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Comparison of Estrogen Responsive Genes in the Mouse Uterus, Vagina and Mammary Gland

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ABSTRACT. Female reproductive organs are mainly regulated by estrogen and progesterone. Specifically, the uterus, vagina and mammary gland show organ-specific mitosis and morphological changes during proliferative events, such as estrous cycle, gestation and lactation. The mechanism underlying these organ-specific estrogen-dependent events is still unknown. We examined, therefore, global gene expression in the mature uterus, vagina and mammary gland of ovariectomized adult mice 6 hr after an injection of 5 μg/kg 17β-estradiol (E_2) using a microarray method in order to identify primary E_2-responsive genes. Half of the E_2 up-regulated genes in the uterus were similar to those in the vagina. E_2 up-regulated the expression of *Insulin-like growth factor 1 (Igf-1)* genes in the uterus and vagina. In the vagina, E_2 up-regulated the expression of IGF binding proteins (*Igfbp2* and *Igfbp5*). In the mammary gland, unlike the uterus and vagina, no gene showed altered expression 6 hr after the E_2 exposure. These results suggest that expression of *Igf-1* and morphogenesis genes is regulated by E_2 in an organ-specific manner, and it is supported by the results of BrdU labeling showing E_2-induced mitosis in the uterus and vagina except the mammary gland. The differences in organ specificity in response to E_2 may be attributed by differences in gene expression regulated by E_2 in female reproductive organs. The candidate estrogen-responsive genes in the uterus and vagina identified by profiling provide an important foundation understanding functional mechanisms of estrogen regulating morphogenesis and maintenance of each reproductive organ.

KEY WORDS: estrogen responsive genes, microarray, tissue specificity.

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Female reproductive organs vary their morphology during reproductive events, such as differentiation, development, estrous cycle, gestation and lactation. Estrogen is known to have differential developmental effects widely on the uterus, vagina, mammary gland, bone, liver, thymus and brain as its target organs. Although the proliferation of uterine and vaginal epithelia, and ductal elongation of mammary gland in mice could be regulated by estrogen alone [20], the mammary gland requires progesterone and prolactin in addition to estrogen to complete the architecture [12, 17, 33]. Ovariectomy and termination of weaning induce apoptosis in epithelial cells in the uterus, vagina and mammary gland [19, 30]. These estrogen target organs are controlled by estrogen receptors (ERα and ERβ) in the epithelial and stromal cells [9, 21, 24]. It is known, moreover, that growth factor(s) from the stroma are involved in epithelial proliferation of these organs [2, 6, 9]. An increase of epithelial and stromal cells in the uterus and vagina is mediated through *Insulin-like growth factor I (IGF-1)* and *Epidermal growth factor (EGF)* [3, 6, 14, 18]. Prolactin also induces expression of *Igf-2* mRNA in the developing mammary gland [13].

Tamoxifen, a selective ER modulator (SERM), acts as an estrogen agonist in the uterus and vagina, but acts as an estrogen antagonist in the mammary gland [22, 32]. The ligand-dependent effect on the mammary gland supports the idea of tissue specificity of gene expression by estrogen. Profiling of estrogen-regulated gene expression is reported recently in the estrogen target cells, tissues and organs [10, 34–36]. However, any comparisons of gene expression in the estrogen target organs have not not been reported. Gene expression reached a maximum 6 hr after E_2 administration in the uterus of ovariectomized adult mice without any histological changes [34]. Thus, we examined global gene expression 6 hr after a single injection of E_2 in order to identify early estrogen-responsive genes in the uterus vagina and mammary gland as the estrogen target organs in ovariectomized adult mice.

MATERIALS AND METHODS

Animals: C57BL/6J mice (CLEA, Tokyo, Japan) at 2 months of age, 20–23 g body weight, were used for mating. Mice were maintained under 12 hr light/12 hr dark at 23–25°C, fed with a commercial diet (CE-2, CLEA, Tokyo, Japan) and provided tap water *ad libitum*. All experiments

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and animal husbandry protocols were approved by the animal care committee of National Institutes of Natural Sciences.

