identify the target genes of ERs by sequence motifs alone; filter binding (Inoue *et al.* 1993), computational approaches (Bourdeau *et al.* 2004; Kamalakaran *et al.* 2005) and ChIP on Chip (Carroll *et al.* 2005; Laganiere *et al.* 2005) have been applied to their identification. As these studies focused primarily on cultured cells, no information regarding whole organisms was available. Previously, we demonstrated the differential expression of estrogen response genes between tissues and throughout various stages of maturation (Watanabe *et al.* 2004). Thus, identification of the direct gene targets of ERα in whole organisms will contribute to our understanding of the function of estrogen during development and maturation.

Although transcription factor binding sites can be determined using ChIP on Chip, this technique requires a large set of arrays, whereas our study did not. A recent report described the use of ChIP with a paired-end ditag sequencing strategy (Wei *et al.* 2006) and this technique may be useful for increasing the numbers of known binding sites and obtaining a genome-wide perspective.

We were unable to estimate the amount of nonspecific contamination of DNA fragments that occurred with the ChIP procedure because it was not possible to map any of the fragments precipitated from the untreated uterus, a result that suggests that our protocol had a very low background. In order to reduce background we introduced a density gradient to remove uncoupled DNA fragments prior to immunoprecipitation. In addition, we introduced steps such as perfusion of the mouse tissue, sonication, and washing of the bound samples, all of which contributed to the reduced background.

Previously, we investigated the temporal regulation of uterine genes by estrogen (Watanabe *et al.* 2003), but were unable to distinguish whether the activation was a direct effect of ERα or a secondary effect of estrogen. In combination with the results from ChIP, previous DNA microarray data may now be interpreted in greater detail.

Aquaporin in the uterus

The effect of estrogen can be observed clearly in the uterine response. Within 1–2 h of estrogen administration, uterine wet weight begins to increase via water imbibition. Although vascular endothelial growth factors are considered to be crucial to this process (Cullinan-Bove & Koos 1993), the contribution of actual water channels

(AQPs) remains poorly understood. However, AQPs are considered to play an important role in the periimplantation stage and it is known that *AQP1,4* and *5* are expressed at that stage (Li *et al.* 1994; Jablonski *et al.* 2003; Richard *et al.* 2003; Lindsay & Murphy 2004).

In this study, we demonstrated that ERα regulates *AQP5* directly. This result does not concur with the findings of some other studies. For example, using *in situ* hybridization, Richard *et al.* (2003) identified *AQP1*, not *AQP5*, as the estrogen response gene. This discrepancy may have arisen through differences in the methods of detection, since in general, quantitative PCR is more accurate for the evaluation of changes in mRNA than *in situ* hybridization. Actually, recent study using mouse cervix detected the expression of *AQP5* by Northern hybridization (Anderson *et al.* 2006). In addition, Jablonski *et al.* (2003) did not detect expression of *AQP5* at the protein level in the uterus; again, these discrepancies may be a result of differences in experimental design. However, our study clearly detected activation of *AQP5* via both DNA microarray and quantitative PCR, strongly supporting the suggestion that *AQP5* is an estrogen target gene in the uterus. One of the prominent effects of estrogen is water imbibition and for the regulation of water transportation, *AQP5* may play critical role.

Estrogen response and aquaporin

As indicated in Table 2, half ERE could be found in 5′ flanking regions of several AQP genes, but only the ERE motif found in *AQP5* was functional. Our chromatin immunoprecipitation analysis suggested that the half EREs except for *AQP5* were not functional. One possibility is that the EREs were not accessible to ER, but this is less likely because we could detect acetylation of Lys 9 of histon H3 in the region. Although requirement of the *cis* element necessary for ER binding is still not clear, accumulation and analysis of functional EREs *in vivo* such as this study may be important. On the other hand, we could not identify functional ERE of *AQP8* in its 5′ flanking region. As we could not detect genome DNA fragment adjacent to *AQP8*, it is possible that other functional ERE exist in other regions or this gene was activated in secondary effect of estrogen. Recently, we found that adrenomedulin (ADM) gene is a direct target gene of estrogen. *ADM* is activated 1–2 h after estrogen administration and begins to decrease its expression before 6 h. Interestingly, this temporal gene expression profile is very similar to *AQP5*. On the other hand, the temporal gene expression profile of *AQP8* was slightly different and its gene expression level did not change over 12 h. Relevance of direct ER binding and temporal gene expression pattern is one of the intriguing questions.