Treatments: 17β-Estradiol (E_2, Sigma, St. Louis, MO) was dissolved in sesame oil. Sixty-day-old mice were ovariectomized and injected with 5 μg E_2/kg body weight after a 10-day recovery period to ensure that endogenous E_2 levels were reduced. Six hr after E_2 injection, 4 mice were killed by decapitation and the uteri, vaginae and mammary glands were collected. Four mice injected with oil vehicle only were used as controls. The tissues were pooled for DNA microarray analysis and the analyses were done on two independent experiments. Two other groups of 4 ovariectomized mice were likewise given E_2 or oil and killed 24 hr after the injection for bromodeoxyuridine (BrdU)-labeling study.

DNA microarray analysis: Total RNA was extracted from tissues (4 mice each) 6 hr after a single injection of 5 μg E_2/kg b.w. or the oil vehicle alone. Ten μg of total RNA were used to synthesize cDNA, which was then used to generate biotinylated cRNA. The cRNA was hybridized to murine U74A version 2 GeneChip expression arrays (Affymetrix, Applied Biosystems (APB), Tokyo, Japan) as described [34]. Total RNA was extracted using TRIzol reagent (Invitrogen, Tokyo, Japan) and purified with an RNeasy total RNA purification kit (Qiagen, Tokyo, Japan). Ten μg of total RNA were converted into double stranded cDNA using the Superscript Choice System (Invitrogen) with a T7-$(dT)_{24}$ primer (APB). Biotin-labeled cRNA was synthesized using the ENZO BioArray High Yield RNA transcript labeling kit (APB). The cRNA was purified by RNeasy (Qiagen). The purified cRNA was fragmented with fragmentation buffer (40 mM Tris, 100 mM K-acetate and 30 mM Mg-acetate) at 94°C for 35 min. Fragmented cRNA was mixed with hybridization buffer containing 100 mM MES [2-(N-morpholino)ethanesulfonic acid], 1 M NaCl, 20 mM EDTA, 0.01% Tween 20 and control oligonucleotides. The quality of cRNA was first assessed by analysis with Test 2 array (Affymetrix). cRNA was hybridized to Murine U74A version 2 GeneChip Expression Arrays (Affymetrix) for 16 hr at 45°C. All preparations were performed following manufacturer's instructions. Arrays were washed and stained with streptavidin-phycoerytherin, and scanned with an Argon-ion Laser Confocal Scanner (APB). Microarray analysis was performed twice on independent samples [34] and these raw data were loaded into NCBIs Gene Expression Omnibus as the dataset GSM159919-GSM159930 (GEO, http://www.ncbi.nlm.nih.gov/geo/). The putative target genes were validated by quantitative RT-PCR (QRT-PCR).

Statistical analysis: Signals in 2 experiments were detected using the robust multichip average (RMA) algorithms, and normalized using Genespring (Silicon Genetics, Redwoods City, CA). Expressed genes more than 40% raw signal of average raw signals in all genes on chip were selected as detected genes for next analysis. Selected genes showing more than 2-fold alterations by E_2 as compared to the tissue-matched oil controls were analyzed further using Genespring software.

Quantitative RT-PCR: One μg total RNA was reverse transcribed using Super Script II reverse transcriptase (Invitrogen) and random primers at 42°C for 50 min. PCR was performed using PE Prism 5,700 Sequence Detection System (PE Biosystems, Tokyo, Japan) with SYBR Green I dye (Molecular Probes, Eugene, OR) and primers selected by Primer Express *ver 1.0* (APB). Primer sets are described in Table 1.

PCR amplification was performed for 2 min at 50°C, for 10 min at 95°C and continued to 40 cycles at 95°C for 15 sec and at 60°C for 1 min. Data were normalized to ribosomal protein 28S RNA using delta Ct method for each primer set. The ratio was calculated as compared with oil controls of uterus.