AQP5 is expressed at high levels in the salivary and lachrymal glands (Raina *et al.* 1995), which are target tissues of Sjogren's syndrome, an autoimmune disorder that occurs primarily in females. A relationship between *AQP5* and this syndrome has been reported (Steinfeld *et al.* 2001; Tsubota *et al.* 2001). In addition, a recent study has suggested that estrogen may affect lymphopoiesis (Shim *et al.* 2004). Therefore, although it remains unclear whether *AQP5* expression in the salivary and lachrymal glands is activated by estrogen, the presence of an ERE motif in the promoter region of this gene suggests the possibility of a functional relationship under certain conditions.

Experimental procedures

Animals

Female C57BL/6 J mice were housed under a 12 h light/dark cycle at 23–25 °C, fed laboratory chow (CE-2; CLEA, Tokyo, Japan) and provided with access to tap water. In order to assess the effect of estrogen on uterine gene expression, 8-week-old mice were ovariectomized and two weeks later injected intraperitoneally with 50 μg/kg body weight of 17β-oestradiol (E2) (Sigma-Aldrich Japan, Tokyo, Japan) or sesame oil (Nacalai Tesque, Kyoto, Japan) as a vehicle control. The whole uterus was collected immediately or at the following times after treatment 1, 2, 6, 12 or 24 h. All animal experiments were approved by the institutional Animal Care Committee.

Chromatin-immunoprecipitation-mediated target cloning

The mouse uterus was fixed with 1% formaldehyde and homogenized in phosphate buffered saline (PBS) containing 0.125 M glycine, using a Physcotron (NS-310E, Microtec, Chiba, Japan). The homogenates were centrifuged at 700 *g* for 5 min at 4 °C and the pellets incubated in lysis buffer (10 mM Tris-HCl [pH 8.0], 10 mM EDTA [pH 8.0], 0.5 mM EGTA and 0.25% Triton X-100) for 10 min. The samples were harvested by microcentrifugation, the pellets resuspended in sonication buffer (10 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA [pH 8.0], 0.5 mM EGTA), and sonicated using a Bioruptor sonicator (Cosmo Bio, Tokyo, Japan), resulting in DNA with an average length of ~500 bp. Sonicated samples were centrifuged at 15 000 r.p.m. to remove debris and loaded on to a cesium chloride step gradient containing 1.5 mL of 1.75 g/mL CsCl, 1.5 mL of 1.5 g/mL CsCl, and 1 mL of 1.3 g/mL CsCl supplemented with 0.5% (v/v) N-lauroylsarcosine. Samples were centrifuged at 44 000 r.p.m. in a SW55Ti rotor for 24 h. 0.1 mL fractions were collected from the bottom of the gradient and fractions containing cross-linked chromatin were

combined and dialyzed vs. TE buffer. Then the samples were precleared with protein G Sepharose for 1 h at 4 °C and incubated with either 10 μg anti-ERα polyclonal rabbit antibody (H-184; Santa Cruz, CA, USA) overnight at 4 °C. The sample was precipitated with protein G Sepharose, washed 5 times with RIPA buffer (10 mM Tris-HCl [pH 8.0], 140 mM NaCl, 1 mM EDTA [pH 8.0], 0.5 mM EGTA, 1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate), then recovered by incubation in elution buffer (0.1 M sodium bicarbonate, 1% SDS). Cross-linking was reversed by incubation for 6 h at 65 °C, followed by incubation with proteinase K for 4 h at 45 °C. Samples were extracted with phenol/chloroform and ethanol precipitated. The precipitated DNA fragments were cloned into pGEM-T vactor (Promega, Tokyo, Japan) by ligation-mediated PCR (LMPCR). Briefly, the DNA fragment was blunt ended and phosphorylated with T4 DNA polymerase (Takara Bio Inc., Shiga, Japan) and T4 polynucleotide kinase (Takara Bio Inc., Shiga, Japan), respectively. After ligating oligonucleotide linkers, the DNA fragment was amplified by PCR and cloned. Oligonucleotide sequences of the linker were F 5′-GCGGTGACCCGGGAGATCTGAATTC-3′ and R 5′-GAATTCAGATC-3′. PCR amplification was performed in the presence of 0.1 nM primer "F," 0.2 mM of each nucleotide (dATP, dCTP, dGTP and dTTP), 1 × PCR buffer and 1 U of LA Taq (Takara Bio Inc., Shiga, Japan) in 20 μL of reaction buffer. After 30 amplification cycles, the PCR products were purified with QIAquick PCR purification kit (QIAGEN, Tokyo, Japan) and cloned into pGEM-T easy vector. Colonies were randomly picked up after transformation and inserted sequences were analyzed.