BrdU-Labeling and Immunostaining: A single injection of 200 mg BrdU (Roche, Grenzacherstrasse, Switzerland)/kg b.w. was given to mice (4 mice each) 1 hr before sacrifice. Uterus, vagina and mammary gland were fixed with neutral-buffered 10% formalin, embedded in paraffin and sectioned at 6 μm. Sections were dipped in PBS and endogenous peroxidase activity was blocked with 3% H_2O_2 in methanol for 30 min. After washing in 0.5% Tween 20 in PBS twice, sections were dipped in 2N HCl for 20 min, then, neutralized sections borate buffer (0.1 M NaB_4O_7, pH 8.5) twice. After Tween/PBS washing, sections were dipped in 1% BSA/PBS for 20 min. Then, sections were incubated with 1:20 anti-BrdU (Roche). Sections detected with diaminobenzidine staining were analyzed. The BrdU labeling index (%) was estimated by counting BrdU positive cells in 2,000–3,000 epithelial and 10,000–20,000 stromal cells in the uterus and vagina, and in 300–500 epithelial or stromal cells in the mammary glands.

RESULTS

Gene expression in the uterus, vagina and mammary gland exposed to E_2: Approximately 12,400 genes were ana-

Table 1. Primer sets for QRT-PCR

Genebank accession No.	Name	Forward primer	Reverse primer
M13500	Klk 1	ATGGATGGAGGCAAAGACACTT	ACCTTGGAGAACACCATCACAGA
X04480	IGFI	CTACAAAAGCAGCCCGCTCTA	TCCTTCTGAGTCTTGGGCATGT
X81580	IGFBP2	GGAACATCTCTACTCCCTGCACAT	TTGTACCGGCCATGCTTGT
NM_010518	IGFBP5	GGTGTGTGGACAAGTACGGAATGA	ACGTTACTGCTGTCGAAGGCGT
X00525	Ribosomal 28S	AGACCGTCGTGAGACAGGTTAGTT	GCAGGATTACCATGGCAACAA

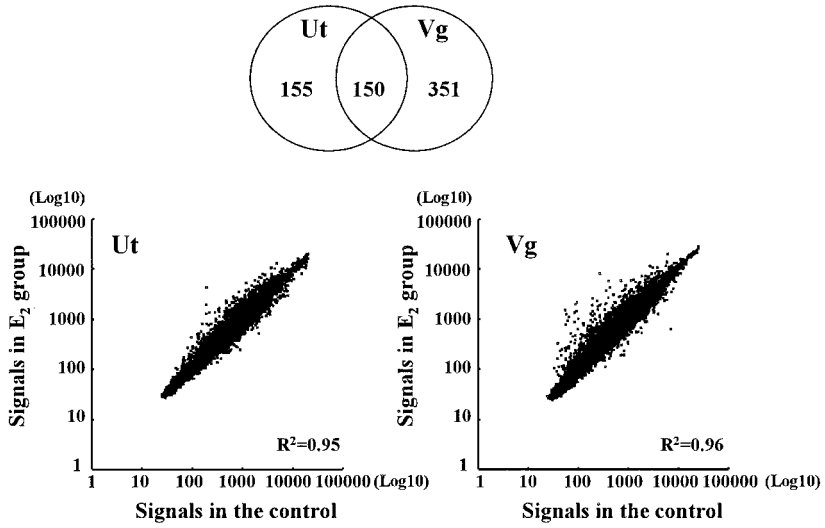

Fig. 1. Gene expression profiles in the uterus and vagina 6 hr after a single injection of 5 μg E_2/kg body weight. The Venn diagram indicatess detected genes in the oil-controls. Scatter plots indicate gene expression levels and the correlations between oil and E_2 groups in all genes. R_2 is correlation between oil and E_2 groups. Ut, uterus; Vg, vagina.

lyzed in the uterus, vagina and mammary gland. The total normalized signals between controls and E_2-exposed mice exhibited high correlations (R^2=0.95–0.96) (Fig. 1). The total number of genes showing at least 2-fold expression change 6 hr after a single injection of E_2 was 656 in all samples (Fig. 1). E_2 did not alter any gene expression more than 2-fold change in the mammary gland in the present study (data not shown). Genes showing organ-specific expression were 155 and 351 in the uterus and vagina, respectively (Fig. 1). Among them, 150 genes were regulated commonly in the uterus and vagina (Fig. 1).