Chromatin immunoprecipitation

Mouse uterine genomic DNA was prepared and sonicated as described above. The sonicated samples were precleared with protein G Sepharose for 1 h at 4 °C, then incubated with either 10 μg anti-ERα polyclonal rabbit antibody (Santa Cruz, CA, USA) or 10 μg anti-acetylated histone H3 (Lys9) antibody (Cell Signaling, Danvers, MA, USA) overnight at 4 °C. The samples were precipitated with protein G Sepharose, washed 5 times with RIPA buffer (10 mM Tris-HCl [pH 8.0], 140 mM NaCl, 1 mM EDTA [pH 8.0], 0.5 mM EGTA, 1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate), then recovered by incubation in elution buffer (0.1 M sodium bicarbonate, 1% SDS). Cross-linking was reversed by incubation for 6 h at 65 °C, followed by incubation with proteinase K for 4 h at 45 °C. Samples were extracted with phenol/chloroform and ethanol precipitated. In general, 1/30th of the precipitated DNA was used in a PCR amplification reaction. PCR amplification was performed in the presence of 0.1 nM primers, 0.2 mM of each nucleotide (dATP, dCTP, dGTP and dTTP), 1 × PCR buffer and 1 U of AmpliTaq Gold (PerkinElmer Japan, Tokyo, Japan) in 20 μL of reaction buffer. After 35 amplification cycles, the PCR products were analyzed using agarose electrophoresis. As a negative control, the same experiments were performed with IgG. Primer sequences used to amplify the precipitated DNA are listed in Tables 1 and 2.

Preparation of labeled cRNA and microarray analysis

Total uterine RNA was extracted using TRIzol reagent (Invitrogen, Tokyo, Japan) and the RNeasy total RNA purification kit (Qiagen, Tokyo, Japan). cRNA probes were prepared from purified RNA using a CodeLink Expression assay reagent kit (Amersham Bioscience K.K., Tokyo, Japan). The amplified cRNA (10 μg) was hybridized to oligonucleotide DNA microarrays (CodeLink Uniset Mouse I Bioarray, Amersham Bioscience K.K., Tokyo, Japan), which were scanned and processed using a GenePix 4000B scanner and GenePix Pro software (Axon Instruments, Union City, CA, USA), respectively. In order to confirm the estrogen-related changes in gene expression revealed by DNA microarray analysis, the experiment was repeated independently at least twice. The expression data were analyzed using GeneSpring software (Agilent Tech. Japan, Tokyo, Japan).

Quantitative real time-PCR

cDNA was synthesized from total RNA purified using Superscript II RT(-) (Invitrogen) and random primers at 42 °C for 60 min. Quantitative PCR reactions were performed according to the manufacturer's instructions, using the PE Prism 7000 sequence detector (Applied Biosystems, Tokyo, Japan), SYBR-Green PCR Core Reagents (Applied Biosystems) and primers designed for amplification of short PCR products (< 100 bp; Table 3).

Plasmid construction

A DNA fragment encoding mouse ERα was amplified from uterine cDNA using the primers 5′-GGCGAATTCATGACCATGACCCTTCACAC-3′ and 5′-GCAGTCGACTCAGATCGTGTTGGGGAAGC-3′, digested with *Eco*RI and *Sal*I, then ligated into pcDNA3.1(+) (Invitrogen), generating pERαcDNA3.1.