In the uterus, 228 genes were up-regulated and 77 genes were down-regulated by E_2 as compared to the controls (Fig 2). In the vagina, 446 genes were up-regulated and 35 were down-regulated by E_2. In the uterus, 63% of E_2 up-regulated genes were overlapped those in the vagina. E_2 down-regulated common genes in the uterus and vagina were only 6 (Fig. 2). We further analyzed genes related to cell growth and organogenesis to find tissue-specific genes. E_2-responsive genes related to development, cell growth and apoptosis in the uterus and vagina were listed in Table 2.

Expression of Igf-1 family and Kallikrein 1 genes: Since clustering analysis revealed many E_2-regulated genes, we compared expressions of *Kallikrein 1 (Klk1)* genes and *Igf-1* family genes in each tissue using QRT-PCR.

In the controls, expression of *Klk1* mRNA was similar between uterus and vagina, while those of *Igf-1* and *Igfbp5* were lower in the vagina than in the uterus (Fig. 3). In the mammary gland, unlike the uterus and vagina, expressions of all mRNAs examined were very low or undetectable in the ovariectomized mice with or without E_2. In the uterus and vagina, expression of *Igf-1* mRNA was markedly increased by E_2. However, expressions of *Igfbp2* and *Igfbp5*

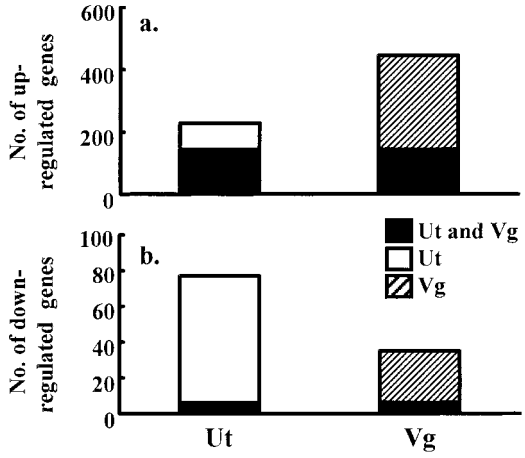

Fig. 2. Number of estrogen up-regulated genes (a) and down-regulated genes (b) in the uterus (Ut) and vagina (Vg). "Ut and Vg" indicates genes showing commonly altered expression by E_2 both in the uterus and vagina, while Ut and Vg indicate organ specific genes, respectively.

mRNAs were increased by E_2 in the vagina (Fig. 3). *Klk1* mRNAs was significantly increased as by E_2 in the vagina.

BrdU incorporation in the uterus, vagina and mammary gland, vagina 24 hr after E_2 injection: BrdU labeled cells were barely detected in epithelial cells of the uterus and mammary gland (0% and 0.05%) in the controls, as compared to those of the vagina (0.24%) in the controls (Fig. 4). The BrdU labeling index in the epithelial cells of the uterus and vagina was significantly increased 24 hr after the E_2 injection as compared with each control. In the mammary gland, however, BrdU-positive cells were not evident in the

Table 2. List of estrogen responsive genes related development, cell growth and apoptosis in the uterus and vagina by microarray analysis

Accession No.	Name	Ratio Ut	Ratio Vg
M60523	Inhibitor of DNA binding 3	**0.42**	0.71
AI840339	Ribonuclease, RNase A family 4	**0.42**	1.32
AA838868	Latent transforming growth factor beta binding protein 4	**0.42**	1.07
L31532	B-cell leukemia/lymphoma 2	**0.43**	0.59
X70298	SRY-box containing gene 4	**0.45**	**0.36**
U88567	Secreted frizzled-related protein 2	**0.45**	0.89
AI843106	Sestrin 1	**0.45**	**0.43**
AW123618	Frizzled homolog 2 (Drosophila)	**0.46**	0.64
AV092014	Peptidoglycan recognition protein 1	1.54	**0.37**
AI834950	Nuclear receptor subfamily 1, group D, member 1	0.58	**0.37**
AF076482	Peptidoglycan recognition protein 1	1.10	**0.40**
AF056187	IGF1 receptor	0.59	**0.41**
AF099973	Schlafen 2	1.02	**0.46**
X07750	Thyroid hormone receptor alpha	0.72	**0.49**
X81580	Insulin-like growth factor binding protein 2 (IGFBP2)	1.00	**2.00**
AW123099	Chromosome segregation 1-like (S. cerevisiae)	1.76	**2.01**
AF003695	Hypoxia inducible factor 1, alpha subunit	1.57	**2.01**
AI747899	Phosphatidylinositol transfer protein, beta	1.24	**2.02**
X03491	Keratin complex 2, basic, gene 4	1.03	**2.08**
X62154	similar to DNA replication licensing factor MCM3 (P1-MCM3)	1.48	**2.09**
AW124529	Tumor necrosis factor superfamily, member 5-induced protein 1	0.97	**2.16**
AF011644	CDK2 (cyclin-dependent kinase 2)-associated protein 1	1.81	**2.19**
AW048763	NMDA receptor-regulated gene 1	1.66	**2.20**
D49382	Septin 2	1.48	**2.20**
AW125478	HtrA serine peptidase 1	1.46	**2.22**
X02452	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	1.95	**2.26**
D10214	Prolactin receptor	1.12	**2.30**
AF014117	Glial cell line derived neurotrophic factor family receptor α 1	1.70	**2.34**
AF041476	Actin-like 6A	1.96	**2.36**
M73329	Protein disulfide isomerase associated 3	1.92	**2.38**
U35846	Apoptosis inhibitor 5	1.76	**2.43**
AF058798	Stratifin	1.32	**2.44**
D12780	S-adenosylmethionine decarboxylase 1	1.89	**2.47**
Z23077	S-adenosylmethionine decarboxylase 1 and 2	1.74	**2.49**
J04766	Plasminogen	1.02	**2.62**
D00613	Matrix Gla protein	0.63	**2.68**
X59846	Growth arrest specific 6	1.14	**2.79**
L12447	Insulin-like growth factor binding protein 5 (IGFBP5)	1.18	**3.09**
AI847054	Phosphatidic acid phosphatase type 2B	1.69	**3.32**
M35523	Cellular retinoic acid binding protein II	1.02	**3.47**
AI837110	Protein arginine N-methyltransferase 1	1.86	**3.49**
M74570	Aldehyde dehydrogenase family 1, subfamily A1	1.31	**4.11**
AV028204	Plasminogen	0.75	**4.38**
AI553024	Zinc finger and BTB domain containing 16	0.52	**4.44**
AW124889	Aldehyde dehydrogenase 18 family, member A1	**2.02**	1.45
AF100777	WNT1 inducible signaling pathway protein 1	**2.03**	1.38
AW260482	NMDA receptor-regulated gene 1	**2.10**	1.50
X13986	Secreted phosphoprotein 1	**2.17**	1.23
U00937	Growth arrest and DNA-damage-inducible 45 alpha	**2.18**	**3.08**
AF079528	Neuropilin 1	**2.20**	1.57
AB003502	G1 to S phase transition 1	**2.21**	1.66
D63784	DnaJ (Hsp40) homolog, subfamily C, member 2	**2.24**	1.96
AI645561	NMDA receptor-regulated gene 1	**2.29**	**2.10**
U53208	DnaJ (Hsp40) homolog, subfamily C, member 2	**2.34**	1.79
AW046181	Serum/glucocorticoid regulated kinase	**2.38**	1.02
AA529583	Mortality factor 4 like 2	**2.39**	**3.56**
V00756	Interferon-related developmental regulator 1	**2.40**	**2.63**
U88327	Suppressor of cytokine signaling 2	**2.42**	1.07
AB012276	Activating transcription factor 5	**2.43**	1.96
M13500	Kallikrein 1	**2.43**	0.92