The 5′ flanking regions of AQP genes were amplified using the primers listed in Table 3, then digested with *Mlu*I and *Bgl*II and ligated into pGL2-basic (Promega, Tokyo, Japan). Inserts for the shorter *AQP5* reporters, p103AQP5-luc and p13AQP5-luc (5′ regions flanking *AQP5*, -103 to +74 and -13 to +74, respectively), were amplified using the forward primers 5′-cgacgcgtTGGGTGAGACCGACCGGGTCAAGATG-3′ and 5′-cgacgcgtAAAGGCCGGCCGGAGAGGGA-3′, respectively. The primer 5′-cgacgcgtTGGGTGAGACCGACCGaaTCAAGATGCTCC-3′ was used for amplification of the mutated ERE reporter (pm103AQP5-luc).

Luciferase reporter assay

HEK293T cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum. For ligand-dependent transcription assays, cells were seeded in 24-well tissue culture plates and grown in phenol red-free DMEM supplemented with 5% charcoal/dextran-treated fetal calf serum (JRH Biosciences, Inc. Lenexa, KS, USA). HEK293T cells were

Table 3 Primer sequences for quantitative PCR and cloning

Gene	RefSeq number	Q-PCR Direction	Position	Sequence	Reporter position	Sequence
AQP1	NM_007472	F	2246	TCACAGCTGCACACTCGTCTC	−932	cgacgcgtCCACTTTCCACCATCACTCG
		R	2319	TGGGTCCTCAGTGCCCTTAT	28	gaagatctCTCGACTTAACCGCTGGATG
AQP2	NM_009699	F	1237	GGGTGTAAAAGTGCTCGTCCAT	−917	cgacgcgtCTGTGGTAAGGGTGGCTCTG
		R	1287	TCTGCACGTGAGGAAAGAAACA	30	gaagatctGCTCTTCCTCCCTCCCTCTC
AQP3	NM_016689	F	1535	GGCTGAAGTCCAGGTCGTAAGT	−868	cgacgcgtGCACTGCTATGAACGTGTGG
		R	1634	GGAGTTTCCCACCCCTATTCC	66	gaagatctCAACTCCTTCTGTCGACCCA
AQP4	NM_009700	F	1030	GAGGACAGCACTGAAGGCAGA	−939	cgacgcgtTTAAGAGCCAGAGAACCTAC
		R	1097	TCCTTAATGGGTGGCAGGAA	74	gaagatctGTGCTGAGCATTGTTTCCTG
AQP5	NM_009701	F	1527	TTGTGAAGGCAGTGCAAGCT	−611	cgacgcgtCGCAGAAACGCAGGAACACA
		R	1579	CACCCCTTTCTGGGATGGT	74	gaagatctGTGCGTGCTGGGCTCTCCTG
AQP6	NM_175087	F	1436	TGGATCCCTGTCTTGGAGAAA	−998	cgacgcgtAGACAGGGAGGGTGGCATTT
		R	1517	TGGGCTCTGAAGCTCCTTCAT	70	gaagatctGCCAGGAACTCAGCAAAAAG
AQP7	NM_007473	F	2206	TCAAGACAGGGTTTCTCGGTG	−988	cgacgcgtCATGGGTGCAGGCTACAGAC
		R	2294	AGGCAGGCGGATTTCTGAG	63	gaagatctTGTCTTTCAGCCTCCGTCTC
AQP8	NM_007474	F	1220	GGGATTAGAAGGGCTGAGAAGG	−943	cgacgcgtTCAGACAAGAAGCGGCAGAG
		R	1311	GAATTGGGTTCCAAACCCAAC	47	gaagatctTCTAGGTCACAGACTGGAGG
AQP9	NM_022026	F	2061	TGACCTGAGCAAGTTGCCCT	NT	
		R	2140	CAGTCGGCTAGCAAGCTTCTG		
AQP11	NM_175105	F	1110	CAGTCAAGCTGGATGCGACA	NT	
		R	1172	AAGCTGAAGCAGGAGGCGT		
AQP12	NM_177587	F	924	CCGGCAGAAAAGCAAATACC	−981	cgacgcgtTTTGCTTAGCCTGTCCTGTG
		R	1019	ACGGCCCTTTGCCACTACT	54	gaagatctTGGGTTCTACAAGGAGCAAG