Table 2. Continued

Accession No.	Name	Ratio Ut	Ratio Vg
U84411	Protein tyrosine phosphatase 4a1	**2.46**	**2.34**
AW048937	Cyclin-dependent kinase inhibitor 1A (P21)	**2.51**	**2.45**
AF055638	Growth arrest and DNA-damage-inducible 45 gamma	**2.53**	**5.03**
AI596034	Receptor tyrosine kinase-like orphan receptor 2	**2.64**	**3.31**
V00727	FBJ osteosarcoma oncogene	**2.66**	**3.22**
L32751	RAN, member RAS oncogene family	**2.77**	**2.36**
D50086	Neuropilin 1	**2.95**	1.27
X99273	Aldehyde dehydrogenase family 1, subfamily A2	**2.99**	1.90
M63801	Gap junction membrane channel protein alpha 1	**3.02**	1.98
AI785289	Guanine nucleotide binding protein-like 3 (nucleolar)	**3.82**	**2.57**
X04480	Insulin-like growth factor 1 (IGF-I)	**4.67**	**4.82**
U83902	MAD2 (mitotic arrest deficient, homolog)-like 1 (yeast)	**4.75**	**4.57**
AF053232	Nucleolar protein 5	**5.09**	**3.32**
X69620	Inhibin beta-B	**10.29**	**7.60**

Bold means more than 2-fold alterations by E_2.

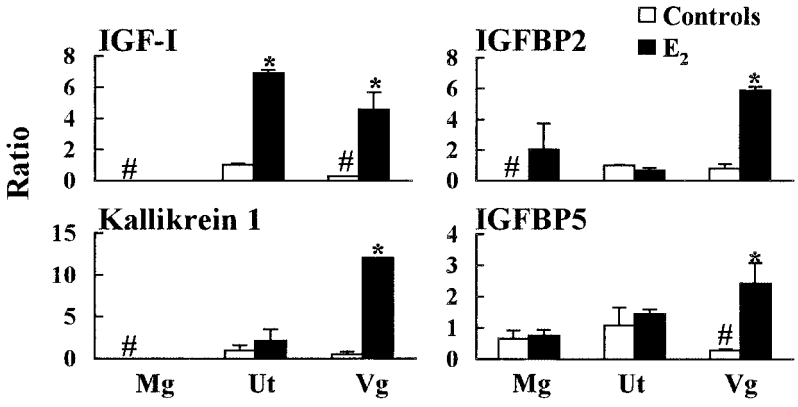

Fig. 3. Ratio of mRNA gene expressions of *Igf-1* family and *Kallikrein 1* in the three organs 6 hr after the E_2 injection using QRT-PCR. *, $P<0.05$ v.s. the control of each tissue; #, $P<0.05$ v.s. the control uterus.

epithelium 24 hr after the E_2 injection. The index was significantly increased by E_2 in the uterine stroma (Fig. 4).

DISCUSSION

Estrogen regulates mitosis and morphological changes in female reproductive organs during proliferative events, such as estrous cycles, gestation and lactation. In order to understand the underlying mechanisms of estrogen functions in reproductive organs, detection of estrogen responsive genes in each reproductive organs are is essential. Effects of estrogen are different in each reproductive organ, therefore we investigated a global gene expression in uterus, vagina and mammary gland after a single injection of E_2.

In the present study, BrdU labeled cells were remarkably increased in the uterine and vaginal epithelia, and in the uterine stroma after the E_2 injection, but not in any parts of the mammary gland. Gene expression in response to estrogen is different among these organs. *Igf-1* is a key epithelial mitogen induced by estrogenic chemicals [28, 31], whereas IGFBP prevents signal pathway by binding to *Igf-1*, and inhibits phosphorlylation of Insulin receptor substrate-1 (IRS-1), Phosphatidylinositol 3-kinase (PI3K), Protein kinase B (PKB) and Forkhead transcription factors (FKHRL1) [23]. *Igfbp2* and *Igfbp5* promote apoptosis in the prostate cancer cells and mammary gland cells [23, 26, 27, 29]. Hence, proliferations of uterine and vaginal cells was appear to be regulated by estrogen via *Igf-1* and receptor complex, and its modulator. In the present study, estrogen increased *Igf-1* mRNA and mitosis in the uterus and vagina. However, the *Igf-1* modulators and *Igfbp* mRNAs were also increased in E_2-exposed vagina. This may be accounted for by the suppression of stromal cell proliferation caused by increase of *Igfbp* mRNAs in the stroma rather than the epithelium. Up-regulation of *Klk1* was reported by E_2 in the uterus [34]. *Klk* plays an important role for the release of bradykinin from kininogen, activation of growth factors and alteration of the extracellular matrix in the uterine epithelium [7]. *Klk* is regulated hormonally [7, 8] and the gene expression was found in human ovarian, prostate

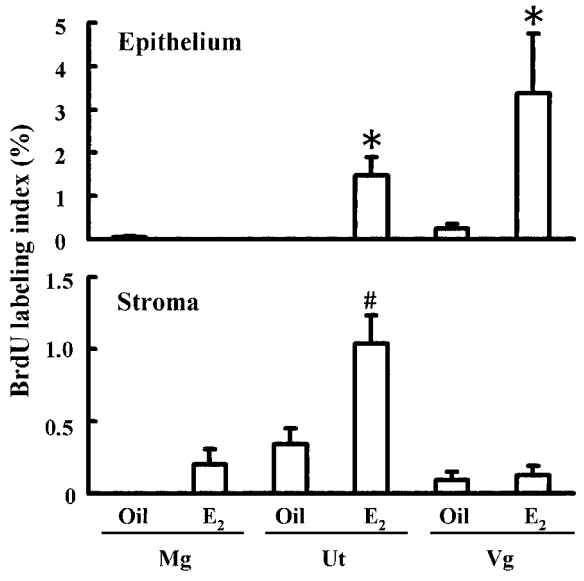

Fig. 4. BrdU labeling index (positive cells/counted cells, %) in epithelium and stroma of the mammary gland, uterus and vagina. Mg, mammary gland; Ut, uterus; Vg, vagina; *, $P<0.05$ v.s. the control of each tissue.

and breast cancer cells and in mouse vagina [15, 25, 37]. We found the up-regulation of *Klk1* gene expression in the vagina by QRT-PCR. Thus, *Klk1* may be related to epidermal proliferation and their expressions can be used for markers of acute response to estrogen in the uterus and vagina.

We found that estrogen regulated genes were markedly limited in the mammary gland as compared to those in the uterus and vagina 6 hr after the E_2 exposure. Global gene expression in E_2-exposed mammary gland has not been reported. Only gene expression in human breast cancer MCF-7 cells treated E_2 *in vitro* was reported [4, 10, 11]. MCF-7 cells treated with E_2 revealed the major down-regulation (70%) of gene expression including transcriptional repressor, antiproliferative and proapoptotic genes, such as *Bcl-2, Cyclin G2* and *TGF-β* family [11]. Moreover, using the serial analysis of gene expression (SAGE) method, 3 up-regulated genes were reported in MCF-7 cells 10 hr after E_2 treatment *in vitro* [4]. The genes reported as E_2 down-regulated genes in MCF-7 cells were not found in the mammary gland in the present study. MCF-7 cells are a single species of mammary cancer cells and show precise response of time- and dose-dependent proliferation to estrogen. Normal mammary gland may need longer than 6 hr to respond to E_2 *in vivo*. Mammary gland has various types of cells, such as epithelial cells stromal cells and adipocytes. Thus, we need further precise experiment to understand estrogen responsive genes in the mammary gland.

Although the mammary gland is known to be one of the target organs of estrogen, ER-α knockout (αERKO) mice showed proliferation and morphogenesis of the mammary gland in adulthood [24]. The mammary gland seems to be regulated by progesterone and prolactin rather than estrogen [5, 12, 15, 17, 24, 33]. The up-regulation of gene expression, such as *IRS-1, Msx-2, C/EBPβ* and *Stat5*, by progesterone was reported in human breast cancer cells [15]. Prolactin induced expression of *Igf-2* mRNA in the developing mammary gland [13]. Thus, mammary gland is possibly regulated largely by progesterone and/or prolactin. This may account for no expression of estrogen responsive genes observed 6 hr after the E_2 exposure and absence of definite mitogenic response in the mammary gland of ovariectomized adult mice 24 hr after the E_2 exposure.

In conclusion, E_2 regulates expression of a number of genes in the vagina and uterus, but not in the mammary gland. Half of E_2-regulated genes in the uterus were in common with the vagina including *Kallikrein* and *Igf* family genes. Differences in expression of these genes in response to E_2 may be leased on the tissue specificity to estrogen exposure. The candidate estrogen responsive genes in the uterus and vagina identified by profiling provide an important foundation to understand functional mechanisms of estrogen regulating morphogenesis and maintenance of the reproductive organ.

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